Title
Effects of different bacterial growth modes and of Bangladeshi aquaculture practices on environmental contamination by mercury and arsenic

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Publication Date
2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Effects of different bacterial growth modes and of Bangladeshi aquaculture practices on environmental contamination by mercury and arsenic

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Civil Engineering

By

Tiffany Yi-Ling Lin

2014

ABSTRACT OF THE DISSERTATION

Effects of different bacteria growth modes and of Bangladeshi aquaculture practices on environmental contamination by mercury and arsenic

by

Tiffany Yi-Ling Lin
Doctor of Philosophy in Civil Engineering
University of California, Los Angeles, 2014
Professor Jennifer A. Jay, Chair

Environmental contamination is an important public health concern as As in groundwater has led to the largest environmental poisoning in history. The work in this dissertation addresses the effects of differing growth modes on mercury methylation by SRB as well as the effects of Bangladeshi aquaculture practices on As mobilization.

Methylmercury is a known neurotoxin that bioaccumulates, particularly in the aquatic system. When people eat fish that have consumed contaminated lower species, people then take in the mercury that has bioaccumulated in the fish leading to skin problems, sensory impairment, and internal damage (Barringer et al 2005). Studies have investigated mercury methylation in planktonic cultures of SRB but bacteria exist primarily as biofilms in the environment. Thus, it is important and more applicable to study methylation by biofilm cultures. Our study uses inihibition and gene expression to compare mercury methylation by SRB strains

ND132 and M8 and specifically addresses a less studied acetyl-CoA pathway. Results showed that biofilm cultures methylate up to four times more than planktonic cultures and through different and possibly more pathways. The acetyl-CoA pathyway was found to be important for both biofilm and planktonic cultures of ND132.

Arsenic in Bangladesh groundwater results in chronic exposure to the population, leading to millions of diagnosed cases of arsenicosis and other health concerns each year (Yu et al 2003). Using sequential extraction, solid-phase As host fractions were characterized for sediment collected from a new aquaculture pond in Bangladesh upon initial excavation and one year later. Sediment from the newly excavated pond was unfertilized and showed that As was found predominantly in the recalcitrant fraction with up to 35% of solid As found in phosphate- and hydrochloric acid-extracted fractions. However, extraction from one-year old sediment showed no As in the phosphate-extracted fraction and all in hydrochloric acid-extractable and recalcitrant fractions. This shift in host fractions in addition to an overall decrease in total arsenic from initial excavation to one year later suggests that treating the pond with cow manure altered host fractions and mobilized As. Dialysis experiments were performed with As, Ca^{2-}, and NOM to investigate binding capabilities of NOM, as NOM is key to As mobilization. Cow dung and sediment treated with cow dung showed the greatest ability to complex As, which further suggests that fertilization practices are important and problematic. As speciation mostly complexed with NOM when NOM was present in geochemical models utilizing constants derived from experimental work with field samples. It should be noted that these studies imply that current practices of treating aquaculture ponds with cow manure is potentially exacerbating arsenic contamination in Bangladesh groundwater.

The dissertation of Tiffany Yi-Ling Lin is approved.

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Jennifer A. Jay, Committee Chair

University of California, Los Angeles

2014

DEDICATION

I dedicate this work to my family, my friends, and Eric, all of whom have helped and supported me in so many ways. Without them, I could not have accomplished this.

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ACKNOWLEDGEMENTS

I would like to acknowledge and thank everyone in the Jay lab for being the best lab anyone could ask for. Your support, ideas, time, and even just your willingness to listen have been invaluable. More importantly, I want to thank Dr. Jenny Jay for being such an incredible advisor, mentor, and friend. Without you, I would never have thought a Ph.D. possible. Thank you for exposing me to this amazing field and for always supporting me.

This work has been supported by a number of sources including the National Science Foundation (NSF) CAREER grant BES-0348783, NSF grant CCR-012077, and NSF SGER grant BES-0605515.

Chapter 2 is a version of: "Lin, TY, R Kampalth, CC Lin, M Zhang, K Chavarria, J Lacson, and JA Jay (2013). Investigation of mercury methylation pathways in biofilm versus planktonic cultures of *Desulfovibrio* desulfuricans. ES&T 47(11)."

Chapter 3 is a version of "Lin, TY, L. Rice, J. Lee, A. Maki, T. Sevilla, M. Stahl, R. Neumann, C. Harvey, I.M. Suffet, and JA Jay. Arsenic binding to NOM in cow and chicken fecal matter: relevance to mobilization of arsenic in groundwater." The manuscript is prepared for submission.

Chapter 5 is a version of: "Mika, KB, TY Lin, M Ferreira, J Lacson, CM Lee, C-C Lin, K O'Byrne, W Sandoval, V Thulsiraj, JA Jay (2012). Incorporating service-learning in traditionally lecture-based environmental engineering courses through researching bacterial contamination at a local beach. Global Journal of Engineering Education 14(2)."

Thank you again to all of my dear family and friends for everything they bring to my life.

VITA

Education

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Publications

- Lin, T.Y. et al. (2013) Investigation of mercury methylation pathways in biofilm vs planktonic cultures of *Desulfovibrio desulfuricans*. Environmental Science & Technology.
- Mika, K.B. et al. (2012) Incorporating service-learning in traditionally lecture-based environmental engineering courses through researching bacterial contamination at a local beach. Global Journal of Engineering Education.
- Ramanathan, N. et al. (2007) Sensor-based investigation of biogeochemical controls on arsenic mobilization in rural Bangladesh. In Procs. of American Chemistry Society, Gen. Mtg.
- Lee, C.M. et al. (2006) Persistence of fecal indicator bacteria in Santa Monica Bay beach sediments. Water Research.

Chapter 1 – Introduction

The presence of metals in aquatic environments is a serious issue that continues to be ongoing environmental poisoning. Understanding the variables that contribute to such contamination is important, as both arsenic and mercury are found in groundwater and surface water worldwide and are harmful to public health.

It is important to investigate the fate and transport of mercury in the environment because mercury is particularly difficult to control and able to persist is atmospheric, soil, and aquatic systems. Exposure to different forms of mercury can result in different symptoms and illnesses, however, organic forms with methylmercury in particular are more toxic than inorganic forms (Barringer et al 2005, Bernhoft 2011). Not only can cardiovascular, respiratory, and renal systems be affected, methylmercury is a neurotoxin that can eventually lead to death (Barringer et al 2005, Bernhoft 2011).

Methylmercury in aquatic environments are especially troubling for public health as microbes transform inorganic forms of mercury into methylmercury, which can be easily take in by organisms and then bioaccumulated through the food web (Mason et al 1995/1996, Pinheiro et al 2009). Humans are consequently exposed to methylmercury through fish consumption after it has been biomagnified (Mason et al 1995, Barringer et al 2005, Pinheiro et al 2009).

Previous studies have investigated the mechanisms by which mercury is methylated, recognizing dissimilatory sulfate-reducing bacteria and iron-reducing bacteria as important methylators in aquatic systems (1,2). These and other studies have primarily explored microbial methylators in their planktonic forms despite widely recognizing that bacteria exists predominantly as biofilms in natural environments (9,10). Biofilm and planktonic forms of the same microbe have been shown to have phenotypic and metabolic differences, therefore, it is reasonable to hypothesize that mercury methylation differs between biofilm and planktonic bacteria (9,58-60).

Our study, explained in chapter 2, utilized inhibition and genetic expression tests to not only compares methylating abilities by planktonic and biofilm forms of a particular strain of sulfur-reducing bacteria, *Desulfovibrio* desulfuricans ND132, but also investigates whether multiple methylation pathways, including the less studied acetyl-coenzyme A pathway, are utilized by this strain.

Tens of millions of people in the Ganges Delta consume arsenic-contaminated groundwater everyday leading to the largest environmental poisoning in history (Yu et al 2003). In Bangladesh alone, more than two million people are diagnosed with arsenic-induced health problems each year (Yu et al 2003). While the World Health Organization (WHO) has limited the maximum allowable limit of arsenic to 10 µg/L, eight million wells in Bangladesh pump groundwater with arsenic exceeding this limit. As groundwater is their main source of drinking water due to pathogenic contaminated surface water, Bangladeshis generally experience chronic exposure that could lead to death (Yu et al 2003, Smedley and Kinniburgh 2003). Despite the tragic public health implications of this problem, the question of why concentrations are so high in the groundwater of the Ganges Delta has not been completely answered. To develop more effective treatment strategies that could improve the quality of life in critical populations, it is necessary to investigate further what contributes to contamination and what could be changed to lessen such effects.

The mechanisms and controlling factors for As mobilization in groundwater are complex and not yet fully understood. While it is currently accepted that As release is related to microbilaly-driven reduction of iron hydroxides (Swartz et al 2005, Smedley and Kinniburgh 2003, Saalfield and Bostick 2009, Kirk et al 2010). Other mechanisms including microbially-driven mineral dissolution have been shown to be important at some sites (Mailloux et al 2009?). Abiotic processes can also influence As mobilization significantly through complexation, secondary mineral formation, and As^{V} reduction to less sorptive As^{III}, and ionic competition (Burnol and Charlet, 2010, Smedley and Kinniburgh, 2003).

Natural organic matter (NOM) is an important factor in both microbial and abiotic processes that influence arsenic mobilization. Not only does NOM stimulate organisms involved in iron hydroxide reduction and mineral dissolution, NOM also interacts with mineral surfaces, bridging, and As itself (Harvey et al. 2006, Bauer and Blodau 2006, Redman et al 2002). However, the origin of the water and NOM involved with contaminated aquifer recharge has not been well studied.

It was previously believed that rice paddies in Bangladesh were the site of As mobilization and transport into aquifer recharge, however new studies suggest the aquaculture ponds, which cover less than 10% of the land in Bangladsh, are actually important to arsenic release. Labile NOM utilized by microbes have been traced to the aquaculture ponds and flow paths of recharge from the ponds have been linked to the contaminated aquifer (Neumann et al 2009). However, aquaculture ponds for fish production provide the majority of meat in Bangladeshi diet (FAO 2001, Hossain et al 2006, Yi et al 2004) and are fertilized with cow manure once excavated to stimulate the food chain for improved fish population. Cow manure not only contains desired nutrients such as nitrogen and phosphorus, it also contains NOM that can increase As mobilization. Despite the impacts that fertilization with manure could have on groundwater levels of As, studies are only beginning to investigate how they are related.

Through As and NOM dialysis experiments, chapter 3 investigated the binding capabilities of NOM extracted from manure and Bangladeshi sediment, measuring and comparing condition distribution coefficients and apparent stability constants. NOM was characterized and experimental data was utilized in a geochemical model to predict As-NOM complexes under different fertilization schemes. Chapter 4 determines host fractions of solid-phase As through sequential extraction and compares that for a new pond and the same pond one year later to explore how aquaculture practices of fertilization may have resulted in shifts in host fractions, possibly indicating increased As mobilization.

Chapter 5 presents a service-learning project implemented as a course in the Department of Civil and Environmental Engineering, where undergraduate and graduate students were instructed on how to teach K-12 students about environmental science topics. In particular, UCLA students taught K-12 students about climate change and beach water quality through a collaborator quarter-long project including hypothesis development, field sampling, sample processing, data analysis, and presentation of the work. The service-learning component was created to augment classroom learning and allow UCLA students an opportunity to mentor and affect young students in the field of science.

Chapter 6 presents overall results and conclusions of this dissertation.

Chapter 2 – Investigation of mercury methylation pathways in biofilm versus planktonic cultures of *Desulfovibrio* desulfuricans

Abstract

Biofilms can methylate mercury (Hg) at higher rates than unattached bacteria and are increasingly recognized as important Hg methylation sites in the environment. Our previous study showed that methylation rates in biofilm cultures were up to an order of magnitude greater than those in planktonic cultures of a sulfate-reducing bacterium. To probe whether the differential Hg methylation rates resulted from metabolic differences between these two cultures, Hg methylation assays following molybdate or chloroform inhibition (a specific inhibitor of the acetyl-coA pathway) were conducted on biofilm and planktonic cultures of *Desulfovibrio desulfuricans* strains M8 and ND132. Molybdate was as effective in inhibiting Hg methylation as well as growth in both planktonic and biofilm cultures. Addition of chloroform only impacted Hg methylation in biofilm cultures, suggesting that different pathways are used for methylation in biofilm as compared to planktonic cultures. To investigate this further, expression of the *cooS* gene, which encodes for carbon monoxide dehydrogenase, a key enzyme in the acetyl-CoA pathway, was compared in biofilm and planktonic cultures of ND132. Biofilm cultures showed up to four times higher expression of *cooS* than planktonic cultures. Based on these results, the acetyl-CoA pathway appears to play an important role in methylation in biofilm cultures of this organism, possibly by supplying the methyl group to Hg methylating enzymes; methylation in planktonic cultures appear to be independent of this pathway. This observation has important implications, particularly in developing reliable models to predict Hg methylation rates in different environments and perhaps eventually in being able to control this undesirable chemical transformation.

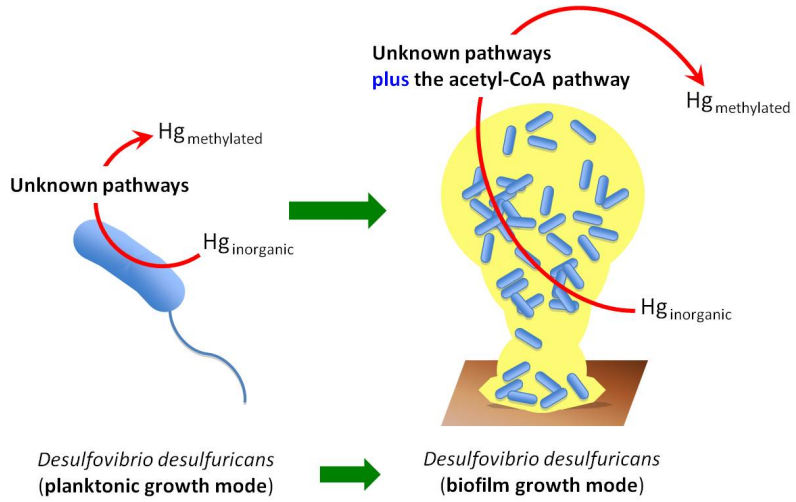

Figure 2-1 Abstract art

Introduction

Elevated concentrations of methylmercury (MeHg) in natural ecosystems, mostly produced *in situ* by specific microbial guilds, are of vital environmental concern because MeHg is a potent neurotoxin that can readily accumulate in aquatic and terrestrial food chains, posing a threat to wildlife and human health.[1,2] Dissimilatory sulfate-reducing bacteria (SRB) and iron-reducing bacteria are now widely recognized as the primary methylators of mercury (Hg) in most aquatic systems;[3-8] however, nearly all previous mechanistic methylation studies to date have been solely conducted with planktonic cultures of these methylating microbes, even though many acknowledge that the majority of bacteria in both natural and engineered environments (including those in soil) live as sessile cells, or biofilms.[9,10] Indeed, attached microbial communities, such as periphyton in wetlands and microbial mats, are increasingly recognized as important sites for environmental Hg methylation.[11-14]

While much remains to be learned about the role of environmental biofilms in the Hg methylation process, at present biofilms are known to provide conditions conducive to the growth of anaerobic organisms (including methylating microbes) in systems that would be considered oxic at the bulk scale owing to reduced microzones that form during biofilm

growth.[15-18] The nutrient cycling and symbiotic relationships among organisms that occur in biofilms may also drive Hg methylation.[19-21] For example, coexistence of SRB with phototrophic sulfur-oxidizing bacteria may be of particular importance in MeHg formation because sulfur-oxidizers consume sulfide,[4,22-25] which may maintain favorable chemical speciation for Hg uptake by SRB and thus ultimately increase Hg methylation.[26] The biofilm growth mode itself may be another determinant of a cell's methylation rate, possibly due to different gene expression or metabolic activities that may occur in organisms growing as biofilms as opposed to unattached cultures. In fact, previous work with pure cultures of two strains of *Desulfovibrio desulfuricans* showed that Hg methylation rates were an order of magnitude higher when the cultures were grown as biofilms compared to when the same organisms were grown as planktonic cultures.[27] However, the biochemical mechanism of *in vivo* Hg methylation remains elusive, particularly in biofilms.[28-30]

Earlier planktonic-culture studies on the mechanism of Hg methylation, conducted on one model strain of SRB, *Desulfovibrio desulfuricans* LS, indicated that MeHg synthesis was an enzymatically catalyzed process associated with the acetyl-coenzyme A (acetyl-CoA) pathway.[31-34] In this carbon metabolism pathway, carbon monoxide dehydrogenase (CODH), encoded by the *cooS* gene*,* is involved in the cleavage of acetyl-CoA and the further oxidation of the formed carbon monoxide to carbon dioxide.[35] Other work showed that although a B_{12}-containing methyl-transferase plays a key role in MeHg formation in complete-oxidizing SRB, Hg methylation in incomplete oxidizers (such as *D. desulfuricans*) appeared to be independent of the acetyl-CoA pathway.[36,37] The recent study by Parks et al. used rigorous genomic, genetic and biochemical approaches to identify two genes, *hgcA* (which encodes a putative corrinoid protein) and *hgcB* (which encodes a 2[4Fe-4S] ferredoxin), necessary for Hg methylation.[38] These genes were proposed as key components of the bacterial Hg methylation pathway, with the HgcA protein hypothesized to facilitate transfer of a methyl group to inorganic Hg(II) and the HgcB protein serving for HgcA turnover. The acetyl-CoA pathway is shown as a potential

source of C1 units for the methylating enzymes in the proposed mechanistic model.[38] While available evidence from these studies with planktonic cultures suggests that there may be multiple pathways for Hg methylation,[36] pathways for methylation in biofilms have, to date, not been studied, and it is unknown whether there may be a metabolic basis for the high methylation rates observed in environmental biofilms of the two tested strains of *Desulfovibrio desulfuricans*.[27]

To investigate whether differential methylation rates in biofilm versus planktonic cultures of incomplete-oxidizing SRB resulted from metabolic differences between these two modes of growth, we extended our study by performing enzyme inhibition assays on planktonic and biofilm cultures of two strains of *D. desulfuricans*: the strain M8 is a brackish water strain of SRB used in our previous work,[27] and the strain ND132 is a well-known Hg-methylating sulfate-reducer whose genome has recently been sequenced and thus can be examined genetically.[38-41] Both molybdate, a known inhibitor of sulfate reduction,[3] and chloroform ($CHCl_3$), a known inhibitor of corrinoid-containing enzymes, which play a critical part in the acetyl-CoA pathway,[35] were included in this study to specifically probe the role of the acetyl-CoA pathway in Hg methylation. In addition, expression of *cooS*, which encodes for a key enzyme involved in the acetyl-CoA pathway, relative to reference genes and cell count in biofilm and planktonic cultures of this organism was compared.

Materials and Methods

Cultures and growth media. Both strains were cultured at room temperature (~22°C) in a medium containing basal salts, vitamin mixture, selenite-tungstate, thiamine, and non-chelated trace element mixture, buffered at pH 7.2 with 30 mM bicarbonate.[42] Lactate (35 mM) and sulfate (28 mM) were chosen as the electron donor and acceptor, respectively. The salinity of the medium was adjusted to suit each strain: 10 and 1 g/L of NaCl for M8 and ND132,

respectively. The medium was reduced with 0.25 mM Ti(III), which has been shown to not affect the growth of SRB.[43,44] Medium was prepared anoxically under a gas stream passed through heated copper beads (for inhibition assays, CO_2 and N_2 in a ratio of 20:80 (%) was used, and for gene expression assays, H_2, CO_2, and N_2 in a ratio of 5:15:80 (%) was used to match the headspace in the anoxic chamber). Planktonic cultures were maintained in serum bottles sealed with butyl rubber stoppers. Biofilm cultures were grown by inoculating sterile 50 mL Falcon tubes filled with 30 mL of Hg-free medium and containing acid-washed, autoclaved glass microscopic slides. The Falcon tubes were kept in an anoxic chamber (Coy Labs) in an atmosphere consisting of H_2, CO_2, and N_2 in a ratio of 5:15:80 (%). While the experimental biofilms were being established (over the course of one week for inhibition tests and two weeks for gene expression studies), 50% of the medium was replenished every 2 days to provide sufficient nutrients to maintain growth.

Prior to inoculation of experimental bottles, planktonic cultures were grown to exponential phase, pelleted and re-suspended in fresh medium formulated as described in the previous paragraph but without sulfate in order to minimize sulfide formation prior to the start of the experiment. One milliliter of inoculum was then transferred to 50 mL acid- washed serum bottles containing 30 mL of sulfate-containing assay media (again, formulated as described in the previous paragraph). Active biofilm-coated slides were transferred to acid-washed, sterile 50 mL Falcon tubes containing 30 mL of assay media after being rinsed three times in fresh sulfate-free medium to eliminate unattached cells. Sulfate-free medium was used to eliminate sulfide transfer during inoculation, as sulfide is known to inhibit methylation. During inhibition experiments, planktonic cultures were maintained on an orbital shaker at 160 rpm in the dark, while unstirred Falcon tubes containing biofilm-coated slides were covered with aluminum foil and kept in the anoxic chamber. For gene expression assays, all cultures were unstirred and uncovered, and kept at room temperature.

Enzyme inhibition tests. Stock chloroform solutions were first prepared in absolute (>99%) ethanol and then diluted twice (at least a 30-fold dilution each time) in Hg-free medium. 50 μM chloroform was chosen as a final concentration.[35,36] Similar to chloroform, molybdate was added from stock solutions and then diluted in the medium to the final concentration of 28 mM.[36,45] Solutions of chloroform and molybdate were prepared and filter-sterilized immediately before being spiked into assay media.

Triplicate bottles/tubes of planktonic and biofilm cultures were prepared. An initial test was conducted using ND132 with cultures incubated for 7 h at room temperature, followed by 48 h Hg methylation assays (spiked with 50 ng L^{-1} inorganic Hg(II) standard). A second set of tests was conducted with both M8 and ND132 with all inhibitor-assayed cultures incubated for 7 h at room temperature, followed by 12 h Hg methylation assays (spiked with 50 ng L^{-1} inorganic Hg(II) standard). Each bottle was sampled for MeHg and cell density measurements at the beginning and the end of the methylation assays. Killed cultures spiked with Hg standard served as negative controls, while live cultures spiked only with Hg (i.e., no inhibitors added) served as positive controls and were included in each batch. Cultures that did not receive Hg and inhibitors served as blank assays to determine the background level of MeHg.

Gene expression tests. Assay medium, as previously described, in half of the bottles/tubes were amended with 50 ng L^{-1} inorganic Hg(II) and equilibrated overnight prior to inoculation with ND132. For each of the four conditions (Live ± Hg, Killed ± Hg), bottles and tubes were sacrificed in triplicate for each timepoint. Four replicate biofilm slides were scraped into one new tube with phosphate-buffered saline (PBS), thereby "pooling" the biofilm cells for a higher cell count (12 total biofilms per timepoint per condition). Inside the anoxic chamber, biofilm cells were collected by rinsing the slide with sterile PBS, vortexing for 30 s, scraping, rinsing, and vortexing again, as previously described by other researchers.[9,46] The slide was removed once the PBS solution contained the biofilm cells. Samples from each of the new

triplicate tubes were collected for MeHg, RNA, DNA, and cell counts. Samples were preserved and stored as follows: Hg samples were syringe-filtered, acidified (0.5% HCl v/v for total Hg, 0.2% H_2SO_4 v/v for MeHg) and stored at 8°C; RNA was stabilized using 2:1 Qiagen RNAprotect Bacteria Reagent and refrigerated at 8°C; DNA samples were unamended and frozen in -80°C; samples preserved for cell counts received 5% v/v formaldehyde and were refrigerated at 8°C.

RNA extraction, design of degenerate primer sets and reverse transcription-qPCR. RNA samples unamended with stabilizing reagent were immediately extracted for RNA content. mRNA was extracted (Qiagen RNeasy kit, Qiagen RNase-Free DNA Digestion kit), quantified (Nanodrop 2000), and amplified (Qiagen SYBR Green One-Step RT-qPCR kit, Applied Biosystems StepOnePlus Real-Time PCR System). The location of the *cooS* gene in ND132 was located through the EMBL database, and 130 bp amplicon primers were obtained using GeneBlast. Two sets of primers (150 bp and 190 bp) for 16S rRNA in ND132 were designed similarly (see Table 1) and are used to normalize *cooS* gene expression. It is noted that although the use of reference genes is desirable, reference genes can vary significantly and can adapt to growth conditions.[47,48] In such cases, geometric means of multiple references are used.[49-51] Cycling conditions were as follows: 95°C for 10 min followed by 45 cycles of 94°C for 15 s, 60°C for 1 min, and 72°C for 30 s. Relative quantification of the qPCR results use the $2^{-\Delta\Delta Ct}$ method to compare differences in gene expression.[52,53] Both amplification plots and melt curves were obtained during RT-qPCR. This expression was normalized to both cells (counted) and the geometric mean of expression of two primer sets from 16S rRNA.

MeHg and cell density determination. MeHg analysis was performed via distillation/ethylation/GC-CVAFS.[54] Prior to distillation, interference resulting from sulfide was eliminated by acidifying samples with 9N sulfuric acid and then purging with gold-coated sand-trap-filtered nitrogen (45 mL min^{-1} for approximately 25 minutes).[27] Cell density from both planktonic and biofilm cultures was determined by direct counts using 4',6-diamidino-2-

phenylindole (DAPI) staining and epifluorescent microscopy in accord with our previous protocols.[27]

Results

Effects of inhibitors in both planktonic and biofilm cultures. Our previous study comparing Hg methylation rates on a per cell basis between planktonic and biofilm cultures of a methylating sulfate-reducer, *D. desulfuricans* M8, indicated that the specific Hg methylation rate in biofilm cultures was approximately an order of magnitude higher than that in planktonic cultures.[27] In the current study, the importance of the acetyl-CoA pathway in both planktonic and biofilm cultures of this strain, as well as another known Hg-methylating strain, *D. desulfuricans* ND132, was tested using specific inhibitors.

As expected, a similar and effective inhibition of growth and Hg methylation was observed in both planktonic and biofilm cultures of M8 and ND132 amended with molybdate, a known competitive inhibitor for sulfate in SRB.[55] However, results show that chloroform had no significant influence on either growth or methylation in planktonic cultures. Growth rates were exactly the same in cultures with and without chloroform addition for ND132 (0.040 h^{-1}) and M8 (0.051 h^{-1}). Hg methylation was also not affected significantly ($p>0.05$, t-test) by the presence of chloroform (Fig. 1).

Yet, unlike planktonic cultures, chloroform inhibition on Hg methylation was observed in biofilm cultures. Both ND132 and M8 produced lower MeHg concentrations in cultures incubated with chloroform, and the specific rates of Hg methylation in chloroform-amended cultures decreased by a factor of greater than two compared to cultures in the absence of inhibitors ($p<0.05$, t-test; Fig. 1). Interestingly, chloroform had no influence in growth of biofilm cultures, as seen in 0.025 h^{-1} (chloroform-free cultures) vs. 0.025 h^{-1} (chloroform-amended cultures) of ND132, and 0.063 vs. 0.069 h^{-1} of M8, respectively.

Fig. 2 depicts a longer (2 day) inhibition experiment with ND132, in which an even greater difference in rates can be observed between biofilm and planktonic cultures. Because it is unclear whether cells which detached from biofilms during the experiments should be treated as biofilm or planktonic cells, specific Hg methylation rates illustrated in Fig. 2 were calculated according to the Case 1 method described in an earlier study, which assumed that the detached cells still methylate Hg at the same rate as the attached cells.[27] It should be noted that Case 1 calculations resulted in more conservative numbers as compared to Case 2 calculations, which account for methylation by the detached cells at the rate determined for the planktonic system so that only attached cells are regarded as true biofilm cells.

Comparison of cooS gene expression. Given that results from chloroform inhibition assays indicated a potential role of the acetyl-CoA pathway in biofilm cultures, the importance of Hg methylation by this pathway was further explored by comparing gene expression of *cooS* in biofilms and planktonic cultures of *D. desulfuricans* ND132. Expression of *cooS* normalized to cell number (i.e., copies/cell) was approximately four times higher in biofilms compared to planktonic cultures (Fig. 3A). Cell count error, as calculated by the square root of total cells by the number of total cells, was less than 6.5% for live biofilm and planktonic cultures, as well as for killed planktonic cultures. Error was as high as 13% for killed biofilm cultures, as a result of significantly fewer cells present to be counted. Primer dimerization did not appear to occur based on melting curve analysis. Similar results were observed when normalizing to the geometric mean of expression of two primer sets from 16S rRNA (Fig. 3B).

Discussion

While significant progress has been made towards identifying the biochemical mechanism that leads to MeHg production in bacterial cells, to date this mechanism has not been definitively identified.[30,38,56] The recent study by Parks et al identified genes necessary for Hg

methylation in SRB, though the exact pathway(s) by which Hg is methylated in these organisms is not known for certain.[38] Results of previous studies have suggested that the acetyl-CoA pathway is involved in this microbially-mediated environmental process in methylating SRB, particularly when this pathway is used in methylating SRB for primary carbon metabolism (i.e., in complete-oxidizing SRB).[31-34,36] Considering that other pathways of Hg methylation occurring independently of the acetyl-CoA pathway are still unclear,[36,37,57] an understanding of whether metabolic differences between planktonic and biofilm cultures of Hg-methylating *D. desulfuricans* strains, in particular the role of the acetyl-CoA pathway in the production of MeHg in biofilm versus planktonic cultures, could help elucidate the differential methylation rates observed in these two growth modes. Thus, enzyme inhibition and reverse transcription-qPCR assays specifically targeting the gene and enzyme required in the acetyl-CoA pathway were conducted in this study.

Data from chloroform inhibition tests with the strains M8 and ND132 revealed that Hg methylation was only inhibited in biofilm cultures. Consistent with work done by Ekstrom et al. methylation was not inhibited by chloroform in planktonic cultures of both of these incomplete-oxidizers.[36] In their work, Ekstrom et al. showed that with 50 μM chloroform, Hg methylation was effectively inhibited (lower than the detection limit) in planktonically-growing cultures of the complete-oxidizer, *Desulfococcus multivorans* 1be1. In another set of their experiments, neither Hg methylation nor growth of an incomplete-oxidizing strain, *Desulfovibrio africanus*, was inhibited over a shorter incubation (5 h) with chloroform. Longer incubation (3 days) eventually affected culture growth; however, no significant difference in specific Hg methylation rates was observed between shorter- and longer-incubated cultures.[36] The result that addition of chloroform only impacted Hg methylation in biofilm cultures in this study indicated a role for the acetyl-CoA pathway for methylation in biofilm but not planktonic cultures of the organisms tested. Further, while Hg methylation in chloroform-amended biofilm cultures was inhibited, it was not inhibited completely, suggesting that multiple pathways for Hg methylation may take

place in biofilm cells. Parks et al. proposed a mechanism by which the proteins encoded by the genes they identified as necessary for methylation (*hgcA* and *hgcB*) might methylate Hg, which may involve the acetyl-CoA pathway as well as other pathways to provide a C1 source. Since it is unknown whether the proteins encoded by *hgcA* and *hgcB* are involved with additional metabolic pathways in SRB, our findings that an unknown pathway may also be involved is not inconsistent with these results.

The importance of the acetyl-CoA pathway in biofilm cultures relative to that in planktonic cultures was also supported by the result of the *cooS* expression tests, as biofilm cultures showed up to four times higher expression of *cooS* than planktonic cultures on a per-cell basis. While similar results were observed when copy numbers were normalized to the geometric mean of expression of 16S rRNA using two primer sets (Fig. 3B), it should be kept in mind that standardization of mRNA in gene expression studies has been a complex issue that has not been fully resolved. Ideally, expression would be normalized to cell count but the accuracy of cell count can be a concern.[50] Although use of reference genes is preferable, expression of reference genes can vary significantly. It has been suggested that reference genes can adapt to growth conditions, which is especially important for biofilms as their metabolic processes may differ significantly from planktonic cultures.[47,48] Desirable reference genes would express equally despite experimental treatments, however, in cases when they are not, geometric means of multiple reference genes are used.[49-51] The use of reference, or housekeeping, genes for biofilms, in particular, is complex and has not yet been thoroughly investigated.

It is known that in response to environmental signals, biofilm bacteria can express new phenotypes that distinguish themselves from their planktonic counterparts.[9,58-60] This is illustrated by data that have shown higher resistance to toxic compounds, including antimicrobials, metals and metalloids, in biofilm cultures than in planktonic cultures.[61-63] In particular, work on susceptibility of planktonic and biofilm cells of the same microorganism to antibiotics has shown that the structure of biofilms do not simply provide a diffusion barrier to

these compounds; instead, the biofilms employ distinct resistance mechanisms, and exhibit differential gene expression as compared to planktonic modes of growth.[64-66] In biofilms, significant upregulation of multiple genes related to cellular function and metabolic pathways may occur;[67,68] thus the formation of biofilms may favor one pathway over another. Presumably, it is possible that such alternation of gene and protein expressions may result in different metabolic pathways between biofilm and planktonic cultures, thus causing differences in Hg methylation. Indeed, results of our work showed differences in expression of a gene are likely important in methylation between biofilms and planktonic cultures, suggesting a *metabolic basis* for the high methylation rates observed in environmental biofilms.

A likely connection between Hg methylation and the acetyl-CoA pathway in biofilm cultures can also be inferred from the significantly higher methylation rates observed in biofilm versus planktonic cultures for both M8 and ND132 (Fig. 1 & 2). King et al. have suggested that Hg methylation potential may be related to genetic composition and/or carbon metabolism in SRB.[69] Results from their pure culture experiments showed that Hg methylation rates on a per-cell basis were up to 3 orders of magnitude higher in the family *Desulfobacteriaceae*, which is the family of SRB that use the acetyl-CoA pathway for complete oxidation of carbon substrates as compared to the other family of SRB, *Desulfovibrionaceae*. The same pattern was also observed by Ekstrom and Morel from their cobalt limitation assays which showed that *D. multivorans*, a complete-oxidizer that uses the acetyl–CoA pathway for major carbon metabolism, made 10–50 times more MeHg per cell than a *Desulfovibrio* strain, *D. africanus* (DSMZ 2603).[37]

It is noted that the process of intracellular MeHg synthesis has been postulated to be most likely a metabolic mistake rather than a mechanism that confers Hg resistance in SRB[30.70]; however, a tight coupling between Hg methylation and MeHg export from the cell of Hg-methylating strains observed in a recent study has brought up the question of whether methylation may be a strategy for bacteria to avoid build up and subsequent toxicity of cellular

Hg, or possibly a part of a Hg detoxification process.[29] In the present study, it was observed that 1) chloroform only inhibited Hg methylation and not growth in biofilms, and 2) the acetyl-CoA pathway seems to influence the rate of formation of MeHg in biofilm cultures only, possibly by providing C1 for Hg methylation.[38] These results, combined with the observation that, in general, biofilm cells typically show higher resistance to toxic metals than planktonic cells, suggest that whether methylation may serve as a mechanism for resistance to or reduction of Hg(II) toxicity for *D. desulfuricans* biofilms warrants future study.

One of the major goals of Hg methylation research is to determine factors influencing Hg transformation potentials in order to develop quantitative, reliable models to predict Hg methylation rates in different environments. Theoretically, such a model should rely on parameters that adequately describe (i) the microbial community composition, (ii) the bioavailability of inorganic Hg(II), and (iii) the principal biochemical pathways responsible for Hg methylation.[71,72] Recent research has attempted to predict in situ methylation rates using information on SRB activity and methylation.[69,73] Our study contributes to a deeper understanding of the actual methylation rates in sediments and in attached communities. Extending this work to field samples would indicate the environmental importance of a metabolic basis for increased methylation in attached communities. Whether detached biofilms cells would retain increased methylation capability is an open question. If so, events such as rain could disturb attached communities and transport cells with higher Hg methylation rates. In addition, having an understanding of the mechanisms by which SRB methylate mercury holds promise for informing remediation strategies in engineered treatment systems.

Acknowledgments

This work was supported through National Science Foundation CAREER grant BES-0348783. We thank all of our lab colleagues for their constant support, and we are appreciative of lab

assistance from Saeedreza Hafenezami, Myfanway Rowlands, Jonathan Lucio, and Ahana Mukherjee.

Table 2-1 Primers used in RT-qPCR

Primers	Forward Sequence (5' → 3')	Reverse Sequence (5' → 3')
cooS	AGGGCGAGACCAAGGATTAC	GCGAAAAAGCACTCCATGAC
16S rRNA primer set 1	GGGGGAAACCCTGACGCAGC	TGCTGGCACGGAGTTAGCCG
16S rRNA primer set 2	CGACGCCGCGTGTAGGAAGA	ACGCACGCTTTACGCCCAGT

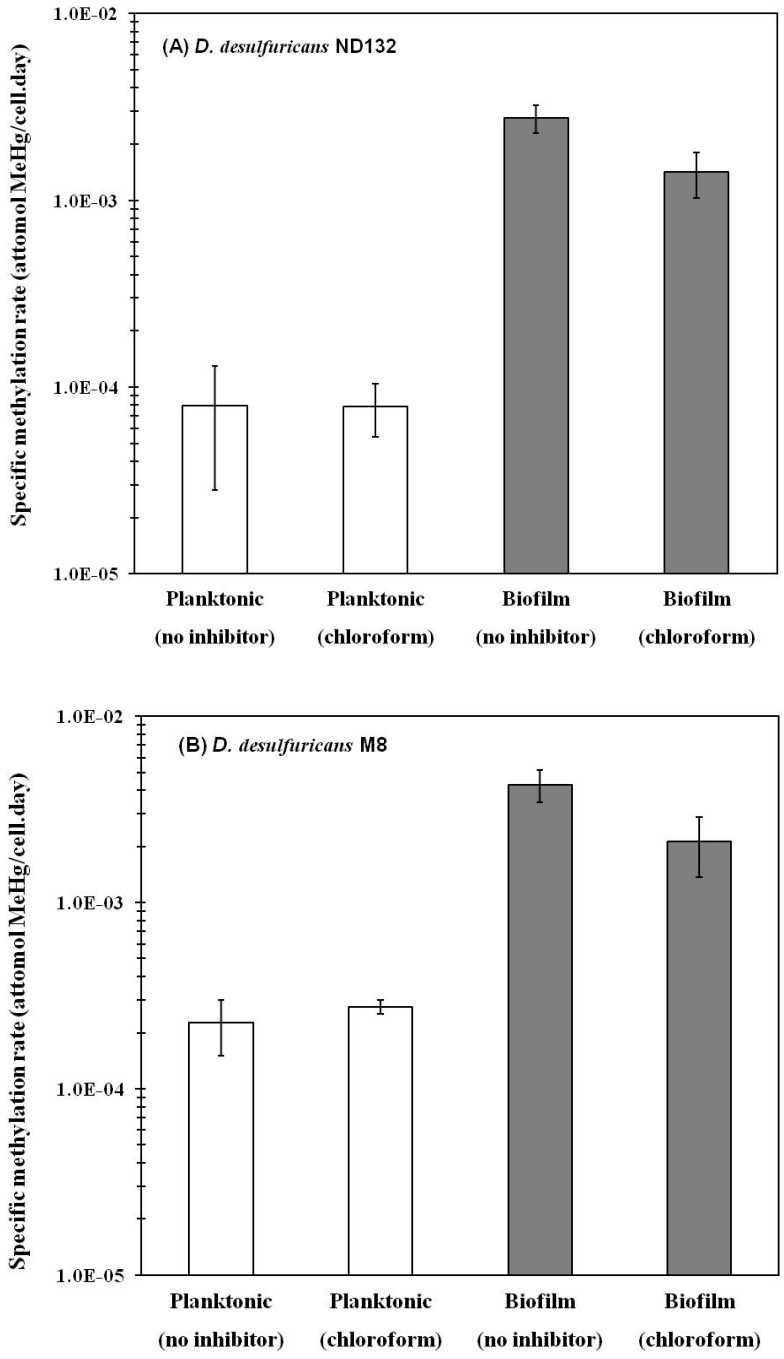

Figure 2-1 Observed methylation rates after a 12-h methylation test following a 7-h inhibition test in planktonic and biofilm cultures (A) *Desulfovibrio desulfuricans* ND132 and (B) *D. desulfuricans* M8. Error bars represent the standard deviations of triplicate assays.

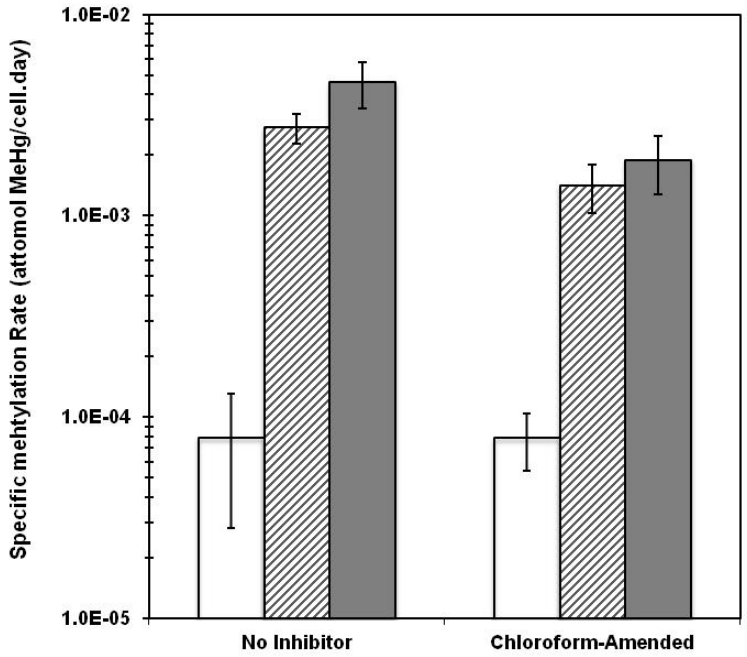

Figure 2-2 Methylation rates after a 2-day methylation test following 7-h exposure to inhibitors in planktonic and biofilm cultures of *D. desulfuricans* ND132. Blank columns represent rates of planktonic cultures. Light-filled columns are the rates of biofilm cultures according to Case 1 calculation, assuming the detached cells still methylate mercury at the same rates as the biofilm cells. Dark-filled columns are the methylation rate of biofilm cells, accounting for methylation by detached cells at the rate determined for planktonic cultures (Case 2). Error bars represent the standard deviations of triplicate assays.

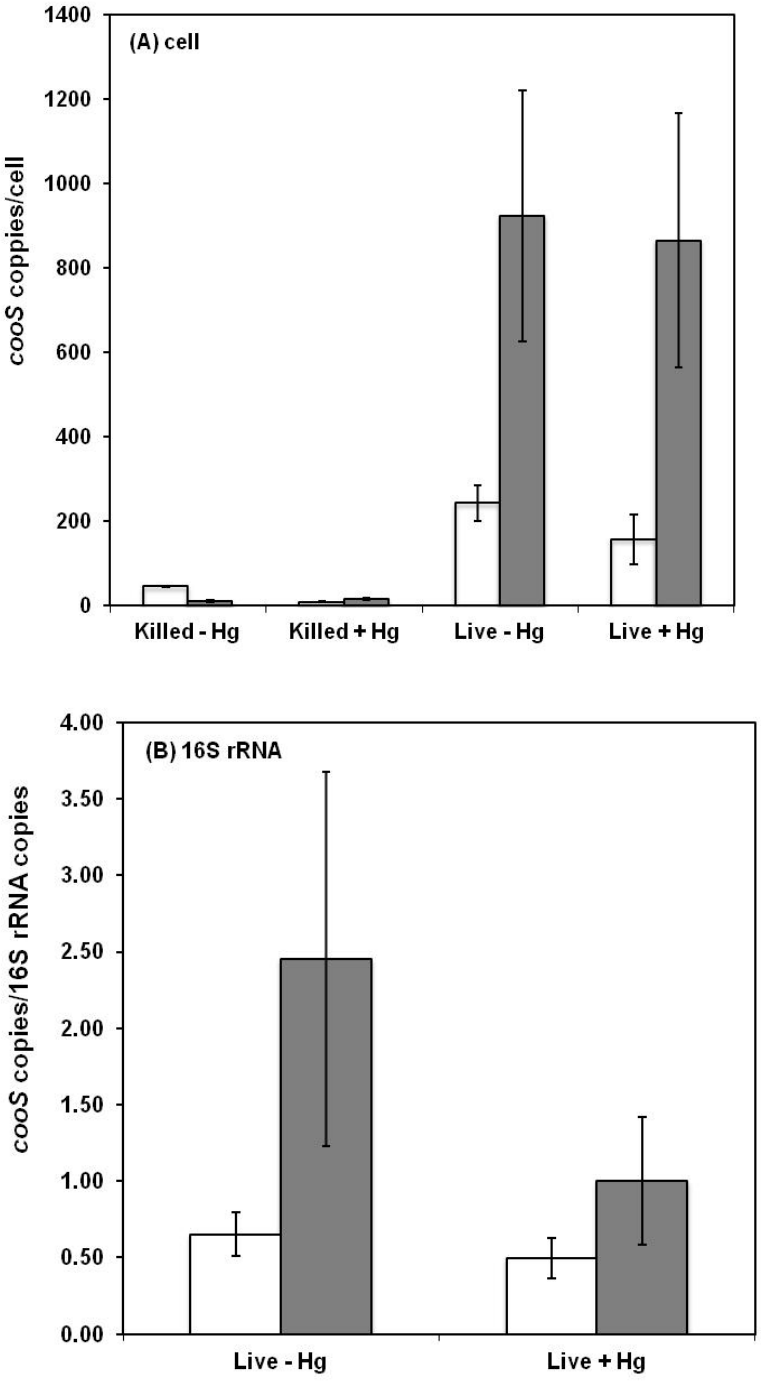

Figure 2-3 Comparison of cooS gene expression, based on the $2^{-\Delta\Delta Ct}$ method for relative quantification, between planktonic and biofilm cultures of *D. desulfuricans* ND132 in the presence and absence of Hg. (A) Copies per cell are relative values based on changes in the threshold cycle. (B) Copies of *cooS* per copies of 16S rRNA are also based on relative values

based on changes in the threshold cycle. Blank columns represent planktonic cultures. Dark columns represent biofilm cultures. Error bars represent the standard error of triplicate experimental bottles (each bottle was subject to triplicate analysis).

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Chapter 3 – Sediment characterization of a new Bangladeshi aquaculture pond and a one-year old pond

Introduction

While arsenic contamination of groundwater is a worldwide problem, in a tragic nexus, regions of very dense population along river deltas in India, Bangladesh, Cambodia, Vietnam, China, and Nepal overlie areas of high arsenic contamination (VanGeen 2008), affecting tens of millions of people and resulting in the largest environmental poisoning in history.

Each year in Bangladesh, millions of people are diagnosed with arsenicosis and other illnesses, resulting in hundreds of thousands of deaths. Four million wells in Bangladesh alone pump arsenic-contaminated groundwater exceeding the WHO maximum allowable limit of 10 µg/L (Smedley and Kinniburgh 2003). Despite the tragic public health implications of this problem, the question of why dissolved arsenic concentrations are so high in the groundwater of the Ganges Delta has not been completely answered. To more effectively develop treatment strategies that could improve the quality of life in critical populations, it is necessary to know where the arsenic originates and what conditions contribute its mobilization.

It is known that arsenic is of geological original and is mobilized into the groundwater primarily through the oxidation of organic matter. The location of the mobilization and the source of organic matter have sparked much debate in scientific literature. It is now widely acknowledged that the arsenic is released from near-surface sediments from the oxidation of organic matter that is of anthropogenic origin (Polizzotto et al. 2005; Harvey et al. 2006; Polizzotto et al. 2008). In anaerobic conditions, bacteria oxidizing the organic matter will reduce arsenic-bearing minerals, thus releasing arsenic into the water phase.

Gaps remain in the understanding of the origin of water and organic matter recharging contaminated aquifers. Rice paddies cover approximately 90% of the area in many parts of Bangladesh and were assumed for many years to be the source of recharge to the groundwater

that released arsenic. However, recent work has shown that ponds constructed for aquaculture, while covering less than 10% of land in the study area, recharge the groundwater much faster than paddies (Neumann et al. 2010), and may be an important source of recharge water that mobilizes arsenic. Further, the chemistry of the pond water appears to be much more conducive to reductive processes that release arsenic than that of paddy water (Neumann et al. 2010).

Additionally, chemistry in the ponds is engineered through additions designed to maximize fish production but impacts of aquacultural practices on subsurface arsenic mobilization are completely unstudied. Notably, cow dung is routinely added to ponds as it is readily available, but it may result in unintended enhanced arsenic mobilization due to the organic matter in the dung (Williams et al 2011). As NOM is a key factor in arsenic mobilization, the practice of adding manure to ponds as well as the anaerobic conditions created by flooding during the monsoon season can exacerbate arsenic problems in the region.

The goals of this study are: 1) determine the host fractions of arsenic in pond sediments by sequential extraction, and 2) compare the solid-phase arsenic in a new pond and a one-year old pond to observe possible aquaculture and weathering impacts. The outcomes of this research will contribute to solving the critical scientific problem of widespread arsenic exposure to humans.

Methods

Field Site. The Bangladesh field site is located in the Munshiganj district near the village of Srinigar, which is approximately 25 km south of the capital city of Dhaka. Land use of the main study area is rice cultivation, where the surface is composed of organic-rich clay and silt. The site's subsurface contains layers of clay, a Holocene aquifer of gray sand, clay aquitard, and a Pleistocene aquifer of deep, burnt-orange sand (Polizzotto et al, 2006). A number of local

excavated ponds are located at or near the study area and may be up to several meters deep. They generally have low-permeability clay bottoms, enabling ponding of irrigation and floodwater. Also nearby are villages and roads that rise 2 to 3 meters above the level of the fields. The composition, layout, and land use of this field site is typical of the region. During the dry season when Boro rice is cultivated, irrigation occurs on average twice a week, where the entire paddy is flooded with arsenic-laden water. During the wet season, the site is under meters of rainwater. At this site, arsenic levels are up to 400 ppb in the irrigation water and up to 1.2 ppm in the groundwater.

Sediment Collection. Sediment cores were collected using AMS Multi-Stage Soil Core Sampler. Cores were capped, sealed, double bagged in low gas permeable Escal bags containing RP oxygen absorbing agents (Mitsubishi Gas Company), and shipped on ice to UCLA. Cores were collected from a newly constructed pond and the same pond one year later. Cores were collected in 6-inch intervals down to 3 feet in depth for the new pond and 5 feet in depth for the pond one year later.

Arsenic Sequential Extraction. Solid-phase arsenic mobility and transport was evaluated using a sequential extraction procedure modified from Keon et al (2001) (See Table 3-1). Cores were extruded in a Coy anaerobic chamber equipped with a flushable core entrance port, homogenized, and portioned out for characterization. In the anaerobic chamber, 25 ml of extractant was added to 1 g of wet sediment in a 30 ml Oak-Ridge polypropylene copolymer centrifuge tube. Slurries were tumble-shaken for the duration of each step, then centrifuged at 7,800g for 40 minutes. Aliquots of syringe-filtered (0.22 um) supernatant were preserved for arsenic(total), Fe(total), and Fe(II) analyses, described below.

Table 3-1 Sequential extraction method modified from Keon et al 2001

Step	*Extractant*	*Target Phase*
Mg	1M $MgCl_2$, pH 8, 2hr, 25^{o}C	Ionically bound As

	2 repititions, 1 water wash	
PO_4	1M NaH_2PO_4, pH 5, 16 & 24 hr, 25°C 1 repetition each time duration, 1 water wash	Strongly-adsorbed As
HCl	1N HCl, 1hr, 25°C 1 repitition, 1 water wash	As coprecipitated with acid-volatile sulfides, carbonates, Mn oxides, very amorphous Fe oxyhydroxides
Oxalate	0.2M ammonium oxalate/oxalic acid, pH 3, 2hr, 25°C in dark 1 repetition, 1 water wash	As precipitated with amorphous Fe oxyhydroxides
Hot HNO_3	15N HNO_3 + 30% H_2O_2 based on EPA method 3050B	As oxides, As coprecipitated with silicates, pyrites, and amorphous As_2S_3, orpiment & other remaining recalcitrant As minerals

Aqueous Phase Analyses. Samples were quantified for arsenic using Graphite Furnace Atomic Absorption Spectrometry (GFAAS). Dissolved Fe(II) was quantified using the ferrozine method and total dissolved iron was determined using a spectrophotometric method by the Hach Company with TPTZ reagent. Analysis of total dissolved concentration of hydrogen sulfide species, $(H_2S)_T = (H_2S) + (HS^-) + (S^{2-})$, referred to as total sulfide concentration, was conducted using the methylene blue method.

Results

Sediment was characterized for solid-phase arsenic to obtain a depth profile. Speciation of the new pond showed the majority of extracted arsenic is recalcitrant for all depths with arsenic also found in the strongly-adsorbed and co-precipitated fractions (Figure 1). Total arsenic levels extracted ranged from 5 to 24.5 µg/g and the maximum of 24.5 µg/g occurs at 1.5 feet in depth (Figure 3).

One-year old sediment from the same pond has a similar range of arsenic with a slightly lower maximum of 22 µg/g at 2 feet in depth (Figure 6). However, all of the arsenic is found only in recalcitrant and co-precipitated fractions (Figure 4). At most depths, arsenic levels in the recalcitrant fraction in one-year old sediment decreased from 0-year old sediment, suggesting that some of the arsenic in that fraction has shifted to other, less recalcitrant fractions. In addition to the shift in recalcitrant arsenic, the easily-mobilizable fractions have decreased to below detectable limits, possibly having been mobilized (Figure 2, Figure 5).

While the fraction accounted for less than 3.2 µg/g of arsenic at all depths, it can account for significant levels of As released into the groundwater. Equation 1, where ρ is density in g/cm^3, Φ is the aquifer porosity, V is the aquifer volume in L, and C is the concentration of arsenic in sediment (µg/g) and arsenic mobilized into the groundwater (µg/L), predicts how much arsenic is mobilized based on sequential extraction results. In a conservative calculation using approximately 10% of the initial easily-mobilizable fraction with an assumed sediment density of 2.5 g/cm^3 and an aquifer porosity of 0.3, sediment containing 0.1 µg As/g at a 1 L aquifer will result in 175 µg/L of arsenic mobilized.

$$\rho_{SEDIMENT} \times (1 - \Phi_{AQUIFER}) \times V_{AQUIFER} \times C_{SEDIMENT} = C_{MOBILIZED} \quad (1)$$

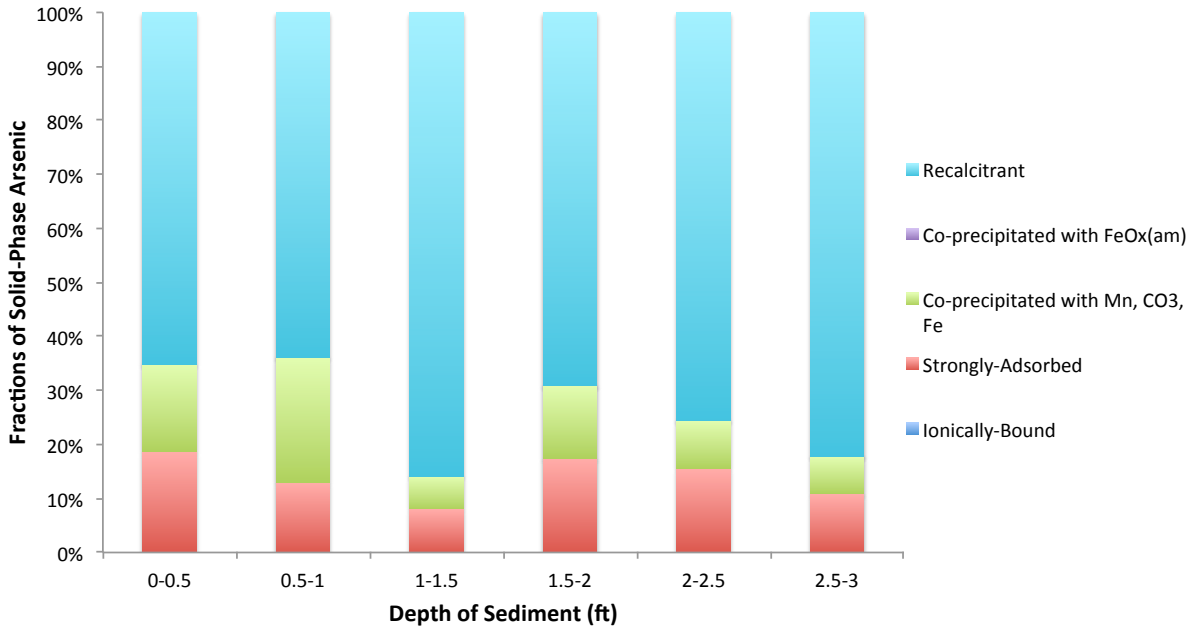

Figure 3-1 Solid-phase arsenic speciation in sediment from the newly excavated pond

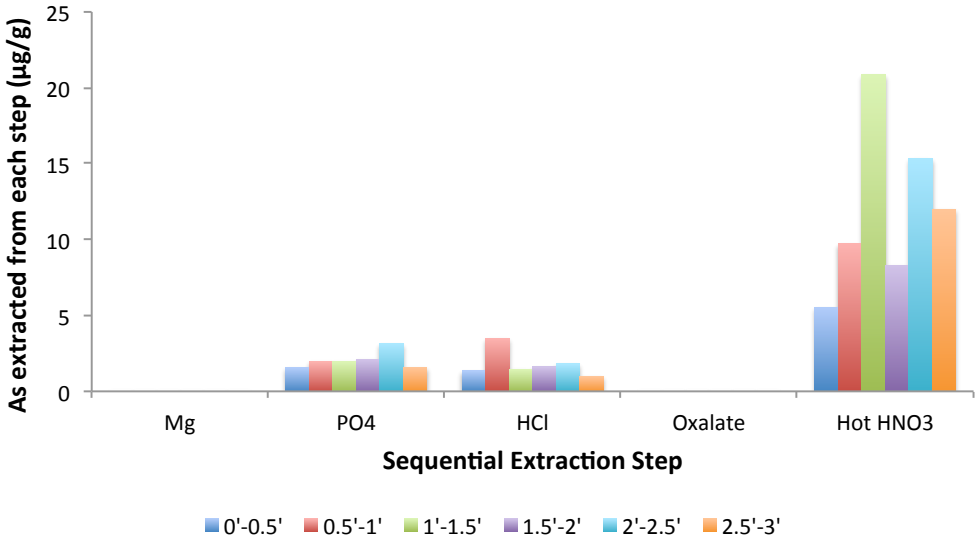

Figure 3-2 Arsenic extracted by sequential extraction from sediment from the newly excavated pond

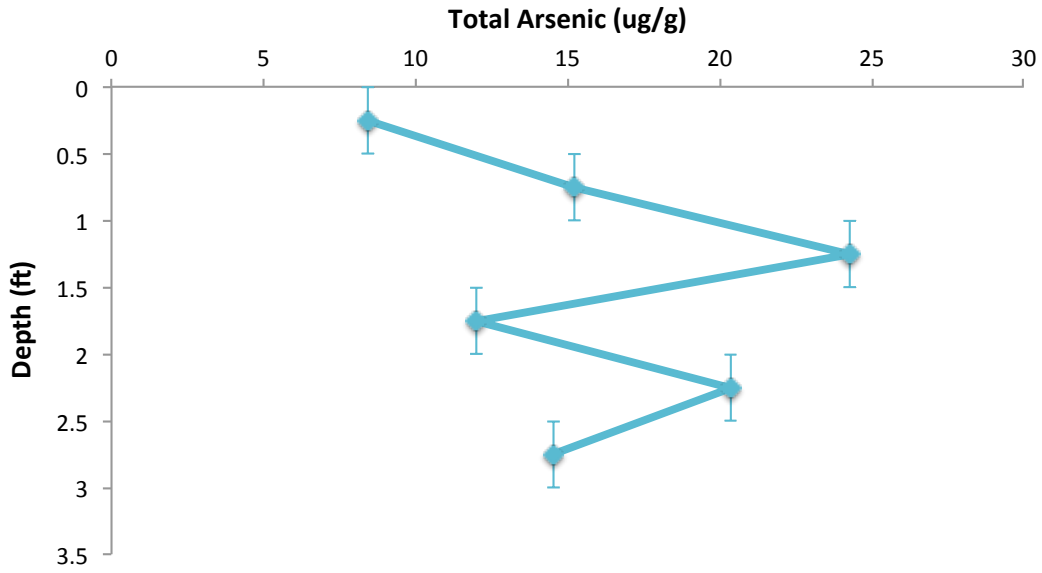

Figure 3-3 Depth profile of arsenic in sediment from the newly excavated pond, where the bars represent the section of homogenized sediment represented by the diamond

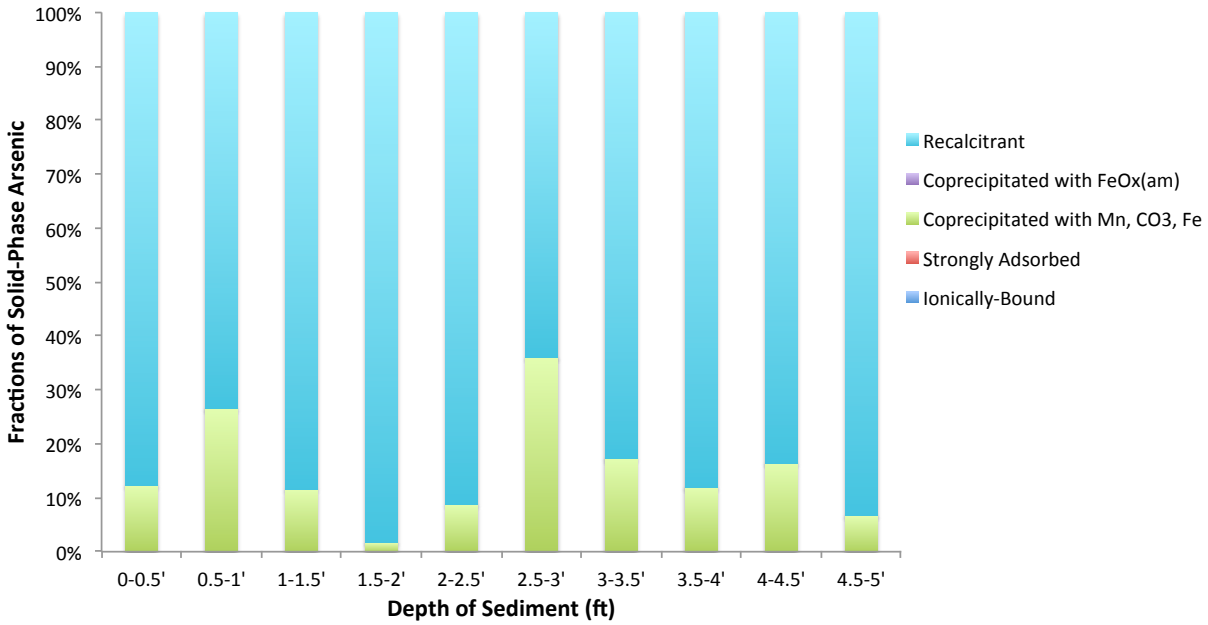

Figure 3-4 Solid-phase arsenic speciation in sediment from the one-year old pond

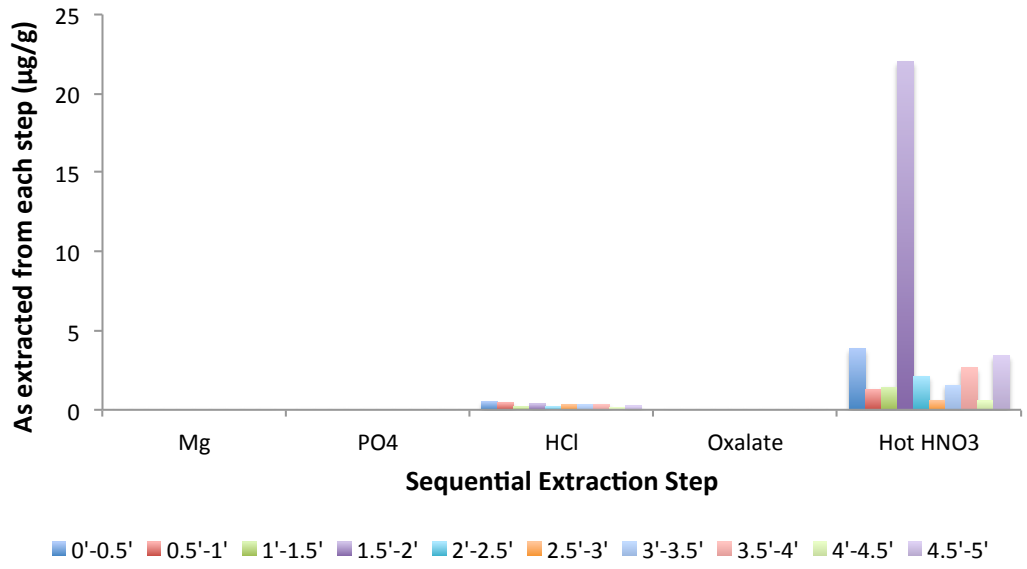

Figure 3-5 Arsenic extracted by sequential extraction from sediment from the one-year old pond

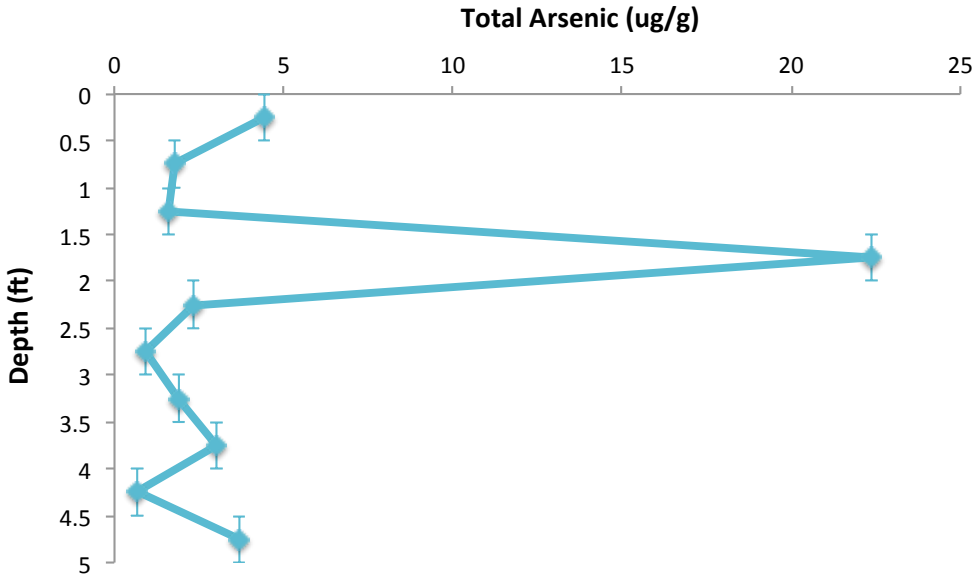

Figure 3-6 Depth profile of arsenic in sediment from the one-year old pond, where the bars represent the section of homogenized sediment represented by the diamond

New pond sediment was treated with various fertilizers and extracted for easily mobilizable arsenic showed that using cown manure released more arsenic than using chicken manure (Figure 3-7). Stimulating microbes by supplying a carbon source without any additional

bacteria or nutrients did not release as much arsenic as other treatments, suggesting that the aquaculture fertilization practice is important to arsenic mobilization. The treatment with nutrients only, no additional carbon or bacteria, resulted in the most arsenic released, however, this treatment contained more nutrients than any other treatment, not allowing it to be a direct comparison with either manure treatment. Nonetheless, this treatment shows the competitive desorption through phosphorus and other ions, may be an important mechanism for arsenic mobilization.

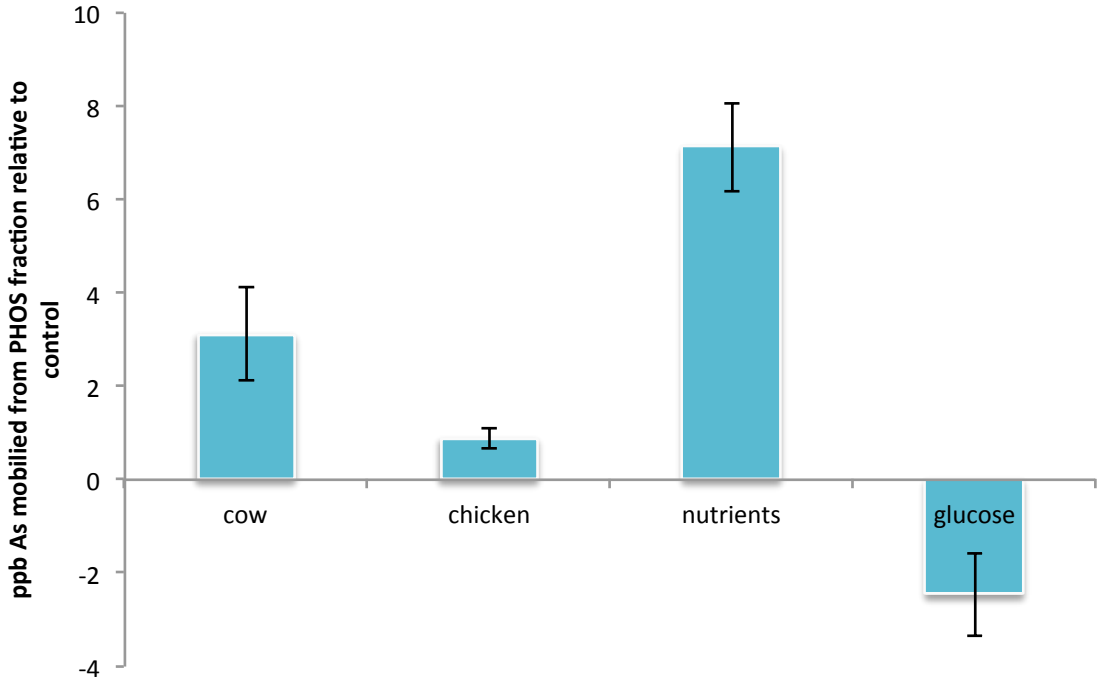

Figure 3-7 Arsenic extracted by phosphate from sediment treated with different fertilization regimes

Discussion

During the year between the two sediment collections, the pond was fertilized with manure and endured a monsoon season that filled the pond, both of which can contribute to arsenic mobilization. When ponds are flooded and filled, the pond becomes anaerobic and

particularly conducive to iron hydroxide reduction, which has been established as an important mechanism for arsenic mobilization (Smedley and Kinniburgh 2003; Harvey et al, 2005…). Previous studies have observed an increase in dissolved arsenic under anoxic conditions brought on by flooding and irrigation. Microcosm experiments by Burton et al saw that microbial iron hydroxide reduction correlated with flooding events and resulted in iron and arsenic mobilization (Burton et al 2008). Similar results were observed by Andreas-Weber et al when they flooded soil microcosms and achieved anoxic environments (Andreas-Weber et al 2010).

Because aquaculture plays an important role in Bangladesh economy and nutritional needs of the people, it is important to study the effect of aquaculture practices on groundwater contamination so that proper changes can be made to minimize arsenic release. Typically aquaculture is practiced with applications of manure and/or other fertilizers to supplement the system with nutrients (N and P) for maximum fish production by stimulating planktonic growth (Peker and Olah 1990, Yi et al 2004). While the nutrient content of poultry manure has been shown to be more effective at promoting fish production through increased phytoplankton and zooplankton counts (Hossain et al. 2006, FAO 2001, Yi et al 2004). However), cow dung is the most common fertilizer in use due to availability.

Not only does manure contain nutrients, it also contains NOM that can further arsenic mobilization. It is accepted that reductive dissolution of Fe(III) oxyhydroxides and consequent mobilization of sorbed arsenic is the major mechanism of arsenic accumulation in groundwater in many regions. Similarly, other work shows that reductive dissolution of Mn oxyhydroxides can also be an important mechanism for arsenic release. In phosphorus (P)-limited conditions, mineral dissolution for P solubilization (with concurrent arsenic release) is an alternative mechanism (Mailloux et al. 2009). Regardless of which of these microbial processes is taking place, NOM is needed as a C and energy source for the microbes. Studies have suggested that ponds and wetlands may provide the labile organic carbon required to As mobilization at sites in

Bangladesh (Polizzotto et al. 2006; Neumann et al. 2010) and Cambodia (Brenner et al. 2008; Kocar et al. 2008; Polizzotto et al. 2008; Neumann et al. 2010).

While NOM plays an important role in stimulating the organisms that mobilize As, it also affects the fate and transport of As through interactions with mineral surfaces and with As itself. NOM can enhance the solubility of As through complexation, and the extent of complexation varies greatly among NOM samples depending on the origin and cationic metal content of the NOM (Redman et al. 2002). Cow manure characterized for NOM showed a notable presence of ionically-exchangeable functional groups including carboxyl groups, which has been shown to drive complexation and result in higher levels of aqueous arsenic (Lin et al 20XX, Tessema & Kosmus 2001, Sharma 2011). Purified fulvic and humic acids (FA, HA) have been studied and their affects on arsenic compared, showing FA being more effective in mobilizing arsenic, partially due to higher carboxylic content in FA than in HA (Weng et al 2009, Sharma et al 2011).

While few studies are conducted with environmental NOM, our previous research has also shown that both manure, in particular cow manure, increased As-NOM complexation. Similarly experiments using NOM extracted from one-year old sediment that has been treated with cow manure resulted in higher complexation than that using NOM from new untreated sediment. Additonally, Williams et al conducted a study specifically in Bangladesh investigating the effects of cow dung being added to rice paddies and showed that arsenic was notably released and suggested this release was unrelated to iron hydroxide reduction. Sharma et al 2011 also compared the effects of environmental and purified NOM and both types of NOM resulted in decreased arsenic adsorption. Specifically, environmentally-extracted NOM was the most efficient in mobilizing arsenic and implies that studies using purified NOM is significantly underestimating NOM effects. NOM is added to contaminated sites as a remediation approach and studies on such practices have shown additional arsenic released into porewater (Bernal 2007, Moreno-Jiminez 2013).

Ionic competition has been well documented to contribute to mobilizing arsenic by displacing arsenic from binding sites in minerals and numerous reports document competition between NOM and As for sorption sites on minerals. In general, in the presence of NOM, sorption of As to solid surfaces was reduced, thereby increasing the mobility of As (Xu et al. 1998; Takahashi et al. 1999; Davis et al 2001; Grafe et al. 2002, Bauer et al 2006). Similarly, phosphorus, one of the main nutrients sought after in aquaculture practices, is a strong competitor due to its similarities with arsenic and is found in varying levels in manure (REF). Studies have shown increased released arsenic in the presence of phosphorus (Acharyya et al., 1999, Polizzotto 2006)

Conclusion

Results from this study showed significant change in host fractions for arsenic in sediment collected from an aquaculture pond in Bangladesh one year apart. The pond was fertilized and flooded during the year between sediment collections. It is likely that both occurrences affect arsenic mobilization. The aquaculture practice of fertilizing the pond to stimulate fish plays an important role in mobilizing arsenic as studies have shown increased arsenic release in the presence of manure. Due to recent implications that aquaculture ponds supply the necessary labile carbon for microbial iron hydroxide reduction, it is important to further investigate the role of ponds as a possible origin for contaminated aquifer recharge and labile organic matter contributing to higher dissolved arsenic levels. It is important to delve further into aquaculture techniques and other variables that affect these ponds to better develop agricultural and aquacultural practices that do not exacerbate arsenic contamination in the groundwater.

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Chapter 4 - Arsenic binding to NOM in cow and chicken fecal matter: relevance to mobilization of arsenic in Bangladesh groundwater

Abstract

Groundwater contamination by As is a serious public health concern, particularly in Bangladesh. This study experimentally determined conditional distribution coefficients and apparent stability constants between As^{III} and NOM from cow dung, chicken dung, Bangladeshi unfertilized pond sediment, and Bangladeshi fertilized pond sediment. The data showed that cow dung resulted in the most As^{III}-NOM complexes. Cow dung fertilized sediment from Bangladesh showed greater binding abilities than unfertilized sediment from Bangladesh, suggesting practices of treating aquaculture ponds with cow dung contributes to As^{III} mobilization. Phreeqcl modeling using constants derived from the experiments also support significant As^{III}-NOM complexation. Applying site-specific constants to geochemical models will better predict As speciation for the field site as previous studies that used purified NOM considerably underestimate actual effects by environmental NOM.

Introduction

Arsenic (As) contamination of groundwater is a worldwide problem that has affected millions of people. This is particularly true in the West Bengal region of Bangladesh, resulting in health concerns such as arsenicosis and cancer (VanGeen 2008). Arsenic mobilization is particularly affected by the presence of natural organic matter (NOM), thus it is important to study ways in which NOM interacts with As.

While NOM plays an important role in stimulating the organisms that mobilize As, it also affects the fate and transport of As through interactions with mineral surfaces and with As itself.

NOM has been observed to bind As and reduce As mobility by forming colloids, however some NOM can clearly enhance As mobilization. Research is just beginning to unravel the intricacies of the effects of NOM on As, including stimulating microbially-mediated As-bearing mineral reduction, complexation, competition for mineral sorption sites, alteration of available surfaces through coating minerals, and redox chemistry (Harvey et al. 2006, Bauer and Blodau 2008, Redman et al 2002, Davis et al 2001).

Recent studies have shown that NOM can enhance As solubility through complexation (Buschmann et al 2006, Wang et al 2009, Liu et al 2013, Redman et al 2002). Not only can negatively-charged NOM bind with cations including arsenic, functional groups in NOM such as carboxyl groups can also complex with arsenic (Stevenson 1992, Mcknight et al 1992, Wang and Mulligan 2006, Tessema & Kosmus 2001, Sharma et al 2011, Kar et al 2011). Additionally, there are very few studies on the role of other ions on the binding between As and NOM, and it appears that other ions can either decrease As binding to NOM through competition or increase it by acting as bridging ions. While studies have mentioned cation bridging of As and NOM could enhance complexation, little experimental evidence have been shown to illustrate the potential importance of such interactions.

Arsenic is present in the environment typically as arsenate (As^{V}) or arsenite (As^{III}). In anaerobic conditions such as Bangladesh, arsenite is more relevant and exists mostly as uncharged H_3AsO_3 until the first pKa at 9.2. Arsenate exists mostly as anionic H_3AsO_4 in environmental waters as the first pKa is 2.2. The second pKa for As^{V} is 6.9 so at circumneutral pH, arsenate will be a mix of $H_2AsO_4^-$ and $HAsO_4^{-2}$. A mix of oxidation states is often seen, partly due to aerobic waters and to the presence of microbes that can both reduce and oxidize arsenic. Minerals with solid arsenic can be reduced and release dissolved As^{III}. Regardless of speciation, both As^{V} and As^{III} are known to sorb to iron minerals including ferrihydrite and

goethite. Aluminum, manganese, and other ions can compete for sorption sites as well as bridge arsenic and NOM to increase mobilization. A charged species of arsenic can also contribute to NOM and As interactions that lead to arsenic mobilization. This study will focus on complexation of As^{III} and DOM.

Gaps remain in our understanding of the origin of water and organic matter recharging to contaminated aquifers. Recent work has shown that ponds constructed for aquaculture in Bangladesh and may be an important source of recharge water containing biologically degradable organic carbon (BDOC) that may mobilize As (Neumann et al. 2009, 2010). Additionally, cow manure is added to the ponds to supply nutrients to stimulate fish production, however fertilizers, such as cow dung, contain significant amounts of OM, which can promote As release. However, little research yet has investigated the role of different types of organic matter in pond recharge on As mobilization from pond sediments (REFS?).

Geochemical modeling can be an important and useful tool in predicting how various factors may impact As release. It is important to apply experimental data to models to better predict how existing agricultural practices and other variables may be enhancing As mobilization into groundwater. Existing models mostly use idealized systems with data from purified NOM and are currently lacking environmental binding constants, which could significantly underestimate actual effects of environmental NOM (Sharma et al 2011).

Studies have implied that arsenic mobilization and the presence of organic matter are related, as is exhibited in many countries worldwide (Liu et al 2013). Studies in Taiwan have suggested that arsenic and humic substance binding lead to health conditions, specifically Blackfoot diease (Lu et al 1990, Wu et al 1978). Consequently, it is important to further investigate the role of NOM in As mobilization.

In this study, we (1) measure and compare conditional As binding constants distribution coefficients (K_D) and binding constants (K_S) for As^{III} with NOM originating from cow dung, chicken dung, and pond sediments from a constructed pond in Bangladesh pre and post fertilization. These parameters will provide complementary information regarding the quantitative capacity of DOM to bind As^{III} and the quantitative stability of the resulting complexes based on a stoichiometric view of As^{III} and DOM interaction, respectively. We will also (2) characterize functional groups in the various types of NOM and (3) test importance of the predicted As^{III}-NOM complexes in speciation models of pond charge at a Bangladeshi field site under differing fertilization schemes.

Methods

Field Site. Environmental NOM samples were extracted from a constructed pond at the village of Srinigar in the Munshiganj district of Bangladesh, approximately 25 km south of Dhaka. Land use in the area is primarily agricultural, rice cultivation in particular. Rice fields and aquaculture ponds cover the majority of the land area. Collaborators from MIT excavated and constructed a new pond to serve as an important site for studying the importance of ponds in arsenic mobilization.

Sediment cores were collected using an AMS Multi-Stage Soil Core Sampler. Cores were capped, sealed, double-bagged in low gas permeable Escal bags containing RP oxygen absorbing agents (Mitsubishi Gas Company), and shipped on ice to UCLA. Cores were collected in six-inch section reaching a depth of five feet after it was first constructed (prior to a monsoon season) and again after 1 year (after the pond was fertilized with cow dung and a monsoon season has passed).

NOM Extraction. NOM was extracted from four samples (two dung, and two environmental). Dung was collected fresh from chicken and cow located at a local animal

sanctuary (Gentle Barn, CA). Sediment collected at the depth of 2 to 2.5 feet from the Bangladeshi aquaculture pond one year apart was also used. Aliquots of each organic matter source were shaken for 5 minutes with ultrapure Milli-Q water (Millipore) to extract loosely-bound NOM between the range of 90 and 110 mg C/L. The supernatant was filtered (0.45 um), deoxygenated, and stored in serum bottles capped with Teflon-coated stoppers at 4^{o}C.

NOM Characterization. In order to probe the nature of humic substances present, NOM samples were dissolved and separated using ultrafiltration. Then, the dissolved fractions larger and smaller than 5kDa, referred to as gf and uf, respectively, were probed using the polarity rapid assessment method (PRAM) (Rosario-Ortiz et al. 2007; Rosario-Ortiz et al. 2009, Singer et al. 2007), in which samples are run in parallel through solid phase extraction (SPE) cartridges: here C18 was used as a nonpolar SPE representing hydrophobic substances, while diol and amino were used as polar (hydrophilic) and anionic (charged/ion exchange) SPEs, respectively. In each case, fractions of substances similar to respective SPEs are retained on the cartridges and carbon levels of all fractions are measured via UVA_{254}. Total organic carbon (TOC) analysis was also conducted using catalytically-aided Pt 680° C combustion/non dispersive infrared (NDIR) method and a Shimadzu 5050 TOC analyzer. Retention coefficients (RC) were calculated (Equation 1) for each extraction and represents the fraction retained by each sorbent. $C_{breakthrough}$ is the maximum breakthrough concentration of the sample and C_0 is the initial sample concentration.

$$RC = \frac{1 - C_{breakthrough}}{C_0} \quad (1)$$

Nutrient Analysis. Nitrogen and phosphorus content in cow and chicken dung were quantified through Hach Company TNTplus analytical kits and spectrophotometric methods. Nitrogen associated with nitrate and ammonia were measured using dimethylphenol and salicylate methods respectively, while phosphorus from phosphates was measured using an ascorbic acid method (Hach Company, 2005).

Dialysis Experiment. Conditional distribution coefficients (K_D) and apparent stability constants (K_S) were determined through a modified method from Bushmann et al 2006 using dialysis experiments with DOM and As^{III}. 500 Da dialysis tubing (Spectra/Por Biotech Cellulose Ester (CE) membrane) containing 100 mg DOC/L DOM (size > 500 Da) extracted from one of the four NOM sources was equilibrated inside acid-washed serum bottles filled with a deoxygenate 0-2000 ug/L As^{III} solution (Fisherbrand sodium arsenite, pH 7, 0.05M NaCl, 0.15 mM NaN_3, and 1 mM $NaHCO_3$ and then capped with Teflon-coated stoppers to hold the two ends of the dialysis tubing in place, while maintaining an anaerobic environment.

DOM and As^{III}-DOM complexes remain inside the tubing, unable to pass through the 500 Da membrane. Total As inside and outside of the tubing were quantified by Graphite Furnace Atomic Absorbance Spectometry (GFAAS).

K_D, which represents the ability for As and DOM binding, was calculated by Eqn. 2 (Buschmann et al, 2006), where K_D is given in L/kg, $[As^{III}]_f$ and $[As^{III}]_b$, the free and bound concentrations of As^{III}, in µg/L, and [DOC], the concentration of DOC in solution, in kg/L.

$$K_D = \frac{[As]_b}{[As]_f \cdot [DOC]} \quad (2)$$

K_S, which characterizes the stability of the complexes in solution, was estimated by Eqn. 3 where K_S is given in M^{-1}, C_f and C_b, the free and bound concentrations of As^{III}, in M, and C_{DOM}, the concentration of effective ligands in DOM, in M.

$$K_S = \frac{C_b^{As}}{C_f^{As} \cdot C_{DOM}} \quad (3)$$

However, C_{DOM} is typically unknown because the molecular structure of DOM is typically unclear. The Scatchard plot, given as Eqn. 4, is often used to overcome the uncertainty of C_{DOM}. In this form, C_b/C_f is plotted against C_b, allowing K_S and B_{max}, the maximum binding capability of DOM towards As^{III} in mol/L, to be estimated.

$$\frac{C_b^{As}}{C_f^{As}} = -K_S \cdot C_b^{As} + K_S \cdot B_{\max} \quad (4)$$

Geochemical Modeling. Observed K_S from the dialysis experiments were used to geochemical model As speciation in pond water in the presence and absence of DOM using the Phreeqcl software. Pond water chemistry was obtained from the field site by Neumann et al 2009.

Cation Competition and Bridging Experiment. Experiments were conducted in the presence of calcium chloride (Fisher) to explore cation effects on arsenic binding in a system with DOC (10 mg C/L) and 0-1000 ug/L As^{III} and As^{V}. Dialysis experiments, as described above, were modified to include three environmentally-relevant concentrations of calcium (10, 50, 100 mg Ca/L) in the exterior solution. Free and bound As^{III} and As^{V} were quantified by GFAAS.

Results

NOM Characterization. This work presents a comparison of the As binding capability of NOM in cow dung, poultry dung and sediment; further, we evaluate characteristics of the NOM in order to explain observed differences in complexation constants.

Characterization of chicken dung and cow dung suggest that chicken dung is richer in carbon content than cow dung, however sediment-extracted DOM contains notably less carbon than either types of dung (see Table 1). Nutrient analysis showed that cow dung had nearly five times as much P and just over five times as much NO_3-N as chicken dung, which had 60% more NH_3-N (see Table 2).

Table 4-1 Organic Carbon Content of Dissolved Organic Matter

Organic Matter	TOC/OM (mg/g)

Cow Dung	28.0
Chicken Dung	62.5
Sediment Year 0	0.89
Sediment Year 1	0.98

Table 4-2 Nutrient Content of Dissolved Organic Matter from Dung (mg/L)

	Nitrate-N	Ammonia-N	Phosphate-P
Cow Dung	0.69	1.53	3.18
Chicken Dung	0.13	2.47	0.66

PRAM analysis showed that there are functional differences between cow and chicken dung larger than 5 kDa. The size fractions below 5 kDa show relatively similar polarity signatures according to retention coefficients, defined as 1- ($C_{breakthrough}/C_0$), for each type of SPE cartridge. Dissolved cow dung greater than 5kDa had six times more polar fractions than dissolved chicken dung. In the same size fraction, dissolved chicken dung contained 1.5 times more anionic fractions and twice as much nonpolar fractions than dissolved cow dung.

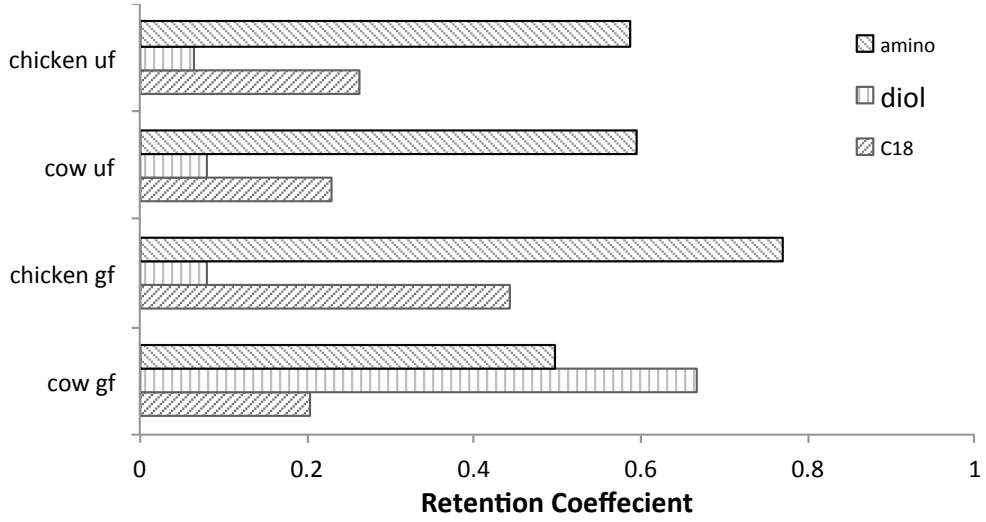
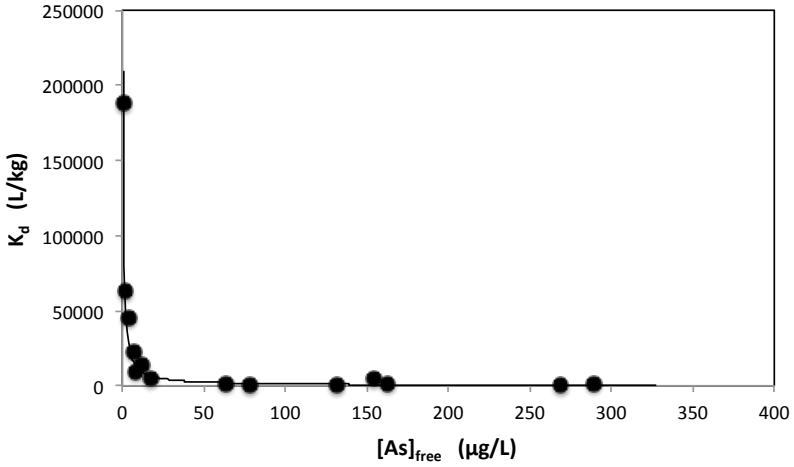
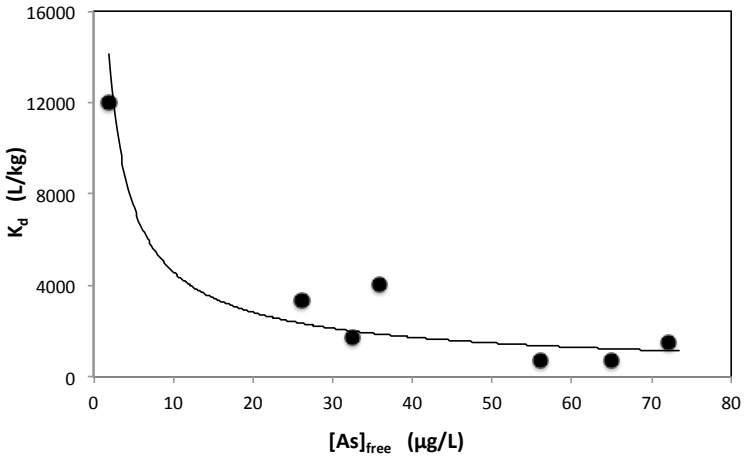


Figure 4-1 PRAM analysis of cow and chicken dung

Dialysis Experiment. Conditional distribution coefficients (KD) of AsIII and DOM ranged four orders of magnitude. Chicken dung showed the lowest ability for binding AsIII, approximately ten times less than cow dung and sediment collected from a 1-year old pond. KD for DOM extracted from sediment at a newly excavated pond is also low, nearly three times less than that of sediment from the same pond 1 year later. KD decreases as the ratio AsIII to DOC increases in all cases, however KD decreases most rapidly in cow dung and 1-year old pond sediment.





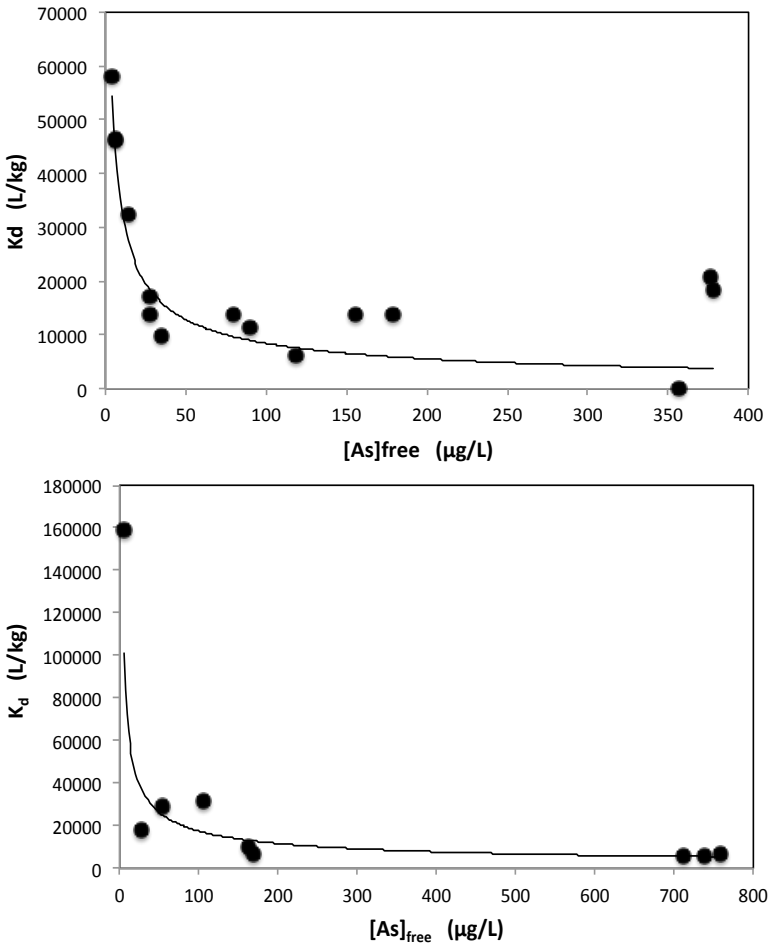
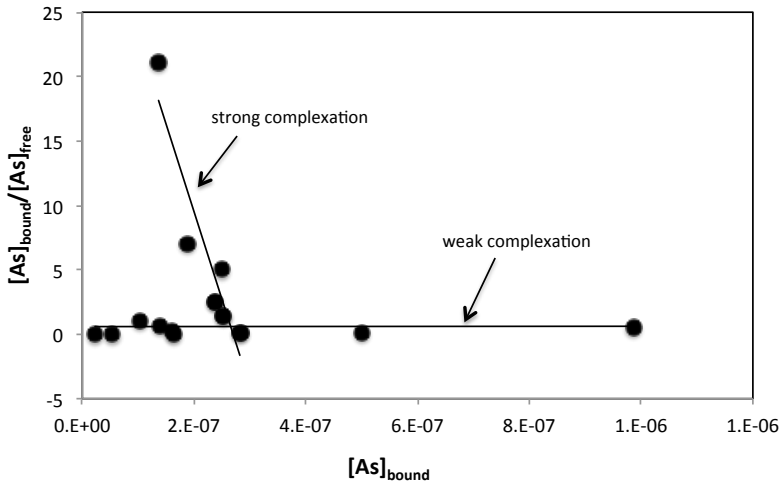
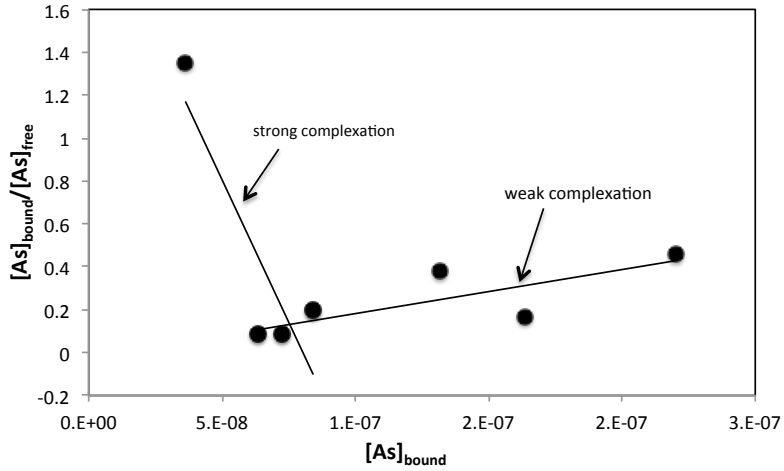


Figure 4-2 (a-d) Conditional distribution coefficients as a function of unbound AsIII for (A) cow dung, (B) chicken dung, (C) sediment-extracted NOM from a new pond, and (D) sediment-extracted NOM from the same pond 1 year later. Solid lines are fitted lines based on nonlinear regression.

When bound and free As^{III} data was linearized according to Scatchard plots, the plots showed that a single-site binding is insufficient to model the nonlinearity of the data. This suggests that a multiple-site model is necessary. A two-site model captures the data, as can be seen by two lines in Figure 3. Lower concentrations of As^{III} resulted generally in higher K_S, indicating stronger complexation between As^{III} and DOM. Lower apparent stability at higher As^{III} concentrations suggests the presence of weak complexation. In the case of 1-year old

sediment where K_S and B_{max} were both available, there is an order of magnitude more weak binding sites than strong binding sites. This is consistent with less stability in weak complexation by As^{III} and DOM. Similar to the study by Liu and Cai, 2010, the inflection point from strong to weak complexation and its surrounding region is affected by both strong and weak complexation.





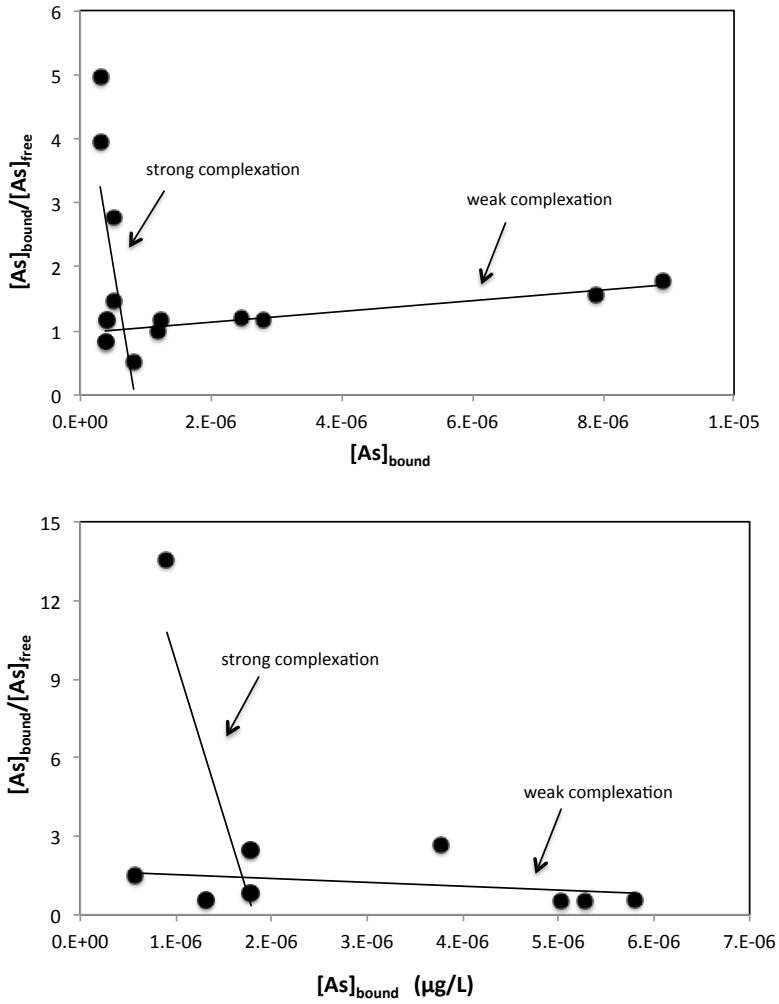


Figure 4-3 (a-d) Scatchard plots as a function of bound AsIII for (A) cow dung, (B) chicken dung, (C) sediment-extracted NOM from a new pond, and (D) sediment-extracted NOM from the same pond 1 year later. Solid lines are Scatchard lines based on nonlinear regression for two types of complexation, weak and strong.

Table 4-3 Conditional distribution coefficients (K_D), apparent stability constants (K_S), and maximum binding capabilities of As^{III} to different sources of DOM based on nonlinear regression

Organic Matter	Log K_D	Strong Complexation		Weak Complexation	
		Log K_S	Log B_{max}	Log K_S	Log B_{max}
Cow Dung	2.06 - 5.27	8.13	-6.57		

Chicken Dung	2.87 - 4.08	7.42	-7.09		-7.94
Sediment Year 0	1.63 - 4.76	6.79	-6.08		
Sediment Year 1	3.79 - 5.20	7.07	-5.74	5.17	-4.94

Cation Competition and Bridging Experiment. Results show that calcium affects arsenate and arsenite differently and effects are sensitive to calcium levels. Dialysis experiments using arsenite suggests that the medium concentration of Ca^{2+} increased arsenic binding, while higher and lower levels of Ca^{2+} did not (Figure 3). Experiments with arsenate showed little effect by all levels of Ca^{2+} on arsenic binding (Figure 3).

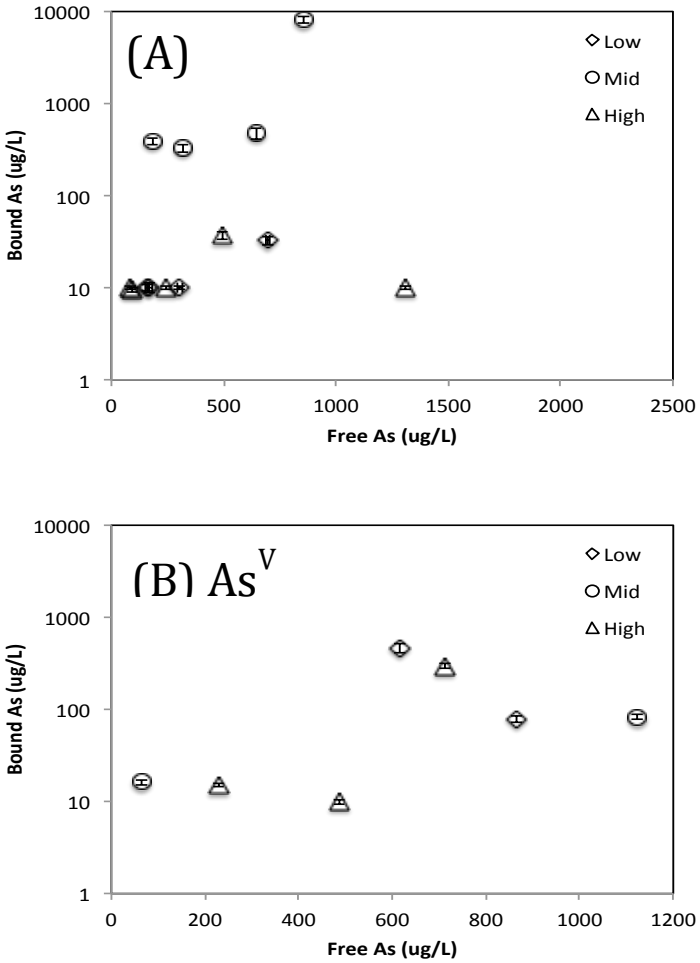


Figure 4-4 Calcium effects on NOM binding by (A) As^{III} and (B) As^{V}

Geochemical Modeling. As speciation was determined through Phreeqcl using previously characterized pond water chemisty and K_S observed from the dialysis experiments for strong complexation. Results show that in the presence of any NOM, the dominant form of As is bound to NOM by a minimum of 4 orders of magnitude. The different forms of added NOM subsequently varied the levels of As in other forms, however negligibly. When cow dung was added to the system already containing NOM from the newly excavated pond (T0) to simulate the common practice of fertilizing a new pond with cow dung in Bangladesh, Phreeqcl results showed that the As is bound mostly to cow NOM (5.2E-7 mol), then to sediment NOM (1.8E-8 mol), which is still 5 orders of magnitude greater than As not bound to either form of NOM. Similar results were obtained by inputting chicken NOM to sediment NOM from year 0. The sum of NOM-bound As in both cases total that of NOM-bound As using NOM from 1-year old sediment.

Table 4-4 Speciation of As (mol) in the Presence of Different Fertilization Schemes based on Phreeqcl Analysis

As Speciation	*Unfertilized Pond with no NOM*	*NOM from Cow Dung*	*NOM from Chicken Dung*	*Sediment NOM from Unfertilized Pond*
$H3AsO3\text{-}DOM_{sed1yr}$				
$H3AsO3\text{-}DOM_{sednew}$				5.33E-07
$H3AsO3\text{-}DOM_{chicken}$			5.33E-07	
$H3AsO3\text{-}DOM_{cow}$		5.33E-07		
H3AsO3	5.32E-07	4.25E-13	2.16E-12	1.21E-11
H2AsO3-	1.02E-09	8.12E-16	4.13E-15	2.32E-14

H4AsO3+	8.56E-14	6.83E-20	3.47E-19	1.95E-18
HAsO3-2	4.17E-15	3.33E-21	1.69E-20	9.51E-20
AsO3-3	8.74E-22	6.98E-28	3.55E-27	1.99E-26

As Speciation	*Sediment NOM from One-Year Old Pond*	*Cow Dung added to Unfertilized Pond*	*Chicken Dung added to Unfertilized Pond*
$H3AsO3\text{-}DOM_{sed1yr}$	5.33E-07		
$H3AsO3\text{-}DOM_{sednew}$		1.81E-08	8.07E-08
$H3AsO3\text{-}DOM_{chicken}$			4.53E-07
$H3AsO3\text{-}DOM_{cow}$		5.15E-07	
H3AsO3	5.78E-12	4.11E-13	1.84E-12
H2AsO3-	1.11E-14	7.84E-16	3.50E-15
H4AsO3+	9.29E-19	6.60E-20	2.95E-19
HAsO3-2	4.53E-20	3.22E-21	1.44E-20
AsO3-3	9.50E-27	6.74E-28	3.01E-27

Discussion

Mobilization of As by additions of NOM. NOM can abiotically impact As mobilization through complexation. Results from the dialysis experiments support that the presence of chicken dung and in particular cow dung and Bangladeshi sediment can increase aqueous levels of As, through binding with NOM. Studies have shown increased levels of As in solution in the presence of organic matter such as fulvic acid (FA) and humic acids (HA) (Weng et al 2009, Sharma et al 2011).

A two-site binding model was used to capture the data, suggesting weak and strong complexation occurs in the presence of As^{III} and NOM. Other studies have suggested a need for multiple-site models with Sharma et al 2011 also using a two-site model. The slopes for weak complexation by chicken dung and pond sediment at year 0 could indicate the need for more than two sites for ligand binding.

Additionally, both cow and chicken dung have notable fractions of ionically-exchangeable functional groups including carboxylic groups, which has been shown to be important to As release (Tessema & Kosmus 2001, Sharma et al 2011). FA has been indicated to be more effective than HA in releasing As due to its smaller size, higher polarity, and more carboxylic groups (Tessema & Kosmus 2001, Sharma et al 2010, Sharma et al 2011). High K_D's using cow dung in this study could be partially attributed to the large polar and amino fractions in cow dung.

The extent of complexation varies greatly among NOM samples depending on the origin and cationic metal content and functional characteristics of the NOM (Redman et al. 2002). Cow dung showed a high affinity for binding As so it is reasonable to say that the addition of cow dung can change the groundwater system and increase the binding affinity of pond soil. Yet, there have been studies where the addition of manure had little effect on As levels (Sisr 2007, Grafe 2002).

Aquaculture in Bangladesh. Aquaculture studies in Bangladesh have been optimizing type, amount, and frequency of addition of manure to deliver the most nutrients with the least risk of anoxia (Hossain et al 2006, FAO 2001, Yi et al 2004). However, as the connection between ponds and As contamination was only recently discovered, current aquaculture practice recommendations do not take into account whether conditions could favor As mobilization. It is important to note the fertilizers, such as cow dung, contain significant amounts of OM, which can promote As release.

Because NOM plays a key role in As partitioning, the amount and type of NOM in the ponds may be a determinant of downstream As mobilization. Cow dung in particular has low nutrient content and contains tannins that darken the water leading to increased light attenuation and low dissolved oxygen. Recent studies have shown that the addition of cow dung resulted in increased mobilized As, including a study by Williams et al conducted at a Bangledeshi paddy with high levels of DOC and was fertilized with cow manure. The study showed considerable increase in mobilized As not attributed to reductive dissolution of iron minerals (Williams et al 2011).

Environmental levels of NOM are often increased anthropogenically due to aquaculture practices, particularly in South and Southeast Asia, however, fertilization with NOM is also a common remediation technique that contributes to possible additional mobilizable As-NOM complexation (Bernal 2007, Moreno-Jimenez 2013). The Moreno-Jimenez study has shown that more As is released into porewater where contaminated soil is treated with compost and/or nutrients, implying ionic competition, flooding, resulting pH change, and complexation with As and other bridging ion all impact As release.

Cation Competition and Bridging Experiment. In general, in the presence of NOM, sorption of As to solid surfaces was reduced, thereby increasing the mobility of As (Xu et al. 1998; Takahashi et al. 1999; Grafe et al. 2002). In the presence of cations, such as Ca^{2+}, cation-bridging may occur further promoting As in solution (Buschmann et al 2006, Mijutta & Kretzschmar 2011). Buschmann et al conducted As and NOM sorption experiments in the presence of varying concentrations of Al (Buschmann et al 2006). These authors saw a modest decrease in K_{DOM} for As when aluminum was present, which could be due to competition for binding sites on NOM (Buschmann et al 2006). On the contrary, the mobilization of As from mine tailings has been shown to correlate strongly with co-mobilized heavy metal concentrations; the authors attribute this to heavy metals potentially acting as bridging ions (Wang and Mulligan 2009). Other studies have seen increases in As-NOM complexation in the

presence of additional cations, including Fe, Ca, Mg, Al, and Mn (Redman et al 2002, Lin et al 2004). However, the As and NOM system is complex, particularly in the presence of other cations as they can compete and complex. More studies are necessary to better understand the role of cation-bridging and to determine what ions are important such interactions.

Conclusions

In this study, levels of bound As is higher in 1-year old pond sediment than in new 0-year old pond sediment, likely due to the pond having been treated with cow dung prior to the monsoon season in the yearlong gap. It is implied that human practices to improve agriculture and nutrition in Bangladesh could have consequential impact on arsenic contamination and subsequent public health ramifications due to chronic exposure. The outcomes of this research will contribute to solving the critical scientific problem of widespread arsenic exposure to humans. Aquaculture practices have increased both the food supply and the standard of living in Bangladesh, and while there is a growing body of evidence that aquaculture ponds are linked to increased levels of arsenic in groundwater, the effects of these practices have not been well studied. Before steps are taken to alter aquaculture practices and/or fertilization remediation techniques, the link between that and As mobilization should be fully elucidated. Furthermore, recent studies have indicated that environmentally-extracted NOM is more effective than purified NOM at promoting As release, implying that current studies based on purified NOM is likely underestimating effects. Whichever mechanism is at play, the source of organic matter driving the mobilization of As is crucial to understand.

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Chapter 5 – Incorporating Service-Learning in Traditionally Lecture-based Environmental Engineering Courses Through Researching Bacterial Contamination at a Local Beach

Abstract

The objective of this study was to determine the efficacy of an optional 1-2 unit service learning (SL) course added onto two undergraduate Civil and Environmental Engineering classes: C&EE 154: Fate and Transport of Chemicals in the Environment and C&EE 166/266: Environmental Microbiology. The SL add-on aimed to increase participant understanding of and interest in local environmental science issues relevant to the course material and consisted of classroom visits to a partnering middle school class, collaborative environmental field research to test student-generated hypotheses, and presentations of the results at the university. Letter writing about environmental issues was included as a political engagement opportunity for the middle school students some years. While the add-on has been offered for since 2002-2011, pre- and post- surveys were administered to assess the effect of the SL on middle school and university student awareness of different issues in 2008 and 2009. The SL component resulted in self-reported increase in undergraduates feeling more informed about politics, feeling more that they have a say in government, and feeling more interested in management as a career choice. A similar survey was administered to middle school students, whose results were less consistent across both years. In the post-survey in 2008, middle school students' thought that an understanding of science was more important, that they were more interested in the local environment, that they were better informed about politics, and the public officials cared more about issues than in the pre-surveys. These results show that even the addition of an optional service learning component can provide value to both the community and the undergraduate students through increased understanding of local environmental issues, and how scientific topics such as microbiology or chemical fate and transport can answer questions about broadly important topics such as recreational water quality.

Introduction

Over the past ten years (From 2002- 2012), we have offered service-learning (SL) opportunities for undergraduate students in Civil and Environmental Engineering at the University of California, Los Angeles (UCLA) through collaborative research partnerships with local K-12 classrooms. The SL involvement has taken two forms. In the first form, which was offered from 2002 to 2011, SL was offered an optional add-on to a traditional lecture-based course for any enrolled students who were interested in partaking. In two of those years, 2008 and 2009, pre- and post-activity surveys were administered to assess the efficacy of these add-ons as an added benefit to the undergraduate and middle school students. Human Subjects Internal Review Board approved the survey for both university undergraduates and middle school students. The other SL form was begun in Winter Quarter 2012, in which we offered a designated SL course (which, as of 2012, was the available SL course in the Henry Samueli School of Engineering at UCLA) in which the SL was an integral and required part of the course (Jay, 2012, submitted).

Service learning (SL) is a form of experiential learning that integrates academic subject matter with community needs; key elements include reciprocity, reflection, and a community voice in projects (Shastri, 2000; Brandell and Hinck, 1997). SL courses have been shown to improve mastery of technical objectives and critical thinking skills (Shastri, 2000; Cawthorn et al., 2011; Mpofu, 2007; Peters, 2011; Strage, 2000). In addition, students in SL courses have reported a higher course satisfaction (Evangelopoulos et al., 2003) and improved attitudes about the subject matter (Packer, 2009). Having been well-established over the last three decades in other disciplines, SL is now increasingly being adopted in engineering (Coyle et al., 1997; Mehta and Sukumaran, 2007; Beach et al., 2007; Zhang et al., 2007; Riley and Bloomgarden, 2006; Cline and Kroth, 2008). Partnerships between universities and K-12 classrooms offer educational and mentoring benefits for students at all levels, particularly when

the SL addresses a community needs and material is integrated into the curricula (Lima, 2004; Moskal et al., 2007; Moskal and Skokan, 2011).

One important benefit of SL is that it helps students navigate complicate novel concepts through educating others and experiencing a real-world context for the course material (Zlotkowski, 2012). In pre- and post- activity surveys, SL components have been shown to increase students' understanding of course concepts (Cawthorn et al., 2011) or to increase their feeling that they made progress toward gaining knowledge (Packer, 2009). In addition to greater understanding of the course material, SL can increase more intangible benefits for students as well. Student ratings of the field of study, course, and instructor all increased after an SL component (Packer, 2009), and SL components show some impact on behavior and appreciation of the environment when that is included as a focus of the SL activity (Cawthorn et al., 2011; Packer, 2009).

One additional aspect that was incorporated into these SL add-ons was that of community-based research (CBR), which involves collaboration on research projects between faculty, students, and community partners. CBR is a growing and significant component of the community-engagement efforts at institutes of higher learning (Stocking and Cutforth, 2006; Polanyi and Cockburn, 2003; Weinberg, 2003). One of the basic principles of CBR is that it is a true collaboration between the involved parties, rather than the community serving as a "lab" for academic research.

In order to bring in the practices of both CBR and SL into our department, the add-on course was structured so that university students would visit middle school classes to develop research ideas in collaboration with the middle school students rather than providing the hypotheses. Under the guidance of university students, middle school students worked together in teams to develop hypotheses, conduct research at local field sites and make posters of the results. Middle school students were then invited to UCLA to tour the campus and present their results at a poster session. The objectives of the SL program were to increase interest in

science and environmental issues among the middle school students. There were mentoring benefits as well for both the middle school students and the university students, due to the focus on small group work with consistent mentors. This study focuses on the addition of an optional service learning lab that can be added to a required course, which is different from other studies that look at a service learning component integrated into a course.

In this paper, we will: 1) give an overview of this add-on model for incorporating SL in traditionally lecture based environmental engineering courses at UCLA; 2) present some of the experimental results obtained at a field site by middle school students involved in this SL project; 3) describe two years of results of an IRB-approved survey of participating middle school students; and 4) present excerpts from reflections from university students working with middle school students.

Methods and Program Overview

Partnering schools. For the optional add-on model for SL, we worked with two middle schools: St. Francis X. Cabrini and St. Anne's Schools. Both schools serve a population that is over 95% Latino with 70% of the students qualifying for reduced hot lunch. Schools were chosen based on both demographics, as one goal of the program was to encourage students from minority groups underrepresented in science and engineering to pursue this field, and the need for supplementary resources for science education.

Description of Add-on Model of SL. For 10 years, a subset (between 12-30) of UCLA students in CEE166A Environmental Microbiology or CEE154 Chemical Fate and Transport in Aqueous Environments also enrolled in optional one- or two-unit course to work with middle school students on an environmental research project. First, these university students were given some background in local coastal water quality and required to read a literature paper on this topic. They were then trained by graduate student researchers in the instructor's lab in the techniques they would be required to teach to the middle school students. The main technique

required for this project was to measure fecal indicator bacteria (FIB), which are used to assess the microbial quality of recreational waters. After learning the needed laboratory techniques and background information, university students participated in a series of four weekly sessions with middle school students to share this knowledge with them.

Session 1. At the first meeting, which occurred at the middle school, undergraduate students were joined with small groups of middle school students who would be their students for the remainder of the service-learning component. For the first twenty minutes of the hour-long visit, undergraduates discussed concepts in microbiology with their groups and led the microbe safari, which is a self-directed experiment where middle school students collected bacteria from the environment and cultured (further described below). This 'icebreaker' activity provided a hands-on or tangible opportunity to familiarize middle school students with general microbial techniques prior to the water quality-oriented project of collecting and culturing specific bacteria from the environment to answer a scientific question.

For the microbe safari exercise, each seventh grader was given a Petri dish to grow bacteria from two samples, such as from their hand before and after washing, or from the bottom of their shoes and socks. Middle school students were encouraged to propose simple hypotheses, such as "My hands will have fewer microbes after I wash my hands compared to before" or "the bottom of my shoes will be much dirtier than my hands". Creativity was encouraged and prizes were sometimes offered for the plates that grew the most bacteria or that showed the most interesting results.

For the remainder of the one-hour session after middle school students collected their microbe safari samples, each group of university and middle school students discussed the beach water quality project, that would be the main focus of the class thereafter. Together, they studied aerial photos of the field sites that would be sampled (locations included Mother's Beach in Marina del Rey, the Santa Monica Pier, and a large storm drain on Santa Monica Beach) and discussed known information about bacteria levels in water and sand at that site. Previous field

research in the lab had found the areas underneath Santa Monica Pier and close to the storm drain to be consistently contaminated with FIB, with concentrations generally decreasing with distance from the Pier or storm drain. Undergraduates also presented general information about FIB, including their increased ability to survive in sand over water due to factors such as decreased solar inactivation (Sinton, 1999) or increased protection from predators (Brettar and Holfe, 1992).

Following these presentations, each middle school group brainstormed (with guidance from undergraduate group leaders as needed) to come up with their research question, hypothesis (See Table 5-1), and research approach. University students familiarized middle school students with the materials that would be used during sample measurement at the beach field site (field kits containing measurement materials were brought to the class). Field kits were comprised mainly of easily purchasable items such as small spray bottles to sterilize with ethanol, measuring spoons to add the correct amount of sand for processing, a plastic box with lid to hold kit, and instant hand sanitizing gel to clean up after processing the samples.

Session 2. For the next session, university students met the middle school students at the field site where they spent two hours collecting and analyzing samples. Middle school students gained hands-on experience processing and analyzing samples at the beach. To measure *Escherichia coli* and enterococci in water samples, middle school students diluted water collected from the sites and added a reagent packet (Colilert or Enterolert, IDEXX) to the bottle. Each reagent packet contained a species (or genus) specific formulation of nutrients that would initiate growth, which could then be used to quantify concentration of bacteria in a sample. For sand samples, students weighed and transferred a known quantity of sand into a sample container, and eluted bacteria from the sand by adding a saline buffer to the container and shaking it in a manner similar to that described by Cao et al. (2012). This standardized protocol essentially mobilizes bacteria into the buffer, which can then be mixed with the IDEXX

reagent packet and induced to grow. After an overnight incubation step, university students were able to complete the analysis in lab and determine bacterial density for all samples.

Session 3. The following week, university students shared sample results and assisted middle school students with data analysis and poster-making. Middle school students worked with university students to graph the data their group had obtained. In some cases, data were shared among groups for related research questions so data could be analyzed together. Students also chose photos from the field site and sample-processing events, wrote the text to put into the PowerPoint slides, and decided the format and layout of the information and data on the poster. Each group created one PowerPoint slide to be printed as a large (2' by 3') poster.

Session 4. Middle school students visited UCLA to present results from the field day. A typical schedule for the visit was as follows: 9:30-10:30: Panel on college life. University students served as the panel and middle school students were free to ask questions on any topics, including pathways to college or what life was like as a college student. Even though the students had been getting to know each other informally all quarter, this provided an opportunity for a lively discussion guided by the interests of the middle school students. 10:30-11:30: Students toured campus together in their small groups. Tours were tailored to the students' interests, with locations including the dorms, Powell library, and research laboratories. 11:30-12: Lunch. 12-1: Poster session at which the middle school students were able to present their findings to a broad range of university faculty and students from many departments.

Post-activity letter writing. Middle school students wrote letters to the mayor about the importance of coastal water quality and walked the letters to City Hall. Both the letter writing and the response from the mayor came well after the end of the course; thus, any potential benefit from this engagement activity would not be reflected in the course surveys.

Survey Questions. Pre- and post-activity surveys were developed and administered for two years of the program (See Appendix A). Undergraduate students were asked about the likelihood they would pursue one of the following careers: Engineer for a private company;

Engineer in the public sector; K-12 teacher; College/university professor; or management. Middle school students were asked to rate their agreement with the following science-themed statements: I consider myself to be very interested in science; I feel that an understanding of science is important for just about everyone; I feel that clean drinking water (same question was also asked re: recreational water) is an issue that concerns me and that is worth my time to be involved in; I am interested in local environmental issues; I am interested in global environmental issues. Both undergraduate and middle school students were asked to rate their agreement with the following policy-themed statements: 1) I consider myself to be well qualified to participate in politics and/or community issues; 2) I feel that I have a pretty good understanding of the important political issues facing the country; 3) I feel that I could do as good a job in public office as other people; 4) I think that I am better informed about politics and government than other people; 5) Public officials don't care much about what people like me think; 6) People like me don't have any say about what the government does; 7) Sometimes politics and government seem so complicated that a person like me can't understand what's going on.

Statistics. Survey answers from undergraduate students regarding career choices were transformed into numbers for the purposes of data analyses on a scale from 1-5 (See Table 5-2.) Differences between pre- and post-activity answers were determined through a Repeated Measures ANOVA with a Greenhouse Geisser correction using SPSS statistical software (SPSS Inc., Chicago, IL).

Results and Assessment of SL program

Quantitative Evidence: Pre- and Post- Survey Results. Pre- and post- activity survey results were examined in each year to assess the impact of the SL component on the students' opinions of various scientific and political issues as well as impacts on their visions of future careers (Figure 5-1). Of the 12 questions asked of undergraduate students in both years, only

four were statistically significantly different, two in each year. Undergraduate students in 2008 felt that they were better informed about politics and government than most (p=0.056) and that they had more say in the government (p=0.069) after the SL activity. In 2009, undergraduate ratings (n=10) for both their likelihood to go into management ($F(1,9) = 6$, $p= 0.037$) and their feeling that politics was very complicated ($F(1,9) = 9$, $p= 0.015$) increased significantly from the initial to the final survey. It is interesting to note that undergraduate students both felt that they were now better informed than their peers about politics and that politics was more complicated after the SL activity. Often a better understanding of material leads to a feeling that it is more complicated because more of the nuances become visible to a better-informed party. For all four of these categories, the same trends were observed across both years, although they were only statistically significant in the one discussed above.

Interestingly, although trends were consistent across years for the undergraduate students, that was not the case for middle school students. In 2008, middle school student pre- and post-activity survey results (n = 20) were analyzed, and ratings for both the importance of science (p=0.054) and for interest in the local environment (p=0.072) had increased significantly from the initial to the final survey. In 2008, middle school students felt they were better informed about politics (p=0.058) and thought that public officials cared more (p=.061) after the session. In 2009, middle school students (n=25) felt that politics was less complicated after completing this activity (p=0.076).

Qualitative Evidence – Excerpts from Undergraduate Open-Ended Survey Questions 2009. In order to glean a qualitative understanding of the effects of this project on the students, surveys were read through to assess overall student opinions. Although middle-school students were also asked about future career choices and what, if anything, had changed in their answers, no visible trends emerged in reading through career choices. Additionally, none of the middle-school students addressed the question about changes through the course of the SL experience. The following statements are about the undergraduate responses to the surveys in

2008 and 2009. Overall, students had a positive view of the course and the SL portion. Among other questions, students were asked to read through their pre-survey answers after answering the post-survey and analyze their answers. If anything had changed, they were asked what had changed and what, if anything, they did in the class they thought might have changed their answers. Students who responded to that question on the survey were very pleased with the results of the lab class, regardless of whether their answers had changed over the course of the class or not. One undergraduate wrote, "nothing changed in my answers [to the survey questions], but the lab class was a very rewarding experience and I would do something like it again, even if there was no school credit." In some cases, this course helped students refine their career goals from broadly within the environmental sector to more specifically environmental engineering or water resources. The reflection question in the post-survey also offered undergraduates a chance to think about what had changed in their survey answers after participation in SL, and why they thought their answers might have changed. One student wrote: "My likelihood of being a K-12 teacher increased slightly. Maybe the fact that we worked with students and my interaction with them helped change this. For some students, this course also fostered an understanding and appreciation of the course material because they learned it in an application that was interesting and relevant to an environmental / public health question. "[After this experience] I have a new appreciation for microbiology because I see a lot of how the environment is affected by microbiological things."

Another interesting facet that emerged from looking through the undergraduate pre- and post-surveys resulted from the question: Do you think you will be a civically engaged citizen in the future (Yes/No)? If yes, what kinds of things will you do as a civically engaged citizen. Most undergraduates responded yes to this question, although a few said no both before and after the SL activity. Undergraduate responses in terms of what they would do reflected their enjoyment of the course. Responses included an increased interest in working with K-12 students from seeing their enthusiasm, a feeling that they understand better how much there is

to know about microbiology and water quality, and that working on this project with kids increased their interest in the research and material. The results in politics were mixed, with some students feeling more informed about politics and others feeling like they were less qualified for politics or public office. This mixed result was likely an artifact of less time spent on the political outreach portion of the SL project than the scientific research portion. Nonetheless, that even a few students felt more empowered to engage in local politics and more informed even with such a small element of policy-oriented activity is very promising to further develop this portion of the SL component. It can also be observed that participating in a service learning / community outreach project helped some students clarify and refine the ways in which they wanted to be a civically engaged citizen in their lives (See Table 5-3).

Conclusion

This work shows that a SL program can be added as an optional component to a traditionally lecture-based class and that even a modest addition resulted in beneficial impacts on both undergraduates and middle school students. UCLA students were able to gain mentoring experience and develop relationships with middle school students through repeated sessions working in the same small groups. Hypothesis-driven environmental research was the focal point of the work, and students gained experience in analyzing and presenting results. Even a relatively small intervention was able to show statistically significant gains in the level of interest in science of participating middle school students.

Table 5-1 Research hypotheses generated by student groups after a brief background on contamination of the site

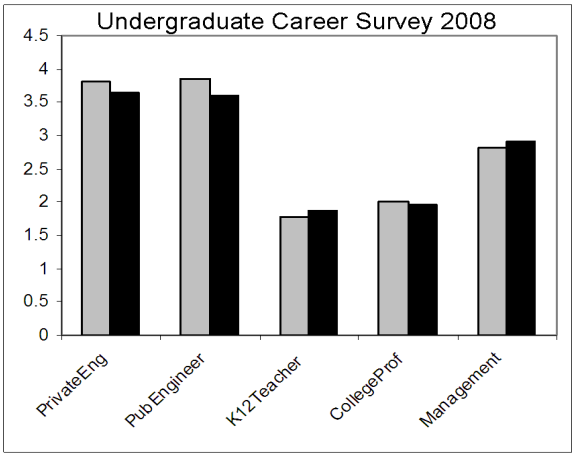
Group	Hypotheses Created by Student Groups for Research Projects
1	We investigated the hypothesis that bacterial levels would be higher near the storm drain outlet. We also wanted to find out if bacterial levels in the sand under the pier, since it is always shady, were higher than sand exposed to sunlight.
2	Bacteria levels will decrease with depth because it exposure to the water decreases.
3	We believe that bacteria levels are higher underneath the pier because there is no sunlight and there are bird droppings adding to the contamination.
4	We investigated the hypothesis that bacteria were coming from a small pool under the pier, caused by a storm drain.
5	We investigated the question: are there any bacteria in the sand and how much is in the sand?

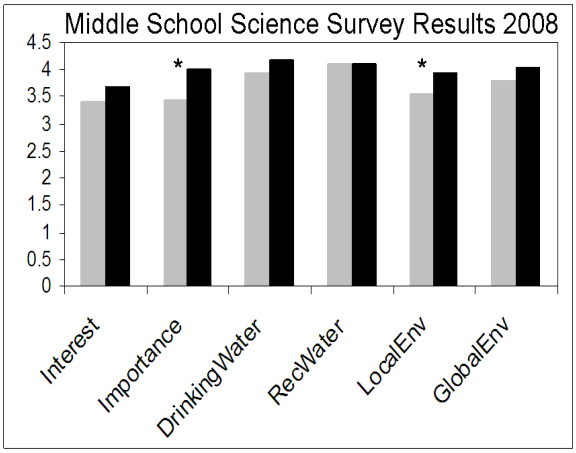
Table 5-2 Numerical ranking of qualitative survey answer options

Career answer options	Science/Policy answer options	Number
Highly unlikely	Strongly disagree	1
Unlikely	Somewhat agree	2
Maybe	Neither agree nor disagree	3
Likely	Somewhat disagree	4
Highly likely	Strongly Disagree	5

Table 5-3 First and Last Week responses to the Survey Question: What kinds of things will you do as a civically engaged citizen?

Student	First Week	Last Week
1	Strive to make socially and environmentally responsible choices in both my personal and professional lives.	Try to stay informed on political/social issues. Take action when I can, volunteer to help.
2	Help the underpriveledged (SIC) / those in need.	Help out in the community and vote.
3	I will likely be working with members / leaders of cities to help build infrastructure.	Mentoring students and aware of societal concerns as an engineer.
4	Vote, work on community projects, be involved.	Get involved and work on community projects that benefit everyone, especially environmentally.
5	Health care.	I would help the underserved community of LA or whichever city I end up residing in.





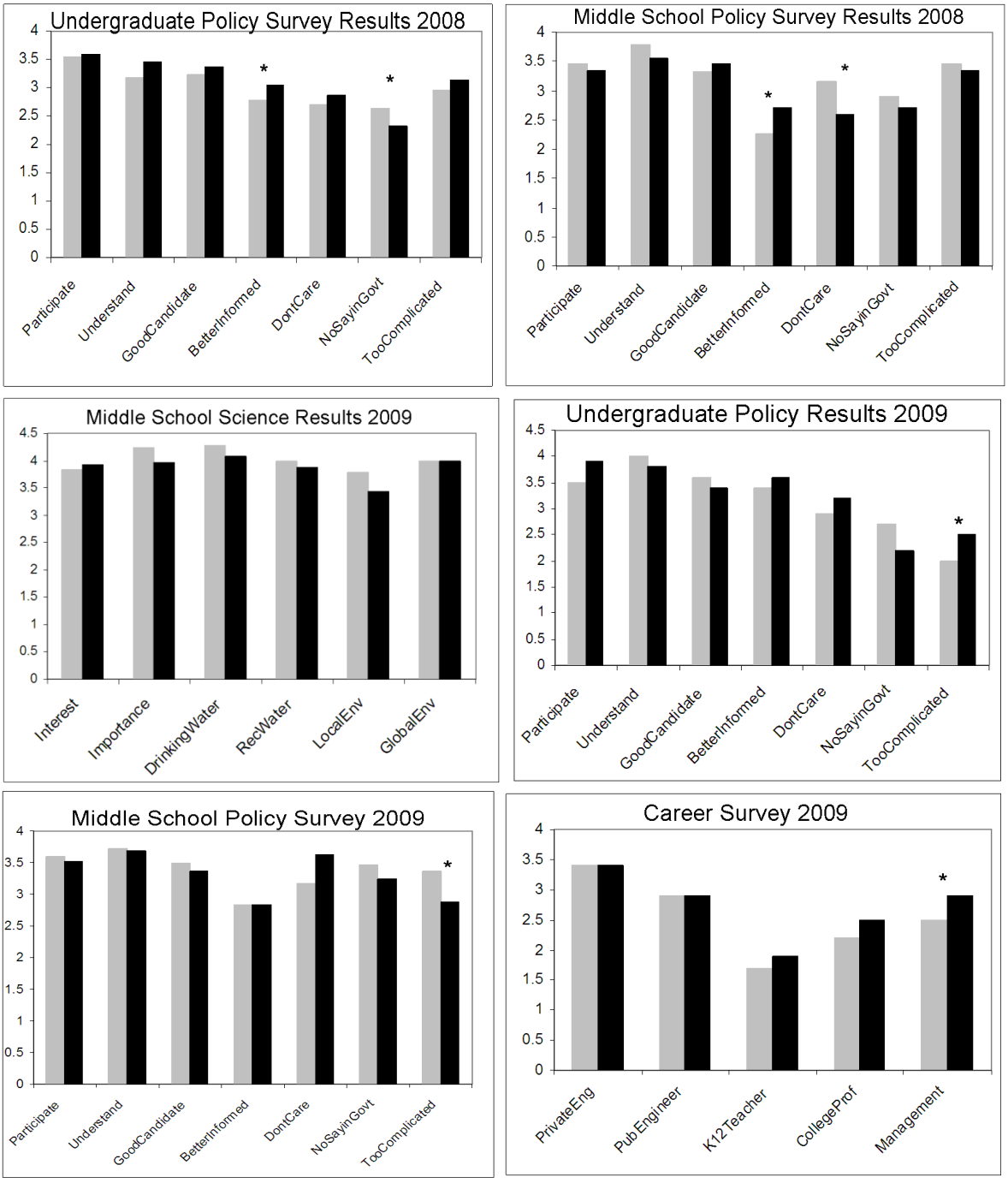


Figure 5-1 Average results from surveys. * denotes statistically significant result. Y-axis is average score (out of 5).

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Chapter 6 – Conclusion

Environmental contamination by metals is a serious issue for public health concern. Both mercury and arsenic are ubiquitous in the environment and factors and conditions that contribute to their mobilization are complex. Currently tens of millions of people are consuming contaminated food and water and millions are diagnosed with arsenic or mercury related conditions. As mercury and arsenic are both potentially fatal cancer-causing agents, it is imperative to explore variables that may exacerbate contamination in atmospheric and aquatic environments to gain insight on how exposure may be reduced.

In chapter 2, mercury methylation by sulfur-reducing bacteria (SRB) was investigated, particularly to ascertain whether the acetyl-coenzyme A (acetyl-CoA) pathway was utilized in methylation. Inhibition studies were performed for two strains of SRB, *Desulfovibrio* desulfuricans ND132 and M8. Molybdate and chloroform were used to inhibit mercury methylation and methylation by the acetyl-CoA pathway respectively. Molybdate successfully inhibited mercury methylation in both planktonic and biofilm cultures of ND132 and M3 while chloroform only inhibited methylation is biofilm cultures for both strains. This partial inhibition suggests that biofilm cultures employ different methylation pathways than planktonic cultures of the same strain of SRB. Gene expression studies were used to further study mercury methylation by the acetyl-CoA pathway. Specifically, expression of the *cooS* gene, which encodes a key enzyme, carbon monoxide dehydrogenase, for the pathway was measured by RT-qPCR and compared between planktonic and biofilm cultures of ND132. Normalizing cooS expression to both cell density and a reference gene, 16S rRNA, biofilm cultures showed up to four times more expression than planktonic cultures suggesting that the acetyl-CoA pathway is important to mercury methylation by biofilm cultures. Bacteria exists mostly as biofilms in the environment and these results suggests that biofilm cultures in particular should be paid

significant attention and that more pathways should be studied. These results will contribute to developing more reliable models for predicting mercury methylation.

Chapter 3 and 4 explores how current aquaculture practices in Bangladesh might be detrimental to arsenic contamination in their groundwater. Chapter 3 specifically studies binding capabilities of various sources of organic matter, cow dung, chicken dung, Bangladeshi sediment from a new unfertilized pond, and Bangladeshi sediment from a fertilized, one-year old pond. Dialysis experiments showed that cow dung bound an order of magnitude more than cow dung did. Unfertilized sediment showed higher levels of bound As than chicken dung but in the same order of magnitude, whereas fertilized one-year old sediment bound As in the range where cow dung bound As. It is interesting to see that cow dung and sediment that has been treated with cow dung complexed similar levels of As and that their levels of As-NOM complexes are notably higher than that from chicken dung and untreated sediment. This suggests that using cow dung to fertilize ponds might not be the best source of adding nutrients as it could potentially result in increased As mobilization. Similarly, K_D and K_S were higher for cow dung and fertilized than sediment and had more strong binding sites. Applying these constants to PhreeqcI, geochemical modeling results suggests that in the presence of any NOM the majority of As will be bound to NOM by at least two orders of magnitude. Both experimental and modeling results support significant As-NOM complexation, particularly in cow dung and cow dung-treated sediment. This implies that practices to improve fish production in Bangladesh could lead to increased contamination of their groundwater and continue to tragically impact public health for the Bangladeshi population. Effects of aquaculture practices need to be further studied for alternative fertilization schemes that can still promote fish populations but minimize related As release. This study is a forward step in developing better mitigation strategies for As mobilization.

Chapter 4 focused on characterizing the host fractions of solid-phase arsenic in unfertilized and fertilized Bangladeshi sediment. Sequential extraction of the unfertilized

sediment showed that most of the solid-phase As, 64% to 86%, was found in recalcitrant minerals, with As found in only two other fractions. 6% to 23% of total As was extracted hydrochloric acid and 8% to 19% extracted by phosphate, representing co-precipitated with very amorphous iron hydroxides, manganese and carbonates and strongly-adsorbed fractions. In fertilized one-year old sediment, As was found only in the recalcitrant and co-precipitated fractions, 74% to 98% and 2% to 36% respectively. This shows a significant shift in host fraction's after the aquaculture pond has been treated and flooded by the monsoon season. More importantly, it suggests that the easily-mobilizable fractions were mobilized, which is also supported by total As extracted from the sediments decreasing after one year. While the phosphate-extracted fraction contained what seems like a small amount of As, 3 μg As/g sediment, calculations show that that is more than enough to contaminate the groundwater to well over 100 μg As/L. This study also suggests that aquaculture practices be further studied for possible impacts on groundwater contamination in search of alternative methods that exacerbates less the As contamination.

Through this research, I would suggest that future studies focus on biofilm cultures of bacteria regarding mercury methylation. Not only is mercury poisoning a terrible health problem, current studies with planktonic bacteria cultures underestimate their roles and abilities in mercury methylation. Research should be broadened include biofilm cultures, particularly of SRB, even if they previously have been shown to not participate in methylation when in planktonic form. Studies should also aim to discover other pathways for mercury methylation as this research has indicated multiple pathways being utilized by SRB. The arsenic research indicates that current aquaculture practices in Bangladesh contribute to their groundwater contamination, even within the first year after fertilizing the pond. It would be prudent to alter some of their methods, including not using cow manure to fertilize the aquaculture ponds. Based on the results, using chicken manure might be a better option than cow manure to

stimulate fish production, however, it would be best altogether to avoid manure due to the additional carbon inputs from any form of manure.

This work indicates the research must be advanced to include previously unstudied areas, whether it be about pathways and mechanisms for contamination or conditions and variables that contribute to environmental problems. Both mercury and arsenic are serious toxins where through high levels or prolonged exposure, millions of people could die. It is imperative to understand better how contamination is occurring and then to develop better, more applicable mitigation strategies for preventing environmental poisoning.