MICROBIAL CARBON SOURCE CYCLING IN BANGLADESH AQUIFERS

EXAMINING MICROBIAL CARBON SOURCE CYCLING IN ARSENIC CONTAMINATED BANGLADESH AQUIFERS THROUGH LIPID AND ISOTOPIC ANALYSES

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Abstract

Understanding how organic matter is microbially cycled through Bangladesh aquifers is a key component in understanding the spatial and temporal patterns of arsenic release into groundwater occurring on wide regional scales. There is a current gap in the literature for how overall microbial carbon cycles are functioning in Bangladesh aquifers, how these microbial metabolisms factor into arsenic release, including methodology as to approach these questions *in situ*. This research aimed to provide insight into carbon sources and cycling of the microbial communities in Bangladesh aquifers through a complimentary applied suite of lipidomic, isotopic and inorganic analytical approaches on *in situ* sediments and groundwater from Bangladesh aquifers.

Through radiocarbon analyses of phospholipid fatty acids (PLFA's), bacterial populations in a shallow Holocene-aged and high arsenic aquifer were found to be predominantly utilizing younger organic matter as their carbon source rather than older sedimentary carbon. At the sites studied, the sources of younger organic matter that coincide with zones where increased reductive dissolution of iron and arsenic release is occurring were consistent with human and livestock waste identified through sedimentary sterol distributions (phytosterols and coprstonaol) and Cl/Br mass ratios in groundwater. Since poor sanitation is widespread across Bangladesh, sewage-derived waste should be considered a prevalent potential microbial carbon source is these systems. An examination of sediment- versus groundwater-associated microbial communities in Bangladesh aquifers (through PLFA analysis) suggested that the former is 5-6 orders of magnitude more abundant than the latter. Archaeal communities, examined through both groundwater methane and sedimentary archaeal lipid (archaeol and glycerol dialkyl glycerol tetraether (GDGT)) analysis, are suggested to be highly active (depths 5-240 m) but to varying degrees in Bangladesh aquifers. Methanogenesis, dominantly being carried out through CO_2 reduction, appears to be spatially associated at some sites with zones of iron/arsenic reductive dissolution in the Bangladesh aquifers. The analytical approaches and conceptual frameworks applied throughout this dissertation have been demonstrated to be effective strategies to understand how microbial carbon cycling is occurring at a community level and intimately involved in arsenic release.

Acknowledgments

" I would rather see the world from a different angle..." Jewel

" The real voyage in discovery consists not in seeking new landscapes, but having new eyes " Marcel Proust

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List of Abbreviations and Symbols

CL: core lipid

DIC: dissolved inorganic carbon

DOC: dissolved organic carbon

FAME: fatty acid methyl ester

GC-MS: gas chromatography coupled to mass spectrometry

GDGT: glycerol dialkyl glycerol tetraether lipids

HCL: hydrochloric acid

HPLC-APCI-MS: high performance liquid chromatography coupled to atmospheric chemical ionization mass spectrometry

GC-IR-MS: gas chromatography coupled to isotopic ratio monitoring mass spectrometry

IPL: intact polar lipid

PLFA: phospholipid fatty acid

NVOC: non-volatile organic carbon

SOC: sedimentary organic carbon

WHO: World Health Organizat

Chapter 1. Introduction

In South and Southeast Asia, estimates of greater than 100 million people are being chronically exposed to arsenic through the regular consumption of contaminated groundwater (Ravenscroft et al. 2009). This issue is of significant and immediate concern in Bangladesh where estimates ranging between 35 and 77 million inhabitants (Dhar et al. 1997; Smith et al. 2000; Argos et al. 2010) are currently at risk of chronic arsenic exposure through drinking contaminated water. Exposure of rural Bangladeshi populations to arsenic occurs through water used for drinking, food preparation and crop irrigation obtained from shallow wells. Approximately ten million inexpensive shallow tube wells installed in Bangladesh in the 1970's facilitate access to the shallow (typically at depths <100m) Holocene aged-aquifers and avoid the acute toxic effects of consuming microbial pathogens present in surface water (Argos et al. 2010; Smith et al. 2000; Ahsan et al. 2000). However, these groundwater sources commonly contain inorganic arsenic concentrations well above the World Health Organization and the water quality standards of the (<10μg/L)(WHO 2011) and in many cases above the less conservative Bangladesh water drinking quality guidelines (<50μg/L) (UNICEF 2009). Recently, deeper tube wells (>100m) have been installed to allow access to the low arsenic groundwater from the underlying Pleistocene-aged aquifer (Ahsan et al. 2000). Concerns regarding the sustainability of this groundwater source have arisen in regards to whether the Pleistocene aquifers may be vulnerable to arsenic contamination(van Geen et al. 2013) from the same biogeochemical mechanisms causing the Holocene sediments to be affected.

The original source of arsenic in aquifer sediments across South and Southeast Asia (specifically West Bengal and Bangladesh) are hypothesized to be geogenic, due to the erosion of the Gondwana coal seams in the eastern Himalayas (contain as high as 200 mg/kg total As) and outcrops of sulphides in the Darjeeling Himalayas (contain as high as 0.8% total arsenic)(Chowdhury et al. 1999). The primary arsenic-bearing minerals contained within these

deposits become oxidized and arsenic is transferred to secondary mineral phases of iron (hydr)oxides (Lowers et al. 2007; Kocar et al. 2008). Subsequent transport of these arsenic-bearing suspended particles occurs through the Mekong, Red and Ganges-Brahmaputra-Meghna river systems (Swartz et al. 2004; Zheng et al. 2005; Berg et al. 2008; Postma et al. 2007; Fendorf et al. 2010). Deposition over time, in commonly deltaic environments, makes up the sediments comprising the aquifer systems. Subsequently, under reducing conditions, reductive dissolution of iron (oxy)-hydroxides occurs removing the solid matrix to which the As was adsorbed and releasing it to the groundwater. Direct arsenate reduction as also been noted to occur within these systems (Ahmann et al. 1997; Postma et al. 2007). While there is currently a strong consensus that this release of arsenic in the shallow aquifers across South and Southeast Asia is microbially mediated (Nickson et al. 1998; Nickson et al. 2000; Islam et al. 2004; Swartz et al. 2004; Dhar et al. 2011; McArthur et al. 2001; Dowling et al. 2002; Harvey et al. 2002; Postma et al. 2007; Postma et al. 2012) there is not a complete understanding of the biogeochemical factors that control arsenic release. This impedes the development of strategies to mitigate arsenic release, as well as prediction of the future sustainability of the deeper water sources.

There is a current gap in the literature of a conceptual framework for how overall microbial carbon cycles are functioning in Bangladesh aquifers, how these microbial metabolisms factor into arsenic release, and methodology to approach these questions *in situ*. The overall aim of the five studies presented within this PhD dissertation is to provide insight into carbon sources and cycling of the microbial communities in Bangladesh aquifers through a complimentary applied suite of lipidomic, isotopic and inorganic analytical approaches on *in situ* environmental sediments and groundwater (as outlined in further detail section 1.7).

1.1 Microbial Degradation of Organic Matter and Thermodynamic Redox ladders in Natural Environments

Anaerobic degradation of organic matter is carried out through a series of metabolic pathways by primary/secondary fermenting bacteria, dissimilatory reductive bacteria and methanogenic archaea (Worm et al. 2010). Electron acceptors which can be coupled to organic carbon oxidation (in this case the electron donor) by reductive bacteria include O_2, NO_3^-, Mn(IV), Fe(III) and SO_4^{-2}. Simply outlined and illustrated in Figure 1.1, primary fermenting bacteria break down complex hydrocarbon polymers such as heterocyclic aromatic compounds to simpler hydrocarbon species (i.e. alcohols and short-chain fatty acids) making them bioavailable to secondary fermenters. Secondary fermenters then break down these hydrocarbon molecules to substrates such as acetate and hydrogen that dissimilatory reducers and methanogens can utilize. This allows acetoclastic methanogens to metabolically mineralize the organic carbon into CO_2 and methane (Worm et al. 2010). While the complex dynamics between microbial consortia which form these syntrophic relationships will not be examined at length here, extensive and recent reviews of the interactions are found within the literature (i.e. Sieber et al. 2010; Worm et al. 2010; Gieg et al. 2014).

The amount of energy available to organisms by coupling reduction reactions to a redox reaction comes from the difference between the energy stored within the bonds of the ultimate products and those in the reactants. Thus, in order for a reaction to be metabolically useful, there must be a positive net amount of usable energy released from the reactants to form the products (Bethke et al. 2011). The relative difference in the net amount of energy obtained between electron accepting processes results in an "energetic hierarchy" also termed a thermodynamic or redox ladder (O_2 > NO_3^- > Mn(IV) > Fe(III) > SO_4^{-2}). In groundwater systems, Champ et al. (1979) formalized the idea of redox ladder by demonstrating a series of redox zones in which electron acceptance (reduction) of a particular oxidized form of an element yields progressively less energy

(Bethke et al. 2011). When available, oxygen and nitrate are reduced readily by microorganisms in surface/near-surface sediment and soil horizons. However, at depth, oxygen and nitrate generally are not available as electron donors and thus iron, manganese and sulphur redox metabolisms are capable of occupying a competitive niche. Manganese oxides, while energetically more favourable for microbes to reduce, are generally present in smaller concentrations than iron in most sub-surface systems and is not a dominant metabolic substrate. Also, it is abiotically quickly oxidized to Mn^{2+} when reacted with Fe^{2+} and H_2S (Postma & Appelo 2000) and therefore not available in this case for biotic reduction. Thus, within sub-surface environments, iron and sulphate reducers along with methanogens dominate the microbial metabolic functional groups. These particular metabolisms occupy the "lowest rings of the thermodynamic ladder" (Bethke et al. 2011) utilizing metabolic strategies which have smaller energetic gains than oxygen and nitrate but are able to able to be successful in these environments.

Methanogenesis, the last stage of the redox ladder, occurs through two dominant pathways: hydrogenotrophic (Equation 1) and acetoclastic methanogenesis (Equation 2). As reflected in their relative pathway names, hydrogentophic methanogensis carries out carbon dioxide reduction using hydrogen as an electron donor and alternatively acetogensis reduces acetate with hydrogen. Dolfing et al. (2008) investigated the thermodynamic constraints of the possible pathways through Gibbs Free Energy calculations in respect to effects of temperature, pH, acetate and H_2 concentrations.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_20 \quad (1)$$

Carbon dioxide reduction with hydrogen to methane: Hydrogenotrophic methanogenisis

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \quad (2)$$

Acetate reduction with hydrogen to methane: Acetoclastic methanogenisis

Acetoclastic methanogenisis has been stated to have a larger "window of opportunity" (the point at which both of the individual pathways carried out by the fermenting bacteria and

methanogens involved both become exergonic). Thus, oxidation of organic matter to acetate and H_2, linked to syntrophic acetate oxidation and hydrogenotrophic methanogenesis may be the most likely to occur in environmental settings (Dolfing et al. 2008). The calculated Gibbs free energy changes ($\Delta G°`$) at defined standard conditions (pH 7.0, 298 K, 105 Pa pressure and 1M concentration of products and reactants) for acetoclastic and hydrogenotrophic methanogenesis was -35.8 and -130.7 kJ/reaction respectively). It should be noted that the theoretical work of Dolfing *et al.* (2008) remains largely speculative but recent work later examined in this discussion have begun to substantiate some of these inferences while notably also finding discrepancies between the theoretically determined thermodynamic pathways that are dominant and those that are actually observed within environmental samples (Gray et al. 2011; Siddique et al. 2012). For instance, in carbon limited systems where acetate may be exhausted, CO_2 methanogens may outcompete acetoclastic methanogens. Through analysis of the stable carbon ($\delta^{13}C$) and hydrogen (*δD)* isotope signatures of methane, these pathways can be discerned, as discussed in more detail below in this introduction and Chapter 4.

1.2 Microbial Carbon Cycling in Arsenic Contaminated Bangladesh Aquifers

The aquifers comprising the Bangladesh sub-surface sediments are limited in terms of organic content, while abundant in iron-hydroxides. Therefore, the nature of carbon sources available to the anaerobic heterotrophic bacteria carrying out reductive dissolution of FeIII and/or AsV by coupling to organic carbon can be expected to be a major thermodynamic driver of arsenic release. The reduction of FeIII is represented in equation (1) with acetate acting as the major electron donor. In natural environments, acetate does not have to be the electron donor driving this reaction and is likely to comprise only a small proportion of complex organic pools.

$$Acetate^- + 8Fe(III) + 4H_2O \rightarrow 2HCO_3^- + 8Fe(II) + 9H^+ \quad (3)$$

Microcosm experiments have used the pure compounds lactate (Dhar et al. 2011) and acetate (i.e. Islam et al. 2004; Hery M. et al. 2010; Lear et al. 2007) as the organic electron donors to stimulate iron reduction in sediments from Bangladesh and surrounding regions to stimulate the release of arsenic into groundwater (Table 1.1). However, as alluded to above, in the environment, organic carbon pools are comprised of complex pools of thousands of organic molecules rather than individual compounds. This creates analytical and logistical challenges in attempting to trace the *in situ* activity of heterotrophic bacteria. In addition to the activity of heterotrophic bacteria in these systems, the coinciding presence of methane with higher iron/arsenic dissolved concentrations that had been noted in a few studies ((Liu et al. 2009; Harvey et al. 2002)) suggests that methanogenic archaea are playing an active role in the carbon cycles alongside the heterotrophic bacteria.

Table 1.1 Microcosm studies that stimulated FeIII/AsV reduction and arsenic release by adding labile carbon to South/Southeast Asian sediments

	Experiment Type	Geographic Location/Origin of Sediments	Author(s)/Year	Carbon Source added vs "Natural" Carbon	Microbial Community profile analyzed?
Studies that stimulated iron reduction and arsenic release by adding labile carbon to sediments	Field	Bangladesh	Harvey et al. 2000	Molasses amended groundwater	No
	Microcosm	Bangladesh, Araihazar	Islam et al. 2004	Acetate	Yes, sediment
	Microcosm	Bangladesh, Araihazar	Dhar et al. 2011	Lactate	No (but added a known culture to sediment)
	Microcosm	India, West Bengal	Hery et al. 2010	Low Acetate and Natural	Yes, sediment
	Microcosm	Cambodia	Lear et al. 2007	Acetate	Yes, sediment
	Microcosm	Cambodia	Rowland et al. 2007	Acetate (also added AQDS for enhanced electron shuttling)	Yes, sediment
	Microcosm	Bangladesh	Akai et al. 2004	Glucose and Polypepton	No
	Microcosm	Bangladesh, Araihazar	van Geen et al. 2004	Acetate	No

The presence of biogenic methane in shallow aquifers throughout South and South-east Asia, has been noted in a few previous studies (Inner Mongolia China (Wang et al. 2015), Bangladesh (Harvey et al. 2002) and Taiwan (Liu et al. 2009). This demonstrates that methanogenesis, the final reductive stage of biologically driven organic matter degradation, is active in these systems. Microbially mediated processes of methanogenesis and the reductive

dissolution of Fe (and arsenic release) can be expected to be interconnected, but there is a current lack of understanding and research into these processes in these systems.

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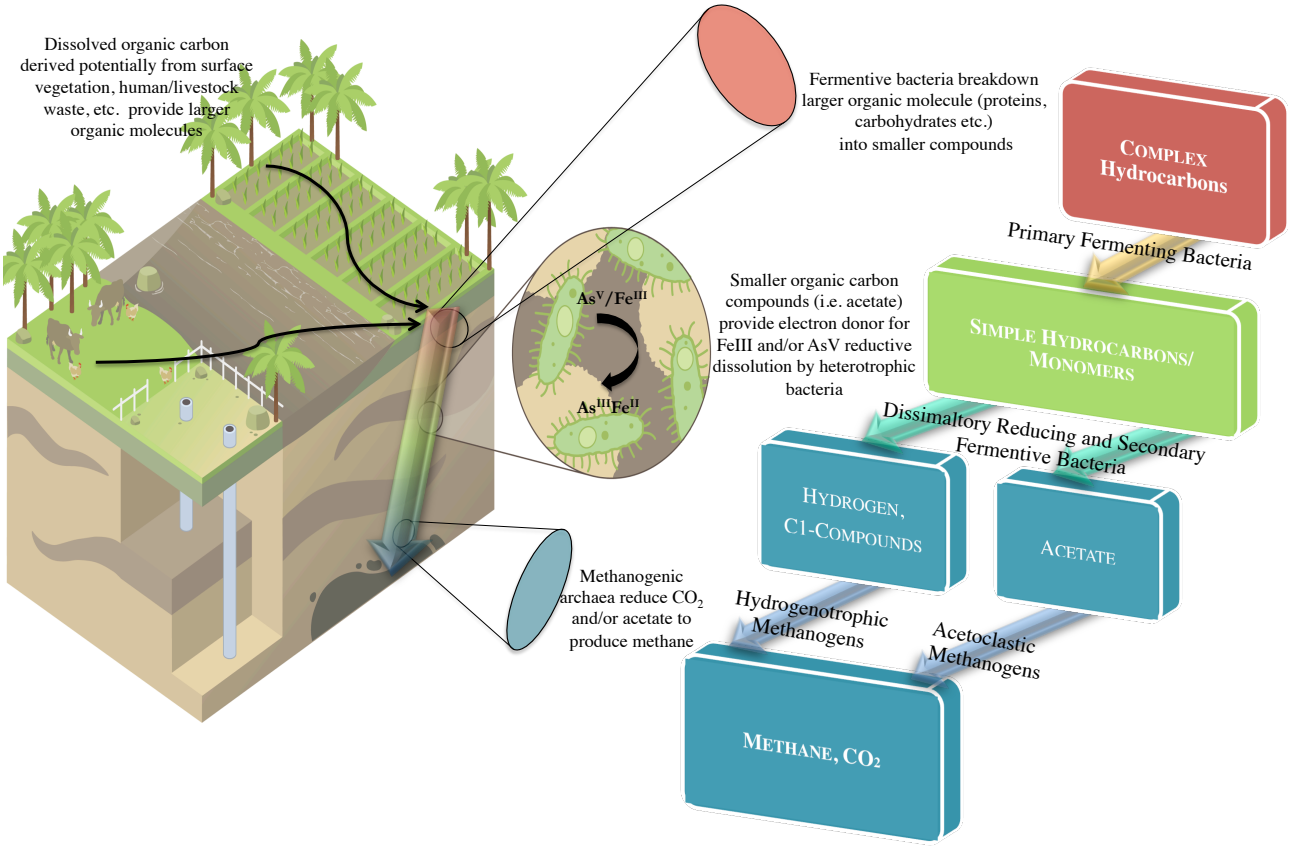

Figure 1.1 Conceptual overview of microbial carbon cycles that expected to be present and active in Bangladesh aquifers and potential relevance to iron and arsenic biogeochemical cycling

1.3 Examining *In situ* Microbial Communities through Bacterial and Archaeal Lipid Distributions

Characterization of the abundance and distribution (along with isotopic composition when possible) of bacterial and archaeal cellular membrane lipids can allow some insight into community structure in environmental settings. Archaeal and bacterial membranes are comprised of differing biochemical composition. Archaeal cellular membrane lipids are exclusively comprised of

hydrocarbon chains bound to the glycerol moiety exclusively through ether linkages (Koga & Morii 2007). Contrary to this, bacterial composition of cellular membranes is generally comprised of fatty acids bound to glycerol through ester linkages (exceptions to this have been observed in a few species of extremophile bacteria (Weijers et al. 2006; Weijers et al. 2007). In addition, a significant number of archaeal species possess membrane lipids with a tetra-ether core forming a membrane monolayer rather than a membrane bilayer. These characteristics are two of the four structural characteristics possessed by archaeal lipids outlined by (Koga & Morii 2007) (Figure 1.2) that distinguish them from their bacterial counterparts.

The overarching purpose of cellular lipid membranes is to provide a semi-permeable barrier capable of maintaining homeostatic conditions of internal cell environments. Archaea and bacteria achieve this by moderating the composition of their lipid membranes in response to environmental fluctuations (Koga 2012). Both archaeal and bacterial organisms are known to respond to external fluctuations by changing the hydrocarbon chain composition/structure within their membranes (i.e. (Kaneda 1991; Nichols et al. 2004; van de Vossenberg et al. 1998; Chong et al. 2012; Koga 2012; Boyd et al. 2013). Thus, it may be possible to observe adaptations/shifts by the microbial community through membrane structure, which have not resulted in taxonomic changes (i.e Hazel 1995; Chintalapati et al. 2004; Guerzoni et al. 2001; Heipieper et al. 2003; Tymczyszyn et al. 2005; Munoz-Rojas et al. 2006; Unell et al. 2007; Schoug et al. 2008). In addition, since lipid analysis does not rely on the amplification of biomolecules, a lipid distribution and abundance can be assumed to be reflective of the original community. In contrast, metagenomics requires sequencing and amplification of the 16s RNA subunit for sufficient quantification which can introduce artefacts into the overall delineation of the community distribution (Schloss et al. 2011).

Examination of the literature reveals that the use of specific bacterial or archaeal biomarker lipids for taxanomic identification is appropriate for very specific cases but needs to be carried out

with caution (Frostegård et al. 2010). Discrepancies are especially evident for PLFA's from bacterial communities since they have been the focus of relatively more research studies to date than archaeal membrane lipids but in all probability remains true for both domains. For instance, cyc19:0 has been considered as an indicator of gram-positive bacteria, however (Schoug et al. 2008) found this lipid to have fairly high proportions (~25%) in the overall lipid distribution of the gram-negative bacteria, *Lactobacillus coryniformis*. In environmental archaeal communities, two main categories of archaeal lipids comprise the distribution in varying proportions: glycerol dialkyl glycerol tetraether lipids (also referred to as GDGT's) and diether lipids (also referred to as GDGD's or archaeol) (Figure 1.3). Archaeol is considered a distinctive lipid biomarker for methanogenic archaea (Lim et al. 2012; Pancost et al. 2011) due to its presence in relatively high abundance in these organisms. However, it should be noted that the relative proportion of archaeaol and GDGT's in methanogens has been demonstrated to change rapidly in response to external temperature. Sprott et al. (1991) found that when the growth temperature of the methanogenic archaeaon *Methanocaldcoccus jannaschii* was raised from 45°C to 60°C, the proportion of diether (archaeol) based lipids decreased significantly from 80% to 20% while the tetra-ether lipids and the cyclic archaeol-based lipids increased from 10% to 40% of the overall lipid distribution

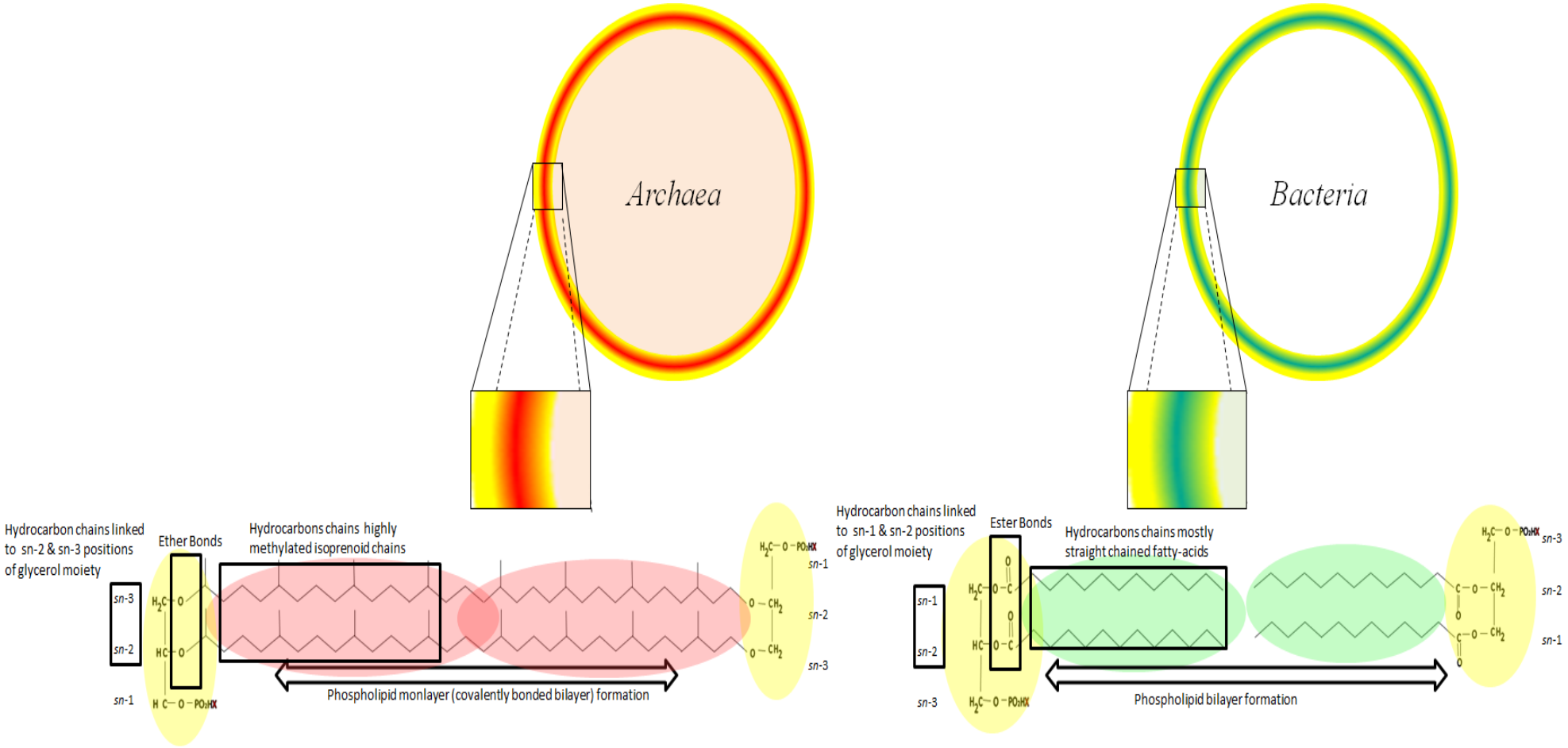

Figure 1.2 Diagram illustrating the four main distinguishing biochemical properties of the cellular lipid membranes of the archaeal and bacterial domains including differences in the position of linkage between the hydrocarbon chains and glycerol moiety, the ether vs. ester bonds linking the hydrocarbon chains to the glycerol moiety, the methylated isoprenoids chains vs. the predominantly straight changed fatty acids and the phospholipid layer formations

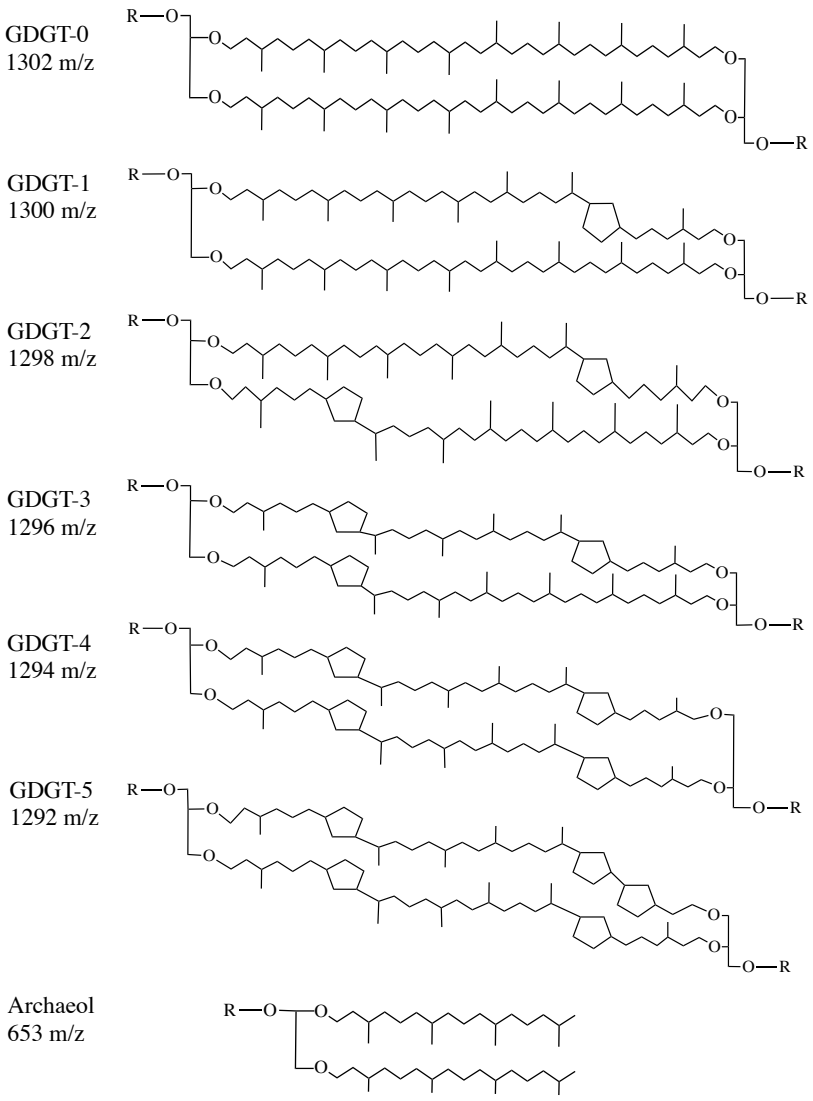

Figure 1. 3 Chemical structures and molecular weights of core archaeal membrane lipids GDGT 0-5 (1302, 1300, 1298, 1296, 1294, 1292 m/z) and archaeal (653 m/z) where R=polar headgroup.

1.3.1 Using Intact Microbial Cellular Membranes Lipids as a Measure of Live Community Abundance

Well-established conversion factors allow for *in situ* live bacterial cell density estimates directly from phospholipid fatty acid concentrations. A conservative conversion factor applicable to

sub-surface environments, provided by White et al. (1979) and reviewed in depth by Green & Scow (2000), is one picomole of phospholipid fatty acids equates to 2.4 x 10^4 live bacterial cells in an environmental sample. The use of intact polar lipids from cellular membranes of archaea as a measure of live biomass cannot be simply used an analogous method to how phospholipid fatty acids (PLFA) are used a measure of live biomass for bacterial communities. As mentioned**,** bacterial and archaeal cellular membrane lipids possess distinct structural differences that result in significantly different properties in intact polar lipid (IPL) stability upon cell death. The ester- and ether- linkages between polar head groups and the hydrocarbon chain which dominate the bacterial and archaeal domains respectively possess different levels of stability. The ester linkages are more suseptible to hydrolysis and as result, the polar head groups of bacterial IPL's are rapidly lost soon after cell death. Since PLFA's of bacterial organisms have been reported to degrade on the order of days to weeks, analysis of PLFA's are a usable measure of the live bacterial community (White et al. 1979; Harvey et al. 1986; Logemann et al. 2011). Alternatively, the ether linkages present in polar lipids from archaeal membranes are more stable and resistant to biotic and abiotic degradation processes. Thus, difficulties arise when attempting to use the analysis of the intact polar lipids of archaea as a measure of live biomass. Recent research suggests that archaeal IPL's degrade at rates orders of magnitudes slower than bacterial IPLs (Harvey et al. 1986; Schouten et al. 2010; Logemann et al. 2011; Xie et al. 2013). The exact rate of archaeal IPL degradation is still in debate but recent degradation half-life rates range from estimates range between 100 days to 2000 years in biotically active near-surface sediments (<1 metre below sea level) ((Harvey et al. 1986; Lipp & Hinrichs 2009; Schouten et al. 2010; Logemann et al. 2011; Xie et al. 2013). Xie *et al*. (2013) used radiocarbon labeled headgroups of archaeol (di-ether) lipid to monitor degradation rates of intact archaeal lipids in marine sediments over a time span of 300 days and used modeling strategies to

extrapolate the data over geological timescales. While the study was focused on elucidating the fate of intact lipids in deep sub-surface environments, estimate half-lives of total archaeal intact polar lipid degradation in near-surface environments (approximately 0.1 metres below surface) were provided and were one to two magnitude slower than bacterial IPLs (~ 1000 years). This was based on only 0.5% of the diether compound degrading after 300 days. However, Harvey *et al*. (1986) reported much higher degradation rates of archaeal lipids in low carbon environments (51% was degraded after 75 days) perhaps due to limited carbon sources resulting in the microbial degradation of archaeal lipids of dead cells as an energy source. While some arguments exist that intact polar lipids of archaea provide information on predominantly living populations (e.g. (Pitcher et al. 2009), this view needs to be exercised with caution as recent evidence has indicated that intact polar lipids from archaea can be molecular fossils derived from dead organisms (Harvey et al. 1986; Lipp & Hinrichs 2009; Schouten et al. 2010; Logemann et al. 2011; Xie et al. 2013). Despite these limitations, within the current suite of analytical strategies, analysis of archaeal IPLs opposed to their degraded counterparts remains a useful strategy for examining the activity and behaviour of live archaeal communities (Pitcher et al. 2009).

1.4 Using $\delta^{13}C$ and $\Delta^{14}C$ of Microbial Lipids to Examine Carbon Cycling

The use of stable carbon isotopic analysis ($\delta^{13}C$, notation defined in Figure 1.4) in lipids allows insight into microbial carbon cycling within the environment. This is possible because the $\delta^{13}C$ isotopic signature within cellular membrane lipids is directly determined by the both the isotopic composition of the carbon source as well as by the mechanism of carbon assimilation (Pancost & Sinninghe Damsté 2003). Delineating a specific carbon substrate that a microbial community is utilizing in a laboratory setting, either with pure cultures or microcosm experiments, consists of measuring the isotopic composition of amended carbon substrates and that of the

microbial lipid. In laboratory settings this is relatively simple in that the ^{13}C is generally derived from a specific carbon source (i.e. lactate, acetate, etc.). However, many available carbon sources do not have distinctive ^{13}C signatures in natural settings and carbon substrates available to micro-organisms are numerous and complex. The ^{13}C of natural organic matter derived from C3 plants has been estimated to have a wide range from -24 to -34 ‰ (Faure 1986). In these instances, the isotopic composition of carbon pools (dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), sedimentary organic carbon (SOC), particulate organic carbon (POC), etc) may be compared with cellular components to try to demonstrate carbon uptake.

$$\delta^{13}C\ (‰) = \left(\frac{(^{13}C/^{12}C_{sample})}{(^{13}C/^{12}C_{standard})} - 1 \right) - 1000$$

$$\delta D\ (‰) = \left(\frac{(^{2}H/^{1}H_{sample})}{(^{2}H/^{1}H_{standard})} - 1 \right) - 1000$$

Figure 1.4 Delta ("δ") notation definitions stable carbon and hydrogen isotopes where $\delta^{13}C$ is relative to the Pee Dee Vienna Belemite standard and δD is relative to the Vienna Standard Meteroic Ocean Water (VSMOW)

An additional level of complexity in utilizing $\delta^{13}C$ in a cellular component such as lipids to delineate carbon sources, is that biosynthetic pathways will induce changes to the signature (Hayes 2001). This is discussed further on as it requires first an explanation of the reason for fractionation in biological pathways. The mass-dependent fractionation of ^{13}C and ^{12}C results in a kinetic isotopic effect. ^{13}C has a greater mass than ^{12}C and forms slightly stronger bonds because greater mass will

result in a smaller vibrational energy within the bond. Vibrational frequency is inversely related to an atom's mass as seen in the equation ($v=1/(2\pi\sqrt{k/\mu})$ where v = vibrational frequency of the bond, k = force constant and $u = m_a m_b/m_a + m_b$) (Anbar & Rouxel 2007). Thus, the weaker bond will dissociate faster (^{12}C) resulting in an enrichment of this isotope in the product (Anbar & Rouxel 2007). Thus, research has been invested in understanding the factors in lipid biosynthesis that can influence fractionation, causing lipids to be depleted in ^{13}C relative to carbon sources and overall cell biomass. Generally, lipids derived from aerobic bacteria are 2-3 ‰ depleted than overall biomass, while in anaerobic bacteria depletion is estimated to be 5-10% (Hayes 2001). DeNiro & Epstein (1977) identified that in the lipid biosynthetic pathway, the oxidation of pyruvate to acetyl co-enzyme A, which is subsequently converted to the acyl chains of phospholipid fatty acids, causes a significant fractionation effect.

In addition, specific metabolic pathway (autotrophic versus heterotrophic (Scholl & Harvey 1992; Londry et al. 2008) , fermentive versus acetogenic (Blaser et al. 2013) will determine the extent of fractionation (discussed below). Through these pathways, mass-dependent fractionation on stable carbon isotopes ultimately leading to a progressive enrichment of ^{12}C and corresponding depletion of ^{13}C as carbon is cycled metabolically. The initial fixation of inorganic carbon (CO_2) into organic metabolites is carried out by autotrophic organisms resulting in a pronounced fractionation of carbon. This requires an initial fractionation step associated with CO_2 diffusion into the cell and a carboxylation step. The enzyme which catalyzes this reaction is ribulose-1,5 biphosphate carboxylase oxygenase or Rubsico. This process creates a total fractionation approximated to be $\delta^{13}C = -29‰$ (Hayes 2001).

As carbon is subsequently metabolized by heterotrophic microorganisms additional (but not as pronounced fractionation) occurs. Using an overgeneralized view of the entire carbon microbial

cycle: autotrophic (CO_2 to organic substrate) to heterotrophic (complex organic substrate to simple organic substrate) to methanogenic (simple organic substrate to methane) to methanotrophic (methane to CO_2), the extent of fractionation within an isotopic signature can then be used to gain insight into metabolisms utilized within an environment. This is complicated by the fact that autotrophs can include autotrophic methanogens that fix CO_2 which $\delta^{13}C$ ranges from -10 to -6‰ (Pancost & Sinninghe Damsté 2003).

Schidlowski (2001) provides a useful model showing $\delta^{13}C$ signatures that would be expected from a variety of metabolisms showing that the most depleted signatures would arise in methanotrophic organisms where the carbon has been through multiple fractionation steps. Londry et al. (2004) demonstrated that in a cultured strain of sulphate reducing bacteria, the extent of fractionation in the lipids were different in those grown autotrophically (on CO_2) and those grown heterotrophically on acetate. Specifically, the lipids of autotrophic bacteria were significantly depleted compared to those grown heterotrophically exhibiting the pronounced fractionation of Rubisco. Similarly, Blaser et al. (2013) showed strong fractionation in lipids of acetogenic strains of bacteria grown on CO_2 and H_2 (autotrophic bacteria that produce acetate) compared to fermentive bacteria. Thus, in order for $\delta^{13}C$ in lipids to be a useful tracer of carbon cycling within the environment there must a measurable and distinctive difference between carbon sources present in the environment and the fractionation associated with lipid biosynthesis needs to be well defined.

The third isotope of carbon is ^{14}C (6 protons and 8 neutrons) and is produced through cosmic ray bombardment of ^{14}N in the atmosphere. This particular isotope is only present in trace amounts (~one per trillion) making it technically difficult to measure (Mollenhauer & Rethemeyer 2009). However, ^{14}C is also is radioactive with a half-life of 5568 years. Since radiocarbon (denoted

as $\Delta^{14}C$ to show a change in concentration compared to a known standard (oxalic acid) decays overtime, it can serve as useful measure of the age of carbon. The use of natural abundance radiocarbon measured in lipids can provide a complimentary technique to $\delta^{13}C$ measurement for two major reasons. The first is that the mass-dependent fractionation effects are corrected for in radiocarbon measurements so the amount of ^{14}C is specifically a function of it's age (Ziolkowski et al. 2013). Secondly, $\Delta^{14}C$ values have a wide range with -1000‰ for ancient carbon to modern carbon values having between ~ 0 and +50 ‰. Comparing the radiocarbon signature of available carbon pools within the environment with those of assimilated/biosynthesized carbon is particularly useful where the radiocarbon age of various carbon pools is commonly easily distinguishable from one another. Also, since CO_2 has a modern age due to being recently fixed from the atmosphere, autotrophic metabolisms can be easily identified. Measuring the radiocarbon signatures of PLFA biomarkers of autotrophic versus heterotrophic biomarkers, Slater et al. (2006) were able to discern $\Delta^{14}C$ signatures of ~ +30‰ (modern aged CO_2 fixation) and ~ -230 ‰ (organic matter metabolization) respectively. Ingalls et al. (2006) measured the radiocarbon values of archaeal lipids in a marine environment to determine whether the archaeal communities were heterotrophic or autotrophic. $\Delta^{14}C$ values in the archaeal (GDGT) lipids ranged from -60 to -127 ‰ and using a mass-balance approach determined that 86% of the carbon was derived from modern sources (CO_2).

1.5 Tracing Microbial Carbon Sources in Bangladesh Aquifers

Conflicting hypotheses regarding microbial carbon sources proposed to date can be found on either side of two over-arching arguments. The first proposes that the indigenous bacteria in the aquifers are metabolizing older carbon pools naturally found in the sediment strata such as buried peat layers (Ravenscroft et al. 2001; McArthur et al. 2001; Anawar et al. 2003; Yamazaki et al. 2003; McArthur et al. 2004; Mladenov et al. 2010; Planer-Friedrich et al. 2012), petroleum

(Rowland et al. 2006; Dongen et al. 2008; Rowland et al. 2002; Rowland et al. 2007) or ambient carbon buried at the time of sediment deposition (Nickson et al. 2000; Meharg et al. 2006; Postma et al. 2007; Postma et al. 2012) (Figure 1.5). The second proposes that microbial communities will preferentially utilize younger carbon sources such as human/livestock waste (Harvey et al. 2002; McArthur et al. 2001), constructed ponds (Neumann et al. 2014), or wetland and rice paddy environments (Meharg et al. 2006; Polizzotto et al. 2008) transported from the surface to the Holocene-aged sediments in the aquifer (Figure 1.5).

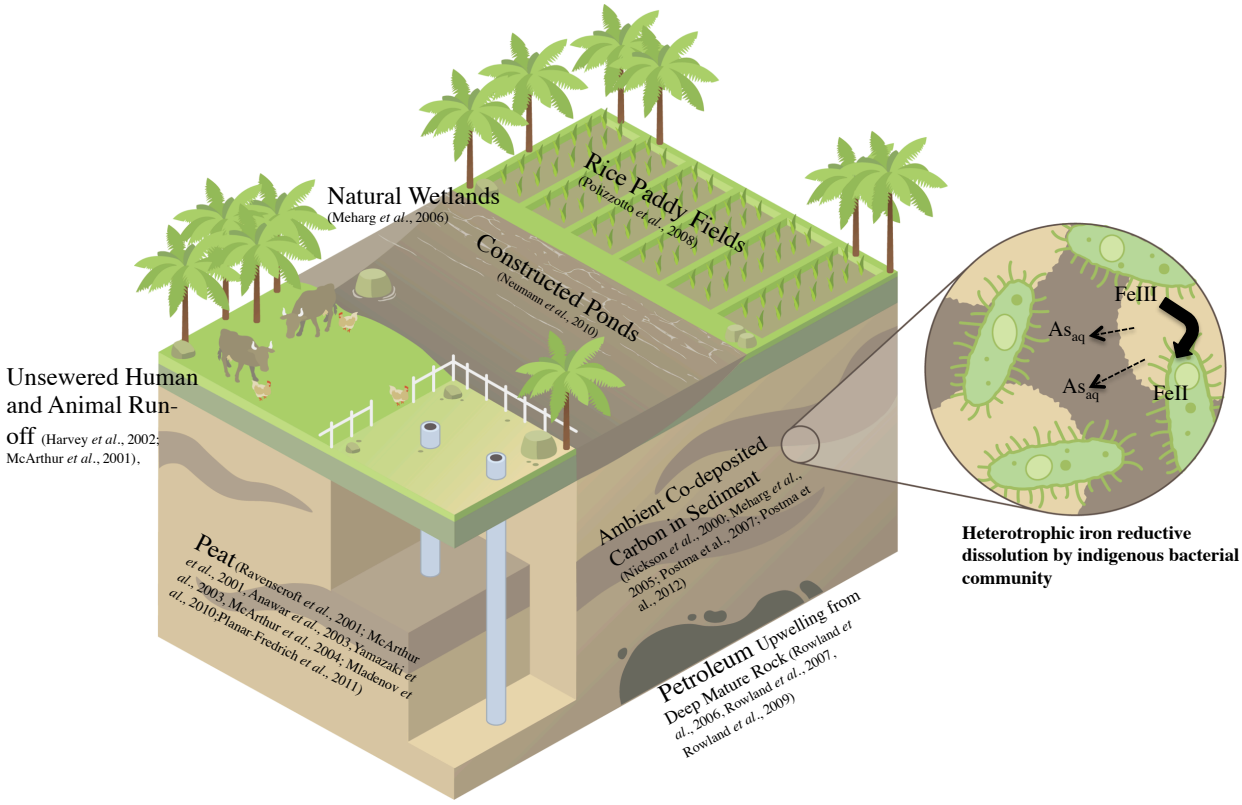

Figure 1.5 Conflicting microbial carbon source hypotheses across South and South-East Asia that heterotrophic bacteria in shallow aquifers are utilizing to drive iron reductive dissolution and arsenic release.

There is an expected difference in reactivity between younger versus older organic matter. Over time, carbon sources can be expected to have undergone degradation and as such are comprised in higher proportions with more recalcitrant molecules relative to the original material. Thus, younger organic matter will have more reactive moieties and microbial usage would be expected to be more energetically favorable. Prior to this work, the age of organic carbon driving the dissimilatory iron reduction in shallow Bangladesh aquifers has only been directly determined at one site (Mailloux et al. 2013), where modern-aged $\Delta^{14}C$ signatures in microbial DNA confirmed the microbial metabolization of young surface-derived carbon sources. Further work is necessary as the radiocarbon analysis of DNA carried out targeted groundwater-associated micro-organisms but not sediment-associated microorganisms. This is an important aspect for two reasons in Bangladesh aquifers. The sediment-associated microbes are likely present at abundances approximately 5 orders of magnitude higher than groundwater concentrations (i.e. Hazen et al. 1991). Also, it cannot be assumed that these two groups of organisms are using the same carbon sources. In fact, sediment-associated organisms would have far greater potential to access sedimentary organic carbon than groundwater organisms. The latter might be expected to only be able to utilize DOC carbon sources. Third, since the proposed mechanism for As release is reduction of iron oxides present in the solid phase in these systems, it is critical to examine the sediment associated microorganisms as they may be far more important than the groundwater community in mediating As release

The organic carbon pools available to microorganisms in the environment are complex mixtures and microbial substrate input and utilization is dynamic. Therefore, tracing organic carbon cycling by microbial communities in the environment is challenging. A direct approach to deconvolute the organic matter micro-organisms are metabolizing is through comparison of the

radiocarbon isotopic carbon signatures ($\Delta^{14}C$) in their organic cellular components, such as lipids or DNA, with the $\Delta^{14}C$ of potential carbon sources. Essentially, this analytical technique exploits that natural organic matter, recently fixed via photosynthesis, contains modern levels of radiocarbon and will progressively loose ^{14}C over time (as discussed above). In environmental settings, the carbon sources of indigenous microbial communities has been determined in previous studies through compound-specific radiocarbon analysis of lipid cellular membrane analysis (Pearson et al. 2001; Shah et al. 2008; Ingalls et al. 2006; Slater et al. 2005; Morrill et al. 2006; Slater et al. 2006; Cowie et al. 2010; Ahad et al. 2010; Ahad & Pakdel 2013). As mentioned, this direct and *in situ* approach allows the sources of carbon that bacterial and archaeal populations are metabolizing in environmental settings to be delineated. Complimentary analyses of lipid biomarkers characteristic of particular carbon sources (i.e. plant vs. human/livestock organic carbon as discussed in Chapter 3) can further elucidate the origins of metabolized carbon pools and concurrent stable isotope analysis (discussed above) can be applied.

1.6 Study Region: Araihazar Upazila, Bangladesh

The studies presented throughout this dissertation in Chapter 2-6 were carried out within Araihazar Upazila (23.7917°N 90.6500°E), Bangladesh (Figure 1.6). This rural region in Bangladesh is approximately 25 km east of the capitol city Dhaka and hosts villages where the local populations are highly dependent on local groundwater wells for drinking/cooking water and crop irrigation. Through ongoing monitoring programs in the region for over fifteen years, numerous studies (i.e. (van Geen et al., 2006; Stute et al., 2007; Dhar et al., 2008; Legg et al., 2012) have identified prevalent arsenic contamination of the shallow aquifer groundwater. Thus, this research dissertation was conducted to help address outstanding research questions that have emerged from

the previous body of studies conducted and through a collaborative effort between researchers from McMaster University, Columbia University, Barnard College and the University of Dhaka.

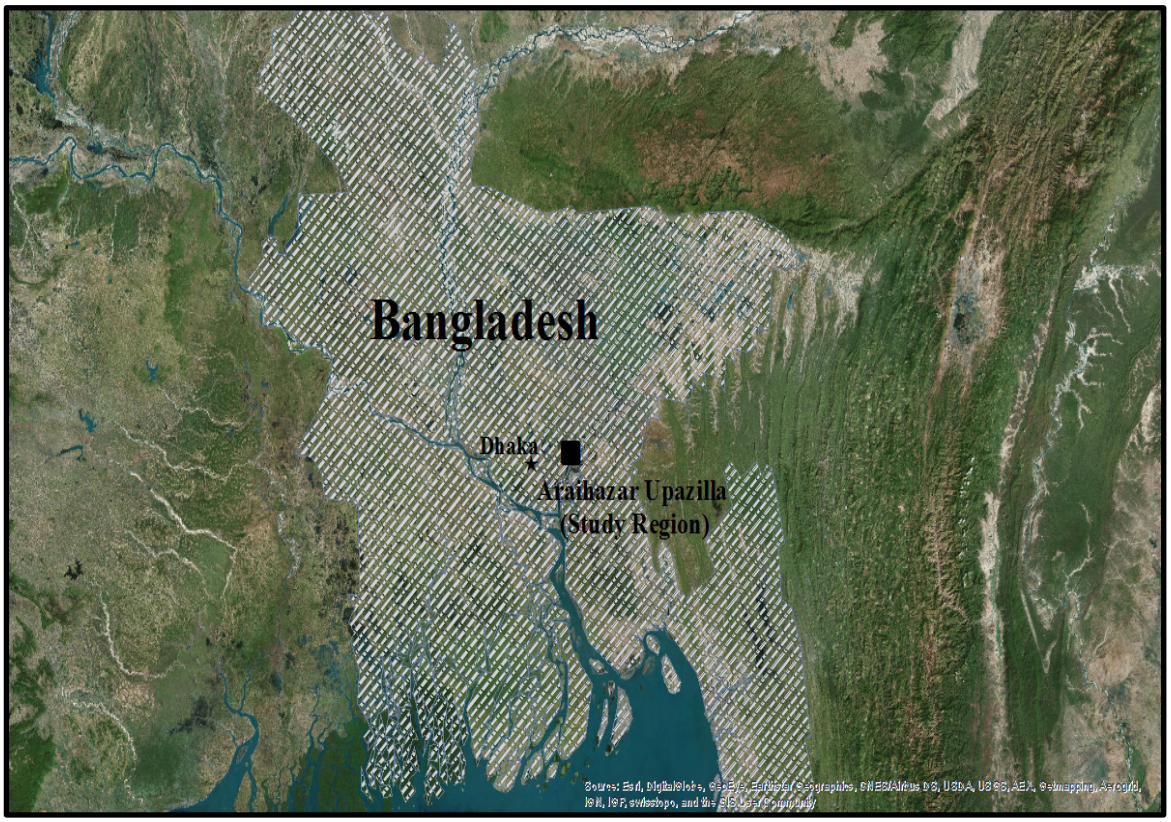

Figure 1.6 Aerial satellite image of Bangladesh (shaded in white) and research study region (Araihazar Upazila) indicated by black square approximately 25 km east of Dhaka (image created with ARC MAP GIS software).

1.7 Overall Research Aims for Examining Microbial Carbon Cycling in Bangladesh Aquifers

This research dissertation aimed to address three major outstanding questions regarding microbial communities and carbon cycling in Bangladesh aquifers. The first two studies presented in Chapter 2 and Chapter 3 aimed to discern the age and origin of carbon sources that sedimentary bacterial communities are metabolizing within shallow arsenic contaminated aquifers. As mentioned, this has important implications in terms of effective management policy (such as the governance of massive pumping practices) that can affect carbon flow regimes throughout Bangladesh aquifers. The study presented in Chapter 4 examined distinctions between sediment-

associated versus groundwater associated microbial communities in Bangladesh aquifers through PLFA analysis. The findings of this work may be particularly pertinent to guide future work that aims to specifically target the communities that drive arsenic release (i.e. sediment versus groundwater associated bacteria). Finally, the fourth and fifth study, presented in Chapters 5 and 6 respectively, aimed to explore the roles that archaeal communities are playing in microbial carbon cycling and ultimately arsenic release.

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Chapter 2: Stimulation of Microbially-Mediated Arsenic Release in Bangladesh Aquifers by Young Carbon Indicated by Radiocarbon Analysis of Sedimentary Bacterial Lipids

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2.1 Abstract

The sources of reduced carbon driving the microbially-mediated release of arsenic to shallow groundwater in Bangladesh remain poorly understood. Using radiocarbon analysis of phospholipid fatty acids (PLFA's) and potential carbon pools, the abundance and carbon sources of the active, sediment-associated, *in situ* bacterial communities inhabiting shallow aquifers (<30 m) at two sites in Araihazar, Bangladesh were investigated. At both sites, sedimentary organic carbon (SOC) $\Delta^{14}C$ signatures of -631 ± 54‰ (n=12) were significantly depleted relative to dissolved inorganic carbon (DIC) of +24 ± 30‰ and dissolved organic carbon (DOC) of -230 ± 100‰. Sediment-associated PLFA $\Delta^{14}C$ signatures (n=10) at Site F (-167‰ to +20‰) and Site B (–163‰ to +21‰), were highly consistent and indicated utilization of carbon sources younger than the SOC, likely from the DOC pool. Sediment-associated PLFA $\Delta^{14}C$ signatures were consistent with previously determined $\Delta^{14}C$ signatures of microbial DNA sampled from groundwater at Site F indicating the carbon source for these two components of the subsurface microbial community is consistent and is temporally stable over the two years between studies. These results demonstrate that the utilization of relatively young carbon sources by the subsurface microbial community occurs at sites with varying hydrology. Further they indicate that these young carbon sources drive the metabolism of the more abundant sediment-associated microbial communities that are presumably more capable of Fe reduction and associated release of As. This implies that an introduction of younger carbon to as of yet unaffected sediments (such as those comprising the deeper Pleistocene aquifer) could stimulate microbial communities and result in arsenic release.

2.2 Introduction

Across South and Southeast Asia, an estimated 100 million people regularly consume arsenic contaminated groundwater (Ravenscroft et al. 2009). In Bangladesh, between 35 to 77 million inhabitants (Dhar et al. 1997; Smith et al. 2000; Argos et al. 2010) use groundwater from shallow (<30 m) Holocene-aged-aquifers containing arsenic concentrations above the World Health Organization (WHO) water quality standards of 10µg/L (WHO 2011) for drinking, food

preparation, and crop irrigation. Recently installed deeper tube-wells (usually >100m) facilitate access to low arsenic groundwater in the underlying Pleistocene-aged aquifer (Ahmed et al. 2006). There are concerns about whether this deep, Pleistocene aquifer also may be vulnerable to future arsenic contamination attributed to the same microbially-mediated processes that have affected the shallow aquifer systems (Zheng et al. 2005; Burgess et al. 2010; Dhar et al. 2011; Radloff et al. 2011; van Geen et al., 2013). A fundamental understanding of the electron donors utilized during arsenic release in the shallow aquifers of Bangladesh is required to understand present day and future distributions of arsenic.

Strong evidence indicates that anaerobic micro-organisms mediate arsenic release in deltaic sediments throughout the Bengal Basin (Nickson et al. 1998; Nickson et al. 2000; Islam et al. 2004; Swartz et al. 2004; Dhar et al. 2011; McArthur et al. 2001; Dowling et al. 2002; Harvey et al. 2002; Postma et al. 2007; Postma et al. 2012). These organisms release arsenic by coupling the oxidation of organic carbon to the reductive dissolution of As-bearing Fe (oxy)-hydroxides (Nickson et al. 1998; Nickson et al. 2000; Islam et al. 2004; Swartz et al. 2004; McArthur et al. 2001; Dowling et al. 2002; Harvey et al. 2002; Postma et al. 2012). The microbial reduction of sorbed arsenate (AsV) to arsenite (AsIII) can also enhance its mobility (Ahmann et al. 1997; Postma et al. 2007). The abundance and metabolic activity of the microorganisms within these groundwater environments thus can control dissolved arsenic concentrations. Microbial abundance in such oligotrophic systems is often limited by nutrient availability. As such, fundamental controls on organic carbon cycling within Bangladesh aquifers should control microbial activity and subsequently arsenic release(Kocar & Fendorf 2012).

The predominant source of organic carbon stimulating microbially driven arsenic release in Bangladesh aquifers remains controversial. A number of studies in Bangladesh and the surrounding

regions have proposed that the bacteria are utilizing sedimentary organic carbon (SOC) present in the aquifers such as buried peat layers(Ravenscroft et al. 2001; McArthur et al. 2001; Anawar et al. 2003; Yamazaki et al. 2003; McArthur et al. 2004; Mladenov et al. 2010; Planer-Friedrich et al. 2012), petroleum(Rowland et al. 2006; Dongen et al. 2008; Rowland et al. 2002; Rowland et al. 2007) or ambient carbon buried at the time of sediment deposition(Nickson et al. 2000; Meharg et al. 2006; Postma et al. 2007; Postma et al. 2012; Desbarats et al. 2014). Conversely, it has been suggested that dissolved organic carbon (DOC) sources derived from human/animal waste in unsewered runoff water(Harvey et al. 2002; McArthur et al. 2012), constructed ponds(Lawson et al. 2013; Neumann et al. 2010), wetland and rice paddy environments(Meharg et al. 2006; Polizzotto et al. 2008; Stuckey et al. 2015) and/or river-derived organic carbon(van Geen, Benjamin C Bostick, et al. 2013) are transported downwards from the surface to the Holocene-aged sediments in the aquifer and are the primary drivers of bacterial activity. Recently, radiocarbon analysis of DNA from filtered groundwater samples provided the first direct evidence of microbial carbon sources in Bangladesh at one site (Mailloux et al. 2013). The relatively young $\Delta^{14}C$ contents of DNA derived from microorganisms present in groundwater samples indicated utilization of younger dissolved inorganic carbon (DIC) or DOC carbon sources rather than sedimentary sources. This is consistent with the proposal by Harvey *et al*.(2002) that indigenous methanogens were primarily using younger DIC carbon sources based on the isotopic composition of DIC and methane in southern Bangladesh. However, neither study was able to assess the carbon sources being utilized by the sediment-associated microbial communities. Such assessment is of fundamental importance as sedimentary microbial communities are more likely to be involved in As release by virtue of their association with the solid phase iron oxides. Sediment-associated microbial communities are also expected to have much greater impact on subsurface biogeochemical cycling as they are generally

present in greater abundances than groundwater-associated microorganisms(Hazen et al. 1991). Further, given their close association with sedimentary materials, these microbial communities have much greater potential to access organic carbon that is a component of, or sorbed to, solid matrix materials. These factors imply that there is the potential that sediment-associated microorganisms have a greater influence on As release in Bangladesh aquifer sediments, but also that they may utilize different organic carbon sources that are not easily accessible to their groundwater-associated counterparts.

Analysis of *in situ* phospholipid fatty acid (PLFA) concentrations is often used to assess microbial bacterial abundances in subsurface systems and thus to identify zones of high levels of bacterial activity associated with increased cellular abundances(Green & Scow 2000). PLFA degrade within days to weeks after cell death, thus they effectively represent the viable biological community(White et al. 1979; Harvey et al. 1986; Logemann et al. 2011). PLFA concentrations can be converted to cellular abundances via conversion factors that are based on a consistent relationship between membrane PLFA concentrations and cell abundances(White et al. 1979; Green & Scow 2000). In addition, compound specific radiocarbon analysis (CSRA) of phospholipid fatty acids (PLFA) can elucidate the carbon sources supporting *in situ* bacterial communities(Abraham et al. 1998; Pancost et al. 2000; Slater et al. 2005; Ingalls et al. 2006; Morrill et al. 2006; Slater et al. 2006; Cowie et al. 2009; Ahad et al. 2010; Bray et al. 2012; Ahad & Pakdel 2013; Mahmoudi et al. 2013; Ziolkowski et al. 2013; Mailloux et al. 2013). Comparing the $\Delta^{14}C$ signatures of PLFA to those of potential carbon sources (e.g. DIC, DOC, SOC) can directly identify the carbon pools being utilized by the bacterial community(Abraham et al. 1998; Pancost et al. 2000; Slater et al. 2005; Ingalls et al. 2006; Morrill et al. 2006; Slater et al. 2006; Cowie et al. 2009; Ahad et al. 2010; Bray et al. 2012; Ahad & Pakdel 2013; Mahmoudi et al. 2013; Ziolkowski et al. 2013; Mailloux et

al. 2013) assuming different sources of distinct signatures because $\Delta^{14}C$ signatures are normalized to remove biosynthetic fractionation effects during data processing(Stuiver & Polach 1977). This approach has been used to identify microbial carbon sources in a number of environments(Abraham et al. 1998; Pancost et al. 2000; Slater et al. 2005; Ingalls et al. 2006; Morrill et al. 2006; Slater et al. 2006; Cowie et al. 2009; Ahad et al. 2010; Bray et al. 2012; Ahad & Pakdel 2013; Mahmoudi et al. 2013; Ziolkowski et al. 2013; Mailloux et al. 2013) including contaminated soils(Cowie et al. 2009), coastal environments(Slater et al. 2005; Slater et al. 2006; Mahmoudi et al. 2013) and groundwater systems(Slater et al. 2008; Ahad & Pakdel 2013).

The goal of this study was to elucidate the predominant carbon sources being used by the sediment-associated bacterial communities in Bangladesh aquifers, to extend our understanding beyond one location and to characterize the relationship of bacterial abundance to As concentrations. Two sites with distinct hydrogeologic and geochemical conditions, specifically having distinct aqueous arsenic distributions and ranges, were compared: Site F, a sandy site with faster recharge and lower arsenic, and Site B, a clay capped site with slower recharge and higher arsenic concentrations(Stute et al. 2007). Concentrations of sediment-associated PLFA's were determined at both sites. In addition, groundwater-associated PLFA's concentrations were determined at Site B in order to assess relative abundances between the two communities. Carbon sources driving metabolisms by the sedimentary bacterial communities were then determined via CSRA of PLFA and comparison to potential carbon sources (SOC, DIC, DOC) in the aquifer.

2.3 Methods

2.3.1 Field Sites

Field sampling for this study was focused in Araihazar Upazila, Bangladesh (Site F (Lashkardi Village), Site B (Baylakandi Village) (Figure S1) where geochemical and hydrological

parameters have been well characterized over the past decade(van Geen 2003; van Geen et al. 2004; Stute et al. 2007; Zheng et al. 2005; Dhar et al. 2008; Mailloux et al. 2013). Additional samples that were collected from nearby locations (Site O, Site N, Site S, Site M and Site T) were included in this study to provide a regional and depth profile context (Figure S1). Briefly, the study area is ~25 kilometers east of Dhaka and the shallow (Holocene-aged aquifer) groundwater commonly contains arsenic concentrations exceeding the WHO's drinking limit of 10µg/L with local variation in the depth concentration gradients(van Geen 2003; Zheng et al. 2005; Stute et al. 2007; Dhar et al. 2008) (Figure S1). In the shallow groundwater wells tested in the area, ~38% contain arsenic concentrations up to 10ug/L, ~53% contain up to 50 ug/L with the remainder above(Van Geen et al. 2014). All site names within this study were kept consistent with the existing literature(Stute et al. 2007; Dhar et al. 2008; Mailloux et al. 2013; Zhang et al. 2012). Site F and Site B were chosen as the focus of this study because while being relatively close in proximity (~3.5 km), they contain distinct depth profiles of arsenic concentrations(Stute et al. 2007; Zheng et al. 2005; Dhar et al. 2008; Mailloux et al. 2013). Groundwater at Site B contains significantly higher arsenic concentrations (as high as ~500 µg/L) than Site F (as high ~200 µg/L) and the highest arsenic concentrations at Site B occur at shallower depths (~14m) than Site F (~20 m)(Stute et al. 2007; Zheng et al. 2005; Dhar et al. 2008; Mailloux et al. 2013). The arsenic concentrations are correlated with $^{3}H/^{3}He$ ages and Site B has slower recharge rates than Site F (Stute et al. 2007). In addition, radiocarbon signatures from microbial DNA at Site F (Mailloux et al. 2013) enabled comparison of the two methods and potentially any differences or similarities between carbon sources of the sediment- and groundwater-associated bacterial populations.

2.3.2 Sediment and Groundwater Sampling

A detailed timeline outlining all sample collection events is included within the Supporting Information (SI) (Figure S2). Sediment samples for $\Delta^{14}C$ analysis of PLFA and SOC were taken in January 2013 from Site F (PLFA only) and Site B. SOC values for Site F were derived from values reported at the same site by Mailloux *et al*.(Mailloux et al. 2013) sampled in 2012. Sediment samples were taken in 2011 and 2012 from Sites O, N, M, S and T (Figure S1). Sediment cores were taken using a gravity corer (see description in SI), sectioned directly into whirl packs bags, immediately placed on ice and frozen at -20°C at a local clinic. Sediments were kept frozen until further processing/analysis. Sediment samples were subsequently freeze-dried for 48-72 hours and homogenized.

Groundwater samples (each 250 mL) for DOC and DIC analyses were taken from pre-existing well nests(Stute et al. 2007) at Site F and B from multiple depths in January 2015 with submerged pumps (methods outlined in Mailloux *et al*.(Mailloux et al. 2013)). PLFA in groundwater was sampled in 2013 from Site B by pumping large volumes (1800-8600 L) of groundwater from the three wells through glass-wool filters (poresize 0.7µm, burnt at 400°C overnight) from Site B and freezing at -20°C on site for transport until being freeze-dried for 48-72 hours prior to extraction. Groundwater samples for DOC radiocarbon analysis were acidified in 250 ml glass bottles with hydrochloric acid (HCl) on site until further processing.

2.3.3 DIC and DOC Groundwater Concentration Analysis

DOC concentrations were measured using a Shimadzu OC analyzer 5000A as non-volatile organic carbon (NVOC) from acidified samples collected in baked glass vials (500°C for 4 hours) to avoid external contamination. DIC concentrations were measured in water fixed with $HgCl_2$ and the samples were shipped to National Ocean Sciences Accelerated Mass Spectrometry Facility

(NOSAMS) at Woods Hole Oceanographic Institution (Maine, Massachusetts) using standard protocol described at www.whoi.edu/nosams/page.do?pid=40135.

2.3.4 Bacterial Phospholipid Extraction, Purification and Analysis through Gas Chromatography-Mass Spectrometry (GC-MS)

Preliminary extractions using ~50 grams of sediment were carried out to determine the amount of sediment required to obtain enough mass of PLFA for $\Delta^{14}C$ analysis. Sediments (ranging from ~300 grams to ~1 kg depending on biomass) and glass-wool filters were extracted twice overnight using a modified Bligh and Dyer(Bligh & Dyer 1959; White et al. 1979) and phospholipids were separated through silica gel chromatography (F_1=dichloromethane (DCM), F_2=acetone, F_3=methanol (MeOH)). The phospholipid/methanol fraction was evaporated to dryness under N_2 and reacted to become fatty acid methyl esters (FAME) via the mild alkaline methanolysis reaction(White et al. 1979) allowing the compounds to be amenable to GC-MS analysis. FAMEs were purified through a secondary silica gel chromatography (F_1=4:1 hexane:DCM, F_2=DCM (contains FAME's) and F_3=MeOH). All methanol used in methanolysis reactions are characterized for both its $\delta^{13}C$ and $\Delta^{14}C$ values to allow PLFA isotope values to be corrected for the addition of an extra methyl group.

All samples were analyzed for PLFA concentrations using gas chromatography on an Agilent 6890N GC (30 m × 0.32 mm DB-5 MS column, 0.25 μm film thickness) coupled to a 5973 quadrupole mass spectrometer monitoring for masses (50-450 m/z). Operating GC-MS conditions included a temperature program with an initial hold for 1 minute at 40 °C ramped to 130 °C at 20 °C/min to 160 °C at 4 °C/min and finally to 300 °C at 8 °C/min. QA/QC, standards and reagents used for PLFA analysis are described in SI.

2.3.5 Radiocarbon analysis of PLFA, DOC, DIC and SOC

PLFA extracts from sediments were run through a final purification procedure using a five fraction elution scheme (F_1=hexane, F_2=3:1 hexane:DCM, F_3=5:1 hexane:DCM, F_4=DCM (containing FAME's), F_5=MeOH) through ~1 gram of activated silica gel to remove non-PLFA carbon and purity was checked with GC-MS. DOC samples were obtained by filtering ~250 ml of groundwater samples (Durpore® PVDP 0.22µm (Millipore), freeze-drying the filtered water and subsequent acid-treatment with HCl to liberate residual inorganic carbon. Sedimentary PLFA extracts, DOC, DIC, and freeze-dried sediment samples (SOC) were shipped to NOSAMS for radiocarbon analysis through accelerated mass spectrometry (AMS). All samples were analyzed using Oxalic Acid II and Vienna Pee Dee Belemnite (VPDB) standards. An error ±20‰ was assumed for all PLFA $\Delta^{14}C$ values which is a conservative and appropriate estimate of error for microscale $\Delta^{14}C$ measurements(Pearson et al. 1998).

2.3.6 Statistical Analyses

All statistical analyses was carried out using SYSTAT software using 95% confidence intervals and detailed results of the statistical analysis are provided in SI.

2.4 Results

2.4.1 Sediment/Groundwater Bacterial Community Abundances and Arsenic Groundwater Concentrations

PLFA concentrations in sediments varied with depth, and ranged from 20 to 1300 picomoles/gram of sediment at both Site F (540 ± 280 picomoles/gram) and Site B (520 ± 360 picomoles/gram) (SI Table S1 and S2). Corresponding bacterial cell abundances, calculated using a conversion factor of 2 x 10^4 cells/picomole PLFA(Green & Scow 2000), averaged 1.1 x 10^7 ± 7 x 10^6 and 1.0 x 10^7 ± 6 x10^6 cells/gram of sediment (dry wt.) for Site F (n=8) and Site B (n= 11)

respectively (Figure 1, Table S1 and S2). At Site B, the bacterial cell abundances in the groundwater at 7.3 m, 14.3 m and 45.4 m were found to be approximately 5 orders of magnitude less abundant at 7.7×10^1, 1.3×10^2 and 1.0×10^2 cells/ml of groundwater respectively (Table S3). The sedimentary cell abundances are typical of nutrient/carbon limited aquifer sediments(Griebler & Lueders 2009; Smith et al. 2015; Amalfitano et al. 2014) and the groundwater-associated bacterial communities are consistent with a previously reported direct cell count of shallow Bangladesh groundwater(Islam et al. 2001). No significant correlation was found between the total bacterial abundance estimates of the sediment and the average dissolved arsenic concentrations within the coinciding groundwater at either Site F (unparametric Spearman Rank Correlation coefficient $\rho = 0.112$) or Site B (unparametric Spearman Rank Correlation coefficient $\rho = 0.217$ (Figure 2.1).

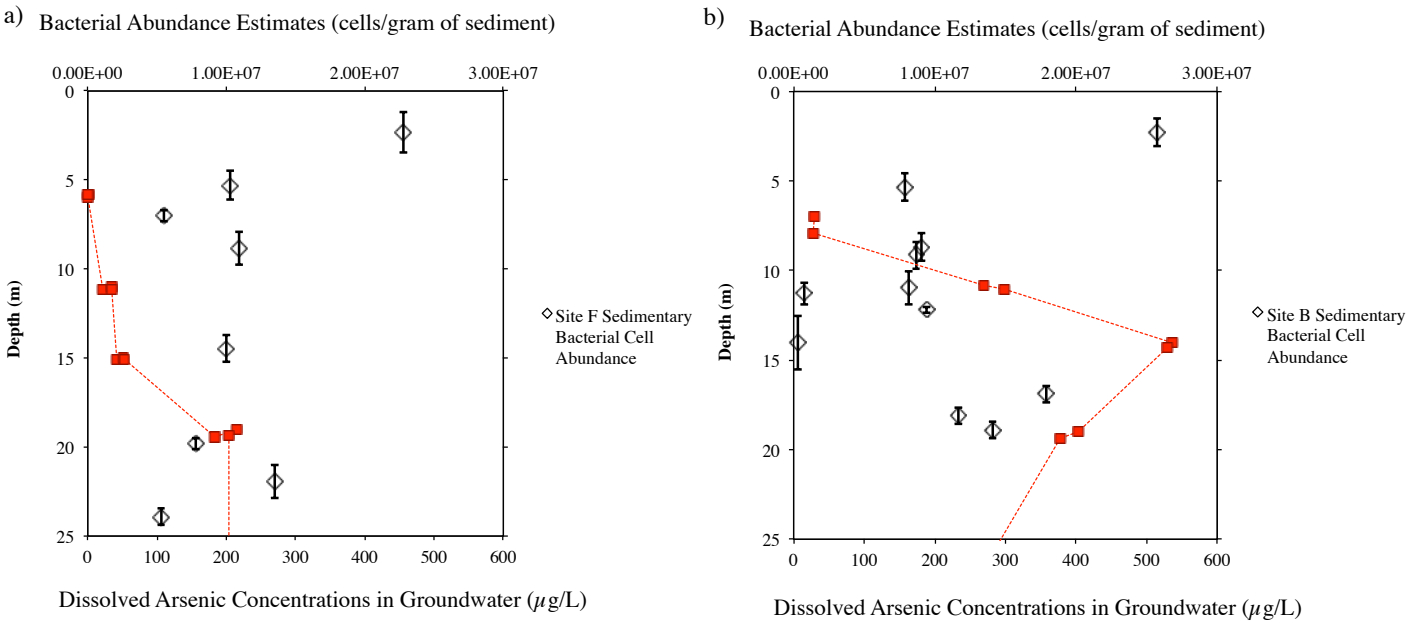

Figure 2.1 Depth profiles at (a) Site F and (b) Site B of sedimentary bacterial abundance (cells/gram of sediment) and dissolved arsenic concentrations measured in coinciding groundwater. Vertical error bars on PLFA indicate the depth range of composite sediment samples.

2.4.2 $\Delta^{14}C$ Signatures in Shallow Aquifer Carbon Pools

The average sedimentary organic carbon $\Delta^{14}C$ signature at the shallow depths corresponding to those analyzed for PLFA (<30 m) of Site F (Lashkardi Village)(Mailloux et al. 2013) and Site B (Baylakandi Village) was -631 ± 54 ‰ ($\Delta^{14}C$ range =-147‰ to -906‰, Figure 2a,b, Table 1). The youngest SOC signature (Site F, 7 m depth, $\Delta^{14}C$ = -147‰(Mailloux et al. 2013)) was a statistical outlier from all other SOC signatures found at Site B and Site F. Without this point, the mean is -675 ± 140 ‰. There was no significant trend with depth for these shallow sediments at Site F and B. These values were consistent with radiocarbon signatures of SOC at Sites M, N, O, S and T (all within a ~4 km radius of Site F and Site B) from depths ranging from 35 m to 67 m (Table 1). $\Delta^{14}C$ of SOC at these deeper depths ranged from -613‰ to -989‰ with the most positive values occurring at the shallowest depths and overlapping with the ranges observed at Site B and F (Figure

3, Table 1). These SOC $\Delta^{14}C$ signatures are consistent with the expectations based on the sediment deposition history of rapid infilling of the Bengal Basin(Alam et al. 2003) after the last glacial maximum.

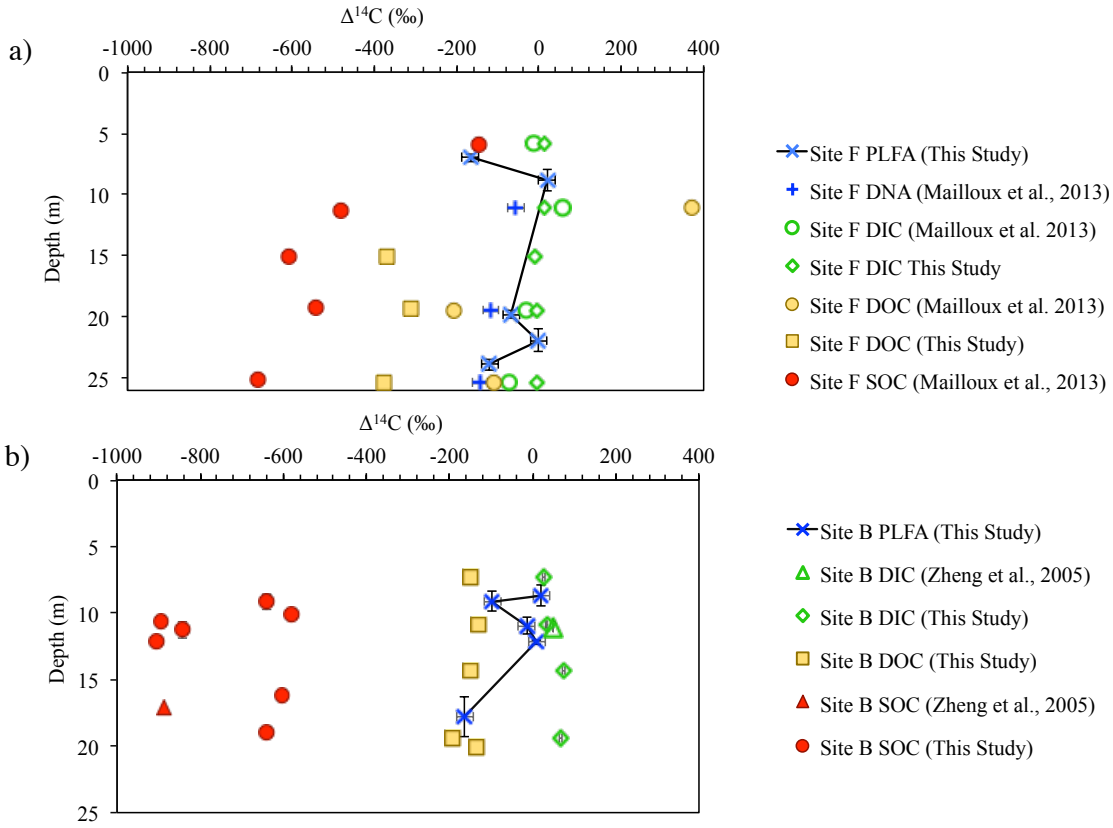

Figure 2.2 Radiocarbon signatures of microbial cellular components (sedimentary PLFA and groundwater microbial DNA) and carbon pools (dissolved inorganic carbon (DIC), dissolved organic carbon (DOC), sedimentary organic carbon (SOC) in the shallow aquifer portion of a) Site F and b) Site B. Horizontal Error bars of SOC, DIC and DOC indicate instrumental error reported by NOSAMS and if not visible were smaller than the marker. Horizontal error bars on PLFA assume a conservative error estimate of ±20‰ considered appropriate for microscale $\Delta^{14}C$ measurements (Pearson et al. 1998). Vertical error bars on PLFA indicate the depth range of composite sediment samples.

Table 2.1 Radiocarbon values of sedimentary phospholipid fatty acids (PLFA's) and sedimentary carbon sources (SOC) at study sites in the Araihazar Region, Bangladesh.

Bangladesh Site	Sample Description	PLFA Depth Interval (m)[a]	PLFA Depth Midpoint (m)	PLFA $\Delta^{14}C$ (‰) ± 20[b]	SOC Depth (m)	SOC $\Delta^{14}C$ (‰) ± Error[c]
Site F (Lashkardi Village)	Sediment	6.7 - 7.3	7	-167	-	-
Site F (Lashkardi Village)	Sediment	7.9 - 9.8	8.8	20	-	-
Site F (Lashkardi Village)	Sediment	19.5 - 20	19.8	-67	-	-
Site F (Lashkardi Village)	Sediment	21 - 22.9	21.9	-1	-	-
Site F (Lashkardi Village)	Sediment	23.5 - 24.4	23.9	-119	-	-
Site B (Baylakandi)	Sediment	7.6 - 9.8	8.7	21	9.1	-640 ± 1.6
Site B (Baylakandi)	Sediment	8.5 - 9.8	9.1	-96	10.7	-896 ± 1.4
Site B (Baylakandi)	Sediment	10 - 11.9	11	-14	10.1	-580 ± 1.5
Site B (Baylakandi)	Sediment	12 - 12.4	12.2	10	11.3	-842 ± 1.5
Site B (Baylakandi)	Sediment	16.5 - 19.2	17.8	-163	12.2	-906 ± 1.5
Site B (Baylakandi)	Sediment	-	-	-	16.2	-604 ± 1.6
Site B (Baylakandi)	Sediment	-	-	-	18.9	-640 ± 1.6
Site M	Buried Peat	-	-	-	35.4	-664 ± 1.5
Site M	Sediment	-	-	-	35.4	-663 ± 1.8
Site M	Charcoal	-	-	-	37.2	-613 ± 1.8
Site M	Buried Peat	-	-	-	37.2	-652 ± 2.2
Site M	Sediment	-	-	-	37.2	-654 ± 1.5
Site M	Buried Peat	-	-	-	37.8	-658 ± 1.5
Site M	Sediment	-	-	-	50	-777 ± 1.3
Site M	Sediment	-	-	-	50.6	-754 ± 1.4
Site M	Sediment	-	-	-	73.8	-883 ± 0.9
Site M	Sediment	-	-	-	78	-989 ± 2.2
Site N	Sediment	-	-	-	50.3	-710 ± 1.5
Site O	Sediment	-	-	-	56.4	-843 ± 1.6
Site S	Sediment	-	-	-	33.5	-663 ± 1.5
Site S	Sediment	-	-	-	44.2	-950 ± 1.6
Site S	Sediment	-	-	-	44.2	-923 ± 2.6
Site S	Sediment	-	-	-	68.6	-973 ± 1.4
Site S	Sediment	-	-	-	73.2	-884 ± 1.5
Site T	Sediment	-	-	-	42.7	-642 ± 1.7
Site T	Sediment	-	-	-	57.9	-805 ± 1.6
Site T	Sediment	-	-	-	67.1	-955 ± 1.6

[a] PLFA depth intervals represent the range of depths in the composite sediment samples for PLFA

[b] A conservative error of ± 20‰ was applied to each PLFA radiocarbon measurement which is considered appropriate for <100 microgram sample size run with AMS (Pearson *et al.*[65])

[c] Error provided for the sedimentary organic carbon is the instrumental error reported by NOSAMS

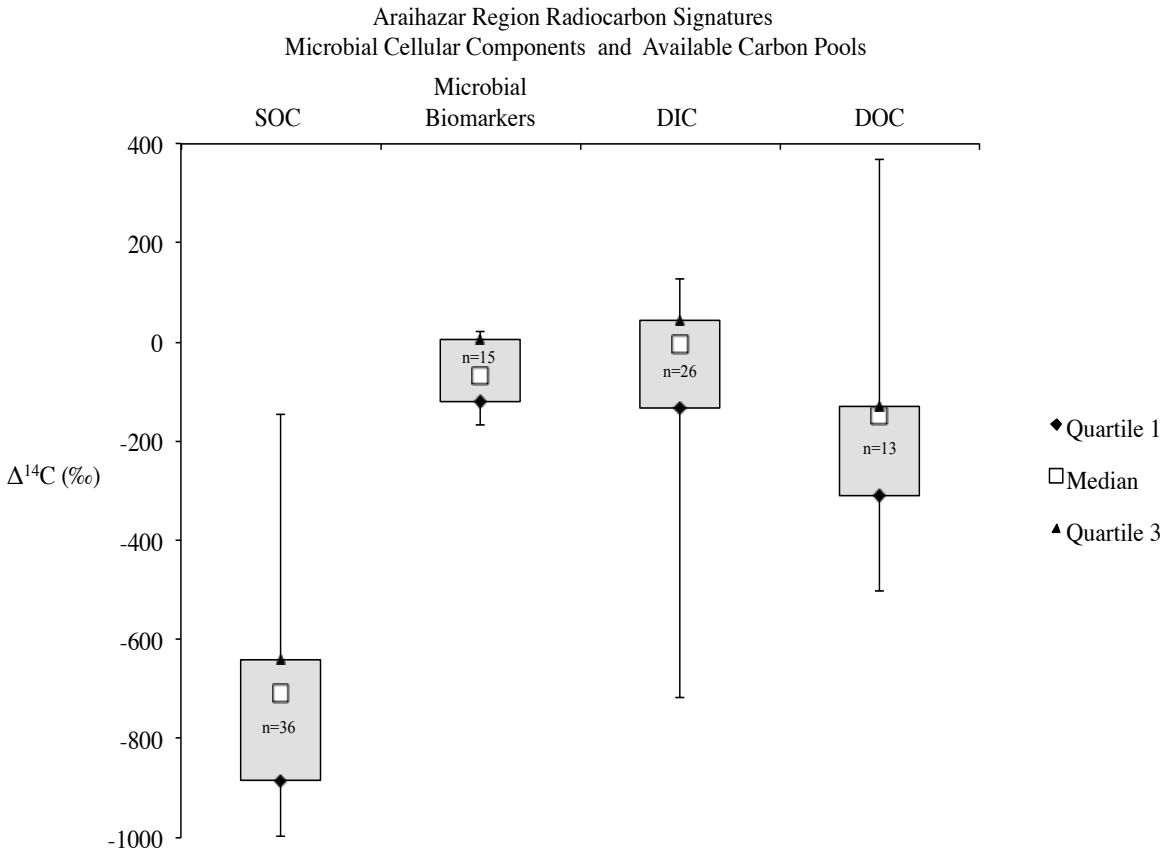

Figure 2. 3 Box-plot summary of available radiocarbon signatures in the Araihazar Region in Bangladesh from sedimentary organic carbon (SOC), microbial biomarkers (PLFA and DNA), dissolved inorganic carbon (DIC), dissolved organic carbon (DOC) reported in this study, (Mailloux et al. 2013) and (Zheng et al. 2005). Error bars indicate range of $\Delta^{14}C$ signatures.

Average DIC groundwater concentrations at Site F and Site B of 5 ± 2 mmol/L and 9 ± 2 mmol/L were orders of magnitudes higher than corresponding DOC concentrations (0.05 ± 0.02 mmol/L and 0.2 ± 0.02 mmol/L) (Figure 4; Table S4). DIC $\Delta^{14}C$ signatures from all measured samples <30 m depth were significantly more modern than SOC ranging from -8 ‰ to +12 ‰ at Site F (+1 ± 10‰) and from +27 ‰ to +75 ‰ at Site B (+48 ± 20) (Figure 2a,b; Table S5;S6;S7). DOC $\Delta^{14}C$ signatures were likewise more modern than SOC but significantly more depleted than DIC. $\Delta^{14}C$ DOC ranged from -310 ‰ to -370 ‰ at Site F (-353 ± 40‰) and -194‰ to -131‰ at

Site B (-151 ± 25‰) ((Figure 2.3 a,b, Table S5;S6;S7). At both sites, the $\Delta^{14}C$ of DIC was found to be statistically more positive than that of the DOC (Site F: p=0.002, Site B: p=0.039) (Table S6;S7). Notably, the DOC $\Delta^{14}C$ signatures measured in groundwater collected for this study in 2015 from Site F were significantly lower and had a smaller range than the DOC measured in 2012 samples ($\Delta^{14}C$ = +19 ± 308) from the same depths by Mailloux *et al*.(Mailloux et al. 2013).

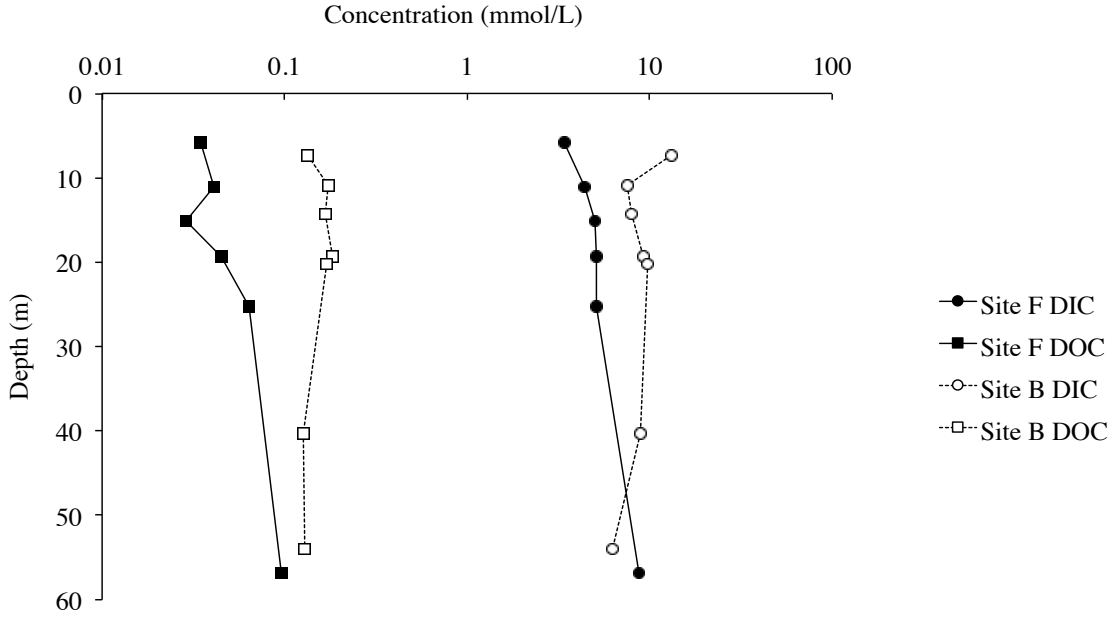

Figure 2. 4 Depth profile of dissolved inorganic and organic carbon concentrations (mmol/L) measured in groundwater at Site F (black symbols) and Site B (open symbols).

2.4.3 Δ ^{14}C Signatures of PLFA Relative to Available Carbon Pools

$\Delta^{14}C$ signatures of PLFA extracted from sediment of both Site F and Site B ranged from -167‰ to +20‰ and -163‰ to +21‰ respectively, varying with depth (Figure 2a,b; Table 1). When a comparison between $\Delta^{14}C$ of PLFA and the $\Delta^{14}C$ of DOC/DIC was possible for a given depth (sample depths were within 1.5 m of each other) (n=7), the $\Delta^{14}C$ of PLFA were in agreement

(within error (±20‰)) with the $\Delta^{14}C$ of DIC for three sample sets (Site B at 8m, 11m and 12m). In the remaining four samples, the $\Delta^{14}C$ of PLFA fell within an intermediate range between the DOC and the DIC. At Site F where Mailloux *et al*.(Mailloux et al. 2013) had previously measured the $\Delta^{14}C$ of DNA from the groundwater microbial community, the $\Delta^{14}C$ signatures of PLFA correlated (Pearson R=0.995) with those measured in microbial DNA (Mailloux et al. 2013) but had a slightly younger signature (average difference of +50‰, just outside of analytical precision). Given that the samples were collected two years apart and may have been affected by slight variation in the age of the microbial carbon sources between 2013 to 2015 or differences between sediment- associated (this study) vs. groundwater(Mailloux et al. 2013) bacteria. (Figure S3, Table S8) this agreement between the two methods is remarkably good. To examine which carbon source the indigenous sedimentary bacteria were utilizing, post-hoc Tukey pairwise statistical comparisons of $\Delta^{14}C$ between all of the PLFA, DOC, DIC and SOC at each site from this study combined with the results from Mailloux *et al*.(Mailloux et al. 2013) (Site F: DOC, DIC, SOC) were carried out. The DOC and DIC by Mailloux *et al*.(Mailloux et al. 2013) were specifically included within the statistical analysis with the 2015 DIC and DOC values to ensure the analysis was representative as the sediments for PLFA were collected at an intermediate time point (2013). This analysis revealed no significant difference between the bulk DOC and PLFA at Site B ($p=0.38$) but did reveal a statistical difference at Site F between the bulk DOC and PLFA ($p=0.04$) (Table S6;S7). At either site, no statistical difference was observed between the radiocarbon signatures between the bulk DIC and the PLFA (Site F: $p=0.78$, Site B: $p=0.67$) (Table S6;S7). The PLFA values are significantly more enriched than the SOC values for Site B ($p=<0.001$) and Site F ($p=0.0003$) (Figure 2a,b; Table S6;S7) or the region (Figure 2.3, Table 1).

2.4.4 Carbon Source Age and Bacterial Community Abundance

If Site F is considered in isolation, a positive correlation (R=0.91, p=0.032) between the bacterial community abundance (calculated using conversion factors from total PLFA concentrations) and the $\Delta^{14}C$ of PLFA is observed. When Site B is examined independently, no significant positive correlation was observed. However, this lack of correlation is largely controlled by a single sample where the indigenous populations with the highest bacterial abundance (~1.5 x 10^7 cells/gram of sediment) also had the most depleted $\Delta^{14}C$ signature at the site. Exclusion of this sample's data and a combined Site F and Site B regression analysis between bacterial cell abundance estimates and radiocarbon signatures gives a positive correlation of Pearson R=0.72 (p=0.028) (Figure 5).

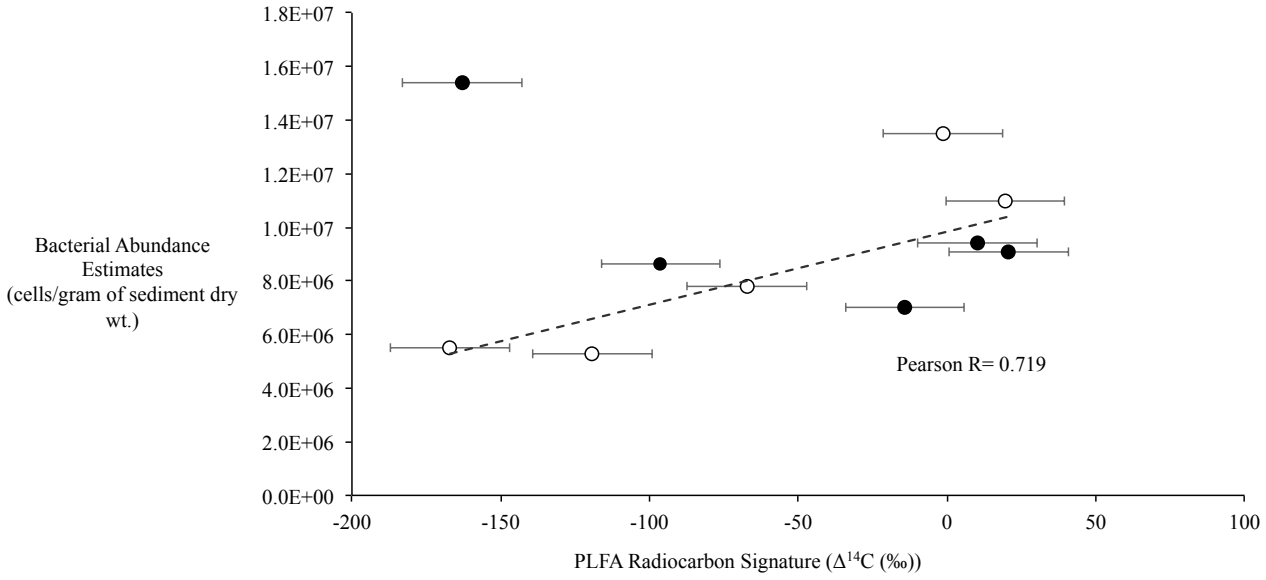

Figure 2. 5 Regression plot of sedimentary bacterial abundance estimates (cells/gram of aquifer sediments (dry)) and corresponding PLFA radiocarbon signature at Site F (open circles) and Site B (filled circles). Regression analysis (Pearson R=0.719) excluded a single outlier point from Site B ($\Delta^{14}C$=-163‰, bacterial abundance = 1.54 x 10^7 cells/gram dry sediment). Horizontal error bars on PLFA assume a conservative error estimate of ±20‰ (Pearson et al. 1998).

2.5 Discussion

2.5.1 Arsenic Groundwater Concentrations and Sediment Bacterial Community Abundance

The observed lack of correlation between arsenic concentrations in the groundwater and the sedimentary bacterial community abundances indicates that it is not an increase in the total bacterial population size that is responsible for the observed high As concentrations (Figure 1). While the sedimentary bacterial community abundances are not changing coincident with the occurrence of high levels of As, an increased proportion of the active community carrying out Fe and/or As reduction has been observed previously in the areas of high arsenic(Islam et al. 2004; Mailloux et al. 2013). However, this observation would also be consistent with As release occurring close to recharge points and being transported with water as has been suggested recently(Stuckey et al. 2015). The approaches used within this study are unable to differentiate between these two possibilities and thus warrants future research.

2.5.2 $\Delta^{14}C$ Shallow Aquifer Carbon Pools and Carbon Cycling

The modern DIC values were consistent with the expectations based on tritium dating(Stute et al. 2007) indicating that this DIC is partially atmospherically derived and has undergone vertical transport with the water and/or is the product of mineralization of modern organic carbon being transported vertically. The more positive range observed at Site B, including points above the current $\Delta^{14}C$ of the atmosphere, is consistent with the presence of DIC influenced by atmospheric weapons testing ("bomb carbon") and the slower infiltration rates at this site(Stute et al. 2007). DOC is likely a mixture of relatively modern components derived from vertical recharge co-transported with the DIC and dissolution/mobilization of some SOC carbon from shallower depths (~<30 m) during transport. The cause of temporal fluctuation observed in DOC at Site F compared

to Mailloux *et al*.(Mailloux et al. 2013), while the $\Delta^{14}C$ of DIC at Site F remained consistent, is not known. At Site F, more depleted $\Delta^{14}C$ values of DOC suggest a higher proportion of SOC dissolution may be occurring in 2015 compared to 2012. At Site B, SOC is not the predominant source of carbon contributing to the bulk DOC pool in 2015. Variations in the proportions of these sources may be responsible for the observed $\Delta^{14}C$ variations.

2.5.3 Radiocarbon Signatures of Bacterial PLFA and Available Carbon Pools

$\Delta^{14}C$ values of the bacterial lipids (PLFA) generally fell between DIC and DOC and trended closer to the age of the DIC pool. Recent research has demonstrated the presence of autotrophic bacterial genes in the subsurface(Kellermann 2012; Wrighton et al. 2014) which may indicate that DIC utilization could be occurring, likely in combination with more predominant heterotrophy. The agreement between the PLFA and DIC $\Delta^{14}C$ signatures could also in part be explained through heterotrophic bacteria mineralizing a younger component of the DOC, adding to the DIC pool. However, with the DOC far less abundant than the DIC, the DIC signatures are unlikely to be produced predominantly from DOC mineralization. To examine microbial metabolization of a mixture of potential carbon sources (Figure 2a,b), a mass balance approach (example in equation 1) was carried out twice assuming in each case only two major carbon pools (DIC versus DOC aged carbon (equation 1, Figure 6a) and DIC versus SOC aged carbon (Figure 6b) contributing to the PLFA $\Delta^{14}C$ signature (Table S9).

Equation 1 $$\Delta 14C_{PLFA} = \Delta 14C_{DOC} * (f) + \Delta 14C_{DIC} * (1 - f)$$

where (f) equals the proportion of sedimentary bacteria metabolizing DOC aged carbon and (1-f) equals proportion of sedimentary bacteria using DIC aged carbon. The mass balance results between DIC and DOC (Figure 6a) indicate that in this scenario the bacterial community would be

predominantly (>60%) using the DIC or alternatively, the microbes could be using a component of the DOC pool of equivalent $\Delta^{14}C$ age to the DIC. Utilization of a component of the DOC pool is consistent with the idea that heterotrophic communities dominate sub-surface aquifer systems. In this scenario, DOC potentially comprised of more polar, bioavailable organics is transported with surface recharge and heterotrophic bacteria utilized this subcomponent of DOC to respire CO_2 contributing some younger carbon to the DIC pool.

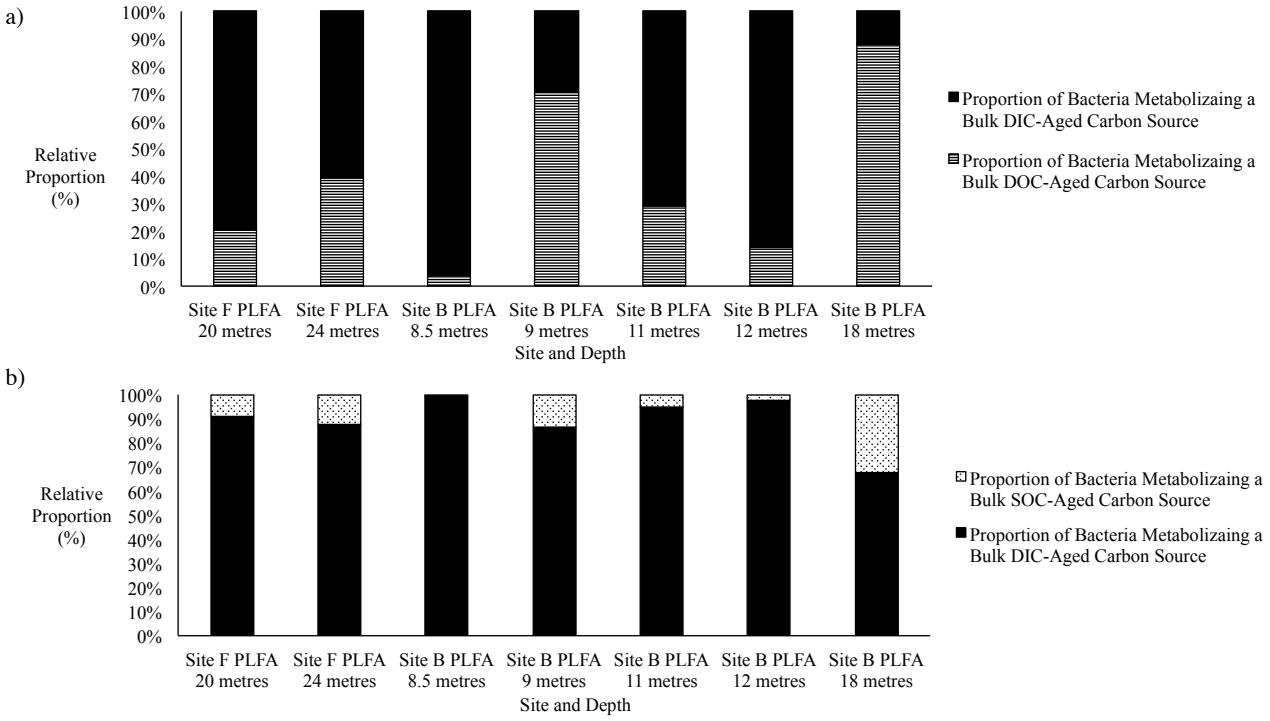

Figure 2. 6 Mass balance approach using PLFA radiocarbon signatures with a) DOC and DIC-aged carbon sources as two predominant sources at Site F and Site B and b) SOC and DIC-aged carbon sources as two predominant sources at Site F and Site B.

The results of the mass balance between the $\Delta^{14}C$ signatures of DIC and SOC at both Site F and Site B suggested that negligible (<10%) utilization of SOC derived carbon was occurring. (Figure 6b, Table S9). The PLFA were consistently more modern than the DOC and SOC, so a mass balance approach using the bulk $\Delta^{14}C$ DOC and SOC signatures could not result in the

observed PLFA values. These results imply that sedimentary bacterial communities in Bangladesh aquifers are not primarily utilizing older sedimentary derived organic carbon sources such as petroleum-derived carbon(Rowland et al. 2006; Dongen et al. 2008; Rowland et al. 2002; Rowland et al. 2007), peat(Ravenscroft et al. 2001; McArthur et al. 2001; Mladenov et al. 2010; Planer-Friedrich et al. 2012), or carbon buried at the time of sediment deposition(Meharg et al. 2006) to drive their metabolisms. These results do not preclude the possibility of minor amounts of SOC being utilized as suggested previously(Mailloux et al. 2013; van Geen, Benjamin C Bostick, et al. 2013). At the shallowest depth measured at Site F (~7 m), PLFA $\Delta^{14}C$ values were within the 20‰ error of the sedimentary-derived carbon, and therefore metabolization of younger peat layers or other similarly aged sedimentary carbon sources cannot be ruled out for this depth. Overall, these findings strongly suggest a predominant source of carbon that is significantly younger than the SOC.

Rather than a primary carbon source, peat and/or other sedimentary derived carbon sources may contain humic substances acting as electron shuttling substrates. This would allow SOC to that facilitate enhanced microbial reduction and iron dissolution(Lovley et al. 1996; Lovley et al. 1999; Jiang & Kappler 2008; Bauer & Kappler 2009; Mladenov et al. 2010; Planer-Friedrich et al. 2012; Mladenov et al. 2015) while more modern carbon sources in the dissolved phase serve as the carbon and electron donors for the microbial community. This indirect role for SOC rather than as the carbon source might explain the co-occurrence of high arsenic concentrations and peat layers reported at some sites(Ravenscroft et al. 2001; McArthur et al. 2001; Anawar et al. 2003; Yamazaki et al. 2003; McArthur et al. 2004; Mladenov et al. 2010; Planer-Friedrich et al. 2012), but arsenic release occurs in the absence of peat layers indicating this is a secondary requirement (Harvey et al. 2002; Swartz et al. 2004; Charlet et al. 2007).

2.5.4 Preferential Microbial Utilization of Younger Carbon Sources by Sedimentary Bacteria

A preferential utilization of younger dissolved carbon pools over older sedimentary derived carbon by the *in situ* bacterial communities may be explained by younger carbon pools still containing higher proportions of labile carbon compounds that are more bioavailable than older and more recalcitrant mixtures present in the sedimentary carbon pool(Slater et al. 2005). There is also a possibility the older sedimentary pool is labile but somehow protected in environmental settings from microbial degradation. The latter is supported through recent microcosm experiments with Bangladesh sediments, where Neumann *et al*.(Neumann et al. 2014) reported a promoted mobilization of SOC followed by microbial utilization of sedimentary organic carbon after sampling and homogenization of the sediment. Preferential degradation of younger carbon sources by bacteria in soils and sediments has been reported in other environments through radiocarbon analysis(Cowie et al. 2010; Slater et al. 2005; Trumbore 2009). The correlation between the age of metabolized carbon sources and the overall size of the sedimentary bacteria communities (Figure 5) supports that younger carbon sources are more labile and may support a larger (more active) bacterial community. Preferential microbial metabolization of younger-aged DOC over older SOC is in agreement with a recent study by Al Lawati *et al*. (2013) Al Lawati *et al*. (2013) reported that no correlational relationship was found between the carbon species distribution within sedimentary carbon and arsenic release in a microcosm experiment using South-East Asian aquifer sediments (from Taiwan). The authors inferred that an additional electron donor (such as dissolved carbon sources) is providing the electron donors facilitating iron reduction and arsenic release. The single outlier from Site B where the sedimentary bacteria with most depleted $\Delta^{14}C$ also had the highest abundance is a reminder that carbon sources and/or controls on bacterial community abundance can vary locally due to numerous ecosystem factors such as nutrient limitation, organic carbon

availability and/or characteristics, changing redox conditions, predatory micro-eukaryotic populations, etc.

2.5.5 Implications of Carbon Cycling Effects on Arsenic Contamination in Bangladesh

The hydrologic system within Bangladesh has been rapidly changing including increases in irrigation pumping, water withdrawal for municipal pumping causing large scale drawdowns, communities switching to deeper community wells to avoid arsenic exposure, and the installation of local piped water supplies. All of these changes are increasing the demand for groundwater and will increase flow rates while decreasing residence times. The results of this study, Mailloux *et al*. (2013) and Harvey *et al*. (2002) all suggest that utilization of relatively modern carbon is driving microbial metabolism in the Holocene-aged sediments and that this carbon can be advected through the aquifer sediments. The changes in the hydrologic regime could redistribute the reactive organic carbon pools throughout both the shallow and deep aquifer sediments and could lead to changes in the microbial communities, geochemistry of the groundwater and the distribution of As.

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Supporting Information

A field site photograph and map, chemicals and reagents, PLFA data, DOC and DIC concentrations, $\Delta^{14}C$ data, mass balance results and statistical analyses are included in SI.

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2.6 Chapter 2. Supporting Information: Stimulation of Microbially-Mediated Arsenic Release in Bangladesh Aquifers by Young Carbon Indicated by Radiocarbon Analysis of Sedimentary Bacterial Lipids

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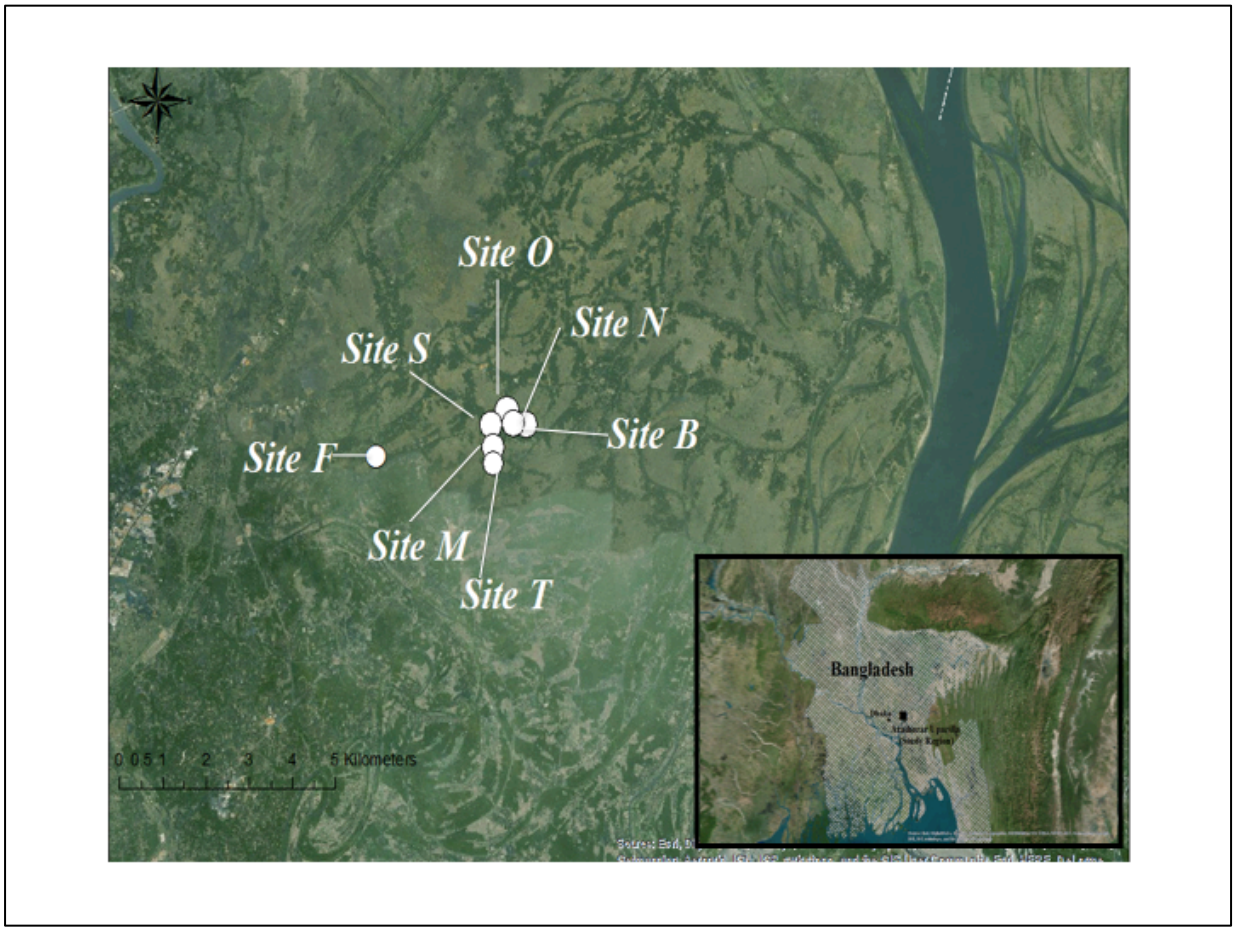

Figure S1 Field site map of Arzaihazar Upazila, Bangladesh (inset) and study sites B, F, O, N, S, M and T constructed using ArcMap©10.3.1. (Site F (Lashkardi Village, 23.774 N 90.606 E), Site B (Baylakandi Village, 23.7800 N 90.6400 E), Site O (23.7823 N 90.6356 E), Site N (23.7801 N 90.6372 E), Site S (23.7799 N 90.6320 E), Site M (23.7760 E 90.6325 S), Site T (23.7731 E 90.6326 S).

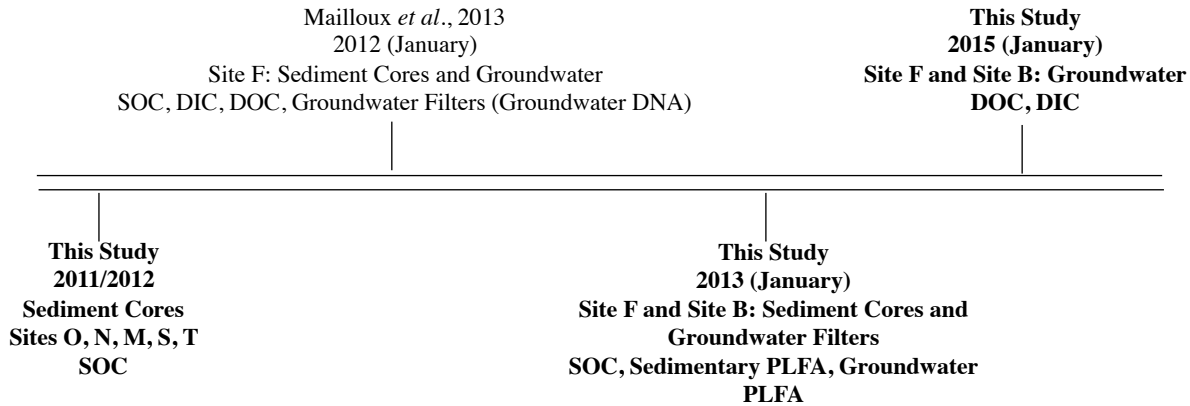

Figure S2 Field sampling timeline of sediment cores, groundwater and groundwater filters used for $\Delta^{14}C$ analysis at Site F, Sit and study sites B, F, O, N, S, M and T

Sediment Collection with Gravity Corer

An AMS corer (commonly used to collect soil cores from shallow depths) was used to collect sediments cores. The corer collects an undisturbed core from the base of a pilot hole drilled using local hydraulic drilling techniques. This pilot hole was purged, and a gravity corer (5 cm) diameter core sleeves were used to get enough material for analysis) was placed in the bottom of the hole by attaching it to >20m of steel extension rods to bring it to the bottom of the hole. A slide hammer or sledge was then used to push the corer into the soil the length of the core sleeve (30 cm), and was manually lifted out of the hole, removing sections of the extension rods as it is pulled up. To ensure that only undisturbed material is obtained, the top few centimeters of the core is removed to make sure that any contamination at the bottom of the hole is eliminated. Core recoveries were found to be typically in excess of 70%, but are problematic for some sands. Smaller diameter tubing was used in some instances (2.5 cm) to improve recovery but these cores obtain much less material for analysis.

PLFA Extraction and Analysis through Gas-Chromatography-Mass Spectrometry

Sediment samples and glass-wool filters were sequentially extracted twice over night at room temperature using a 1:2:0.8 DCM: methanol: phosphate buffer solution. Extracts were combined in a seperatory funnel, adjusted to a final ratio of 1:1:0.9 DCM: Methanol: Aqueous Solution and the organic phase/total lipid extract (TLE) was separated and blown down under N_2 gas to 1-2ml. Phospholipids were isolated from other components in the sedimentary TLE's using ~40 grams of activated silica gel chromatography and elution of 300 ml of DCM, 300 ml Acetone and 500 ml MeOH. Preliminary elution tests demonstrated this volume of solvent was sufficient for recovery of phospholipids from the silica gel (Aldrich, 63–200 μm, 60 Å poresize). Phospholipids were isolated from other components in the groundwater filters TLE's using ~6 grams of activated silica gel chromatography and elution of 60 ml of DCM, 60 ml Acetone and 60 ml MeOH. After

methanolysis of the dried MeOH fractions, containing fatty acid methyl esters (trans-esterified PLFA's), secondary silica gel chromatography was used to further purify the fatty acid methyl esters (FAME's) on ~1 gram of fully activated silica gel eluting with 3 ml each of 4:1 hexane: DCM, DCM (contains FAME's) and MeOH. Extracts were blown down under N_2 to 250 µl. The quantification limit of the GC-MS was 1 mg/L based on the lowest concentration of alkane standard used within the calibration curves. QA/QC tests included instrumental and method extraction blanks and triplicate analysis of calibration standards. FAME's were identified using two FAME Mix reference standards (Bacterial Acid Methyl Esters CP Mix, Supelco Inc. and 37 Comp Fatty-Acid Methyl Ester Mix, Supelco Inc.), mass fragmentation patterns and relative retention times. FAME's were quantified by calibration to alkane standards C_{14} (methyl myristate, $C_{15}H_{30}O_2$, MW 242 g/mol, ≥99%, GC), C_{16} (methyl palmitate, $C_{17}H_{34}O_2$, MW 270 g/mol, ≥99 % capillary GC (Sigma Aldrich), C_{18} (methyl stearate, $C_{19}H_{38}O_2$, MW 298 g/mol, 99% capillary GC (Sigma Aldrich) and C_{20} (methyl arachidate, $C_{21}H_{42}O_2$, MW 326 g/mol, 99% GC (Sigma Aldrich). FAME's were less than the LOQ in method and instrumental blanks. All GC-MS data was processed using the Enhanced MSD ChemStation Software (Copyright© Agilent Technologies).

Groundwater Arsenic Concentrations

Site F and B arsenic concentrations (µg/L) profiles are average values from Stute *et al.* (2007), Dhar *et al.* (2008); Mladenov *et al*. (2010) and Mailloux *et al.* (2013) (Site F only). While measured previously, the average arsenic concentrations used for this analysis for both sites were considered representative of the conditions at the time of sediment sampling for this study as groundwater parameters have been demonstrated to remain stable at these sites over the past eight years ((Stute et al. 2007; Dhar et al. 2008; Mladenov et al. 2010; Mailloux et al. 2013)).

Table S1 Site B phospholipid fatty acid total concentrations and bacterial abundance estimates (cells/gram of sediment (dry weight). Arsenic concentrations (μg/L, ppb) for specific depths were derived from concentration profiles for Site B provided by Stute *et al*. (2007), Dhar *et al*. (2007), and Mladenov *et al* (2010).

Bangladesh Araihazar Upazila Site B (Baylakandi Village, 23.7800 N 90.6400 E)	**Depth Interval (m)**	1.5-3	4.6-6.1	7.6-9.8	8.5-9.8	10-11.9	10.7-11.9	12.2	12.5-15.5	16.5-17.3	17.9-18.3	18.6-19.2
	Depth Midpoint (m)	2.3	5.3	8.7	9.1	11.0	11.3	12.2	14.0	16.9	18.1	18.9
	Depth Interval (ft)	5-10	15-20	25-32	28-32	33-39	35-39	40	41-51	54-57	59-60	61-63
	Amount of Sediment Extracted (g)	51	48	367	1000	311	566	538	750	141	85	140
	Total pmol PLFA/mL of extract	66098	18789	166620	432617	126929	21737	253122	12608	126740	49789	98330
	pmol PLFA/gram sediment	1288	393	454	433	408	38	471	17	897	585	704
	Bacterial cells/ gram of sediment	2.58×10^7	7.86×10^6	9.09×10^6	8.65×10^6	8.15×10^6	7.68×10^5	9.41×10^6	3.36×10^5	1.79×10^7	1.17×10^7	1.41×10^7
	[As](ppb)	N/A	N/A	100	140	305	345	395	530	460	430	410

[a] Bacterial abundance was calculated using the conversion factor (2×10^4 cells/picomole of PLFA) appropriate for aquifer sediments (Green and Skow, 2000)

Table S2 Site F sedimentary phospholipid fatty acid total concentrations and bacterial abundance estimates (cells/gram of sediment (dry weight). Arsenic concentrations (μg/L, ppb) for specific depths were derived from concentration profiles for Site B provided by Stute *et al*. (2007), Dhar *et al*. (2007), Mladenov *et al* (2010) and Mailloux *et al*. (2013).

	Depth Midpoint (m)	2.3	5.3	7.0	8.5	14.5	19.8	21.9	23.9
Bangladesh, Araihazar Upazila Site F (Lashkardi Village, 23.774° N, 90.606° E)	**Depth Interval (m)**	1.5-3.8	4.6-6.1	6.7-7.3	7.9-9.8	13.7-15.2	19.5-20.1	21-22.9	23.5-24.4
	Depth Interval (ft)	5-10	15-20	22-24	26-32	45-50	64-66	69-75	77-80
	Total pmol PLFA/mL of extract	71339	34815	125544	290358	60562	281122	368872	122257
	pmolPLFA/gram sediment	1139	514	276	548	501	391	676	264
	Amount Extracted (g)	62.6	67.7	455.5	530	120.9	718.9	545	464
	Bacterial cells/gram of sediment	2.28×10^7	1.03×10^7	5.51×10^6	1.10×10^7	1.00×10^7	7.82×10^6	1.35×10^7	5.27×10^6
	[As] (ppb)	N/A	N/A	5	10	45	200	200	200

[a] Bacterial abundance was calculated using the conversion factor ($2 * 10^4$ cells/picomole of PLFA) appropriate for aquifer sediments (Green and Skow, 2000)

Table S3 Site B groundwater phospholipid fatty acid total concentrations and bacterial abundance estimates (cells/mL of groundwater)

	Site B 7.29 m	**Site B 14.3 m**	**Site B 45.4 m**
Pumping Volume (L)	8637	1821	4917
Total pmol PLFA/mL of extract	33240	11654	26983
Bacterial Cell Abundance Estimate For whole Pumping Volume	664796485	233076461	539653071
Bacterial Cell Abundance Estimate/ ml of groundwater (cells/ml)	77	128	110

Table S4 Dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) groundw concentrations (mmol/L)

	Depth	Site F DIC (mmol/L)	Site F DOC (mmol/L)
F1	5.8	3.4	0.035
F2	11.1	4.4	0.041
F3	15.1	5.0	0.029
F4	19.4	5.1	0.045
F6	25.3	5.1	0.064
F5	56.8	8.7	0.096
	Depth	Site B DIC (mmol/L)	Site B DOC (mmol/L)
B7	7.3	13.2	0.14
B8	10.9	7.5	0.18
B3	14.3	8.0	0.17
B4	19.4	9.3	0.19
B9	20.1	9.8	0.17
B5	40.2	8.9	0.13
B6	53.9	6.3	0.13

Table S5 Radiocarbon ($\Delta^{14}C$ (‰)) values of dissolved organic carbon (DOC) and dissolve inorganic carbon (DIC) in groundwater taken from Site F and B of the Araihazar Region, Bangladesh in January 2015.

Bangladesh Site	Sample Description	DOC Depth (m)	DOC $\Delta^{14}C$ (‰)	DIC Depth (m)	DIC $\Delta^{14}C$ (‰)
Site F (Lashkardi Village)	Groundwater	-	-	5.8	12 ± 2.2
Site F (Lashkardi Village)	Groundwater	-	-	11.1	12 ± 2.2
Site F (Lashkardi Village)	Groundwater	15.1	-372 ± 7	15.1	-8.2± 2.1
Site F (Lashkardi Village)	Groundwater	19.4	-310 ± 6	19.4	-5.8± 2.1
Site F (Lashkardi Village)	Groundwater	25.4	-379 ± 12	25.4	-5.5± 2.2
Site B (Baylakandi)	Groundwater	7.0	-148 ± 2	7.3	27 ± 2.2
Site B (Baylakandi)	Groundwater	14.3	-149 ± 2	10.9	33 ± 2.3
Site B (Baylakandi)	Groundwater	10.9	-131 ± 2	14.3	75 ± 2.2
Site B (Baylakandi)	Groundwater	19.4	-194 ± 2	19.4	68 ± 2.4
Site B (Baylakandi)	Groundwater	20.1	-135 ± 3	28.1	36 ± 2.3
Site B (Baylakandi)	Groundwater	-	-	39.7	52 ± 2.4
Site B (Baylakandi)	Groundwater	-	-	53.2	-169 ± 1.8

Statistical Analysis

Table S6 Statistical results (SYSTAT Software) of a post-hoc pairwise comparison of $\Delta^{14}C$ signatures of Site F samples using Tukey's Honestly-Significant Difference Test with a 95% confidence interval. Results from DOC and DIC groundwater samples collected from Site F in January 2015 were pooled with the DOC and DIC respectively $\Delta^{14}C$ (‰) values reported by Mailloux *et al*. (2013) from the same sample wells collected in 2012, SOC radiocarbon values are derived from Mailloux *et al*., (2013) and sediments for PLFA analysis were collected in January 2013.

Sample	Sample	Difference	p-value	95.0% Confidence Interval	
				Lower	Upper
Site F DIC	Site F DOC	269	0.0022	90.6	446.9
Site F DIC	Site F PLFA	61	0.78	-117.3	239.0
Site F DIC	Site F SOC	487	6.3E-06	308.4	664.7
Site F DOC	Site F PLFA	-208	0.042	-409.9	-5.9
Site F DOC	Site F SOC	218	0.032	15.8	419.8
Site F PLFA	Site F SOC	426	5.4E-05	223.7	627.7

Table S7 Statistical results (SYSTAT Software) of a post-hoc pairwise comparison of $\Delta^{14}C$ signatures (‰) of Site B samples using Tukey's Honestly-Significant Difference Test with a 95% confidence interval. DOC and DIC groundwater samples were collected from Site B in January 2015 and sediment samples for SOC and PLFA analysis was collected in January 2013.

Sample	Sample	Difference	p-value	95.0% Confidence Interval	
				Lower	Upper
Site B DIC	Site B DOC	169	0.039	6.8	330.9
Site B DIC	Site B PLFA	66	0.672	-96.2	227.9
Site B DIC	Site B SOC	747	<0.001	599.2	895.1
Site B DOC	Site B PLFA	-103	0.376	-278.0	72.0
Site B DOC	Site B SOC	578	<0.001	416.3	740.3
Site B PLFA	Site B SOC	681	<0.001	519.3	843.3

Table S8 Comparison between radiocarbon values derived for Site F from both PLFA (this study) and microbial DNA (Mailloux *et al*., 2013).

PLFA Sediment Depth Interval (m)	PLFA Sediment Depth Midpoint (m)	DNA Water Filter Depth (m) Mailloux *et al.* (2013)	PLFA $\Delta^{14}C$ ‰	DNA $\Delta^{14}C$ ‰	Difference $\Delta^{14}C$‰ (PLFA-DNA)
7.9 - 9.8	8.8	11.1	19.7	-56	75.7
19.5 - 20.1	19.8	19.4	-67.1	-117	49.9
23.5 - 24.4	23.9	25.4	-119.1	-143	23.9
				Average ± SE	**50 ± 12**

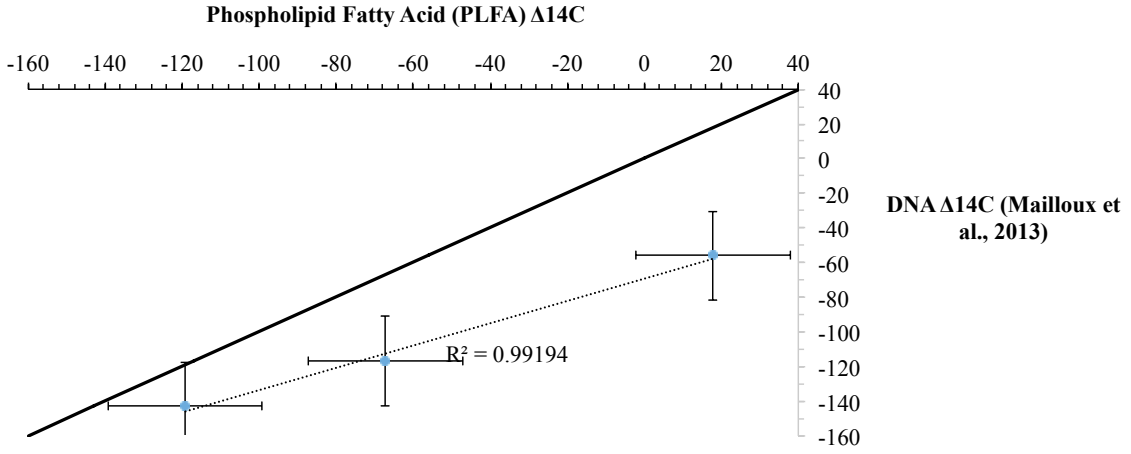

Figure S3 Regression analysis between the radiocarbon signatures of Site F PLFA (this study) and radiocarbon signatures of Site F microbial DNA (Mailloux *et al*., 2013) (R^2=0.99194).

Table S9 Mass balance calculation results for the microbial phospholipid fatty acid (PLFA) $\Delta^{14}C$ (‰) at Site F and Site B using dissolved organic carbon (DOC) versus dissolved inorganic carbon (DIC) $\Delta^{14}C$ (‰) and sedimentary organic carbon (SOC) versus dissolved inorganic carbon (DIC) $\Delta^{14}C$(‰).

										DOC aged versus DIC aged carbon		SOC aged versus DIC aged carbon	
Site and Approxim-ate Depth (m)	**PLFA Sediment Mid-point Depth (m)**	**PLFA $\Delta^{14}C$ (‰)**	**Ground-water Depth (m)**	**DOC $\Delta^{14}C$ (‰)**		**DIC $\Delta^{14}C$ (‰)**		**SOC Depth (m)**	**SOC $\Delta^{14}C$ (‰)**	**Proportion of Bacteria Metabolizing a Bulk DOC-Aged Carbon Source**	**Proportion of Bacteria Metabolizing a Bulk DIC-Aged Carbon Source**	**Proportion of Bacteria Metabolizing a Bulk SOC-Aged Carbon Source**	**Proportion of Bacteria Metabolizing a Bulk DIC-Aged Carbon Source**
				2012[a]	**2015**	**2012[a]**	**2015**						
Site F	19.8	-67	19.4	-205	-310	-31	-6	19.2	-541[a]	0.20	0.80	0.09	0.91
Site F	23.9	-119	25.4	-108	-379	-72	-6	25.1	-684[a]	0.39	0.61	0.12	0.88
Site B	8.7	21	7.3	N/A[b]	-148	N/A	27	9.1	-640	0.04	0.96	0.01	0.99
Site B	9.1	-96	7.3	N/A	-148	N/A	27	10.7	-896	0.70	0.30	0.13	0.87
Site B	11.0	-14	10.9	N/A	-130	N/A	33	11.3	-842	0.29	0.71	0.05	0.95
Site B	12.2	10	10.9	N/A	-130	N/A	33	12.2	-906	0.14	0.86	0.02	0.98
Site B	17.8	-163	19.4	N/A	-194	N/A	68	18.9	-640	0.88	0.12	0.32	0.68

[a] DOC, DIC and SOC $\Delta^{14}C$ values from Site F from 2012 are derived from Mailloux *et al*. (2013)

[b] N/A indicates where measurements from 2012 were not available

Chapter 3. Human and Livestock Waste as a Reduced Carbon Source Contributing to the Release of Arsenic to Shallow Bangladesh Groundwater

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3.1 Abstract

Recent studies have demonstrated that the supply of relatively young organic carbon stimulates the release of arsenic to groundwater in Bangladesh. This study explores the potential role of human and livestock waste as a significant source of this carbon in a densely populated rural area with limited sanitation. Profiles of aquifer sediment samples were analyzed for phytosterols and coprostanol to assess the relative contributions of plant-derived and human/livestock waste-derived organic carbon at two well-characterized sites in Araihazar. Coprostanol concentrations increased with depth from non-detection (<10 m at Site B and <23 m at Site F) to maxima of 1.3 and 0.5 ng/g in aquifer sands recovered from 17 m (Site B) and 26 m (Site F), respectively. The commonly used sewage contamination index ([5β-coprostanol]/([5α-cholestanol]+[5β-coprostanol])) exceeds 0.7 between 12 and 19 m at Site B and between 24 and 26 m at Site F, indicating input of human/livestock waste to these depths. Urine/fecal input within the same depth range is supported by groundwater Cl/Br mass ratios >1000 compared to Cl/Br<500 at depths >50 m. Installed tube wells in the area's study sites may act as a conduit for DOC and specifically human/livestock waste into the aquifer during flood events. The depth range of maximum input of human/livestock waste indicated by these independent markers coincides with the highest dissolved Fe (10-20 mg/L) and As (200-400 μg/L) concentrations in groundwater at both sites. The new findings suggest that the oxidation of human/livestock waste coupled to the reductive dissolution of iron-(oxy)-hydroxides and/or arsenate may enhance groundwater contamination with As.

3.2 Introduction

Chronic exposure to arsenic (As) contained in groundwater poses a considerable human health risk across rural Bangladesh and surrounding regions, where most villagers

depend on shallow wells as their primary source of drinking water. The release of As from uncontaminated sediments into groundwater has been shown to be mediated through microbial mechanisms(Islam et al. 2004; Nickson et al. 1998; Nickson et al. 2000; Dhar et al. 2011; Swartz et al. 2004; Harvey et al. 2002; Postma et al. 2007; Postma et al. 2012). Heterotrophic bacteria couple the reduction dissolution of iron (oxy)-hydroxide bearing particles (Islam et al. 2004; Nickson et al. 1998; Nickson et al. 2000; Dhar et al. 2011; Swartz et al. 2004; McArthur et al. 2001; Dowling et al. 2002; Harvey et al. 2002; Postma et al. 2007; Postma et al. 2012; Cummings et al. 1999) or directly of As(V) (Ahmann et al. 1997; Postma et al. 2007) to the oxidation of organic carbon resulting in the release of adsorbed As from the sediment to groundwater. The supply and reactivity of the organic carbon driving this release is the ultimate thermodynamic driver of these processes. However, after more than a decade of research, the relative importance of advected carbon to sedimentary carbon, as well as the role of natural versus anthropogenic carbon sources, remains unclear. These distinctions are important because they have very different implications for the evolution of the distribution of As in Bangladesh aquifers under the influence of massive groundwater pumping (Burgess et al. 2010; Michael & Voss 2008).

A number of potential sources of organic carbon including human waste (Harvey et al. 2002; McArthur et al. 2012), man-made constructed ponds(Lawson et al. 2013; Neumann et al. 2010), wetland/rice paddy environments(Meharg et al. 2006; Polizzotto et al. 2008; Stuckey et al. 2015), river-derived flows (van Geen et al. 2013) , buried peat layers(Ravenscroft et al. 2001; McArthur et al. 2001; Anawar et al. 2003; Yamazaki et al. 2003; McArthur et al. 2004; Mladenov et al. 2010; Planer-Friedrich et al. 2012) and

organics deposited with sediments (Nickson et al. 2000; Meharg et al. 2006; Postma et al. 2007; Postma et al. 2012; Desbarats et al. 2014) have been proposed based on studies conducted at various sites. Recent evidence based on $\Delta^{14}C$ analysis of microbial lipids, DNA and biogenic methane from a few sites in Bangladesh has indicated that reductive dissolution of iron (Fe) oxides at depths <30 m may be driven primarily by relatively young surface-derived carbon sources as opposed to older sedimentary carbon (Harvey et al. 2002; Mailloux et al. 2013; Whaley-Martin et al. 2016). Determining which of the potential sources of surface derived reactive carbon, e.g. plant versus human/livestock waste, is a crucial next question as the implications to management and mitigation efforts of these two potential sources would be very different.

Potential sources of plant-derived carbon that have been proposed include ponds (Neumann et al. 2014; Lawson et al. 2013), local wetlands (Meharg et al. 2006) or rice-paddy crops (Polizzotto et al. 2008) which are all abundant in land coverage across Bangladesh (Islam & Rahman 2010; Rahaman 2012; Meharg & Rahman 2003). In a recent field experiment, Stuckey *et al*. (2015) stimulated Fe reduction and As release from Cambodian sediments by adding large quantities of local grass as a microbial carbon source. In contrast, Neumann *et al*. (2014) found dissolved organic carbon (DOC) carried from rice-paddy fields (plant-derived) shows limited biological degradation under natural conditions and instead proposed DOC carried with recharge water from ponds as a predominant microbial carbon source at one site. While the origin of the DOC from ponds Neumann *et al*. (2014) referred to is unclear, many ponds in Bangladesh are extensively contaminated with latrine discharge and animal waste (Knappett et al., 2012; Knappett et al., 2012).

McArthur *et al*. (2012) suggested widespread contamination of shallow aquifers across the Bengal basin with human waste on the basis of Cl/Br ratios and Cl concentrations in groundwater (McArthur et al. 2012; Davis et al. 1998). However, the relationship between these indicators and fecal contamination or As concentrations in groundwater was unclear. Monitoring of a substantial number of shallow wells in Bangladesh has documented an inverse relationship between the fecal indicator *E. coli* and As concentrations (Leber et al. 2011; van Geen et al. 2011). This could be seen as an indication that human waste, if anything, inhibits the release of As to groundwater (McArthur et al. 2012). An alternate explanation supported by tritium-helium dating of groundwater is that enhanced recharge through more permeable surface soil favours the downwards transport of *E. coli* while at the same time diluting As released by aquifer sediments (Stute et al. 2007; Aziz et al. 2008).

Analysis of C_{29} sterols could potentially address this question as they can serve as biomarkers for complex pools of carbon that are travelling through the aquifer sediments (such as organic matter derived from plants versus human/livestock waste in sediments) (Martins et al. 2011; Lee et al. 2011; Tse et al. 2014; Biache & Philp 2013; Tolosa et al. 2013; Chikaraishi et al. 2005; Furtula et al. 2012). The relative distributions of these biomarkers can provide an indication of how much input from each source has occurred.

The octanol-water partitioning coefficients of these hydrophobic compounds are high, indicating that they are likely to sorb strongly onto aquifer sediments and will be retarded relative to the movement of groundwater (Froehner & Sánez 2013). Phytosterols (i.e. β-sitosterol, stigmasterol and campesterol) are reliable biomarkers of plant matter in

sediments. In contrast, coprostanol (5β-cholestan-3β-ol, logKow ≈ 8.2) is biosynthesized from cholesterol exclusively within the mammalian gut and comprises 25 to 90% of total steroids in feces (Leeming et al. 1996). It is therefore a widely accepted biomarker for sewage/fecal matter of human or animal origin (Hussain et al. 2010; Furtula et al. 2012; Chou & Liu 2004; Martins et al. 2011; Lee et al. 2011). Under aerobic conditions in sandy sediments, Pratt *et al*. (2008) observed that coprostanol degrades more slowly than fecal bacterial indicators. Under anaerobic conditions, coprostanol can persist in sediments for years to centuries (Reeves & Patton 2005; Nishimura & Koyama 1977; Bartlett 1987; Tse et al. 2014).

This study documents the vertical distribution of C_{29} sterols at two well-characterized sites in Araihazar upazila, Bangladesh (Stute et al. 2007; Dhar et al. 2008) that are impacted by high levels of As in groundwater. The sterol distributions are compared with other indicators of unsewered wastes (Cl/Br mass ratios), as well as As and Fe concentrations. The organic extracts used for the present study are the same that were previously used for $\Delta^{14}C$ analysis of bacterial lipids (Whaley-Martin et al. 2016) and showed that young carbon was driving microbial metabolisms.

3.3 Methods

3.3.1 Field Sites

The area surrounding the first of the two sites, Site B in Baylakandi Village (23.7800 N 90.6400 E), is very densely populated and groundwater in most wells contains >50 μg/L As (Figure 3.1b). In contrast, the area around Site F in Lashkardi

Village (23.774 N 90.606 E) is less densely populated and groundwater from most surrounding wells contains <50 μg/L, and in many cases <10 μg/L, which is the World Health Organization guideline for As in drinking water (Figure 3.1a). At Site B, a 7-m thick clay/silt layer caps the sandy aquifer whereas the sandy formation extends essentially to the surface at Site F (Dhar et al. 2008). As described in detail in Whaley-Martin *et al*. (2016), sediments were collected in January 2013 by gravity coring, sectioned directly into whirlpacks, immediately frozen, shipped on ice and frozen until future analyses. Data for groundwater samples collected between 2002 and 2015 from pre-existing well nests at the two sites(Dhar et al. 2008) using a battery operated downhole pump (Groundwater Essentials ©) is also presented. Wells were purged until the conductivity stabilizes before groundwater was collected in two scintillation vials, without filtration.

3.3.2 Sterol Lipid Extraction, Separation and Derivatization

Prior to extractions, all sediments were freeze-dried for 48-72 hours. A detailed description of the organic compound extraction and separation is provided in Whaley-Martin *et al*. (2016). Sediment samples (~300 g to 1 kg) were extracted twice overnight using a modified Bligh and Dyer extraction and the organic phase was removed after phase separation in separatory funnels. A three-fraction ($Fraction_1$=dichloromethane (DCM), $Fraction_2$=acetone, $Fraction_3$=methanol) silica gel chromatographic elution was carried out. Methanol fractions were utilized for phospholipid fatty acid analyses reported in a previous study (Whaley-Martin et al. 2016). Preliminary tests revealed elution of sterols occurred in both the $Fraction_{1\&2}$ in agreement with a recent study(Birk et al. 2012)

showing high recoveries using elution with DCM and acetone. The DCM and acetone fractions containing the sterols were blow down to dryness under N_2 and underwent a BSTFA (N,O-bis-(trimethylsily)-trifluoroacetamide, Sigma-Aldrich) derivatization using pyridine as a catalyst for 30 minutes at 65°C to create trimethylsilyl (TMS)-derivatives of the original sterols. TMS-derivatives were found to be better resolved through the chromatographic separation than the original steroid precursors, as seen in previous studies (Marcos & Pozo 2015).

3.3.3 Sterol Analysis through Gas Chromatography-Mass Spectrometry (GC-MS)

Sterol standards used for identification and quantification with GC-MS were cholestanol (5-α-cholestan-3β-ol, ≥95%, Sigma Aldrich), cholesterol (cholet-5-en-3β-ol, ~94%, Sigma Aldrich), stigmasterol (24-ethylcholesta-5,22E-dien-3β-ol, ~95%, Sigma Aldrich), campesterol (24-α-methyl-5-cholesten-3β-ol, ~65%, Sigma Aldrich), sitosterol (24-ethylcholest-5-en-3-β-ol, ≥97%, Sigma Aldrich) and coprostanol (5β-cholestan-3β-ol, ≥98%, Santa Cruz Biotechnology). All standards underwent the BSTFA derivatization at the same time as samples and their TMS-derivatives were used to compare to sample extracts (Table A1). Concentrated samples (run in 30 µl of BSTFA + 10 µl of pyridine) were run with 1µl injections on an Agilent Technologies 6890N GC with an Agilent DB5-MS capillary column (30m x 0.32µm, 0.25 µm film thickness) coupled to a 5973 quadrupole mass spectrometer scanning for masses from 50-500m/z. Operating GC-MS conditions included a temperature program with an initial hold for 1 minute at 40°C ramped to 300°C at 4°C/min and held at 300°C for 24 minutes (Biache & Philp 2013). The limit of quantification (0.5µg/mL) was determined based on linearity of the sterol

calibration curves. Final sterol LOQ's for each particular sample (ng/g of sediment) was dependent on the total mass of sediment weight extracted (Table S2).

3.3.4 Inorganic Groundwater Analysis

Cl, Br and other anion concentrations were determined using a DIONEX-500 ion chromatograph system equipped with an IonPac® AS18 anion-exchange column using standard EPA methods. QA/QC included duplicates (<1% RPD), blanks, lab standards and Certified Reference Materials to ensure that recoveries and analytical accuracy and precision were within 5%. Concentrations of As and Fe were determined using high resolution-inductively coupled plasma mass spectrometry (HR-ICP-MS) using previously described methods (Cheng et al. 2004). Reference materials NIST1640A and 1643A and internal consistency standards are included with each run. The long-run reproducibility of this method is on the order of 5% and the detection limit <0.1 μg/L.

3.4 Results

3.4.1 Profiles of Phytosterols and Coprostanol in the Sediment

Concentrations of the phytosterol plant biomarkers in the two sediment profiles extending to 19 m depth at Site B (n=11) and to 26 m at Site F (n=7) ranged from < 2 ng/g to <LODs (Table A2). Concentrations of phytosterols are the highest at ≤5 m depth at both sites, decline to non-detectable levels at intermediate depths, and return to values that are almost as high at 17-19 m depth at Site B and 24-26 m at Site F (Figures 3.1c-d). In contrast, concentrations of the sewage biomarker coprostanol remained below detection to a depth of 10 m at Site B and to 24 m at Site F, respectively. Coprostanol

concentrations of 0.5 ng/g or higher were measured in four intervals between 12 and 18 m depth at Site B, and at 26 m at Site F.

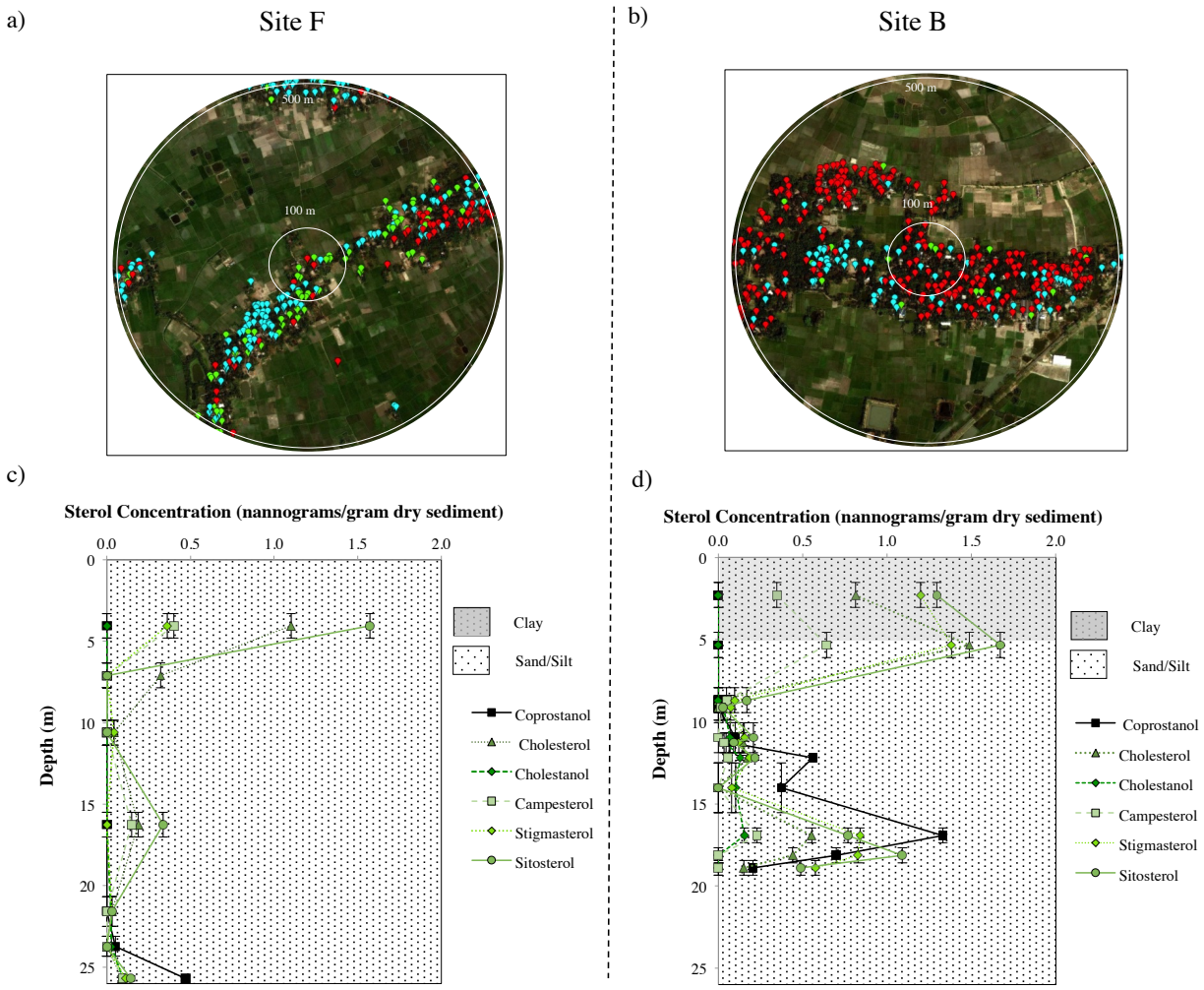

Figure 3.1 Local well distributions and groundwater arsenic concentrations from a 2012-13 survey within 100 m (inner white circle) and 500 m (outer white circle) from Site F (a) and Site B (b) where [As] in groundwater are indicated by blue circles <10 µg/L, green circles 10 to 50 µg/L and red circles >50 µg/L. Depth profiles of phytosterols (green symbols: cholesterol, cholestanol, campesterol, stigmasterol and sitosterol) and sewage biomarker coprostanol (black symbols) in shallow aquifer sediments (ng/g sediment) of the Araihazar Upazilla, Bangladesh of (c) Site F and (d) Site B.

The sewage index ([5β-coprostanol]/([5α-cholestanol]+[5β-coprostanol]) is a commonly used measure to determine the presence, extent and distribution of fecal/sewage contamination(Bull et al. 2002; Vane et al. 2010; Martins et al. 2011; Reeves & Patton 2005). At 12-19 m depth at Site B and 24-26 m depth at Site F, this ratio is >0.7 and provides a clear indication of human/livestock waste input to the sediment(Bull et al. 2002; Vane et al. 2010; Martins et al. 2011).

Table 3.1 Calculated sewage contamination indexes ([5β-coprostanol]/([5α-cholestanol]+[5β-coprostanol]) for each sediment depth at Site B and Site F. Bold values indicate the sewage contamination index was >0.7.

Site B				Site F			
Depth (m)	Sewage Contamination Index [5β-coprostanol]/([5α-cholestanol]+[5β-coprostanol])	$[As]_{aq}$ [a] (μg/L)	$[Fe]_{aq}$ [a] (mg/L)	Depth (m)	Sewage Contamination Index [5β-coprostanol]/([5α-cholestanol]+[5β-coprostanol]	$[As]_{aq}$ [a] (μg/L)	$[Fe]_{aq}$ [a] (mg/L)
2.3	<LOD	N/A	N/A	4.1	<LOD	N/A	N/A
5.3	<LOD	N/A	N/A	7.1	<LOD	20	0.6
8.7	<LOD	25	8	10.6	<LOD	72	1.4
9.1	<LOD	90	7	16.3	<LOD	130	1.4
11	0.6	245	5	21.6	<LOD	190	2.6
11.3	0.6	255	5	23.7	**0.7**	200	4
12.2	**0.8**	370	6	25.7	**0.8**	205	6.5
14	**0.8**	430	8	-	-	-	-
16.9	**0.9**	420	18	-	-	-	-
18.1	**1**	410	20	-	-	-	-
18.9	**1**	380	21	-	-	-	-

[a] Arsenic and iron concentrations were estimated for each particular sediment depth based on the groundwater depth profiles measured in the 2014-2015 groundwater samples.

3.4.2 Profiles of Cl and Br in Groundwater

At depths >50 m, concentrations of Cl in groundwater at Site F are about twenty times higher than at Site B. However ratios of Cl/Br (278 $\pm$ 40) in groundwater from

these deeper well depths are comparable at both sites. In contrast, Cl concentrations in the shallow aquifer are up to an order of magnitude higher than at depth at Site B (Figure 2). The higher Cl concentrations are paired with particularly high Cl/Br ratios (1528 ± 719, n=157). There were several outliers at Site B between April and October 2005 (n=6) indicating input from an unidentified dilute bromide source at 11.6 m depth. This temporarily increased the Cl/Br mass ratio to ~97300 (Figure A4a and A4b). Considered an anomaly, they were excluded from average concentrations reported above for Site B. At Site F, Cl concentrations in the shallow aquifer are lower than in the shallow aquifer at Site B but Cl/Br ratios (726 ± 636, n=249) are still elevated compared to deeper groundwater. Binary mixing relationships for Cl/Br as a function of Cl that consider various end members reported in previous studies suggest a significant contribution of human/livestock urine and/or waste throughout the shallow aquifer at Site B and primarily within the 20-25 m depth range at Site F (Figure 3.2).

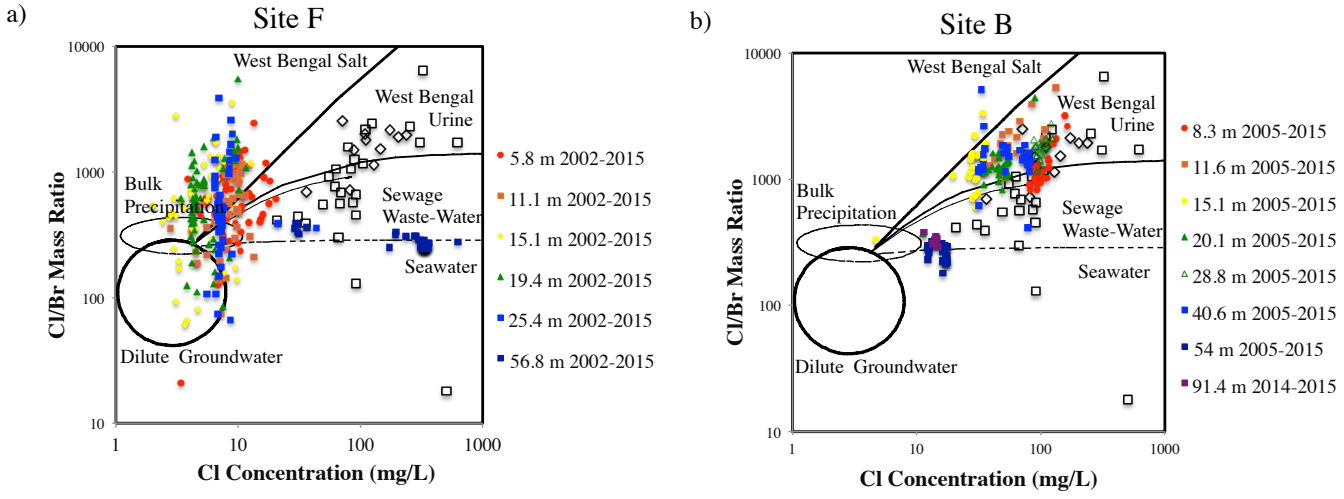

Figure 3.2 Groundwater Cl/Br mass ratios and Cl concentrations (mg/L) at (a) Site F: 5.8 to 56.8 m depth in 2002-2015 b) Site B: 8.3 to 91 m depth 2005-2015. Open white square

markers are derived from USA septic leachate samples (Panno *et al*. 2005; 2006), open white diamond markers are septic tank outfall samples measured in the West Bengal, Bangladesh (McArthur *et al*., 2012). Graphs are modified from Katz *et al*. 2011 and McArthur *et al*., 2012 basing binary mixing lines with Cl/Br and Cl concentrations using end members based on literature values for dilute groundwater, bulk precipitation, West Bengal salt, West Bengal urine, sewage waste-water and seawater.

3.4.3 Profiles of Iron and Arsenic in Groundwater

Consistent profiles of dissolved Fe and As have been monitored for over a decade at Sites B and F (Stute et al. 2007; Dhar et al. 2008). Below the thick clay layer that caps the shallow aquifer at Site B, concentrations of Fe and As in groundwater rapidly increase to maximum values of about 25 mg/L and 500 μg/L in the 15-20 m depth range (Figure 3). At Site F, where the sandy aquifer extends almost to the surface, concentrations of Fe and As increase with depth as well, but much more gradually and only to 7 mg/L and 200 μg/L, respectively.

The contrast between the two profiles explains the marked difference in the As content of water pumped from private wells, most of which are <50 m deep (Figure 1). Below 20 m at Site B, the aquifer is interspersed with several relatively thin clay layers and concentrations of Fe and As decline gradually. Even at 55 m depth, however, groundwater As concentrations are still >50 μg/L. At Site F, groundwater from the aquifer below the thick layer contains As concentrations <10 μg/L, even if groundwater Fe concentrations remain high at 20 mg/L.

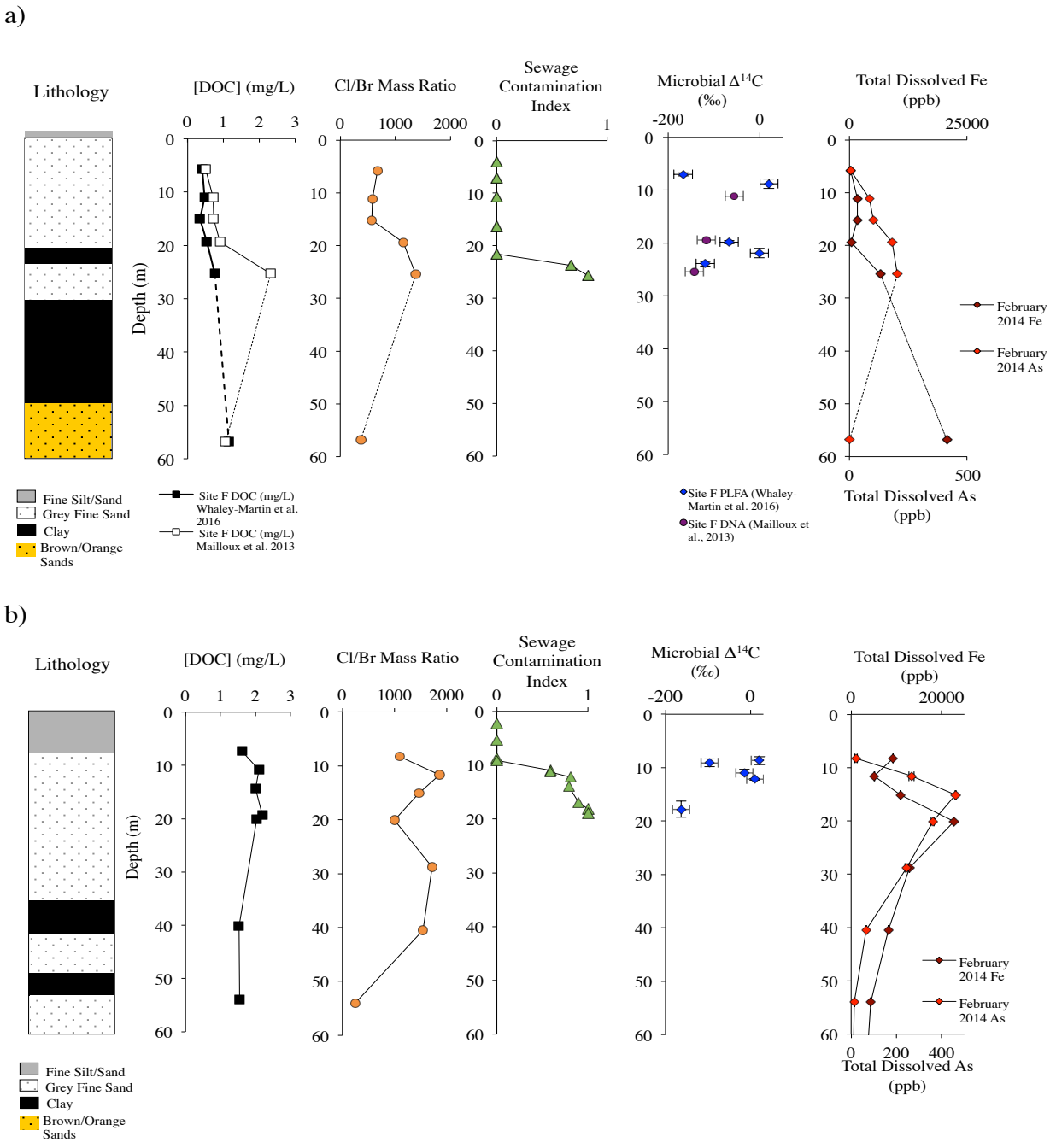

Figure 3.3 Biogeochemical depth profiles from 0 to 60 m of a) Site F and b) Site B from available 2013/2014 data for lithology (Dhar *et al*. 2007), DOC concentrations, Cl/Br mass ratios, sewage contamination indexes, PLFA $\Delta^{14}C$ (Whaley-Martin et al., 2016) and total dissolved Fe and As (January to February 2014) in groundwater.

3.5 Discussion

3.5.1 Pathways for Plant Sterols and Human/Livestock Waste

Plant-derived sterols are produced primarily above ground and it is therefore not surprising that their highest concentrations were measured within the upper 5 m of sediment at both sites (Figure 1c,d) in agreement with the observations of Al Lawati *et al*.(Al Lawati et al. 2013) at a study site in Vietnam. Concentrations of plant-derived sterols were not markedly different in shallow silt/clay at Site B compared to the shallow sandy sediment collected at Site F. At a depth of about 10 m, concentrations of the sterols declined by at least an order of magnitude at both sites. This is not inconsistent with the relatively recalcitrant nature of these biomarkers, given that radiocarbon dating shows that the sediment at this depth is several thousand years old (Zheng et al. 2005; Mailloux et al. 2013). Perhaps more surprising is a return to higher concentrations of plant-derived sterols below 17 m depth at Site B and below 14 m at Site F. There is no obvious change in the nature of these deposits that could support a sedimentological explanation for a return to higher plant-derived sterol concentrations at depth.

Although coprostanol is also produced by humans and livestock above-ground, it was not detected down to a depth of 11 m at Site B and 22 m at Site F (Figure 3.1c,d). This is unexpected given the continued practice of open defecation in South Asia, the very basic nature of the pit latrines, and the abundance of cattle in the study area. The imprint of human activities may be restricted to the very top layer of soil at these sites, which was not sampled. In addition, the shallowest samples were of the lowest mass and therefore had higher limits of detection than deeper samples (Table S2). However, these limits of detection did not prevent successful detection of the phytosterols. Therefore,

coprostanol and phytosterols can be assumed to not be co-occurring in similar abundances within the shallowest sediments. Differences in the degradation rates of coprostanol versus the phytosterols(Pratt et al. 2008) could explain the absence of coprostanol in shallower sediments, although this seems unlikely given the similarities in structure of the sterols. The strikingly higher coprostanol concentrations within the same deeper intervals that contain plant-derived sterols at Sites B and F suggest a common input mechanism to these depths.

There are several possible scenarios that could explain how these sterols are supplied to aquifer sediments in the 10-25 m depth range while bypassing shallower intervals. The first is depositional and invokes surface input by vegetation and the local population and livestock several thousand years ago, followed by the rapid deposition of sediment that was relatively free of plant material and human/livestock waste. This cannot be ruled out given that the Bengal basin has long supported a high population density and is a highly dynamic depositional environment. A large branch of the Brahmaputra River, for instance, is believed to have passed through the area as little as a few hundred years ago(Weinman et al. 2008). Its avulsion undoubtedly perturbed previous sediment erosion and deposition patterns. Under this scenario, the elevated Cl/Br mass ratios in groundwater from 2002 to 2015 at these sites (strongly at Site B), an independent indication of recent input of human/livestock waste to the same depth intervals, would be coincidental.

Another possible explanation is lateral inflow from a shallower area where both plant-derived sterols and human/livestock waste entered the recharge water and were subsequently advected to greater depth. The correlation observed in the underlying

sediments between phytosterol and coprostanol distributions would be expected from sewage-derived carbon from animals with omnivorous or herbivorous diets that have both sterol groups within their fecal components(Leeming et al. 1996; Bull et al. 2002) or from plant and sewage matter travelling along similar flow paths. There are several difficulties with this explanation, however. Any migration of the sterols through groundwater movement would be slowed considerably by adsorption. The $K_{OW's}$ of the sterols in combination with the range of total organic carbon contents estimated in the sediments (Legg et al. 2012) results in calculated soil/solution distribution coefficients ranging from 10^4 to 10^7.These values imply retardation factors of 10^5 to 10^8 relative to conservative constituents of groundwater such as Cl and Br. Tritium-helium dating has shown the groundwater to be 20 and 5 years old relative to the time of recharge at Sites B and F, respectively (Stute et al. 2007). According to this scenario, the accumulation of sterols observed between 10-25 m depth would therefore have to reflect an input hundreds of thousands of years ago, which is well before the formation was deposited. However, faster lateral transport rates from potential colloidal flow through the sand units may be occurring since organic phases of sewage discharges have been associated with colloidal transport during heavy rainfall (Eganhouse & Sherblom 2001). A much shorter flow path through zones of high horizontal conductivity, possibly enhanced by irrigation pumping, cannot be excluded although it would have to be minor fraction of the flow given the gradual increase in groundwater age with depth (Stute et al. 2007).

The third explanation requires a preferential path that leads directly from the surface to the 10-25 m depth intervals where plant and human/livestock sterols have accumulated in the sediment. The large number of tubewells installed in the area may

provide such a conduit. A total of 337 wells constructed of PVC pipe, without the use of any grout, were installed within a radius of 500 m of Site B when the most recent survey was conducted in 2012-13 (Figure 3.1a,b). The screened intervals of most of these wells span the 10-25 m depth range (Figure 3.4). On the basis of the high rate and replacement of household wells (van Geen et al., 2014), at least a comparable number were previously installed by the same households within the area and abandoned without taking any measures to seal them. Given that the fields surrounding the village are flooded every monsoon and that the village itself is flooded roughly once a decade, transport of plant-derived sterols and human/livestock waste down these wells, or along their annulus, and into the sediment is conceivable. Knappett et al.(2012) also invoked transport along a well's annulus to explain the higher frequency of *E. coli* in groundwater pumped from unsealed wells compared to wells that had been grouted. However, transport of human/livestock waste along a well's annulus would not explain the absence and reappearance of coprostanol at depth. Therefore, organic matter input through the well's screen during flooding may be a more dominant pathway. According to these scenarios, the lower population and well density could possibly explain the generally lower concentrations of sterols at Site F compared to Site B. Generally lower Cl/Br ratios in shallow groundwater at Site F compared to Site B and a broad resemblance to vertical profiles of the sewage contamination index may suggest that the input of a non-adsorbing indicator of human waste such as Cl also occurs predominantly in flooded wells through screens or year-round along a well's annulus.

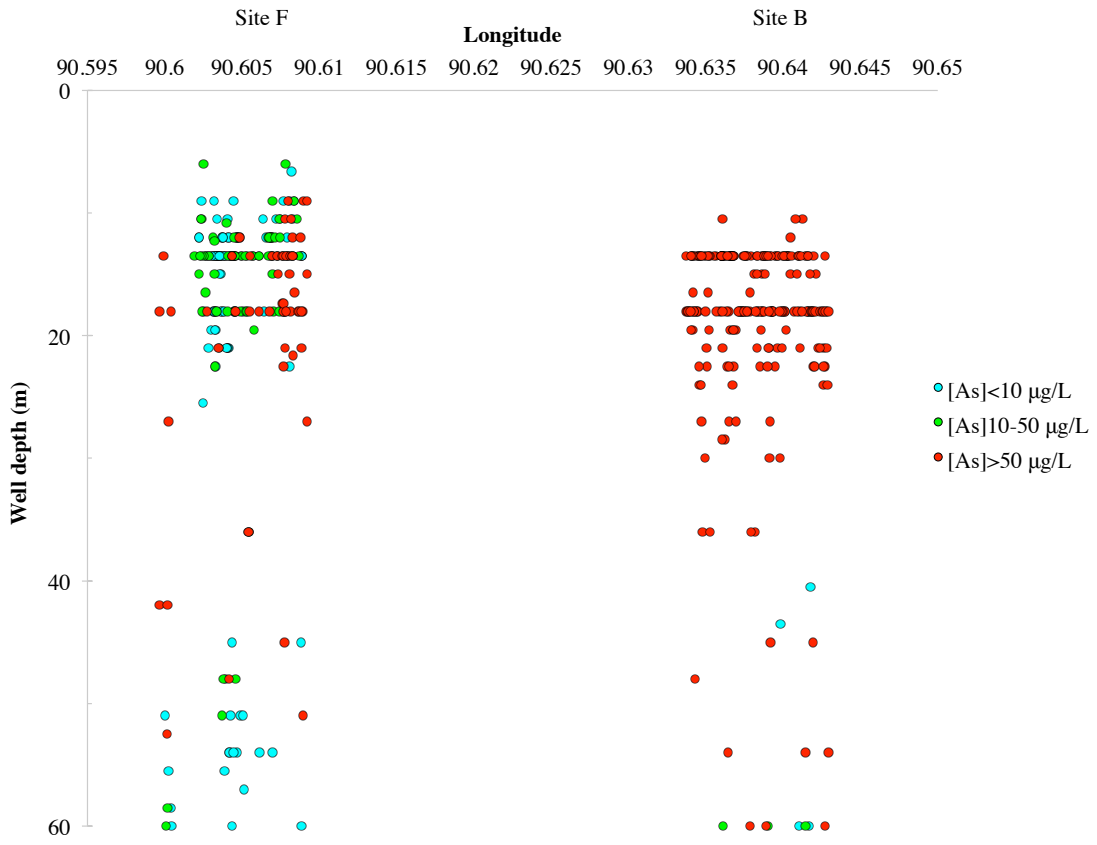

Figure 3.4 Longitude, latitude and depths (m) of screening wells and associated groundwater total arsenic concentrations within 500 m of Site F and Site B.

3.5.2 Reactivity of Plant Sterols and Human/Livestock Waste

Profiles of the sterol-based sewage contamination index bear a remarkable resemblance to dissolved Fe and As profiles in groundwater at the two study sites, including more pronounced features at Site B compared to Site F (Table 1; Figure 3). The strong correlations found between the sewage contamination and arsenic concentrations (Figure A2) support that human/livestock waste may provide the carbon source driving microbially mediated arsenic release at these sites. In addition, the installation of large numbers of tubewells, by providing a pathway for plant- and human/livestock-derived

sterols and other organic matter, might enhance the release of As to groundwater in already grey (reduced) Holocene sands. This would create a pathway that appears to shunt sterols including coprostanol (plant and/or sewage), from the surface to aquifers sediments in the 10-25 m depth range. The transport of these sterols and associated DOC components present in the water may enhance the reductive dissolution of Fe oxides and the release of As to groundwater at these depths.

Understanding how the organic nature of human/livestock waste may enhance microbial Fe reduction in Bangladesh aquifers is important to determine whether this mechanism is plausible in these systems. Bangladesh aquifer sediments are oligotrophic containing low carbon and inputs of labile carbon sources has been shown experimentally(Stuckey et al. 2015; Islam et al. 2004; Dhar et al. 2011) to stimulate the *in situ* sedimentary bacterial communities and ultimately arsenic release. Islam *et al*.(2004) detected a major shift in the sediment associated community with iron-reducing (*Geobacter*) bacteria increases when a small amounts of carbon (acetate) was added to Bangladesh sediments in a microcosm experiment. Sewage waste-water contains higher proportions of organic compounds (ie. short-chained fatty acids such as acetate, propionate, and butyrate)(Wong et al. 2006) produced through fermentation in the animal gut that that are readily degraded by anaerobic reducing microbial communities(Smith et al. 2008) compared to other sources (i.e. unfermented plant material and recalcitrant sedimentary organic carbon). In agreement with this notion, in a sewage-contaminated aquifer with similar geochemical characteristics to Site F and Site B, ($Fe(OH_3)$ coated mineral grains, circum-neutral pH and anoxic groundwater), Lee & Bennett(1998) reported enhanced reductive dissolution and mobilization of Fe at depth where anaerobic

indigenous bacterial communities were utilizing sewage-derived organics as their carbon sources.

Previous work conducted at our two study sites has shown that the microbial community, including presumably the ubiquitous Fe and As reducers, is fueled in large part by relatively young advected carbon (Mailloux et al. 2013; Whaley-Martin et al. 2016). Unlike the microbial radiocarbon ages which show no systematic different between the two sites, the radiocarbon age of DOC in these shallow aquifers is considerably younger at Site B ($\Delta^{14}C_{DOC}$ -151 ± 25‰) compared to Site F ($\Delta^{14}C_{DOC}$ -353 ± 40‰) (Whaley-Martin et al. 2016), possibly because of larger contributions of relatively young organic matter. In addition, DOC concentrations are 1.5 times higher and coprostanol concentrations almost 3 times higher at Site B compared to Site F. These observations, while not definitive, suggest that whatever the pathway that shunts sterols, including coprostanol, from the surface to aquifers sediments in the 10-25 m depth range may have implications for As in shallow aquifers and deserves further study.

3.6 Conclusion

Our new observations from two well-characterized sites in Bangladesh document the supply of plant- and human/livestock-derived organic matter to the depth range where both Fe and As are released to groundwater. The presence of coprostanol, in particular, which can be uniquely traced to the gut of higher animals, suggest the supply of a reactive carbon pool that could potentially stimulate the release of As from aquifer sediments. There is no evidence at the regional scale that such input has led to widespread Fe oxide reduction and As release in similarly populated areas where shallow aquifers are composed of orange Pleistocene sands. This does not mean a supply of

reactive carbon associated with human and livestock waste could not potentially enhance the release of As from grey Holocene sediments. Given the indication of the potential role of human/livestock waste, resolving the mechanisms of transport of these sources to depth is a crucial next step.

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Supporting Information

Included in the SI are aerial photographs of the study sites, correlational relationship between the sewage contamination index and As concentrations, tables of sedimentary sterol concentrations and groundwater total [As] and [Fe] concentrations.

3.6 Supporting Information

Human and Livestock Waste as a Reduced Carbon Source Contributing to the Release of Arsenic to Shallow Bangladesh Groundwater

Whaley-Martin, K.J.[1], Mailloux, B. J. [2], Bostick, B.C. [3] , van Geen, A. [3], Ahmed, K. M. [4], Choudhury, I. [4], Slater, G.F.[1]

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Figure S4. Bangladesh study sites "F" and " B "in the Arzaihazar Upazila, Bangaladesh (inset).

Table S10. Common and IUPAC names of sterol standards and molecular weights of TMS-derivatives used for identification and quantification through GC-MS

Common Name	IUPAC Name	Sterol TMS-Derivative MW
Coprostanol	5β-cholestan-3β-ol	370 g/mol
Cholesterol	cholest-5-en-3β-ol	458 g/mol
Cholestanol	5α-cholestan-3β-ol	460 g/mol
Campesterol	24-methylcholest-5-en-3β-ol	472 g/mol
Stigmasterol	24-ethylcholesta-5,22(*E*)-dien-3β-ol	484 g/mol
β-Sitosterol	24-ethylcholest-5-en-3β-ol	486 g/mol

Statistical Analysis

All statistical tests were carried out using 95% confidence intervals and Shapiro-Wilk Normality Tests were carried out prior to parametric statistical testing (Pearson R Correlation Coefficient). At both sites, As concentrations and Cl/Br ratios were found to

be normality distributed ($p>0.05$) and the sewage contamination indexes were assumed to be approaching normality.

Table S11. Sterol concentrations (ng/g) (coprostanol, cholesterol, cholestanol, campesterol, stigmasterol, sitosterol) in Site B (n=11) and Site F (n=7) shallow aquifer sediment

	Amount Extracted (g (dry wt))	Depth (m)	Coprostanol (ng/g)	Cholesterol (ng/g)	Cholestanol (ng/g)	Campesterol (ng/g)	Stigmasterol (ng/g)	Sitosterol (ng/g)
Site B	51	2.3	<LOQ	0.81	<LOQ	0.35	1.20	1.30
	48	5.3	<LOQ	1.49	<LOQ	0.64	1.38	1.67
	367	8.7	<LOQ	<LOQ	<LOQ	0.05	0.09	0.17
	1000	9.1	<LOQ	<LOQ	0.03	0.01	0.07	0.03
	311	11.0	0.10	0.15	0.07	<LOQ	0.15	0.21
	566	11.3	0.06	0.14	0.04	0.03	0.12	0.09
	538	12.2	0.56	0.17	0.13	0.06	0.19	0.22
	750	14.0	0.37	<LOQ	0.10	<LOQ	0.08	<LOQ
	141	16.9	1.33	0.55	0.15	0.23	0.84	0.77
	85	18.1	0.70	0.44	0.00	0.00	0.83	1.09
	1140	18.9	0.20	0.15	0.00	0.00	0.57	0.49
Site F	63	4.1	<LOQ	1.10	<LOQ	0.40	0.36	1.57
	68	7.1	<LOQ	0.32	<LOQ	<LOQ	<LOQ	<LOQ
	530	10.6	<LOQ	0.04	<LOQ	<LOQ	0.04	<LOQ
	121	16.3	<LOQ	0.19	<LOQ	0.15	<LOQ	0.34
	719	21.6	<LOQ	0.03	0.02	<LOQ	0.03	0.03
	546	23.7	0.05	<LOQ	0.03	<LOQ	<LOQ	<LOQ
	464	25.7	0.47	0.09	0.10	0.10	0.11	0.14

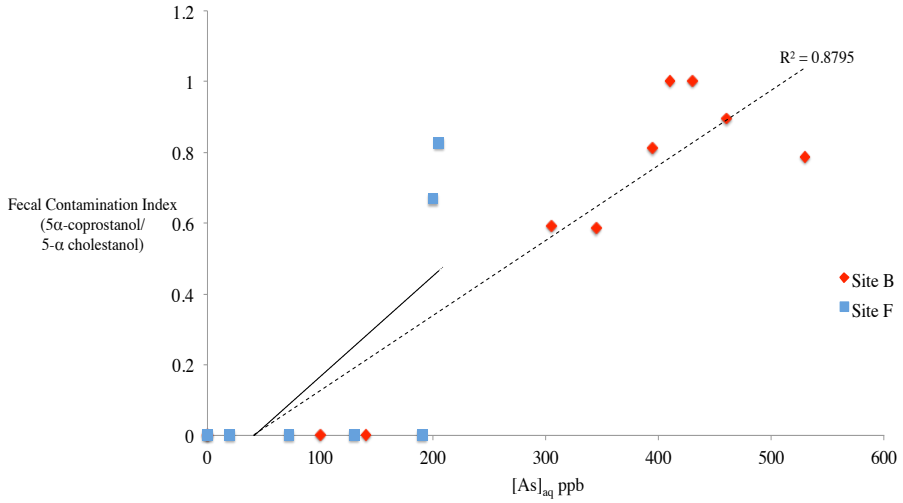

Figure S5. Correlational relationships between the fecal contamination index ratio ([5-α-coprostanol]/[5-α-cholestanol] and inferred groundwater As concentrations (μg/g) in the coinciding groundwater (based on measured 2014 $[As]_{aq}$ depth profiles) at Site B (red diamonds) and Site F (blue squares). Data points with a fecal contamination index of zero are sediment samples where sedimentary coprostanol concentrations were <LOQ.

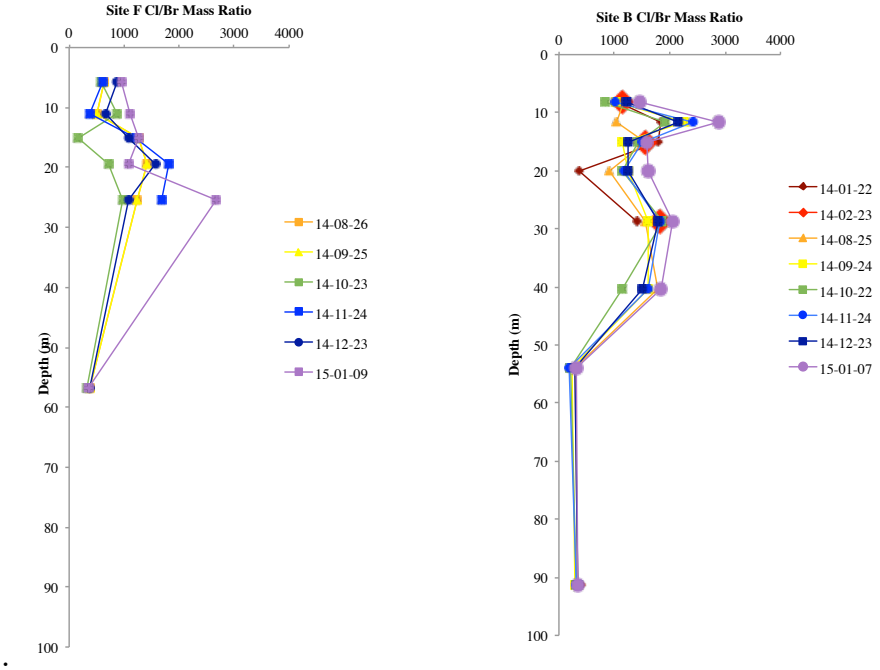

Figure S6. 2014-2015 groundwater depth profiles of (a) Site F and (b) Site B of Cl/Br mass ratios over time.

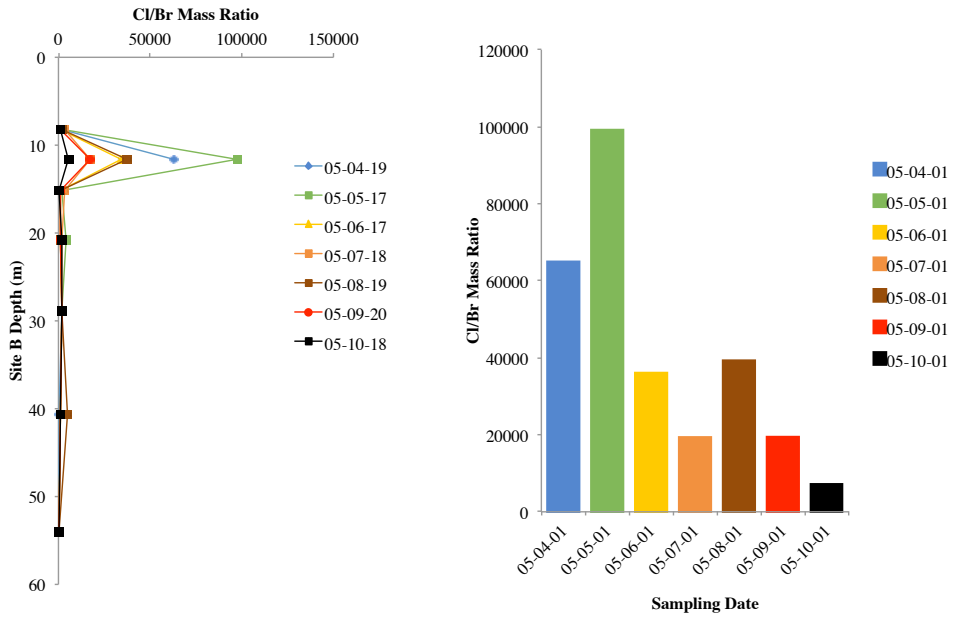

Figure S7. Site B Cl/Br mass ratios between April and November 2005 (a) groundwater depth profiles and (b) 11.6 m depth

Table S12. Groundwater total arsenic concentrations (µg/L) at Site F (January-February 2014) (HR-ICPMS)

Date	Well	Depth (m)	As (µg/L)
14-01-22	F1	5.8	6.2
14-02-27	F1	5.8	2.6
14-01-22	F2	11.1	79.3
14-02-23	F2	11.1	66.3
14-02-27	F2	11.1	105.0
14-01-22	F3	15.1	103.9
14-02-21	F3	15.1	100.1
14-02-23	F3	15.1	101.0
14-01-22	F4	19.4	184.9
14-02-20	F4	19.4	178.9
14-02-23	F4	19.4	183.4
14-01-22	F6	25.4	202.8
14-02-23	F6	25.4	202.8
14-02-21	F6	25.4	207.8
14-01-22	F5	56.8	0.1
14-02-19	F5	56.8	2.3
14-02-23	F5	56.8	0.1

Table S13. Groundwater total arsenic concentrations (μg/L) at Site B (January-February 2014) (HR-ICPMS)

Date	Well	Depth (m)	As (μg/L)
14-01-22	B7	8.25	18.5
14-02-23	B7	8.25	21.2
14-02-26	B7	8.25	31.7
14-01-22	B8	11.63	275.9
14-02-20	B8	11.63	255.5
14-02-23	B8	11.63	263.8
14-02-26	B8	11.63	244.8
14-01-22	B3	15.1	462.6
14-02-23	B3	15.1	457.2
14-01-22	B9	20.12	367.2
14-02-22	B9	20.12	356.2
14-02-23	B9	20.12	356.2
14-01-22	B4	28.8	236.6
14-02-21	B4	28.8	245.9
14-02-23	B4	28.8	251.5
14-01-22	B5	40.46	64.1
14-02-20	B5	40.46	70.6
14-02-22	B5	40.46	66.0
14-02-23	B5	40.46	65.0
14-01-22	B6	54	15.9
14-02-23	B6	54	14.1
14-02-26	B6	54	13.8
14-01-22	BCW2	91.35	0.3
14-02-19	BCW2	91.35	0.4
14-02-23	BCW2	91.35	0.4

Table S14. Groundwater total Fe concentrations (μg/L) at Site F (February 2014) (HR-ICPMS)

Date	Well	Depth (m)	Fe (μg/L)

14-02-27	F1	5.8	288
14-02-27	F2	11.1	1629
14-02-21	F3	15.1	1737
14-02-20	F4	19.4	309
14-02-21	F6	25.4	6551
14-02-19	F5	56.8	20689

Table S15. Groundwater total Fe concentrations (μg/L) at Site B (February 2014) (HR-ICPMS)

Date	Well	Depth (m)	Fe (μg/L)
14-02-26	B7	8.25	9276
14-02-20	B8	11.63	5198
14-02-26	B8	11.63	5096
14-02-23	B3	15.1	10846
14-02-22	B9	20.12	22716
14-02-21	B4	28.8	12925
14-02-20	B5	40.46	8234
14-02-22	B5	40.46	8207
14-02-26	B6	54	4303
14-02-19	BCW2	91.35	1481

Chapter 4. Exploring Approaches for Examining Sediment and Adjacent Groundwater Microbial Communities in Bangladesh Aquifers through PLFA Distributions and $\delta^{13}C$ Analysis

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4.1 Abstract

Arsenic groundwater contamination throughout shallow aquifer sediments in Southern Asia has resulted in a large-scale human health crisis from chronic arsenic exposure. There is strong evidence that microbial iron reduction coupled to organic carbon oxidation is the predominant mechanism driving this arsenic release. Research studies examining microbial communities composition and functioning across the region are limited. In addition, the relative roles between the sediment versus groundwater bacterial communities in terms of arsenic release from aquifer sediments remains unclear. Analysis of lipid biomarkers of bacterial and micro-eukarayotes (phospholipid fatty acids (PLFA)) provide a useful approach to examine the *in situ* distributions and abundances of these respective communities. The overall aim of this research study was to use microbial lipid biomarker distributions and $\delta^{13}C$ analysis to compare the indigenous sedimentary-associated with the groundwater-associated microbial communities in Bangladesh aquifers in terms of relative cellular abundance, PLFA distributions and $\delta^{13}C$ signatures of PLFA. Field sampling was carried out in the Araihazar Upazila, Bangladesh in 2013 and 2015. A significant difference ($p<0.00001$) was found between the cell abundances in the groundwater-associated (2.8×10^1 to 3.0×10^2 cells/mL) (n=9) and the sediment associated communities (averaging ~1.1×10^7 cells/gram (n=19)). Long-chain FAME's (C_{22}-C_{29}) derived from micro-eukaryotes were within the sediments of both Site B and F comprising up to ~17 and ~7 mole % of the total FAME distribution respectively but not detected in groundwater. Shallow Site B sedimentary PLFA showed a progressive depletion in $\delta^{13}C$ with depth from -24 to -31 ‰ while Site F PLFA from similar depths did not show a similar trend. Groundwater at 14m had a bulk FAME signature of $\delta^{13}C$ value of -41‰, which may indicate methanotrophic activity at these depths.

4.2 Introduction

Arsenic groundwater contamination throughout Southern Asia has resulted in a large-scale human health crisis from chronic arsenic exposure. There is a strong consensus that microbial iron reduction is the predominant mechanism driving the arsenic

release in the shallow Holocene-aged aquifers of Bangladesh (Nickson et al. 1998; Nickson et al. 2000; Harvey et al. 2002; Islam & Rahman 2010). This biogeochemical mechanism currently does not affect the deeper Pleistocene-aged aquifer (~>50m) allowing the usage of deeper wells to facilitate access to low arsenic groundwater. However, serious concerns have arisen regarding whether the sustainability of deep aquifers in Bangladesh may be compromised in the near future (Dhar et al. 2011; Burgess et al. 2010; Feighery et al. 2013; Radloff et al. 2011). Documented drastic increases in water withdrawal rates from the deep aquifer for irrigation and municipal water use (Khan et al. 2016) has led to speculation that downward migration of dissolved carbon sources could stimulate deep microbial communities and ultimately lead to arsenic contamination of the deeper groundwater. Stimulation of iron reducing bacteria and arsenic release through amendments with labile carbon sources (i.e. acetate, lactate) has been demonstrated through several microcosm (van Geen et al. 2004; Akai et al. 2004; Islam et al. 2004; Rowland et al. 2007; Lear et al. 2007; Hery M. et al. 2010; Dhar et al. 2008) and field (Harvey et al. 2002) experiments. Thus, knowledge of the indigenous microbial communities' composition and carbon cycling dynamics inhabiting Bangladesh aquifers is necessary to better understand the biogeochemical mechanisms governing arsenic release.

Research studies in South and Southeast Asia that have examined the indigenous microbial communities abundance, composition and functioning in arsenic contaminated aquifers are limited (Islam et al. 2004; Sutton et al. 2009; Legg et al. 2012; Mailloux et al. 2013; Li et al. 2015). In addition, the types of microbial communities that have been studied (sediment versus groundwater) have varied and comparative analysis between

studies is challenging. However, results from these limited studies suggests that in South and South-east Asian aquifers, sediment-associated microbial communities and/or the adjacent groundwater microbial communities may possess distinct composition and potential differences in metabolic activity (Islam et al. 2004; Sutton et al. 2009; Legg et al. 2012; Mailloux et al. 2013; Li et al. 2015). For example, in sedimentary microbial communities, microbial species associated with iron and/or arsenic reduction has been reported (Hery et al. 2010; Legg et al. 2012) while in groundwater microbial communities genes associated with arsenic resistance were dominant(Sutton et al. 2009). In a field study of arsenic contaminated shallow aquifers in China, Li *et al*. (2015) reported that the species richness and diversity was significantly higher in the sedimentary versus the groundwater microorganisms. This observation aligns with well-established research that demonstrates microbial communities in aquifer sediments are unique compared to those in the the adjacent groundwater communities (Hazen et al. 1991) and may not be interchangeable proxies for the each other. In Bangladesh aquifers a similar type of comparative analysis between sediment and groundwater communities has not been carried out to date. This has significant implications for appropriately characterizing the microbial communities directly governing arsenic release in Bangladesh aquifers.

Phospholipid fatty acid (PLFA) analysis provides a complimentary tool to genetic analyses to gain information on *in situ* bacterial and micro-eukaryotic communities both in groundwater and sediment. Due to an anticipated ratio between PLFA concentrations and microbial abundance, *in situ* cellular-abundances can be estimated (Green & Scow 2000). The PLFA distributions can give insight into the composition of the microbial

communities due to particular fatty acid chains acting as biomarkers for certain organisms. Physiological shifts may be observable in the PLFA distributions, as microorganisms may change the composition of their cellular membrane in response to changes in their external environment. Stable carbon ($\delta^{13}C$) compound specific isotopic analysis (CSIA) of PLFA can provide insight into microbial metabolic pathways because of a pronounced kinetic isotope fractionation of ^{12}C and ^{13}C (Hayes, 2001) of organic substrates that is recorded in their cellular components. This fractionation continually re-occurs as carbon is cycled by heterotrophic organisms resulting in successive enrichment in ^{12}C and depletion in ^{13}C as carbon pools are cycled through metabolic receptors (i.e. autotrophs to fermenting bacteria to respiring bacteria to methanogens to methanotrophs, etc.).

This research study utilized abundance, distribution and $\delta^{13}C$ analysis of *in situ* PLFA to compare the indigenous groundwater and sediment microbial communities in Bangladesh aquifers. PLFA abundances and distributions were found to be significantly different between sediment-associated versus groundwater-associated communities and $\delta^{13}C$ PLFA signatures, which supports the notion that there is a co-existence of distinctive communities in these systems. The importance of these results were considered in the context of future research studies attempting to explore microbial communities governing arsenic release in Bangladesh and surrounding regions.

4.3 Methods

4.3.1 Bangladesh Study Sites

Field sampling was carried out in the Araihazar Upazila, Bangladesh in 2013 and 2015 (Figure S1: Field study map: need co-ordinates). Groundwater filter samples were

collected at sites with pre-existing shallow (<40m) and deep wells (<240m). In 2013, using submerged pumps, groundwater was pumped through glass wool filters (0.7 μm poresize, burnt at 450°C for 4 hours) for a range of pumping volumes depending on the site (Table 4.1) and placed in whirlpacks. In 2015, a slightly different method was attempted which pumped groundwater through glass wool filters followed by back flushing on to polyether sulfone (PES) filters (pore-size) and placed in 50 mL polyeythylene tubes. Subsequent to pumping, all filters were kept on ice until within the same day being frozen at a local health clinic at -10°C. All samples remained frozen through shipments on dry ice until stored at McMaster University at -10°C until further processing. All glass wool filters were subsequently freeze-dried prior to extraction while the PES filters were not. Sediment cores were collected from Site F and Site B using a gravity corer method as described in detail previously (Whaley-Martin et al., 2016).

4.3.2 Phospholipid Fatty Acid Analysis through Gas Chromatography-Mass Spectrometry (GC-MS)

Organic extractions of sediments and filters were carried out using a modified Bligh and Dyer (1959) as described in more detail in Whaley-Martin *et al*. (2016). Briefly, silica gel chromatography ($fraction_1$ = dichloromethane (DCM), $fraction_2$ = acetone, $fraction_3$ = methanol (MeOH) (contained PLFA) was carried out prior to the trans-esterification methanolysis reaction to create fatty acid methyl esters (FAME's) from the PLFA. The reaction was carried out using previously $\delta^{13}C$ characterized methanol for later corrections. A secondary silica gel purification step ($fraction_1$ = 4:1 hexane:DCM, $fraction_2$ = DCM (contains FAME's) and $fraction_3$ = MeOH) was used to purify the FAME's prior to GC-MS analysis.

4.3.3 Phospholipid Fatty Acid (PLFA) Distribution Analysis through GC-MS

PLFA concentrations and distributions of samples were analyzed on an Agilent 6890N GC (30 m × 0.32 mm DB-5 MS column, 0.25 μm film thickness) coupled to a 5973 quadrapole mass spectrometer. GC-MS conditions had a temperature program with an initial hold for 1 minute at 40 °C ramped to 130 °C at 20 °C/min to 160 °C at 4 °C/min and to 300 °C at 8 °C/min monitoring for masses (50-450 m/z). QA/QC, standards and reagents used for PLFA analysis are described in Supporting Information (SI).

4.3.4 Compound Specific Stable Carbon Isotopic ($\delta^{13}C$) Analysis of PLFA's through GC-IRMS

Stable carbon isotope ratio analysis of individual PLFA's (FAMES) was carried out with an Agilent 6890 GC (30 m × 0.32 mm DB-5 MS column, 0.25 μm film thickness) coupled to a Thermo Delta Plus XP isotope ratio mass spectrometer via a Conflo III interface. The GC program was 50 °C for 1 min; 10 °C/min to 150 °C; 1.5 °C/min to 180 °C for 20 min; 10 °C/min to 280 °C and finally 15 °C/min to 320 for 15 min. The GC-IRMS data was processed using Enhanced MSD ChemStation Software (Copyright © Agilent Technologies). All carbon isotope ratios were normalized to the Vienna Pee Dee Belmenite (VPDB) standard and triplicate analyses of samples and standards were carried out (RSD ≤10%).

Stable Carbon Isotopic ($\delta^{13}C$) of DOC, DIC and SOC

Stable carbon isotopic analyses of DOC, DIC and SOC were determined at the same time as previous radiocarbon measurements (Whaley-Martin et al. 2016) of these various carbon pools at Woodshole Oceanographic Institute through National Ocean

Sciences Accelerator Mass Spectrometry (NOSAMS) through methods available in detail at www.whoi.edu/nosams/page.do?pid=40135.

4.4 Results/Discussion

4.4.1 Estimating Groundwater Microbial Cell Abundances through Unconventional Filtering Method

The total PLFA extracted from all of the groundwater filters ranged from 1.4×10^{-3} to 1.5×10^{-2} picomoles/mL (Table 4.2). Total PLFA abundances for three of the nine groundwater samples that were reported in Whaley-Martin *et al*.(Whaley-Martin et al. 2016) are included within this analysis. Total cell abundances were estimated using a conversion factor 2×10^{4} cells/ pmol PLFA that is appropriate for aquifer environments(Green & Scow 2000) and ranged from 2.8×10^{1} to 3.0×10^{2} cells/mL of groundwater. Through comparison to previous cell counts directly on groundwater within the same region in Bangladesh (magnitudes of $\sim 10^{2}$ cells/mL of water) (Islam et al. 2001), capture efficiency of the glass-wool filters was considered equitable. However, these estimates may include an inherent underestimate of the actual bacterial abundance in the groundwater at these sites since bacterial cells less than approximately the 0.7µm pore-size of the glass-wool filter in diameter may pass through the filters and recent research has reported ultra small bacterial cells ($\sim 0.01 \mu m^3$) in groundwater (Luef et al. 2015). Nevertheless, the cell abundance estimates from the filters were considered useful to allow comparison between groundwater with adjacent sediments in these aquifers.

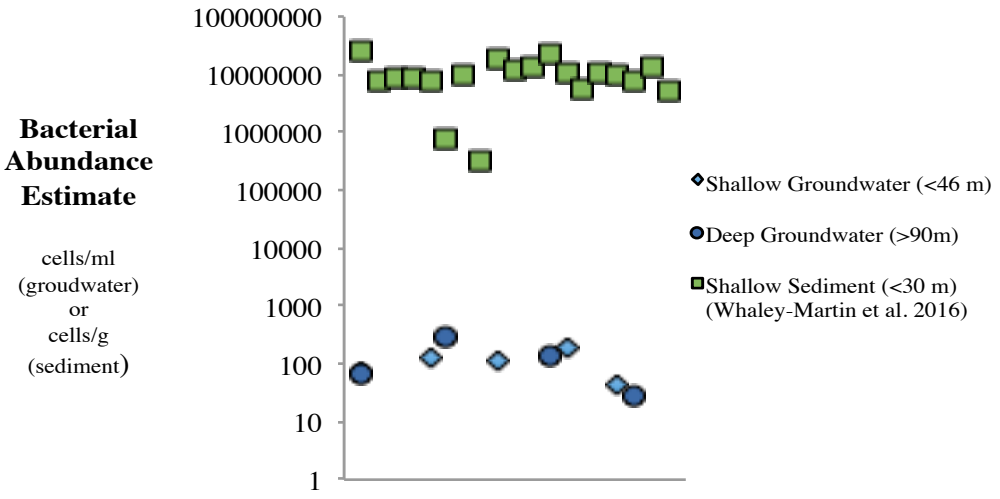

Figure 4.1 Bacterial abundance estimates in shallow groundwater (<46 m), deep groundwater (>90 m) and shallow sediments (<30 m) collected from Araihazar Upazila, Bangladesh.

4.4.2 Comparison of Microbial Cell Abundances Estimates Between Shallow Sediment and Shallow/Deep Groundwater in Bangladesh Aquifers

Shallow groundwater (<45 m) contained cell abundances estimated ranged between 4.5 x 10^1 and 2.0 x 10^2 cells/ml of groundwater and deep groundwater (91-240 m) ranged from 2.8 x 10^1 to 3 x 10^2 cells/ml (Table 4.1). No significant difference was found between the cell abundance between shallow and deep groundwater ($p=0.772$) and therefore are considered as part of the same group for comparison with shallow sediments. A significant difference ($p<0.00001$) was found between the cell abundances in these groundwater-associated communities (n=9) that were approximately five to six orders of magnitudes lower than the sediment associated cell abundances reported previously averaging ~1.1 x 10^7 (n=19) (Whaley-Martin et al. 2016). In terms of determining which microbial community is predominantly driving geochemical

conditions in these systems (i.e. Fe and As redox), the sediment-associated bacteria being far more abundant than groundwater bacteria, are more likely candidates.

Table 4.1 Total phospholipid fatty acids (picomoles) and cell abundance estimates of shallow and deep groundwater pumped through glass wool and PES filters in 2013 and 2015 in Araihazar Upazila, Bangladesh.

			2013 Glass Wool Filter			2015 PES Filter				
	Site (Co-ordinates)	**Depth (m)**	**Pumping Volume (L)**	**Total PLFA (Picomoles) /pumping volume**	**Total PLFA (Picomoles) /mL**	**Cell Abundance/ ml of Groundwater**	**Pumping Volume (L)**	**Total PLFA (Picomoles) /mL**	**Total PLFA (Picomoles)/pumping volume**	**Cell Abundance/ml of Groundwater**
Shallow Ground water	Site B	7.3	8637	33240	3.8×10^{-3}	7.7×10^{1}	-	-	-	-
	Site B	14.3	1821	10963	6.0×10^{-3}	1.2×10^{2}	16387	1.0×10^{-2}	167493	2.0×10^{2}
	Site B	45.5	4917	26983	5.5×10^{-3}	1.1×10^{2}	-	-	-	-
	Site F	19.3	-	-	-	-	20119	2.2×10^{-3}	45602	4.5×10^{1}
Deep Ground water	Site B (Community Well)	91.4	1805	5676	3.1×10^{-3}	6.3×10^{1}	-	-	-	-
	Site CAT	240	1673	24870	1.5×10^{-2}	3.0×10^{2}	15793	1.4×10^{-3}	22150	2.8×10^{1}
	Site SAM	240	2593	18355	7.1×10^{-3}	1.4×10^{2}	-	-	-	-

[a]Cell abundance estimates for 2013 Site B (7.3, 14.3 and 45.4m) were reported previously in Whaley-Martin *et al*. (2016)

4.4.3 PLFA Distribution of Sediment and Adjacent Groundwater in Bangladesh Aquifers

In the shallow sediments of Site B (<20 m, n=11), the most abundant fatty acid methyl esters in decreasing order of mole percentage were 16:0 (16.7 ± 1.2%), 18:1 (12.2 ± 0.7%), a-15:0 (6.3 ± 0.4%), 18:0 (6.2 ± 0.2%), 16:1 (6.0 ± 0.5%) and i-15:0 (5.9 ± 0.5%) (Figure 4.2) comprising ~53% of the total FAME distribution. A similar profile was found in shallow Site F sediments (<24m, n=8) with 16:0 (19.7 ± 0.8%), 18:1 (13.1 ± 2.0%), a-15:0 (6.9 ± 0.7%), 18:0 (6.0 ± 0.6%), i-15:0 (6.1 ± 0.8%) and 16:1 (5.2 ± 1.3%) (Figure 4.2). The location of double bonds (i.e. through subsequent derivatization using dimethyl disulfide (DMDS)) was not determined. Therefore, the reported proportion of

unsaturated FAME's (i.e. 18:1, 16:1) represents the sum of all the FAME's with the same chain length and number of double bonds. Within all of groundwater derived PLFA, the FAME's found on average to be the most predominant were 16:0 (21.8 ± 3.2%), 18:1 (16.9 ± 1.3%), 16:1 (12.9 ± 7.9%), a-15:0 (7.6 ± 4.7%) and 18:0 (5.5 ± 1.6%) and Br-17:0 (5.0 ± 0.9%) comprising ~65% of the total FAME's. Thus, sedimentary and groundwater communities were similar in the distributions of the most abundant FAME's which is expected as these lipids are common major components of bacterial and micro-eukaryal cellular membranes. Long-chain FAME's (C_{22}-C_{29}) were detected within the sediments of both Site B and F comprising up to ~17 %and ~7% (mol %) of the total FAME distribution respectively (Figure 4.1). Contrary to this, long chain FAME's were not detected in any of the groundwater filters, including those taken from Site B (Figure

S1c;d).

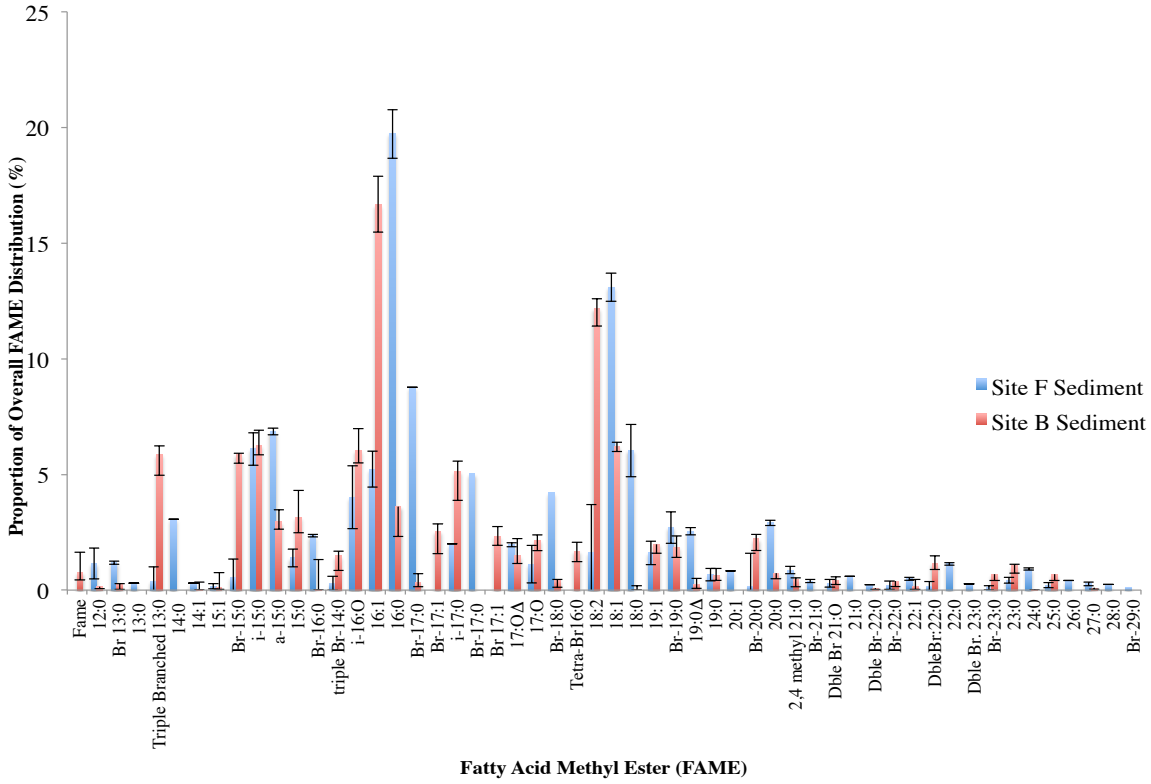

Figure 4. 2 Average profiles of fatty acid methyl esters derived from phospholipid fatty acid of Site F (blue) and Site B (red) sediments

Principle component analysis (PCA) on the relative proportions (mole %) of individual fatty acid methyl esters (FAME's) derived from PLFA in groundwater (n=9) and shallow aquifer sediment (n=19) was carried out using Multibase2015 Microsoft®Excel® add-in program. In the PCA, only 34% of variance within the samples could be attributed to first three principle components (PC1 (13.7%), PC2 (10.3%) and PC3 (9.5%). However, when all three components were plotted for each sample, distinctive clustering patterns between the PLFA distributions in the groundwater

compared to the sediment were found (Figure 4.3). The variables (FAME's) with the highest loading in the PCA analysis were found to be mainly long chain fatty acids (23:0, 24:0, 26:0, double-branched 23:0). This strongly suggests that the presence of long chain fatty acids in sediments and not in groundwater is likely driving the clustering of the samples in the PCA.

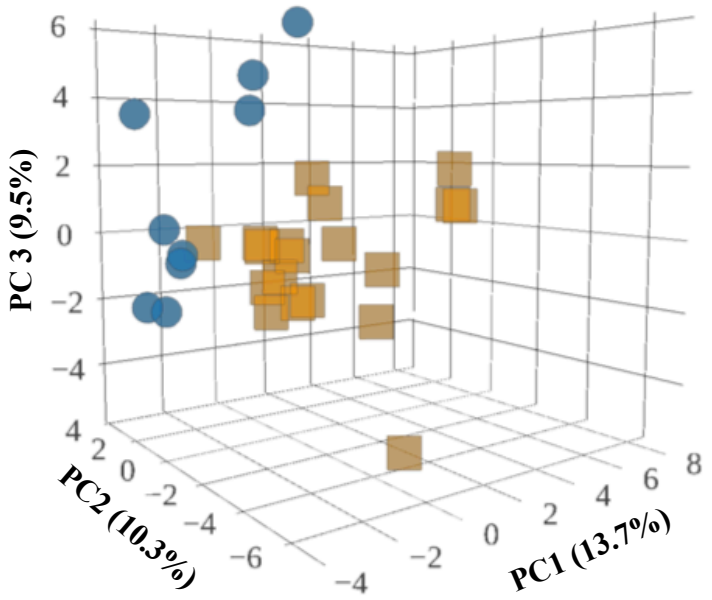

Figure 4.3 Principle component analysis (PCA) (on relative proportions (mole %) of individual fatty acid methyl esters (FAME's) derived from PLFA in groundwater (n=9) (blue circles) and shallow aquifer sediment (n=19) (orange squares) in Araihazar upazilla. The three-dimensional scatter plot was created from the PCA derived values for each sample using Ployly1©2016).

While not routinely reported possibly due to their later elution in a GC run (Summit et al. 2000), long-chain FAME's have been suggested as biomarkers typical of

micro-eukaryotic populations rather than bacteria in the environment(Rajendran et al. 1995). No significant correlation was observed between concentrations of the long chain fatty acids and the relatively low and consistent concentrations of the possible fungal biomarker 18:2 at either Site F ($p=0.38$) or Site B ($p=0.09$) suggesting that there is a non-fungal micro-eukaryotic population inhabiting the sediments. However, since no derivitation was carried out to determine the double bond locations and only the 18:2ω6,9 is considered a useful biomarker for fungi, this can not be ascertained using only this dataset(Frostegård et al. 2010). Very long chain PLFA's (21-30 carbons) found previously in hydrothermal marine sediments were suggested to be a signature of oligotrophic (Summit et al. 2000) conditions. The focus of studies characterizing microbial communities in Bangladesh aquifers has thus far been on the bacterial communities comprising the sediment and groundwater. While one previous study has reported the presence of micro-eukaryotic organisms (zooplankton) in Bangladesh groundwater (Islam et al. 2001) through direct zooplankton counting methods, none have reported micro-eukaryotes in sediment through PLFA or 18S rRNA analysis. However, the PLFA profiles of shallow sediments at Site F and Site B suggest their presence and a presence in high arsenic groundwater zones (Figure 4.1).

A comparison between FAME distributions of the three deep groundwater samples, indicate that between 2013 and 2015, a change was observed in the proportion of mono-unsaturated and cyclic FAME's present. SAM and CAT PLFA profiles in 2013 were similar in their relative proportion of FAME classes. However, in 2015 CAT PLFA profiles, a decrease in relative proportions of mono-unsaturated FAMES occurred by 22% (38.5 to 16.1%) and an increase in 21% (2.6 to 23.7%) in cyclic FAME's occurred

(Figure 2). Moneonoic PLFA transformations into cyclopropyl products by bacteria has been observed as a physiological indicator of slow growth/starvation effects(Navarrete et al. 2000). These may indicate a change in the microbial community's environmental conditions between 2013 and 2015 at Site CAT within deep zones (~240m) of the aquifer.

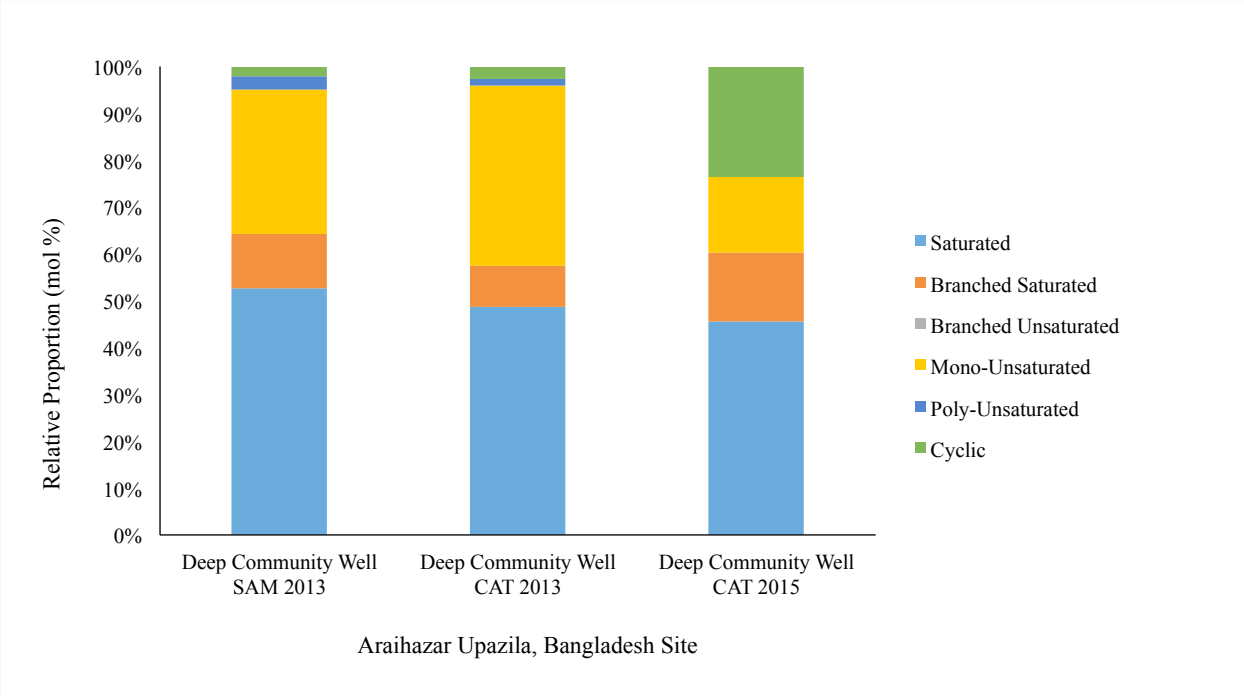

Figure 4.4 Relative proportion (mol %) of FAME distributions categorized as saturated, branched saturated, branched unsaturated, mono-unsaturated, poly-saturated or cyclic of the three deep groundwater samples from SITE SAM (2013) and Site CAT (2013 and 2015).

4.4.4 $\delta^{13}C$ Analysis of PLFA of Sediment and Adjacent Groundwater in Bangladesh Aquifers

Shallow Site B sedimentary PLFA's showed a progressive depletion in $\delta^{13}C$ in major FAME's (i-15:0, a-15:0, 16:0 and 18:0) with depth from -24‰ to -31‰ while Site F PLFA from similar depths did not show the same trend in $\delta^{13}C$ within a similar depth interval of i-15:0, a-15:0, 16:0, 18:0 and Br-17:0) but did possess a similar range (-24‰

to -30‰) (Figure 4.5). The progressive depletion of Site B sediment PLFA did not correspond to a depletion in the bulk signals of the coinciding carbon pools (DOC, SOC, DIC) (Figure 4.5) but previous radiocarbon analysis of PLFA on these samples indicate a predominant utilization of DOC and/or DIC-aged carbon sources (Whaley-Martin et al. 2016). Changing microbial metabolic activity (as reflected in the changing overarching geochemical conditions throughout the depth profile) may explain a change in the $\delta^{13}C$ PLFA signatures at Site B. Previous studies in Bangladesh show preliminary evidence of an increase in methane production correlating with increases in dissolved iron and arsenic (Harvey et al. 2002; Horneman et al. 2008). If present, upon cell death methanogens at these depths would provide depleted carbon compounds to reducing bacteria as a substrate showing up as a more reduced signal in their PLFA. In addition, an increased proportion of methanotrophic bacteria in the indigenous community would explain the subsequent depletion in in $\delta^{13}C$ of PLFA at these depths. Within groundwater collected at 14 m from Site B in 2015, the bulk PLFA had a $\delta^{13}C$ of -41‰ that is possibly indicative of increased methanogenic activity. This level of $\delta^{13}C$ depletion was not observed in the FAME's derived from sediments at a similar depth and may indicate that proportionally the groundwater community may contain more bacteria involved in methanotrophic activity than in the sediment-associated community. The $\delta^{13}C$ PLFA signatures of deep groundwater samples ranged from -21‰ to -32‰ consistently falling within a similar range as the shallow sediment PLFA signatures. Previous radiocarbon measurements of microbial DNA from deeper groundwater at Site F was significantly older than microbial DNA from shallower groundwater highlighting that at these sites, microbial carbon sources at these sites cannot be discerned through $\delta^{13}C$ signatures. Overall the $\delta^{13}C$

signatures of PLFA from shallow groundwater communities suggest some variation from the consistent $\delta^{13}C$ signatures of PLFA from shallow sediment communities and deep groundwater communities. This indicates that there may be differences in the carbon metabolisms/cycling within sediment and groundwater microbial communities.

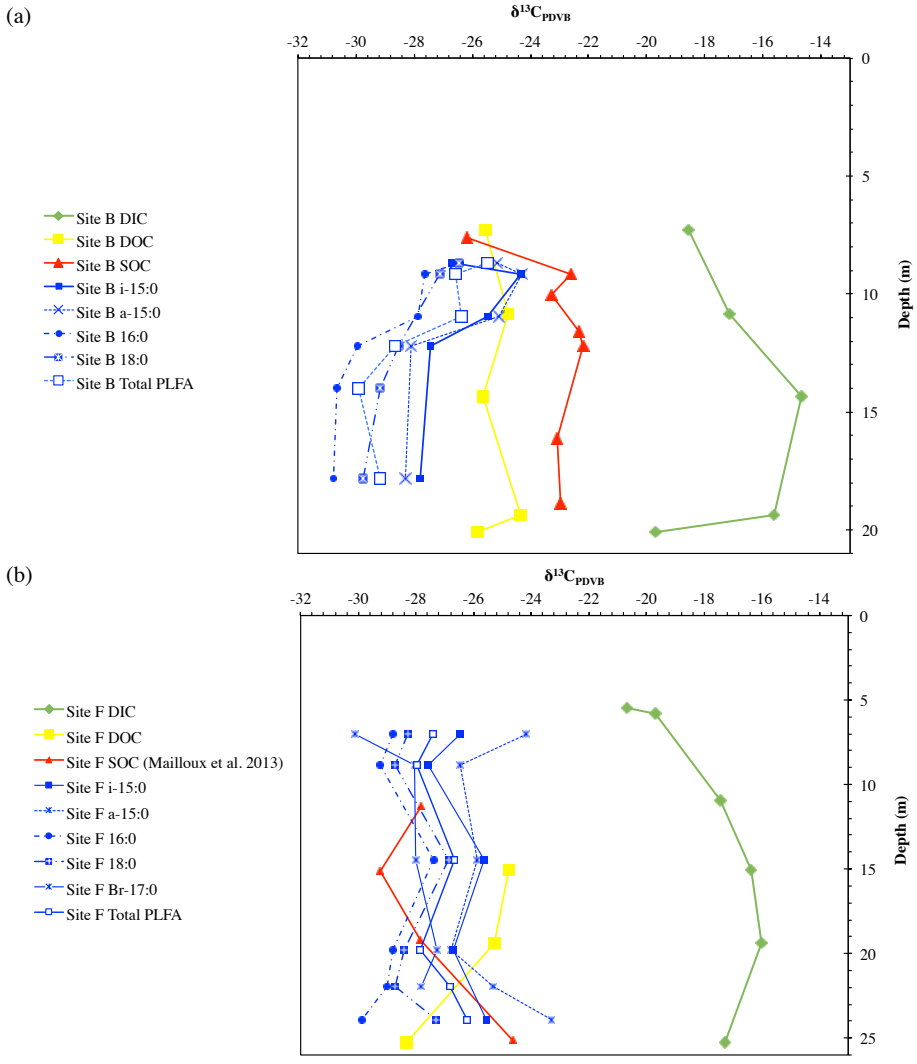

Figure 4. 5 $\delta^{13}C$ PLFA signatures in shallow sediments relative to DOC, DIC, SOC at (a) Site B and (b) Site F.

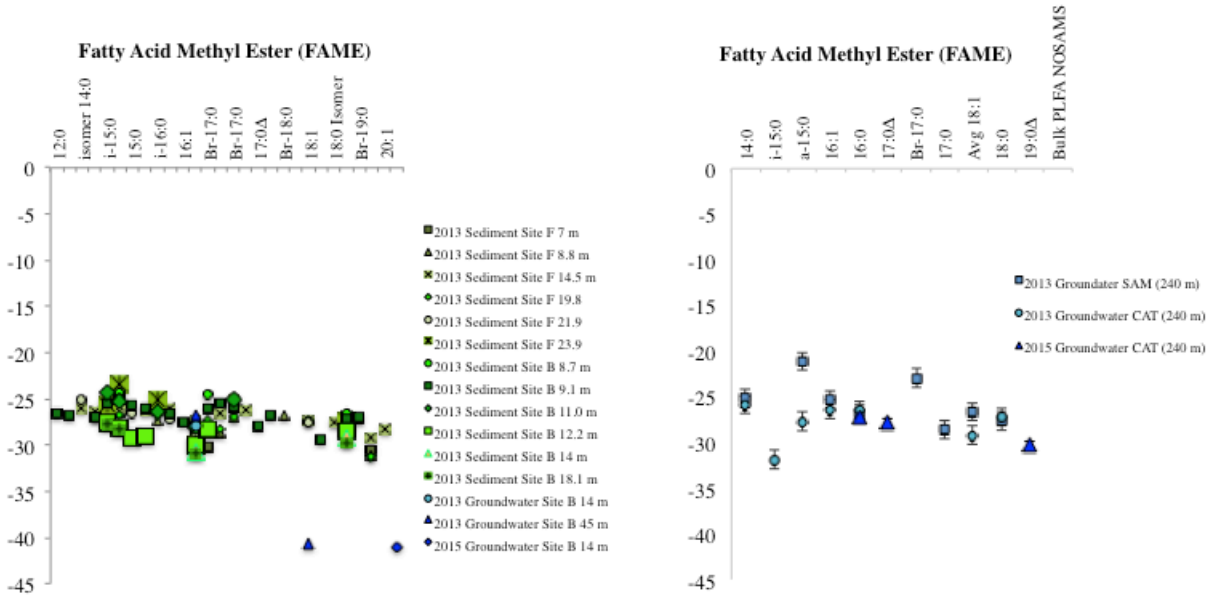

Figure 4.6 2013 and 2015 $\delta^{13}C$ PLFA signatures of (a) shallow Site B and F sediments and (b) SAM and CAT deep groundwater (~240 m).

4.5 Conclusions

There are significant differences between the abundance of microbial communities that inhabit Bangladesh aquifers in shallow sediments versus shallow and deep groundwater. Lipid distributions and some differences between PLFA $\delta^{13}C$ signatures support that these communities should be not be used interchangeably in future work exploring the microbial communities driving iron reduction and arsenic release. Thus, while proximal, these communities are distinct from each other certainly in terms of abundance and possibly in composition and metabolic activity and their relative roles in microbial arsenic release in Bangladesh aquifers may be very different.

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Chapter 5. Examining Methanogenesis in Shallow and Deep Bangladesh Aquifers Through Groundwater Methane Concentration, $\delta^{13}C$ and δD Analysis

Whaley-Martin, K.J., Mailloux, B., van Geen, A., Bostick, B., Harvey, C., Ahmed, K.M., Choudhury, I., Slater, G.F.

5.1 Abstract

Microbially driven arsenic release had resulted in widespread groundwater contamination throughout Bangladesh. The coinciding presence of methane within the same aquifers has been commonly found but limited work has examined the relationship between the iron and arsenic reductive bacteria in the systems and methanogens. This study examined the methane distributions and isotopic signatures ($\delta^{13}C$ and $\delta^{2}H$) of groundwater collected in 2016 and 2017 from five sites (Sites F, C, E, B and SS, ranging in depth from ~5 to 90 m) in Araihazar Upazila, Bangladesh, an area with high dissolved arsenic concentrations. Five deep wells from an arsenic contaminated Plesitocene aquifer (190 to 240 m) in the village of Roynadi Kalagachi were also analyzed. Groundwater CH_4 concentrations (SRI-GC analysis) ranged from 0.2 to 1240 μmoles/L. Methane concentrations in deep well groundwater ranged from 0.2 to 124 μmoles/L. Methane concentrations were generally higher in groundwater where higher arsenic concentrations were present. Methane $\delta^{13}C$ signatures measured by GC-IRMS ranged from -49 to -90 ‰ and at Site B where δD of methane ranged from -110 to 210 ‰ which was indicative of methangenesis through CO_2 reduction.

5.2 Introduction

In Bangladesh aquifers, the indigenous microbial communities govern the overarching geochemical conditions and stimulate groundwater arsenic contamination (Nickson et al. 1998; Nickson et al. 2000; Islam et al. 2004; Swartz et al. 2004; Dhar et al. 2011; McArthur et al. 2001; Dowling et al. 2002; Harvey et al. 2002; Postma et al. 2007; Postma et al. 2012). While the iron-reducing bacteria have been the focus of studies across Bangladesh (Nickson et al. 1998; Nickson et al. 2000; Islam et al. 2004; Swartz et al. 2004; McArthur et al. 2001; Dowling et al. 2002; Harvey et al. 2002; Postma et al. 2012), the co-inhabiting archaeal populations and their potential roles within the anaerobic microbial communities have to date been under-addressed. However the presence of biogenic methane has been reported in arsenic contaminated aquifers in South and Southeast Asia in Bangladesh (Harvey et al. 2002), Taiwan(Liu et

al. 2009), Inner Mongolia China(Wang et al. 2015). This demonstrates that methanogenic archaea are present and active. This raises questions as to how methanogenesis might be associated with and/or affecting other parameters that are related to microbial carbon cycling (Mailloux et al. 2013; Whaley-Martin et al. 2016), such as iron reductive dissolution and arsenic release.

Understanding the community dynamics and metabolic activity of carbon cycles is important, as they maintain an intimate connection to the iron/arsenic cycling into groundwater. Heterotrophic bacteria couple the oxidation of organic carbon to the reduction of FeIII and/or AsV. Lovley and Phillips (1986) demonstrated that over time the reactivity/availability of iron oxides to the reducing bacteria becomes depleted leaving behind more recalcitrant mineral phases of iron oxides. In addition, the carbon products of these reactions (i.e. acetate and CO_2/H_2) become available and under reducing conditions, methanogenesis can be stimulated. This results in a common spatial zonation of iron reduction preceding methanogenesis in natural environments. Thus, the presence of biogenic methane indicates highly reductive conditions. In Bangladesh aquifers, how methanogenesis and microbial FeIII/AsV reduction are related (i.e. zonation versus co-occurrence) is unclear and whether these processes have a homogenous versus heterogeneous distribution has not been reported. The interconnectivity between the iron-oxide reduction and methanogenesis has been studied for over twenty years (i.e. Lovley and Phillips, 1986) but recent work has brought into question the traditional models of simple microbial metabolic zonation thought to govern the spatial distribution of these organisms ((Sivan et al. 2016). Understanding the role of methanogens may be particularly pertinent for monitoring conditions in oxidized deep Pleistocene sediments

(hosts low arsenic groundwater) where a delivery of reactive organic carbon to depth could stimulate microbial reduction and arsenic release (Zheng et al. 2005; Burgess et al. 2010; Dhar et al. 2011; Radloff et al. 2011; van Geen, Benjamin C Bostick, et al. 2013).

The origins of methane can be elucidated through predictable signatures of stable carbon ($\delta^{13}C$) and hydrogen (δD) isotopes that are distinctive for the two dominant biogenically produced methane pathways (CO_2 reduction (equation 1) and acetate fermentation (equation 2), as well as thermogenic methane.

(1) $CO_2 + 4H_2 \rightarrow 2H_20 + CH_4$

(2) $CH_3COOH \rightarrow CH_4 + CO_2$

Biogenic methane will have $\delta^{13}C_{CH4}$ signatures between ~-100 to -40 ‰ and δD_{CH4} will range between ~-450 to -150‰ (Whiticar 1999). Hydrogenotrophic methanogens will produce methane through the CO_2 reduction pathway that is more depleted in $\delta^{13}C$ (~-60 to -100 ‰) than methanogens utilizing the acetate fermentation pathway (~-40 to -60‰) (Whiticar 1999). The use of CO_2 versus acetate, which has two carbon atoms present, is the main reason for these differences. CO_2 reduction pathways result in δD_{CH4} signatures ranging from -170 to -250‰ whereas acetogenic methanogens produce methane ranging from -250 to -400‰ (Whiticar 1999). Thermogenic methane that is formed at higher temperatures has more enriched $\delta^{13}C$ signatures (~ -50 to -20 ‰) and δD signatures ranging from ~ -275 to -100 ‰ (Whiticar 1999). Through the predicted fractionation between $\delta^{13}C_{CO2}$ and $\delta^{13}C_{CH4}$, Liu et al. (2009) reported that in an arsenic contaminated aquifer in Taiwan, methanogens were utilizing the CO_2 reduction pathway at depth. This type of investigation has not currently been extended to examine methanogens in Bangladesh aquifers. This research study uses complimentary measures of groundwater

methane concentration and isotopes ($\delta^{13}C/\delta D$) to examine methanogenesis within Holocene and Pleistocene aged aquifers (ranging from ~5 to 240 m depth) in Araihazar Upazilla in Bangladesh. The relationships between the activity of methanogenic archaea and Fe/As microbial cycling will be explored in this system.

5.3 Methods

5.3.1 Field Sampling

Groundwater samples were collected from Araihazar Upazilla in Bangladesh in June 2016 (Site B and Site F) and January 2017 (Site C, Site E, Site SS) from pre-existing well nests (depths ~5 m to 90 m depending on site) that have been the focus of previous research studies (van Geen 2003; Stute et al. 2007; Dhar et al. 2008; Mailloux et al. 2013; Whaley-Martin et al. 2016). In January 2017, five deep wells (188 m to 236 m) in Roynadi Kalagachi (23125, 24477, ND01, ND02, ND03) that lie within Pleistocene-aged sediments were also targeted. Field kites were used to screen for dissolved arsenic concentrations above the WHO guidelines of 10 μg/L (50 to 300 μg/L). Using submerged pumps, 60 ml (2016) and 100ml (2017) samples of groundwater were injected through septa into pre-evacuated and burnt glass serum bottles fixed with Hg_2Cl and stored upside until further analysis.

5.3.2 Methane Concentration and Isotopic Analysis

Methane concentrations in equilibrated groundwater samples were measured by injecting of 50 to 1000 μL of headspace from the bottles on a SRI 8610C gas-chromatographer (silica gel column 0.91 m x 2.1 mm) coupled to a flame ionization detector (GC-FID). Methane calibration curves were created directly from gas tanks with

pre-characterized methane concentrations. All samples were measured in triplicate (RSD ≤10%) and PeakSimple 3.29 software was used for peak analysis/integration.

Stable carbon isotope ratios of CH_4 were determined with an Agilent 6890 GC (GSQ; 30m × 0.32 mm) coupled to a Thermo Delta Plus XP isotope ratio mass spectrometer via a Conflo III interface. Injections were made with a isothermal GC temperature program. GC-IRMS data was processed with Enhanced MSD ChemStation Software (Agilent Technologies©). Triplicate analyses of samples and standards were carried out (RSD ≤10%). Deuterium analysis of CH_4 was also carried out on an Agilent 6890N GC coupled to a Delta V Plus IRMS via a GC Combustion III interface (30m x 0.32mm x 15 μm, Carboxen 1010 column). The GC temperature program was 110 °C for 5.5 min; to 180 °C @ 35 °C/min and a final hold for 2 minutes. The temperature conversion reactor was held at 1450°C.

5.4 Results/Discussion

5.4.1 Depth Profiles of Methane Concentrations in Araihazar, Bangladesh

At Site F, E, C, B and SS, methane was consistently present but widely varying in concentrations (range from 0.2 to 1239 μmoles/L) depending on depth and location (Figure 5.1: arranged from right to left with lowest to highest peak methane concentrations). However, the general depth profile of low [CH_4] at shallow depths (0-30 m) followed by a peak in [CH_4] and subsequent decrease at the greatest depths was consistent for each of these sites (Figure 5.1). Methane concentrations in deep well groundwater ranged from 0.2 to 124 μmol//L (Table 5.1). Of the deep wells sampled (>188m depth), BD 24477 (236 m) and BD NDO3 (204 m) had the highest methane concentrations present at 124 and 16 μmol//L respectively.

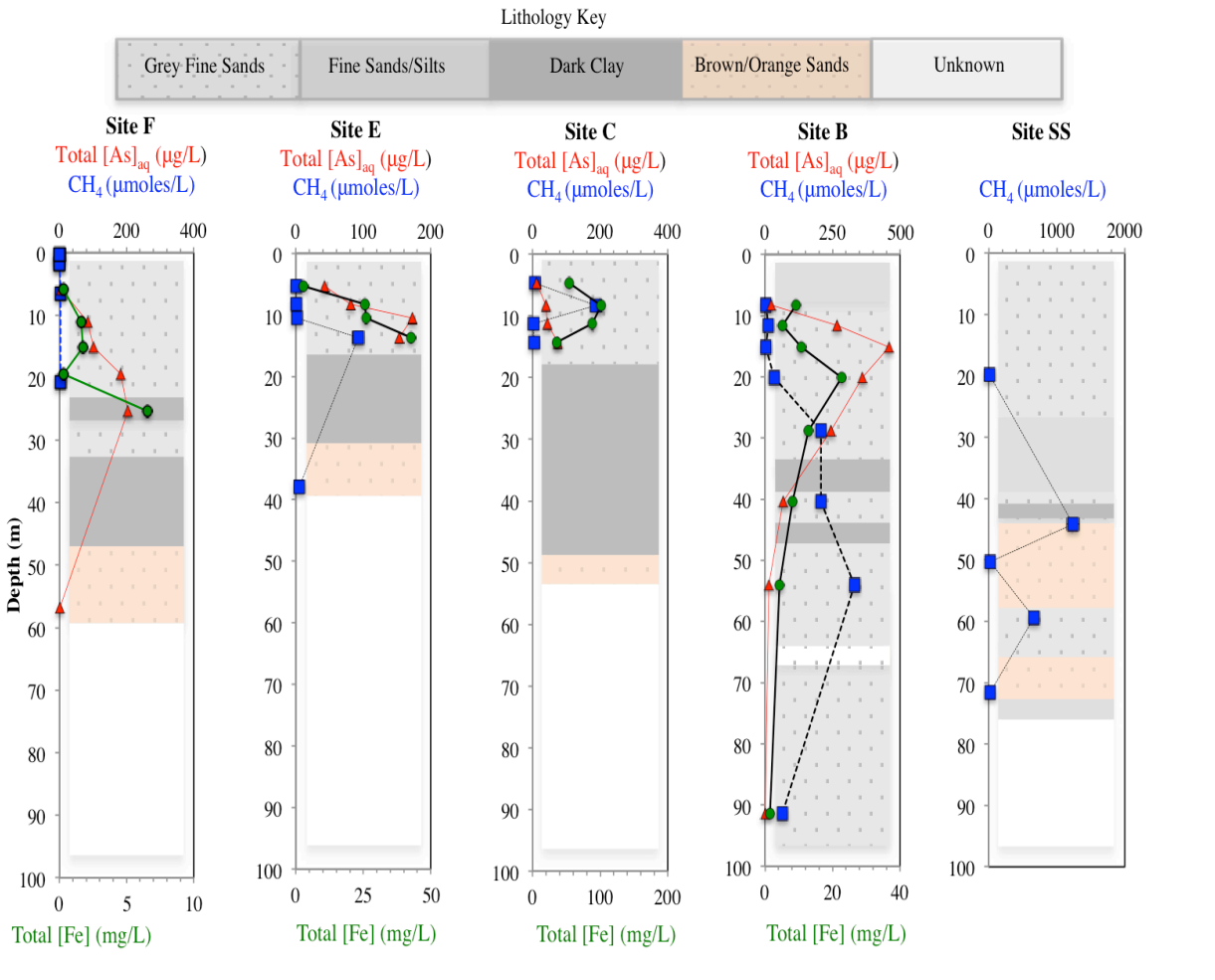

Figure 5. 1 Groundwater depth profiles of total methane (this study) (blue squares), dissolved As (red triangles) and Fe (green circles) concentrations at Sites C and E (Stute et al., 2007) and Sites F and B (Whaley-Martin et al., 2017).

Table 5.1 Groundwater methane concentrations (µmol//L) and $\delta^{13}C_{CH4}$ signatures from deep wells (188 to 236 m depth) Araihazar, Bangladesh

	Depth (m)	µmol//L CH_4	$\delta^{13}C_{CH4}$ (‰)
BD 24477 Deep Well	236	124 ± 4.9	-90.1
BD "23125" Deep Well	188	0.2 ± 0.005	<LOD
BD ND01 Deep Well	216	7 ± 0.05	<LOD
BD ND02 Deep Well	204	0.5 ± 0.003	<LOD
BD ND03 Deep Well	204	16 ± 0.24	-78.6

The lithological profiles of the Site F, E, C, B and SS varied in stratigraphy (Figure 5.1), which is expected due to the dynamic deltaic depositional environments of these aquifer sediment. General relationships between the spatial distribution of different lithological units and groundwater methane distributions could be observed for the five sites. Sites F, E and C sediments contained a relatively thick (>10m) clay unit, methane concentrations peaked in shallower sediments above the clay layers. In Site B and SS, which were largely comprised of fine-grained sand units (with some thinner interbedded clay units), methane concentration profiles were more gradual and higher at greater depths (Figure 5.1). Site SS contains a reduced grey stratigraphic unit between 60 and 65 m that is bound on the upper and lower ends by yellow oxidized sediments. Radiocarbon dating of these sediments has revealed all of these sediments are of Pleistocene ages (personal communication with Dr. Alexander van Geen (Columbia University, 2017). Therefore grey reduced units from 60-65 m could have been produced through induction of post-depositional iron reductive conditions. This unit also hosts groundwater that has higher methane concentrations than the upper and lower oxidized units.

5.4.2 Relationships between Methanogenesis and Microbial Iron/Arsenic Reduction in Bangladesh Aquifers

Where available from previous studies (Site E and C (Stute et al. 2007)) and Site B and F ((Whaley-Martin et al. 2017) total dissolved As and Fe were examined alongside methane concentration depth profiles (Figure 5.3). Within the depth profiles of the four

sites examined, methanogenic zones appear associated with zones with iron/arsenic reductive dissolution. Site F had very low groundwater methane concentrations (<3.6 μmoles/L) but relatively high As and Fe. At Site E and C, CH_4 concentrations were also relatively low (<6 μmoles/L) but each had a localized spike in concentration to 93 and 187 μmoles/L respectively occurring within the high As and Fe zones. This may be attributed to local "lenses" of carbon sources such as buried peat layers that can stimulate methane production. A wider distribution of CH_4 was found through Site B's depth profile and methane concentrations reach 330 μmoles/L. The zone of highest methane concentrations resides below the groundwater with the highest arsenic and iron concentration. Site SS groundwater had methane concentrations that peak an order of magnitude higher (~1240 μmoles/L) than any of the other sites examined. The reduced grey sediments between 60 and 65 m that is bound by oxidized sediments (as mentioned previously) also hosts groundwater that has higher methane concentrations than the upper and lower oxidized units.

The traditional conceptual model of microbial redox ladder predicts (in a non-marine environment where sulphate concentrations are low), dissimlatory iron-reducing zones will consistently precede methanogenic zones due to iron-reducing bacteria out competing methanogenic archaea (Bethke et al. 2011). However, examining the depth profiles of Sites F, E, C and B suggest potential spatial co-occurrence in the aquifers of methanogenesis and iron reduction. Recent research has strongly suggested the traditional model of a cascading microbial metabolic zonation specifically between iron reducers and methanogens is likely an oversimplification (Sivan et al. 2016). Since the methanogenic pathways being used by the indigenous archaea has been shown through

isotopic analyses to be primarily operating through CO_2 reduction rather the using acetate, it's possible that there is not a direct competition between the iron reducing bacteria and methanogens for substrate. In addition the iron reduction may in fact be producing the CO_2 required for the methanogens, while competition for the hydrogen sources are more likely the component that these organisms compete for. Sivan et al. (2016) were recently able to demonstrate that methanogens themselves can reduce iron minerals to produce methane, which would explain the commonly seen coinciding increase of iron and methane concentrations in natural environments. With this particular data set, conclusive differentiation between these potential processes is not possible but both seem at least plausible in this system. Certainly, the examined sites in Araihazar demonstrate that methanogen distributions in these aquifers are heterogeneous in their distribution and activity depending on site specific conditions.

Within the deep wells (~200 m) around the village, Roynadi Kalagachi (23125, 24477, ND01, ND02, ND03), methane concentrations were strongly and positively correlated with dissolved arsenic concentrations (Figure 5.2). This suggests that at these depths, where Pleistocene sediments in the region hosts low arsenic groundwater, microbial reduction of Fe/As has been stimulated and methanogenesis is co-occurring under these reductive conditions. Reductive dissolution of iron may be occurring due to reactive organic carbon sources migrating downwards and infiltrating the deep aquifer. The vulnerability of the deep aquifers to arsenic contamination has been hypothesized in recent studies due to a potential "draw-down" of reactive dissolved carbon from high rates of municipal and rice irrigation pumping in the region (Zheng et al. 2005; Burgess

et al. 2010; Dhar et al. 2011; Radloff et al. 2011; van Geen, Benjamin C Bostick, et al. 2013).

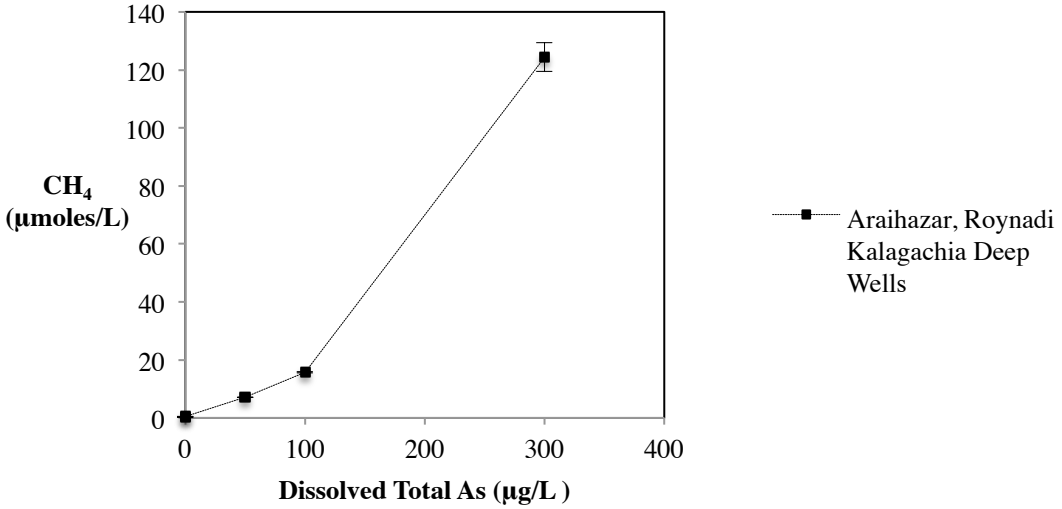

Figure 5. 2 Correlational relationship between groundwater CH_4 concentrations (µmoles/L) and total dissolved arsenic measured through field kits in five deep wells (~200 m) around the village, Roynadi Kalagachi (23125, 24477, ND01, ND02, ND03).

5.4.2 Isotopic $\delta^{13}C_{CH4}$ signatures of Shallow and Deep Groundwater in Araihazar, Bangladesh

Methane $\delta^{13}C$ signatures in groundwater from Araihazar ranged from -49 to -90 ‰ with a general trend to more depleted values at greater depth (Table 5.1; Figure 5.2 a). Due to higher methane concentrations, depth profiles of $^{13}C_{CH4}$ and δD_{CH4} were able to be determined for Site B and SS ($^{13}C_{CH4}$ only) (Table 5.1, Figure 5.2c). Depletion in the $\delta^{13}C_{CH4}$ (-57 to -81 ‰) and δD_{CH4} (-91 to -209 ‰) signatures negatively correlated with the methane concentration increases (Figure 5.2b). While the Pearson R = -0.69 between $\delta^{13}C_{CH4}$ and $[CH_4]$ was not found to statistically significant (p=0.129, most likely due to small sample size), a strong negative and statistically significant correlation (R=-0.90,

p=0.03) was found between methane concentrations and deuterium depletion at Site B. In addition a strong positive correlation (R=0.95, p=0.013) (Figure 5.3d) was found between the $\delta^{13}C_{CH4}$ and δD_{CH4} in the methane. Alternatively, signatures at Site SS, while showing a similar range of $\delta^{13}C_{CH4}$ (-63 to -80‰) did not have the same relationship of progressive depletion with increased concetrattions. The most depleted groundwater $\delta^{13}C_{CH4}$ signature (-91‰) was found in the deepest (236 m) groundwater sampled at well 24477 (Table 5.1)

Table 5.2 Groundwater methane concentrations (μmoles//L) and $\delta^{13}C_{CH4}$ signatures from Site B, F, C, E, and SS in Araihazar, Bangladesh.

	Depth (m)	μmol//L CH_4	$\delta^{13}C_{CH4}$ (‰)[a]	ΔD_{CH4} (‰)[a]
Site F	5.8	0.8 ± 0.01	-	N/A
	11.1	0.2 ± 0.02	-	N/A
	15.1	0.2 ± 0.003	-	N/A
	19.4	0.2 ± 0.01	-	N/A
	25.3	3.6 ± 0.142	-	N/A
Site E	5.3	0.3 ± 0.01	-	N/A
	8.3	0.6 ± 0.02	-	N/A
	10.5	1.0 ± 0.02	-	N/A
	13.7	93 ± 3.65	-68.8	N/A
	38.0	4.8 ± 0.19	-	N/A
Site C	4.7	5.6 ± 0.2	-	N/A
	8.3	187 ± 11.9	-49.1	N/A
	11.3	2.0 ± 0.1	-	N/A
	14.3	3.9 ± 0.2	-	N/A
Site B	11.1	0.2 ± 0.02	-	N/A
	15.1	0.2 ± 0.003	-	N/A
	19.4	0.2 ± 0.01	-	N/A
	25.3	3.6 ± 0.142	-	N/A
	27.9	208 ± 10	-84.1	-160.8
	40.1	207 ± 14	-85.8	-209.0
	52.7	331 ± 27.4	-84.4	-200.5
	91.4	65 ± 2.1	-80.6	-109.2
Site SS	19.8	11 ± 0.1	-70.5	N/A
	44.2	1239 ± 93	-67.3	N/A
	50.3	21 ± 1.1	-72.4	N/A
	59.4	663 ± 59.6	-79.6	N/A
	71.6	22 ± 1.8	-62.6	N/A

[a] **Missing (-) data indicates that methane concentrations were <LOD to obtain $\delta^{13}C_{CH4}$ (‰) or ΔD_{CH4} (‰). N/A indicates that the deterium values that were not measured.**

The $\delta^{13}C_{CH4}$ and the $\delta^{2}H_{CH4}$ signatures suggest that biogenic methane is being produced through hydrogenotrophic methanogens carrying out CO_2 reduction (Table 5.1, Table 5.2, Figure 5.3a). This has additional support at Site B, where additional $\delta^{2}H_{CH4}$ measurements fall also within the range of CO_2 Figure 5.2c,d). These results are in agreement with the previously mentioned Liu et al. (2009) study at a comparable As contaminated shallow aquifer in Taiwan, where CH_4 was inferred to be produced CO_2 reduction through $\delta^{13}C_{CH4}$ and $\delta^{13}C_{CO2}$. While in freshwater environments acetotrophic

methanogens are thought to dominate, CO_2 reduction can be expected to become more prominent as organic carbon becomes depleted (Whiticar 1999)) as in the oligotrophic conditions of Bangladesh sub-surface.

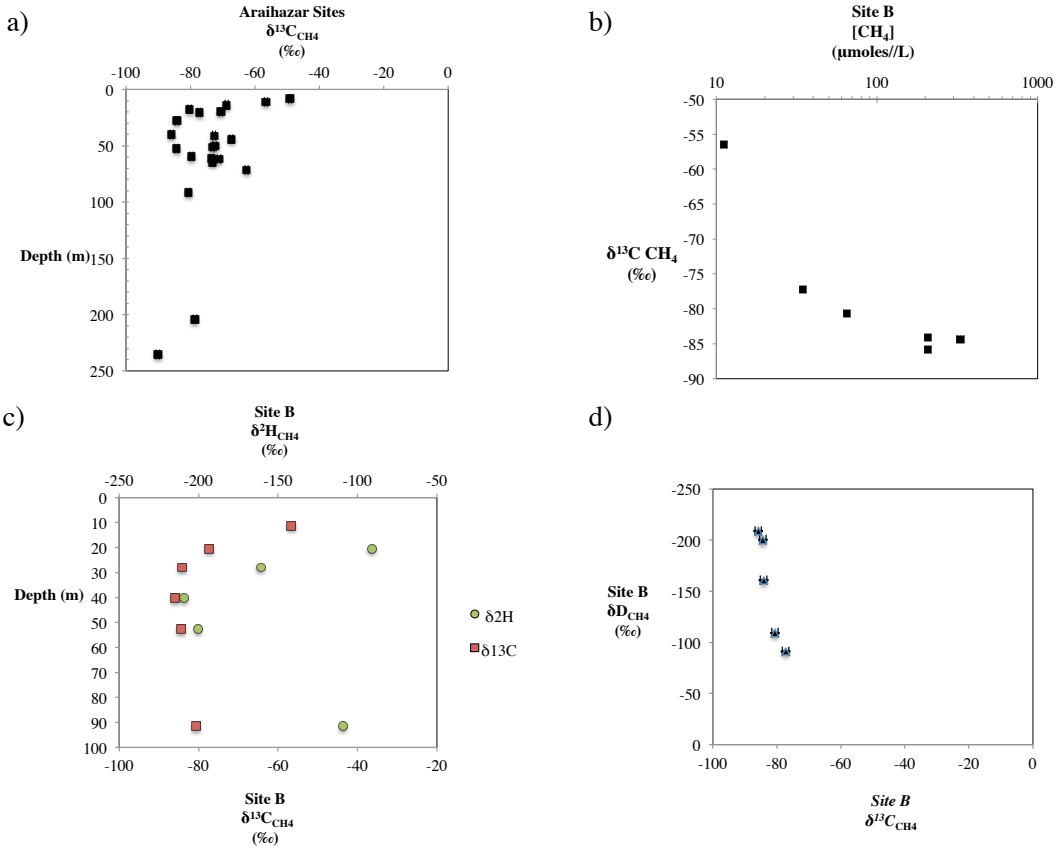

Figure 5. 3 Isotopic characterization of groundwater methane (a) $\delta^{13}C_{CH4}$ at Site F, E, C, B, SS and deep wells 23125, 24477, ND01, ND02, ND03 within Araihazar, Bangladesh (b) Site B correlational relationship between methane concentrations and $\delta^{13}CC_{H4}$ signatures (R=-0.69) (c) Depth profiles of $\delta^{13}C_{CH4}$ and $\delta^{2}H_{CH4}$ (d) Regressional relationship between $\delta^{13}C_{CH4}$ and $\delta^{2}H_{CH4}$ (R=0.95).

5.5 Conclusions

The results of this study demonstrate that methanogenesis is actively occurring across various sites in Bangladesh aquifers and that there is a general trend of higher methane concentrations being associated with higher Fe and As concentrations. The stable isotopic carbon signatures of methane were indicative that methane is being

produced through CO_2 reduction by methanogenic archaea and this was further confirmed at one site with CH_4 deuterium signatures. Methane groundwater concentrations are a useful indicator of highly reductive conditions and heterogeneous in distribution depending on site. The potential relationship between higher arsenic and methane concentrations may allow CH_4 to be useful proxy to monitor where reducing conditions have been induced and arsenic release may occur.

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Chapter 6. Examining Archaeaol and GDGT Microbial Lipids in Shallow Bangladesh Aquifer Sediments

Whaley-Martin, K.J., Mailloux, B., van Geen, A., Bostick, B., Ahmed, K.M., Choudhury, I., Slater, G.F.

6.1 Abstract

The lipids that comprise the cellular membranes of microorganisms provide a useful proxy for examining the presence and activity of these communities within *in situ* environmental conditions. Within aquifer sediments, the use of phospholipid fatty acids (PLFAs) have been used to examine bacterial populations, yet the analogous approach of examining the di-ether and tetra-ether lipids (archeaol and glycerol dialky glycerol tetraethers (GDGTs)) has not been extended to the coinciding archaeal communities in these settings. Archaeal lipids may be particularly useful proxy in Bangladesh aquifers, where microorganisms govern the release of high concentrations of arsenic into groundwater and methanogens seem to have a ubiquitous presence. An aquifer sediment core was collected in January 2016 from a well-studied site (Site B) in the Araihazar Upazila and through high performance liquid chromatography coupled to atmospheric pressure chemical ionization (HPLC-APCI), archaeaol and glycerol dialkyl glycerol tetra-ether (GDGT) lipids were successfully identified in these aquifer sediments. Archaeaol derived from intact polar lipids were >LOD in the sediments but only quantifiable in sediments at 3-9 m and 17-19.5 m (2 and 0.9 ng/g respectively). Of the isoprenoid GDGTs (exclusively biosynthesized by archaea), GDGT-0 consistently had the highest relative abundance in the sediments but GDGT 1-5 were also found at varied abundances. Branched GDGTs were also detected in both the core lipid and intact lipid fractions of Site B sediments. The approaches used to extract, separate and identify these lipid biomarkers in aquifer sediments may be useful for future work where isotopic analyses of these lipids can provide insight into microbial carbon cycling by methanogenic populations.

6.2 Introduction

Characterization of archaeal membrane lipids has been used to provide information on the composition of living microbial communities by providing a measure of biomass and biomarkers specific to certain taxa (i.e. Schouten et al., 1998, 2013; Sturt et al., 2004; Lipp and Hinrichs, 2009). Archaeal intact polar lipids (which consist of an isoprenoid chain and glycerol moieities ether-bound to polar headgroups) have been proposed to be analogous to the well-established usage of phospholipid fatty acids

(PLFA) to investigate living bacterial and micro-eukaryl (Pitcher et al. 2009). However, while the rate of degradation of IPL's to the more recalcitrant core lipids (CL's) upon cell death remains some matter of debate (Harvey et al. 1986; Schouten et al. 2010; Logemann et al. 2011; Xie et al. 2013), they remain a useful indicator of the living community. Complimentary analysis of core lipids (where the polar head-group has been cleaved off from the central tetraether core), provides knowledge of past archaeal communities and environmental conditions. This is highlighted in research where CL distributions and ratios are useful as a paleo-proxy (i.e. the TEX86 index; Schouten et al. 2007)).

In environmental archaeal communities, two main categories of archaeal lipids comprise the distribution in varying proportions: glycerol dialkyl glycerol tetraether lipids (also referred to as GDGTs) and diether lipids (also referred to as GDGDs or archaeol). The GDGTs are subdivided into structurally similar but distinctive major classes: isoprenoid GDGTs (isoGDGTs) and branched GDGTs (brGDGTs). IsoGDGTs are comprised of two C_{40} isoprenoid chains, while brGDGTs are made up of two C_{30} alykyl chains with only 4-6 branching methyl groups (Tierney 2012). The isoGDGT's are exclusively biosynthesized by archaea and are therefore definitive biomarkers of this domain. BrGDGTs alternatively are bacterial in origin (Weijers et al. 2009; Sinninghe Damste et al. 2011). Within both classes of GDGTs, cyclohexane and cyclopentane moieties within the chains can be present and vary in number and type. Archaeol is considered a distinctive lipid biomarker for methanogenic archaea (Lim et al. 2012; Pancost et al., 2011) due to its presence in relatively high abundance in these organisms. However, the relative proportion of archaeaol compared to GDGTs in methanogens has

been demonstrated to change rapidly in response to external temperature (Sprott et al. 1991). Nevertheless, the presence of archaeaol serves a useful positive biomarker of methanogens.

The usage of archaeal lipids to examine these communities has been mainly focused on marine settings (Lipp & Hinrichs 2009; Beman et al. 2008; Hopmans et al. 2000), thermosprings (Boyd et al. 2013) and bogs (Pancost, Mcclymont, et al. 2011). Through a literature, this research area has not been carried out in aquifer settings to the best of our knowledge. The focus of this study was to apply established analytical techniques for extraction, separation and detection of archaeal lipids but in the novel environmental matrix of aquifer sediments. This has a potential application to future work attempting to examine archaeal communities in these systems, which as demonstrated in Chapter 5, are active and important contributors to overall carbon cycling.

6.3 Methods

6.3.1 Field Site Sample Collection and Archaeal Lipid Extraction

A sediment core (~5-20 m depth) was collected from "Site B" (Baylakandi Village; 23.7800 N 90.6400 E) in the Araihazar Upazila, Bangladesh in January 2015 using methods previously described in detail (Whaley-Martin et al., 2016). Sediments were sectioned into whirlpacks on site, placed on freezer packs and frozen the same day at a local health clinic. Frozen sediment samples were subsequently shipped on dry ice from Araihazar to Hamilton, Ontario, Canada (McMaster University) and stored in freezers (-4°C) until future analysis. Sediment samples were freeze-dried for 24-48 hours depending on individual moisture content. Five composite samples (ranging from ~140 -

380 g) of different depth ranges were created by combining weighed sediment samples. Sediment samples underwent two sequential extractions (1) the traditional modified Bligh & Dyer (1959) procedure and (2) an acidified Bligh and Dyer (Nishihara & Koga 1987) Sediments placed in large (250 mL) glass centrifuge tubes and extracted overnight at room temperature with 2:1:0.8 v/v/v of methanol (MeOH), dicholoromethane (DCM) and aqueous phosphate buffer. Samples were centrifuged at 3100 rpm for one hour and the total lipid extract (TLE) was pipetted directly into separatory funnel. Residues in centrifuge tubes were set aside for second extraction. The first extracts were decanted into separatory funnels brought to final ratios of 1:1:0.9 v/v/v of MeOH, DCM, and double distilled water (DDH_2O) to achieve phase separation and drained through 0.7 μm poresize filters collected in glass jars. The second extraction repeated all steps of the first overnight extraction but with an original extraction solvent mixture of 2:1:0.8 v/v/v of MeOH, DCM and 5% triochoroacetoc acid (TCA)(Nishihara & Koga 1987). The acidified organic extract was neutralized in the separatory funnel using methanolic ammonia. The organic layer was washed three times with DDH_2O (each time draining the organic layer into a jar, each time discarding the aqueous layer, to "rinse" any residual TCA out of the extract. The final (4^{th}) aqueous phase was shaken with the organic phase and allowed to sit for ten minutes to allow equilibration and phase separation. The pH of the aqueous phase was tested with pH strips and methanolic ammonia was added until neutral pH was obtained. The neutralized organic (lower) phase was passed through the 0.7 μm poresize filters into a jar ensuring no aqueous phase enters vial.

6.3.4 Separation of Core-lipids from Intact Lipids

The shallow sediment TLE's were separated into core lipids (Fraction 1) and intact lipids (Fraction 2) through elution using silica gel chromatography with 3:1 hexane: ethyl acetate and MeOH respectively based on methods developed by Pitcher *et al*. (Pitcher et al. 2009) and adapted by Wu *et al*. (2013). The core-lipid fraction was evaporated to dryness under N_2 and re-dissolved in 99:1 hexane to iso-propanol. The intact lipid fraction was evaporated to a small volume, transferred to 10 ml round bottom glass flask and evaporated to dryness. These fractions were taken through an acid reflux for three hours at 70°C with 5% HCL in MeOH for 3 hours to achieve acid hydrolysis of head-groups creating core-lipids amendable to analysis by high performance liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI (Pitcher et al. 2009). Refluxed samples were neutralized with 1 N KOH_{aq} and adjusted to achieve a final ratio of 1:1 MeOH:H_2O. DCM was added to achieve phase separation and the DCM layer was removed, and the remaining phase rinsed an additional three times with DCM and combined. Fractions 1 and 2 were blown down to dryness and re-dissolved 100 µl in 99:1 hexane: isopropanol.

6.3.5 Analysis and Quantification of Archaeal Lipids with HPLC-APCI-MS

Analysis of core archeaol and GDGT's was carried out using HPLC-APCI-MS. (Hopmans et al. 2000; Schouten et al. 2007). Samples were run on an Agilent 1200 series 6120 quadrapole LC-MS and separated with a Prevail Cyano column (2.1 mm x 150 mm x 3µm; Alltech, Deer-field, IL, USA) at a constant temperature of 30°C. Archaeaol and GDGT's and were eluted at 0.2ml/min isocratically with 99% hexane and

1% iso-propanol followed by an a linear gradient to 1.8% isopropanol over 45 minutes. Every run included a 15 minute backflush at 0.4ml/min and a column re-equilibration for 10 minutes with initial run conditions. Detection with APCI-MS of the eluent was achieved with the conditions as follows: nebulizer pressure 60 psi, vaporizer temperature 400°C, flow and temperature of drying gas (N_2) was 6 L/min at 200°C, capillary voltage 3kV and corona 3.2 kV.

A GDGT core lipid standard containing GDGT's 0 (1302 g/mol), GDGT-1 (1300 g/mol), GDGT-2 (1298 g/mol), GDGT-3 (1296 g/mol), GDGT-4 (1294 g/mol) and GDGT-5 (g/mol) was created by acid hydrolysis of headgroups of the the main phospholipid (MPL) mixture of *Thermoplasma acidophilum* (>95%, Matreya LLC) (Figure S1) (Pitcher et al. 2009). This GDGT standard allowed positive identification of sedimentary GDGT's based on retention time and mass spectra and semi-quantitative characterizations based on relative peak area abundance. A calibration curve was constructed for archaeaol from 0.2 to 50 ppm (1,2-di-O-phytanyl-*sn*-glycerol, Avanti Polar lipids, MW=653.16 g/mol). An archaeaol standard of 0.1 ppm was also prepared and could be detected but linearly was lost in this interval and therefore 0.2 was considered the limit of quantification while 0.1 ppm was considered the limit of analytical detection. The ultimate limit of detection for archaeaol in the sediments was dependent on the mass of sediment extracted and averaging 6 ng/g (6 ppb) of dry sediment. A synthesized internal standard (Patwardhan & Thompson, 1999) C_{46} GDGT (743 m/z) was added to each sample correct for ionization variance of core archaeal lipids within each sample due to matrix effects or instrument fluctuations (Huguet et al. 2006). Ion extractions of individual iso-prenoid core lipids (1302, 1300, 1298, 1296, 1294, 1292

m/z) and branched core-lipids (1022, 1020, 1018, 1036, 1034, 1032, 1050, 1048 and 1046 m/z) were carried out to obtain relative abundances of each lipid in sample fractions.

6.4 Results/Discussion

6.4.1 Di-ether (archaeol) lipid distributions in shallow aquifer sediments

Archaeaol that was derived from intact polar lipids (F2) was present at abundances greater than limits of detection in all extracted sediment samples (retention time = ~4.5 min) but was only quantifiable for two samples (Figure 6.1. Archaeol in sediments sample 3-9 m and 17-19.5 m were 2 and 0.9 ng/g respectively. Archaeaol in the core-lipid fraction was <LOD in all of the extracted sediment samples.

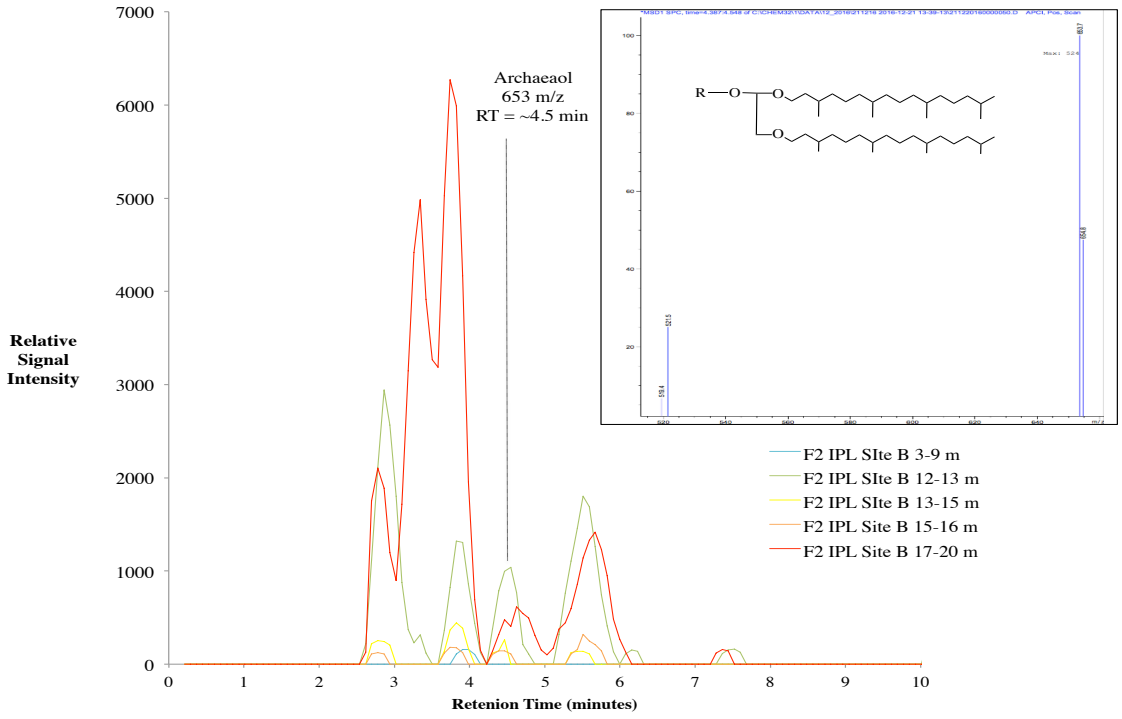

Figure 6.1 HPLC-APCI chromatographic traces of Site B sediment extracts from retention time 0-10 minutes indicating the archaeaol peak (RT-~4.5 min). Inset shows mass spectrum of the archaeaol peak at 4.5 min of sample Site B 12-13 m with a prominent fragment ion at 653 m/z.

6.4.2. Tetra-ether (Iso-GDGT's and Br-GDGT's) lipid distributions in shallow aquifer sediments

In each aquifer sediment at Site B both isoprenoid GDGT and branched GDGT's were present in CL and IPL fractions. Isoprenoid GDGT-0 (1302 m/z) consistently had the greatest relative abundance (determined from peak area) in both the CL and IPL fractions. GDGT's 1-5 were present at lower abundance and at varying concentrations depending on the sample. Branched GDGT's Ia (1022 m/z), Ib (1020 m/z), Ic (1018 m/z), IIa (1036 m/z) and IIIa (1050 m/z) were also detected in the core-lipid and IPL fractions depending on the sample (Figure 6.1).

A PCA analysis was carried out on the relative abundances (peak areas/area C_{46} standard) of the GDGT distributions in the CL and IPL fractions of the five sediment samples (N=10). PCA analyses (where each core lipid was treated as individual variable) resulted in a three-component analysis accounting for ~99% of the variation within the samples PC_1 (67%), PC_2 (28%) and PC_3 (4.4%) (Figure 6.2). Eight of the ten groups clustered into main groups when principle component 1 and principle component 2 were plotted (Figure 6.2): Sediments with relatively low abundance of archaeaol, isoGDGTs and BrGDGTs (red circle) and Site B CL's 12.5 m and Site B CL's15-16.5 m (blue circle). The latter two samples had highly similar lipid distributions including relative abundances of the individual lipids (Figure 6.3a). A significant positive correlation (Pearson R=0.79, p=0.0003) was found between the relative abundance of the CL's and IPL's Site B 12.5 m and 15-16.5 m. This is suggestive that these two pools of lipids are connected and that once intact polar lipids degrade (loose their polar headgroups), they form a dominant component of the core (fossil) lipids.

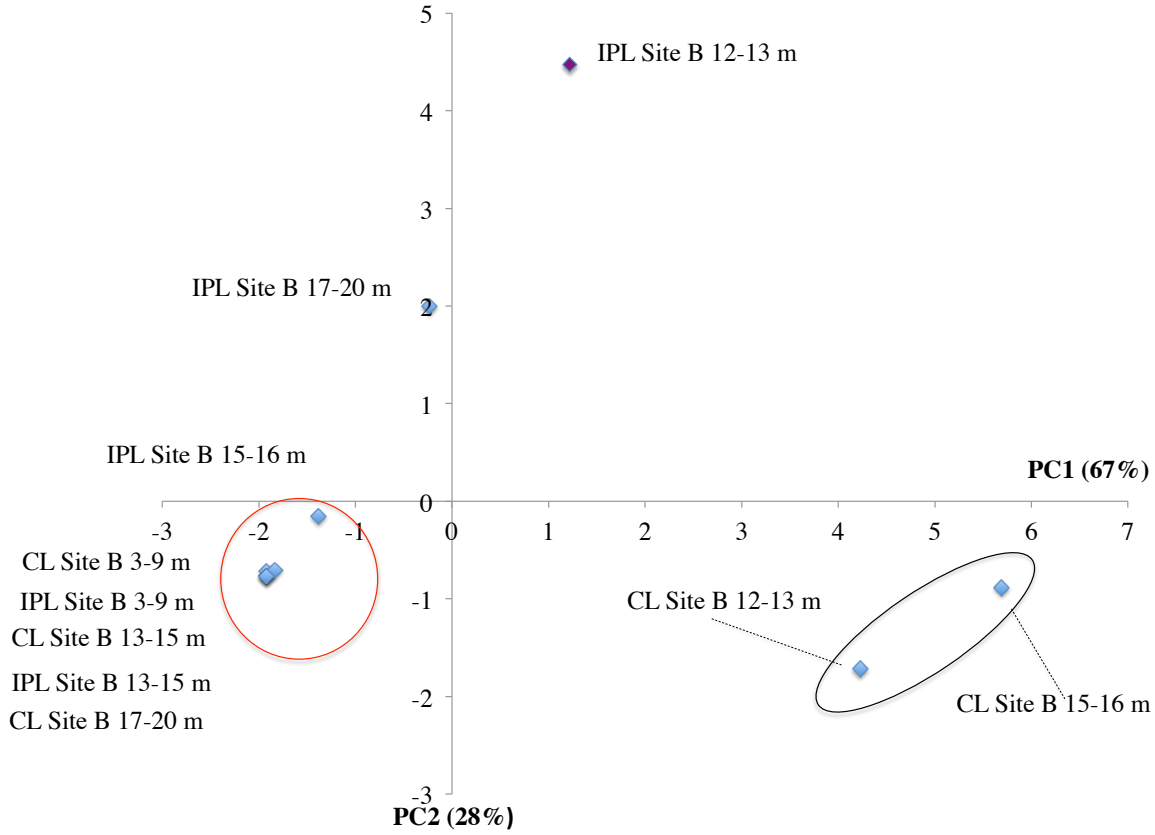

Figure 6.2 Principle component analysis (PCA) showing PC1 (67%) and PC2 (33.8%) of Site B sediments of archaeaol, isoprenoid and branched GDGT distribution from both core and intact lipid fractions.

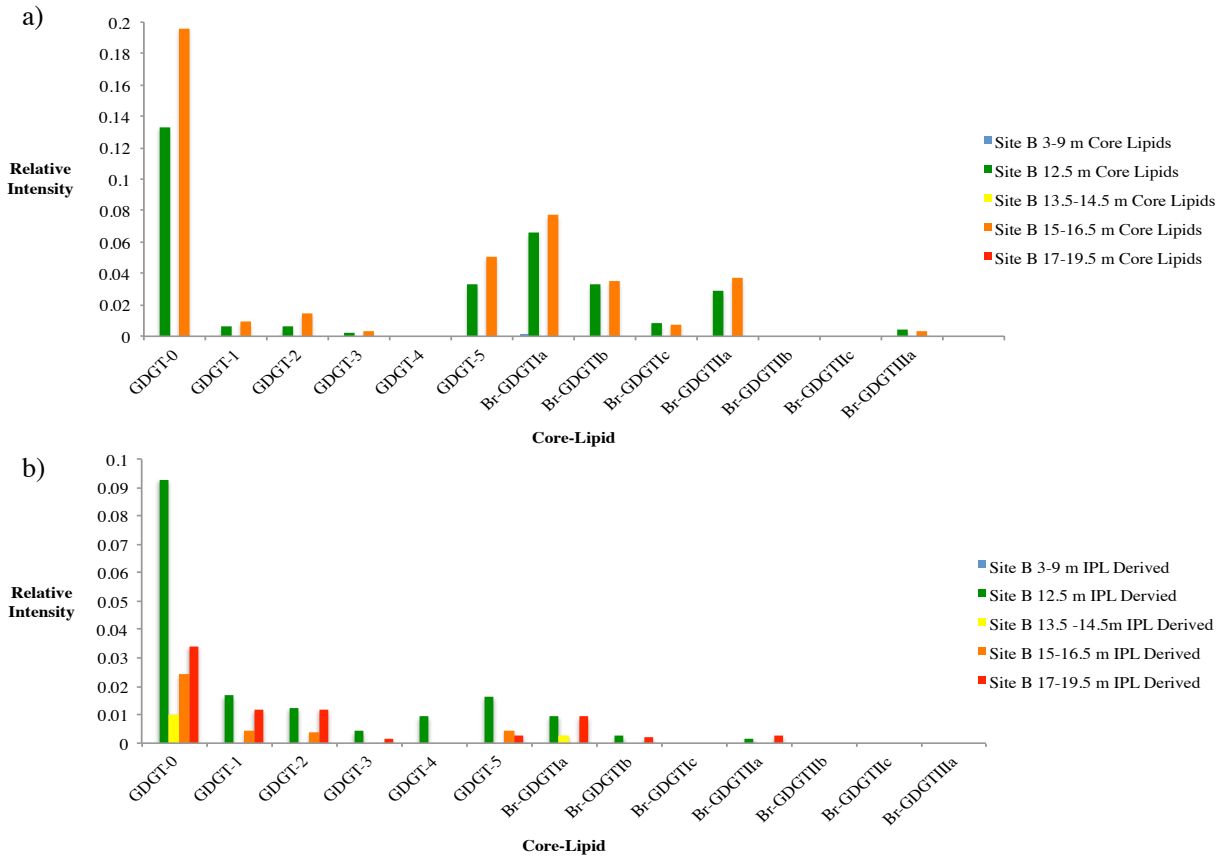

Figure 6.3 IsoGDGT and BrGDGT distributions in shallow (<20m) Site B sediments present as (a) core-lipids (F1) and (b) derived from intact polar lipids upon acid hydrolysis of polar headgroups

6.5 Discussion

6.5.1. Archaeol derived from IPL's provide useful biomarker of methanogens in Bangladesh sediments

The sediments analyzed within this study are from a relatively shallow (<20m) and low biogenic methane region at Site B (refer to Chapter 5) where methane concentrations were as only as high as 34 µmoles/L but reached 331 µmoles/L at depths of 52 m. Since methane is relatively low at these depths, this suggests that the detection of IPL-derived archaeaol is a relatively sensitive indicator of the of the presence of live

methanogenic communities. Deeper sediment cores to depths where methane concentrations were much higher at Site B indicating more abundance in methanogenic communities would hypothetically provide higher concentrations of archaeaol. This may have a potential application for future work where isotopic characterization (i.e. $\delta^{13}C$ or $\Delta^{14}C$) of archaeaol could reveal more information on carbon cycling in these systems.

6.5.2. Isoprenoid and Branched GDGT's Distribution suggest a ubiquitous distribution of archaeal and acidiobacteria in shallow Aquifer Sediments

The presence of isoGDGTs in the CL and IPL fractions of Site B sediments are indicative of past and present archaeal communities inhabiting these aquifers respectively. Contrary to archaeol, the isoGDGTs present (GDGT 0-5) are not known to be specific to any particular species of archaea but are rather biosynthesized across methanogenic, thermophillic and mesophillic phylums. However, the increased abundance of GDGT-0 (contains no cyclic moieties) over that highly cyclic counterparts is consistent with current (~25-28°C) conditions of the aquifers. Highly cyclic iso-GDGT's are have been found to be associated with thermophillic archaea who produce these lipids to maintain structure in higher temperatures (Boyd et al. 2013). Branched GDGT"s are biomarkers of bacteria rather than archaea (Weijers et al., 2006) commonly found in soils, peats, deltaic and lacustrine environments but are absent from marine settings. Non-uniform peat layers are present throughout the stratigraphy at Site B (peat samples were collected during the coring of Site Band the deltaic deposition of these sediment support the presence of bacteria from these settings that produce Br-GDGT's. Of all the branched lipids only one group has been suggested to be specific to a particular

phylum of bacteria. Sinninghe Damsté et al. (2011) found that Br-GDGT's from group I(a,b c) (which were all found to be the dominant Br-GDGT's in Site B's sediments) are produced specifically by acidobacteria. In a recent metagenomic survey of sediments at a nearby site with similar conditions, Legg et al. (2012) found the sub-phylum acidobacteria to comprise ~11% of the overall bacterial community composition.

6.6 Conclusion

This study demonstrated that previously developed analytical approaches used to extract, separate and analyze archaeal and GDGTs can be successfully applied to aquifer sediments where low biomass may be expected. This provides a complimentary tool for researchers to examine the activity of archaea in aquifers as these lipid biomarkers can be used to gain insight into community composition. Perhaps more interesting is their potential use in isotopic analyses to provide insight into microbial carbon cycling by methanogenic and other archaeal populations.

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Chapter 7. Conclusions and Implications for Future Research

The human-health crisis of arsenic exposure across South and South-east Asia that currently affects >100 million people warrants significant efforts and advancements in scientific research that can elucidate the biogeochemical mechanisms governing arsenic in the groundwater. While the coupling of organic matter to the iron-reductive dissolution has been demonstrated to be the predominant microbial process governing arsenic release, limited research has been aimed specifically on understanding microbial carbon cycles in these systems. Thus, this research dissertation aimed to advance our understanding as to how organic matter is being microbially cycled through Bangladesh aquifers and demonstrate the analytical approaches that can be used to address these questions. This research highlighted how important it is to consider these carbon cycles, as they are intimately tied to the spatial and temporal patterns of arsenic release. This is immediately relevant to addressing concerns of the sustainability of recently installed deeper tube wells (>100 m) in Bangladesh, where drastic increases in water withdrawal rates for irrigation and municipal purposes (Khan et al. 2016) may cause a migration of carbon sources downwards, stimulating microbes and arsenic release.

Within this dissertation, various and complimentary analytical methods were successfully applied to understand how *in situ* bacterial and archaeal communities utilize organic matter to drive their metabolisms in Bangladesh aquifers (Figure 7.1). Of the five studies presented here (Chapters 2-6), four (Chapters 2, 3, 4 and 6) were solely focused on two study sites within in Araihazar Upazila in Bangladesh. These particular locations were targeted because they are fairly representative of prevalent regional environmental

conditions. Thus, while these findings may be considered "site-specific", these microbial carbon sources and cycling are potentially relevant on widespread scales.

Prior to this work, the carbon sources that drive the sedimentary bacterial populations dominating the microbial communities in shallow Bangladesh aquifers (and control arsenic release) had not been determined. Within the first study (Whaley-Martin et al., 2016) presented within this dissertation, radiocarbon analyses of the lipids of sedimentary bacteria inhabiting shallow Holocene-aged and high arsenic aquifers revealed they are predominantly utilizing younger organic matter rather than older sedimentary carbon (Chapter 2). This was the first application of radiocarbon analyses to PLFA in Bangladesh aquifers and the first study to specifically target the sediment-associated bacteria. These findings have led to a broader understanding of how surface-derived carbon sources are not only travelling to depth but also being preferentially metabolized by the sedimentary bacteria. Given this, the previous suggestion that any fluxes of younger carbon pools to greater depths caused by massive pumping would be likely to stimulate the sedimentary communities have been validated. The sustainability of deeper wells in the region in regards to future arsenic contamination and thus highly connected to deep groundwater pumping management.

Following the findings of Whaley-Martin et al. 2016, a second study (Chapter 3) was undertaken to determine the origin of the younger surface-derived carbon driving the indigenous microbial communities. Specifically, the relative roles that sewage (human/livestock waste suggested in only one previous study) versus plant matter derived carbon have in driving microbial iron reduction in Bangladesh aquifers were unknown. This work applied complimentary sedimentary sterols analyses (phytosterols and the

sewage biomarker coprostanol) along with groundwater Cl/Br mass ratios and As and Fe concentrations. At our sites, the sources of younger organic matter that coincided with zones where increased reductive dissolution of iron and arsenic release were occurring, were consistent (coprostanol and Cl/Br mass ratios) with human and livestock waste. While this study only focused on two sites, poor sanitation is widespread across Bangladesh; sewage-derived waste should be considered a prevalent potential microbial carbon source in these systems. The surprising finding that human/livestock waste is reaching depths of ~25m in the aquifers and that this seems to be related to where arsenic release is most prevalent, highlights an immediate need to understand how this is occurring. Microcosm experiments using site-derived latrine/livestock waste as a carbon source and aquifer sediments with the indigenous microbial communities could provide more conclusive insight. This type of experiment could confirm whether this particular organic matter can not only enhance arsenic release in already reduced sediments, but also whether this carbon source can potentially stimulate arsenic release from currently low-arsenic, oxidized Pleistocene-aged sediments. Through continued collaboration between McMaster (Dr. Greg Slater), Columbia University, Barnard College and University of Dhaka researchers, additional field sites were targeted for sediment cores in January 2017 within the Araihazar Upazilla region to help address this question. The analytical approaches used throughout this dissertation ($\Delta^{14}C$ of sedimentary PLFA, phytosterol/coprostanol and the inorganic suite of analytes) will be applied. If evidence is found that poorly installed tubewells are acting as a conduit for dissolved human/livestock waste to reach depths and enhance arsenic release, potential

management strategies may be employed to limit this mechanism (i.e. training local villagers about proper installation protocols of tubewells).

The study presented in Chapter 4 aimed to compare approaches where sediment-associated microbial communities versus groundwater associated microbial communities are targeted in Bangladesh aquifers to examine microbial-driven arsenic release. To the best of our knowledge, no research had addressed the fact that these two communities are very likely to be distinct and participating in the biogeochemical cycling of arsenic in different capacities. With this gap in mind, a study, which explicitly examined approaches targeting these different communities, was warranted. This study found significant differences in their estimated relative cell abundances (sediment-associated communities approximately 5-6 orders of magnitude more abundant than groundwater microbes) and potential differences in PLFA distributions and $\delta^{13}C$ signatures within the PLFA. This is pertinent to any future research st with the overall goal of exploring microbial communities in these systems, as it highlights the importance of being conscious of which community is the appropriate choice to focus on depending on the research question. This work is suggestive that if a study is aiming to characterize the community that is governing the prevailing geochemical conditions in Bangladesh aquifers, that the community with the significantly higher abundance (in this case the sediment –associated microbes) is likely a better candidate.

Within Chapters 5 and 6, the archaeal communities that co-inhabit Bangladesh aquifers with bacteria were focused on through two different approaches. The overall motivation behind each of these studies was that the continued oxidation of organic matter, ultimately to the final reduced phase of CH_4 by archaea (methanogens), is also

associated with zones of high groundwater arsenic. Despite this, limited work has been carried out prior to this research to investigate the presence and activity of archaea throughout Bangladesh aquifers. Through methane concentration and stable isotopic analyses across six sites and depths reaching 240 m, methanogenesis was demonstrated to be highly active in these systems (Chapter 5) but differing in scale depending on site. As the final reductive stage in the degradation of organic matter, it also appears to be associated in shallow and deep iron/arsenic reductive dissolution zones. A continued sampling program currently underway (through collaboration between researchers from McMaster, Columbia University, MIT and the University of Dhaka) at additional Bangladesh and Vietnam sites aims to better define the relationships between iron/arsenic cycling and methanogens. This will include the analytical approaches (SRI-GC for methane concentration and GC-IRMS for $\delta^{13}C_{CH4}$) used in Chapter 5, along with metagenomic and inorganic groundwater analysis, to elucidate the metabolic capabilities/activity of the microbial communities. Within Chapter 6, the archaeal lipid distributions (archaeol and GDGTs) were determined within shallow Bangladesh aquifer sediments through organic extractions, silica gel chromatography and HPLC-APCI. This is the first study to expand the use of these previously developed analytical techniques to aquifer sediments. Future work aimed at examining carbon cycling by archaea (i.e. methanogens) can use the methods described in Chapter 6 and potentially through fraction collection of these lipids, have a useful microbial biomass component for isotopic ($\Delta^{14}C$ and/or $\delta^{13}C$) characterization. Currently, it appears that the monitoring of groundwater methane and the presence of sedimentary archaeol derived from intact polar lipids, are useful indicators of reducing conditions. This may be particularly applicable

within monitoring efforts of the deep aquifers where oxidative (low arsenic) conditions are dominant, but inducing reductive conditions, through perhaps a flux of labile carbon, could cause arsenic release.

The analytical approaches and conceptual frameworks applied throughout this dissertation have been demonstrated to be effective strategies to understand how microbial carbon cycling is occurring and intimately involved in arsenic release (Figure 7.1). Certainly, new and interesting questions have emerged from the five studies presented here and the use of this framework including the analytical techniques will aid in answering outstanding questions.

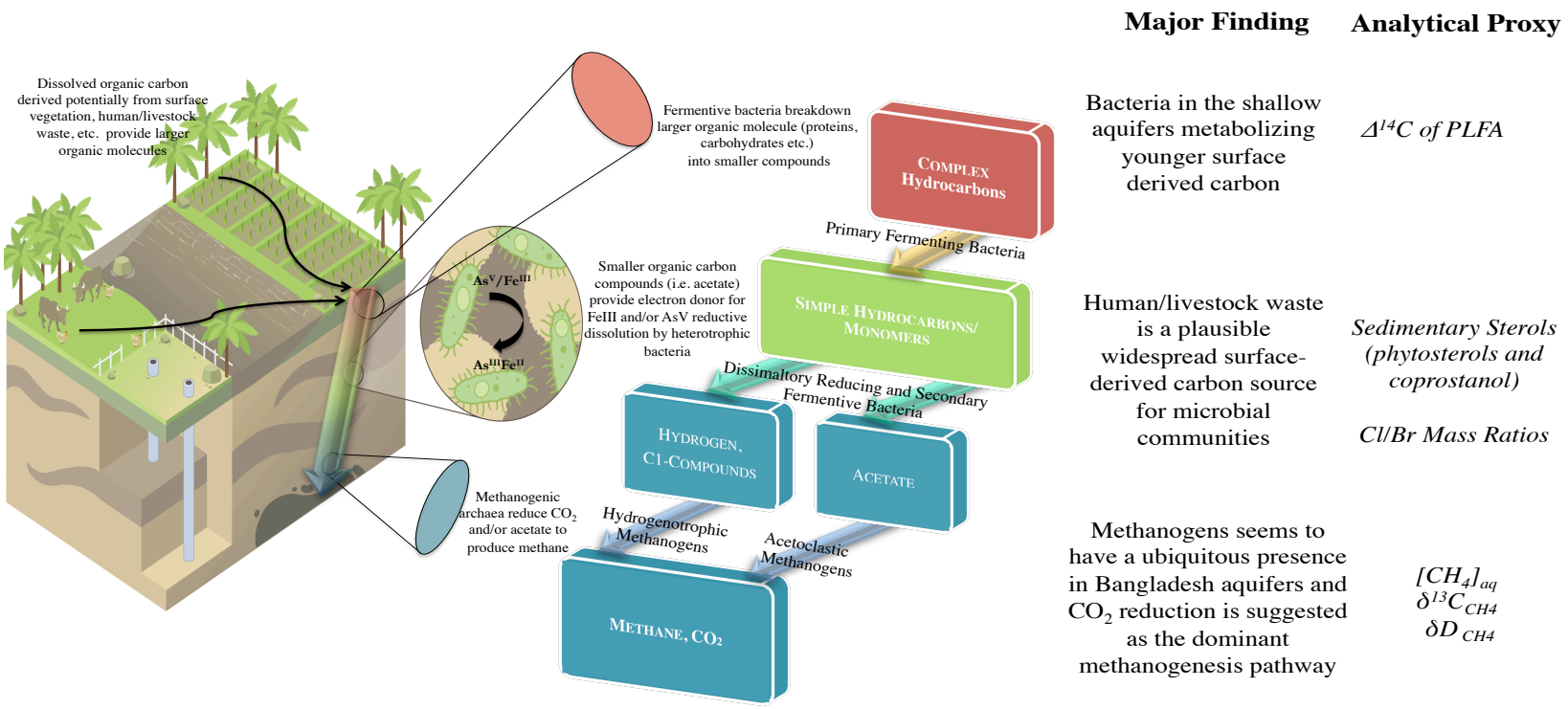

Figure 7.1 Schematic illustration of microbial carbon cycling in Bangladesh aquifers which includes the major findings of this dissertation and the analytical proxies used to discern them.