Anoxic Benzoate Oxidation Coupled to Fe(III) Reduction

And

Side Project:

Identification of mesophilic *Thermatoga*-like Organism from a methanogenic PCE dechlorinating Lactate enricment culture

An Individual Project for the MBL Microbial Diversity Course
Summer 1999

Kelvin B. Gregory

Department of Civil and Environmental Engineering

The University of Iowa

125 Engineering Research Facility

Iowa City, IA 52242

Tel: 319.335.5054

Email: kelvin-gregory@uiowa.edu

Introduction

Aromatic hydrocarbons are widespread in nature and often contribute to contamination of soil and groundwater. Benzene, Toluene, ethylbenzene, and the o,m,p-xylenes (BTEX) are the major constituent of petroleum fuels. Through improper storage or handling, they have been introduced into the environment. Because BTEX are suspected carcinogens (Zedek, 1980), the Environmental Protection Agency (EPA) has established Maximum Contaminant Levels (MCLs) for their concentrations in groundwater. For example, the MCL for benzene is 5 ppb.

Aerobic degradation of BTEX has been extensively studied and documented (Gibson and Subramanian, 1984). In oxic environments O_2 serves as the terminal electron acceptor (TEA) for electrons released during metabolic reactions and as a direct oxidant. However, in many environments O_2 may be limiting, and in these cases anaerobic processes will dominate. Only recently has the microbially mediated anaerobic oxidation of BTEX been elucidated (Harwood and Gibson, 1997). In the absence of oxygen, nitrate (Altenschmidt and Fuchs, 1991), sulfate (Rabus et al. 1993), and metal ions such as iron (Lovely and Lonergan, 1990) will function as terminal electron acceptors.

Previous studies of the anaerobic oxidation of benzene rings using Fe(III) as TEA have relied on the poorly crystaline Fe(III) oxide form (Lovely and Lonergan, 1990) or chelated forms of Fe(III) (Lovely et al., 1996). Rapid oxidation rates seen with chelated Fe(III) forms may be the result of greater bioavailablity of TEA for microbial reduction, and thus a more efficient electron acceptor. The stoichiometry for benzene oxidation coupled to iron reduction is:

$$C_6H_6 + Fe^{3+} + 12\ H_2O \rightarrow 6\ CO_2 + 30\ Fe^{2+} + 30\ H^+$$

In this study, we report the anaerobic oxidation of benzoate in pseudo-fluidized bed reactors seeded with anaerobic marine sediment from Eel Pond in Woods Hole, MA. These results indicate the plausible existence of organisms which may use Fe(III) supplied by iron phosphate as TEA for these oxidations. The selection of iron phosphate to serve as the source of TEA was based on the dual substrate requirement of phosphate and iron for the iron reducing microorganisms. In theory, the organisms will need to use the phosphate in order to access the iron. The light green color of iron phosphate will change as the oxidation state of the iron changes.

Materials and Methods

Column Assembly

This study was performed in two, up-flow, pseudo-fluidized bed columns (Figure 1). The reactors are referred to as "pseudo"-fluidized bed because effluent is 100% recycled as influent. Additionally, the bed was not fluidized in the conventional sense, but suspended by the rotation of a stir bar. The speed of the stir bar determined the suspended height of the bed medium (iron phosphate particulates). So a balance between suspended particles and clear supernatant was established. After several catastrophic pump failures, the pumps were turned off. The columns became continuously stirred batch reactors with a fluidized bed of iron phosphate. Flow was supplied to the columns with a peristaltic

pump at continuous flow rate of 1mL/min. The columns were constructed of glass and had an empty bed volume of 2.25 L. The columns were capped with a rubber stopper that had been wrapped in Teflon tape to minimize adsorption. All connections except those within the peristaltic pump were Teflon. Pump tubing was PharMed NSF-51.

Fluidized Bed and Medium Preparation

Approximately 700 mL of fluidized bed medium as iron phosphate was prepared by dissolving 260 g $FeSO_4$ and 200 g KH_2PO_4 in 9L of deionized H_2O. The reaction was continuously stirred in a10L reactor. Oxygen was supplied to the reactor with 2 aquarium pumps. The initial reaction pH was slowly adjusted from 3.0 to a final pH of 6.0 with concentrated KOH. A lime green precipitate formed that settled in about 10 minutes to approximately 700 mL of wet material. The supernatant was discarded and the precipitate was washed 5 times with 70% seawater. The iron phosphate was divided into 2 equal volume portions and poured into the column reactors. The remainder of column volume was filled with anoxic 70% seawater. The seawater was prepared by dilution with tap water. Oxygen was removed by bubbling the media with N_2 gas for 20 min. The columns were then seeded with 50 mL of black sediment obtained near the MRC boat dock.

Sampling

Samples were removed from the columns via disposable needle that had been pushed through the rubber stopper at the top of the column and fitted with a 3-way valve. Samples were taken in a disposable 3.5 mL syringe and filtered with 0.2 μm syringe-tip filter into autosampler vials with Teflon-lined screw-top caps. Samples were stored at –20°C. Standards and samples were analyzed for parent compound using Waters Model 2690 HPLC. Twenty microliters of samples and standards were separated on a Waters NovaPak C18 reverse-phase column. Sample were eluted in isocratic conditions sing a 50:50 v/v of 50 mM phosphate buffer (pH 4.0) and methanol. The flow rate was 0.5 mL/min. The UV detector was set to a wavelength of 210 nm.

Microscopy

Microscopy was performed on a Zeiss Axioplan2. Slides were captured using a Zeiss MC80 DX camera and auto exposure equipment. Photos were exposed on Kodak Elite Chrome Tungsten T160 film for color slides. Still video images were captured using a Hamamatsu color chilled 3CCD camera and controller. Images were further manipulated using Adobe Photoshop 4.0 and printed on an Epson Stylus Color 640 printer.

Results and Discussion

The benzoate concentrations measured over time are shown in Figure 2. It appears that concentrations decrease over time, and that respikes (on day 6 and13) of 100μM benzoate are also degraded. However, since there are no abiotic control reactors, these decreases in substrate concentration may not necessarily be attributed to biological activity. It is possible that adsorption to $FePO_4$ solids may contribute to the observed losses. Additionally, the concentration observed at day 9 was higher than day 8, though there was no spike of benzoate. This could be an anomalous data point or the result of

adsorption at 210 nm of other organic compounds. Furthermore, the color did not change in the reactor over the 15 day study.

A 1 mL sample was removed from the reactor and diluted 1:10 for microscopy analysis. A photomicrograph was captured from video and is shown in Figure 3. Although not readily apparent from the figure, there was no visible microbial activity. Amorphous green crystals of $FePO_4$ were, however, visible. It is possible, and likely, that microorganisms may have been associated with the surfaces of the solid particles. Although speculative for these enrichments, many organisms which use Fe(III) associate with the solid phase to increase uptake efficiency.

Conclusions

The purpose of this study was to determine if anaerobic marine sediments contained organisms which were capable of oxidizing benzoate and reducing Fe(III) as $FePO_4$. In these experiments, a fluidized medium of $FePO_4$ was established in a batch reactor. The reactor was seeded with anaerobic sediment from the MRC dock. The reactor was repeatedly spiked with 100 µM benzoate. Though benzoate concentrations in the reactor decreased over time, because of the lack of controls, it is not clear weather this was due to microbial activity or external factor(s). Under phase contrast microscopy there was no visible growth in the reactor. It was however possible that organisms were association with the surfaces of $FePO_4$ and were not visible.

SIDE PROJECT:

Identification of mesophilic *Thermagoga*-like Organism from a methanogenic PCE dechlorinating Lactate enricment culture

Because of several pump failures, and since the energy available for growth on benzoate/Fe(III) redox couple is so small as to seriously limit growth yields possible in 2 weeks, it was decided to begin a side project from which genetic techniques could be learned. I decided to study the ecology of a PCE dechlorinating methanogenic enrichment.

A methanogenic enrichment culture (Figure 4), maintained at the University of Iowa has been fed 2mM lactate and 75 µM tetrachloroethene (PCE) for 3 years. This culture as been shown to dechlorinate 250 µM PCE to ethene in about 4 days. It has been established through gene probes that *Dehalococcoides ethenogenes*, a PCE dechlorinatin organisms isolated at Cornell University in the labs of Steve Zinder and Jim Gossett, was present in these enrichments.

The general procedures were; 1) to isolate genomic DNA using the MoBio Soil DNA extraction kit 2) amplify 16S sequences using archaeal and eubacterial primers 3) clone the amplicons in to *E coli* using TOPO TA cloning kit 4) use restriction fragment length

polymorphism (RFLP) to identify unique clones and 5) sequence 16S DNA using ABI 310 sequencer. These sequences were placed on phylogenetic tree using ARB.

Although many different clones were identified, for practical and time considerations, it was decided to sequence only two of the clones. Figure 5 shows the phylogenetic tree developed from these sequences. One of the clones (kelvin24) was identified by ARB and BLAST to be similar to *D. ethenogenes*. The other sequence (kelvin20) was found to be most similar to *Thermatoga*. *Thermatoga* are hyperthermophiles only found in anaerobic geothermal vents. They are among the deepest branches of the eubacterial lineage. They are rod shaped, fermentative organisms with a sheath-like "toga" and are nonsporeforming. The enrichment used for this study was originally seeded from an anaerobic digester in Iowa City, IA. Since seeding the reactor has been maintained at 20°C.

If this is an accurate alignment, this is likely to be the first identification of a mesophilic *Thermatoga*. This would truly be a unique and fascinating finding…

It is hoped that in the future, in-situ hybridization and further attempts to identify this organism will be used to confirm these data.

Literature Cited

Altenschmidt, U. and Fuchs, G., 1991. Anaerobic Degradation of Toluene in Denitrifying *Pseudomonas sp.*: Indication for Toluene Methyl-hydroxylation and benzyl-CoA as Central Aromatic Intermediate. Archives of Microbiology. 156: 152-158.

Harwood, C. S. and Gibson, J., 1997. Shedding Light on Anaerobic Benzene Ring Degradation: a Process Unique to Prokaryotes? Journal of Bacteriology. 179: 301-309.

Gibson, D. T. and Subramanian, V., 1984. Microbial Degradation of Aromatic Hydrocarbons, p. 181-252. *In* D. T. Gibson (ed.) Microbial Degradation ofOrganic Compounds. Marcel Dekker, Inc., New York.

Lovely, D. R., and Lonergan, D. J., 1990. Anaerobic Oxidation of Toluene, Phenol and p-Cresol by the Dissimilatory Iron-Reducing Organism, GS-15. Applied and Environmental Microbiology. 56: 1858-1864.

Lovely, D. R. Woodward, J. C. and Chapelle, F. H., 1996. Rapid Anaerobic Oxidation with a Variety of Chelated Iron(III) Forms. Applied and Environmental Microbiology. 62: 288-291.

Rabus, R., Nordhaus, R., Ludwig, W, and Widdel, F. 1993. Complete Oxidation of Toluene Under Strictly Anaerobic Conditions by a new Sulfate-reducing Bacterium. Applied and Environmental Microbiology. 59: 1444-1451.

Zedek, M. S., 1984. Polycyclic aromatic hydrocarbons- a review. Journal of Environmental Pathology and Toxicology. 3: 537-567.

Figure 1

FIGURE 2.

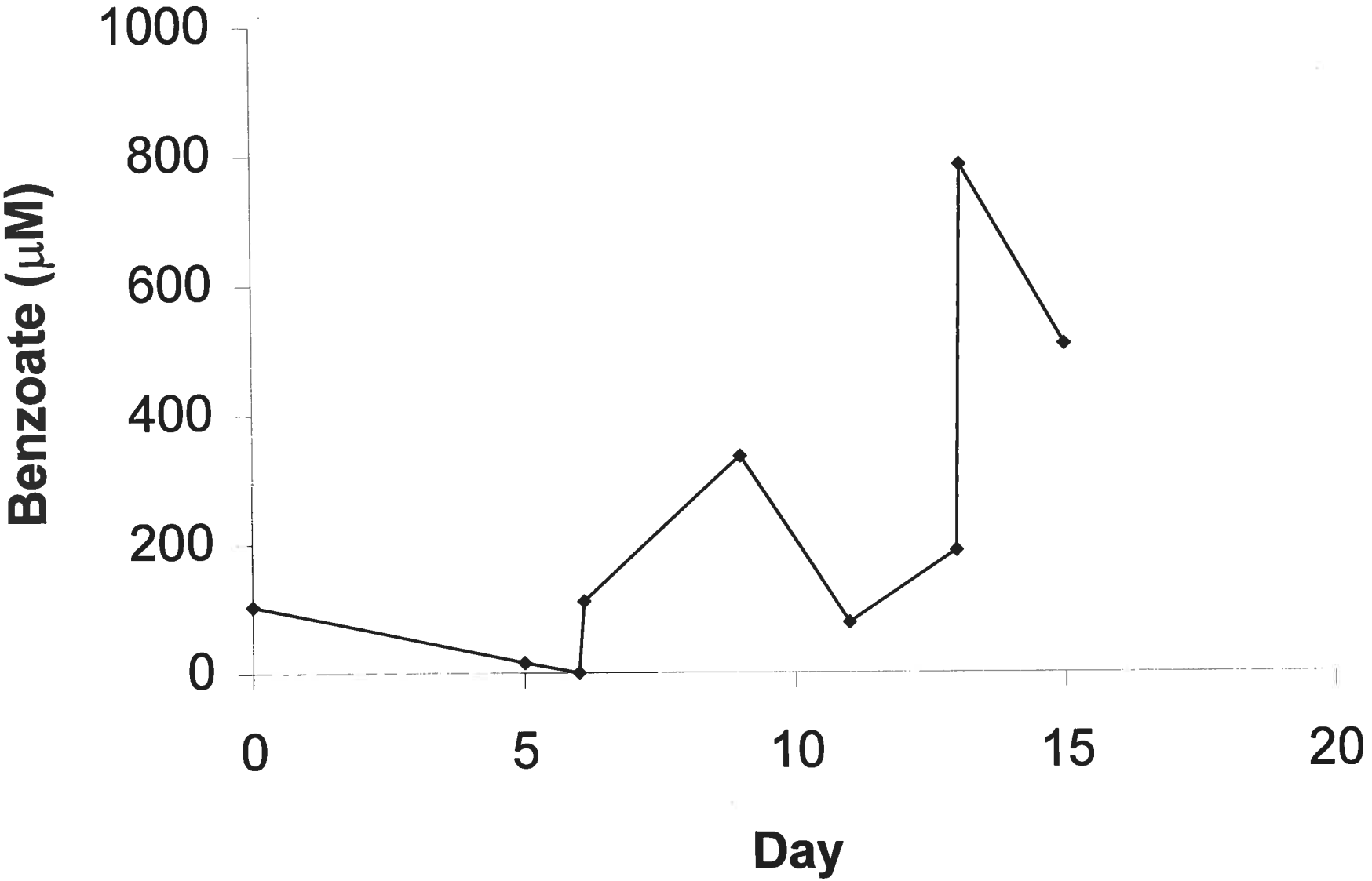


FIGURE 3.

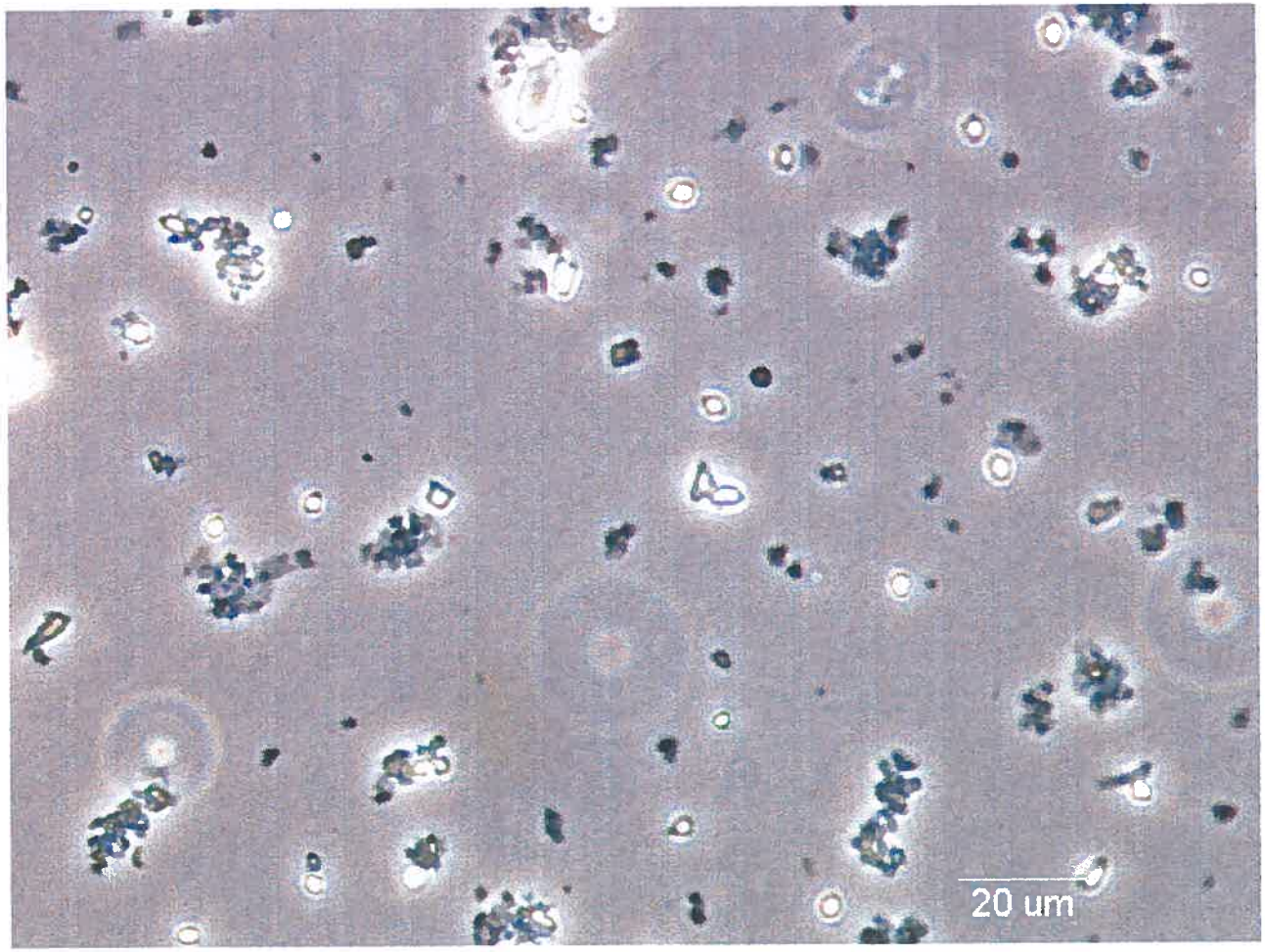
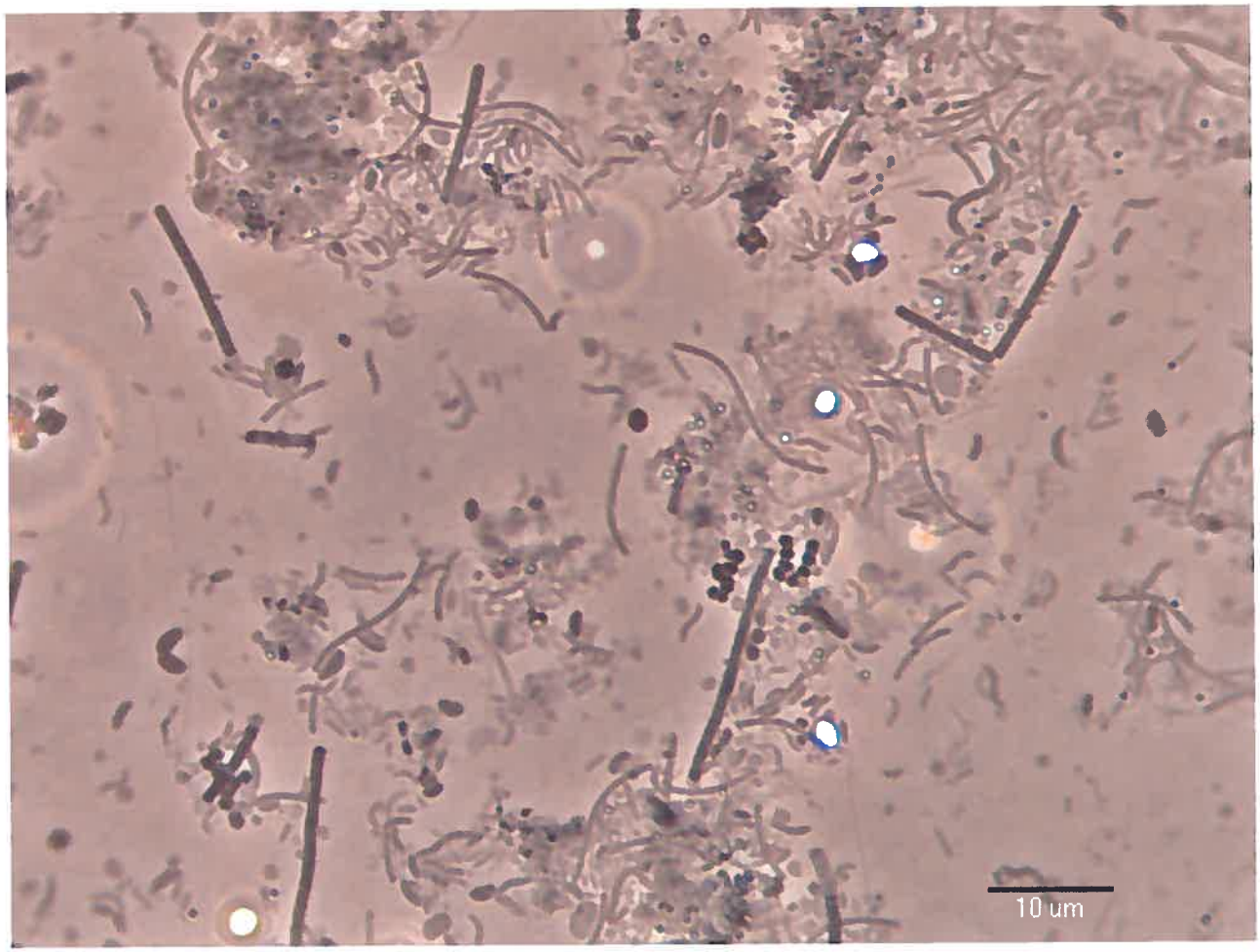
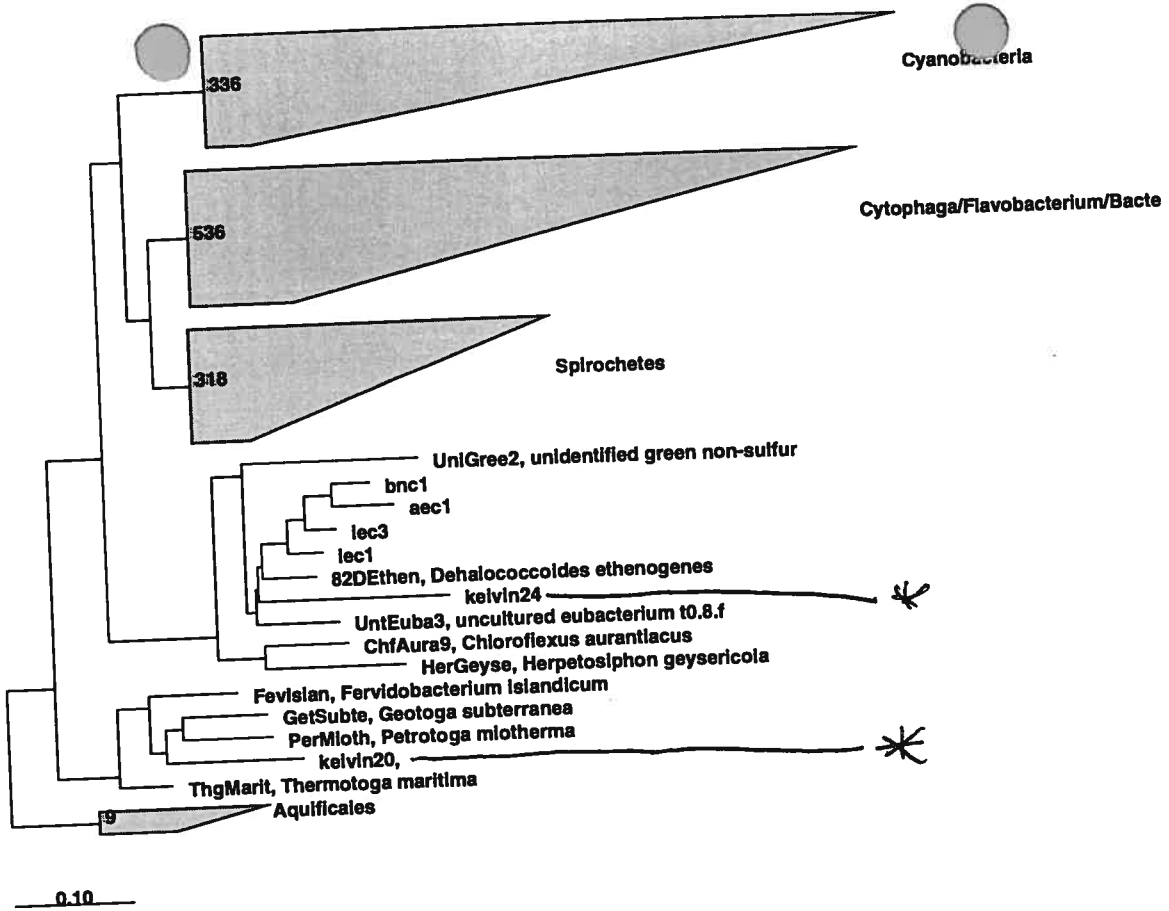


FIGURE 4.




336
Cyanobacteria
536
Cytophaga/Flavobacterium/Bacte
318
Spirochetes
UniGree2, unidentified green non-sulfur
bnc1
aec1
lec3
lec1
82DEthen, Dehalococcoides ethenogenes
kelvin24
UntEuba3, uncultured eubacterium t0.8.f
ChfAura9, Chloroflexus aurantiacus
HerGeyse, Herpetosiphon geysericola
Fevisian, Fervidobacterium islandicum
GetSubte, Geotoga subterranea
PerMioth, Petrotoga miotherma
kelvin20,
ThgMarit, Thermotoga maritima
9
Aquificales
0.10