MOLECULAR TRACKING OF IRON- AND MANGANESE REDUCING ENRICHMENTS FROM EEL POND

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MBL Microbial Diversity 2002

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Many iron-reducing bacteria are able to respire a wide range of compounds as terminal electron acceptors, including insoluble iron and manganese oxides, chelated (soluble) forms of oxidized metals, and humic substances and their analogs such as 2,6-anthraquinone disulfonate (AQDS). In order to evaluate the role of these terminal electron acceptors in selecting for different metal-reducing bacteria, surface sediments from Eel Pond were used to inoculate enrichments containing acetate as the electron donor/carbon source, and various combinations of insoluble Fe(III) or Mn(IV) oxides, soluble Fe(III) chelated to nitrilotriacetic acid (NTA), and AQDS as electron acceptors/shuttles. Microscopy, clone library analysis, and TRFLP data all suggest significant differences in the microbial communities of the enrichments depending on which electron acceptors were present. *Azoarcus* sp. accounted for the most common 16SrRNA sequences in enrichments where Mn(IV) oxides were present, while members of the *Desulfuromonas* group dominated enrichments containing Fe(III) oxides and AQDS.

INTRODUCTION

Insoluble minerals such as iron and manganese oxides have only recently been recognized as important terminal electron acceptors in bacterial respiration. Bacteria that use these minerals for respiration have generated widespread interest due to their significance in biogeochemical cycling of many elements in many environments, potential for bioremediation of organic and inorganic pollutants (4, 7), and unique properties of transferring electrons to insoluble minerals (11). Fe(III)-reducing bacteria (FeRB) are also generally thought to be capable of transferring electrons to Mn(IV) (6) although there are cases where FeRB cannot respire Mn(IV). Bacteria capable of respiring iron and manganese oxides are phylogenetically diverse, falling within the beta, gamma, and delta subdivisions of the proteobacteria. By far the best characterized Fe/MnRB are members of the *Shewanella* and *Geobacter* genera. *Geobacter* is a part of the family *Geobacteraceae* of the delta-proteobacteria which also includes the genera *Desulfuromonas*, *Pelobacter*, and *Desulfuromusa*, all of which are oxidize acetate to carbon dioxide (except Pelobacter) with the reduction of Fe(III). Shewanella are facultative anaerobes that fall within the gamma proteobacteria and are important at oxic-anoxic transition zones. In contrast to *Geobacter*, they prefer lactate and formate as electron and carbon sources rather than acetate.

Because most terminal electrons acceptors are soluble (oxygen, nitrate, sulfate), the discovery of bacterial respiration of insoluble minerals raised new mechanistic questions. Traditionally FeRB were thought to respire by direct contact with the minerals via cytochromes, quinones, and dehydrogenases in the outer membrane of the cell, but recently alternative mechanisms of electron transfer to insoluble minerals have come under study. Two alternative mechanisms have been proposed: solubilization of minerals by producing chelating molecules, and use of soluble shuttles to transfer electrons to the mineral (11). Addition of synthetic chelators to FeRB cultures enhances the transfer of electrons to iron minerals (7), but no microbially produced Fe(III) chelator has been described as a mechanism of accessing iron minerals for respiration. Humic substances have emerged as an important shuttle of electrons from microbes to minerals, and the small quinoid compound 2,6-anthraquinone disulfonate (AQDS) has been commonly used as a humics analog. In fact, all FeRB that have been evaluated can also reduce AQDS (8), and the potential for humics reduction has been demonstrated in a wide range of environments by many Geobacteraceae species (1). In addition to exogenously occurring substances such as humics, microbially produced electron shuttles are now known to play a role in some FeRB but not in others (3, 9,10). There have been few studies of electron shuttling to Mn(IV) oxides, but similar results are expected because of the ability of most FeRB to respire both Fe(III) and Mn(IV) (6).

To determine whether the type of metal supplied as the electron acceptor (amorphous iron oxide, manganese oxide, or Fe(III) chelated and solublized by NTA) or the presence/absence of the humic substance analog AQDS play an important role in selecting for Fe/MnRB, various combinations of these electron acceptors/shuttles were used to enrich for Fe/MnRB from surface sediments of Eel Pond, Woods Hole, MA. Enrichments were followed via microscopy, t-RFLP, and sequence analysis of 16SrRNA gene clone libraries.

MATERIALS AND METHODS

Source and enrichment of microorganisms. Surface sediments (top 5 cm) were obtained with a sterile conical 50 ml-tube from a near shore area just off of a dock in Eel Pond, Woods Hole, MA. The sediment sample was homogenized and ~2ml of sediment was used to inoculate 50-ml Pfennig bottles containing Basal mineral medium (see MBL Microbial Diversity lab protocol, 2002), 5 mM acetate, and the following electron acceptors: (1) 40 mM manganese oxide, (2) 40 mM iron oxide, (3) 5 mM Fe(III) chelated and solublized by to nitrilotriacetic acid (NTA), (4) 20mM iron oxide + 20 mM manganese oxide, (5) 20 mM Mn oxide + 5 mM Fe(III)-NTA. Another set of the above enrichments was amended with 50 μM 2,6-anthraquinone disulfonate (AQDS) for a total of 10 enrichments. Amorphous iron oxide and Fe(III)-NTA were made by the methods of Lovely (6), and manganese oxides were kindly provided by Jochen Mueller.

Reduction of the minerals in the enrichments was followed by observing the change in color of iron oxides from orange to black and of manganese oxides from black to white. Enrichments containing AQDS turned orange after several days, indicating reduction of this quinoid molecule. Cell morphologies of enrichments were followed by phase contrast microscopy on a Zeiss Axioskop 2 Plus microscope. After 5 days 2 ml subsamples of the enrichments were transferred to fresh media: the Pfennig bottles were inverted to mix so that solid mineral phases were transferred in addition to the liquid.

DNA extraction and PCR amplification of 16S rRNA genes. 1.5 ml of enrichments that had been inverted to mix (to ensure inclusion of the mineral phase) were removed at days 0, 5, and 11 for DNA extraction. The 1.5 ml samples were centrifuged, the supernatant removed, and the pellet was resuspened and added to a MoBio UltraClean Soil DNA Isolation Kit. The DNA extraction was done as instructed by the manufacturer. 2 μl of the DNA extraction was used as template DNA for standard PCR amplification of 16S ribosomal RNA genes as previously described (MBL MD 2002 Lab Protocols). Universal primers 8F (5'-agagtttgatcctggctcag-3') and 1492R (5'–ggttaccttgttacgactt-3') were used with Taq DNA polymerase under the following thermal cycling conditions: 1x(95°C 5 min), 25x(95°C 30 sec, 55°C 30 sec, 72°C 1 min), 1x(72°C 12 min). For T-RFLP analysis, a FAM labeled 8F primer was used.

Cloning, sequencing, and phylogenetic analysis of 16S rRNA genes. 16S rRNA genes PCR amplified from enrichment DNA samples were cloned using the TOPO TA cloning kit with the pCR4.0 vector (Invitrogen). TOP10 competent cells were transformed and plated on LB Ampicillin agar plates. Individual colonies were picked and used to inoculate 96-well plates for automated DNA sequencing (Mitch Sogin Laboratory, Marine Biological Laboratory, Woods Hole, MA). 97 clones were submitted total. 16S rRNA sequences were aligned in the ARB software program for sequence data (Lehrstuhl fuer Mikrobiolgie, Technical University of Munich, Germany). Phylogenetic trees were constructed within ARB by neighbor joining and maximum likelihood.

T-RFLP Analysis. FAM-labeled 16S rRNA PCR products were purified with the PCR Purification Kit (Qiagen) and 600 ng was digested with the restriction enzyme HhaI at 37°C for two hours. The restriction enzyme was inactivated by heat treatment at 65°C for 20 minutes, 1.5 μl of TAMARA molecular weight size standards was added to each digestion reaction. The digestions were then dried, frozen, and submitted to the University of Connecticut molecular biology core facility. Data files were analyzed with GeneScan 2.1. Apparently many of the molecular weight size standards were not correctly identified leading to erroneous assignment of lengths to the terminal restriction fragments. The T-RFLP profiles were then manually edited and aligned using ADOBE Illustrator and Canvas using the position of the molecular weight markers that appeared to be correct. Although this seemed to work, there was still some degree of

uncertainty of the exact position of the T-RFLP peaks and the accuracy was not comparable to what should be expected from T-RFLP. ARB was used to predict the terminal fragment lengths of the sequences retrieved from the clone library, and these results were compared to the T-RFLP profile.

RESULTS

Cell morphology and appearance of enrichments. One of the first noticeable differences in the appearance of the enrichments was that within one day of adding Fe(III)-NTA to the basal mineral media, a fluffy orange precipitate was visible in the bottom of the Pfennig bottles, indicating that the chelated Fe(III) had for some unknown reason precipitated out of solution. Because of this unexpected result, all enrichments containing Fe(III)-NTA were excluded from cloning and sequencing of 16S rRNA. Reduction of the iron and manganese minerals and of AQDS was followed by monitoring the color changes of the minerals and the supernatant (AQDS). By day 2 a yellow tint was apparent in the supernatant of all bottles containing AQDS, and by day 3 this color was bright orange indicating reduction of this quinoid molecule. In the enrichments containing iron, the color of the supernatant became dark orange or brown, while in the manganese oxide + AQDS enrichments the color remained bright orange. Color changes of the iron oxides from orange to black and of the manganese oxides from black to white generally began at day two or three and was complete by day 5. The combination iron/manganese oxides remained orange much longer than the iron oxides alone but eventually turned brown. No white reduced manganese minerals were evident in the iron/manganese oxide mix.

As enrichments progressed the dominant cell morphologies changed dramatically and the overall cell density increased. At early stages of the enrichments a wide variety of cell morphologies were observed in all enrichments, including cocci, spirilla, various types of rods, and possibly spirochetes. By day 10 only a few cell morphologies dominated all enrichments: long, thin rods; fat, rounded rods; and highly motile spirilla (Figure 1). Rods seemed to be more prevalent and spirilla were in lower abundance in all enrichments where AQDS was present (Fig. 2, 3) except in enrichments containing Fe(III)-NTA. Rods were often closely associated with minerals in enrichments where AQDS was not present (Fig. 2).

Analysis of 16S rRNA clone libraries. Only 33 of the 97 sequences analyzed were 16S rRNA sequences. Approximately 60 of the sequences were vector, indicating a problem in the cloning of the 16S rRNA PCR products that led to high levels of background in the clone library. This result was unexpected since the Invitrogen pCR 4.0 vector is supposedly a suicide vector that prevents vectors without inserts from replicating. The 33 sequences retrieved were of high quality with > 750 bases of good read in all cases. The sequences were submitted to BLAST, aligned in ARB, and phylogenetic trees were constructed in ARB using both neighbor joining and maximum likelihood methods (Fig. 4,5). 750 bases were considered in all of these alignments, and the distribution of these sequences as identified by BLAST and ARB is shown in figure 6. Three major groups were identified: 14 sequences were nearly identical to each other and are most closely related to the genera *Azoarcus* (Fig. 4), a group of denitrifying microbes known to degrade aromatic compounds such as toluene and phenols (Hurek et. al. 1995, van Schie et. al 1998). 9 of the sequences fell within the family

Geobacteraceae, which includes the genera *Pelobacter*, *Desulfuromonas*, and *Geobacter* (Fig. 5). These microorganisms are commonly recovered from sediments when enriched with acetate and iron oxides (2, 4), and they were the most numerous sequence in the iron oxide + AQDS enrichment. The third major group of sequences (4) retrieved from the clone library was most closely related the *Oceanosprillum*, which are marine aerobic heterotrophic bacteria.

T-RFLP. Terminal Restriction Fragment Length Polymorphisms (T-RFLP) is a technique that allows rapid analysis of a microbial community. The raw data for six of the samples for which T-RFLP were run is presented in Fig. 7. Blue indicates FAM-labeled 16S rRNA signal while red is the TAMARA-labeled molecular weight standards. There was apparently a problem in correctly identifying all of the molecular weight standards, since they were place in different positions in different lanes (Fig. 7). It is suspected that this was due to signal from the FAM-labeled samples leaking into the TAMARA detection and throwing off the assignment of sizes to the molecular weight markers. Due to this error made by the molecular biology core, it was difficult to compare T-RFLP patterns between different lanes and the determination of the length of the terminal fragments was not correct. Because of these complications, only the 6 profiles (out of 23 total profiles) for which there was also sequence data were selected for further analysis. To adjust the scale on the T-RFLP profiles so that molecular weight standards and sample peaks would align correctly between different lanes, the profiles were modified manually using Adobe Illustrator and Canvas. Because multiple molecular weight standards were often wrong, the profiles could not merely be shifted but in many cases had to be stretched and/or condensed in different regions. Similarities in the topology of the sample signals were used as an aid in this process, but there was a significant amount of uncertainty and the resulting data (Fig. 8) is not nearly as accurate as a properly sized T-RFLP profile could be. Nevertheless, patterns could be seen between the different profiles: many peaks were consistent throughout the different enrichments, and some changed dramatically. One of the most salient features of the profiles was a large peak near 387 bases that is present only when manganese oxides are present and not when they are absent. This result is consistent with the dominance of the *Azoarcus*-like sequences that were identified in the clone library as being dominant whenever MnO_2 was present as the sole or partial electron acceptor (Fig. 6). Indeed, the terminal fragment length of these sequences is predicted to be 378 bp when digested with *HhaI* – the difference from the 387 seen in the T-RFLP profile is well within the error of the terminal fragment length assignments due to the erroneous standards. Another potential peak that could be correlated to the sequence data was at 90 bp, which corresponds to the *HhaI* terminal fragment length of many *Geobacter* 16S rRNA genes. No other T-RFLP peaks could be attributed to sequences that were found in the clone library. Due to the problems with accurately sizing the molecular weight standards, TAP-TRFLP could not be used to search the current 16S rRNA database for possible matches.

DISCUSSION

There are several potentially interesting implications of the preliminary results presented here. In all enrichments observations were made consistent with the reduction

of iron and manganese minerals and the humic substance analog AQDS. The presence of AQDS increases the rate at which iron and manganese minerals were reduced, consistent with evidence that many iron reducing bacteria can also reduce humic substances (and AQDS) and that these electron shuttles facilitate transfer of electrons to insoluble mineral terminal electron acceptors (8). Over time and after one transfer the diversity of cell morphologies present in these enrichments went down, presumably indicating an enrichment for microbes respiring iron and manganese oxides.

Analysis of 16S rRNA genes sequences from 6 of the iron- and manganese-reducing enrichments revealed some expected results as well as some surprises. Sequences that fall within the family *Geobacteraceae* were common as would be expected from enrichments where acetate is the electron donor/carbon source and iron and manganese oxides are the electron acceptors (1, 2, 4). Suprisingly, however, only when iron oxide and AQDS sequences were present did *Geobacter* sequences predominate. In all cases where manganese oxide was present as an electron acceptor – even in combination with iron oxide and AQDS - sequences related to *Azoarcus* species predominated. This result is surprising because while *Azoarcus* species are well known for their potential bioremediation applications in anaerobically degrading a host of natural and industrial anaerobic compounds (5, 12, 13), no members of the genus have ever been identified as being able to respire iron or manganese oxides.

There are several potential explanations for *Azoarcus*-related sequences dominating iron and manganese reducing enrichments. First, it should be noted that the overall number of sequences obtained was low and so the significance of the clone library results is questionable. The T-RFLP data however, also suggests that *Azoarcus*-like sequences are abundant in the enrichments containing manganese oxides. Both the T-RFLP data and clone library rely on PCR to identify the members of the enrichment communities and thus are subjected to biases in the DNA extraction and 16S rRNA amplification. Based on sequence analysis the universal 8F and 1492R primers do not favor amplification of the *Azoarcus* sequences over the *Geobacter*, but PCR-independent methods such as Fluorescent In Situ Hybridization (FISH) are required to determine if *Azoarcus*-like species really are abundant in these iron- and manganese-reducing enrichments. A predominance of *Azoarcus*-like species in iron- and manganese-reducing enrichments does not necessarily mean that these microorganisms can respire iron or manganese. They could be "hangers-on" from the original inoculum, which may have contained significant amounts of nitrate that could be used as an electron acceptor. This seems unlikely, however, given that the original incolum was only 4% of the volume of the enrichment, and that only 4% of this enrichment was transferred to fresh media, where the community analysis was conducted. It therefore seems likely that there are *Azoarcus*-like species capable of iron- and/or manganese-reduction in these enrichments, however they will have to be isolated and studied in pure culture to determine conclusively whether this is the case.

The finding of *Azoarcus*-like species capable of dissimilatory metal reduction could have broad implications in the degradation of aromatic compounds in anaerobic environments. Again, isolation of these organisms is required to determine whether they are able to degrade aromatics such as toluene, benzene, and xylene as their closest relatives are. The entire 16S rRNA gene should be sequenced to affirm the phylogeny of these microorganisms, and it may be interesting to use nucleic acid probes (13) to

determine the distribution and abundance of these *Azoarcus*-like microorganisms in Eel Pond sediments.

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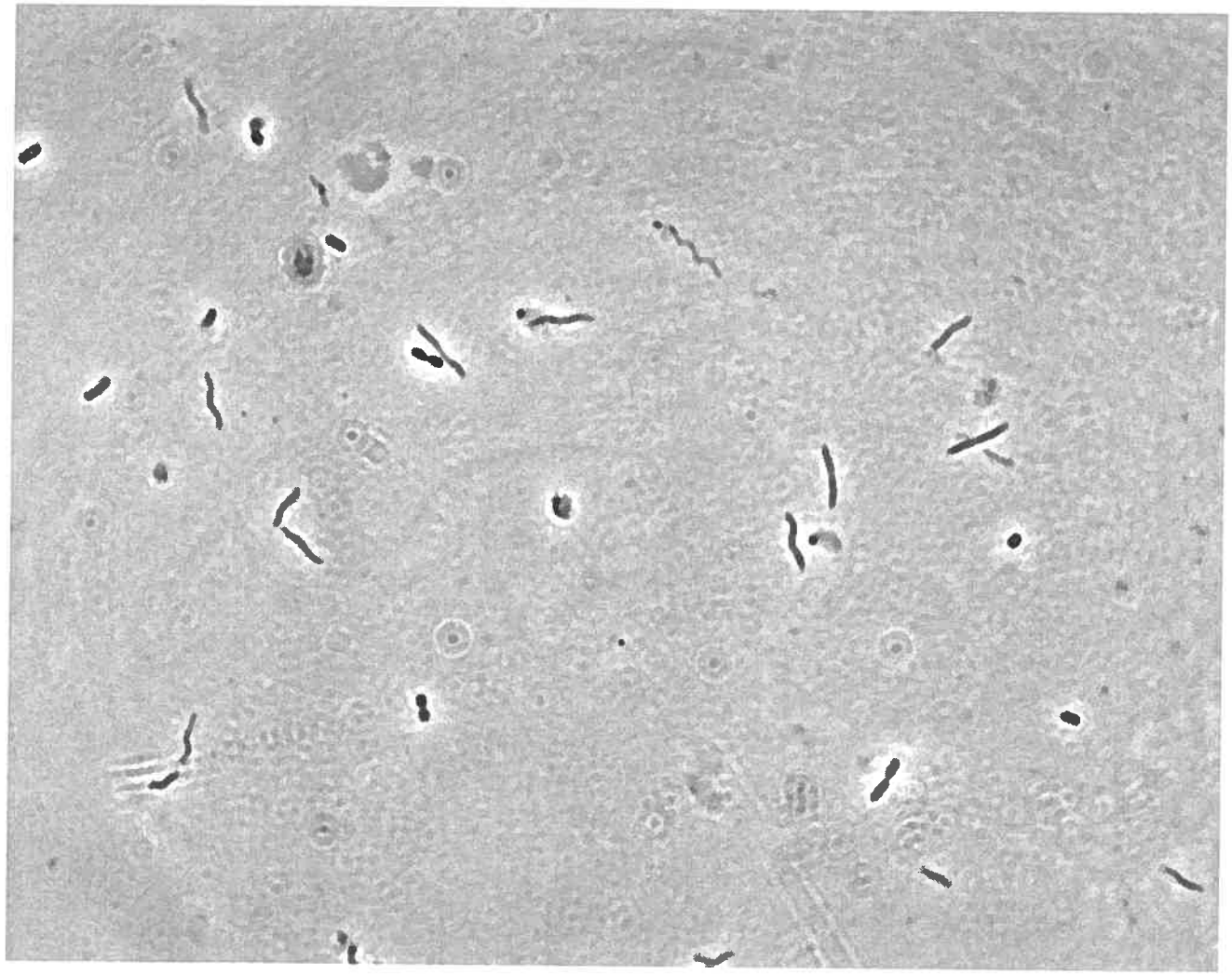


Figure 1. Phase contrast micrograph of manganese oxide, no AQDS enrichment.

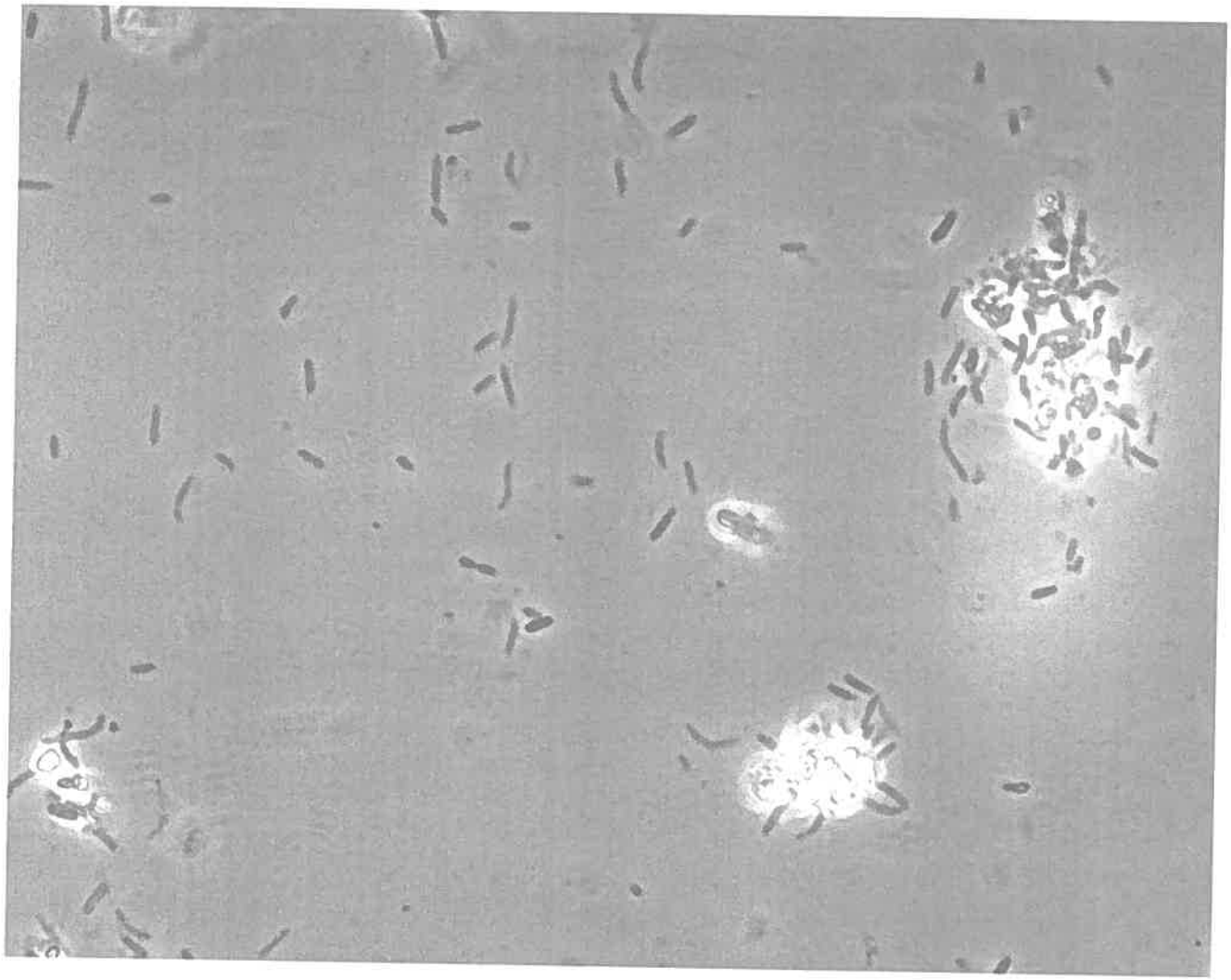


Figure 2. Phase contrast micrograph of iron oxide, no AQDS enrichment.

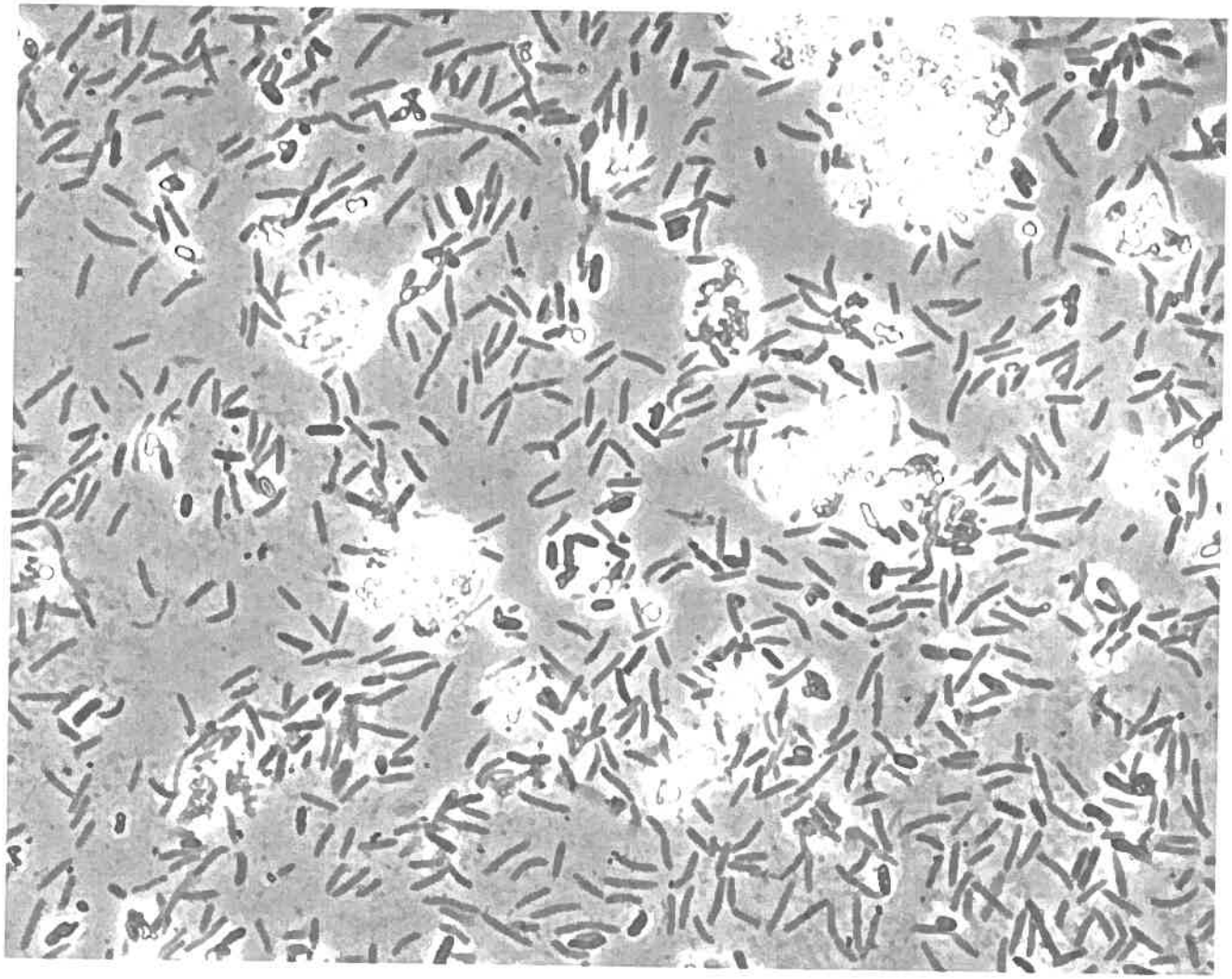


Figure 3. Phase contrast micrograph of iron oxide with AQDS enrichment.

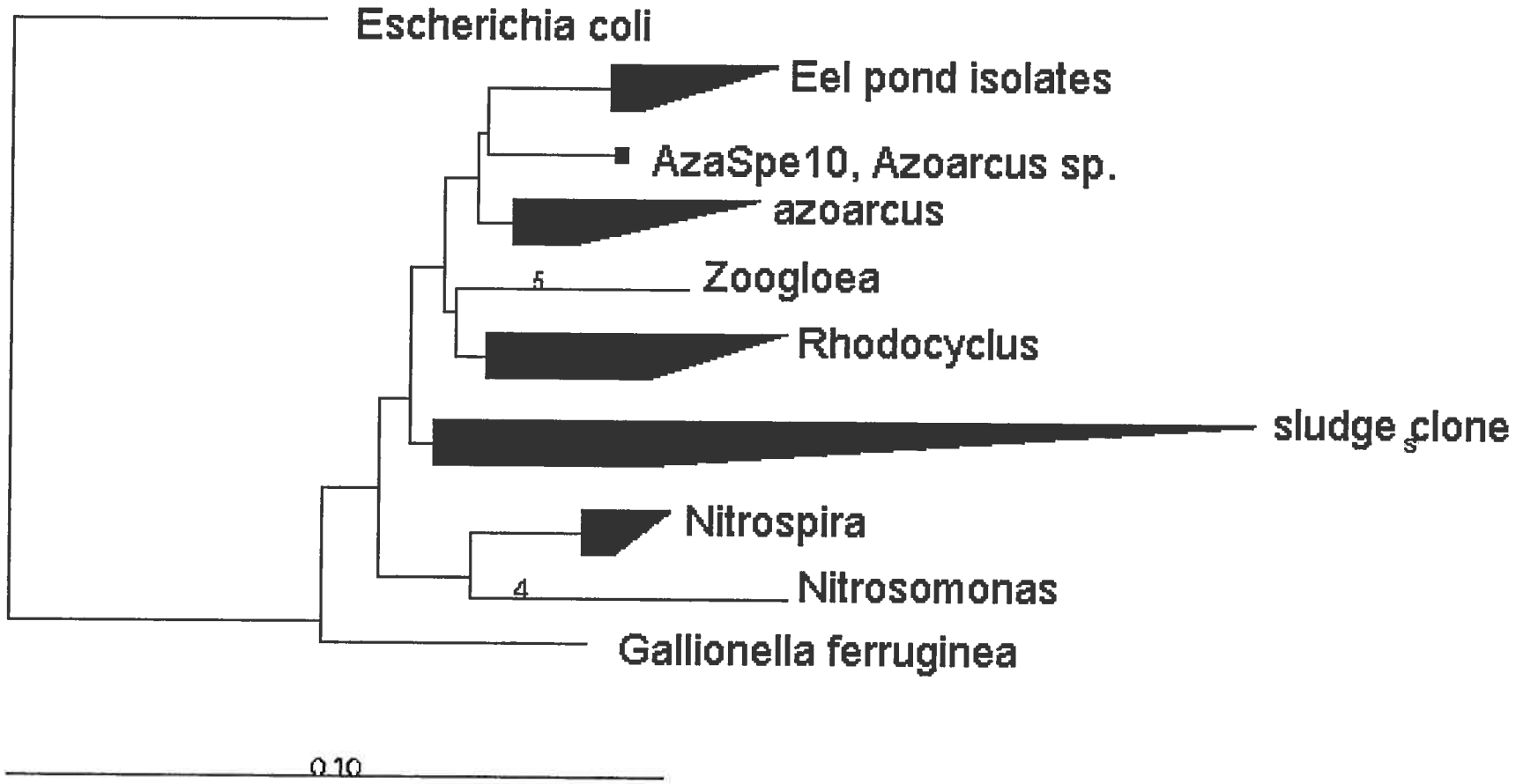


Figure 4. Neighbor-joining tree of Eel pond Azoarcus-like sequences and closest relatives.

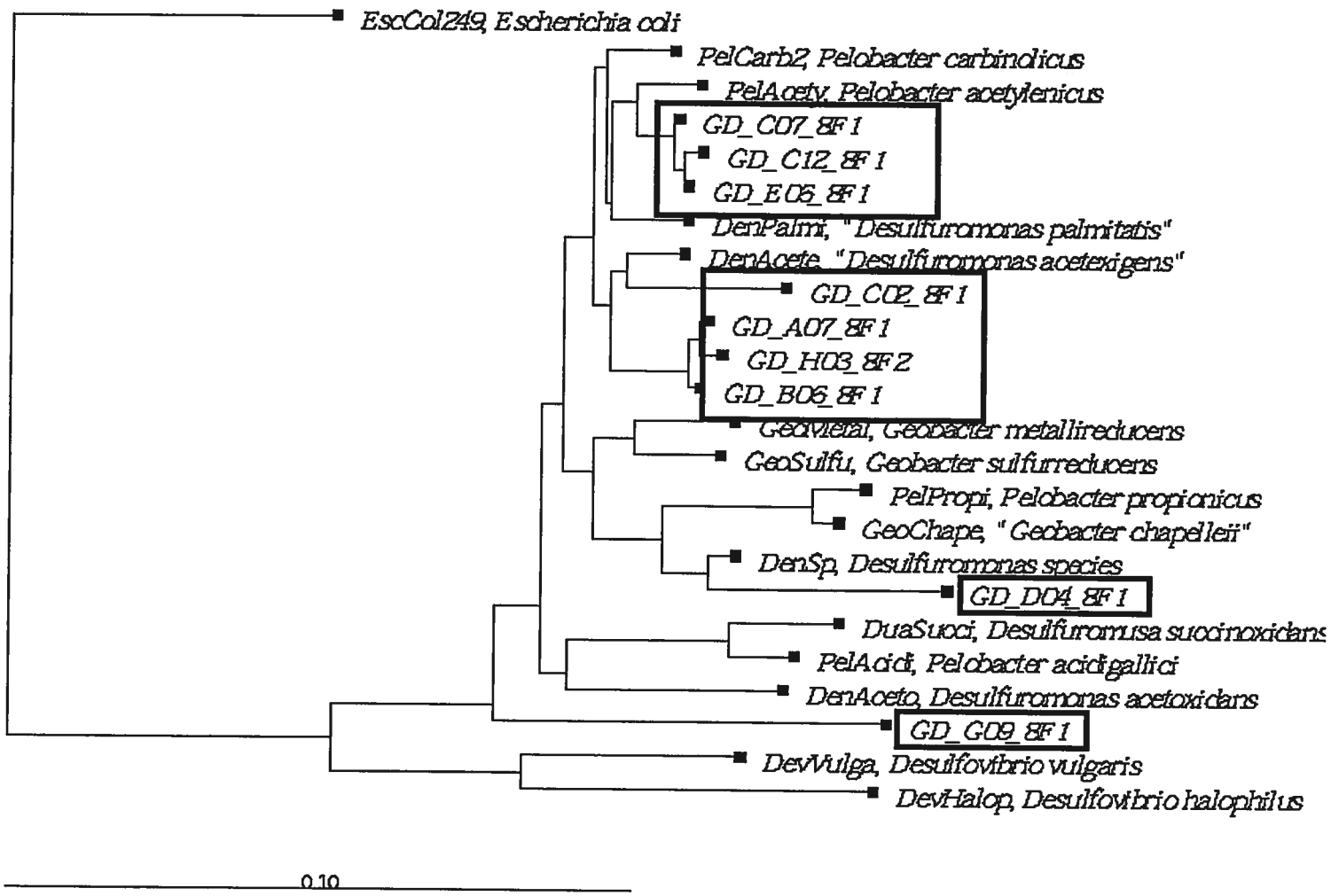


Figure 5. Neighbor joining tree of Eel pond sequences (boxed) and selected *Geobacteraceae* members.

Figure 6
Clone Library Results

MnO_2:

- 4/9 Azoarcus
- 3/9 Oceanospirilla
- 1/9 Geobacter
- 1/9 Mycoplasma

MnO_2 + AQDS:

- 3/7 Azoarcus
- 2/7 Geobacter
- 1/7 Clostridia
- 1/7 Oceanospirilla

$Fe(OH)_3$:

- 2/3 Oceanospirilla
- 1/3 Geobacter

$Fe(OH)_3$ + AQDS:

- 4/5 Geobacter
- 1/5 Pseudomonas

$Fe(OH)_3/MnO_2$:

- 3/4 Azoarcus
- 1/4 Shewanella

$Fe(OH)_3/MnO_2$ + AQDS:

- 4/5 Azoarcus
- 1/5 Geobacter

Figure 7

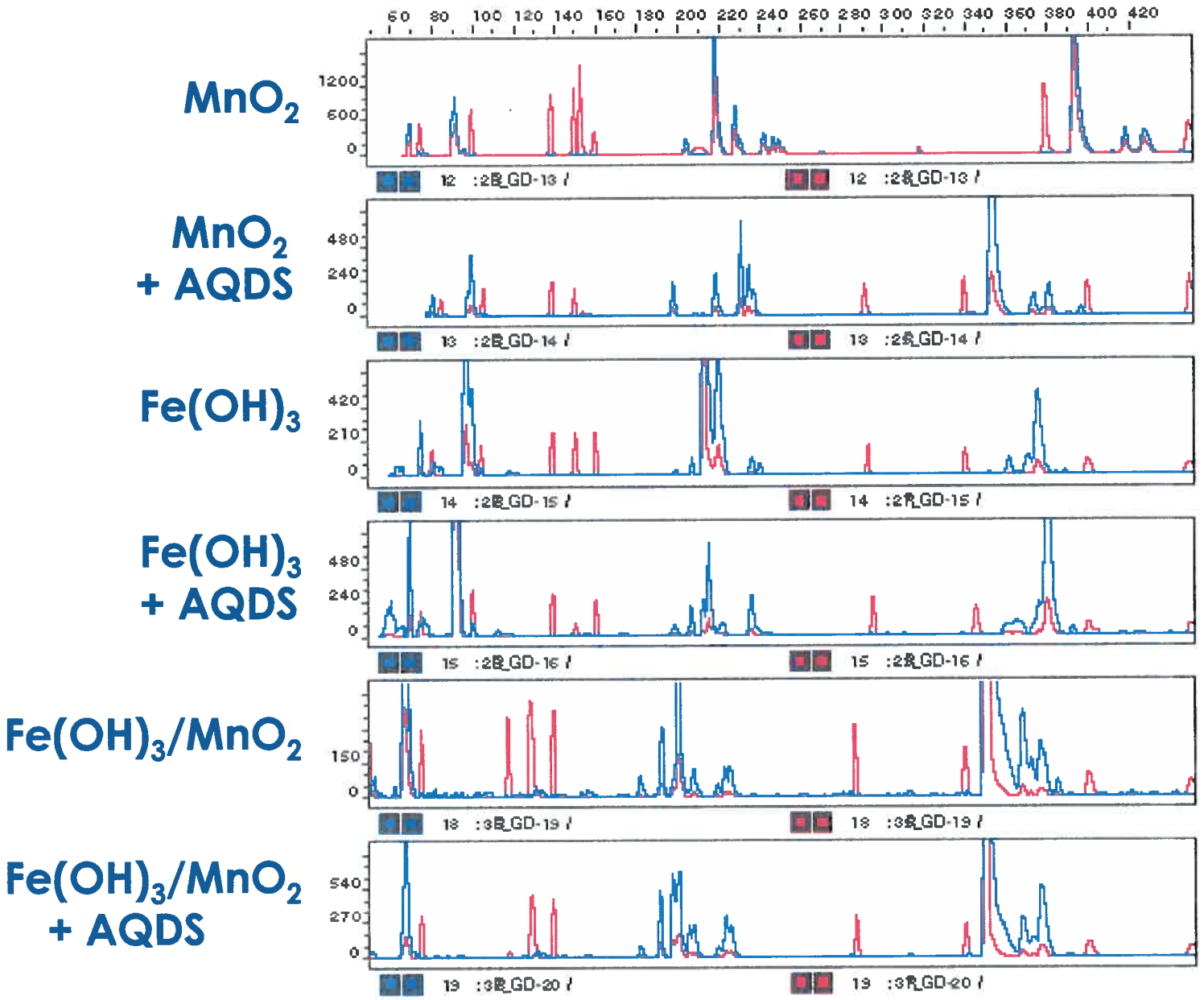
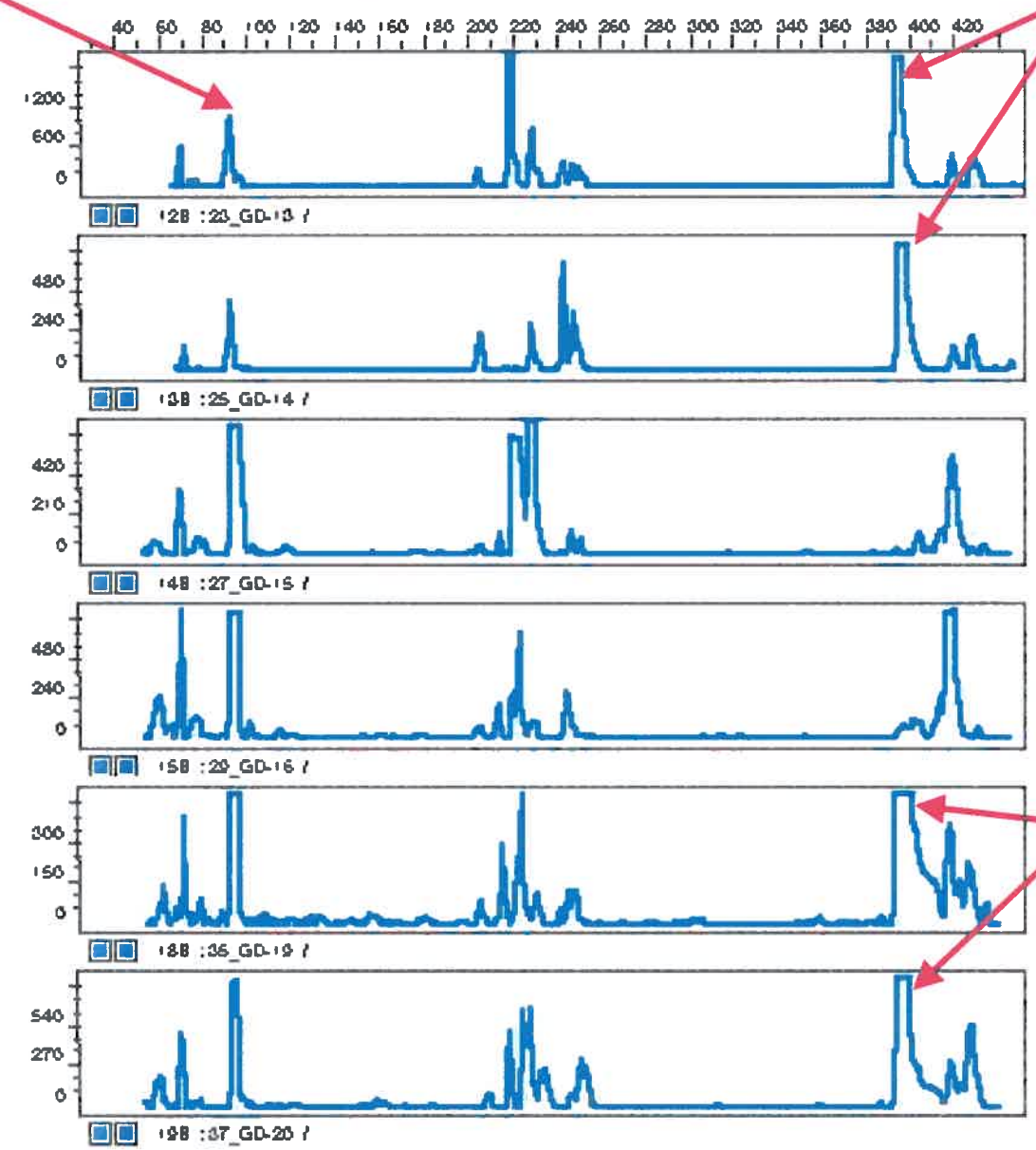

MnO2
MnO2 + AQDS
Fe(OH)3
Fe(OH)3 + AQDS
Fe(OH)3/MnO2
Fe(OH)3/MnO2 + AQDS
50 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420
1200
600
0
480
240
420
210
150
540
270
12 :2R_GD-13 /
13 :2R_GD-14 /
14 :2R_GD-15 /
15 :2R_GD-16 /
18 :3R_GD-19 /
19 :3R_GD-20 /

Figure 8

Geobacter

Azoarcus



MnO_2

MnO_2 + AQDS

$Fe(OH)_3$

$Fe(OH)_3$ + AQDS

$Fe(OH)_3/MnO_2$

$Fe(OH)_3/MnO_2$ + AQDS

Azoarcus