Methanogenic Bacteria and Silicate Weathering at the Bemidji, Minnesota Oil Contaminated Aquifer: A Proposal for Future Work

Name withheld at request of student
Department of Geology

Department of Ecology and Evolutionary Biology and Museum of Natural History
University of Kansas
Lawrence, KS 66045

Introduction.
Microorganisms represent the most ubiquitous and diverse group of life on the planet, comprising all prokaryotic and many eukaryotic species (Beveridge 1989; Palmer and Reeve 1993; Barker et al. 1998). The considerable variation that exists among these organisms is almost exclusively physiological and it is
this variation that allows these organisms to play significant roles in the ecosystem processes of nearly every surficial and subsurficial habitat on earth (Ehrlich 1990). Of these myriad processes, mineral weathering is distinguished as one of the most important, as it is responsible for, among other things, soil formation, changing water composition, and regulation of global climate (Barker et al. 1998; Edwards and Rutenberg 2001). The study of microbially-mediated mineral weathering remains a relatively young science and many of the answers to how and why microbes affect mineral weathering are as yet unknown (Liebermann et al. 2000; Bennett et al. 2001; Dong 2003). One prominent hypothesis states that microbes may be increasing weathering rates as a means to access mineral-bound nutrients, vital to metabolic processes (Bennett et al. 2001). In these cases, changes in mineral solubility may be effected by microbial production of organic acids or ligands, as well as the direct uptake of solubilized mineral-bound nutrients (Holland et al. 1987; Liebermann et al. 2000; Bennett et al. 2001). These biogeochemical interactions may be most evident in systems where other requirements of metabolism (e.g. carbon source) are no longer limiting microbial community growth or activity (Bennett et al. 2001). The introduction of BTEX (benzene, toluene, ethylbenzene, and xylene) into a system, as part of an oil contamination, may result in an influx of metabolically available carbon, as these petroleum constituents are able to be degraded by many microbial organisms (Holland et al. 1987; Ehrlich 1990). As carbon sources become available the size and metabolic activity of a microbial community may increase, thus increasing the demand for mineral-bound nutrients, which will lead to increased mineral weathering rates (Barker et al. 1998).

In 1979 a buried pipeline in Bemidji, Minnesota broke, dumping about 11,000 barrels of hydrocarbon into the surrounding aquifer (USGS 2005). Since that time many geochemical studies have focused on the area, with some addressing issues of accelerated mineral weathering. These studies have found that silicate mineral weathering, particularly in anaerobic environments has accelerated far beyond normal rates (Bennett et al. 2001). While many microorganisms may be contributing to the weathering process, methanogenic bacteria may be taking a leading role. These archaea reduce acetate, CO₂, methanol, CO, formate, and methylamines to methane, however it is their unique requirement of nickel that may be driving the silicate mineral dissolution in the Bemidji aquifer (Holland et al. 1987; Mulrooney and Hausinger 2003). Methanogens require nickel in greater amounts compared to most microorganisms because their most prominent metabolic protein is nickel dependent (Wackett et al. 1989; Mulrooney and Hausinger 2003). Methyl coenzyme M reductase (MR) comprises as much as 10% of a methanogens total cellular protein and this is because the MR enzyme catalyzes the final step in methane metabolism (Palmer and Reeve 1993). The nickel possessing silicate minerals at Bemidji may be experiencing accelerated weathering as a result of methanogenic activity associated with procuring important metabolic nutrients. However, we have no knowledge about if methanogens are able to drive silicate mineral dissolution or how they may be doing this. The focus of the following is to propose a multi-faceted study that will address the question of how does the nickel concentration of silicate minerals affect the weathering of those minerals by a native, methanogenic microbial consortia?
Preliminary Study. Throughout the fall 2005, we conducted a preliminary study addressing the issue raised in the previous section. Results from this study (unpublished data) indicate some support for the hypothesis that microbial activity, specifically methanogenesis, affects silicate weathering in laboratory microcosms. However, these results were confounded by certain aspects of the experimental design, which restricted explicit tests of hypotheses and/or introduced a considerable amount of error into the study. The following is a discussion of each of these aspects:

Microbial Community. The hypothesis of the preliminary study specifically emphasized the role of methanogenic bacteria, as part of the microbial consortium, in silicate weathering. However, we did not conduct any assessment of microbial species identity for the native consortium and it became clear that the community interactions among various types of bacteria (e.g. iron reducers) limited our ability to monitor the activity of methanogens.

Sample Size. The preliminary experiment had eight levels of nickel treatment with 3 replicates at each level. The degree of replication is somewhat low and may have contributed to our inability to find significant differences among treatment levels in some cases.

Microbial Activity. This study did not include any direct measure of microbial activity, particularly as it pertained to nickel uptake. While many other lines of evidence (i.e. iron, CO₂, CH₄, biomass, and pH) all provided indirect measures of this activity it was nonetheless difficult to directly associate changes in microcosm geochemistry to the activity of the microbial community. Additionally, we could only speculate as to what biological processes might be effecting these changes.

Data Collection. Experimenter error was probably a significant force in confounding the results of our preliminary study. Many of the data collectors were untrained in the laboratory and field techniques used in this study. In addition, there was little consistency in data collection among sample periods, as researchers alternated data collection responsibilities. Finally, for some variables (e.g. silica and iron) there were too few data points to be truly informative.

This proposal details the methods and rationale for further study seeking to identify the possible role of microorganisms in the weathering of silicate minerals in the Bemidji, Minnesota contaminated aquifer. This study will be an expanded version of our preliminary study, utilizing methods that will address the criticisms enumerated above and allow for explicit tests of the hypotheses.

Hypotheses. The nickel requirements of methanogenic metabolism and the subsequent uptake of nickel by methanogenic bacteria will result in the increased dissolution of silica in anaerobic environments, while the absence of methanogens in aerobic environments will result in no increase in silica dissolution.
Nickel toxicity at high concentrations inhibits microbial activity, both in aerobic and anaerobic environments, and results in decreased silicate weathering.

**Predictions/ Expected Results.** Methanogenic consortia will be most actively weathering silica at nickel concentrations between 1 – 2% (mole percent) and this will be evidenced by relative changes in the following variables: increased methane concentration in headspace, decreased CO₂ concentration in headspace, increased transcription of MR gene, increased biomass, and increased concentration of silica in solution. Methanogenic consortia at high nickel concentrations will initially experience increased activity, but then will be inhibited by toxic levels of nickel in solution. This will be associated with significant reductions in biomass, methane production, and gene expression in bottles.

The aerobic bottles should exhibit no significant elevation in silicate weathering over that in the absence of microorganisms. In these bottles we should see much less silica in solution as compared to the methanogenic bottles. At high nickel concentrations, aerobic bacteria should also experience severe inhibition of activity and will exhibit reductions in biomass.

**Experimental Design and Analyses.**

**Field Sampling.** The field sample location for this study is the U.S. Geological Survey Bemidji, Minnesota research site. This site is characterized by an oil-contaminated aquifer and has been studied extensively throughout the previous decade. The aquifer has two distinct microbial environments, the anaerobic environment occurs near the oil plume, with much of the rest of the aquifer being oxygenated. Both aerobic and anaerobic environments will be sampled for this study. The aerobic environment will be sampled at USGS well 707A and the anaerobic will be sampled at USGS well 9014. In addition to water samples, field assessments of dissolved oxygen, pH, alkalinity, and silica and iron concentrations will be taken.

**Microorganisms.** This study will involve aerobic and anaerobic microbial consortia, native to the Bemidji aquifer. Anaerobic consortia will be cultured in order to isolate methanogenic species, which will then be genetically characterized and used to inoculate microcosms. These data will allow us to monitor the microbial community composition and activity throughout the study. Because it is effectively a control and as I have not seen (unpublished data) nor do I anticipate that the predicted effects of this consortia on mineral weathering will be altered by its composition, the aerobic consortia will not be subject to culturing or genetic assessments of biodiversity.

**Pure Culture.** In the previous study there was considerable evidence for the activity of non-methanogenic bacterial communities (i.e. iron reducers) in the anaerobic microcosms. These organisms competed with methanogens and their activities were prominently reflected in the result data, which may be one reason we observed little evidence of methanogenesis. To reduce the number of variables in the study and focus on the actions of methanogens, it is necessary to selectively procure,
through pure culturing, methanogenic organisms for the anaerobic portion of this study. This will be accomplished by methods described in Widdel (1986). Formation water samples taken from the anaerobic environment at Bemidji will be used to produce pure cultures of methanogenic bacteria. The methods to be used in culturing follow Widdel (1986) and are as follows. Pure cultures will be grown in anaerobic environments on bicarbonate-buffered, sulfide-reduced media. Sodium acetate will be added as a carbon source. Once pure cultures are established 50% of culture will be suspended in 10mM Tris-EDTA buffer for DNA extraction.

**Inoculation.** Inoculation of aerobic microcosms will be identical to the procedures used in the previous study (unpublished data) and will include approximately 1ml of microbial consortia derived from approximately 30 g of sediment. No more than 6 species of methanogenic bacteria will be used to inoculate the anaerobic microcosms. The cultures of these species will be diluted in de-ionized water prior to inoculation. The initial cell concentration for both aerobic and methanogenic bacteria in the microcosms is about $10^5$ cells/ml.

**Gene Regions.** Each strain or species of methanogen used in the inoculation of microcosms will be sequenced for the 16S ribosomal RNA and Methyl coenzyme M reductase genes. The 16S gene has been sequenced for a number of methanogenic bacteria and I will use it to identify and/or characterize species and strains included in the study (Palmer and Reeve 1993). *Methyl coenzyme M reductase* (MR) is a nickel dependent enzyme, which catalyzes the final step in methane formation (Hughes and Poole 1989; Palmer and Reeve 1993). The transcription of this gene is an indication of the metabolic activity of methanogens as well as of the nickel uptake of these organisms (Mulrooney and Hausinger 2003). *Methyl coenzyme M reductase* has been successfully sequenced for multiple methanogenic organisms and shows moderate to high levels of sequence variation among taxa (Palmer and Reeve 1993). *Methyl coenzyme M reductase* sequences will be analyzed for single nucleotide polymorphisms (SNPs), occurring at the species level. Subsequently, internal, quantitative PCR primers of the MR gene will be designed with SNP sites included in the primer anchor region. Species level variation in the primer anchor region will produce species-specific primers and allow us to monitor the gene expression patterns of individual species throughout the study. We will obtain designed primers from TATAA Biocenter.

**DNA Extraction and Sequencing.** Cell lysis methods follow those described in Francois et al. (2004) and involve sonicating the bottle reactor for five minutes prior to sampling 100 µl of solution. To this solution is added one hundred milligrams of glass beads (Schieritz and Hauenstein, Arlesheim, Switzerland). The solution is then vortexed at maximum speed for 45 seconds and centrifuged to remove cell debris and beads. A 5-µl aliquot of this solution is then used for PCR. Once amplified on a BioRad TempCycler for 16S rRNA and MR, the product will be purified using a GeneClean kit (Bio 101, La Jolla, California). The purified amplification product will be cloned using a CLONEAMP system (Life
Technologies, Gaithersburg Md.; Sakano and Kerkhof 1998). Plasmid DNA from transformed bacteria is purified and amplified in a sequencing reaction using internal sequencing primers. DNA sequences will be determined using an ABI automated sequencer located at the Museum of Natural History. The sequences will be analyzed using the Sequencher and aligned in Se-al. To determine species identity BLAST searches will be performed. The identification of SNP sites will be performed during the alignment and SNPs that varying at the species level will be chosen to help design species-specific qPCR primers.

**Bottle Microcosm Design.**

**Replication.** A power analysis (Box 1) was conducted, using the data from the preliminary study (unpublished data) for iron, silica, alkalinity, CO$_2$, and CH$_4$. The results of these analyses suggest that adequate statistical power for this study requires that we include no fewer than ten replicates of each treatment level. While this increases both the monetary and labor investment of the study, it will provide for a much more accurate assessment of treatment effects. Each level of treatment will have ten replicates and one sterile control for a total of eleven bottles. This study includes six levels of treatment for both the aerobic and anaerobic microcosms, resulting in a total bottle count of one hundred and thirty-two.

**Initial Solution.** The composition of the initial solution for microcosms is slightly changed from the previous study. Each bottle will have 55 ml of formation water (instead of 50 ml), diluted with an equal volume of de-ionized water. Before the biological inoculation of the microcosms, the water will be autoclaved to ensure known microbial community composition. In addition, the solution will be inoculated with approximately 30 ml of water and tween and 10mg/L of toluene.

**Initial Solids.** The initial composition of solids is slightly changed from the previous study. I have removed the copper/nickel treatment levels from this experimental design. While these levels have

---

**Box 1. Power analysis**

The goal of statistical hypothesis testing is often to determine whether or not there is a causal or correlative relationship among measured variables. Typically, such relationships are inferred following the rejection of the null hypothesis, which generally posits that there exists no relationship among the measured variables. The power of a statistical test refers to the probability of correctly rejecting a false null hypothesis and increasing this power may be achieved by increasing the sample size or number of replicates used in a study or experiment. A power analysis generates an estimation of optimal sample sizes, which maximize the power of a test, given preliminary or predicted mean values, measures of error (i.e. standard deviation), and significance level. The power curve (shown in fig. 1) is a common result output for a power analysis and sample sizes associated with powers of 0.8 or greater are usually considered acceptable.

![Power curve produced with preliminary data from iron analysis, using statistical program JMP (SAS; Heidelberg, Germany). Optimal sample sizes occur between the red lines.](image)


---

Jennifer Roberts 12/21/05 12:12 PM

Comment [3]: huh? Why—you lost me.
shown interesting, yet non-intuitive, effects (unpublished data), sample sizes are already quite large and the effects of copper in this system are poorly delimited in the hypotheses and predictions of this study. Given these considerations and that the results of these treatments are not necessary for adequate testing of hypotheses of this experiment, I will not include copper/nickel treatment levels in this study. Those treatment levels that will be included will have the same solid composition as the previous study. The six treatment levels correspond to the following concentrations (mole percent) of nickel in borosilicate glass: 0.0, 0.01, 0.05, 0.1, 1.0, and 2.0. Each bottle microcosm will receive about 0.25 g of nickel-doped glass.

**Initial Headspace.** Initial headspace composition will be similar to the previous study. The headspace for both the aerobic and anaerobic microcosms will consist of gaseous CH₄ and CO₂, derived from toluene inoculation. The average starting concentrations of these gases in the headspace of anaerobic microcosms in the last study was about 25 mmol/L CO₂ and about 580 mmol/L CH₄ (unpublished data). In addition, the aerobic bottles will receive a 500 µl injection of room air.

**Gene Expression.** Gene expression of methanogens will be analyzed using a multiplexed quantitative reverse-transcriptase real-time PCR method (Box 2). This analysis will be conducted once every two weeks for the three-month duration of the experiment. Additionally, only three bottles will be analyzed per treatment level and these will remain the only bottles analyzed throughout the experiment. Each bottle is analyzed in a separate reaction, which includes all the primers and associated fluorescent dyes for each species in the consortium. Quantitative PCR machines are located in the Department of Molecular Biosciences on the University of Kansas campus. A primer and fluorescent dye set can be obtained from TATAA Biocenter (Goteborg, Sweden) for $237.00.

**Silica Analysis.** Prior to the start of this study, we will conduct a preliminary study of background silica dissolution rates. Anecdotal evidence from the previous study suggests that nickel concentration alone affects rates of silica dissolution. The preliminary study will involve three replicate bottles from each treatment level. All microcosm constituents are added to the reactors except microorganisms. This study will last three weeks and will determine the background dissolution rate of silica at each treatment level.
This rate will subtracted from measurement values of silica concentration taken during the primary study. Silica analysis will follow the molybdate method as performed in the previous study. Sampling will occur once a week for the three-month duration of the experiment and will involve all aerobic and anaerobic bottles. Absorbance is measured on a UV-Vis about 12 minutes after the mixing of regents. The sampling of any fluids from the anaerobic bottles requires the use of an anaerobic chamber. I am not changing this analytical procedure because it is cost effective and accurate within the parameters of this study.

Headspace Analysis. Headspace analysis will follow the same methods used in the preliminary study. Once a week, 250 µl of headspace will be removed using a gas chromatograph syringe. This gas is loaded onto a gas chromatography machine (located in the Roberts lab) and analyzed with detection by thermal conductivity on a Haysep Q column. Following the removal of this gas all bottles will be weighed and 500 µl of nitrogen will be injected into the headspace of anaerobic bottles to avoid creating a vacuum. I am not changing this analytical method because I feel that it is really the most direct way to measure these variables. Additionally, CO₂ and CH₄ concentration is a good correlate for microbial activity.

Iron Analysis. Iron analysis will follow the same methods used in the preliminary study. Solution concentration of Fe(II) will be determined by adding reagents to about 1.5 ml of sample solution. A spectrophotometer will be used to quantify the concentration of iron in solution. Iron analysis will be conducted once every two weeks. I am not changing this analytical procedure because it is cost effective and accurate within the parameters of this study.

Biomass Estimates. Estimates of biomass will be made using the same procedures used in the preliminary study. DAPI-staining does exhibit some problems, including differentiating between dead and live organisms. However, I believe that using this method in combination with gene expression data will provide an accurate estimate of community size and activity. Biomass estimates will be made once every two weeks and will include all bottles in the study. This will require 1 ml of solution each sample period. The solution that is filtered may be used for other analysis such as silica, pH, or iron.

pH. The pH of solution will be determined using standard methods employed during the preliminary study. Once a week 1 ml of solution will be combined with 1 ml of de-ionized water and the pH of this solution will be determined using a standard pH probe.

Statistical Analyses. Data resulting from biomass, CO₂, CH₄, pH, Fe(II), and silica will be analyzed using both linear regression and Analysis of Variance methods. Linear regression will be employed to determine significant changes in a measured variable for a single treatment level over time. A significant result indicates that the environment is changing over time. An ANOVA will allow the comparison of multiple treatment level means at a given data point. This will allow us to infer an effect of
treatment, with a significant result indicating differences among the treatments for a given measured variable.

**Sixth Month Anaerobic Predictions.**

**Biomass.** I predict that biomass will be relatively unchanged for all bottles but those at the highest nickel concentrations. The bottles at moderate to low nickel concentrations should be active, but the absence of nitrogen and phosphorous in the reactors may not allow the overall increase in biomass. Organisms must scavenge these nutrients off dead bacteria, effectively limiting population size. Bottles at high nickel concentrations, those that did not experience total depletion of biomass, will be rendered sterile in six months time (Hughes and Poole 1989).

**Population Succession.** As the Fe(III) is reduced to Fe(II) and enters solution iron reducers will begin to lose available electron acceptors and their populations will decline (Ehrlich 1990; Kalinowski *et al.* 2000). Methanogens, in the absence of iron and sulfur reducers, will begin to take over and their populations will grow (Holland *et al.* 1987; Palmer and Reeve 1993).

**Microbial Activity.** As methanogens become more prominent methane will be produced, while CO₂ and toluene is consumed (Palmer and Reeve 1993). Both iron and nickel concentrations in solution will be higher than they are now as all of the iron will have been reduced to Fe(II) and silica will continue to weather releasing nickel into solution (Barker *et al.* 1998). In high nickel treatments, there should be little to no microbial activity as most organisms will have expired.

**Major Electron Acceptors and Donors.** Iron will have been completely reduced in six months. The major electron acceptor is CO₂, which is reduced to CH₄, and the electron donors are H₂ and acetate (derived from toluene; Ehrlich 1990; Palmer and Reeve 1993). In high nickel treatments Fe(III) may still be available for reducers only because these populations died from nickel toxicity before they were able to metabolize the metal, this explanation is supported by the data shown in figure 2.

**pH.** The pH of the solution should begin to increase as CO₂ concentrations are beginning to decline.

**Glass Weathering.** As methanogenic bacteria proliferate glass-weathering rates should increase, due to uptake of nickel by methanogens (Bennett *et al.* 2001). This rate may again reach a steady state as population size of methanogen populations reach maximum. The rate of silica weathering in high nickel treatments should be greater than that in other treatments (shown in figure 1), because of the effect of glass composition.
Secondary Mineral Formation. Silica concentration in solution may begin to reach a saturation point at which time this will begin to precipitate out of solution. Additionally, in high nickel treatments nickel may begin to precipitate out of solution.

Sixth Month Aerobic Predictions.

Biomass. Biomass should be low as the environment has gone from aerobic to anaerobic, killing off much of the original consortia. However, there may be a number of facultative aerobes/anaerobes present in the community and these may have proliferated resulting in similar biomass measurements. In high nickel concentration treatments there will be no biomass as the microbes succumb to nickel toxicity.

Population Succession. Oxygen depletion will result in the decline of obligate aerobes and lead to the proliferation of facultative aerobes/anaerobes (Holland et al. 1987).

Microbial Activity. These bacteria will continue to oxidize the toluene, producing CO\(_2\) (Ehrlich 1990). Fe(II) concentration in solution will begin to increase as iron reducers multiply (Ehrlich 1990). Nickel concentration will also continue to increase as it is released during silicate weathering.

Major Electron Acceptors and Donors. Fe(III) will become the major electron acceptor as oxygen is depleted.

pH. The pH will begin to decrease as CO\(_2\) concentration in the reactor continues to increase, leading to the formation of carbonic acid.

Glass Weathering. Glass weathering rates should be similar to what they are presently as there is no uptake nickel to effect accelerated dissolution. In higher nickel treatments weathering may proceed more swiftly, as indicated in table 1. This is purely because of the composition of the silicate glass.

Secondary Mineral Formation. There may be some silica precipitating out of solution as it reaches saturation.

Twelve Month Anaerobic Predictions.

Biomass. I predict that at 12 months biomass will have decreased, but there will be some methanogens and methanotrophs comprising a syntrophic relationship. However, nickel concentrations may have reached levels too toxic for microbial life.

Comment [6]: Based on experimental design—is there Fe(III) (or Fe(II) for that matter) in these microcosms?
Population Succession. Methanotrophs will have become more prominent with the increase in methane production.

Microbial Activity. CO₂ and CH₄ will both alternatively consumed by methanotrophs and methanogens, respectively. Iron will be in solution in high amounts as it has been completely reduced, while nickel will also be in solution though may have begun to precipitate as it reached saturation levels (Kalinowski et al. 2000).

Major Electron Acceptors and Donors. Methane and carbon dioxide serve as both electron donors and acceptors as part of the syntrophic relationship.

pH. The pH will remain similar or somewhat lower than that of the six month testing as CO₂ levels have increased somewhat in the reactor.

Glass Weathering. Glass weathering will be proceeding at rates slower than the sixth month period. Much of the nickel in the system may no longer be complexed in the silicate matrix, reducing the rate of silica dissolution.

Secondary Mineral Formation. Both silica and nickel may be precipitating out of solution as concentrations continue to increase.

Twelve Month Aerobic Predictions.

Biomass. Biomass will be significantly decreased from the six month testing. This is because iron reducers have used all available iron for metabolism and there are no more electron acceptors. It is possible that we collected methanogens from an aerobic environment, but I think this is doubtful.

Population Succession. There has probably been no succession, instead microbial extinction has occurred.

Microbial Activity. There is no microbial activity. Both iron and nickel will be present in the solution, most likely in relatively high concentrations (Kalinowski et al. 2000).

Major Electron Acceptors and Donors. The major electron acceptors would be CO₂ and primary donors would be methane and H₂ (Holland et al. 1987)

pH. The pH would be lower than the six month testing as the iron reducers introduced more CO₂ into environment, creating carbonic acid.

Glass Weathering. Glass weathering will proceed at a background rate.

Secondary Mineral Formation. It is possible that iron may begin to precipitate, however there has not been a great deal of silica dissolution and this may not yet have reached the saturation point.
Figure 1. Silica concentration ($\mu$M/L) in solution for anaerobic treatment.

Table 1. Silica concentration ($\mu$M/L) in solution for aerobic treatment.
Figure 2. Fe(II) concentration (mM/L) in solution for anaerobic treatment.

References.


