CHARACTERIZATION OF THREE DISTINCT ARSENIC RESISTANT MICROORGANISMS ISOLATED FROM THE AGRICULTURAL SOILS OF MEKONG DELTA IN VIETNAM

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ABSTRACT OF THE THESIS

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Arsenic is a naturally occurring metalloid in the earth's crust. Microbial processes play an evident and vital role in biogeochemical cycling of elements. Arsenic and its forms vary in toxicity and mobility, and its contamination can be a major problem for the living beings and the environment. The Mekong River delta region in Vietnam is a populated area and the fertile fields are used for cultivating rice, wheat and maize. Arsenic accumulation in the agricultural soils and groundwater leads to exposure by direct contact or by being absorbed and accumulated in the food crops. Microorganisms have developed the ability to either utilize arsenic species for metabolism or for bioconversion to reduce its lethality, which affects arsenic mobility, bioavailability in the environment. This study aims to isolate and identify microorganisms in these contaminated soil samples by enrichment culture technique, T-RFLP fingerprinting, genetic analysis through PCR amplification, and HPLC method to monitor arsenate reduction to arsenite. Three distinct arsenate reducing strains were isolated from the rice and maize soil samples and they belong to the genera *Shewanella, Klebsiella* and *Enterobacter*, respectively. Members of these genera are known to transform arsenic species. Arsenic methylation is being explored by researchers

as the methylated forms can be absorbed by food crops, especially by the rice plants. Rice is a staple food crop and high arsenic concentrations in the grain will lead to exposure and bioaccumulation when ingested. The three arsentate-reducing bacterial strains isolated in this study may also possibly methylate arsenite, as methylated forms were detected through HPLC and the presence of the requisite genese by PCR experiments. This work contributes to the current knowledge of arsenic biotransformation by microorganisms, specifically in the Mekong River delta, and this information can then be useful in mitigating arsenic contamination.

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INTRODUCTION

ARSENIC

Arsenic (As) is a metalloid with atomic number 33 and appears in Group 15 of the periodic table below phosphorus. Albertus Magnus is credited with isolating arsenic in the elemental form from arsenic trisulfide in 1250 (Emsley 2001). Arsenic resembles phosphorus in its chemical properties. The primary oxidation states of inorganic arsenic are: arsenide (As^{3-}), arsenite (As^{3+}), arsenate (As^{5+}) forming trioxides (As_2O_3) and pentaoxides (As_2O_5).

The organic forms of arsenic are known as organoarsenicals synthesized by alkylating arsine, a flammable highly toxic gas (AsH_3) or arsenic halides to create $AsH_{3-x}R_x$ (trivalent arsenic) and $RAsO(OH)_2$ or $R_2AsO(OH)$ (pentavalent arsenic), where R is an aryl or alkyl. Some of the commonly found organoarsenicals are monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethylarsine (TMA), trimethylarsine oxide (TMAO), arsenobetaine (AB), arsenocholine (AC) and arsenosugars (Grund et al. 2000). The pentavalent forms can be reduced to the more toxic trivalent states catalyzed by glutathione (GSH). Specific methyl transferases along with the methyl donating cofactor S-adenosylmethionine (SAM) catalyze the methylation of arsenite to pentavalent MMA which is reduced by GSH to trivalent MMA. (Cullen and Reimer 1989). Further methylation and reduction reactions synthesizes DMA and TMA. Arsenosugars are arsenic containing carbohydrates composed of a 5-deoxypentose moiety and an arsinoyl or arsinothioyl group, attached to the C5 atom with a variable side chain at the C1 position.

The 5-deoxy-5-arsinoyl-β-d-riboside derivatives contain a di or tri methylated arsenic group which can be reduced to arsenocholine (AC) and further oxidized to arsenobetaine (AB) (Figure 1) (Niegel and Matysik 2010).

Known as the ‘Poison of Kings’, arsenic was commonly used as a discreet and potent homicidal agent used for murders of powerful people. The metalloid is used in traditional Chinese and Indian medicines as well as for treating leukemia (Espinoza et al. 1995, Saper et al. 2004, Tallman 2007). Paris green and Scheele’s green, two arsenic based chemical compounds were widely used as pesticides, insecticides and green pigments in paints, food, printing, textiles and fireworks. As the world learned about arsenic toxicity and poisoning, the use of these compounds diminished, and DDT emerged as the more popular choice of insecticide (Murphy and Aucott 1998). Arsenic compounds have found uses in wood treatment for preservation, as a feed additive for weight gain and disease prevention, lead components in car batteries, semiconductors, and optical glass (Grund et al. 2000, Rahman et al. 2004). Agent blue, one of the so-called ‘rainbow herbicides’, consisting of two organic arsenical compounds was used by the United States during the Vietnam War to destroy and drying out the paddy fields making them unsuitable for cultivation. 500,000 acres of crops were destroyed by 19.6 million gallons of Agent Blue heavily contaminating the agricultural soil and water sources (Young 2009).

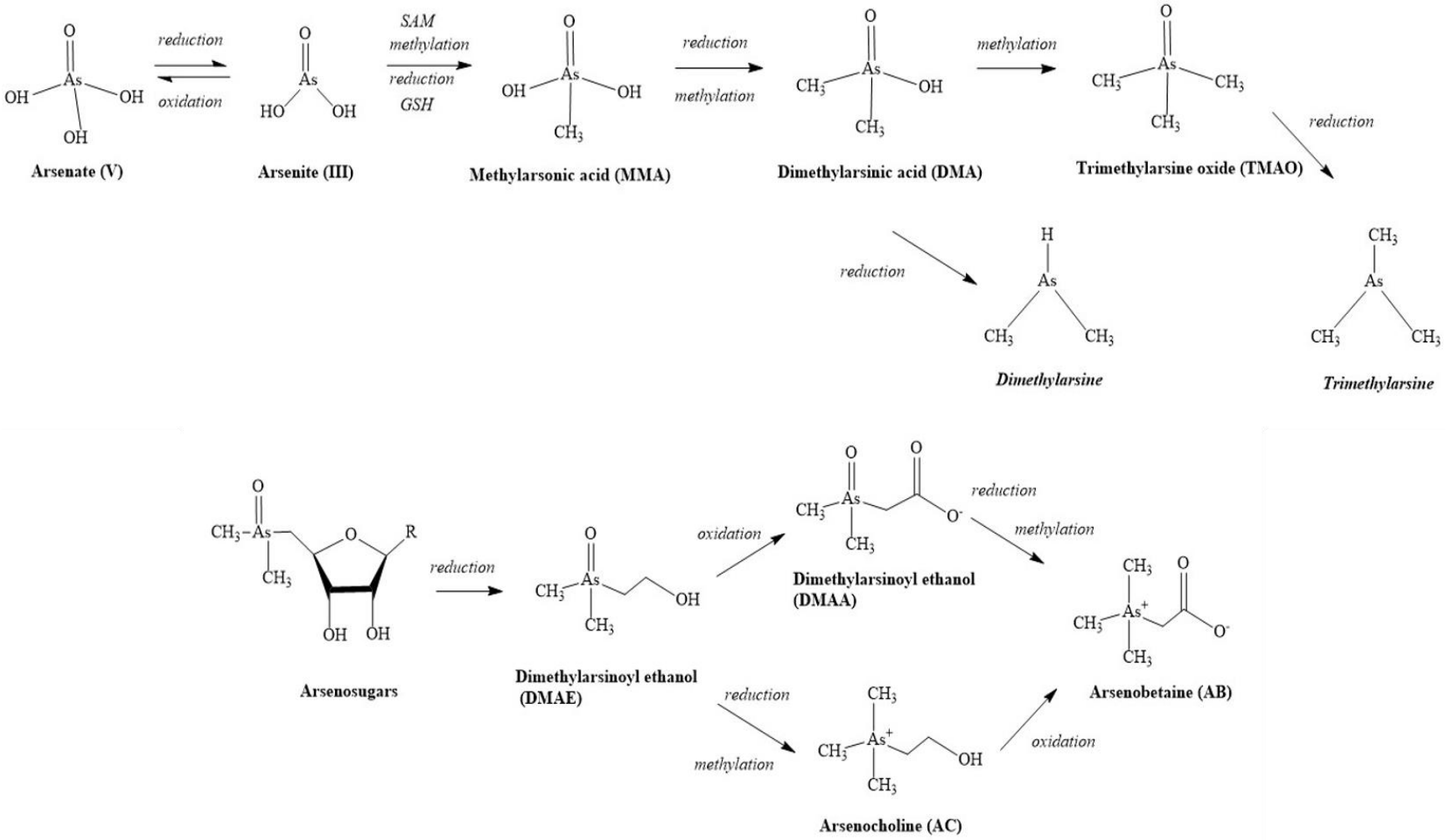
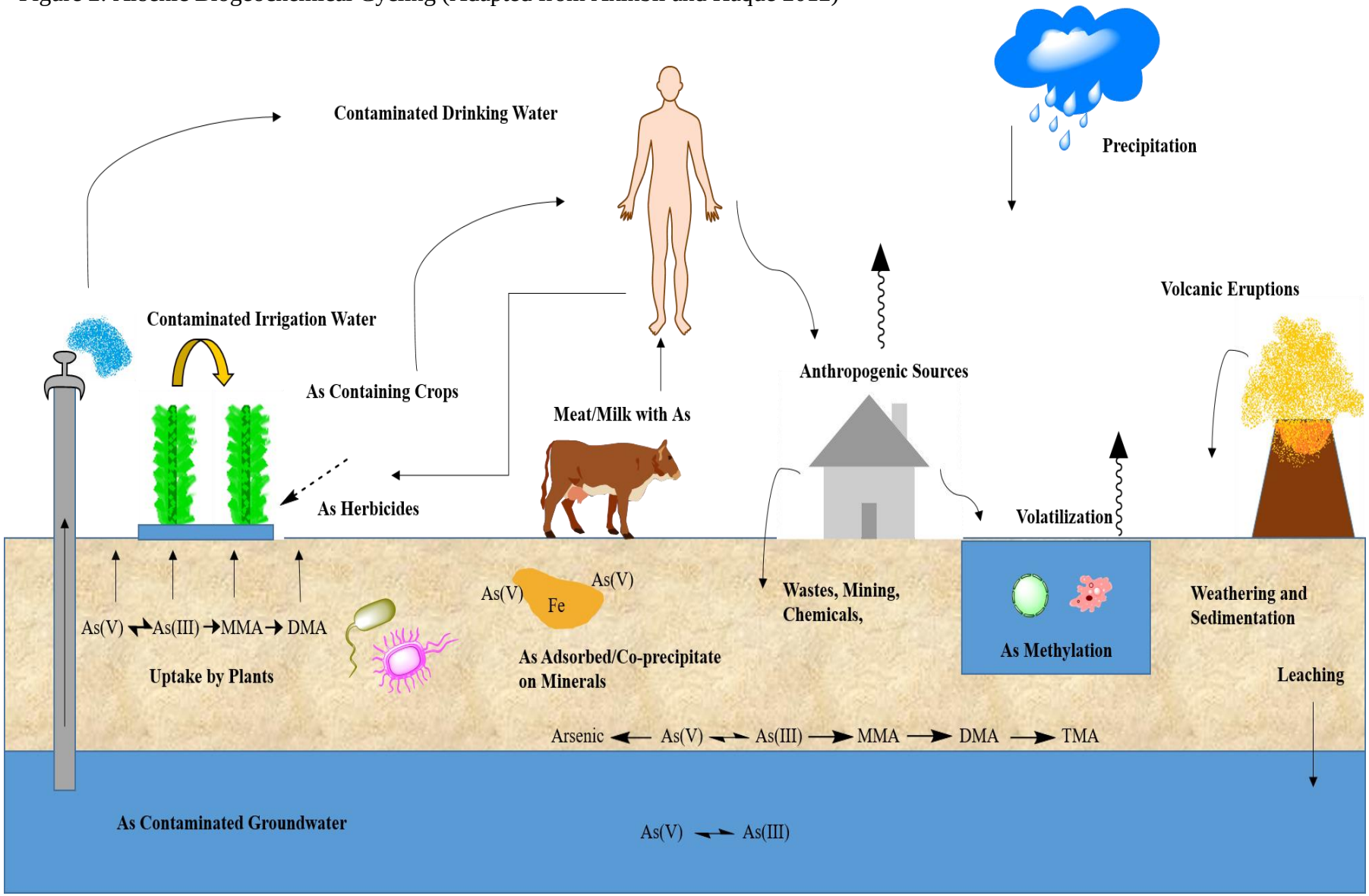


Figure 1: Arsenic Speciation (Adapted from Mukhopadhyay et al. 2002, Nakamura 2011)

ARSENIC IN THE ENVIRONMENT

The total amount of arsenic in the Earth's crust is estimated to be about $4.01x10^{16}$ kg which accounts for 1.5 ppm of the earth's crust, making it the 53rd most abundant element (Emsley 2001). As is liberated from the lithosphere into the exogenic cycle primarily by volcanic eruptions. Arsenic is found in weathered volcanic and marine sedimentary rocks, fossil fuels and various minerals. Anthropogenic sources include mainly copper smelting and coal combustion, in addition to herbicides and pesticides, wood preservation, lead and zinc smelting, glass and steel production, mine tailings, sewage sludge and wastes, landfills, pharmaceuticals and warfare agents. Organoarsenicals and arsenosugars are commonly found in the marine environments often concentrated in sea weeds, algae, marine animals and phytoplankton (Frankenberger 2001). Microbial processes convert arsenate to arsenite which can then be methylated into various organic forms (Hall et al. 1997, Pongratz 1998) which account for up to ~10% of the total arsenic present in the euphotic zone that is the surface of water which receives sunlight (Chester 1993). Arsenic is seen as a nutrient-type element in sea water due to its biogeochemical cycling by phytoplankton and bacteria (Stolz and Oremland 1999, Matschullat 2000, Mukhopadhyay et al. 2002). The cycling of arsenic in the environment is summarized in Figure 2.

Figure 2: Arsenic Biogeochemical Cycling (Adapted from Akinbil and Haque 2012)



ARSENIC METABOLISM

Arsenic absorption in humans and some animals takes place in the small intestine after ingestion. In the liver, it is biomethylated into MMA and DMA which then accumulate in the tissues and cytosol (Aposhian 1997, Vahidnia et al. 2007). Arsenate resembles phosphate in structure and chemical properties and can replace it in important biochemical reactions (Dixon 1996). During glycolysis, it can form glucose 6-arsenate and 6-arsenogluconate instead of glucose 6-phosphate and 6-phosphogluconate (Laguna 1980). The formation of 1,3-bisphosphoglycerate can be replaced by anhydride 1-arsenato-3-phosphoglycerate which is unstable and hydrolyzes to arsenate and 3-phosphoglycerate and thus ATP cannot be produced. In the mitochondria during oxidative phosphorylation, adenosine diphosphate-arsenate is formed from ADP instead of ATP. This interference, called arsenolysis, can disrupt ATP formation both at substrate level during glycolysis and mitochondrial level during oxidative phosphorylation (Gresser 1981, Aposhian 1989). Arsenate can also replace phosphate in the sodium pump and anion exchange transport system (Kenney and Kaplan 1988). Arsenite and other trivalent arsenicals readily react with thiol containing receptors and enzymes like GSH and cysteine (Delnomdedieu et al. 1993). Arsenite binds to the dithiol cofactor, lipoic acid which is required for pyruvate dehydrogenase (PDH) activity. Pyruvate oxidation to acetyl-CoA during the citric acid cycle is catalyzed by PDH. Inhibition of PDH by arsenite leads to decreased ATP production and depletion of intermediates formed during the citric acid cycle which can be utilized in gluconeogenesis (Szinicz and Forth 1988). Trivalent methylated arsenics are potent inhibitors of GSH (Styblo et al. 1997) and thioredoxin reductases (Lin et al. 1999) which changes cellular redox status and cause cytotoxicity (Hughes 2002).

Table 1: Biochemical effects of arsenic (Derived from Hughes et al. 2011)

Arsenic Mode of Action
Phosphorus interaction to form unstable arsenoesters
Sulfur interaction to form As-S complexes
Genotoxicity, chromosomal aberration, genomic instability
Reactive oxygen species, H_2O_2, O_2^-, ROO^-, OH^-, NO
Altered DNA repair due to ligase inhibition
Signal transduction, activation of protein kinase pathway
Cell proliferation, hyperplasia of bladder epithelium
Altered DNA methylation, genomic imprinting

ARSENIC TOXICITY

Trivalent arsenicals are more toxic and mobile in water compared to the pentavalent forms and organic arsenic compounds exhibit much less toxicity compared to the inorganic forms (Table 2) (Fowler 1977). However, studies conducted on human cell cultures show trivalent MMA to be the most cytotoxic (Lomax et al. 2011). Arsenosugars are usually non-toxic, although not much information on their physiological and biochemical significance has been found, it is believed that organisms create arsenosugars to detoxify and eliminate the inorganic arsenics ingested by food or water (Andrewes 2004, Niegel and Matysik 2010). Exposure to arsenosugars is quite high in Asia due to the use of seaweed in food which can contain up to 100 mg/kg arsenic resulting in dietary intakes of 1 mg/day. Seafood also contains about 10-100 mg/kg arsenic (Andrewes et al. 2004).

Acute arsenic poisoning can lead to symptoms such as vomiting, diarrhea, dehydration, hypotension as well as delirium, pulmonary edema, heart failure, encephalopathy and peripheral neuropathy (De Wolff and Edelbroek 1994). Chronic arsenic toxicity in the environment from natural or industrial processes causes health issues in many countries, including India, Bangladesh and Taiwan, where groundwater and drinking water sources are highly contaminated with arsenic (Mukherjee et al. 2006). Skin pigmentation, hyperkeratosis, Bowen's disease, black foot disease, gastrointestinal problems, diabetes, anemia, liver diseases, Mees' lines in nails, lung, bladder and skin cancer are some of the serious problems which develop after 10-15 years of arsenic exposure.

Table 2: Acute toxicity doses for laboratory animals. (Based on Hughes 2002)

Arsenic Form	**Animal Model**	**LD_{50} (mg/kg)**	**Reference**
Arsenic trioxide	Mouse	26	Kaise et al. 1985
	Rat	15	Harrison et al. 1958
Arsenite	Mouse	8	Bencko et al. 1978
	Hamster	8	Petrick et al. 2001
Arsenate	Mouse	22	Bencko et al. 1978
MMA	Mouse	916	Kaise et al. 1989
DMA	Mouse	648	Kaise et al. 1989
TMAO	Mouse	5500	Kaise et al. 1989
Arsenobetaine	Mouse	>4260	Kaise et al. 1985

Urine and blood tests are done to diagnose arsenic levels. Hair samples are also examined by micro-analytical techniques, such as Synchrotron radiation based X-ray fluorescence (SXRF) spectroscopy and Micro-particle induced X-ray emission (PIXE) for monitoring arsenic levels especially in cases where arsenic is used for the treatment of acute promyelocytic leukemia (Nicolis et al. 2009). Arsenic disrupts vital body functions, thus it is of primary concern to restore processes and cure dehydration by detoxification and that can be achieved using the chelators like 2,3-dimercapto-1-propanol (British anti-lewisite, BAL) (Vantroyen et al. 2004), meso-2,3- dimercaptosuccinic acid (DMSA), D-penicillamine and sodium 2,3-dimercapto-1-propamesulfonate (DMPS) (Stenehjem et al. 2007). These chelators however do not provide benefits in cases of neurological diseases (Hall 2002) caused due to chronic exposure (Vahidnia et al. 2007).

ARSENIC LEVELS AROUND THE WORLD

Arsenic is a recognized toxic carcinogen, one of the World Health Organization's 10 chemicals of major public health concern. Thus, to protect public health, WHO has standardized the recommended limit of arsenic concentration in drinking water to 10 µg/l or 10 parts per billion (ppb). About 150 million people in the world are affected by arsenic exposure (Rahman et al. 2014). Most of the affected regions are countries in Southeast Asia which includes India, Bangladesh, Nepal, Pakistan, Iran, China, Taiwan, Cambodia and Vietnam. Arsenic also contaminates large areas in Chile, Argentina, Mexico and parts of the United States (Table 3).

The populous Bengal delta in Bangladesh and India is highly contaminated in arsenic. High levels of arsenic are contributed by the Ganges River which brings down the fluvial deposits from the Himalayas and the coal mine of Gondwana, the tributaries Bhagirathi and Padma transport arsenic from the metal deposit Gorubathan. Arsenic is present in the aquifers, mobilized into water from sediments and is transported in the groundwater circulation. The floodplain is fertile for crop cultivation, mainly rice, and the contaminated groundwater and river water is used for irrigation and drinking purposes, exposing millions of natives to chronic toxicity (Henke 2009).

Table 3: Arsenic levels in different areas. (Reviewed by Nordstrom 2002, Mukherjee et al. 2006, Henke 2009, Rahman et al. 2014)

Country	**Region**	**Arsenic levels in Water (µg/L)**	**Reference**
Afghanistan	Ghazni	10-500	Saltori 2004
Argentina	Encon, San José de Jáchal	9-357	Nicolli et al. 1989, Smedley et al. 1998
	La Pampa	3-1326	
Australia	Victoria	1-220	Hinwood et al. 1998
Bangladesh	Noakhali, Bengal basin	1-4730	Dhar et al. 1997, Chowdhury et al. 1999, Chowdhury et al. 2002, Sengupta et al. 2003, Chakraborti et al. 2010
Belgium	Zenne River	Up to-30	Brunt et al. 2004, Nriagu et al. 2007
Brazil	Minas Gerais	0.4-350 200-860mg/kg (soil)	Matschullat et al. 2000, Bundschuh et al. 2012
Cambodia	PreyVeng, Kandal	Up to 900	Sthiannopkao et al. 2008 Stanger et al. 2005
	Mekong delta	1-1610	
Canada	Nova Scotia	1-5-739	Meranger et al. 1984

Country	**Region**	**Arsenic levels in Water (µg/L)**	**Reference**
Chile	Esquiña	12-74 489mg/kg (soil)	Borgoño and Greiber 1971, Zaldivar 1974, Bundschuh et al. 2012
China	Tianshan plain	40-750	Lianfang and Jianghoung 1994, Zheng and Long 1994
	Yellow River plain	1-2400	
Finland	Southwest	17-980	Kurttio et al. 1998
Germany	Northern Bavaria	10-150	Butzengeiger 1940, Nordstrom 2002
Ghana	Obuasi	1-175	Bowell 1992, Nordstrom 2002
Greece	Fairbanks	Up to 10,000	Smedley and Kinniburgh 2002, Yiannis 2004
Hungary	Danube Basin	2-4000	Egyedi and Pataky 1978, Nagy and Korom 1983, Varsanyi 1989
India	Central	10-3200 16-417mg/kg (soil)	Datta and Kaul 1976, Garai 1984, Das et al. 1995, Chowdhury et al. 1999, Pandey et al. 1999, Chakraborti et al. 2002, Chowdhury et al. 2002, Mukherjee et al. 2003, Das et al. 2013, Srivastava and Sharma 2013
	Uttar Pradesh	44-621	
	Bihar	3-1861	

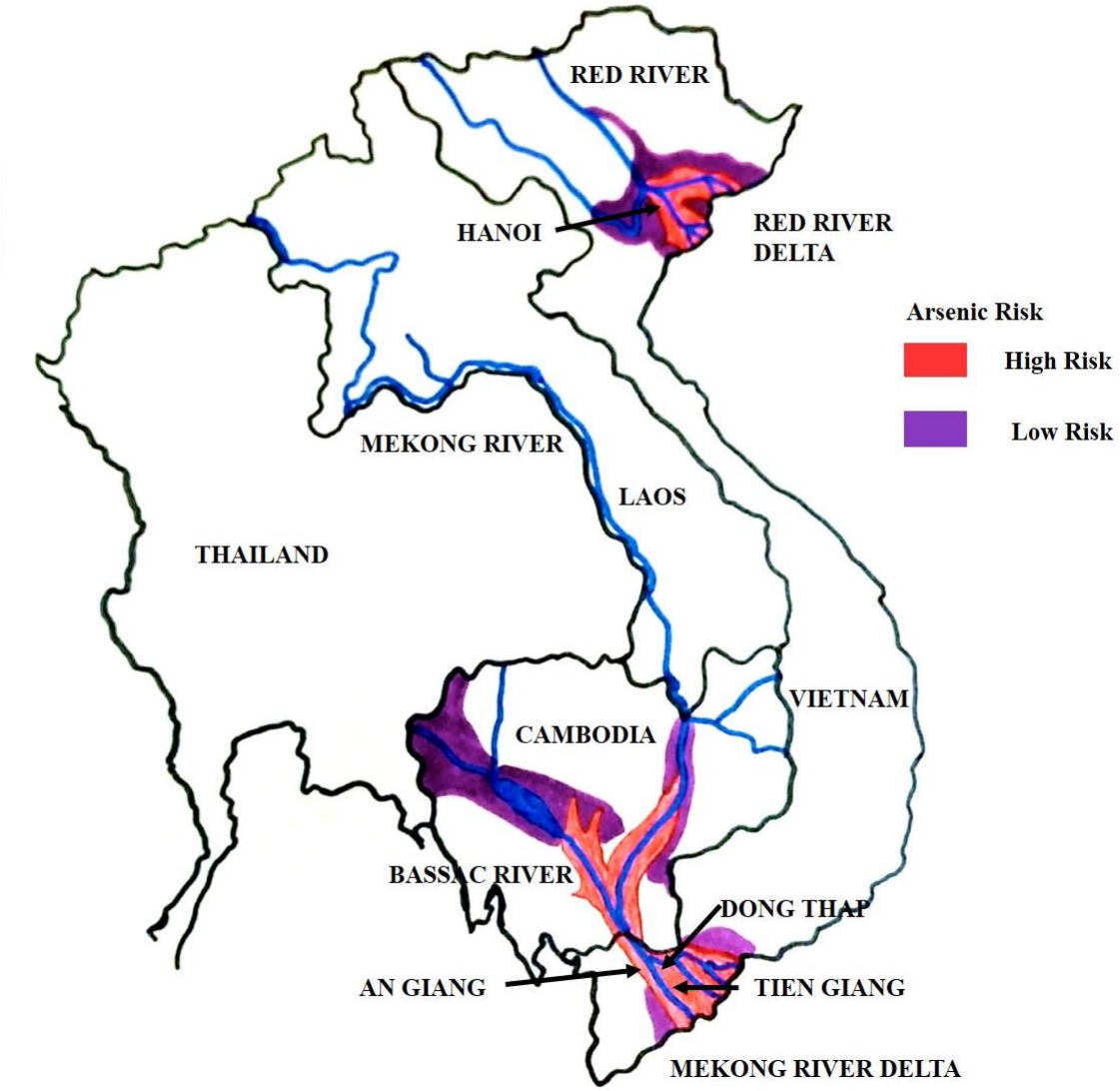
Country	**Region**	**Arsenic levels in Water (µg/L)**	**Reference**
	Bengal Basin	1-4100	
Iran	Kurdistan	Up to 290	Mosaferi et al. 2003
Japan	Fukuoka	1-293	Kondo et al. 1999
Laos		0.5-278	Chanpiwat et al. 2011
Mexico	Lagunera	8-620 2215-2675mg/kg (soil)	Cebrián et al. 1983, Del Razo et al. 1990, Armienta et al. 1997
Mongolia	Hetao Basin	0.6-572	Khan and Ho 2011
Nepal	Terai	10-2620	Halsey 2000, Tandukar et al. 2001, Shrestha et al. 2003
Pakistan	Indus Valley	10-1900	Nickson et al. 2005, Khan and Ho 2011
Poland	Lower Silesia	18100mg/kg (soil)	Karczewska et al. 2007
Romania	Transylvania	2-176	Gurzau and Gurzau 2001
Spain	Duero Cenozoic Basin	41	Gómez et al. 2006, Olías et al. 2006
Switzerland	Jura Mountains, Alps	Up to 170	Pfeifer and Zobrist 2002

Country	**Region**	**Arsenic levels in Water (µg/L)**	**Reference**
Taiwan		10-1820	Tseng et al. 1967, Guo et al. 1997
Thailand	Ronpibool	1-5000	Choprapawon and Rodcline 1977, Pavittranon et al. 2003
Turkey	Simav Plain	Up to 562 660mg/kg (soil)	Gunduz et al. 2010
United Kingdom	Cornwall	11-80	Farago et al. 1997, Thornton and Farago 1997, Palumbo-Roe et al. 2005
United States	Tulare Basin	Up to 2600 280mg/kg (soil)	Welch et al. 2000, Brunt et al. 2004, Twarakavi and Kaluarachchi 2006, Cutler et al. 2013
Vietnam	Red River delta	1-3050	Berg et al. 2001
	Mekong River delta	1-845	

ARSENIC CONTAMINATION IN VIETNAM

Vietnam, an agricultural country, is densely populated mainly in the delta regions of the Red River and Mekong River which comprise of fertile alluvial deposits suitable for crop cultivation. The Mekong and Bassac River flow through An-Giang and Dong Thap provinces (Figure 3). The groundwater is used for irrigation and household purposes as the surface water and shallow well water is prone to bacterial contamination. The groundwater is a reducing environment with high concentrations of iron, manganese and ammonium. It has been suggested that arsenic is released in the groundwater due to reductive dissolution of iron oxyhydroxides present in the aquifer rocks. Microorganisms rapidly consume the dissolved oxygen present in the sediment subsurface by mineralizing natural organic matter (NOM) creating anoxic conditions. These reductive conditions favor the reduction of arsenate As (V) to arsenite As (III) which is mobile and soluble in water (Buschmann et al. 2008, Williams et al. 2011). Mining wastes brought down by the rivers, agricultural pesticides, chemical warfare agents (Agent Blue) and landfill leakages also heavily contribute to arsenic contaminations in these areas. People in Vietnam are not only exposed to arsenite through groundwater used for drinking, but also through food as the water is used for irrigation and it has been observed that crops accumulate methylated forms of arsenic (Agusa et al. 2013). Arsenobetaine is also consumed through seafood.

Figure 3: Map of the Vietnamese area affected with arsenic contamination



The first paper on arsenic contamination (Berg et al. 2001) in the Hanoi region comprising the Red River delta reported average concentrations of 160 µg/L. In a later study conducted (Berg et al. 2007) 112 groundwater samples from the rural areas in the Mekong delta region were analyzed with arsenic concentrations of <1-845 µg/L. It was suggested that alkaline conditions pH (>7) leads to higher arsenic release from the

sediments. They also collected hair samples from inhabitants of two villages, one exposed to polluted groundwater, >50 μg/L arsenic and the other exposed to <50 μg/L arsenic. The levels of arsenic in hair ranged from 0.11–2.92 mg/kg (unexposed hair contains <1 μg/g (Agusa et al. 2013)) and from 1–167 μg/L in groundwater. Exposure to >50 μg/L arsenic levels for 10-15 years can cause chronic toxicity (Smith et al. 2000) thus these results indicate that the people in the Mekong River delta are exposed to chronic levels of arsenic. Similar studies were conducted for the Hanoi region of the Red River delta and the results show that these areas are almost as heavily polluted as the Bengal delta (Berg et al. 2007).

Shinkai et al. (2007) examined the distribution and contamination levels of arsenic and other metals in the groundwater of the Tien Giang Province and Dong Thap Province around the Mekong delta. Arsenic concentrations ranged from 0.9 μg/L to 321 μg/L in the groundwater used for drinking supply and 27% of the shallow-well samples exceeded the WHO limits of 10 μg/L. Manganese (Mn) and barium (Ba) quantities were higher than the WHO guidelines in 91% and 27% respectively of the shallow-well water samples. They did not find such high levels of contamination in deep-well water samples which suggests that As, Mn and Ba are widely present in the shallow aquifers of the Mekong River delta and the people consuming this water are at high risk.

The mechanism of arsenic mobilization was studied in the Red River floodplain near Hanoi by Postma et al. (2007). Their model suggests that arsenic mobilization is a result of the reduction of iron oxides by sedimentary organic matter. They present that iron oxides are not poorly crystalline oxyhydroxides to which arsenic is adsorbed on the surface, but are more stable crystalline phases and the arsenic is firmly bound within the Fe-oxide structure. Only upon bulk reductive dissolution of the iron oxides, arsenic is released to the

groundwater and part of the arsenite gets readsorbed to the surface of the remaining Fe-oxides. The rate control factor of iron-oxide reduction and thus arsenic release is the reactivity of the organic matter in the sediment, and its rate of degradation is low and constant. Therefore, the release of arsenic in the groundwater may continue for thousands of years given the amount of Fe-oxide bound to arsenic in these sediments. The levels of arsenic they measured in the groundwater samples exceeded 500 µg/L which is 50 times the limit suggested by WHO, making this a very serious problem in the Red river floodplain.

Cases of chronic exposure have not been observed in Vietnam mainly because the use of groundwater for household purposes is recent, over the last 10 years. Thus it is expected that in the near future, victims of chronic poisoning will be identified. The early detection of arsenicosis (arsenic poisoning) manifestation is also difficult to diagnose and depends on the awareness of the local people and doctors. The general nutrition of the Vietnamese population is also better when compared to Bangladesh and that could contribute in late appearance of symptoms and manifestation of the disease (Berg et al. 2007).

ARSENIC REMOVAL TECHNIQUES

Technologies for removing arsenic from the environment must be robust, not causing additional harm on the environment, and should be able to sustain water supply systems for irrigation and drinking while maintaining the quality requirements. These can broadly be divided into three categories: physical, chemical and biological. Physical

treatments include soil washing by acids, using cement to immobilize soluble arsenites, filtration, osmosis or electrodialysis and the use of surfactants, co-solvents and cyclodextrin to aid soil flushing. Chemical methods involve adsorption, coagulation, precipitation, stabilization and immobilization using specific chemical reactions. The use of iron and aluminum oxides as coagulation for co-precipitation has been particularly efficient in removing arsenic from the soil (Mahimairaja et al. 2005, Duarte et al. 2009, Komárek et al. 2013). Iron oxide nanoparticles scavenge inorganic pollutants in the soil and due to their high reactivity and surface area, they can be very useful for remediation (Jiang et al. 2012, Lim et al. 2014). Local people living in the Red River delta use sand filter systems, where the iron oxides in sand beds accumulate arsenic (Tobias and Berg 2011) and the water filtered through these sand filters greatly reduces the amount of arsenic (Agusa et al. 2013).

Bioremediation is a potentially cost effective way to reduce organic and inorganic arsenic from contaminated soils. Intrinsic bioremediation involves naturally present microbiota transforming arsenic without any human interference and is appropriate for low concentrations of arsenic. Engineered bioremediation is facilitating the growth and activity of microorganisms by optimizing the environment to promote arsenic degradation (Mahimairaja et al. 2005). Microbial remediation contributes to arsenic biogeochemical cycling, arsenate reduction to arsenite for respiration or detoxification, arsenite oxidation to arsenate and arsenite methylation. Arsenite oxidation to arsenate is crucial in remediation strategies to eliminate dissolved arsenite from water, done by immobilizing the oxidized arsenate in solid form by co-precipitation with iron oxyhydroxides (Leiva et al. 2014). In addition to utilizing microbial oxidation for arsenic attenuation,

phytoremediation is being considered. Floating plants like water hyacinth can adsorb arsenic from contaminated water ponds and aquatic rooted plants can remove arsenic from waterbeds (Gonzaga et al. 2006). Phytoremediation in water environments can also be enhanced using microorganisms which can assist in biomethylation (Yang et al. 2012). Research on genetically transformed microbes to enhance methylation and arsenite oxidation is also being done for bioaugmentation to improve arsenic remediation (Lim et al. 2014).

ARSENIC BIOACCUMULATION IN CROPS

Cereal crops and even horticulture produce can accumulate inorganic arsenic and DMA (Schoof et al. 1999). While the plants themselves don't methylate arsenic, anthropogenic sources such as pesticides, environmental deposition or microbial methylation in soil around plant rhizospheres can contribute to bioaccumulation of methylated arsenics (Figure 4) (Norton et al. 2013). Elevated levels of arsenic are observed in grain crops, such as rice which is a dietary staple for about half the world's population. Rice in particular is efficient in accumulating arsenic in the roots and shoots and translocate it to the grain (Marin et al. 1992, Marin et al. 1993, Williams et al. 2005). Hence, it is very important to understand the metabolism of arsenic in rice to prevent and mitigate widespread contamination and accumulation in the food chain (Arao et al. 2011). Methylated arsenic is associated with the physiological 'straight-head' disease in rice which results in spikelet sterility and yield losses (Zhao et al. 2013).

Xu et al. (2008) examined arsenic accumulation in the rice plant and arsenic speciation in the soil solution under flooded and aerobic conditions. Arsenic rapidly mobilized as arsenite under flooded conditions and arsenic concentrations were higher than aerobic conditions. Arsenic concentrations in the rice grain were 10-15 times higher in flooded rice compared to aerobically grown rice, and inorganic arsenic decreased while DMA proportions increased. This study showed that the bioavailability of arsenic increased under flooded conditions leading to greater arsenic accumulation in rice and that the transfer of arsenic from soil to grain was much lesser in aerobically grown rice. The authors suggested that this conclusion also supports studies which report that arsenic induced 'straight-head' symptoms in rice develop in flooded conditions, and also those that rice grown on raised beds and furrows accumulated less arsenic in the straw and grain compared to flooded rice as a result of higher redox potential in soil.

The efficiency of translocation of DMA in the rice grain was over an order of magnitude greater than inorganic species (Carey et al. 2010). DMA was also more mobile in the phloem and xylem of the plant than arsenite. Arsenic species were pulsed hydroponically, and 90% arsenite and 55% DMA was transported to the grain via the phloem. DMA was found to be dispersed throughout the external parts of the grain and in the endosperm while arsenite was retained in the ovular vascular trace indicating that inorganic and organic arsenic species accumulate differently in the rice plant and the grain.

Arao et al. (2011) investigated whether DMA is synthesized in the plant or taken up from the soil. Rice was grown in soil and in solution culture to study arsenic speciation and the effect of DMA, MMA and arsenite mobilization and also arsenic methylation under suppressed bacterial activity. Under flooded conditions in the soil grown rice, DMA

concentrations increased in brown rice and straw while in solution culture DMA concentrations increased in the grain on MMA and arsenite amendment suggesting MMA is methylated to DMA. Bacterial suppression by using the antibacterial chloramphenicol dramatically decreased DMA levels inferring that bacteria associated with rice rhizosphere is involved in DMA formation and thus arsenic methylation.

Lomax et al. (2012) performed related studies on rice, tomato and red clover to determine if the plants methylated arsenic in-vivo or the methylation was due to bacterial biotransformation. They planted these crops in axenic conditions, non-sterile hydroponic cultures and in flooded soils, to find that when exposed to inorganic arsenic in axenic conditions, there were no methylated arsenicals in the plants, whereas, rice plants were able to take up MMA or DMA but not convert MMA to DMA. In non-sterile hydroponic cultures, methylated arsenicals were found in the shoots and grain of rice. Anaerobic bacterial species and the presence of bacterial *arsM* methyltransferases was detected in paddy soils of Bangladesh along with other genes involved in arsenic cycling (*arsA*, *arsB*, *arsC*, *aoxB*). In comparing soil samples from UK they found that the methylation process can occur in the absence of the plant.

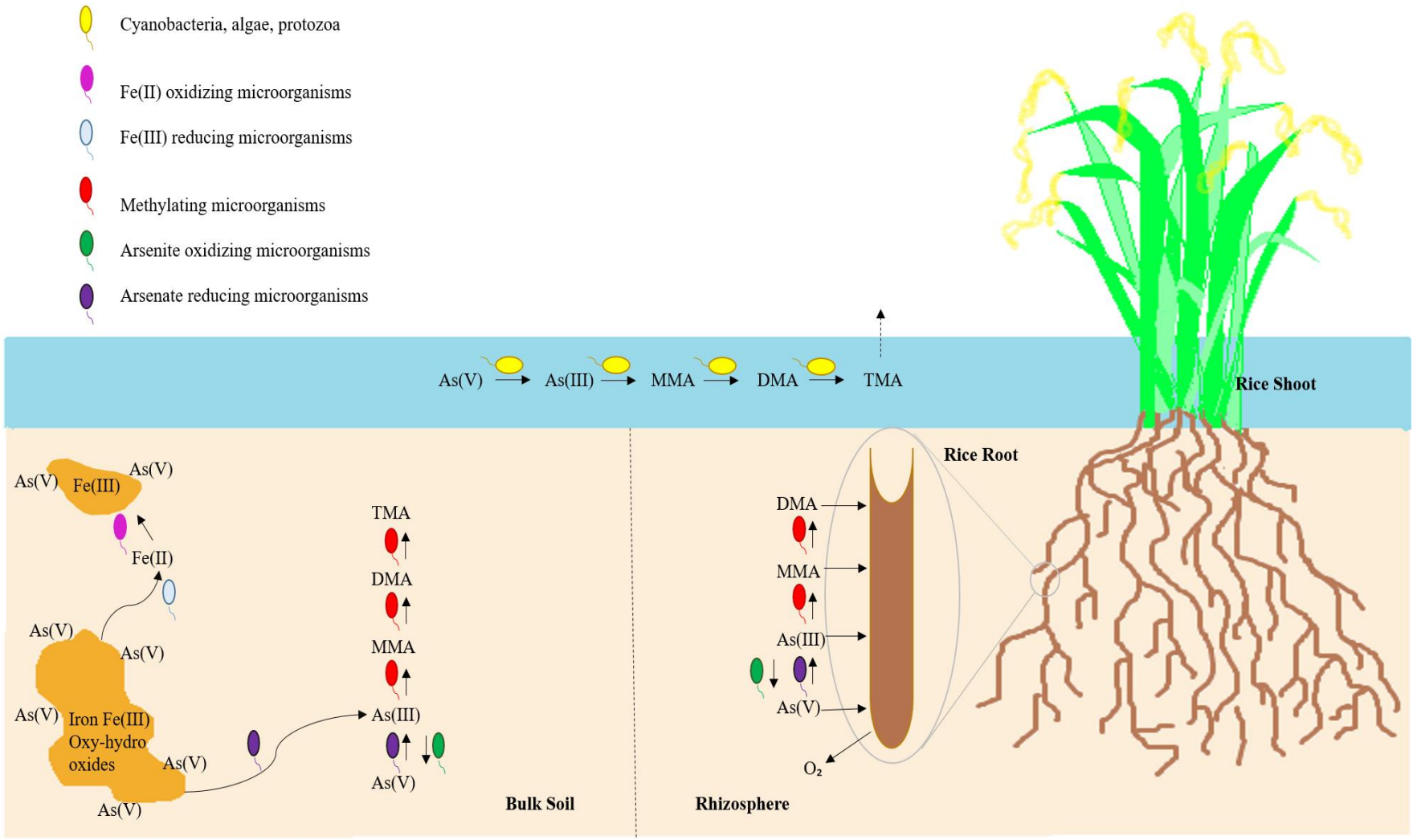
The biogeochemical cycling of elements in the rice rhizosphere is intense and complex and is regarded as a separate system. Jia et al. (2014) explain that rhizospheric chemical processes such as iron redox reactions on the root surfaces affect oxygen release from the roots influencing arsenic uptake by rice. Arsenic precipitates as arsenate with iron and manganese oxides coated on oxygen releasing root surfaces in aerated rice rhizospheres. Arsenic speciation is also greatly affected by microbial processes such as respiration by anaerobic bacteria which reduce arsenate to arsenite through *arrA* reductases

and detoxification by reduction of arsenate to arsenite through *arsC* reductases. Arsenite is weakly bound to soil minerals compared to arsenate and thus mobilizes and is released in the soil solutions mainly under anaerobic or flooded conditions in paddy fields. Some chemoautotrophic and heterotrophic bacteria also can oxidize arsenite to arsenate through *aroA* or *aox* genes which aids arsenic sequestration in metal oxides, reducing arsenic bioavailability and uptake by plants and thus facilitating the remediation of arsenic contaminated soil and water.

An arsenite methylating *Streptomyces* strain was anaerobically isolated from the rice rhizosphere of arsenic contaminated paddy soil (Kuramata et al. 2015). This strain forms MMA and DMA in liquid cultures containing arsenite and has a phylogenetically different *arsM* gene from other known *arsM* containing bacteria. DMA was also detected in the shoots of rice plants grown in liquid medium supplemented with arsenite and the *Streptomyces* strain and the authors deduced that the bacterium inhabits the oxidative zone around the rhizosphere and contributes to DMA accumulation in rice grains by arsenic methylation.

The arsenic content in the rice shoots and straw fed to livestock used for milk and meat production is also an important consideration. Nandi et al. (2005) found significantly increased arsenic levels in cattle hair and described the presence of arsenic related health symptoms in cattle exposed to high arsenic intake in affected West Bengal areas.

Figure 4: Arsenic cycling and biotransformation in paddy soil and rhizosphere (Adapted from Zhao et al. 2010 and Zheng et al. 2012)



ARSENIC CYCLING BY MICROORGANISMS

Prokaryotes play a crucial role in driving and contributing to biogeochemical cycles of elements in the environment. Microbial activity directly or indirectly determines the fate of arsenic release by influencing its mobilization, sequestration and transformation by utilizing arsenic for various metabolic processes such as assimilation, respiration, methylation and detoxification (Stolz et al. 2006). Several phylogenetically diverse microorganisms have been discovered to consume arsenic for 'eating', arsenite functioning as an electron donor, and 'breathing', arsenate being an electron acceptor, for respiration (Silver and Phung 2005). Despite their ability to utilize As, bacteria do not require arsenic for nutritional purposes in the cell cytoplasm, hence a dedicated arsenic uptake system has not been developed or discovered till date. Due to arsenate's analogy to phosphate, it enters the cell through existing membrane transporters such as Pit/Pst (phosphate transporters) or as arsenite (at neutral pH) enters through aquaglyceroporins (GlpF) which is a channel for water and small nonionic molecules such as glycerol (Rosen 2002, Meng et al. 2004, Páez-Espino et al. 2009). To utilize arsenic for energy, chemolithoautotrophic arsenite oxidizers couple the process with oxygen or nitrate reduction, while heterotrophic arsenate respiring bacteria use lactate or such small fatty acids as carbon sources and some may use hydrogen as an electron donor (Huber et al. 2000, Silver and Phung 2005). Apart from energy generation and detoxification, arsenic can be assimilated as organoarsenicals: arsenosugars and arsenolipids (Figure 5) (Stolz et al. 2006). Microbial arsenic biotransformation and mobility has been studied by many researchers (Cullen and Reimer 1989, Pongratz 1998, Turpeinen et al. 1999, Turpeinen et al. 2002, Kim 2014)

Arsenite Oxidization

Arsenite oxidizing bacteria were first described by Green (1918). Chemoautotrophs can oxidize arsenite aerobically, anaerobically by nitrate and selenite dependent respiration, or phototrophy (Rhine et al. 2006, Budinoff and Hollibaugh 2008, Cavalca et al. 2013). These include members of the genera *Alcaligenes* (Philips and Taylor 1976), *Agrobacterium/Rhizobium* (Santini et al. 2000), *Ectothiorhodospora* (Oremland et al. 2002), *Pseudomonas* (Macur et al. 2004) and *Thermus* (Gihring and Banfield 2001). The arsenite oxidase was first isolated by Anderson et al. (1992) from *Alcaligenes faecalis*. Similar enzymes were characterized from *Rhizobium* sp. strain NT-26 (Santini and vanden Hoven 2004) and *Hydrogenophaga* sp. strain NT-14 (vanden Hoven and Santini 2004). These enzymes belong to the dimethyl sulfoxide (DMSO) reductase family of molybdenum enzymes. The genes coding for these enzymes have been assigned as *aox, aso* or *aro* by different authors, but are now being grouped as *aio* (Lett et al. 2012). Zargar et al. 2010 isolated a novel arsenite oxidase, *arxA* from *Alkalilimnicola ehrlichii* MLHE-1 (Hoeft et al. 2007) which couples arsenite oxidation to nitrate reduction, from Mono Lake, California. A similar *arx* operon was found in a photosynthetic purple sulfur bacterium *Ectothiorhodospira* sp. strain PHS-1 which uses arsenite as an electron donor in anoxygenic photosynthesis (Kulp et al. 2008). Another strain, ML-SRAO, anaerobically oxidizes arsenite while reducing selenite (Fisher and Hollibaugh 2008). Arsenite oxidizing gene homologs have also been found in green sulfur and non-sulfur bacteria (Stolz et al. 2006). These findings suggest a possibility that microbial oxidation of arsenite into relatively less toxic arsenate in the absence of oxygen led to partial detoxification of arsenic

rich environments to make it suitable for other microorganisms in the archaic anoxic world (Cavalca et al. 2013).

Arsenic Resistance

Arsenic resistant microbes (ARM's) reduce arsenate to arsenite for detoxification utilizing the *ars* operon. *Ars* genes have been found on plasmids as well as on the chromosomes of *Firmicutes* and *Alpha-* and *Gammaproteobacteria* (Cervantes et al. 1994). The *ars* genes are located as an operon which differs in organisms, the *arsRDABC* located on the *Escherichia coli* plasmid R773 is the most well studied (Hedges and Baumberg 1973, Gladysheva et al. 1994). *ArsC* encodes a cytoplasmic arsenate reductase, *arsB* encodes the arsenite efflux pump, *arsR* encodes a transcriptional repressor that binds to the *ars* operon operator and has an arsenite specific binding site (Wu and Rosen 1991, Xu et al. 1996), *arsD* encodes a secondary trans-acting regulator which binds to arsenite (Wu and Rosen 1993), while *arsA* encodes a membrane associated ATPase unit providing energy to *arsB* (Tisa and Rosen 1990, Kaur and Roden 1992). Another gene cluster of the *ars* operon only consists of *arsRBC. ArsH* found in some gram negative bacteria confers the organism to grow at high arsenic concentrations (Branco et al. 2008). *Acr3* also functions as an arsenite efflux pump in *Actinobacteria* and *Alphaproteobacteria* (Achour et al. 2007). *Acr2* encodes the arsenate reductase in *Saccharomyces cerevisiae* (Messens and Silver 2006). Arsenic reducing microorganisms play a major role in arsenic mobilization and speciation.

Arsenate Respiration

Heterotrophic dissimilatory arsenate respiring prokaryotes (DARP's) reduce arsenate to arsenite anaerobically. These include members of the Gamma-, Delta-, and Epsilon*proteobacteria, Firmicutes, Aquificae, Deferribacteres, Chryosiogenetes,* thermophilic eubacteria, and *Crenarchaetoa* as shown in Table 4 (Macy et al. 1996, as reviewed by Oremland and Stolz 2003, and Cavalca et al. 2013). Ahmann et al. (1994) reported on the first arsenate respiring bacterium, strain MIT-13, which was later named *Geospirillum arsenophilus* (Lovley and Coates 1997). The respiratory arsenate reductase, *arr* was first purified from *Chrysiogenes arsenatis* (Krafft and Macy 1998) and *Bacillus selenitireducens* (Afkar et al. 2003). *Arr* is a heterodimer consisting of two subunits, molybdopterin and iron sulfur center protein (*arrA, arrB*). A third subunit *arrC* has been discovered in *Desulfitobacterium hafniense*, *Alkaliphilus metalliredigens* and *Wollinella succinogenes* and an additional chaperone coding *arrD* is observed in *Alkaliphilus oremlandii, Bacillus selenitireducens* MLS10, strain MLMS-1, *Geobacter lovleyi*, *Desulfitobacterium hafniense* and *Halarsenatibacter silvermanii* (Cavalca et al. 2013). Saltikov et al. (2003), Saltikov et al. (2005), Malasarn et al. (2008) studied and characterized the *arr* operon in *Shewanella* sp. strain ANA-3. The *arr* operon lies immediately downstream of the *ars* operon in *Shewanella* sp. strain ANA-3 (Saltikov and Newman 2003). The molybdenum center and Fe-S cluster work as a biochemically reversible unit functioning as an oxidase or reductase depending upon the redox potential, although the expression of both oxidation and reduction in the same organism is rare (Richey et al. 2009). Reductive reactions of DARP's and Fe(III) reducers contributes in the release of arsenic from sediments to groundwater (Malasarn et al. 2004).

Table 4: Diversity of known arsenic respiring bacteria.

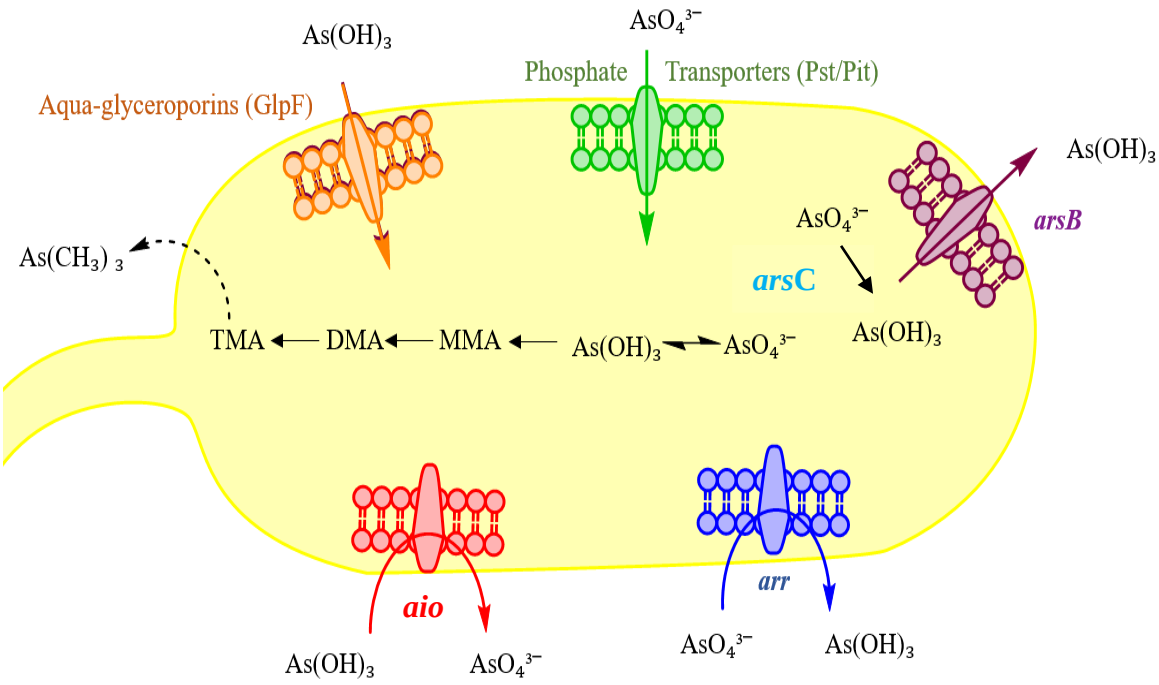
Species	**Phylogeny**	**References**
Bacillus arsenicoselenatis	*Firmicutes*	Oremland et al. 1999
Bacillus beveridgei	*Firmicutes*	Baesman et al. 2009
Bacillus macyae	*Firmicutes*	Santini et al. 2004
Bacillus selenatarsenatis	*Actinobacteria*	Yamamura et al. 2007
Bacillus selenitireducens	*Firmicutes*	Oremland et al. 2000
Bacillus sp. JMM-4	*Firmicutes*	Santini et al. 2002
Chrysiogenes arsenatis	*Chrysiogenes*	Macy et al. 1996
Desulfitobacterium sp. GBFH	*Firmicutes*	Niggemyer et al. 2001
Ferrimonas futtsuensis	*Gammaproteobacteria*	Nakagawa et al. 2006
Ferrimonas kyonanensis	*Gammaproteobacteria*	Nakagawa et al. 2006
Neisseria mucosa	*Betaproteobacteria*	Youssef et al. 2009
Rahnella aquatilis	*Gammaproteobacteria*	Youssef et al. 2009
Seleniivibrio woodruffi	*Deferribacteriacae*	Rauschenbach et al. 2011
Shewanella ANA - 3	*Gammaproteobacteria*	Saltikov at el. 2003
Sulfurospirillum arsenophilum	*Epsilonproteobacteria*	Stolz et al. 1999
Sulfurospirillum barnesii	*Epsilonproteobacteria*	Stolz et al. 1999
Thermus sp. HR13	*Thermus*	Gihring and Banfield 2001
Wollinella succinogenes	*Epsilonproteobacteria*	Tomei et al. 1992

Arsenite Methylation

Arsenic methylation is well understood in fungi and eukaryotes but less in bacteria. The methylation pathway based on *Scopulariopsis brevicaulis* was first proposed by Challenger (1945). It involves arsenate reduction followed by oxidative addition of methyl groups generating gaseous arsines, MMA, DMA, TMA and TMAO (Dombrowski et al. 2005). Glutathione participates in reduction, the source of the methyl groups is S-adenosinemethionine (SAM), and some anaerobic bacteria use methylcobalamin as electron donors (Páez-Espino et al. 2009). In 1971, McBride and Wolfe reported volatilization of arsenicals by *Methanobacterium bryantii. ArsM*, a methyltransferase has been identified in over 100 Bacteria and 16 Archaea, and was characterized in *Rhodopseudomonas palustris* (Qin et al. 2006).

Challenger (1945) also explained the cleavage of As-C to produce trimethyl arsine in *Scopulariopsis brevicaulis* and *Penicillium notatum*. Demethylation of mono- and dimethyl-arsenic compounds has also been reported in *Alcaligenes*, *Pseudomonas*, and *Mycobacterium* species (Bentley and Chasteen 2002). A *Pseudomonas* bacterium uses DMA as carbon source releasing arsenic (Maki et al. 2004).

Figure 5: Arsenic biochemistry in a microbial cell. (Adapted from Páez-Espino et al. 2009)



STUDY OBJECTIVE

The aim of this study was to understand and monitor the microbial reduction of arsenate, which contaminates the agricultural fields in the Mekong River delta of Vietnam. Specific goals were to 1) Enrich and isolate bacterial strains contributing to arsenic transformation in the agricultural fields in the Mekong River delta of Vietnam and 2) Understand the reduction of arsenate by the isolates and detect genes encoding for arsenic transformation.

Arsenic speciation and mobilization is highly dependent upon microbial activity. The toxic effects of high concentrations of arsenic are well known and it is important to identify factors contributing to arsenic release in these contaminated areas to mitigate the toxicity and prevent consumption of arsenic through drinking water and food. The presence of arsenate reducing microbes has been found in these soils and the characterization of bacterial communities will give an insight on microbially mediated arsenic transformation.

MATERIALS AND METHODS

The Study Site

The Khanh An district of An-Giang province in the Mekong delta area was the source of soil samples (Figure 6) (Mishra 2013). The soil properties and the crops grown in these collection sites are different and are irrigated using groundwater (Table 5).

Table 5: Reference names and descriptions of soil samples

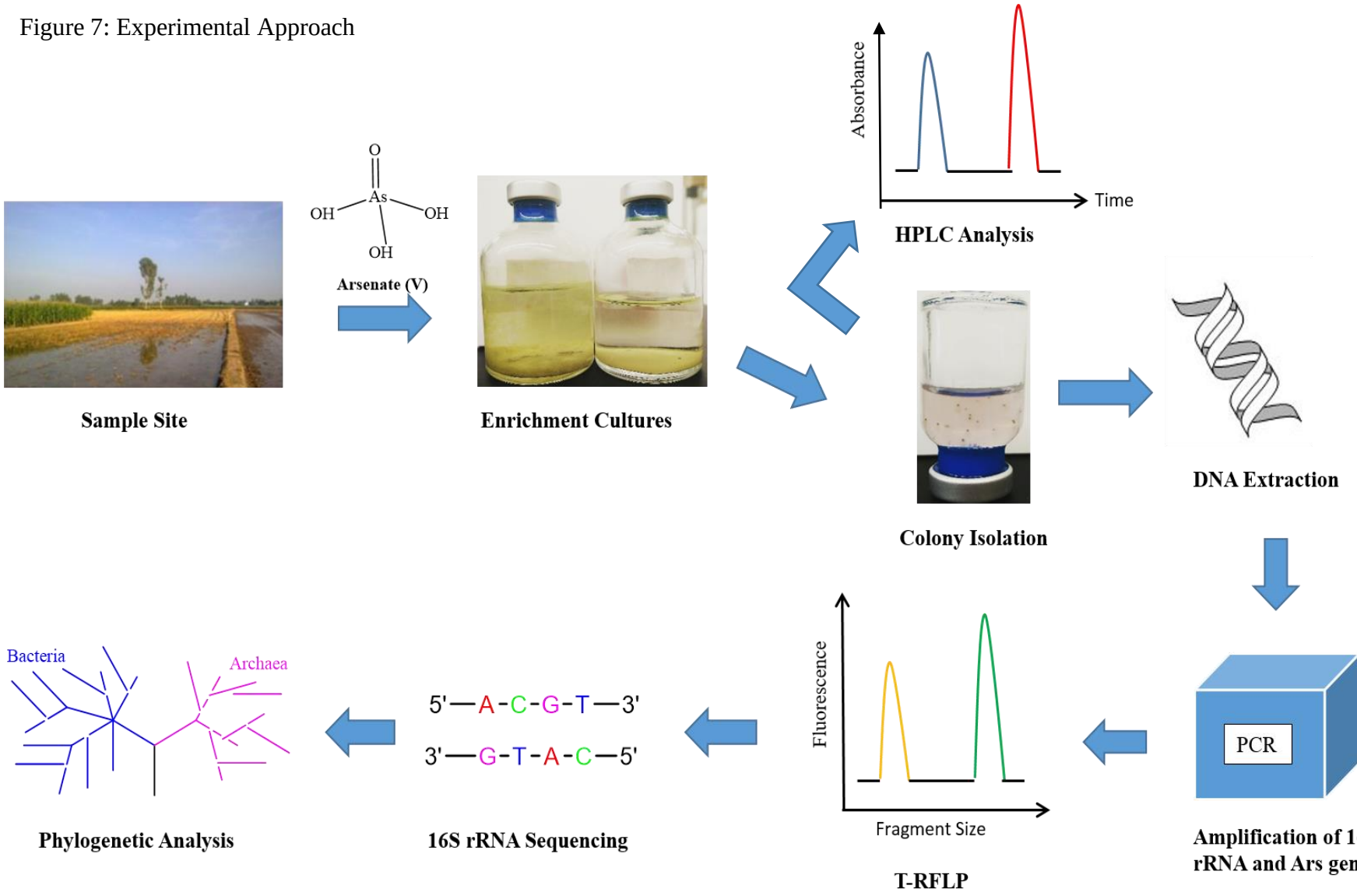
Reference Names	**Irrigation Water Source**	**Crop Cultivated**
G	Groundwater	Rice
E	Groundwater	Rice
C	Groundwater	Maize

Figure 6: Paddy field site in the An-Giang province, the source of rice soil samples used in this study. Photo from Dr. Duong Minh Vien.

To achieve this aim, previously established anaerobic soil enrichment cultures (Mishra 2013) were used to establish fresh enrichments by 1:10 dilution in minimal salt media supplemented with arsenate as the sole electron acceptor and a mix of short chain

organic acids as the carbon donors. Arsenate reduction to arsenite was monitored by High Pressure Liquid Chromatography (HPLC). The enrichment cultures were used to setup agar shake tubes to isolate individual colonies by dilution to extinction. Colonies were picked and genomic DNA was extracted for 16S rRNA gene identification by Terminal Restriction Fragment Length Polymorphism (T-RFLP) fingerprinting and sequencing. To understand the role of these isolated bacterial cultures in arsenate cycling, the presence of arsenic respiration, reduction and transporter genes was analyzed by the PCR technique both in genomic and plasmid DNA. A pictorial representation is shown in Figure 7.

Figure 7: Experimental Approach



Enrichment Culture Preparation

Minimal salt media was prepared by dissolving 1.17 g KCl, 0.2 g KH_2PO_4, 1.7 g NaCl, 0.5 g NH_4Cl, 0.1 g $CaCl_2.2H_2O$, 3g $MgCl_2.6H_2O$ in 1 liter deionized water. The medium was sparged with nitrogen gas for 30-45 min, followed by 15 min with CO_2/N_2 (70:30%) gas mixture along with the addition of 2.5 g $NaHCO_3$. The bottles were capped, autoclaved and cooled. The media was supplemented with anoxic solutions of trace salts, vitamins (Table 7-10), resazurin as redox indicator and reductant sodium sulfide ($Na_2S.9H_2O$). 5 mM arsenate was added as the sole electron acceptor, and 2.5 mM of lactate, acetate, pyruvate and propionate mixture was added as the carbon source. 40 ml of this media mix was injected in each sterile, anoxic serum bottle. 1:10 dilution of previously setup enrichment cultures was prepared to setup fresh enrichments and then incubated at 30 ºC. A yellow precipitate of arsenic trisulfide (As_2S_3) was used as an indication of arsenate reduction. Another set of enrichment cultures was prepared similarly with 5 mM nitrate as the sole electron acceptor.

Soft agar ‘shake tubes’ were prepared to isolate individual colonies. A dilution to extinction scheme was used to serially dilute 1:10 culture in 10 ml tubes. The media was setup as explained above, but 4 g/L agar was also added before autoclaving. It was cooled and stabilized at 55 ºC before the addition of the supplements. The 10 ml tubes were sterilized, sparged and warmed prior to injecting the agar media to avoid solidification, 0.5 ml culture was injected aseptically using CO_2/N_2 flushed syringes in 4.5 ml media and sequentially diluted 1:10 to 10^{-10} dilutions and placed in an ice bath to rapidly solidify the agar. The tubes were inverted, caps facing down and incubated at 30 ºC.

To grow isolates aerobically, Luria Broth and MacConkey agar plates were used. All procedures were carried out under aseptic conditions and for the anaerobic techniques, butyl rubber stoppers and aluminum crisps were used to seal the bottles.

High Pressure Liquid Chromatography (HPLC)

HPLC analysis was performed using a Shimadzu (SPD-M10A VP) equipped with a Hamilton PRP-X100 column, (250 mm long with a diameter of 4.1 mm; 10 μm particle size) and an absorbance detector set at a wavelength of 200 nm. 40 mM ammonium hydrogen phosphate, pH 8.2 was used as mobile phase at a flow rate of 1 ml/min. 10 μl arsenite and arsenate standards and the samples were injected with a runtime of 17 min. Arsenite had a retention time of 3 min and arsenate 15.5 min.

DNA Extraction and PCR amplification for 16S rRNA gene

Colonies from agar shake tubes and samples from liquid enrichment cultures were used for DNA extraction using the MO BIO UltraClean Microbial DNA Isolation Kit. Briefly, cells were lysed using SDS and centrifuged, the supernatant was treated with a reagent which precipitates non DNA organic and inorganic material such as cell debris and proteins, a concentrated salt solution with silica was added to the remaining supernatant containing DNA to allow selective binding on the Spin Filter silica membrane, an ethanol based wash buffer was added to the filter to remove salts and other contaminants and the flow-through was discarded, the filters were centrifuged to remove residual ethanol and 50 μl of sterile elution buffer was added to the center of the membrane to elute the bound DNA and the flow-through after centrifugation contained the extracted DNA.

The 16S rRNA gene was amplified by the PCR technique using the universal 27F and 1100R primer pairs (Lane 1991). The reaction mixture contained 2 µl buffer (10X), 0.2 µl dNTP (10 mM), 0.4 µl 27F primer (FAM labelled for T-RFLP analysis) (10 µM), 0.4 µl 1100 R primer (10 µM), 0.4 µl Taq polymerase, 3 µl DNA template and dH_2O up to a total volume of 20 µl. The PCR reaction was set at 5 min at 94 °C for initial denaturation; 28 cycles of 30 sec at 94 °C for denaturation, 30 sec at 57 °C for annealing and 70 sec at 72 °C for extension; and a final extension at 72 °C for 10 min.

Gel electrophoresis was used to visualize and quantify PCR products. 5 µl product mixed with loading dye was run on a 1% agarose gel made with TAE buffer (1X) and ethidium bromide at 110V. Quantification was done using the lambda ladder on a Bio-Rad Gel Doc EZ Imager.

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

15 ng PCR product was digested at 37ºC for 6 hours with 2 µl buffer (10X), 0.3 µl *MnlI* restriction enzyme and dH_2O up to 20 µl. The DNA was precipitated using 1.9 µl sodium acetate and 0.4 µl pellet paint (6 µl glycogen, 2 µl loading dye) per reaction. Then, 37 µl of freshly made 95% ethanol was added to dissolve the salts and the samples were left at room temperature for 10 min. They were centrifuged at 4 ºC for 15 min and the supernatant was discarded. 125 µl of freshly made 70% ethanol was overlaid to remove residual salt and spun down at 4 ºC for 4 min. After removing the supernatant the tubes were left open to dry the pellet to evaporate remaining ethanol for 25-30 min in a laminar hood. The pellets were resuspended in formamide solution (19.7 µl deionized formamide and 0.3 µl Rox ABI standard. The samples were then denatured at 94 ºC for 2.5 min in a

thermal cycler and then put in ice. 10 µl of sample was loaded for DNA fingerprinting by T-RFLP analysis of the 16S rRNA gene on an ABI PRISM Genetic Analyzer (Applied Biosystems).

16S rRNA Gene Sequencing

A 50 µl PCR reaction mix was setup with 27F (not labelled with FAM) and 1522R primers set at 5 min at 94 °C for initial denaturation; 30 cycles of 45 sec at 94 °C for denaturation, 45 sec at 57 °C for annealing and 1.5 min at 72 °C for extension; and a final extension at 72 °C for 7 min. The PCR products were visualized using gel electrophoresis, cleaned up using the MO BIO UltraClean PCR Clean-up DNA Purification Kit and sent out for sequencing using the 27F, 534R, 704F, 1100R and 1522R primers to Genewiz (South Plainfield, NJ). The sequence contigs obtained were assembled using the BioEdit software (Hall 1999) and the complete 16S rRNA gene sequences were identified by BLAST (Altschul et al. 1990) using the NCBI database. Sequence alignments phylogenetic analysis was done utilizing the MEGA 6 software (Tamura et al. 2013).

Arsenic Genes Analysis

To understand the process of arsenate reduction by the bacterial isolates, genomic and plasmid DNA was used to amplify different genes responsible for arsenate respiration, transportation and reduction using PCR and different primer pairs. Plasmid DNA was extracted using the Thermo Scientific GeneJET Plasmid Miniprep Kit. Briefly, cells were lysed in SDS to liberate plasmid DNA and the lysate was neutralized for optimum binding of the plasmid DNA on the silica membrane of the spin filter. Cell debris and other

precipitated components were centrifuged and the supernatant was loaded onto the column. The plasmid DNA adsorbed onto the membrane and was washed to remove contaminants and was then eluted in elution buffer. A PCR amplification for the 16S rRNA gene was conducted on the plasmid DNA samples as a control to check for chromosomal DNA contamination (Figure 24). The *ars* genes analyzed were *arrA* (arsenate respiration), *arsC* (arsenate reductase), *acr3* (arsenate transporter), *arsB* (arsenate efflux transporter), *arsR* (transcriptional regulator) and *arsM* (arsenite methylation) using different primer sets shown in Table 6. The PCR conditions for the primer sets that produced positive results (marked in asterisk in Table 6) are as follows: *arsC* - initial denaturation at 95 °C for 1.5 min, 40 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s and then a final extension step at 72 °C for 10 min (Daware and Gade 2015); *arsB* - 5 min of denaturation at 94 °C followed by 35 cycles of 45 s of denaturation at 94 °C, 45 s of annealing at 57 °C and 30 s of primer extension at 72 °C and a final extension reaction at 72 °C for 7 min (Achour et al. 2007); *arsR* – denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 45 s, annealing at 56 °C for 45 s, 72 °C for 45 s and then a final extension step at 72 °C for 10 min; *arsM* - 5 min of denaturation at 94 °C, 30 cycles of 30 s at 94 °C, 45 s at 65 °C, and 45 s at 72 °C, followed by 10 min at 72 °C of extension (Meng et al. 2011); *arrA* – 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 50 °C for 40 s, and 72 °C for 1 min, and 72 °C for 10 min (Escudero et al. 2013).

Table 6: The primer sets tested in this study (asterisk marked primers showed positive results).

Gene	**Primer Name**	**Primer Sequence (5' - 3')**	**Reference**
arrA	arrA-F*	AAG GTG TAT GGA ATA AAG CGT TTG TBG GHG AYT T	Malasarn et al. 2004
	arrA-R*	CCT GTG ATT TCA GGT GCC CAY TYV GGN GT	
arsC	amlt-42-F	TCG CGT AAT ACG CTG GAG AT	Sun et al. 2004, Kaur et al. 2009, Escudero et al. 2013
	amlt-376-R	ACT TTC TCG CCG TCT TCC TT	
	smrc-42-F	TCA CGC AAT ACC CTT GAA ATG ATC	
	smrc-376-R	ACC TTT TCA CCG TCC TCT TTC GT	
	arsC-F	GTA ATA CGC TGG AGA TGA TCC G	Saltikov and Olson 2004, Selvi et al. 2014
	arsC-R	TTT TCC TGC TTC ATC AAC GAC	

Gene	Primer Name	Primer Sequence (5' - 3')	Reference
	arsC-F1*	GAG CAT CAC CAT TTA CCA TAA	Daware and Gade 2015
	arsC-R1*	TAT TTC ACA CGC TGC CCT GC	
	arsC-F2*	GAG CAA CAT CAC TCA TTT ATC ACA A	
	arsC-R2*	ACT TTR TCY GTC TTC CTT	
	arsC-F3*	GAG CAA CAT TAC CAT TTA TCA CAA	
	arsC-R3*	TCT CAC CGT CCT CTT TCG T	
	arsC-F4*	GAG CAA CAT MAC YAT YTA TCA CAA	
	arsC-R4*	TTT TCG CCA TCT TCC TTR	
acr3	dacr1-F	GCC ATC GGC CTG ATC GTN ATG ATG TAY CC	Achour et al. 2007
	dacr1-R	CGG CGA TGG CCA GCT CYA AYT TYT T	
	dacr5-F	TGA TCT GGG TCA TGA TCT TCC CVA TGM TGV T	
	dacr4-R	CGG CCA CGG CCA GYT CRA ARA ART T	

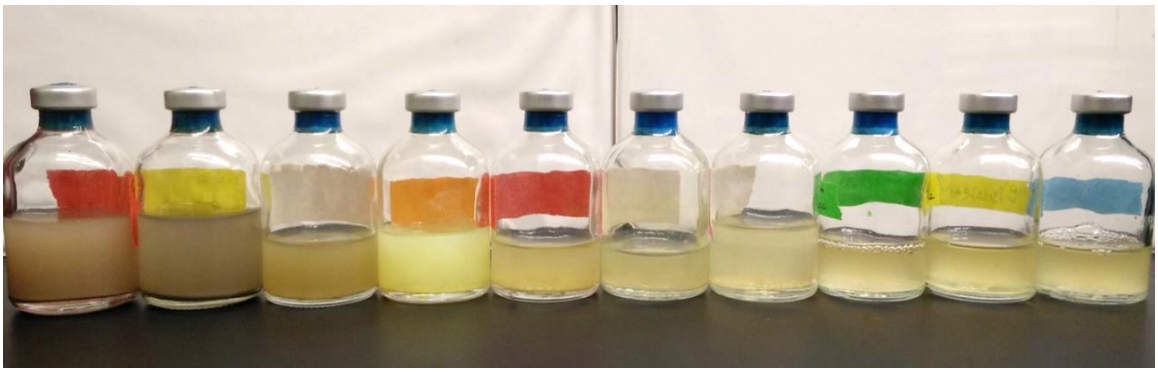
Gene	Primer Name	Primer Sequence (5' - 3')	Reference
arsB	darsB-1F* darsB-1R*	GGT GTG GAA CAT CGT CTG GAA YGC NAC CAG GCC GTA CAC CAC CAG RTA CAT NCC	Achour et al. 2007
arsR	arsR-F arsR-R	ATC AGG AGC GCC ATA TGT C TCC CGG ATA AAA CAC ATC TG	Xu et al. 1996
	arsR-F2* arsR-R2*	CCA GGC CTG TTC AAT CAC CT GGG GAT CGT ATT GCT GCT CA	Designed using Primer design Tool on NCBI (Ye at el. 2012)
arsM	arsM-F arsM-R	TCY CTC GGC TGC GGC AAY CCV AC CGW CCG CCW GGC TTW AGY ACC CG	Zhao et al. 2013
	arsM-F2* arsM-R2*	ATG CCC ACT GAC ATG CAA GAC TCA CCC GCA GCA GCG CGC CG	Meng et al. 2011

RESULTS

Enrichment and Isolation

Enrichment cultures of soil samples from three agricultural sites (rice soil sample 1 and 2 and maize soil sample) in the An-Giang province established in minimal salt media containing lactate mix and arsenate, showed the presence of a yellow precipitate (assumed to be arsenic trisulfide, As_2S_3) which indicated arsenate reduction (Figure 8). The cultures were also able to grow in the presence of nitrate as the sole electron acceptor (data not shown). Third generation transfers of these three enrichment cultures were selected for establishing dilution to extinction agar 'shake' tube cultures yielding individual colonies.

Figure 8: Enrichment cultures in minimal salt media.



Individual colonies grown in agar 'shake' tubes (Figure 9) were picked and designated strains G (from rice soil sample 1), E (from rice soil sample 2) and C (from maize soil sample) (Table 5). These isolates were used for strain identification, genetic analysis and monitoring arsenate reduction by HPLC. The purity of the isolates obtained was confirmed by T-RFLP fingerprinting (data not shown). The strains G, E and C were cultured anaerobically on minimal salt media, aerobically on MacConkey agar plates and

under aerobic and anaerobic conditions in Luria broth (Figure 10). The three strains showed very slow growth in minimal salt media when incubated under anaerobic conditions but grew overnight in rich Luria broth both aerobically and anaerobically. On MacConkey plates, strain G had pale weak growth while strain E and C produced bright pink colonies.

Figure 9: Soft agar ‘shake tubes’ containing individual colonies of samples.

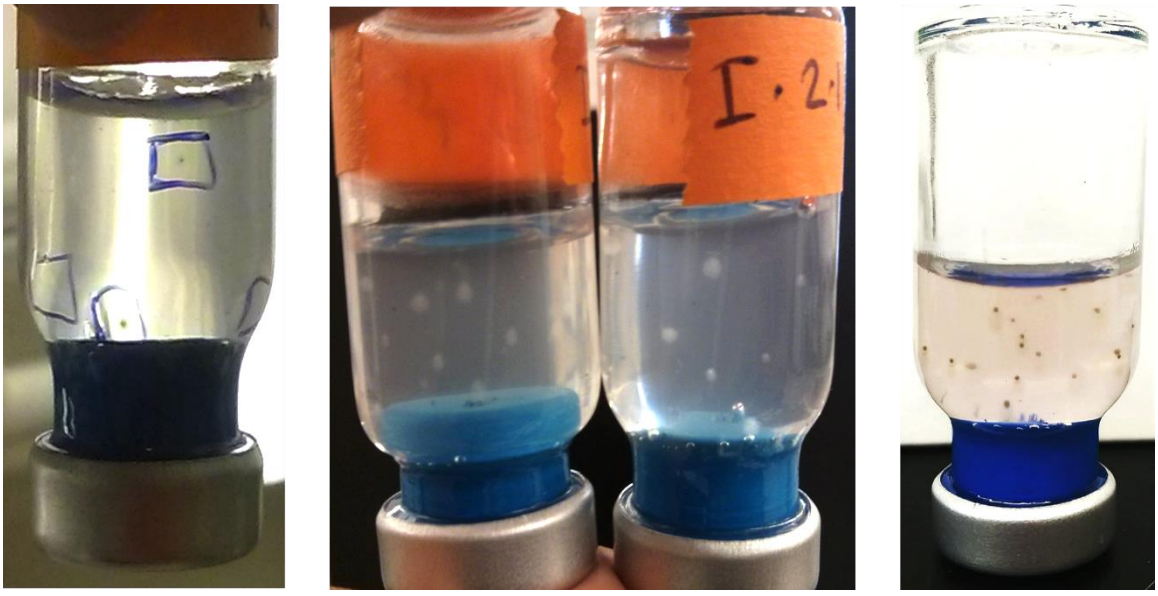
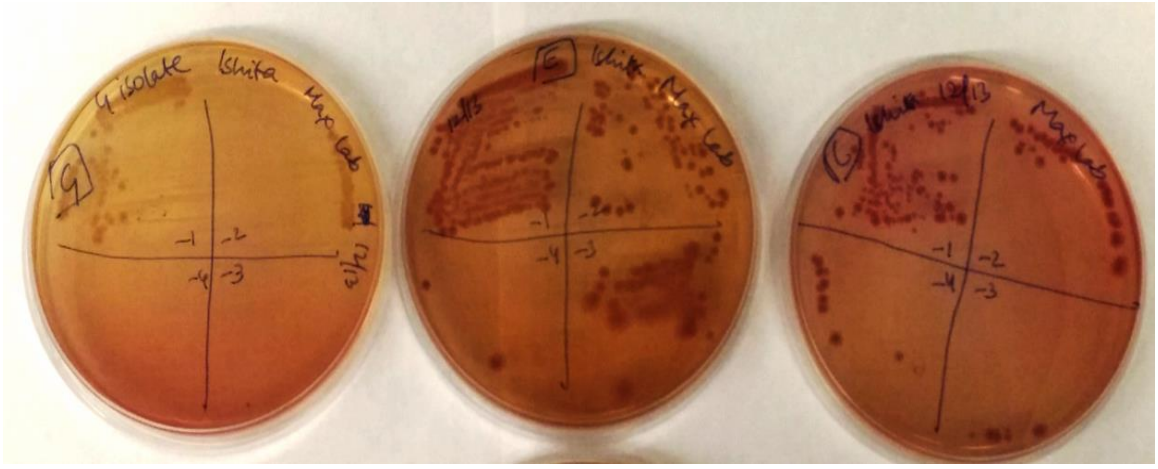


Figure 10: Isolates streaked on MacConkey agar plates.



Transformation of Arsenic

HPLC was used to monitor the reduction of arsenate to arsenite by strains G, E and C set up in replication aerobically and anaerobically in liquid cultures. The detection and separation of arsenite and arsenate was achieved well with the phosphate buffer (Figure 14). For strain G, arsenate was completely reduced to form 1.8 mM arsenite both aerobically and anaerobically. For strain E, 5 mM arsenate was reduced to 3.5 mM aerobically, while 1.2 mM and 0.6 mM arsenite was formed aerobically and anaerobically respectively. For strain C, under aerobic conditions, 5 mM arsenate was reduced to 2.9 mM to form 0.8 mM arsenite, while under anaerobic conditions 5 mM arsenate was reduced to 0.5 mM to form 1.8 mM arsenite. Arsenate was reduced to arsenite in all three cultures as shown in Figure 11 - 13.

Figure 11: Arsenate reduction to arsenite by strain G incubated aerobically and anaerobically in Luria broth.

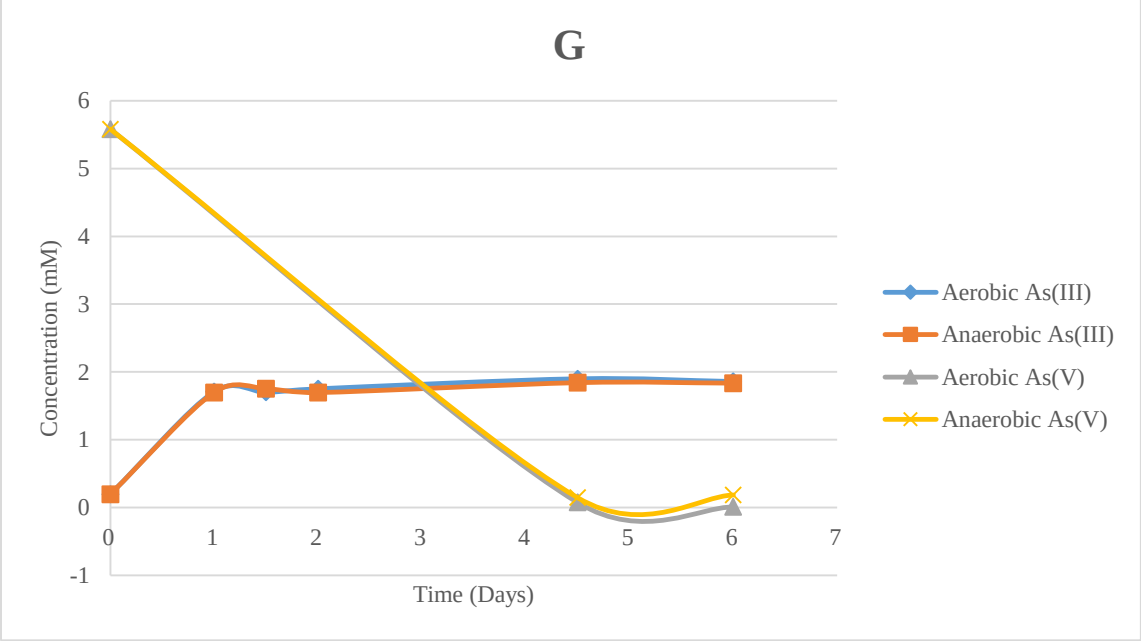


Figure 12: Arsenate reduction to arsenite by strain E incubated aerobically and anaerobically in Luria broth.

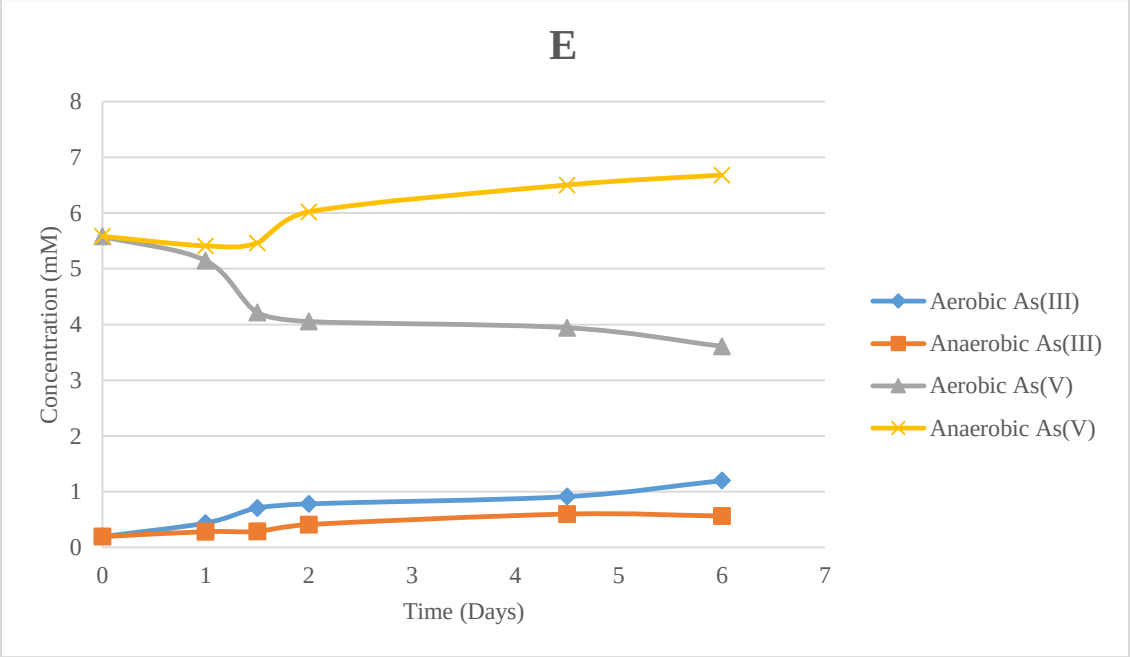
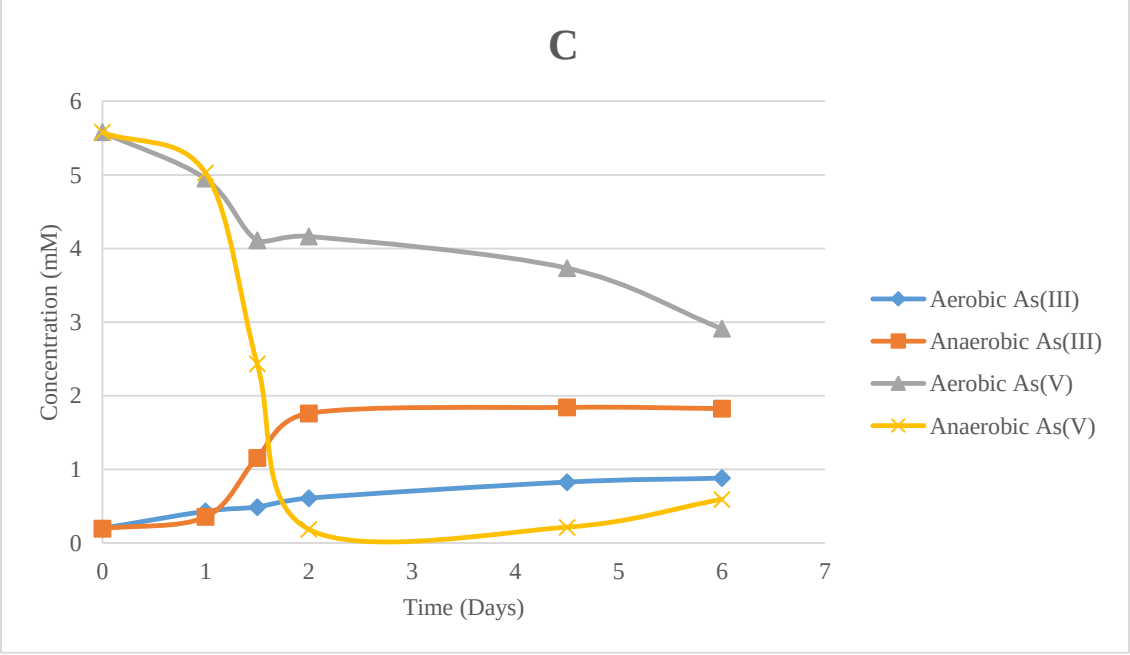


Figure 13: Arsenate reduction to arsenite by strain C incubated aerobically and anaerobically in Luria broth.



Apart from arsenite and arsenate, some other peaks appeared and gradually increased in area over the time of incubation. These could not be quantified due to lack of standards. The chromatograms obtained were compared to previously published papers on arsenic species detection in different samples using HPLC (Gailer and Irgolic 1994, Ellwood and Maher 2003, Ronkart et al. 2007, Huang et al. 2008, Conklin et al. 2013, Maher et al. 2015). The unknown peaks appear to be methylated arsenic species DMA and MMA, and thus arsenite formed from reduction of arsenate is further methylated to these forms (Figure 16 – 18).

Figure 14: HPLC chromatogram of 5 mM arsenite and arsenate standard using ammonium hydrogen phosphate eluent.

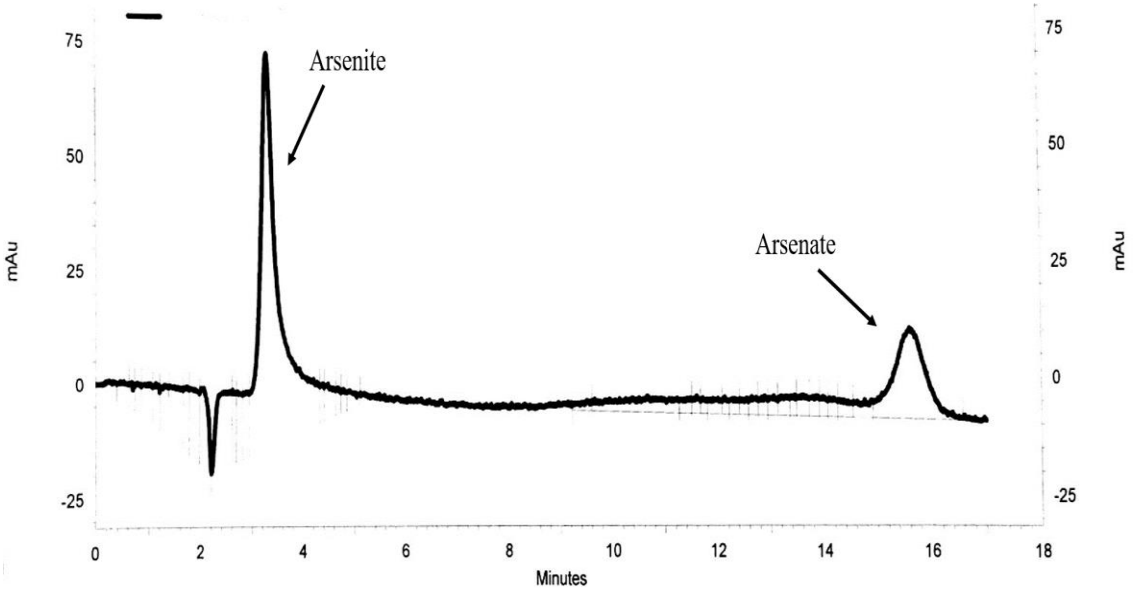


Figure 15: HPLC chromatogram of Control sample containing 5 mM arsenate without bacterial inoculation.

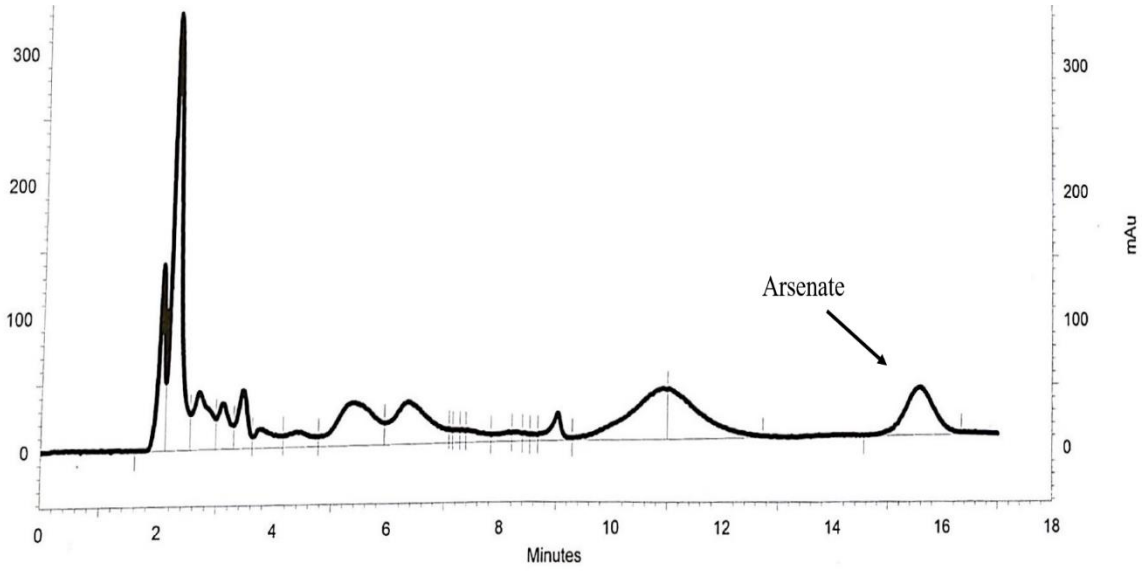


Figure 16: HPLC chromatogram of strain G after 6 days of aerobic incubation.

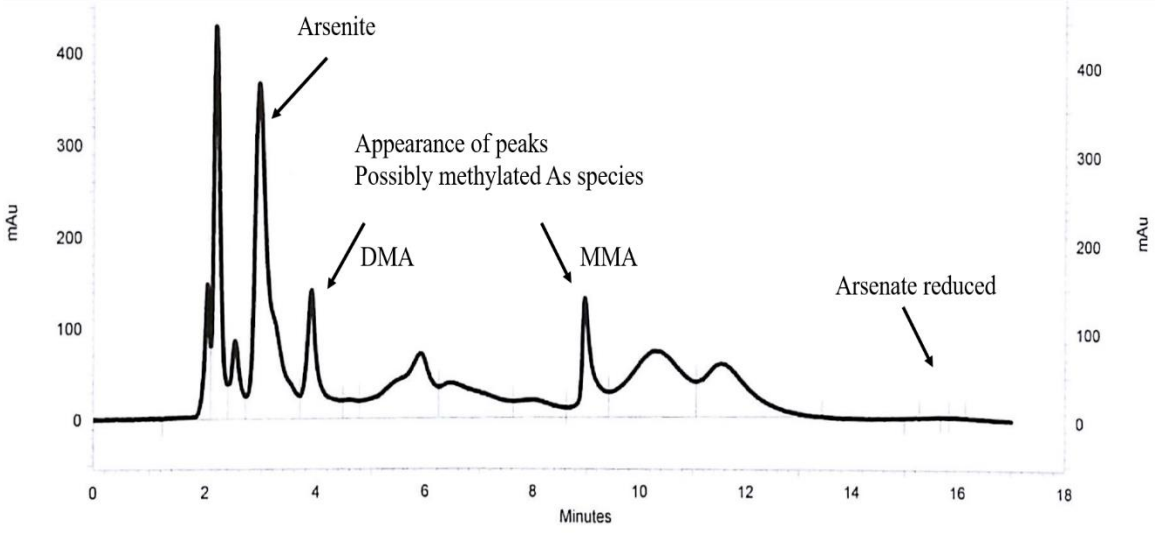


Figure 17: HPLC chromatogram of strain E after 6 days of aerobic incubation.

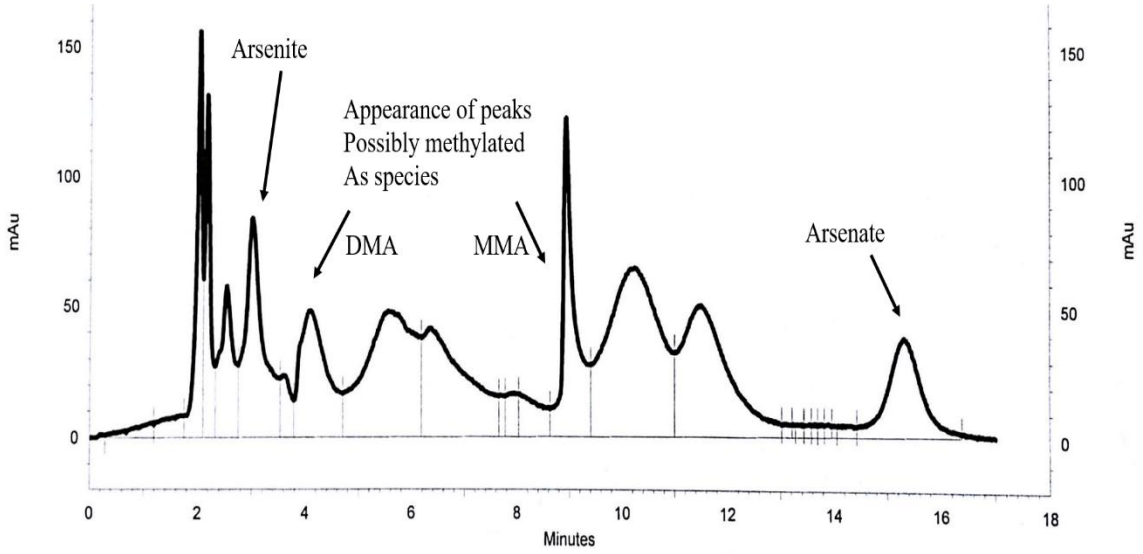


Figure 18: HPLC chromatogram of strain C after 6 days of aerobic incubation.

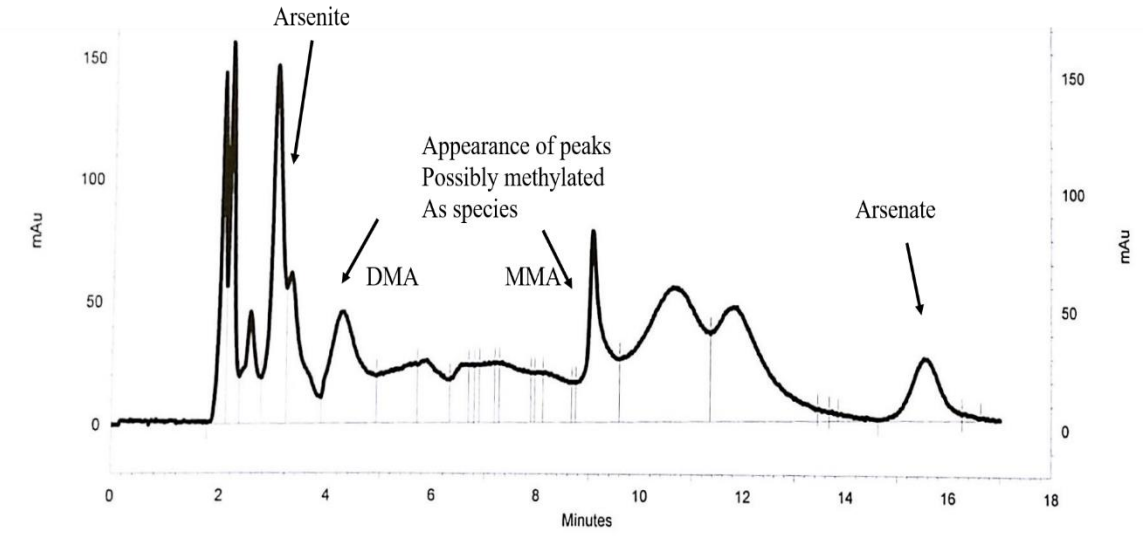
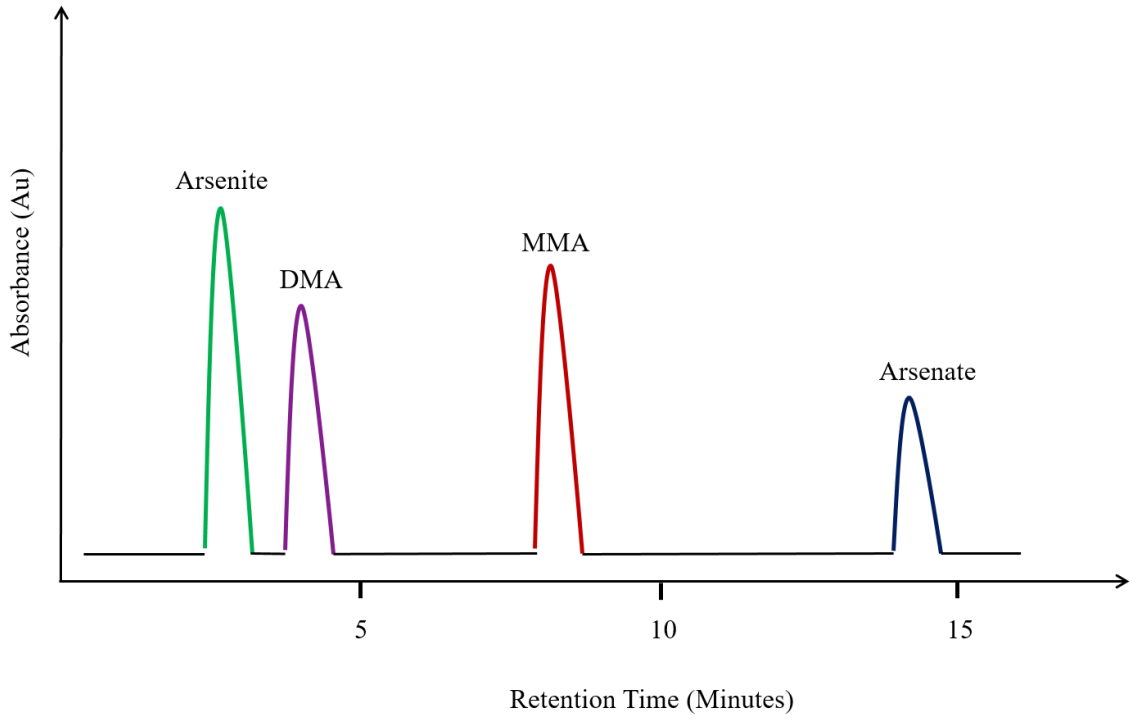


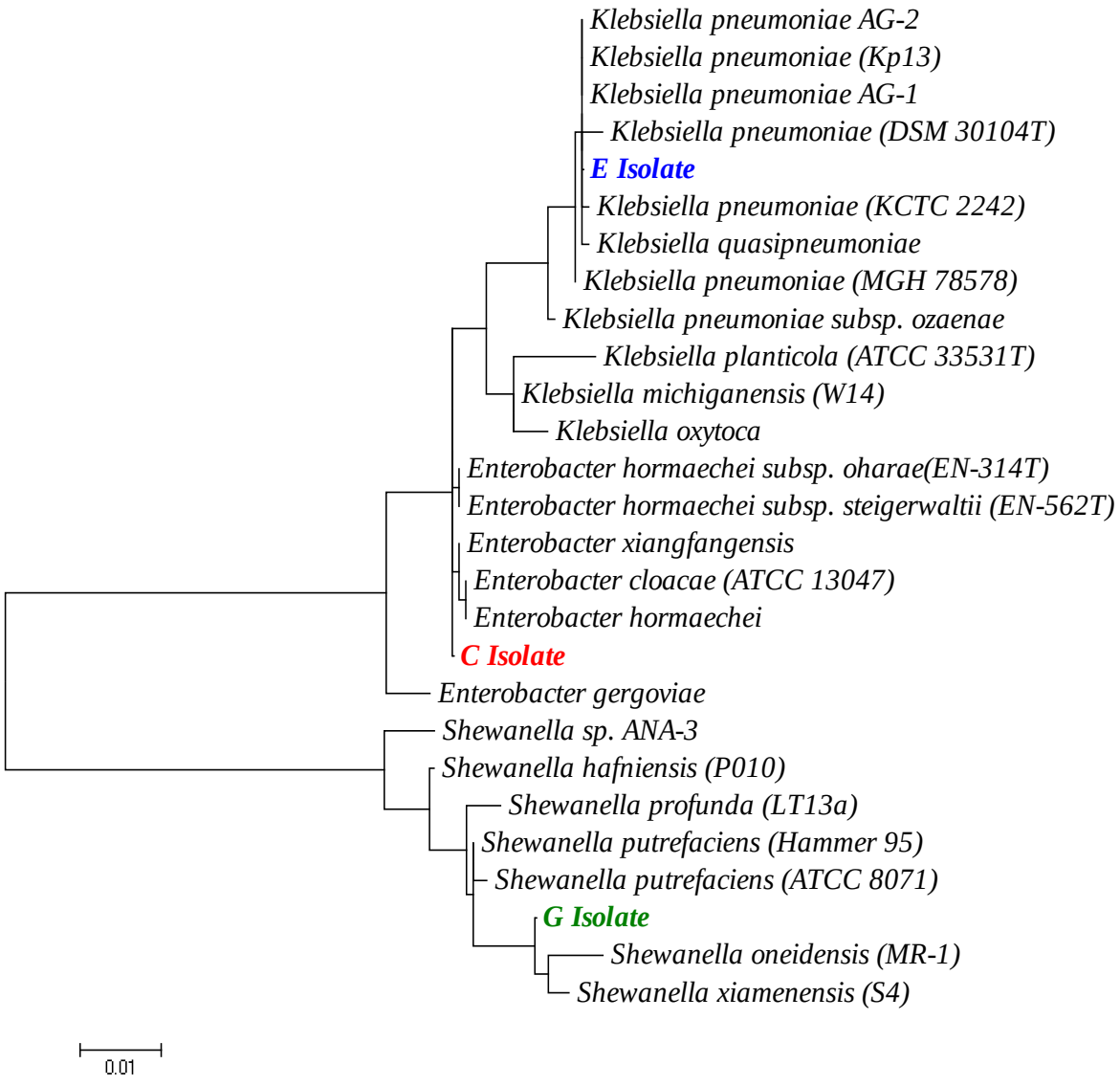
Figure 19: HPLC chromatogram redrawn from previously published papers (Gailer and Irgolic 1994, Ellwood and Maher 2003, Ronkart et al. 2007, Huang et al. 2008, Conklin et al. 2013, Maher et al. 2015) containing arsenic species for comparing and identifying the methylated species.



Strain Identification

The microorganisms responsible for arsenate reduction were identified by 16S rRNA gene analysis. Based on 16S rRNA gene analysis by BLAST and phylogenetic analysis in MEGA 6, strain G clustered closely with *Shewanella oneidensis*, strain E falls with *Klebsiella pneumoniae*, and strain C clustered together with *Enterobacter hormaechei* (Figure 20).

Figure 20: Phylogenetic tree showing relationship of strains G, E and C with other closely related microorganisms based on sequence similarities of the 16S rRNA gene. The sequences were aligned using ClustalW (Larkin et al. 2007) in MEGA 6 and the tree was constructed form 1196 unambiguously aligned positions using the Neighbor joining method in MEGA 6.



Genetic Characterization

To identify the presence of *ars* genes for arsenate respiration (*arrA*), reduction (*arsC*), arsenite transport (*acr3, arsB, arsR*) and methylation (*arsM*) in the chromosomal and plasmid DNA of the three samples, PCR amplification was done using different primer sets. Primer pair for the *arsR* gene was designed based on the gene present in the *Klebsiella pneumoniae* DSM 30104 strain genome, using the Primer design tool on the NCBI website.

Ars genes were detected in the chromosomal and plasmid DNA of the three isolates (G, E and C). For *Shewanella* sp. strain G, *arsC, arsB, arsR* as well as *arrA* was detected in the chromosomal DNA and *arsR* was detected in the plasmid DNA. For *Klebsiella* sp. strain E, *arsB, arsR* and *arsM* was detected in both chromosomal and plasmid DNA of the organism, while *arsC* was only detected in chromosomal DNA. For *Enterobacter* sp. strain C, *arsC, arsR* and *arsM* was detected in both chromosomal and plasmid DNA whereas *arsB* was detected only in the chromosomal DNA. The correct amplified product sizes for the genes were obtained (Daware and Gade 2015, Achour et al. 2010, Meng et al. 2011): 400bp – *arsC*, 750bp – *arsB*, 210 – *arsR*, 850bp – *arsM*. The amplicon size was not stated for *arrA* gene (Malasarn et al. 2004). The results are shown in the Figures 21 - 23. Negative results and controls for all the strains and genes are shown in Figures 24-29.

Figure 21: Key genes encoding for arsenic transformation in *Shewanella* sp. Amplified bands are of the *ars* operon for arsenate reduction (*arsC, arsB, ars R*) and *arrA* (respiration) in chromosomal and plasmid DNA.

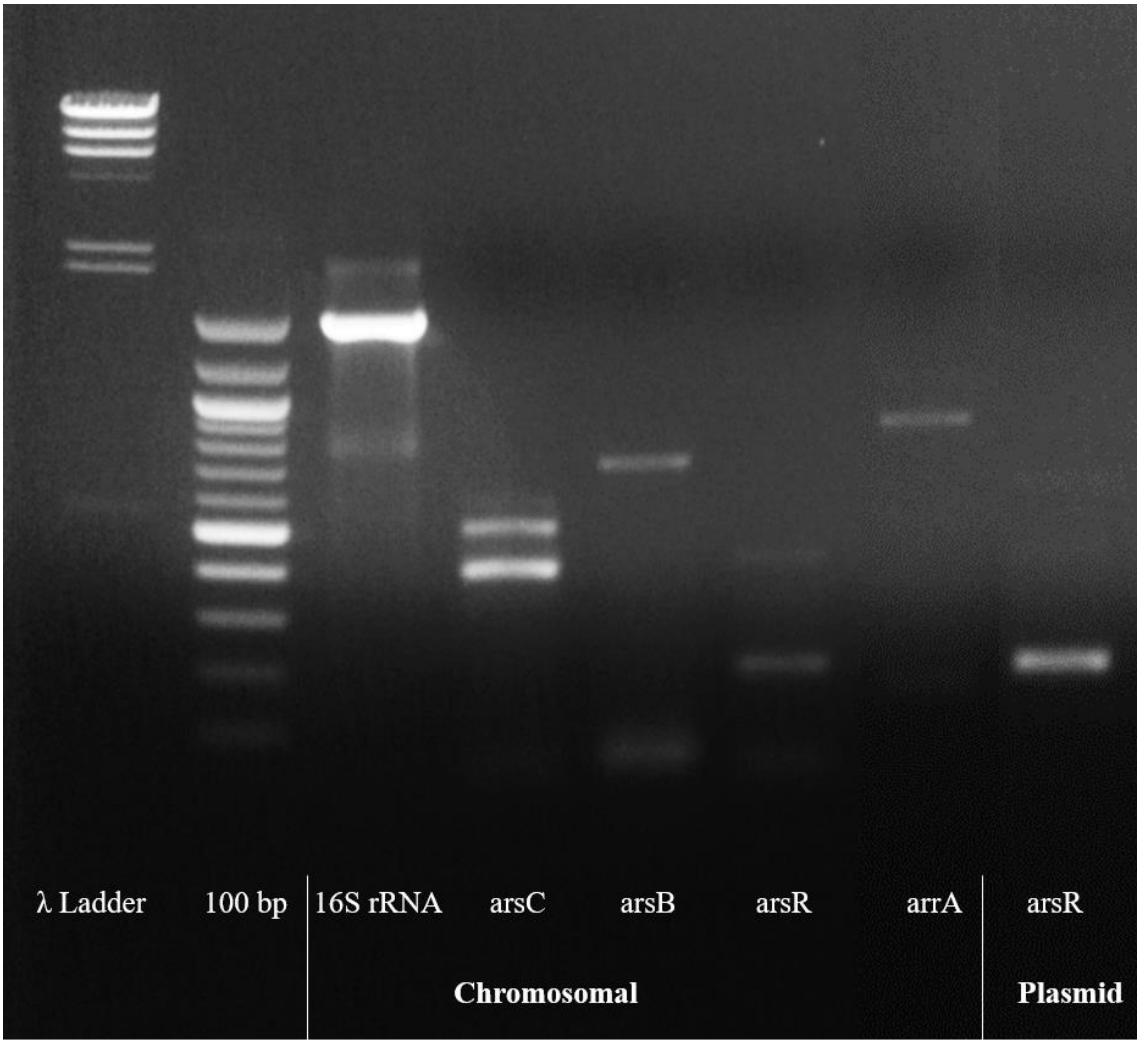


Figure 22: Key genes encoding for arsenic transformation in *Klebsiella* sp. Amplified bands are of the *ars* operon for arsenate reduction (*arsC, arsB, ars R*) and *arsM* (methylation) in chromosomal and plasmid DNA.

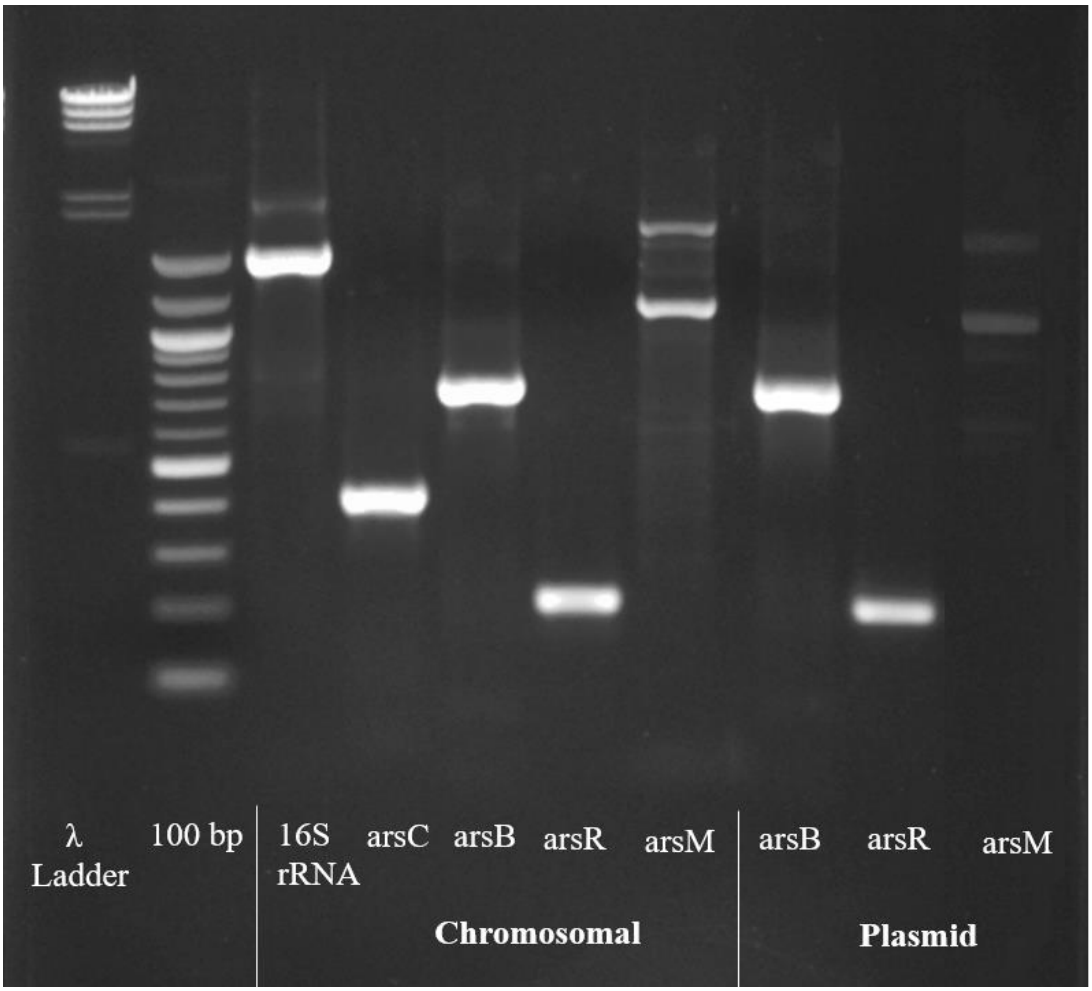
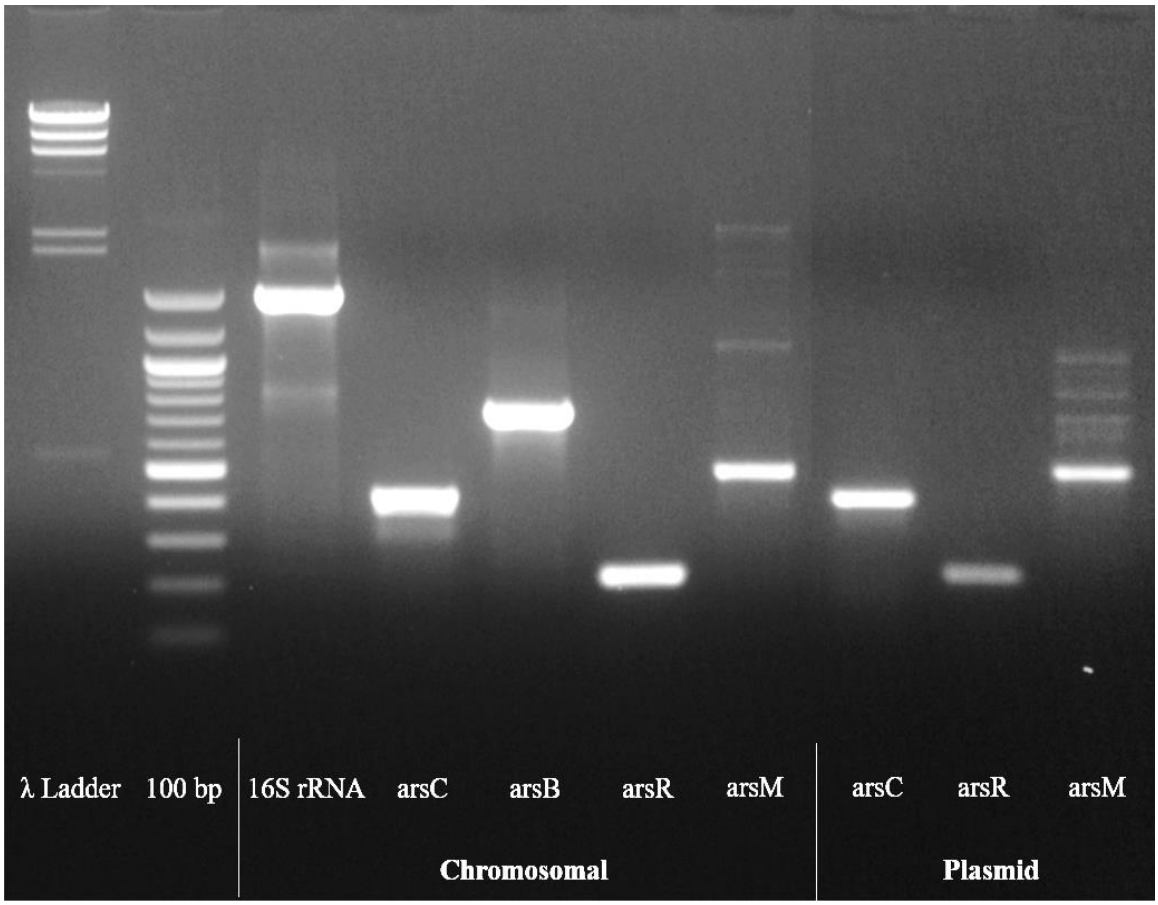


Figure 23: Key genes encoding for arsenic transformation in *Enterobacter* sp. Amplified bands are of the *ars* operon for arsenate reduction (*arsC, arsB, ars R*) and *arsM* (methylation) in chromosomal and plasmid DNA.



DISCUSSION

Isolation and Identification

In this study, three distinct arsenate reducing strains were isolated from rice paddy and maize soil irrigated with arsenic contaminated water from the Mekong River delta in Vietnam. The G, E and C isolates showed high 16S rRNA gene sequence similarity and closely clustered with *Shewanella oneidensis*, *Klebsiella pneumoniae* and *Enterobacter hormaechei*, respectively. Previous studies conducted in our lab on these soil samples correspond with these results. Subramaniam (2013) conducted initial experiments on arsenate and selenate respiration by the microbial community active in these soils along with obtaining a community fingerprint through T-RFLP analysis. Mishra (2013) performed similar studies on the same samples and also identified two isolates responsible for arsenate and selenate respiration, *Klebsiella pneumoniae* strains AG-1 and AG-2. Experiments on selenate respiration were not carried out in this study, however two new strains apart from *Klebsiella pneumoniae* were isolated and PCR amplification of the *ars* operon was done for the three strains.

Arsenic resistance is widespread within the *Enterobacteriaceae* (Cervantes et al. 1994, Diorio et al. 1995). Genome sequences in JGI and NCBI databases show the presence of the *ars* operon in *Enterobacter*, *Klebsiella* and *Shewanella* species. Chaturvedi and Pandey (2014) examined the sequence similarity of the *arsC* protein in *Gammaproteobacteria* for phylogenetic analysis and explain the significance of *Enterobacteriaceae* in arsenic toxicity. Su et al. (2012) isolated an arsenic tolerant strain LSJC7 which showed high similarity to *Enterobacter cloacae* subsp. *dissolvens* and *Enterobacter hormaechei* from an antimony mine-tailing site in Hunan, China. Abbas et

al. (2014) isolated strains similar to *Enterobacter* sp. and *Klebsiella pneumoniae* which could tolerate arsenic levels of 300 mg/L and 370 mg/L, respectively. Liao et al. (2010) isolated arsenic resistant *Enterobacter* sp. strains from arsenic-rich groundwater in Taiwan. Selvi et al. (2014) cultured strains tolerating up to 200 ppm arsenic similar to *Enterobacter asburiae* and *Enterobacter cloacae* from Tamil Nadu, India and also detected the *arsC* gene in their isolates. Daware and Gade (2015) characterized the *arsC* gene, quantify arsenate biotransformation and explain the arsenic tolerance mechanism in *Klebsiella pneumoniae*.

Arsenic respiration and reduction has been extensively studied in *Shewanella* sp. ANA-3 which contains both the *ars* operon for arsenate detoxification and *arr* gene for arsenate respiration (Saltikov et al. 2002). The *ars* system is active under both aerobic and anaerobic conditions, while the *arr* gene is expressed only during anaerobic growth (Saltikov et al. 2005). Drewniak et al. (2015) characterized the dissimilatory arsenate respiratory biotransformation by *Shewanella* sp. O23S isolated from ancient gold mine waters rich in arsenic and other metals. *Shewanella* sp. O23S is a facultative anaerobe which utilized arsenate as a terminal electron acceptor and lactate as an electron donor and produces a yellow precipitate of arsenic sulfide (As_2S_3). Huang et al. (2011) studied the effects of *Shewanella putrefaciens* on arsenite and arsenate adsorption on goethite, ferrihydrite and hematite which in turn has implications in arsenic uptake in the soil rhizosphere. They explained that there are competitive interactions between adsorbed arsenic on the iron oxyhydroxide surfaces and functional moieties (phosphoryl and carboxylate groups) present on bacterial cell surfaces and exopolysaccharides (EPS) which ultimately causes arsenate solubility and thus mobilization in the rhizosphere.

Physiology

The arsenate reducing isolates were initially grown anaerobically in minimal salts media with arsenate as the sole electron acceptor and a mixture of electron donors (lactate, acetate, pyruvate and propionate). Arsenate was reduced for detoxification and no respiration on arsenate was observed, the presence of minimal nutrients and anaerobic conditions resulted in very slow growth of these cultures. The three isolated strains G, E and C were members of the Enterobacteriaceae in the genera *Shewanella, Klebsiella* and *Enterobacter*, respectively. It is possible that these facultative anaerobes fermented the electron donors while detoxifying by reducing arsenic. The isolates grew overnight in rich media Luria broth aerobically and anaerobically. The bright pink colonies of *Klebsiella* and *Enterobacter* strains on MacConkey agar are indicative of their fermenting nature, while the *Shewanella* strain grew poorly showing its weak almost non-fermenting trait.

Arsenic resistance can be both plasmid or chromosomal borne. The R773 *E. coli* plasmid has been well studied for arsenic resistance (Hedges and Baumberg 1973, Gladysheva et al. 1994). These isolates exhibited the presence of both chromosomal and plasmid *ars* genes, which indicates that their ability to reduce and methylate arsenic is not only conferred to the organism due to plasmid acquisition. Correct amplicon sizes obtained by the amplification of *ars* genes in these isolates, as described by Achour et al. (2010), Meng et al. (2011), Daware and Gade (2015) and as evaluated from the primer designing tool for *arsR*, also verifies the positive PCR results.

HPLC analysis using the ammonium phosphate buffer displayed good detection and separation of arsenic species in the samples. Arsenite and arsenate were identified and quantified by standards but the other methylated arsenicals could not be quantified due to

the absence of standards. Qualitative presence of these methylated arsenicals was done by comparing to previously published HPLC chromatograms (Gailer and Irgolic 1994, Ellwood and Maher 2003, Ronkart et al. 2007, Huang et al. 2008, Conklin et al. 2013, Maher et al. 2015) to the chromatograms obtained during arsenate reduction monitoring by HPLC. During aerobic and anaerobic growth, it appears that peak areas of DMA and MMA increase and become prominent. The presence of the *arsM* gene based on PCR amplification shows the potential for arsenite methylation in these cultures. Methylated arsenicals are readily incorporated in rice crops and grains, making arsenic contamination in agricultural soils a major problem causing acute arsenic exposure. Shariatpanahi et al. (1983) identified arsenate methylation to DMA, MMA and TMA in *Enterobacter* sp., while Maeda et al. (1992) observed arsenic biomethylation and some degree of bioaccumulation in *Klebsiella oxytoca.* The increase in arsenate levels in anaerobically grown E isolate culture was unexpected. However, Butt and Rehman (2011) demonstrated the ability of *Klebsiella pneumoniae* and *Klebsiella variicola* to oxidize arsenite to arsenate, the PCR amplification of the arsenite oxidizing *aox/aio* gene was not conducted. The *arr* gene was detected by PCR amplification in isolate G, only a faint band was visible. Non-specific primer amplification may have occurred or these primers were not correct for *arr* gene amplification for this strain. Either way it cannot be determined for sure whether arsenate reduction for this isolate was respiratory or for detoxification. Such gene analysis of the *ars* operon and the identification of *Shewanella, Klebsiella* and *Enterobacter* species in context with arsenic resistance and mobilization has not been done before for these soils in Vietnam.

CONCLUSIONS

In summary, enrichment culture and cultivation approach combined with molecular techniques was employed to analyze the microbial composition of the Mekong delta agricultural soil contaminated with high levels of arsenic. These microorganisms are known to play a critical role in arsenic biogeochemical cycling, so it is important to identify and characterize the microbial community which may enhance the mobility of arsenic in these soils. This knowledge can then be used to mitigate contamination levels along with designing appropriate clean up methods to alleviate the problem and prevent acute and chronic exposure to arsenic. In this study, three phylogenetic and physiologically distinct bacterial strains were isolated, identified and characterized which possibly contribute to arsenic mobilization in the agricultural soils contaminated with high amounts of arsenic in the Mekong River delta in Vietnam. These isolates may also contribute to the formation of methylated arsenic species which are incorporated more efficiently in the crops than inorganic species (Carey et al. 2010, Arao et al. 2011, Jia et al. 2014, Kuramata et al. 2015). Arsenate reduction and methylation was observed both phenotypically through HPLC monitoring and genotypically through PCR gene amplification of the corresponding genes both in chromosomal and plasmid DNA of the three isolates. Further research considerations may include probing the arsenite methylation and arsenate respiration processes, examining different growth conditions to further add to characteristics of these bacterial strains, and exploring sequence variations in the *ars* operons of these cultures when aligned with described *ars* genes in related microorganisms to study possible events of lateral gene transfer and phylogenetic history.

APPENDIX

Media composition:

Table 7: The minimal salt media used to prepare enrichment cultures.

Compound amount per liter-

KCl	1.17g
KH_2PO_4	0.2g
NaCl	1.7g
NH_4Cl	0.5g
$CaCl_2$. $2H_2O$	0.1g
$MgCl_2.6H_2O$	3g
$NaHCO_3$	2.5g
Vitamin solution	5ml/L
Trace salt I	1ml/L
Trace Salt II	0.1ml/L
Resazurin	0.2ml (from 1 g/L stock)
$Na_2S.9H_2O$	0.2 mL (from 500 g/L stock)

Table 8: Vitamin solution components.

5mL/L of Vitamin solution consisted of the following components

Vitamin Mix (5ml/L) – Filter Sterilized and Anoxic Stock	Culture conc. (mg/L)	Stock conc. (g/500mL)
D-Biotin	0.1	0.01
Folic Acid	0.1	0.01
Pyridoxine Hydrochloride	0.5	0.05
Thiamine Hydrochloride	0.25	0.025
Riboflavin	0.25	0.025
Nicotinic Acid	0.25	0.025
DL- Calcium Pantothenate	0.25	0.025
Vitamin B12	0.05	0.005
P-Amino Benzoic Acid	0.25	0.025
Lipoic Acid	0.25	0.025
1,4-Naphthaquinone	0.2	0.02
Nicotinamide	0.5	0.05
Hemin	0.05	0.005

Table 9: Trace Salt I components.

Trace Salt I was added at 1 mL/L concentration, containing the following components (in g/L)

$MnCl_2.6H_2O$	5
H_3BO_4	0.5
$ZnCl_2$	0.5
$CoCl_2.6H_2O$	0.5
$NiCl_2.6H_2O$	0.46
$CuCl.2H_2O$	0.3
$NaMoO_4.2H_2O$	0.1
$FeCl_2.4H_2O$	1.46

Table 10: Trace Salt II components.

Trace Salt II was added at 0.1 mL/L concentration, containing the following components (in g/L):

Na_2SeO_3	0.03
Na_2WO_4	0.08

Table 11: Universal Primers used in this study.

27F	5’- AGA GTT TGA TCM TGG CTC AG - 3’
534R	5’ – ATT ACC GCG GCT GCT GGC – 3’
704F	5’- GTA GCG GTG AAA TGC GTA GA - 3’
1100R	5’- AGG GTT GCG CTC GTT G- 3’
1522R	5’- AAG GAG GTG ATC CAN CCR CA - 3’

Restriction Enzyme used in this study:

MnlI

5’ C C T C N ↓ 3’
3’ G G A G N ↑ 5’

Figure 24: PCR gel image of 16S rRNA gene amplification for strains G, E and C chromosomal and plasmid DNA. No amplification was seen in the plasmids and negative control.

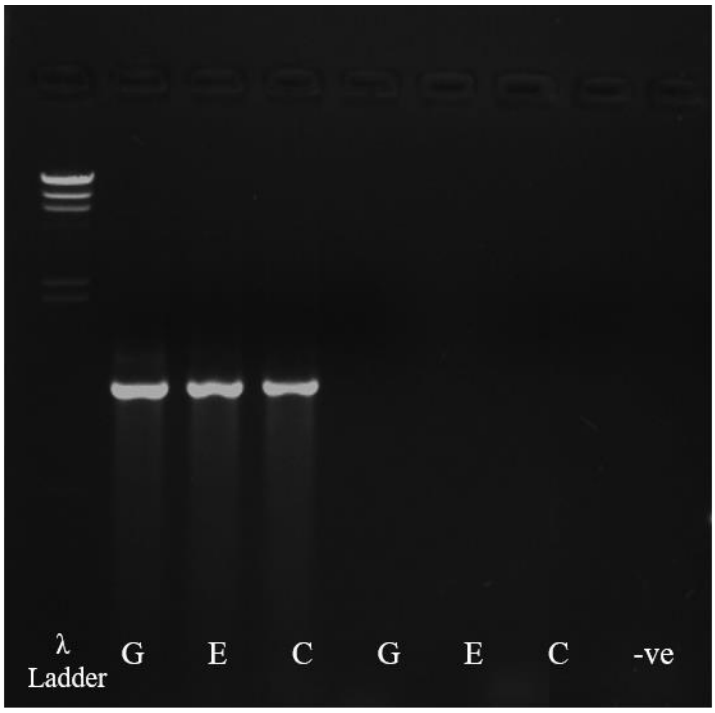


Figure 25: PCR gel image of *arsC* gene amplification for strains G, E and C chromosomal and plasmid DNA. No amplification was seen in strains G and E plasmids and negative control.

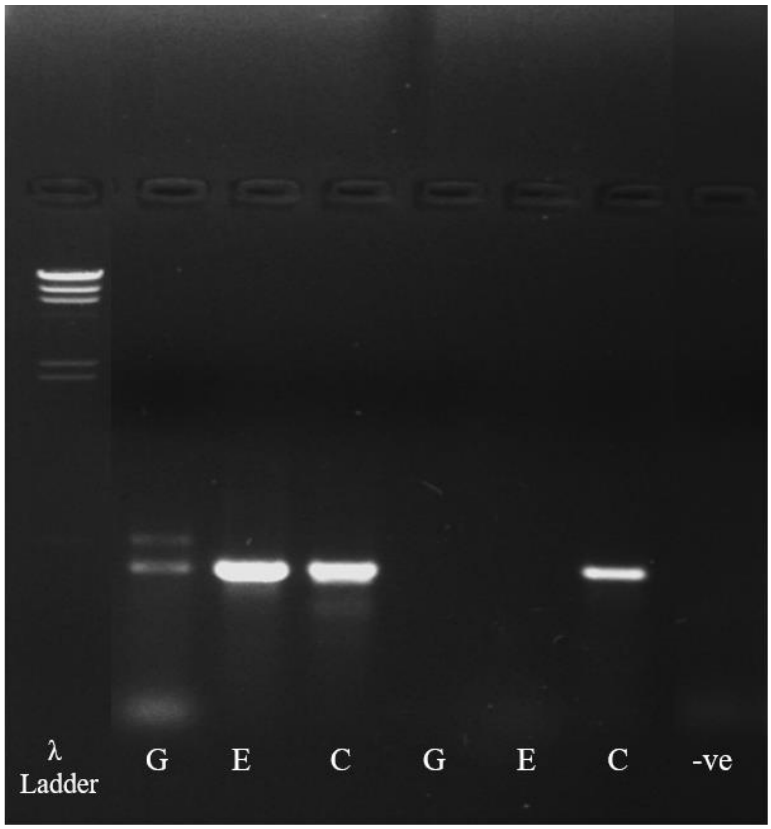


Figure 26: PCR gel image of *arsB* gene amplification for strains G, E and C chromosomal and plasmid DNA. No amplification was seen in strains G and C plasmids and negative control.

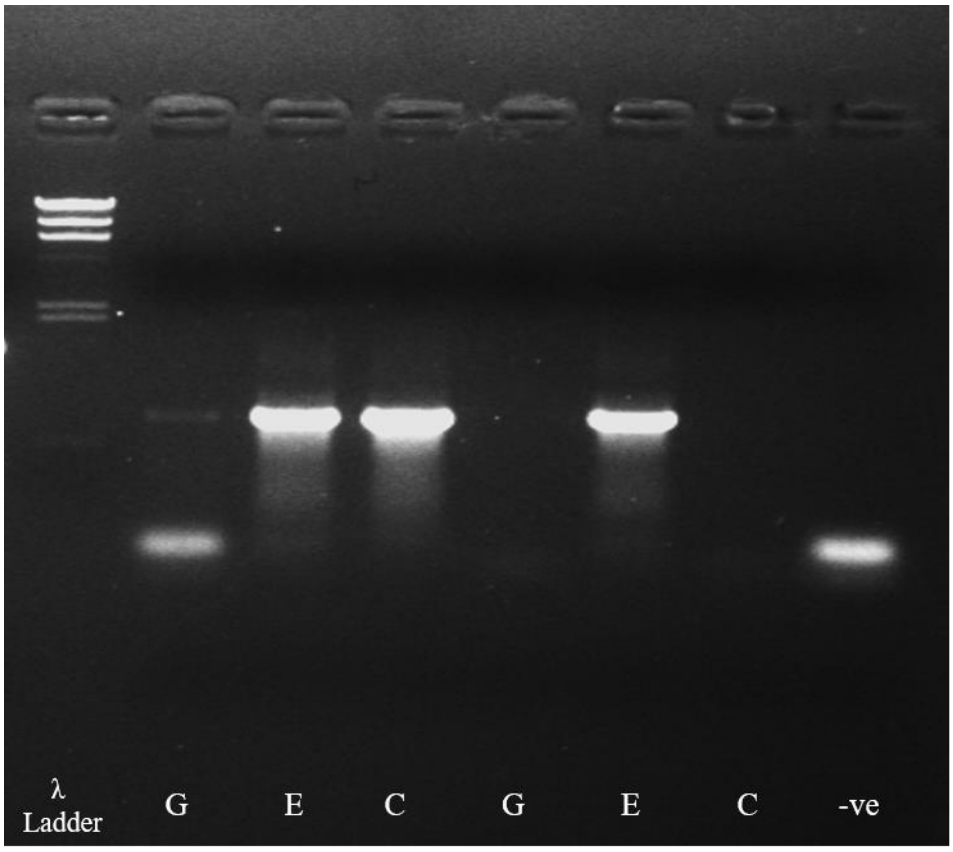


Figure 27: PCR gel image of *arsR* gene amplification for samples G, E and C for chromosomal and plasmid DNA. No amplification was seen in negative control.

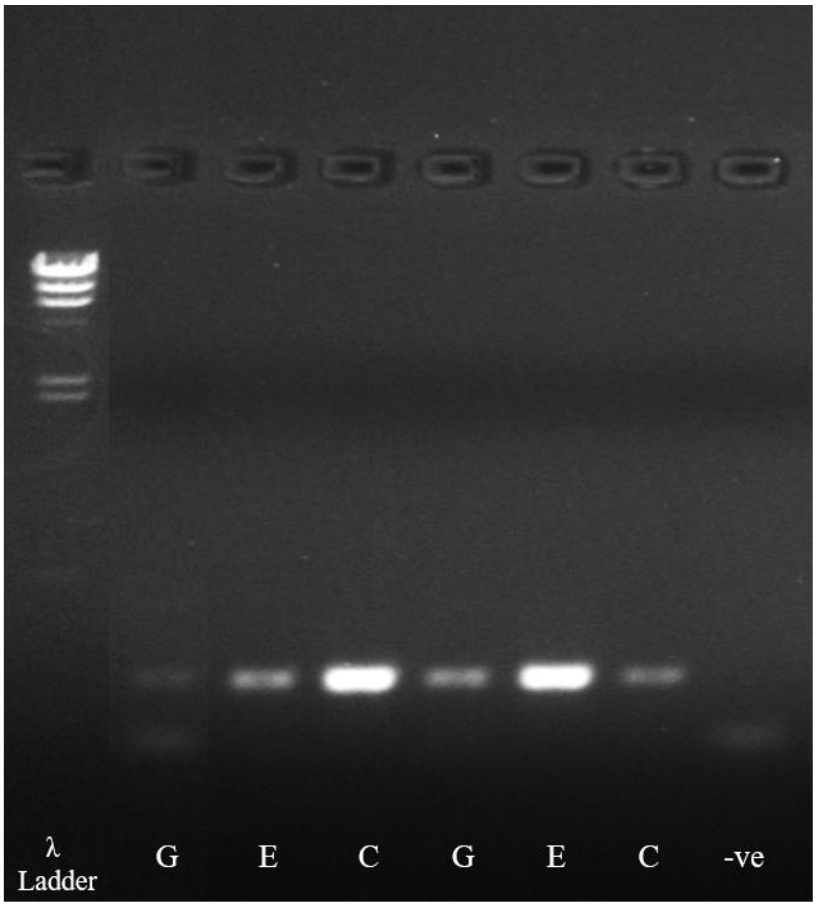


Figure 28: PCR gel image of *arsM* gene amplification for samples G, E and C for chromosomal and plasmid DNA. No amplification was seen in strain G chromosomal and plasmid DNA, and negative control.

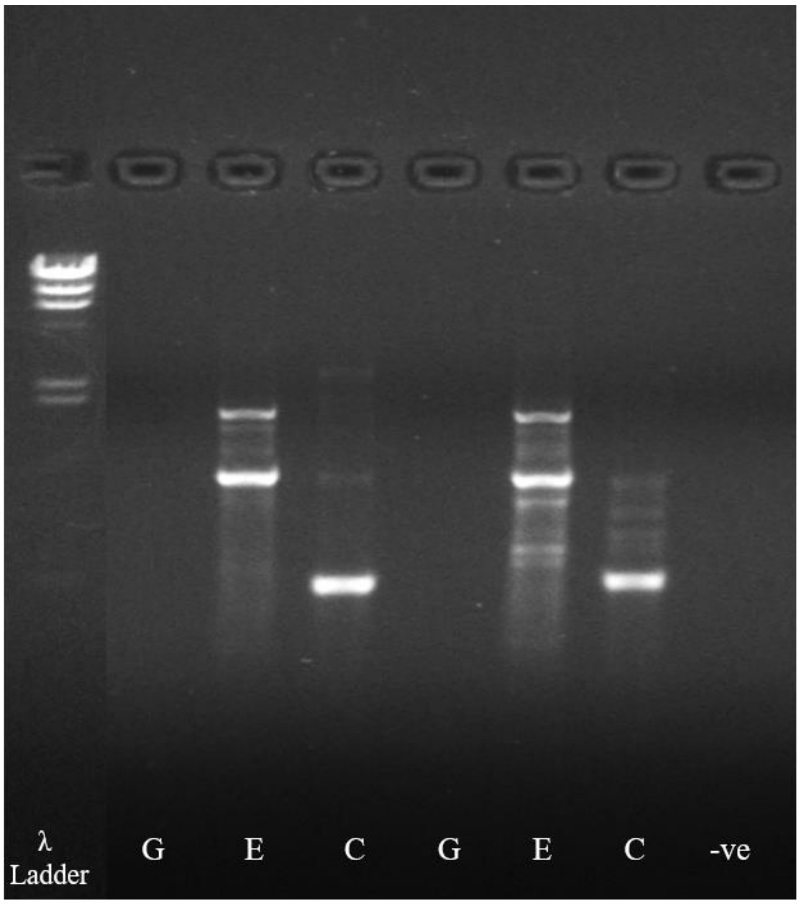
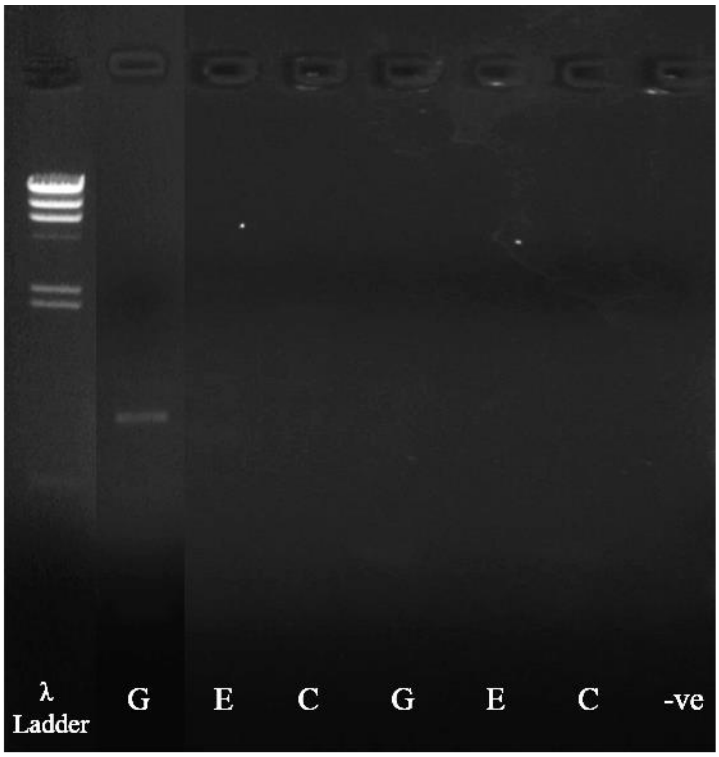


Figure 29: PCR gel image of *arrA* gene amplification for samples G, E and C for chromosomal and plasmid DNA. No amplification was seen in strains G plasmid DNA, E and C chromosomal and plasmid DNA, and negative control.



***Shewanella* sp. strain G 16S rRNA gene sequence**

ATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGC
AGCACAAGGGAGTTTACTCCCTGAGGTGGCGAGCGGCGGACGGGTGAGTAAT
GCCTAGGGATCTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAAT
ACCGCATACGCCCTACGGGGGAAAGGGGGGGACCTTCGGGCCTTCCGCGATT
GGATGAACCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGC
GACGATCCCTAGCTGTTCTGAGAGGATGATCAGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGG
AAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAA
GCACTTTCAGTAGGGAGGAAAGGGTGAGTCTTAATACGGCTCATCTGTGACG
TTACCTACAGAAGAAGGACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
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TTTGTTAAGCGAGATGTGAAAGCCCTGGGCTCAACCTAGGAATAGCATTTCG
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GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGA
CAAAGACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGTCTACTCGGAGTTTGGTGTCTTGAAC
ACTGGGCTCTCAAGCTAACGCATTAAGTAGACCGCCTGGGGAGTACGGCCGC
AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACAG
AAGACTGCAGAGATGCGGTTGTGCCTTCGGGAACTGTGAGACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCCTATCCTTATTTGCCAGCACGTAATGGTGGGAACTCTAGGGAGACT
GCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCT
TACGAGTAGGGCTACACACGTGCTACAATGGCGAGTACAGAGGGTTGCAAAG
CCGCGAGGTGGAGCTAATCTCACAAAGCTCGTCGTAGTCCGGATTGGAGTCT
GCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCC
ACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAG
TGGGCTGCAAAAGAAGTGGGTAGCTTAACCTTCGGGGGGGCGCTCACCACTT
TGTGGTTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCCTAGGGAACCTGC

***Klebsiella* sp. strain E 16S rRNA gene sequence**

GAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAA
GTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGT
GAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGT
AGCTAATACCGCATAATGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCA
TGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCA
CCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAC
TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAA
TGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGT
TGTAAAGCACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTGTCGA
TTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG
TAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGC
AGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGC
ATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGT
AGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCC
CCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCC
TTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTAC
GGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACAT
CCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGT
GCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGG
AGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATG
GCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAG
CGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGG
AGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGA
ATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT
GGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTAC
CACTTTGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAACCGT

***Enterobacter* sp. strain C 16S rRNA gene sequence**

CATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGG
TAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAAT
GTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAAT
ACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATC
GGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGC
GACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACA
CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGC
AAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAG
TACTTTCAGCGGGGAGGAAGGTGATGAGGTTAATAACCTCGTCAATTGACGT
TACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACG
GAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT
CTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAA
ACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTG
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CCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG
CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGC
AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAG
AACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTG
CCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTT
ACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACC
TCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCT
GCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCC
ACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAG
TGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTT
TGTGATTCATGACTGGGGTGAAGTCG

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