CHARACTERIZATION OF THE ARSENATE AND SELENATE REDUCING BACTERIAL COMMUNITY OF AGRICULTURAL SOILS OF THE MEKONG RIVER DELTA, VIETNAM

By

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A Thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Masters of Science

Graduate Program in Microbial Biology

Written under the direction of

Dr. Max M. Häggblom

And approved by

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New Brunswick, New Jersey

October, 2013

ABSTRACT OF THE THESIS

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Arsenic and selenium occur as metalloids in the Earth's crust and microorganisms can utilize the oxyanions of arsenic and selenium as terminal electron acceptors for respiration in the process of dissimilatory reduction. In this study the microbially mediated reduction of selenium and arsenic in agricultural soils of the Mekong River delta, Vietnam was investigated. By employing culture dependent enrichment setup and terminal restriction fragment length polymorphism (T-RFLP) community fingerprinting, we have been able to show the microbially mediated reduction of As and Se oxyanions in agricultural soils and sediments of staple crops such as rice and cabbage. An arsenate and selenate reducing bacterium belonging to the Enterobacteriaceae was isolated from the rice soil irrigated by ground water. Microbially mediated transformations of As and Se may modulate the toxicity of these elements and change their mobility and bioavailability, which affects their translocation and accumulation in

plants. This study is providing preliminary information on the role of bacteria in As and Se biotransformation in agricultural soils of the Mekong river delta.

ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Max M. Häggblom, for all his guidance and mentoring over the past two years. He truly advised me through the process of how to “do science” and insisted on how to ask key research questions and then how to go about answering them. He has a great knowledge of microbial ecology and microbiology. I would always remember the scientific lessons learnt from him throughout in my life and career.

I would like to thank members of the Dr. Häggblom’s Lab, Dam Thuy Hang, Tong Liu, Tiffany Louie, Isabel Horna Gray, Nora Lopez, Dr. Suman Rawat, and Seoyan Sohn. Thanks for all the help; it was great being here with you guys. Bavani Subramanian and I worked on few experiments together for this compilation. I extend my sincere thanks to Dr. Vien M. Duong from Cantho University, Vietnam who provided us the samples for this study. I would like to thank Adam Mumford in Dr. Lily Young’s Laboratory for helping me with HPLC analysis and Joanne Theisen, Madhavi Parikh in Dr. Nathan Yee’s Lab for help with Ion chromatography. Also, I would like to thank Lora McGuiness who guided me through the Molecular Biology protocols for our samples and Dr. Kerkhof in giving valuable advice. I would also like to thank my husband Ruchir for his support and patience throughout my study schedule and child Anvit for helping me sail through this educational endeavor. Last but of utmost importance is blessings from my Parents and Lord Shiva for building patience and faith in me.

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CHAPTER 1-INTRODUCTION

The most important geochemical reactions mediated by microbes in the soil and sedimentary environments are the reduction of terminal electron acceptors such as oxygen, nitrate, Mn (IV), Fe (III), sulfate, and carbon dioxide. A measurement of the rate of these redox reactions provides the most direct determination of predominant processes in a given environment. Microorganisms use various oxyanions of these elements as terminal electron acceptors to support anaerobic growth and this process is known as dissimilatory reduction (Table 1.2). The toxic elements such as Se and As studied in this project are present in groundwater, soil and sediments and pose a threat to wildlife as well as humans. In the redox tower (Table 1.1), the conversion of selenate to selenite has a redox potential of 440 mV and arsenate to arsenite has redox potential of 60 mV. Reduction of Se (VI) to Se (0) is important in the precipitation of selenium in contaminated aquifers and may be important in manipulating the remediation of contaminated environments and waste streams. Selenate is usually present in trace concentrations and is geochemically and environmentally important as its dissimilatory reduction removes toxic selenate (and selenite) from water and results in formation of non toxic, insoluble elemental selenium. On the other hand, arsenic is readily solubilized from toxic oxyanion arsenate to another toxic oxyanion arsenite by arsenate reducing microorganisms (Oremland and Stolz, 1999). Groundwater contamination of arsenate and selenate in Southeast Asia has lead to various diseases and health concerns (Van Geen et al., 2003).

Chemical pair	*E'0* (mV)
O_2/H_2O	+818
SeO_4^{2-}/SeO_3^{2-}	+440
NO_3^-/NO_2^-	+430
Fe^{3+}/Fe^{2+}	+100
$HAsO_4/H_3AsO_3$	+60
SO_4^{2-}/HS^-	-220
CO_2/CH_4	-244
S^0/HS^-	-270

Table1.1. Electrochemical potential of some environmentally important redox pairs (Adapted from Oremland et al., 2004)

Reactions	$\Delta G°(kJmol^{-1})$
$\frac{1}{2} O_2 + H_2 \longrightarrow H_2O$	-237.2
$2/5 NO_3^- + H_2 + 2/5 H^+ \longrightarrow N_2 + 6/5 H_2O$	-240.1
$HAsO_4^{2-} + H_2 + 2H^+ \longrightarrow H_3AsO_3 + H_2O$	-162.4
$2FeOOH(a) + H_2 + 4H^+ \longrightarrow 2Fe^{2+} + 4 H_2O$	-182.5
$SO4^{2-} + H_2 + 1/4H^+ \longrightarrow HS^- + H2O$	-48.0
$\frac{1}{4} HCO^{3-} + H_2 + 1/4 H^+ \longrightarrow \frac{1}{4} CH_4 + 3/4 H_2O$	-43.9
$\frac{1}{2} HCO^{3-} + H_2 + 1/4 H^+ \longrightarrow \frac{1}{4}$ Acetate$^-$ $+ H_2O$	-36.1

Table 1.2. Overview of several H_2-consuming terminal electron accepting processes and their Gibbs Free energies under standard conditions. (Adapted from Heimann et al., 2007)

ARSENIC

Arsenic and its Inorganic forms are classified as carcinogens (Rosen,1971). Arsenic first described by Albertus Magnus in 1250 AD has the atomic number 33 and is situated in Group 15 of the periodic table. The predominant oxidation states of inorganic arsenic are +5 (arsenate: $H_2AsO_4^-$ and $HAsO_4^{2-}$) and +3 (arsenite: $H_3AsO_3^0$ and $H_2AsO_3^-$). As (V) has applications in agricultural pesticides, glass manufacturing, and Cu refining and may be present in soils and sediments under oxidizing conditions. Arsenate adsorbs strongly to mineral surfaces like ferrihydrite, whereas arsenite is much more mobile and toxic (Espino et al., 2009). Soil solution chemistry (pH and redox conditions), solid composition, As-bearing phases, adsorption and desorption, and biological transformations, volatilization, and cycling of As in soil affect arsenic chemistry (Sadiq, 1997). Arsenate in the H_2AsO_4 form is dominant under oxidizing conditions at low pH and as $HAsO_4^{2-}$ at higher pH, whereas under reducing conditions, the uncharged arsenite species $H_3AsO_3^0$ is dominant (Smedley and Kinniburgh, 2002).

SOURCES OF ARSENIC

Anthropogenic sources of arsenic are drainage from abandoned mines and mine tailings, combustion of fossil fuels (especially coal) and municipal solid waste, application of arsenical pesticides, land filling of industrial wastes, sewage sludge, river and irrigation waters, release or disposal of chemical warfare agents, manufacturing of metals and alloys, petroleum refining and

pharmaceutical manufacturing (Ning, 2002; Stolz and Oremland, 2006). Natural sources are derived from hydrothermal leaching or weathering of arsenic minerals in rocks. Arsenic is also geologic in origin, for example deriving from the sediments from the upland Himalayan catchments (Yu et al., 2003; Abul et al., 2001). In these studies, it was also suggested that the chemically reduced environment leads to high dissolved to solid ratios of arsenic. Usage of As has decreased in recent years and to better protect public health, the US-EPA declared a reduction in the Safe Drinking Water Act a Maximum Contaminant Level (MCL) for As from 50 to 10 μg L^{-1} (USEPA 2012).

Arsenic is detected at high concentrations in many drinking water wells especially in South East Asia and Bangladesh. After high As levels were detected in groundwater in the Red River delta in Vietnam in 2001, considerable research has been done in the Hanoi city and Red river delta adjacent sites. The WHO limit is 10 μgL^{-1} and three million people are currently using Red river delta groundwater with As concentrations >10 μgL^{-1} and one million people use groundwater containing >50 μgL^{-1} (Winkel et al., 2010).

BENGAL DELTA REGION CALAMITY

The As calamity in Bangladesh is well known where millions of natives are affected by As poisoning in drinking water from groundwater where concentration as high as 136 μgL^{-1} have been reported (Van Geen et al., 2010). Excessive use of groundwater for irrigation may also release As into groundwater. There is extreme variability in the groundwater As content between boreholes in

Bangladesh and West Bengal only 100 m apart. The hydrology of these flood-plain aquifers that contain paddy rice fields dug ponds, irrigation channels and intensified groundwater pumping for irrigation (Harvey et al., 2006; Yu et al., 2003). In Bangladesh, arsenic occurs naturally in alluvial sediments and is mobilized into the groundwater (Nordstrom, 2002). Arsenic binds to iron oxyhydroxide in the sediments of Bangladesh and due to the reducing nature of groundwater, As is released into groundwater (Ravenscroft, 2001). Dissolved organic carbon may then form aqueous iron and/or arsenic complexes, or inhibit sorption onto mineral surfaces. Fe (II) can then either catalyze formation of secondary minerals such as magnetite or goethite which can mobilize arsenic. If dissolved Fe (II) diffuses into suboxic zones, it could be re-precipitated by reaction with oxygen, resulting in fresh surface sites for arsenic to be re-adsorbed (Nickson et al., 1998; Ahuja, 2008).

Release of As in the groundwater can be combination of i) As being present in aquifers, ii) As mobilized from the sediments to the groundwater, and iii) As transported and flushed in the natural groundwater circulation (Cutler, 2006).

ARSENATE/ARSENITE TOXICITY

High concentrations of As in drinking water (< 50 μgL^{-1}) have been reported in countries including China, Japan, Poland, Nepal, Taiwan, Vietnam, Bangladesh and India (Yu et al., 2003). Rural Bangladesh derives drinking water from shallow tube wells drilled into alluvial and deltaic deposits and south east Bangladesh

have median As concentration in groundwater of 136 μgL^{-1}. Over 60 % of wells in seriously affected districts contain As concentrations exceeding 1000 μgL^{-1} (Winkel et al., 2010). Slow poisoning by arsenic on a regular basis in these regions causes arsenicosis. The symptoms are changes in the color of a person's skin, keratosis of the palms and soles of feet and many forms of cancers (Smith et al., 1992). Another kind of condition is "black foot disease" where the blood vessels in the extremities become diseased and gangrene develops (Chlu, 1994). Organoarsenicals such as monomethylarsinic acid and dimethylarsonic acid also exist in the natural environment, but their toxicities are lower than the inorganic arsenic species as arsenate and arsenite. They are also reported to have been detected more in surface waters than groundwater (Ning, 2002; Pontlus, 1992).

Arsenate is highly toxic to plants as it uncouples phosphorylation and inhibits phosphate uptake and at higher concentrations. As interferes with plant metabolic processes and inhibits growth. Arsenate is adsorbed onto soil particles and its adsorption is dependent on Al and Fe oxides, clay content of soils and soil pH. Microbes can oxidize arsenite to arsenate in aerobic zones and also reduce arsenate to arsenite in flooded anoxic aquifers or sediments. Plants and microbes may methylate As(V) or As(III) forming dimethylarsenic acid (DMAA) and monomethylarsonous acid (MMMA) although these are not abundant in aqueous solutions compared to inorganic forms of arsenic (Semdley and Kinniburgh, 2002; Fendorf, 2010) . Bacteria and fungi can reduce arsenate to volatile methylarsines. Marine algae can transform arsenate to non-volatile

methylated As compounds such as methylarsionic acid and dimethylarsinic acid in sea water (Ning, 2002).

BIOGEOCHEMICAL CYCLE OF ARSENIC

The bacteria that reduce As (V) to As (III) as part of their respiration processes are known as dissimilatory arsenate reducing bacteria and couple As reduction with oxidation of short chain fatty acids such as lactate, acetate as electron donor. The respiratory arsenate reductase (Arr) from *Chrysiogenes arsenatis* and *Bacillus selenitireducens* has been purified and characterized (Krafft and Macy 1998).The respiratory arsenate reductase of *Chrysiogenes arsenatis* is a heterodimer periplasmic protein composed of two subunits (ArrA, 87 kDa and ArrB, 29 kDa). The larger subunit is a molybdopterin subunit containing an iron–sulfur center [4Fe–4S], and the smaller one contains a [Fe–S] center protein (Espino et al., 2009). Arsenate reducing bacteria can be significantly involved in arsenic mobilization through three different pathways 1) the reduction of Fe(III) to Fe(II) and subsequent reduction of As(V) to As(III), 2) the reduction of bound As(V) to As(III), or 3) the simultaneous reduction of As(V) and Fe(III). (Fig.1.1, Islam et al., 2004).

In contrast, chemoautotrophic arsenite-oxidizing bacteria oxidize arsenite to arsenate by using oxygen or nitrate as terminal electron acceptor by fixing inorganic carbon (CO_2) into cell material (Santini et al., 2000). Heterotrophic arsenite oxidizers utilize organic carbon as their source of energy and cell material (Green, 1918; Osborne and Ehrlich, 1976; Rhine et al., 2006). Arsenite

oxidation is carried out by arsenite oxidase (Aox) and is involved in resistance and energy generation (Silver and Phung, 2005). It is a molybdoenzyme, heterodimeric where the catalytic subunit (AoxB) contains the molybdenum center and a [3Fe-4S] cluster. The smaller subunit AoxA harbors a Rieske-type iron-sulfur [2Fe-2S] cluster and contains a twin arginine motif that targets the enzyme for transport to the periplasm (Cutler, 2006.)

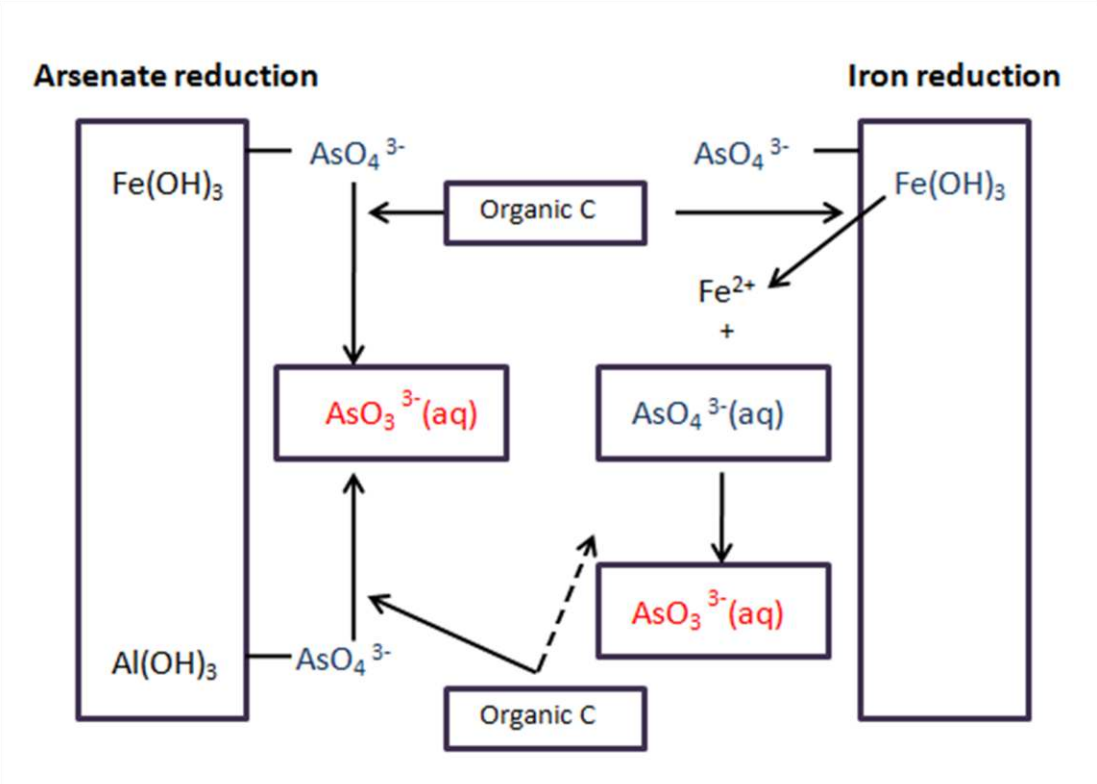


Fig.1.1. Arsenate reducing bacteria and their mechanism (Adapted from Islam et al., 2004)

SELENIUM

Selenium was first discovered by Jons Jacob Berzelius in 1818. Se has the atomic number 34, atomic mass of 78.96 and is a nonmetal with properties that are intermediate between those of the chalcogen elements adjacent to it in the periodic table, sulfur and tellurium (Mehmood, 2012). Selenium is an essential micronutrient for humans; it is a component of amino acids, selenocysteine (Se-Cys) and selenomethionine (Se-Met) and functions as a co-factor for the reduction of antioxidant enzyme, glutathione peroxidases and certain form of thioredoxin reductase. Se enters human and animal systems as selenite or selenomethionine, which are metabolized to various products including methylated derivatives. Monomethylated forms have cancer protective properties while selenides are intermediaries in these processes. Se and various selenoproteins have antioxidant effects by reducing the effect of reactive oxygen species (ROS). Se has been shown to scavenge ROS, while the glutathione peroxidase family of enzymes (GSH-PX) is capable of catalyzing reactions to remove ROS such as H_2O_2 (Phung, 2005).

SOURCES OF SELENIUM

Procurement, processing and combustion of coal for electric power production are some of the sources of selenium contamination and their solid and liquid effluents are highly enriched with selenium. Ash remaining after burning of coal for electricity is 1250 times enriched in selenium. Solid wastes from coal combustion have alkaline pH which results in dissolution of selenium anions

(selenate, selenite) on contact with water (Lemly, 2009). Coal is thus a highly hazardous source of selenium having also influence on bioaccumulation and toxic effects on aquatic life. Rainwater contains dissolved ions that can release selenium oxyanions from mineral lattices and groundwater (Welch, 1987).

SELENIUM DEFICIENCY and TOXICITY

Selenium deficiency in livestock and animals such as lambs and calves can result in nutritional muscular dystrophy, “white muscular disease”. Keshan disease and Kashin-Beck disease are caused by selenium deficiency, where Keshan disease is an endemic cardiomyopathy affecting children and women (Lollar, 2005). Other diseases associated with deficiency of selenium are severe form to less severe forms such as diarrhea, stillbirths, and reduced weight gain (Hansen, 1993).

Selenium and sulfur have similar structural and chemical properties, and in presence of excess selenium, organisms bind it instead of sulfur in the proteins. A dietary intake of 400 µg per day has been recommended by the National Academy of Sciences for adults to prevent the risk of developing toxicity (Frankenberger and Arshadz, 2001). Selenium interferes with the ability to utilize oxygen resulting in deformities in the embryo. Se damages internal organs and the respiratory system on the adults. “Selenosis” is caused by high levels of Se in blood; the symptoms include gastrointestinal upsets, hair loss, white blotchy nails, garlic breath odor, irritability, fatigue and mild lever damage (Raisbeck, 2000).

Selenium enriched Permian coal deposits causing endemic selenium poisoning was documented in the Enshi area of China where concentrations up to 6,470 mg kg^{-1} in the human hairs, blood and nail were reported (Zheng , 2012). Cereal crops (rice and maize) accounted for 65-85% of the selenium intake (Yang et al., 1983). Selenium concentration in surface waters average of 0.2-0.4 μgL^{-1} and 1400 μgL^{-1} concentrations have been reported in subsurface irrigation drainage water in the San Joaquin Valley (Ohlendorf, 1987). Selenium bioaccumulations lead to death of adult birds, dead and deformed embryos in the Kesterson Reservoir, California during 1984-1985. About 40 % of 578 nests monitored between 1983 and 1985 contained at least one dead or deformed embryo or chick (Garone, 1999). Bioaccumulation occurs by direct adsorption or by consumption of contaminated food or water in aquatic ecosystems, whereas it is by ingestion only in terrestrial environments. Selenium enters the aquatic food chain by accumulation in phytoplankton, algae, rooted vegetation and aquatic invertebrates and then it moves to higher trophic levels such as birds and predators being accumulated in organs and tissues. Excessive selenium concentrations in the diet of animals cause acute toxicity, impaired reproduction (developmental abnormalities, embryo mortality, and reduced growth or survival of young), pathological changes in tissues and chronic poisoning of adult animals (Lemly, 2003). Selenium enters the food chain through plants and its metabolic pathways are yet to be discovered in detail.

BIOGEOCHEMICAL CYCLING OF SELENIUM

Selenium is a metalloid with anionic speciation in water and the primary species are selenate (SeO_4^{2-} or Se (VI), selenite (SeO_3^{2-}, or Se (IV)) and organo-selenide e.g. selenomethionine or org-Se (III). Geologic and anthropogenic sources release mostly SeO_4^{2-} which is not reactive with particle surfaces, with bacteria converting SeO_4^{2-} to elemental Se in sediments. SeO_4^{2-}in the water column is taken up slowly, especially if competition with sulfate is involved. SeO_3^{2-} and organo-selenide are much more reactive. If any of these forms are taken up by plants and microbes, it is converted to organo-selenide. Organo-selenide is released back to the water column as these molecules are consumed where some SeO_3^{2-} is formed. Because of hundreds of years of back reaction of conversion of SeO_3^{2-} and organoselenide to SeO_4^{2-}, there is large buildup in the food web of SeO_3^{2-} and organoselenide. For example, in irrigation water in the San Joaquin River watershed in California, Se is nearly 100% in the form of SeO_4^{2-}, whereas in the downstream deltaic region SeO_3^{2-}, SeO_4^{2-} and organoselenides are in equal abundance (USGS, 1996; Ohlendorf, 1987). Se thus poses ecological risks by the buildup of potentially reactive forms in wetlands and estuaries. The oxidation of Se^0 to SeO_3^{2-} and SeO_4^{2-} in soil is biotic in nature, carried out by both autotrophic and heterotrophic organisms and occurs at slow rates (Fig. 1.2). Methylated Se compounds formation occurs in seleniferous soil, sediment, and water from Se oxyanions and organo-Se compounds. Microbes methylate Se as a protective detoxification mechanism (Oremland, 2006).

Microbially mediated bioremediation is an alternative at a pilot-scale level for the treatment of seleniferous wastewater and sediments. For example, *Enterobacter cloacae SLD1a-1*, a facultative bacterium removed added Se(VI) from concentration of 13 µM to 1266 µM up to 94.5% and can also remove Se(IV) in irrigation drainage water to form elemental Se(0) (Losi and Frankenberger,1997).

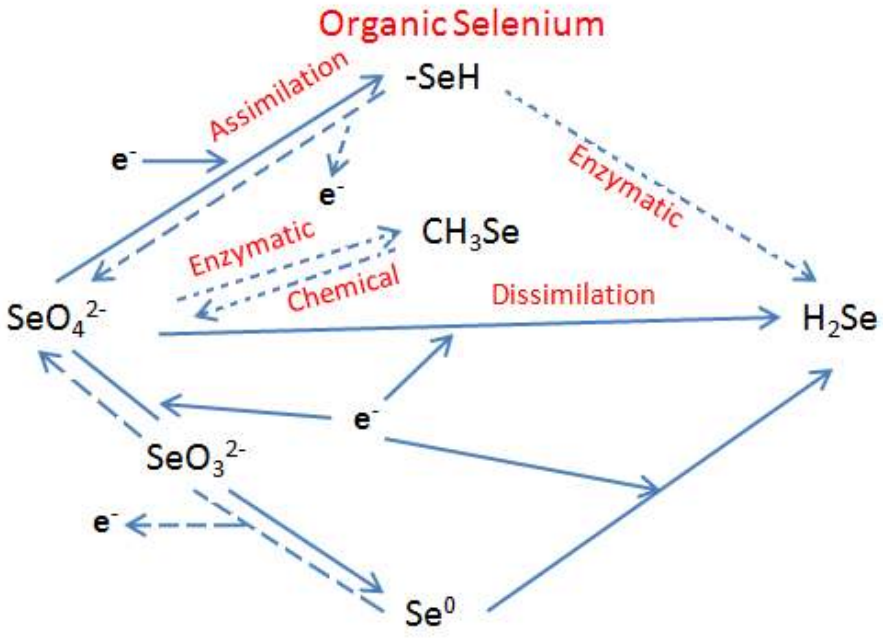


Fig. 1.2 Global selenium cycle with microbial interactions. Solid lines: reduction; dashed lines: oxidation; dotted line: no change in oxidation state of Se. (Redrawn from Barton, 2001)

DISSIMILATORY ARSENATE and SELENATE RESPIRING MICROORGANISMS

Microbes play an important role in transformation of selenium and arsenic and are ubiquitous, found in environments like fresh waters, waste water streams, soils and sediments. These microbes govern the biogeochemical cycling of Se and As mediating oxidation, reduction, methylation, demethylation and volatilization reactions (Stolz and Oremland, 1999). These elements are actively cycled and have roles in carbon mineralization although their significance in microbial ecology is recognized only recently. Lipman and Waksman (1923) found that autotrophic bacteria in soil were capable of growing with selenium and inorganic material and oxidizing selenium, but little work followed for many decades. Oremland et. al. (1989) found a novel dissimilatory selenate reduction process independent of sulfate reduction. This process is known as dissimilatory selenate reduction. Dissimilatory reduction of arsenic was first discovered by Ahmann et al., 1997. Anaerobic microorganism can utilize Se and As oxyanions as terminal electron acceptor for respiration coupled to oxidation of various electron donors. Se and As respiring microbes are ubiquitous, phylogenetically widespread and have varying metabolic and physiological characteristics (Table 1.3). *Thauera selenatis* was the first selenate respiring microbe isolated; it is a facultative anaerobe, and a member of the beta-proteobacteria. First reported as a *Pseudomonas* sp., it is also capable of respiring nitrate and oxygen (Macy et al., 1993)*. Enterobacter cloacae, Desulfovibrio desulfuricans, Thauera selenatis* and several other bacteria have been isolated which are capable of selenate

reduction. The first selenate reductase (Ser) has also been isolated, the enzyme has been purified and characterized and the genes have been cloned and sequenced (Schröder et a., 1997). Ser contains molybdenum, iron, acid-labile sulfur, and heme *b*. Microorganisms such as *Bacillus selenitireducens, Sulfurihydrogenibium subterraneum* respire selenite, another toxic intermediate in the redox reaction (Blum, Jodi Switzer, et al., 1998, Takai et al., 2003). *Bacillus beveridgei* reduces selenate and selenite whereas *Desulfurispirillum indicum* can reduce selenate, selenite, arsenate, nitrate and tellurate (Rauschenbach et al., 2011). *Seleniivibrio woodruffi* is a novel isolate of the Deferribacteriacae capable of arsenate and selenate respiration (Rauschenbach et al., 2013). Also of notable interest are the selenate reducing microbes, *Sedimenticola selenatireducens* strains *AK4OH1* and *KeOH1* isolated from Arthur Kill and Kesterton reservoir respectively, utilizing benzoic acid derivatives as electron donor and producing stoichiometric amount of selenite (Narasingarao and Häggblom, 2006) ;Knight et al., 2002).

Leptothrix sp. strain *MNB-1* and *Thiobacillus sp.*strain *ASN-1* is capable of oxidizing selenium to selenate (Dowdle and Oremland, 1998) and these reactions are relatively slower as compared to Se oxyanion reduction. The arsenite-oxidizing bacteria slowly convert arsenite to arsenate for detoxification as well as energy generation. *Alcaligenes* spp. is capable of arsenite oxidation and arsenite oxidase (Aox), a member of DMSO reductase family of molybdenum enzymes has been purified from it (Williams, 1992). The autotrophic, denitrifying, As(III)-oxidizing microbes *Azocarus sp.* strain *DAO1 and*

Sinorhizobium sp. DAO10 are also of interest mediating detoxification reaction and CO_2 fixation (Rhine et al., 2006). Strain ML-SRAO is a novel, selenate dependant anaerobic arsenite oxidizing bacterium, which is incapable of growth with only arsenite or with arsenite and lactate as carbon source (Fisher and Hollibaugh et al., 2008). A Gammaproteobacterium isolated from Mono Lake, California*, s*train *MLHE-1* oxidizes arsenite under anoxic conditions using nitrate as the terminal electron acceptor (Hollibaugh et al., 2002).

Amongst the first dissimilatory arsenate reducing bacteria are *Sulfurospirillum barnesii* strain SES-3 and *Chrysiogenes arsenatis strain BAL-1* (Stolz, 1999 and Macy et al., 1996). *Neisseria mucosa* and *Rahnella aquatilis* are the most recently isolated facultative anaerobe, capable of respiring arsenate (Youssef et al., 2009). Respiratory arsenate reductase (Arr) has been purified from *Chrysiogenes arsenatis* and *Bacillus selenitireducens* and this enzyme has been identified in *Shewanella sp*. strain ANA-3 through mutagenesis studies and in the genome of *Desulfurispirillum indicum S5* (*Rauschenbach et al., 2011*). *C. arsenatis* Arr is specific for arsenate whereas *Bacillus selenitireducens* can also reduce arsenite, selenate, and selenite (Krafft and Macy, 1998; Afkar et al., 2003).

Methylation of selenate and selenite forms volatile dimethyl selenide (DMSe), dimethyl diselenide (DMDSe) and other methylated selenium species and is a detoxification mechanism. It is carried out by *Enterobacter cloacae*, *Aeromonas* sp., *Pseudomonas* sp., *Rhodocyclustenuis* and *Rhodospirillum rubrum* produce both dimethyl selenide and dimethyl diselenide from selenate while growing

phototrophically (Ranjard et al., 2003). Methylation of arsenic was first observed in *Mathanobacterium bryantii* by McBride and Wolfe (1971). *Desulfovibrio vulgaris, Desulfovibrio gigas, Methanobacterium formicum* have been showed to produce methylated arsenic (Michalke et al., 2000).

Table 1.3. Diversity of Se (VI) and As (V) respiring bacteria

Microorganism	Terminal electron acceptor	Phylogeny	Source	Reference
Anaeromyxobacter dehalogenans	Chlorophenols, fumarate, oxygen, Se(VI) and nitrate	Epsilonproteobacteria	Small stream in Lansing, Michigan	Tiedje et al., 2002 and Yao 2010
Bordetella petrii	Se(VI) ,Nitrate	Betaproteobacteria	River sediments, Germany	Wintzingerode et al., 2001
Citerobacter freundii	Se(VI) , oxygen	Gammaproteobacteria	Stewart Lake, Utah	Frankenberger et al., 2004
Desulfurispirillum indicum	Se(VI) ,Se(IV)	Chrysiogenes	Estuarine canal, Chepauk,Chennai	Rauschenbach et al., 2011
Hafnia alvei	Se(VI) , oxygen	Gammaproteobacteria	Kafr El Dawar, industrial sediments, Egypt	Youssef et al., 2009
Pseudomonas stutzeri	Se(VI), Nitrate, O2	Betaproteobacteria	Lab contaminant	Narasingarao and Häggblom 2007
Pelobacter seleniigenes	Se(VI) , Nitrate, Fe(III), AQDS, S(0)	Deltaproteobacteria	Kearny Marsh, NJ	Narasingarao and Häggblom 2007
Salana multivorans	Se(VI), oxygen	Actinobacteria	Estuarine sediment	Friedrich et al., 2001
Sedimenticola selenatireducens	Se(VI), Nitrate, Nitrite	Gammaproteobacteria	Arthur Kill, NJ	Narasingarao and Häggblom 2006
Selenihalanaerobacter shriftii	Se(VI), Nitrate	Halaanaerobacteriales	Dead Sea sediments	Blum et al. 2001
Stenotrophomonas maltophilia	Se(VI)	Gammaproteobacteria	Seleniferous pond	Frankenberger et al., 2003
Thauera selenatis	Se(VI) ,Nitrate	Betaproteobacteria	Se-contaminated drainage water, CA	Macy 1993
Chrysiogenes arsenatis	As(V), Nitrate, Nitrite	Chrysiogenes	Gold Mine, Australia	Macy 1996
Neisseria mucosa	As(V), oxygen	Betaproteobacteria	Industrial sediments, Egypt	Youssef et al., 2009
Rahnella aquatilis	As(V), oxygen	Gammaproteobacteria	Industrial sediments, Egypt	Youssef et al., 2009

Sulfurospirillum arsenophilum	As(V), Nitrate, fumarate	Epsilonproteobacteria	Arsenic-contaminated freshwater sediments	Stolz 1999
Bacillus macyae	As(V), Nitrate	Firmicutes	Gold mine, Australia	Santini 2004
Bacillus selenatarsenatis	Se(VI), As(V), Nitrate	Actinobacteria	Glass-manufacturing plant in Japan	Yamamura 2007
Bacillus selenitireducens	Se(VI), As(V), Fumarate, Low O2, Nitrate, Nitrite, TMAO	Firmicutes	Mono Lake, California	Oremland et al., 1998
Ferrimonas kyonanensis	Se(VI), As(V), nitrate, citrate, Fe(III), S(0), thiosulfate, Mn(IV) oxide	Gammaproteobacteria	Futtsu beach, Tokyo Bay, Japan	Nakagawa 2006
Ferrimonas futtsuensis	Se(VI), As(V), citrate, Fe(III),S(0),thiosulfate, MnO2	Gammaproteobacteria	Futtsu beach, Tokyo Bay, Japan	Nakagawa 2006
Sulfurospirillum barnesii	Se(VI), As(V), Nitrate, thiosulfate, S(0), fumarate, aspartate and MnO2, TMAO, Fe(III).	Epsilonproteobacteria	Selenium contaminated fresh water marsh	Stolz 1999
Wollinella succinogenes	Se(VI), As(V), Nitrate, Fumarate	Epsilonproteobacteria	Rumen fluid	Tomei et al., 1992
Seleniivibrio woodruffi	Se(VI), As(V)	Deferribacteriacae	Wastewater treatment, Verona, NJ	Rauschenbach et al., 2013
Bacillus beveridgei	Se(VI), As(V), Se(IV), nitrate, nitrite, fumarate, Tellurate, O2.	Firmicutes	Mono Lake, California	Oremland 2009
Bacillus arsenicoselenatis	Se(VI), As(V), fumarate, Nitrate, Fe(III)	Firmicutes	Mono Lake, California	Oremland et al., 1998

STUDY SCOPE AND OBJECTIVES

The goal of this study was to understand microbially mediated arsenate and selenate reduction in different agricultural soils, that differ in their redox environment (upland vs. submerged) and the source of water (river water vs. ground water). Investigation of arsenate reduction, hence mobilization of the toxic arsenite oxyanions through the biota and selenate reduction and non-toxic form of elemental selenium precipitation from these cropped soils would give us insights into these microbially mediated reactions affecting the nutritional quality of the crops. Furthermore, characterization of selenate and arsenate reducing bacterial communities through the 16S rRNA phylogeny from the environmental DNA samples and isolation of bacteria would verify the evidence of arsenate-selenate reducing bacteria in these sites.

APPROACH

1. Detection and characterization of arsenate and selenate reducing activity by employing enrichment culture method of soil and sediments of the Mekong River delta, Vietnam.
2. T-RFLP fingerprinting of 16S rRNA genes of arsenate and selenate reducing enrichment culture will enable preliminary identification of arsenate and selenate reducing bacteria.
3. Isolation and characterization of arsenate and selenate reducing bacteria.

CHAPTER II

As (V) AND Se (VI) REDUCING ACTIVITIES IN ANAEROBIC SOIL ENRICHMENT CULTURES AND CHARACTERIZATION OF THE ARSENATE AND SELENATE REDUCING BACTERIAL COMMUNTIY

INTRODUCTION

Arsenic is a naturally occurring metalloid that is widely distributed in the environment. The predominant inorganic forms are arsenate, As (V) and arsenite, As (III) with arsenate as the most toxic form. Natural and anthropogenic sources both contribute to high amount of arsenic in environment. Arsenic has been detected at high concentrations in drinking water wells around the world such as Bangladesh, India, China, Cambodia and Vietnam (Mukhopadyay, 2002; Berg et al., 2001; Smedley and Kinniburgh, 2002).

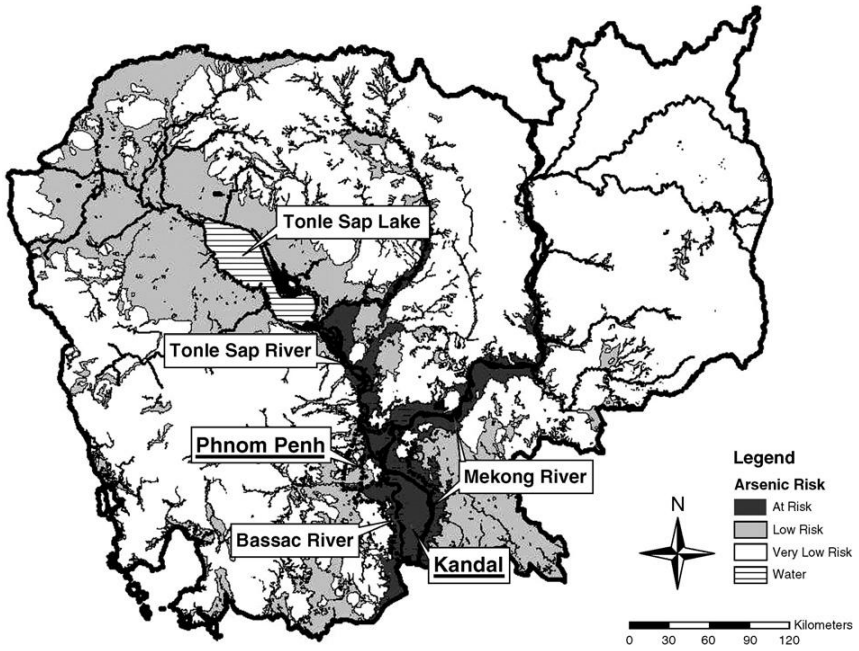


Fig. 2.1 Risk map for arsenic pollution in groundwater of Vietnam. (Berg et al., 2007)

In Vietnam, the Bassac and Mekong rivers flow through the An-Giang and Dong Tha provinces and merge into the Mekong delta flood plain (Fig. 2.1). In a study (Berg et al., 2007) of about 112 groundwater samples in the area, the arsenic concentration ranged from <1 to 845 µg L^{-1} (average 39 µg L^{-1}). Elevated arsenic levels were found in samples with pH values >7. Here arsenic release from sediments was found to be enhanced and the major cause of arsenic pollution was suggested to be the reductive dissolution. To monitor arsenic exposure of people living in the Mekong delta of Vietnam, samples of hair were randomly collected in two villages (one exposed to groundwater arsenic pollution and other having arsenic levels of <50 µg L^{-1}).The arsenic levels found in hair ranged from 0.11–2.92 mg kg^{-1} and from 1–167 µg L^{-1} in groundwater (Table 2.1). The findings of this study indicate that people of the upper Mekong River delta are chronically exposed to high arsenic levels in their drinking water.

Elements	Average Concentration
As	39 µg L^{-1}
Fe	2.6 mg L^{-1}
Mn	3.4 mg L^{-1}
NH_4^+	5 mg L^{-1}
DOC	5.3 mg L^{-1}
HCO_3^-	230 mg L^{-1}
NO_3-N	<0.25 mg L^{-1}
PO_4-P	0.33 mg L^{-1}
Cl^-	690 mg L^{-1}
SO_4^{2-}	41 mg L^{-1}
pH	6.83
Eh	24 mV
Dissolved O_2	0.29 mg L^{-1}

Table 2.1. Soil chemical composition of vietnamese Mekong delta samples: average concentrations and ranges in samples collected on July 2004 (n=112) (Berg et al., 2007)

A maximum contaminant level (MCL) of 10 µg L^{-1} of arsenic in drinking water has been adopted by most countries, which is similar to Asian diets of 50 µg kg^{-1} in grain. Upland crops, such as barley and wheat, have total arsenic levels below 50 µg kg^{-1}. However, the MCL for arsenic has not yet been regulated in many Asian countries except for China. Daily ingestion of the staple crop rice exceeds the arsenic concentrations from 50 µg kg^{-1} to up to ~400 µg kg^{-1} (Berg et al., 2007). In rice, cabbage, maize cropped soil; river water and groundwater are used for irrigation. High levels of As (39 µg L^{-1}) in hair samples have been documented in the Mekong River delta region of Vietnam (Berg et. al 2007). Rice is grown in flooded anoxic soils and is one of the staple crops. Arsenic causes problems because rice can accumulate inorganic and organic forms of arsenic and transport them to the grain. This makes arsenic bio-available for human uptake and its constant exposure causes toxicity problems (Welch, 1998).

A study of groundwater and sediment during 2007-2008 in the Mekong River delta in Vietnam revealed that 26%, 74%, and 50% of groundwater samples were above the US EPA drinking water guidelines for As (10 µg L^{-1}), Mn (0.05 mg L^{-1}), and Fe (0.3 mg L^{-1}) respectively. The range of As, Fe, and Mn concentrations in the Mekong River delta were <0.1-1351 µg L^{-1}, <0.01-38 mg L^{-1}, and <0.01-14 mg L^{-1}, respectively. Elevated levels of As were found in groundwater at sampling sites close to the Mekong River and in wells less than 60-70 m deep. An inverse relationship was found between As and Mn concentrations in groundwater. Sediment samples from An Giang and Dong

Thap had the highest As concentrations (18 mg kg^{-1} and 38 mg kg^{-1}, respectively). Arsenic in sediment occurred mainly in the poorly crystalline Fe oxide phases. It was inferred that reductive dissolution of the Fe oxide phase is not necessarily the dominant mechanism of As release to groundwater (Dang et al., 2010).

Arsenic mobilization has been studied in microcosms of Aberjona sediment where rapid dissolution of arsenic from iron arsenate was observed and 20-28% of arsenic present was mobilized. The Aberjona watershed contains several hundred metric tons of arsenic which migrated south and hence half of the arsenic occurs in dissolved forms. Strain *MIT-13,* a native fresh water arsenate reducing microbe showed much greater activity by dissolving 38% of the arsenic implying that dissimilatory arsenic reduction may contribute to arsenic flux from anoxic sediments in the arsenic-contaminated region of the Aberjona watershed (Ahmann et. al., 1997).

MATERIALS AND METHODS:

The aim of our project is to monitor both As and Se- reducing activity in agricultural soils of the Mekong River delta and to get a perspective of the microbially mediated reactions associated with these elements (Fig. 2.2). Soil enrichment cultures amended with arsenate and selenate were established in duplicate with short chain fatty acids as carbon source to mimic the *in situ* conditions of presence of humic substance and organic matter associated with the agricultural soils. Employing community DNA isolation approach, the

microbial community was characterized. DNA was extracted from both soil and enrichment cultures on arsenate and selenate and two restriction enzymes was chosen for the terminal restriction fragment length polymorphism (T-RFLP) analysis . *HaeIII* restriction enzyme recognizes GG^CC sites and *MnlI* restriction enzyme recognizes CC^TC sites and cuts best at 37° C. Till date, 16S rRNA gene analysis of arsenate and selenate reducing enrichment cultures has not been studied together from the same soils.

Soil Samples were collected in 2012 from various sites within 3-4 miles of Khanh An commune of Khanh An district of An-Giang province of the Mekong river delta in Vietnam. These sites differed in soil properties, crops, source and irrigation of water as described in Table 2.2.

Irrigation mode	**Crop cultivated**	**Sample**
River water	Rice	RW
Ground water	Rice	GW1
Ground water	Rice	GW 2
Ground water	Rice	GW 3
Ground water	Rice	GW 4
Ground water	Cabbage	GW5

Table 2.2 .Codes and details of the samples collected and used in this study

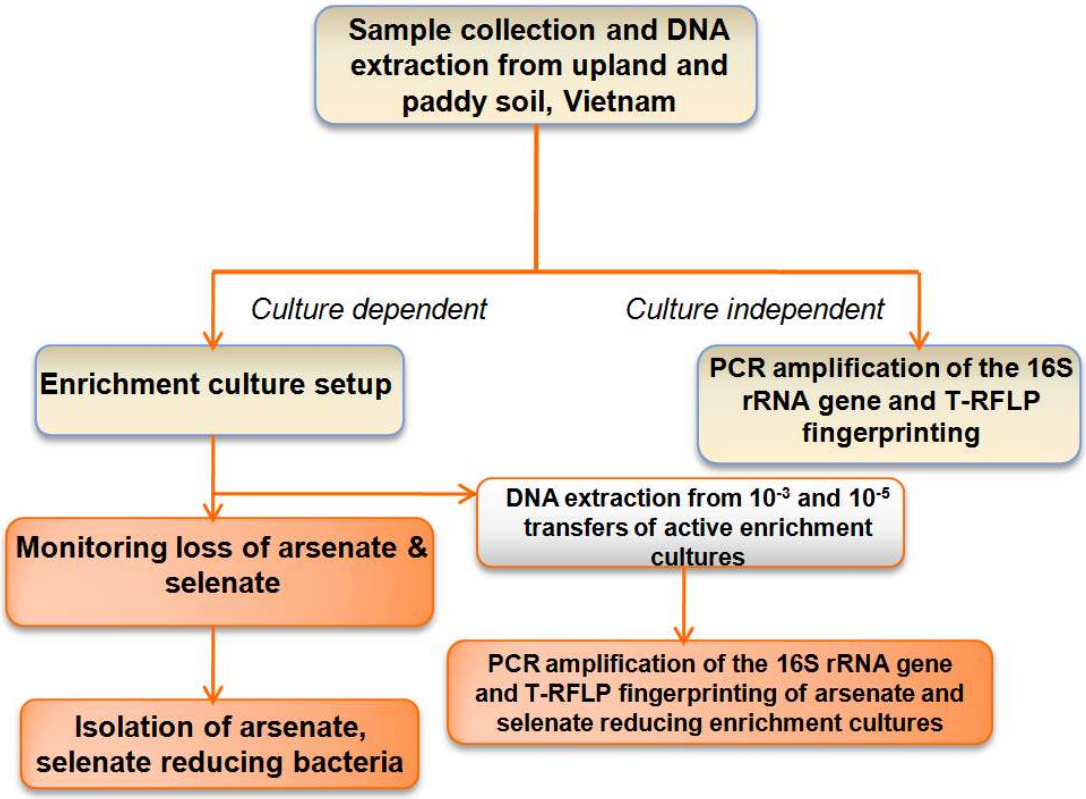


Fig. 2.2. Experimental approach

Enrichment culture setup:

The salts, 1.17 g KCl; 0.2 g KH_2PO_4; 1.7 g NaCl; 0.5 g NH_4Cl; 0.1 g $CaCl_2$. $2H_2O$; 3 g $MgCl_2.6H_2O$ were dissolved in 1 L of DI water (Table 2.3, Fig.2.3). All the chemicals used were of highest grade purity (98%± 0.5). The media was sparged under a N_2 stream for 30-45 minutes. Then it was further sparged with CO_2/N_2 for another 15 minutes followed by the addition of $NaHCO_3$ (2.5 g). The media bottle was sealed, autoclaved and cooled. Anoxic, sterile stocks of Trace Salts I (1mL L^{-1}), II (0.1 mL L^{-1}) Vitamin solution (5 mL L^{-1}), resazurin as redox indicator

(0.2 mL L^{-1}) and Na_2S-$9H_20$ as a reductant (0.2 mL L^{-1}) were added to the cooled minimal salts medium (Table 2.4). pH was checked and adjusted to 6.5-7.

Anaerobic enrichment cultures were established with 5 g to 7.5 g (wet weight) of soil aseptically added with sterile spatula to the serum bottles with 40 mL minimal salts media. Short chain organic acids: lactic acid, pyruvic acid, propionic acid and acetic acid in the final concentrations of 2.5 mM were provided as electron donor and carbon source. The final concentrations were 5 mM electron acceptor (sodium arsenate or sodium selenate respectively). Strict anaerobic technique was utilized throughout the experiment and the serum bottles were sealed with thick butyl rubber stoppers and aluminum seal crimps under a headspace of N_2-CO_2 (70:30%) and incubated at 27° C. Upon visible precipitation of As_2S_3 (yellow color) and Se^0 (red color), in respective cultures, the culture was subsequently transferred to initially 1:10 dilution into fresh medium with electron donors (2.5 mM final concentration) and electron acceptor (5 mM final concentration), and then to 1:100 dilution into fresh medium with electron donors and electron acceptor and finally to 1:100 dilution into fresh medium as described previously with electron donors and electron acceptor (Fig. 2.4). The 1-2 mL samples were withdrawn after 2 weeks of incubation and frozen at -20° C for further analysis.

High Performance Liquid Chromatography (HPLC) Analysis of As (III)

HPLC with UV detection was used to determine the As (III) concentration which is produced by the bacterial reduction of As (V) to As (III) in the enrichment

cultures. The cultures previously established were spiked again with 5mM arsenate. A Hamilton PRP-X100 column, (10 µm particle size, 250 mm long with a diameter of 4.1 mm) was used with an isocratic mobile phase of 40 mM monobasic sodium phosphate, adjusted to pH 6 with NaOH. A 10 µL volume of the As (III) standards and the enrichment culture samples were injected. As (III) was detected at 2.2 min using a flow rate of 1.0 mL/min and a wavelength of 200 nm on a Beckman System Gold 168 detector.

Ion chromatography (IC) analysis of Se (VI) and Se (IV)

Ion chromatography was used for analysis of selenate loss over time. A Dionex ICS-1000 Ion chromatograph equipped with an Ion Pac AS 9-HC anion column and ASRS-ULTRA II 4-mm suppressor was used for Se (VI) and Se (IV) ion analysis. The mobile phase of 0.954 g/L sodium carbonate in Milli-Q water was filtered through a 0.2 µm filter before use. The cultures previously established were spiked again with 5 mM selenate and a 1 mL sample of each enrichment culture was filtered through a 0.45 µm syringe filter and the supernatant was analyzed by IC.

DNA Extraction from enrichment cultures and soil samples:

The active enrichment cultures were transferred first by 1:10 dilution into fresh medium. After approximately 2 weeks, the arsenate amended enrichment cultures (GW 1, GW 2, GW 3, GW 4, GW 5 and RW) were again transferred by 1:100 dilution in to the fresh medium. After about 2 weeks, 1 mL sample were taken for extraction of DNA to characterize the selenate and arsenate reducing

bacterial phylotypes present in these enrichment cultures. After approximately 2 weeks, the GW1 arsenate enrichment culture was again transferred in 1:100 dilution.

A 1 mL aliquot of enrichment culture sample was suspended in 50 µL Solution I (50 mM glucose, 10 mM EDTA and 25 nM Tris-Cl; pH8.0 and vigorously mixed to resuspend the sample. The samples were then freeze thawed at -80°C and 37°C 4-5 times. 175 µL of solution I, 100 µL of lysozyme solution and 75 µL of 0.5 M EDTA was added and kept on roller apparatus for 5-15 min. Freshly made 50 µL 10% SDS was added followed by 800 µL (pH>7.0) of phenol-chloroform-isoamyl alcohol and vortexed for 1-2 min to form an emulsion and then spun in micro centrifuge for 3 min. The top aqueous phase was transferred then to a new centrifuge tube with 800 µL phenol chloroform isoamyl alcohol (25:24:1, v/v), vortexed and spun again for 3 min. Top aqueous layer was then transferred to a new tube and DNA was precipitated by adding 50 µL of 3.0 M sodium acetate and 1000 µL of 100% ethanol. This was then spun in a micro-centrifuge at 23,000 X g for 15 min at 4° C. The supernatant was then removed and sterile water was added to the pellet obtained. The DNA extracted was either used immediately for further downstream processing or kept at -20° C for later analysis (Kerkhof and Ward 1993; Knight et al., 1999).

DNA from the soil sediment was purified by centrifugation through a cesium chloride (CsCl) density gradient. DNA was resuspended in 400 µL of water and/ or brought to at least 400 µL in a 1.5 mL: or 2.0 mL tube. 0.54 grams of cesium chloride was mixed with DNA sample and was allowed to let the

cesium chloride dissolve completely. A mix of 100 µL water and 1 µL ethidium bromide per sample was made and 101 µL of mix was dispensed into sample. Resuspended DNA was mixed with CsCl and loaded on to Beckman TLA 120.1 by placing tubes 180 degrees to each other and then centrifuged for 5-8 hours at 55,000 rpm. The DNA band was collected from the centrifuge tube, extracted with isopropanol to remove the ethidium bromide, and then precipitated with ethanol to recover the DNA.

Gel electrophoresis and PCR reaction:

DNA was quantified by the agarose gel electrophoresis performed in TAE buffer. If DNA was present, the samples were then PCR amplified by using universal 5′-end 6-FAM-labeled 27 F primer (Lane et al., 1991) and 1100 R primer *(*Turner et al., 1999) on Gene amp PCR system 2700.

The reaction mixture consisted of genomic DNA, Taq DNA polymerase, 10X PCR amplification buffer (100 mM Tris-HCl, 500 mM KCl), dNTP, universal primers and 1.5 mM $MgCl_2$. Amplification was done by initial denaturation at 94° C for 3 minutes, followed by 30 cycles of denaturation at 94° C for 30 seconds, annealing temperature of primers was 55°C for 30 seconds and extension at 72° C for 1 minute. The final extension was conducted at 72° C for 10 minutes.

The PCR product was quantified by gel electrophoresis. 10 µL of reaction mixture was analyzed by submerged gel electrophoresis using 1.0% agarose with ethidium bromide at 8 V/cm and the reaction product was visualized under Gel doc/UV trans-illuminator.

Terminal restriction fingerprint length polymorphism (T-RFLP) fingerprinting:

The PCR product was digested for at least 6 h at 37° C. The restriction endonuclease enzymes used for this study were *MnlI* and *HaeIII*. The PCR product was vortexed and centrifuged again, and the entire 10 µL of each sample was then loaded into a well on a tray for DNA fingerprinting. DNA fingerprinting was conducted on an ABI PRISM Genetic Analyzer (Applied Biosystems). Upon completion of fingerprinting, a representative profile is generated where individual peaks ideally represent a single bacterial taxon. The size of each fragment present is indicated in base pairs, and these fragments can be matched to a database of known fragments of DNA to identify what is present with programs such as TriFLe (Junier, 2008).

Fig. 2.3. Agricultural soils sampled from An Giang Province, Vietnam, cropped with a) Cabbage, b) Rice.

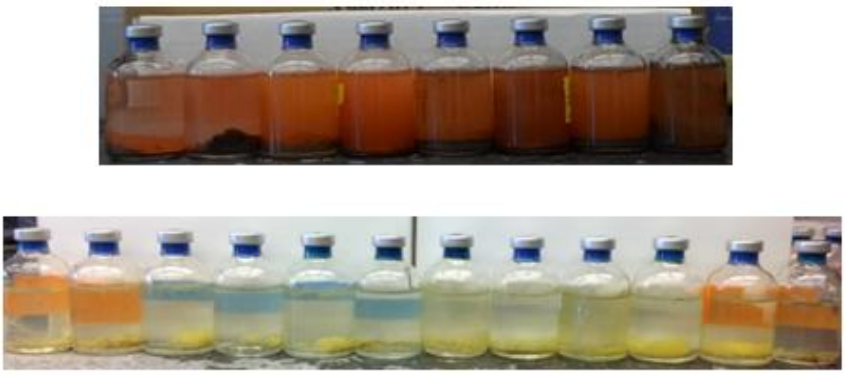


Fig. 2.4. Enrichment cultures showing a) Se (0) and b) As_2S_3 precipitation respectively.

RESULTS

The objective of these experiments was to detect the presence of arsenate and selenate reducing bacteria in agricultural the soils and sediments watered with ground water and river water of the Mekong River delta, Vietnam (Fig. 2.3). In this study, using the enrichment culture approach we have been able to validate the presence of arsenate and selenate reducing bacterial communities at all the sites.

The enrichment cultures were established in duplicate with 5-7 g wet weight sediment samples and 40 mL media with 5 mM of sodium selenate or sodium arsenate as sole electron acceptor and 2.5 mM of electron donor (sodium pyruvate, sodium acetate, sodium propionate and sodium lactate) mix. The reduction of As (V) to As (III) was indicated by the precipitation of As_2S_3 and Se (VI) to Se (0) was indicated by presence to red elemental selenium precipitate (Fig.2.4). The culture was subsequently transferred to initially 1:10 dilution into fresh medium with electron donors (2.5 mM final concentration) and electron acceptor (5 mM final concentration), and then to 1:100 dilution into fresh medium with electron donors and electron acceptor and finally to 1:100 dilution into fresh medium with electron donors and electron acceptor.

The complete reduction of arsenate to arsenite coupled with acetate, for example, is shown below.

$$4\ AsO_4^{3-} + CH_3COOH \longrightarrow 4\ AsO_3^{3-} + 2\ CO_2 + 2\ H_2O$$

The first generation transfer was re-spiked with 5 mM of sodium selenate or sodium arsenate as sole electron acceptor and 2.5 mM of electron donor mix

respectively. As arsenate is respired/ reduced by the bacterial population, there is a concomitant increase of As (III). As (III) produced from the first spike was present at the start of the second incubation. The initial As (III) concentration of 3.5 mM in GW 1 cultures increased to 7.2 mM after 3 weeks (Fig. 2.5). There was an increase in concentration of As (III) from 3.7 mM to 7.8 mM in GW 2 enrichment cultures (Fig. 2.6). An increase of about 3.7 mM As (III) to 7.4 mM was observed in GW 3 (Fig. 2.7). Similarly an increase of concentration of As (III) was observed in GW 4 enrichment cultures where As (III) increased from 3.2 mM to 6.0 mM (Fig. 2.8). In GW 5 soil enrichment culture setup, As (III) increased from 4.2 mM to 6.2 mM (Fig. 2.9). In enrichment cultures from paddy soil irrigated by river water (Fig. 2.10), there was an increase of As (III) from about 3.2 mM to 6.2 mM and remained constant. Through the enrichment culture based approach we obtained a very good evidence of the presence of an arsenate reducing bacterial community in all the agricultural soil samples.

The complete reduction of selenate to selenite and further to elemental selenium coupled with acetate, for example is shown below.

In all the six enrichment cultures established with the sediments and sodium selenate as electron acceptor, a decrease in the selenate concentration was observed. In all the primary enrichment cultures established with selenate, a bright orange red precipitate formed within 7-10 days indicating the precipitation of elemental selenium (Fig.2.4).

In cultures established with ground cabbage soil (Fig. 2.9); about 4.2 mM spiked selenate deleted to 0.7 mM within 13 days. 4.2 mM selenate reduced to below detection limit in GW 1 established cultures (Fig .2.5). In RW enrichment culture 4.7 mM selenate reduced to around 0.2 mM within 13 days (Fig. 2.10). The selenite concentration decreased from 1.9 mM to 1.6 mM and selenate decreased from 4.5 mM to 0.2 mM in RW soil enrichment culture (Fig. 2.10). Only slight increase in selenite concentration for GW 1 was observed from 1.79 mM to 1.81mM and increase from 1.81 mM to 1.83 mM in GW 3 enrichment culture which can be attributed to instrumentation error (Fig. 2.5, 2.7). Selenate concentration depleted rapidly from 5.0 mM to 0.1 mM and the selenite concentration remained unchanged in the GW 4 preliminary enrichment culture (Fig. 2.8). In the GW 5 enrichment culture, Se (IV) concentration increased from 1.6 mM to 1.7 mM (Fig. 2.9), whereas an increase from 1.7 mM to about 1.8 mM was observed in GW 2 enrichment culture (Fig. 2.6).

T-RFLP fingerprinting

We have been able to find very good molecular fingerprint of 16S rRNA genes from An-Giang sediment sites as well as from respective arsenate and selenate amended enrichment cultures.

GW 1 selenate enrichment culture

The selenate amended GW 1enrichment culture of 1:1000 dilution showed 4 dominant peaks. The 1st duplicate showed T-RFs at 105 bp, 120 bp 165 bp and

205 bp with *Mnl1* restriction enzyme digest, whereas 165 bp was absent in the 2nd duplicate. The 105 bp and 205 bp amplicons are also present in the original soil sample suggesting the presence of selenate reducer (Fig. 2.11). Also the 16S rRNA gene electropherogram of GW 1 soil shows a diverse profile, indicating the presence of other different OTUs .Similarly, T-RFs of 198 bp, 206, 298 and 321 bp were detected in 1st enrichment culture set up with *HaeIII* digest and the amplicon 198 bp was also present in the 16S rRNA gene profile of GW1 soil (Fig. 2.14).

GW 1 arsenate enrichment culture

The 1:1000 transfers of GW 1 enrichment cultures of arsenate with *Mnl1* digest showed 240 bp and 275 bp amplicons in the first duplicate whereas an additional 100 bp amplicon was found in the 2nd duplicate (Fig. 2.11). The *HaeIII* digest showed amplicons at 220 bp and 250 bp in the first duplicate whereas additional 90 bp amplicon was detected in the 2nd duplicate. The T-RFLP profile of the GW 1 soil is very distinct suggesting the presence of diverse microbial community (Fig. 2.12).

GW1 in the 1st duplicate enrichment culture on arsenate (10^{-5}) with *Mnl1* restriction showed dominant amplicons at 278 bp and 242 bp (Fig. 2.15). GW1in the 2nd duplicate enriched on arsenate (10^{-5}) set up with *HaeIII* digest showed a single dominant amplicon 253 bp (Fig. 2.16). This suggests that the 278 bp amplicon in the *Mnl1* digest and 253 bp in the *HaeIII* digest are the dominant and active arsenate reducers in the enrichment cultures on arsenate. The 1:1000

GW 1 enrichment culture on arsenate digested with *MnlI* shows T-RFs at 99 bp, 242 bp and 278 bp, whereas the enrichment cultures on selenate shows T-RFs at 99 bp, 105 bp, 120 bp, 165 bp and 205 bp (Fig. 2.15 and 2.16). Hence the microbial community is diverse at this site and further clone library generation can be done to identify the phylotypes and interpret these results more precisely. We then analyzed the arsenate reducing community from other sites as well.

GW 2 Selenate enrichment culture

1:1000 enrichment cultures enriched on selenate and digested with *MnlI* restriction enzyme show amplicons at 99 bp, 120 bp, 278 bp and 285 bp. With *HaeIII* digest, amplicons at 250 bp and 300 bp were observed (Fig. 2.21).

GW 3 Arsenate enrichment culture

GW 3 in the 1st duplicate enriched on arsenate (10^{-5}) with *MnlI* restriction enzyme showed two amplicons at 92 bp, 116 bp and 278 bp. The 2nd duplicate however showed 5 dominant trf's at 59 bp, 92 bp, 99 bp, 116 and 250 bp. However with *HaeIII* restriction enzyme, 1st duplicate showed a single amplicon at 250 bp and 3 major trf's at 60 bp, 100 bp and 250 bp suggesting that the 60 bp and 100 bp amplicons may be the same microorganism reducing arsenic as well as selenium (Fig. 2.23).

RW soil Arsenate enrichment culture

The RW arsenate enrichment culture (1:1000) showed a dominant amplicon at 278 bp as well as an amplicon at 120 bp, digested with *MnlI* restriction enzyme in both the duplicates. The 16S rRNA gene electropherogram of the RW soil is very diverse comprising of amplicons at 120 and 278 bp as well (Fig. 2.22). This might indicate that there arsenate reducers present along with diverse population of bacteria.

GW 4 Arsenate enrichment culture

GW 4 enriched on arsenate (1:1000) and digested with *MnlI* restriction enzyme showed a single amplicon at 278 bp whereas two dominant amplicons at 137 bp and 254 bp were observed in the 2nd duplicate (Fig. 2.24).

It is indeed interesting to observe presence of dominant amplicon 250 bp with *HaeIII* digest in various sites which are few miles apart. It was observed in Ground rice 1, 3, 4 and the amplicon at 278 bp with *MnlI* digest in GW 1, 2, 3 4 and RW (Fig. 2.25). There is a presence of 137 bp amplicon in the sediment site but there is entirely different 16S rRNA profile suggesting the microbial community to be very diverse.

GW 5 Arsenate enrichment culture

1:1000 enrichment culture enriched on arsenate and digested with *HaeIII* restriction enzyme show amplicons at 205 bp, 210 bp, 225 bp in the first duplicate whereas the dominant trf's in the 2nd duplicate were 105 bp, 205 bp,

210 bp and 275 bp (Fig. 2.20). 1:1000 enrichment cultures enriched on arsenate and digested with *Mnl1* restriction enzyme show amplicons at 110 bp, 148 bp, and 155 bp in the first duplicate whereas an additional dominant at 100 bp was observed in the 2nd duplicate (Fig. 2.19).

Terminal Restriction Length Polymorphism combines PCR and rRNA fingerprinting to explore the microbial environments and identify uncultured microorganisms. It is a methodology employed in this study to gain a more complete understanding of the bacterial communities present in a sample (Forney, 1997). PCR products (amplicons) are obtained using primers labeled with fluorescent dye and then digested with restriction enzymes, and the fragments generated are separated by high-resolution electrophoresis. The fingerprint obtained is the set of the lengths of all labeled terminal restriction fragments (T-RFs). This can be applied for 16S rRNA genes and genes of enzymes of interest such as nitrogen fixation, arsenate reduction or mercury resistance (Kitts, 2001; Junier, 2008).

DISCUSSION:

In the GW1 enrichment cultures of the selenate and arsenate reducing T-RFLP electropherogram are very different from each other suggesting the different species represent for As and Se reduction and their rapid reduction in the respective preliminary enrichment cultures verifies the role of bacterial reduction in this agricultural paddy soil watered by groundwater. Arsenite mobilization employing enrichment culture methodology mimicking the in-situ

conditions gives preliminary evidence of arsenate reducing bacterial activity associated with these soil sediments. Similarly, in arsenate enrichment cultures of GW 2, GW 3, GW 4, GW 5 and RW, there was evidence of arsenite production with concomitant As_2S_3 precipitation (Fig. 2.4). Over the past few decades, groundwater wells installed in rural areas throughout the major river basins draining the Himalayas have become the main source of drinking water for millions of people (Schlömann, 2011). Groundwater in this region does not contain microbial pathogens as in surface water, but often contains hazardous amounts of arsenic. Arsenic enters groundwater naturally from rocks and sediment by coupled biogeochemical and hydrologic processes, some of which are presently affected by human activity. Mitigation of the resulting health crisis in South and Southeast Asia requires an understanding of the transport of arsenic and key reactants such as organic carbon that could trigger release in zones with presently low groundwater arsenic levels (Van Geen et al., 2010). The prolonged intake of As contaminated water results in severe health problems in adults and children. The aquifers under Red River and Mekong delta are widely used in drinking water in Vietnam and high concentration of As, Mn and Ba have been reported in tube well water and human hair in suburban areas of Hanoi, Vietnam. The concentrations are much higher than that observed in Bangladesh (Cutler et al., 2006).

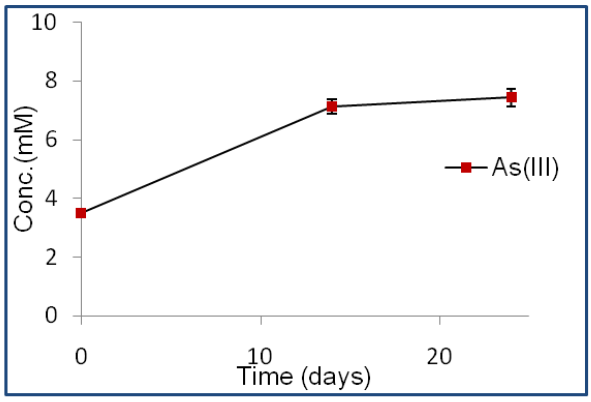
Enrichment culture methodology has been utilized in previous studies to confirm that Fe (III)-reducing bacterial activity plays role in releasing toxic As (III) in the contaminated aquifers of West Bengal sediments (Islam et al., 2004). In

another study, Stable Isotope Probing (SIP) method was employed where ^{13}C–acetate was amended with sodium arsenate in enrichment cultures and arsenic speciation was found in the Mekong river sediments. This study verified the link between carbon utilization and detection of organisms converting As (V) to As (III). Increase in As (V) respiratory reductase genes was also found in these contaminated aquifers of Phnom Penh, Cambodia (Lear et al., 2007). Various short chain fatty acids such as acetic acid (terminal product), pyruvic acid, propionic acid, succinic acid and formic acid to study the Fe (III)-reducing bacteria through enrichment culture method in submerged rice paddy field located in Lengshuijiang, China. Employing t-RFLP as well generation of clone libraries, *Geobacter* species and Firmicutes- related Fe (III) reducing bacteria were enriched. But this study demanded culture independent mRNA based analysis (Li et al., 2011).

In a microcosm study of two different shallow arsenic rich reducing aquifers of Cambodia, microbes were able to reduce Fe (III) and As (V) when provided with electron donor. Members of Betaproteobacteria were found in this As (V) reduction process by probing the arrA gene for As (V) respiration (Lloyd, 2007). In a study of upper, mid and lower bay in the Chesapeake Bay, PCR primers for the α-subunit of *arr* genes were designed and used with PCR amplification to detect uncultured dissimilatory arsenate-respiring bacteria. Arsenate reduction as well as terminal restriction fragment length polymorphism analysis of the putative *arrA* genes showed changes in the community structure in the enrichment cultures. Cloning and sequence analysis of the *arrA* genes

further verified the arsenate-respiring community from this site (Song et al., 2009). The reduction of oxyanions can be mediated both biotically as well as abiotically; nonetheless the initial detection of As (III) as well as reduction of Se (VI) within 10-25 days verifies the evidence of reduction of selenate and arsenate via culture dependent methodology in all the enrichment cultures.

Hence, employing enrichment culture methodology gave us a very good evidence of arsenate and selenate reduction as evidenced by chromatography results and also the evidence of phylotypes when probed in for 16S rRNA gene community structure characterization.



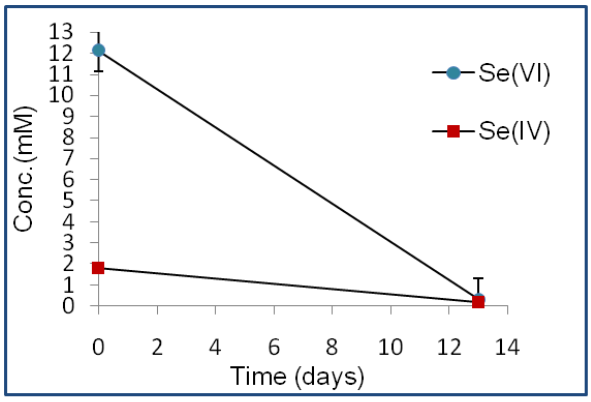
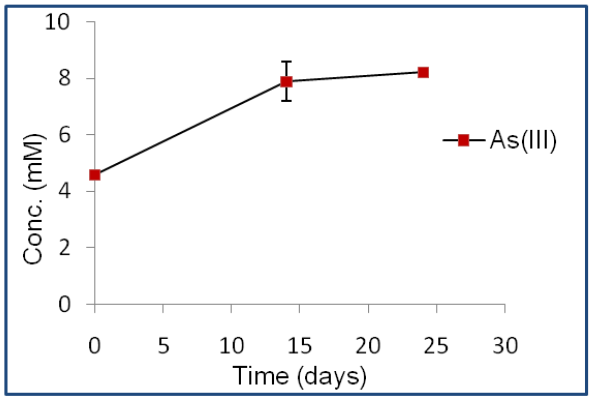


Fig. 2.5. Concentration of a) As(III), b) Se(VI) and Se(IV) in GW1 primary enrichment culture re spiked with As(V) or Se(VI) respectively.



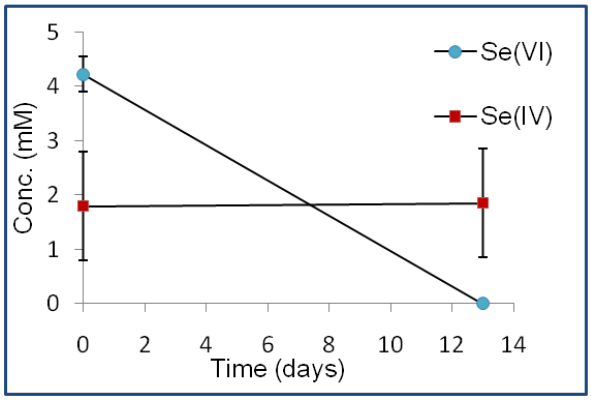
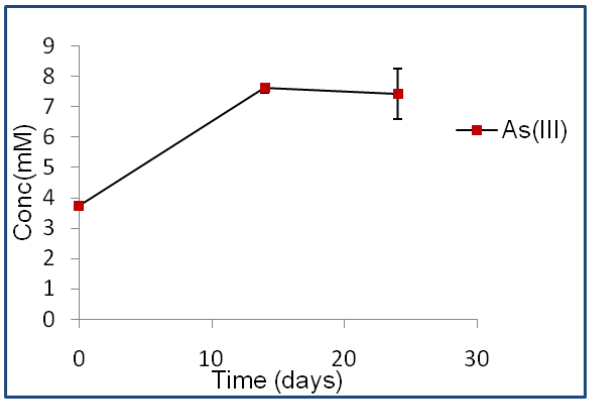


Fig. 2.6. Concentration of a) As(III), b) Se(VI) and Se(IV) in GW2 primary enrichment culture re spiked with As(V) or Se(VI) respectively.



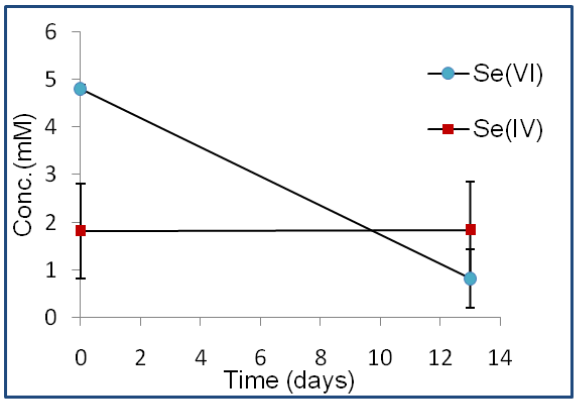
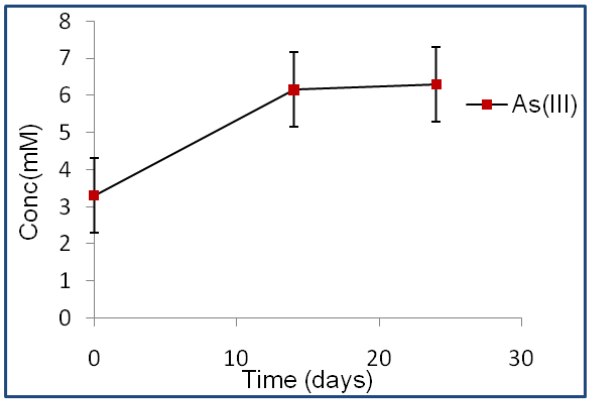


Fig. 2.7. Concentration of a) As(III), b) Se(VI) and Se(IV) in GW3 primary enrichment culture re spiked with As(V) or Se(VI) respectively.



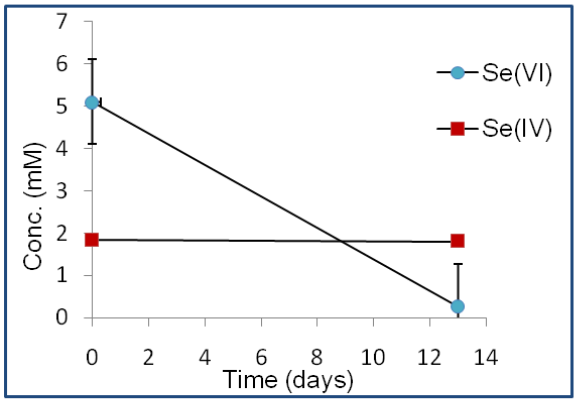
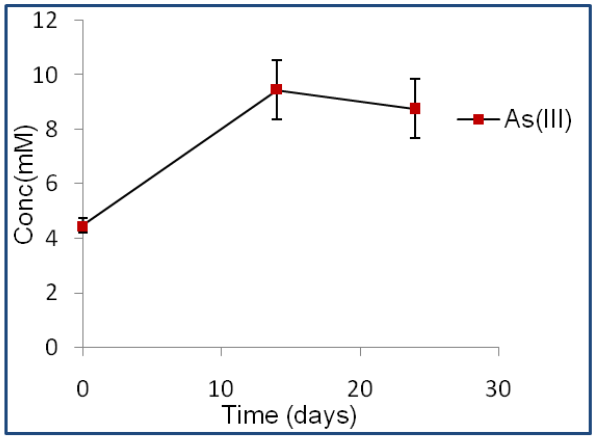


Fig. 2.8. Concentration of a) As(III), b) Se(VI) and Se(IV) in GW4 primary enrichment culture re spiked with As(V) or Se(VI) respectively.



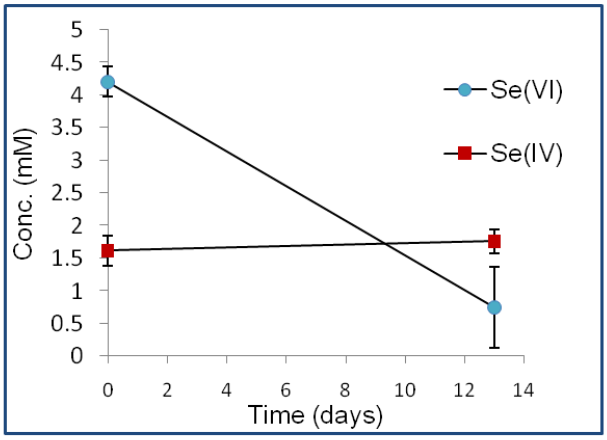
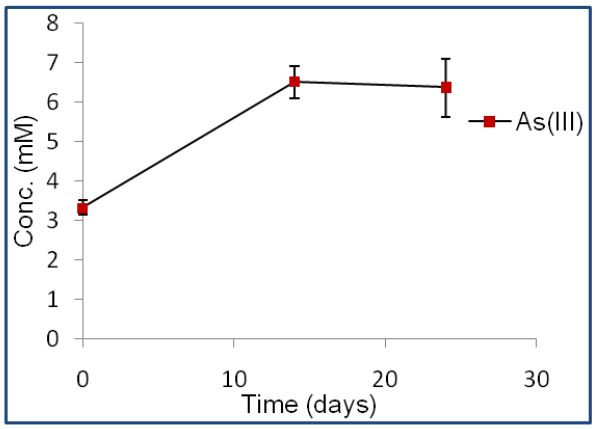


Fig. 2.9. Concentration of a) As(III), b) Se(VI) and Se(IV) in GW 5 primary enrichment culture re spiked with As(V) or Se(VI) respectively.



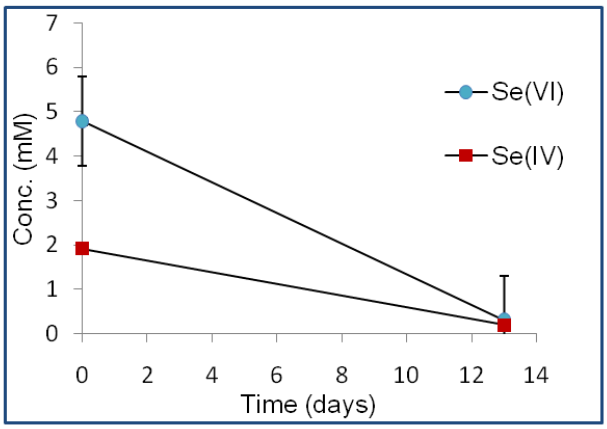


Fig. 2.10. Concentration of a) As(III), b) Se(VI) and Se(IV) in RW primary enrichment culture re spiked with As(V) or Se(VI) respectively.

Fig. 2.11

T-RFLP analysis of the two replicate GW 1 arsenate enrichment cultures (a, b) representing a 10^{-3} dilution of the primary culture compared to the c) original soil sample. DNA was digested with *MnlI* restriction enzyme prior to analysis.

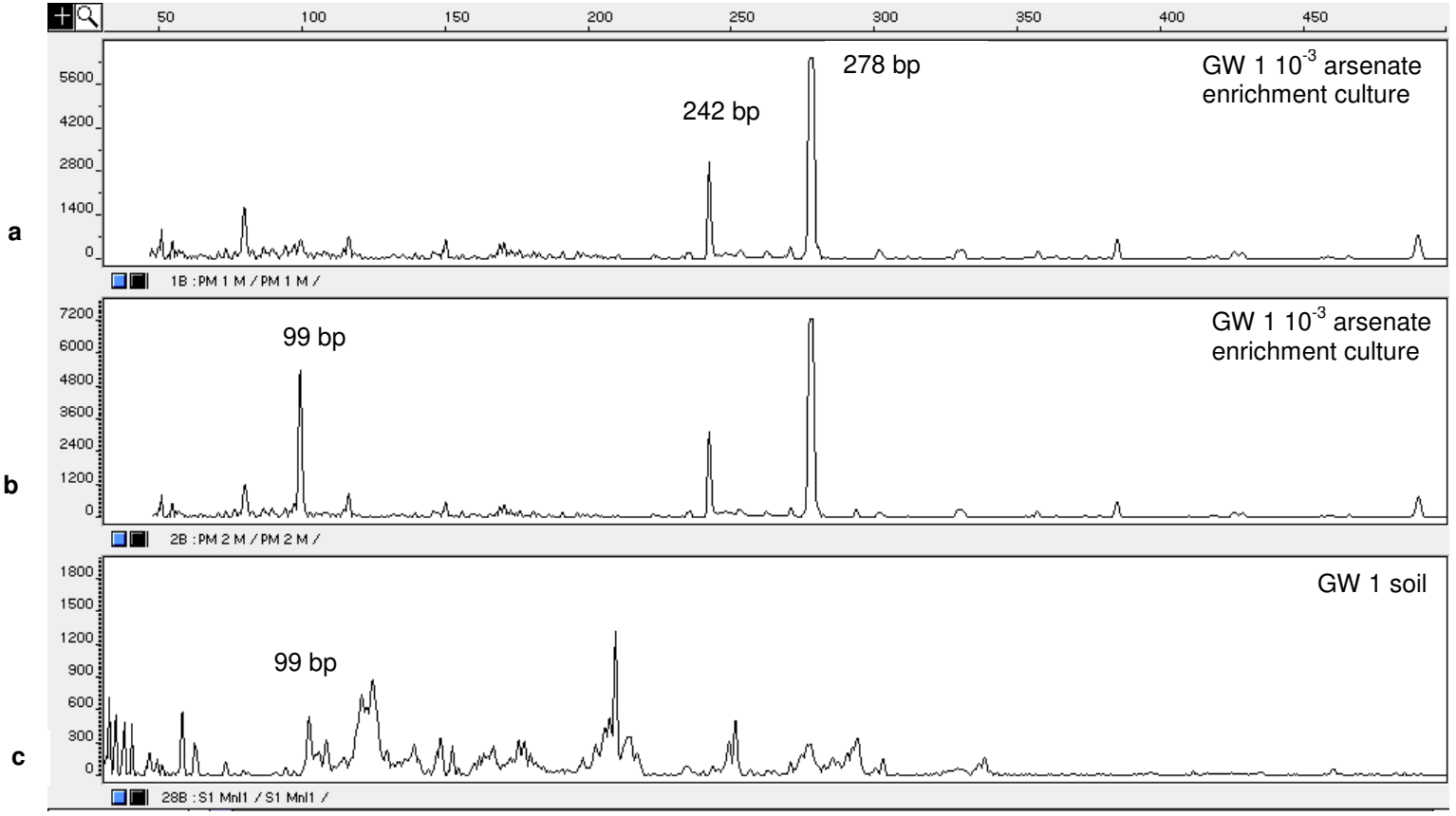


Fig. 2.12

T-RFLP analysis of the two replicate GW 1 arsenate enrichment cultures (a, b) representing a 10^{-3} dilution of the primary culture compared to the c) original soil sample. DNA was digested with *HaeIII* restriction enzyme prior to analysis.

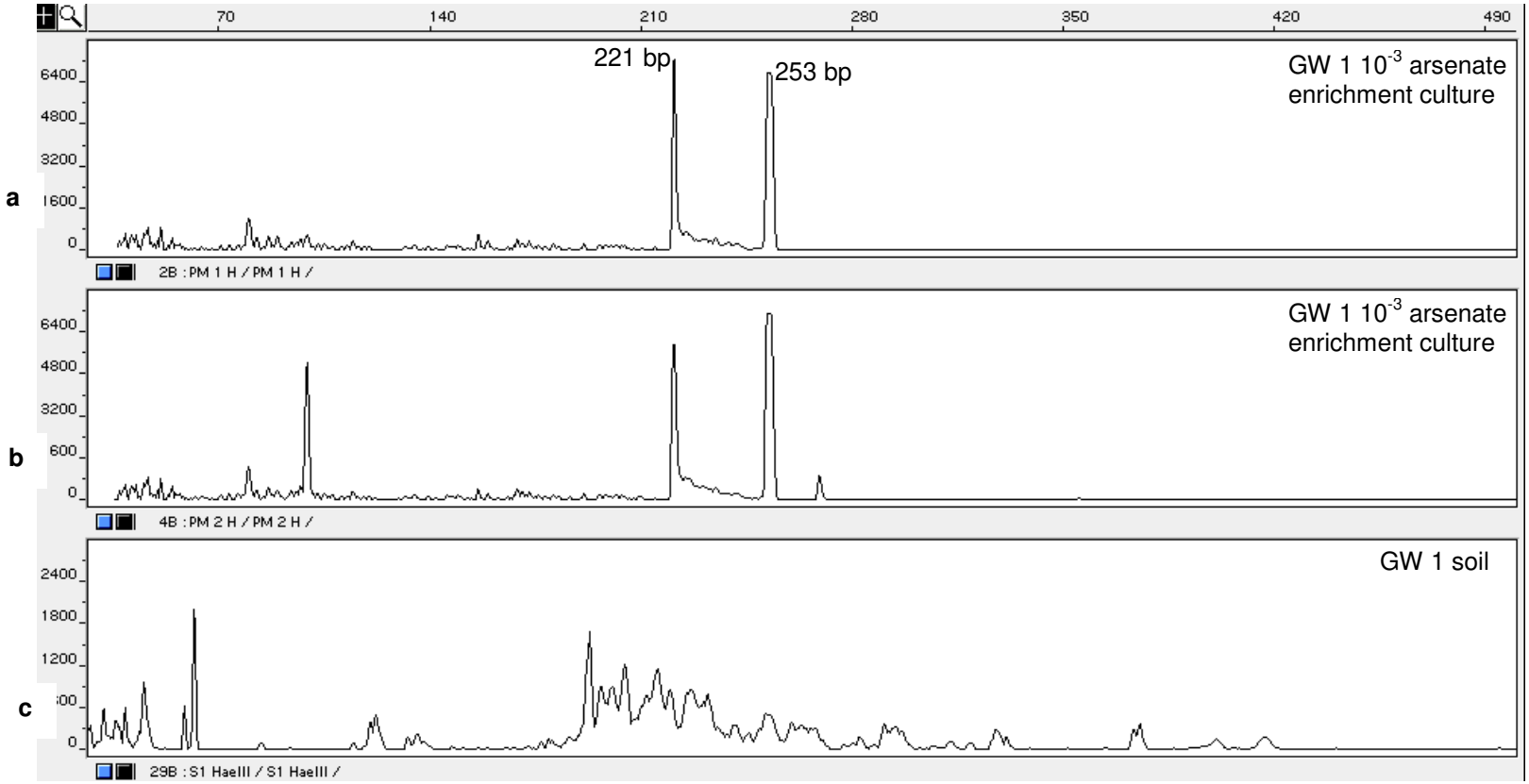


Fig. 2.13

T-RFLP analysis of the two replicate GW 1 selenate enrichment cultures (a, b) representing a 10^{-3} dilution of the primary culture compared to the c) original soil sample. DNA was digested with *MnlI* restriction enzyme prior to analysis.

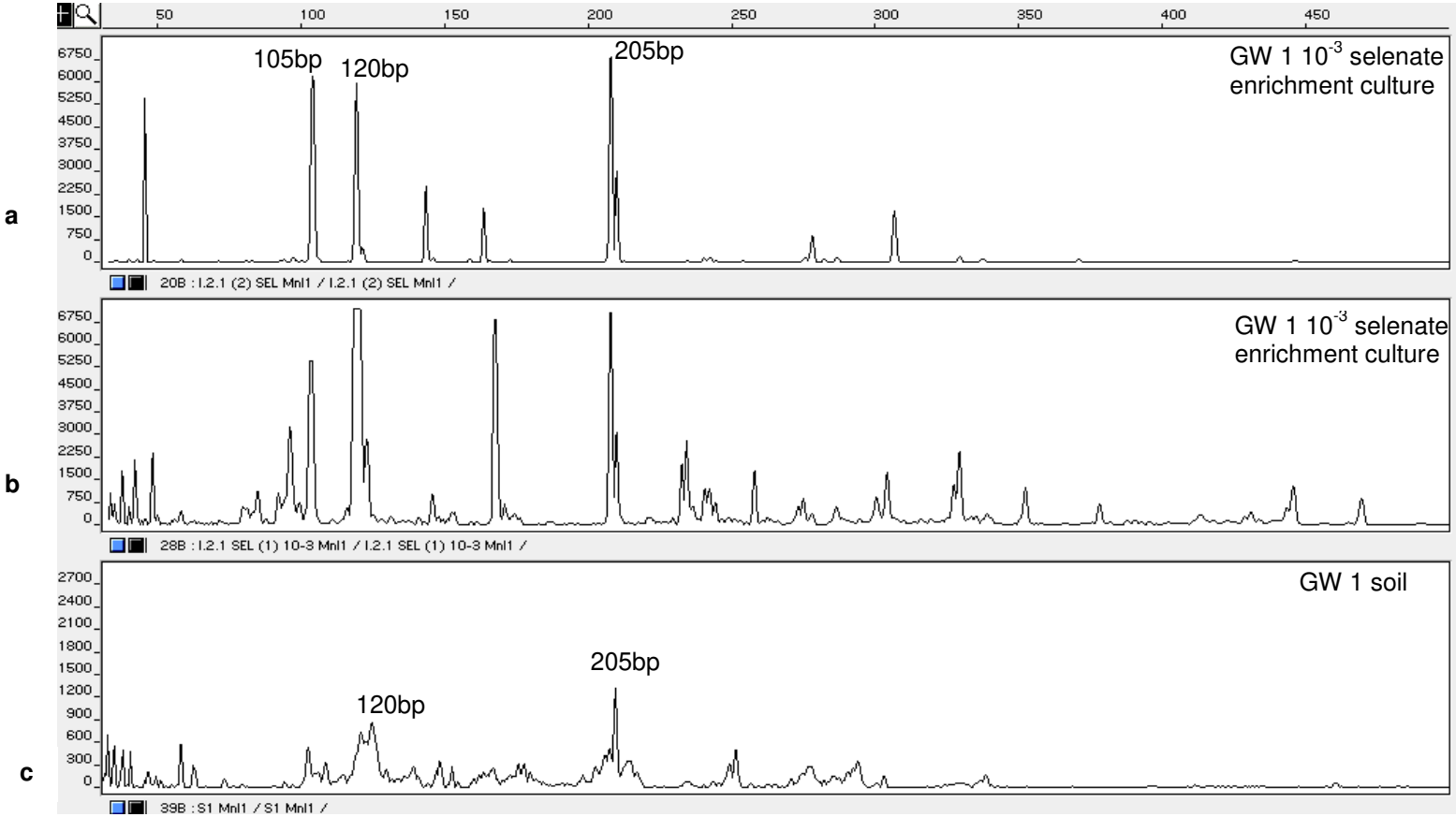


Fig. 2.14

T-RFLP analysis of the two replicate GW 1 selenate enrichment cultures (a, b) representing a 10^{-3} dilution of the primary culture compared to the c) original soil sample. DNA was digested with *HaeIII* restriction enzyme prior to analysis.

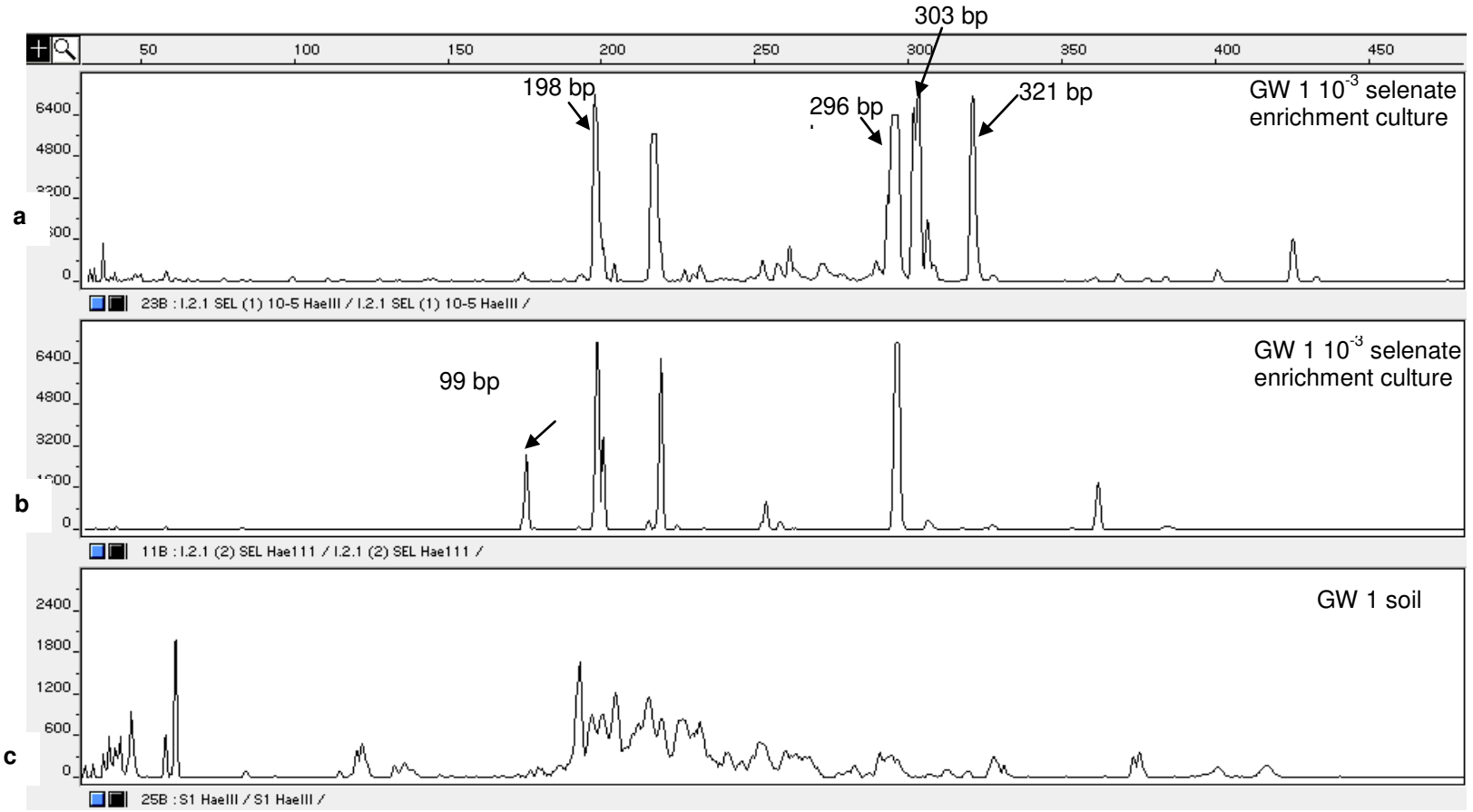


Fig. 2.15

T-RFLP analysis of GW 1 arsenate enrichment cultures (a, b, c) representing a 10^{-5}, 10^{-3}(replicate) dilution of the primary culture compared to the d) original soil sample. DNA was digested with *Mnl1* restriction enzyme prior to analysis.

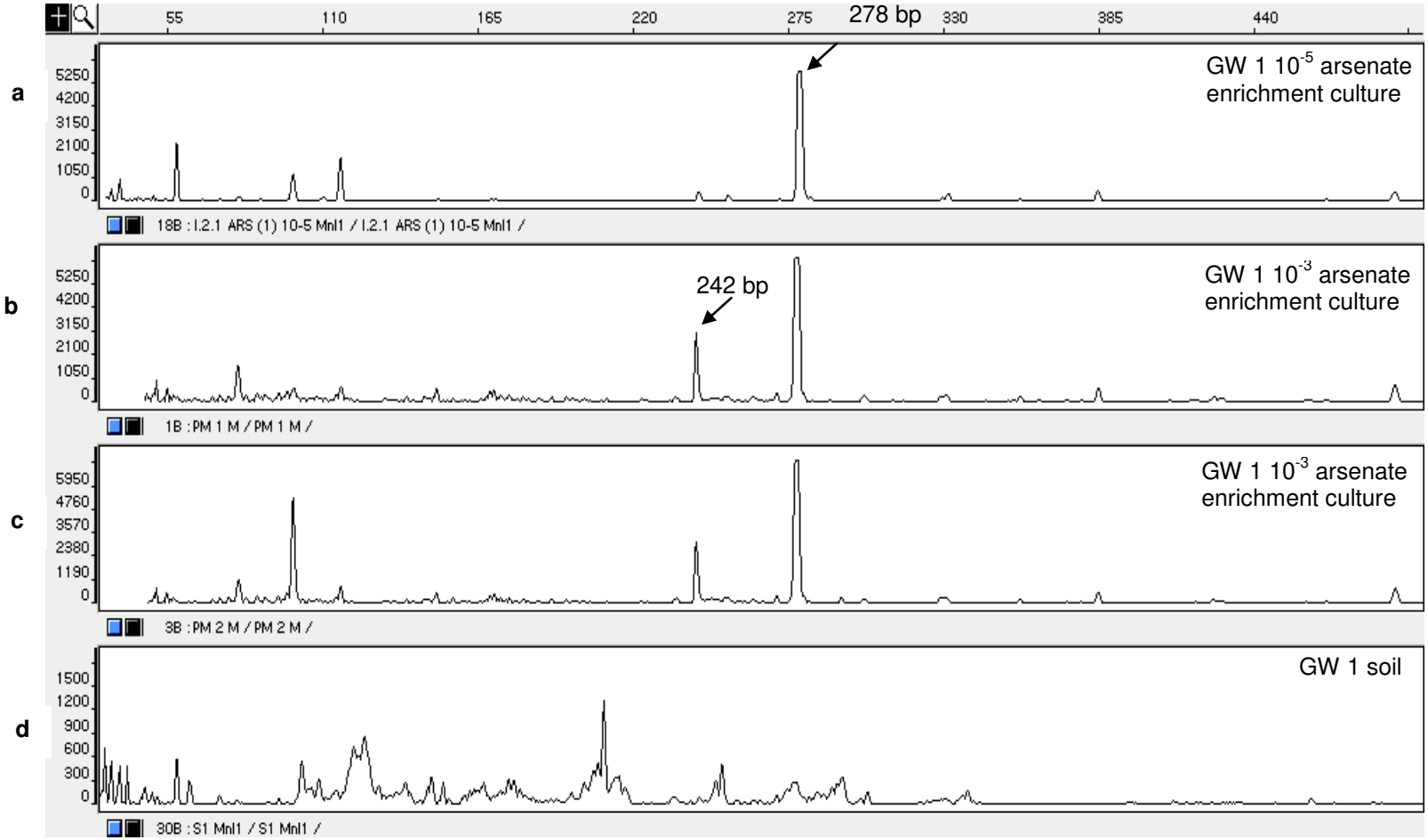


Fig. 2.16

T-RFLP analysis of GW 1 arsenate enrichment cultures representing a 10^{-5} (a), 10^{-3} (b, c) dilution of the primary culture compared to the d) original soil sample. DNA was digested with *HaeIII* restriction enzyme prior to analysis.

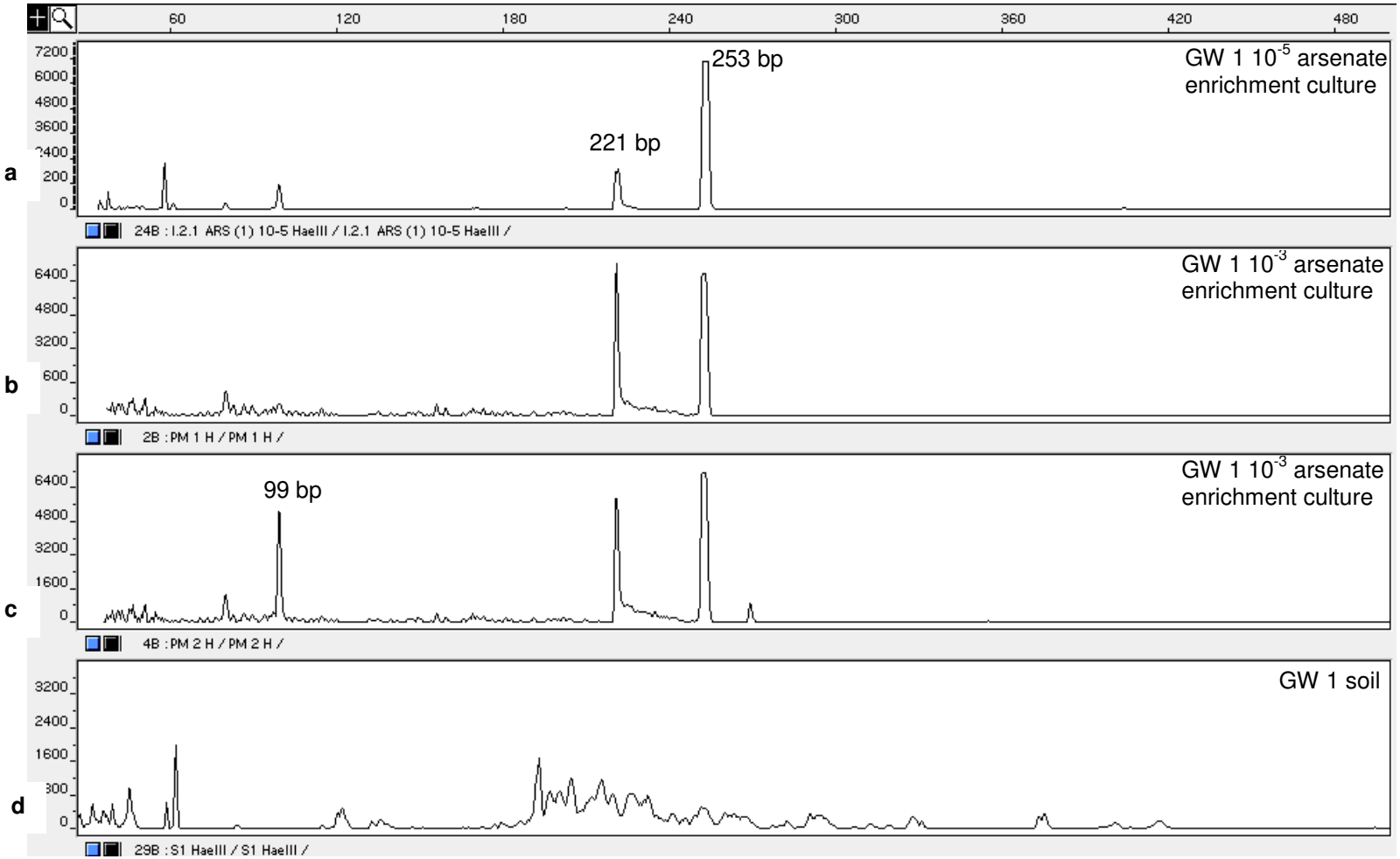


Fig. 2.17
T-RFLP analysis of the two replicate GW 1 arsenate enrichment cultures (a, b) representing a 10^{-3} dilution of the primary culture compared to the (c, d) GW 1 selenate enrichment cultures DNA was digested with *MnlI* restriction enzyme prior to analysis.

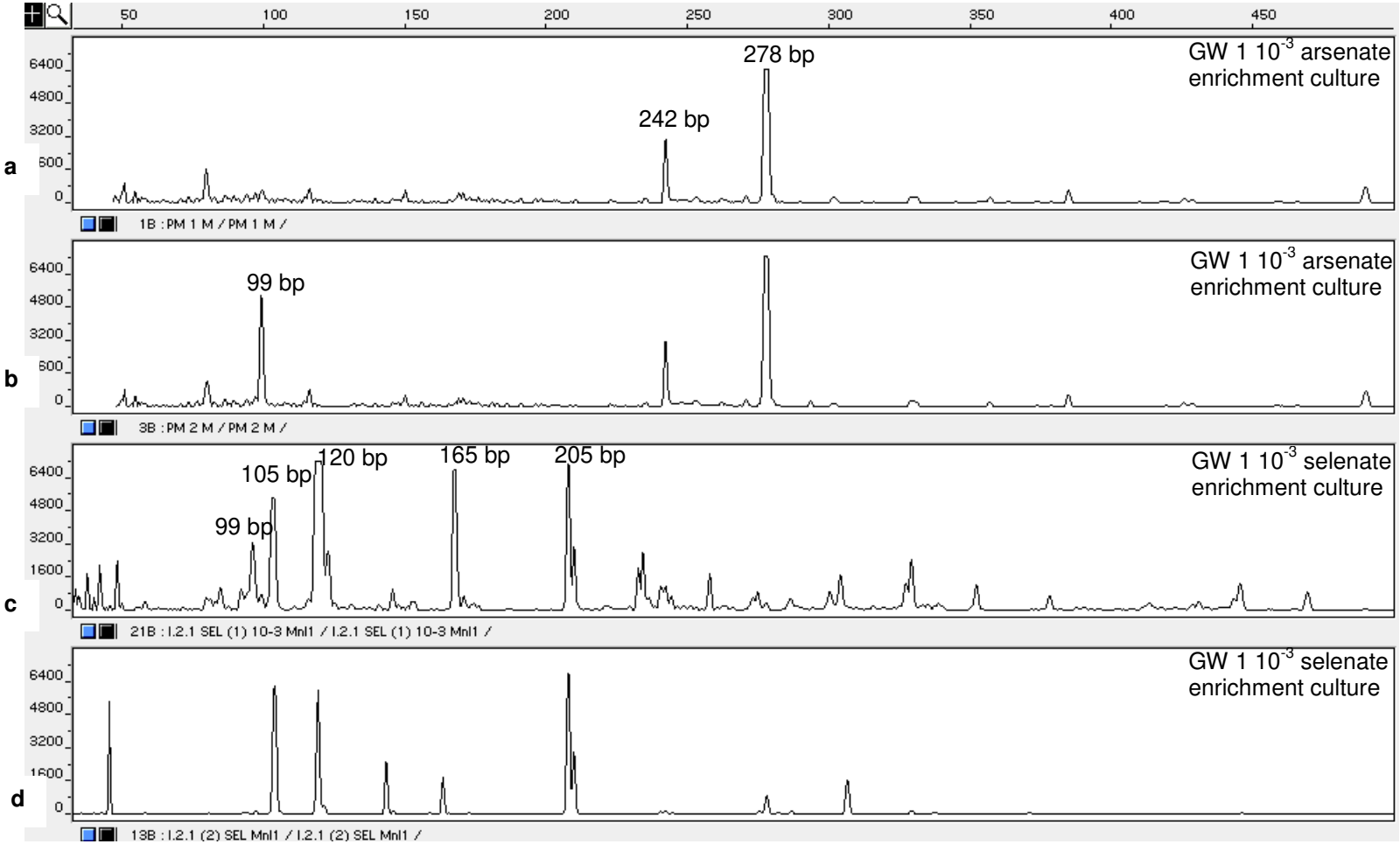


Fig. 2.18

T-RFLP analysis of the two replicate GW 1 arsenate enrichment cultures (a, b) representing a 10^{-3} dilution of the primary culture compared to the (c, d) GW 1 selenate enrichment cultures DNA was digested with *HaeIII* restriction enzyme prior to analysis.

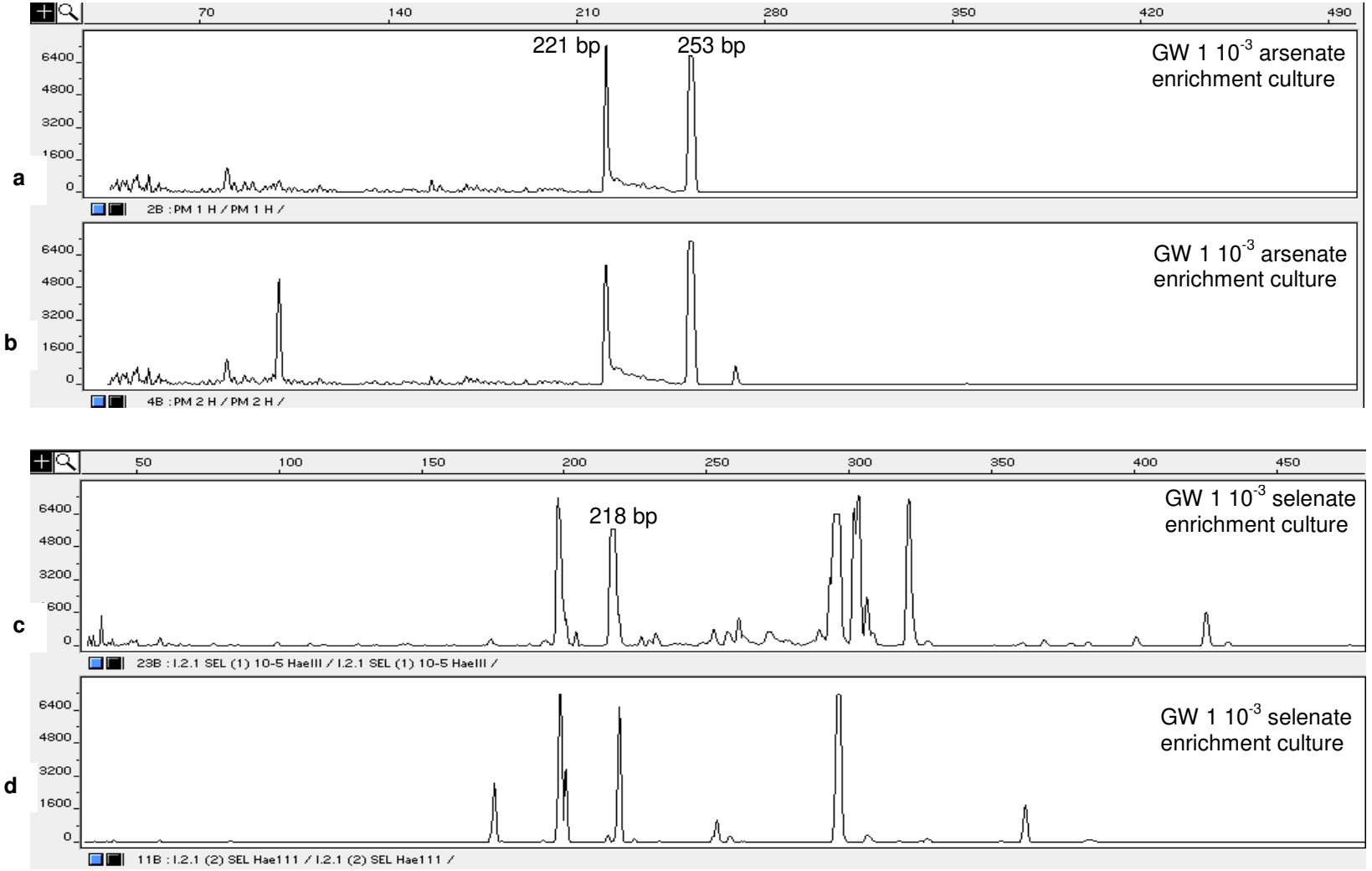


Fig. 2.19

T-RFLP analysis of the two replicate GW 5 arsenate enrichment cultures (a, b) representing a 10^{-3} dilution of the primary culture compared to the c) original soil sample. DNA was digested with *MnlI* restriction enzyme prior to analysis.

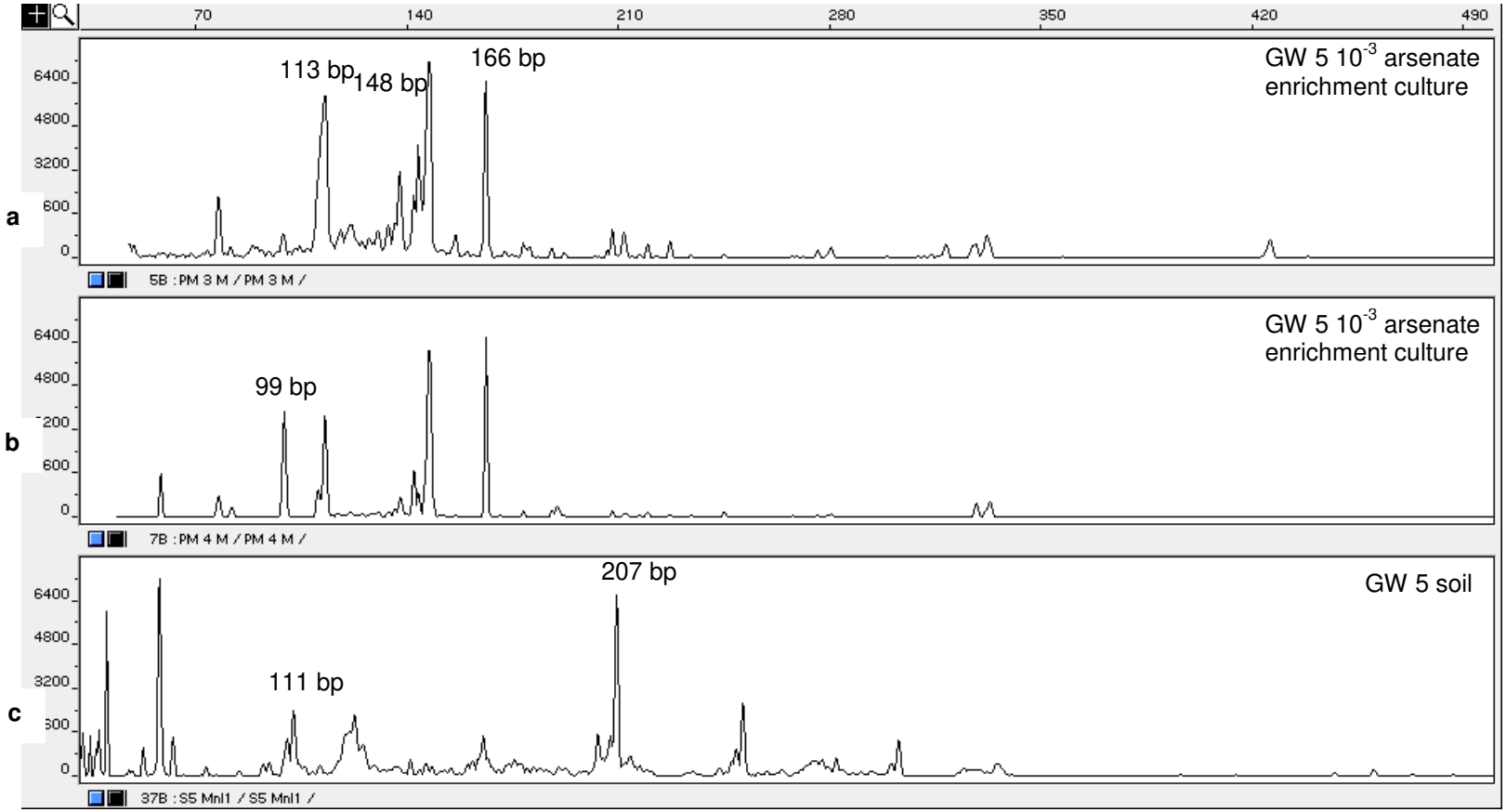


Fig. 2.20

T-RFLP analysis of the two replicate GW 5 arsenate enrichment cultures (a, b) representing a 10^{-3} dilution of the primary culture compared to the c) original soil sample. DNA was digested with *HaeIII* restriction enzyme prior to analysis.

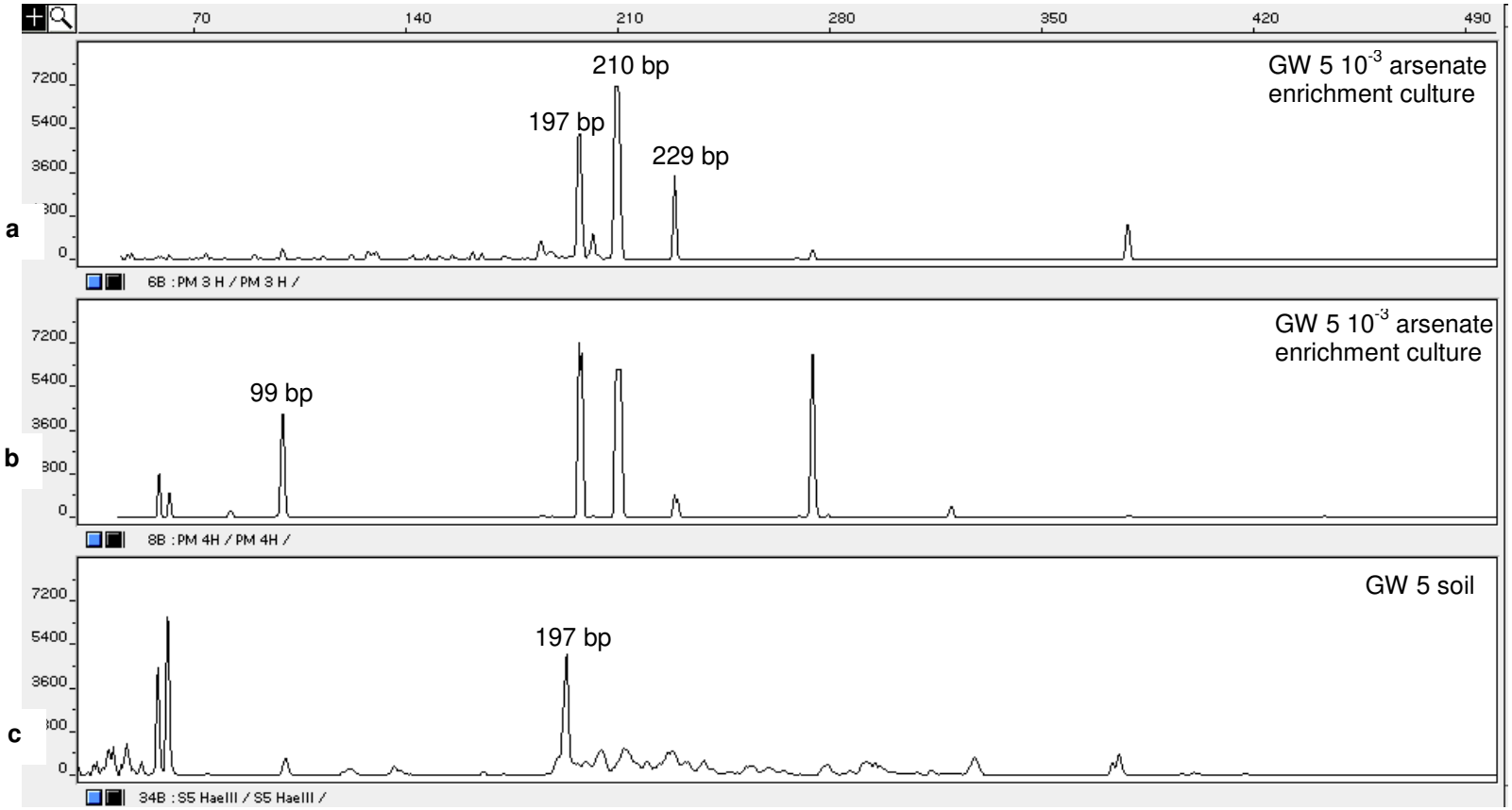


Fig. 2.21

T-RFLP analysis of the GW 2 arsenate enrichment cultures representing a 10^{-3} dilution of the primary culture. DNA was digested with *MnlI* and *HaeIII* (a, b) *restriction* enzyme prior to analysis.

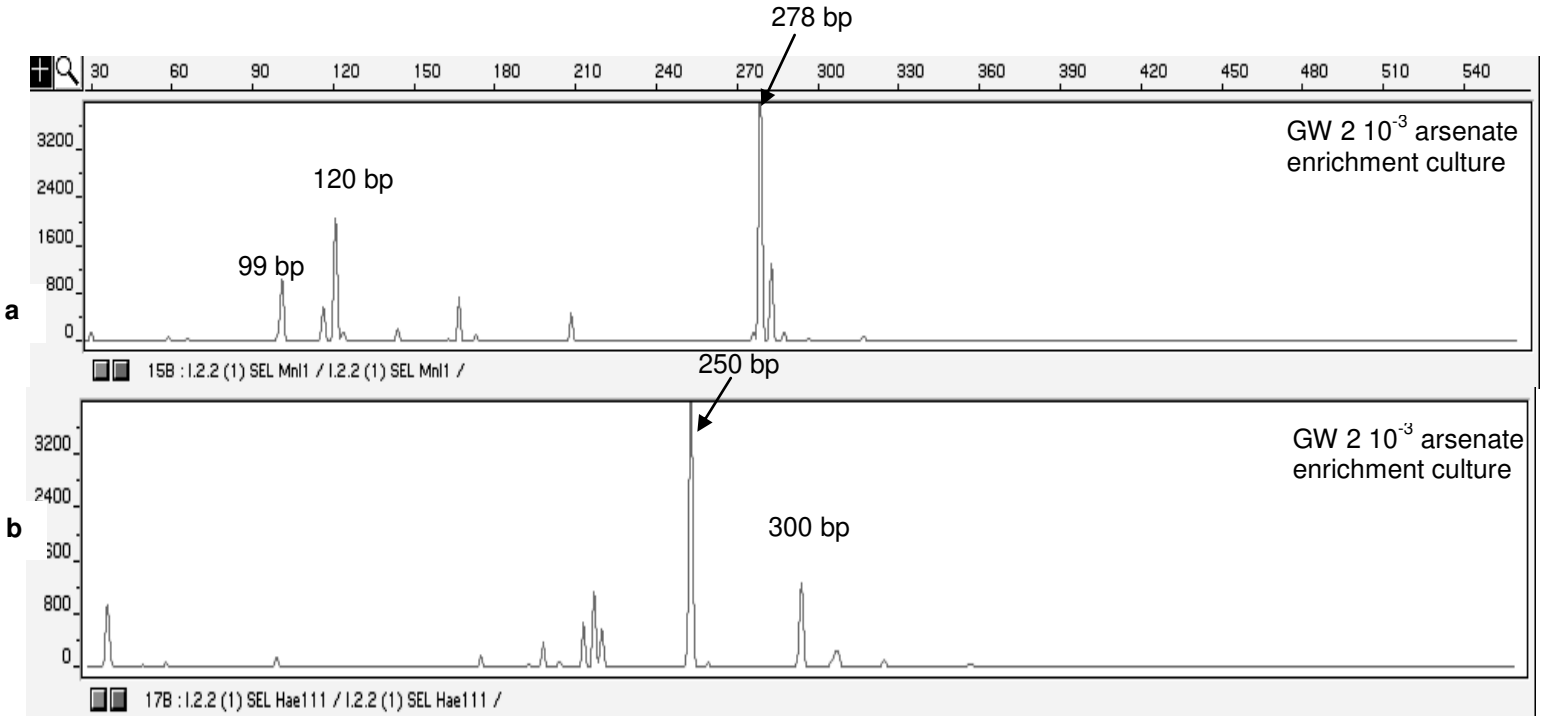


Fig. 2.22

T-RFLP analysis of the two replicate RW arsenate enrichment cultures (a, b) representing a 10^{-3} dilution of the primary culture. DNA was digested with *Mnl1* restriction enzyme prior to analysis.

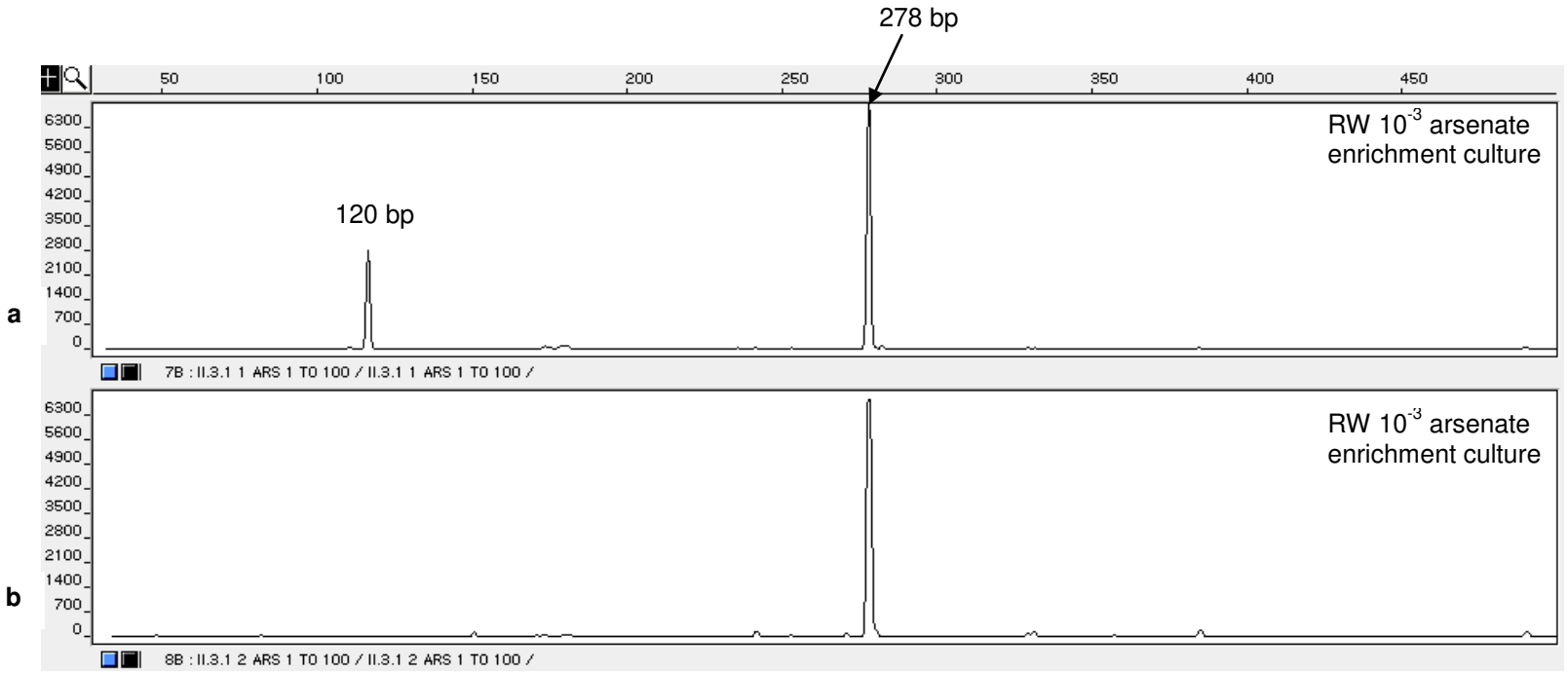


Fig. 2.23
T-RFLP analysis of the GW 3 arsenate enrichment cultures representing a 10^{-5} dilution of the primary culture. DNA was digested with *MnlI* and *HaeIII* (a, b) *restriction* enzyme prior to analysis.

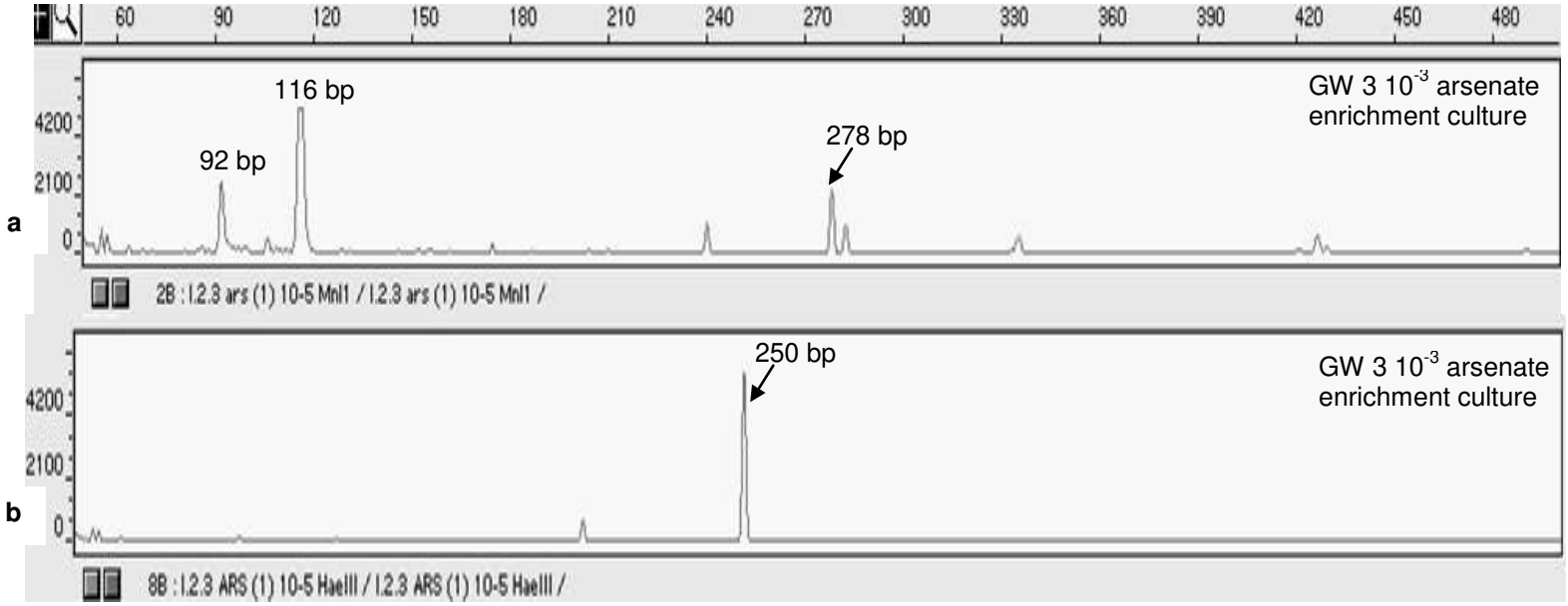


Fig 2.24
T-RFLP analysis of the GW 4 arsenate replicate enrichment cultures representing a 10^{-3} dilution of the primary culture. DNA was digested with *MnlI* restriction enzyme prior to analysis.

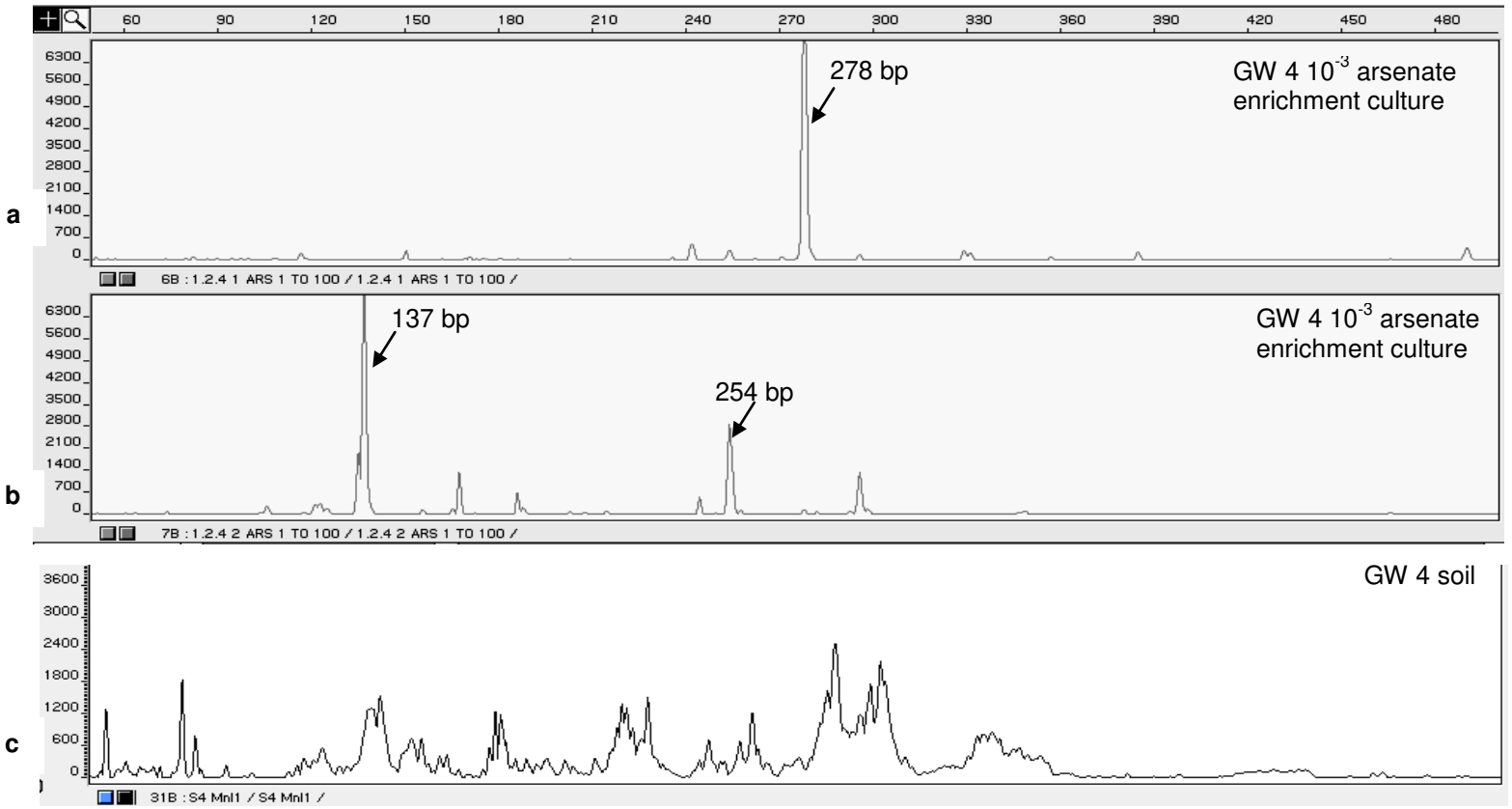
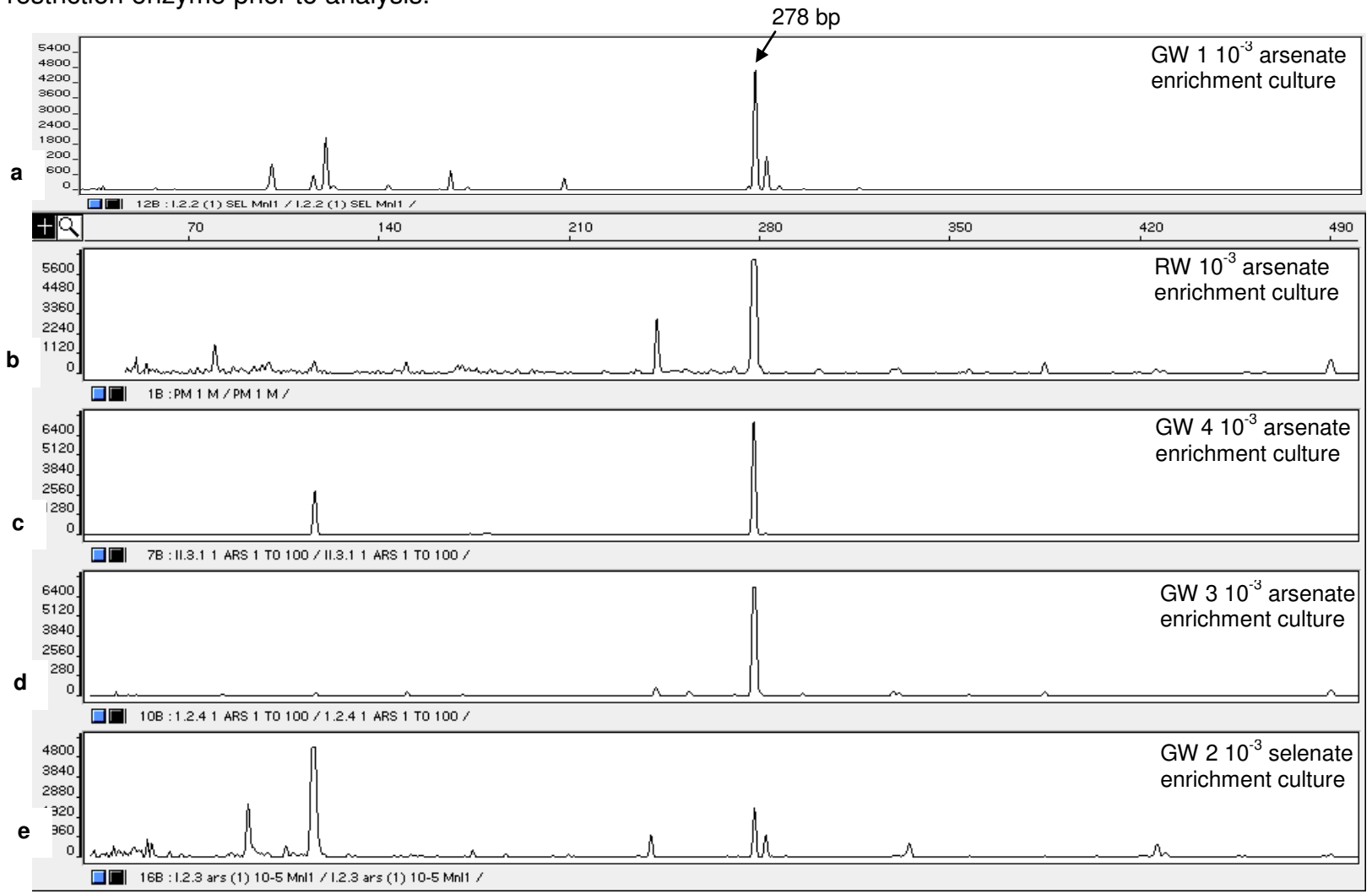


Fig. 2.25
T-RFLP analysis of the GW 1, RW, GW 4 and GW 3 (a.b, c, d) arsenate enrichment cultures and GW 2 (e) selenate enrichment culture representing a 10^{-3} dilution of the primary culture. DNA was digested with *Mnl1* restriction enzyme prior to analysis.



CHAPTER III

ISOLATION AND CHARACTERIZATION OF AN ARSENATE AND SELENATE REDUCING BACTERIUM

Bacteria inhabit diverse range of habitats in part by being able to switch between different respiratory pathways and several As- and Se- respiring bacteria have been isolated. Recently, Aerobic As(V) reducing bacteria, *Bacillus* sp. SXB and *Bacillus* sp. IMH associated with high arsenic groundwater in Shanxi Province and Inner Mongolia has been isolated. The arsenic resistance genes were searched and *arsC* gene was found in strain SXB, while the *arsH* gene was detected in strain IMH (Jing et al., 2012). *Clostridium* sp. BXM is a facultative anaerobic, non-flagellated, non-motile, spore-forming, Gram-positive fusiform rod. It reduces selenate and selenite and is an acetogenic phenotype reducing CO_2 to acetate (Bao et al., 2013).

Our goal was to isolate, identify and characterize bacteria capable of arsenate and/ or selenate reduction/ respiration from these sites which would give us further metabolic, geographic and phylogenetic evidence of selenate/ arsenate reducing bacteria.

MATERIALS AND METHODS

The GW 1 enrichment culture (Chapter II, Fig. 2.11) was used as inoculum for serially diluting into anaerobic agar (0.4 % Difco Noble agar) shake tube (Hungate, 1973), with minimal salt media (Table 2.3) and 5 mM pyruvate as

electron donor and 2.5 mM arsenate as electron acceptor under a head space of N_2:CO_2 (70:30%) followed by incubation at 27°C. Colonies appeared in 5 days and were picked and further grown and sub-cultured several times to obtain a pure culture. Two separate colonies were selected and designated strains *AG-1* and *AG-2,* respectively (Fig.3.1).

PCR amplification of 16S rRNA gene and TRFLP analysis

Pure cultures of the two isolates were grown for 5 days and the genomic DNA was extracted by phenol chloroform method (Kerkhof and Ward, 1993). 27 F (5'-AGAGTTTGATCMTGGCTCAG-3', Lane, 1991) and 1542 R (IDT) primers were used to amplify the near full length 16S rRNA gene sequence. Fluorescently labeled PCR product was analyzed on a 1 % agarose gel and the product was quantified by image analysis. 15 ng of PCR products was digested with *MnI1 endonuclease* (New England Biolab, Beverly, MA) and digested for 6 h at 37°C to verify the purity of the isolate. Precipitation of digested DNA was done by adding 2 mL of 0.75 M sodium acetate solution and 0.4 mL glycogen (100 mg mL^{-1}) with 37 mL of 95 % ethanol. The precipitated DNA was washed with 70 % ethanol, dried and re-suspended in 19.7 mL de-ionized formamide with 0.3 mL ROX 500 size standard (Applied Biosystems, Foster City, CA) for 15 minutes then denatured at 94 °C before analysis. T-RFLP fingerprinting was carried out on ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA) using Genescan software to verify the purity of the isolate.

The primers used for sequencing the 16S RNA gene were 321 F, 704 F, 519 R, 907 R and 1220 R (Table 2.4) which was sequenced at Genewiz, NJ. The sequences obtained were assembled in Geneious software (Drummond et al., 2011). Near full-length 16S rRNA gene sequences of known arsenate and selenate reducing organisms were identified from the Ribosomal Database Project and NCBI websites and used to examine the relationship and build the phylogenetic tree in MEGA 5.2 software (Kumar et. al., 2011).

RESULTS

The strains *AG-1* and *AG-2* grow both in nutrient agar medium aerobically and in minimal salts medium anaerobically (Fig. 3.1). The *AG-2* culture was tested in triplicate for growth with different electron donors and acceptors in minimal salt media (Table 2.3). The electron donors tested were acetate, and pyruvate (10 mM) and the electron acceptor tested were nitrate, arsenate, selenate, selenite (5 mM) (Table 3.1). Controls were established under the same conditions without an electron acceptor or without an electron donor. Cell-free controls were also established to detect any abiotic loss. Cultures were also tested for fermentation of pyruvate (10 mM) without an electron acceptor. Cultures previously grown on pyruvate and arsenate were used for this experiment.

Based on 16S rRNA gene analysis *AG-1* falls within the Gammaproteobacteria and shows 99 % similarity with *Klebsiella pneumoniae* subsp. *pneumoniae* **and** high similarity to K*. pneumoniae K30, K. singaporensis LX-21, K. varricola F2R9, K. pneumoniae subsp.*

pneumoniae strain NTUH-K2044 and 98% similarity with *K. singaporensis LX-3* (Fig 3.4). It also clusters closely together with members of enterobacteriaceae capable of arsenate reduction such *Pantoea agglomerans, Cronobacter sakazakii, Citrobacter freundii* and *Citrobacter braakii* (Fig 3.5).

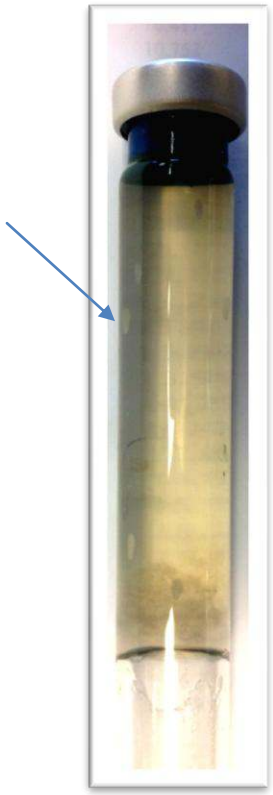


Fig. 3.1. Agar shake tube showing colony of strain AG-1

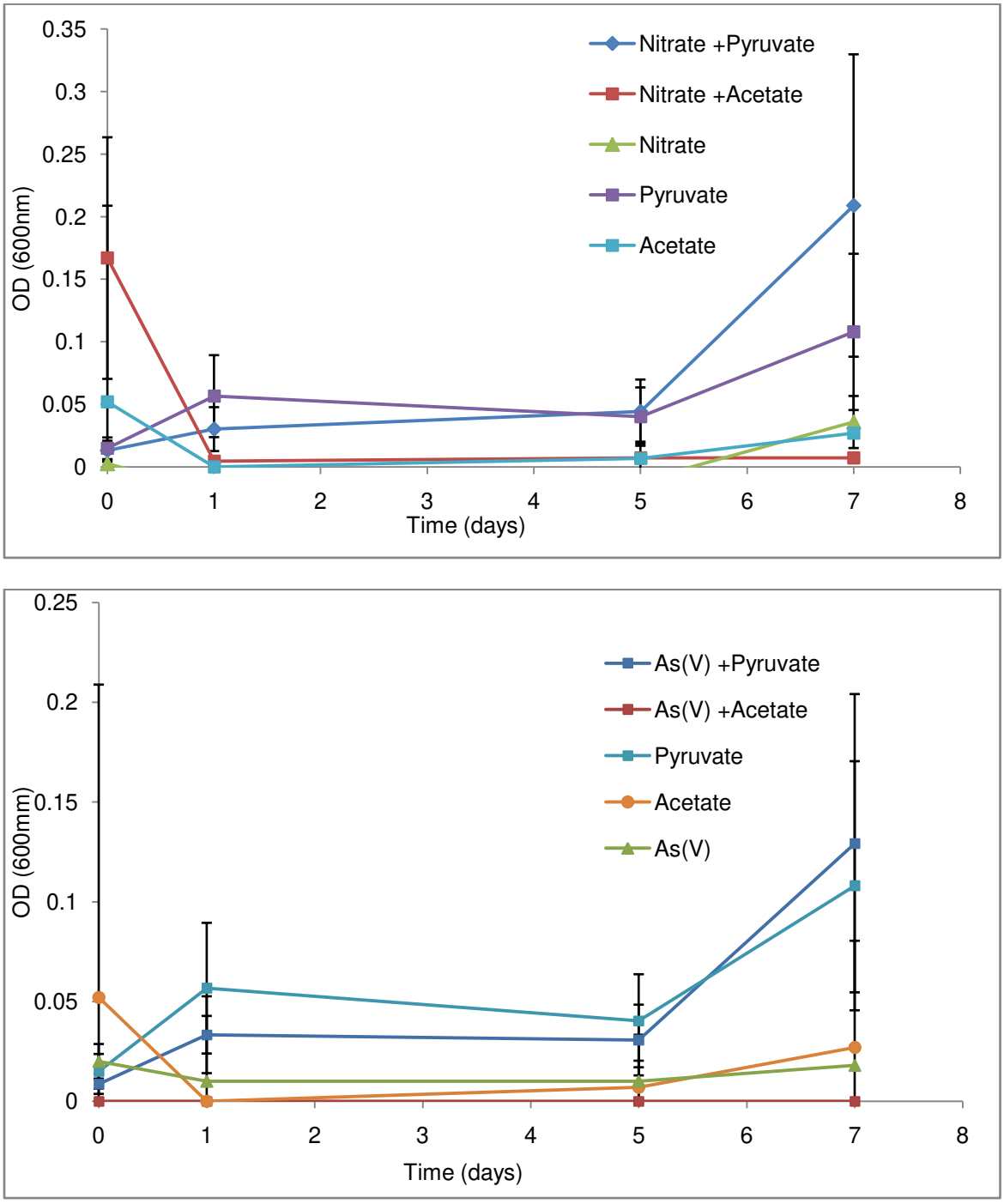


Fig. 3.2. Growth curve of *AG-2* on a) Nitrate+ Pyruvate, Nitrate+ Acetate, Nitrate only, Pyruvate only, Acetate only b) As (V) +Pyruvate, As (V) + Acetate, As(V) only, Pyruvate only, Acetate only.

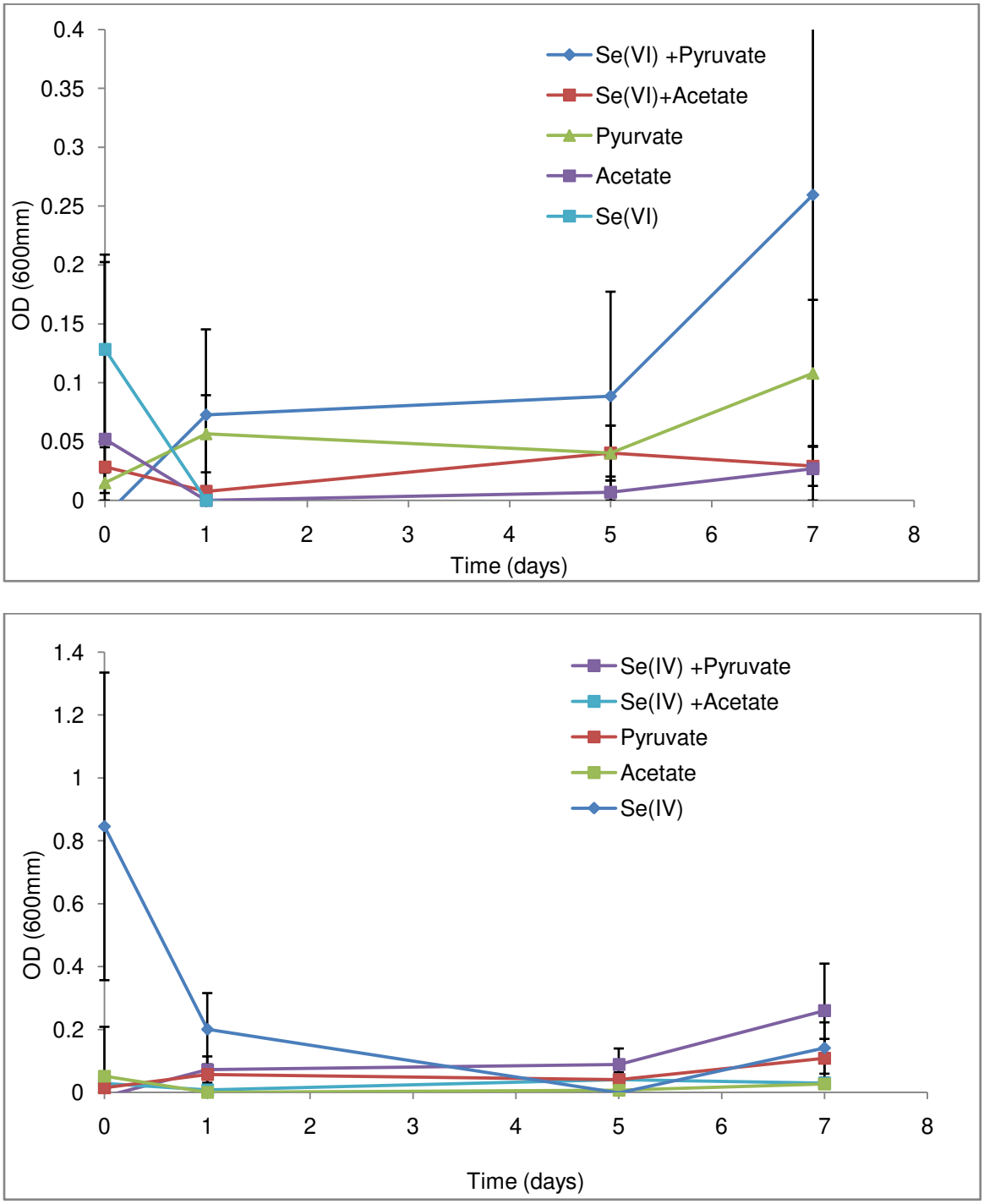


Fig. 3.3. Growth curve of *AG-2* on a) Se (VI) + Pyruvate, Se (VI) + Acetate, Se (VI) only, Pyruvate only, Acetate only b) Se (IV) + +Pyruvate, Se (IV) + Acetate, Se (IV) only, Pyruvate only, Acetate only.

Table 3.1

K. pneumoniae AG-2 and growth assay on various electron donors and electron acceptors.

Electron donor	Electron acceptor	Growth
As(V)	Pyruvate	+
Se(VI)	Pyruvate	+
Se(IV)	Pyruvate	-
Nitrate	Pyruvate	+
-	Pyruvate	+
As(V)	Acetate	-
Se(VI)	Acetate	-
Se(IV)	Acetate	-
Nitrate	Acetate	-
	Acetate	-

Fig. 3.4. Phylogenetic tree showing the relationship between *Klebsiella pneumoniae strains AG-1 and AG-2* with other *Klebsiella sp.* and closely related microorganisms. The tree was constructed by aligning 16S rRNA gene sequences using the Neighbor-joining method in MEGA 5.2. 871 positions in the final dataset and bootstrap values above 70 are shown at the nodes.

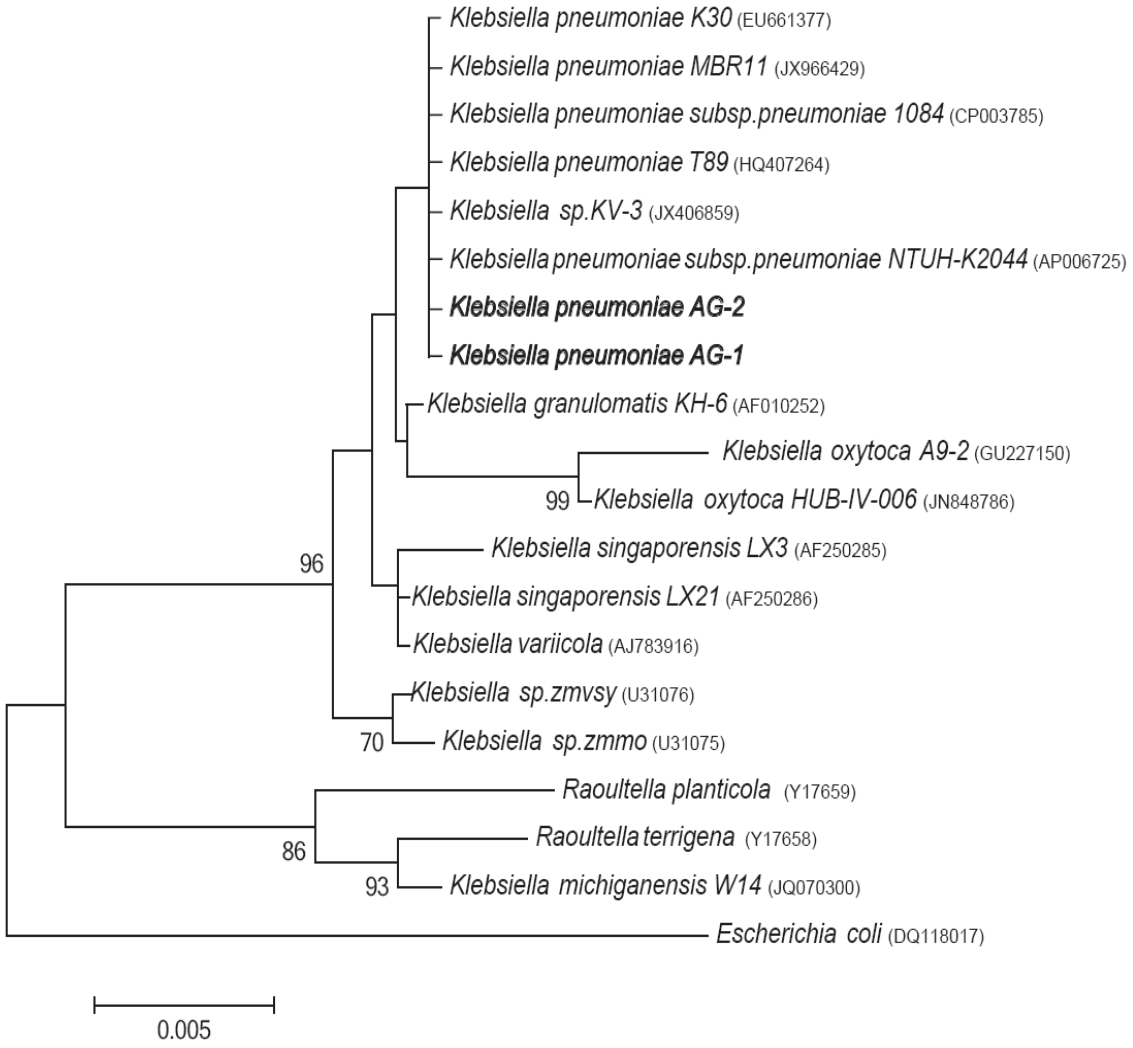
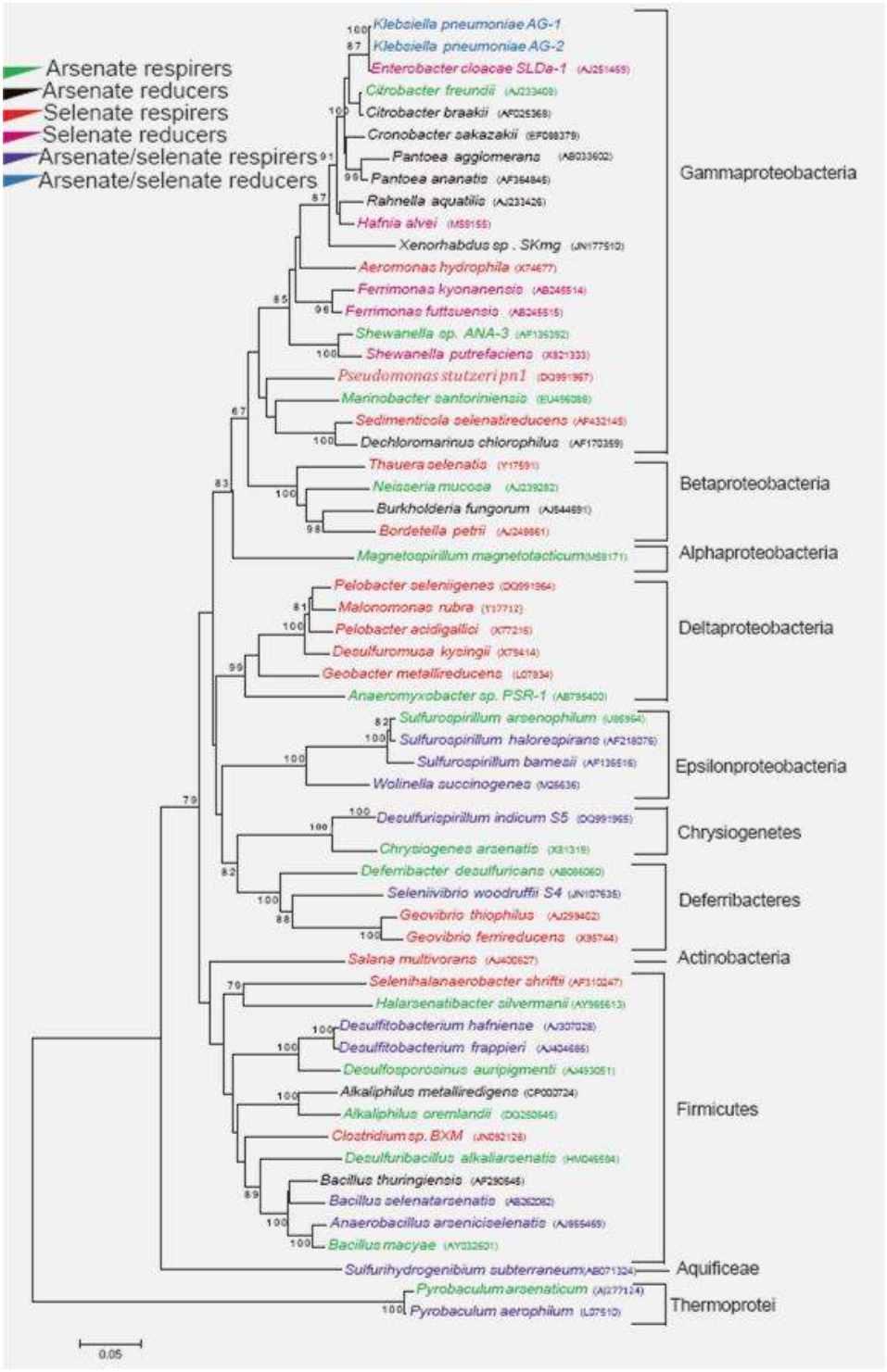


Fig. 3.5. Phylogenetic analysis of the 16S rRNA gene sequences of known arsenate and selenate reducing or respiring microorganisms using Neighbor-Joining method in MEGA 5.2. 1305 positions were used in the final dataset and bootstrap values above 65 are shown at the nodes.



DISCUSSION

We have been able to isolate and characterize two bacterial strains capable of arsenate and selenate reduction from rice paddy soil, Mekong river delta. The *Klebsiella pneumonia strains AG-1and AG-2* isolated in this study show 99 % similarity with many *Klebsiella pneumoniae* strains such as *K. pneumoniae subsp. pneumoniae strain NTUH-K2044* (Fig. 3.2). The 99 bp peak identified in the sediment as well as arsenate and selenate amended enrichment culture clearly indicates the presence of this strain and its possible role in arsenate and selenate reduction in the rice paddy soil (Fig. 2.11). The *AG-2* strain grows on pyruvate as sole carbon source in the media indicating that it is capable fermentative metabolism. The strain *AG-2* also reduces As (V), nitrate, and Se (VI) with pyruvate as electron donor but doesn't grow at all with acetate as electron donor suggesting that it is not a dissimilatory arsenate, selenate reducing bacteria and that it is capable of fermentative metabolism (Fig. 3.2, Fig. 3.3). Similarly, it doesn't show growth with acetate as a carbon source and selenate, arsenate, or nitrate as electron acceptor. The strain is not capable of reducing selenite (Table 3.1, Fig. 3.3.). Thus, for the first time, it has been shown that *Klebsiella* species is capable of both arsenate and selenate reduction.

In a study of two aquifers of Bangladesh of differing arsenic concentrations and redox conditions, *in-situ* bacterial diversity was investigated by T-RFLP and clone library analysis. From the arsenic-affected aquifers, As (III) oxidizing isolates of *Comomonas* and *Microbacterium* were obtained (Tipayno et al., 2012). There was a dominance of putative aerobic, denitrifying populations of

Pseudomonas, Elizabethkingia and *Pantoea,* implying more variable geochemical conditions resulting in arsenic mobilization and sorption (Sultana, 2011). *Thauera selenatis* and *Enterobacter cloacae* are microbes exhibiting different biochemical pathways by either reducing or respiring toxic oxyanion such as Se (Oremland et al., 1999). It has been reported that selenate reductase in *Enterobacter cloacae SLD-1* is truly respiratory in nature and the bacterium grows in the presence of selenate as terminal electron acceptor (Losi and Frankenberger, 1997). The strain was isolated from Se-contaminated drainage water in the San Luis drain (SLD), Joaquin Valley, CA, USA. It reduces Se oxyanion to elemental selenium but cannot grow using selenate as the sole electron acceptor if cultured anaerobically on non-fermentable carbon sources in batch (Butler et al., 2003).

Detoxification of arsenic has been widely studied in *Escherichia coli* and *Staphylococcus aureus* species. The enzymes and proteins involved in this mechanism are encoded by the *ars* operon. This operon is located on the chromosome and plasmid R773 in *E.coli* and in the case of *S. aureues* and *S. xylosus* on plasmids p1258 and pSX26, respectively (Ji and Silver, 1992; Rosen 1997). The functional chromosomal ars operon homologue has been found in *Escherichia coli, Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Salmonella* and *Erwinia* suggesting that it is a widespread arsenate resistance mechanism within Enterobacteriacace *(*Diorio et al.,1995). *Klebsiella pneumoniae* occurs in human feces and clinical specimens, soil, water, grain, fruits and vegetables. *Klebsiella pneumoniae* is a diazotroph,

nitrogen fixer and naturally occurs in soil. The *ter*, *ars*, *cop*, *sil*, and *mer* genes encode products conferring tellurite, arsenic; copper, silver, and mercury resistance have been identified previously in a 269-kilobase conjugative plasmid, pK29, from *K .pneumoniae* (Chen, 2007). In this study, we have shown the evidence of selenate and arsenate reduction by *K .pneumoniae* sp. in the agricultural soil.

CHAPTER IV

CONCLUSIONS

Arsenate and selenate reducing activity and community characterization of associated enrichment cultures through 16S rRNA phylogeny has been studied together for the first time in An Giang agricultural soils and sediments of the Mekong River delta, Vietnam. Isolation of two arsenate, nitrate and selenate reducing bacteria from the family of enterobacteriacace from paddy soil watered with groundwater also provides preliminary evidence of role of arsenate and selenate mobilizing bacterial community in these sites. The arsenate reducing activity by microbes resulting in As (III) production coupled with 16S rRNA gene amplification on arsenate amended enrichment cultures validates our hypothesis of microbially mediated reactions present in these soils and role in mobilization of arsenic in the paddy and cabbage soils watered by either ground water or river water. Similarly, the rapid reduction of selenate in all of the enrichment cultures and generation of very distinct T-RFs from these sites validates our hypothesis that selenate reducing bacteria are present in Paddy soil watered by ground water. This also implies that selenate is effectively removed from the aqueous phase and therefore not likely entering the food chain. We have provided initial evidence of arsenate and selenate reduction and 16S rRNA genes in the associated enrichment cultures. Nonetheless, there are still several open questions which need to be addressed in future. Amplification of arsenate

reductase *(ars C),* selenate reductase *(ser)* genes and /or generation of clone library would give us a better picture of microbial community associated with these agricultural soils and sediments.

APPENDIX

Table 4.1
Media composition:
The media used to study Enrichment cultures consisted of the following components (in g/L):

Component	Compound	Amount per litre
1	KCl	1.17 g
2	KH_2PO_4	0.2 g
3	NaCl	1.7 g
4	NH_4Cl	0.5 g
5	$CaCl_2$. $2H_2O$	0.1 g
6	$MgCl_2.6H_2O$	3 g
7	$NaHCO_3$	2.5 g
8	Vitamin solution	5 ml/L
9	Trace salt I	1 ml/L
10	Trace Salt II	0.1 ml/L
11	Resazurin	0.2 ml (from 1 g/L stock)
12	$Na_2S.9H_2O$	0.2 mL (from 500 g/L stock)

5mL/L of **vitamin solution** consisting of the following components (in mg/L) was added:

Vitamin Mix (5 ml/L) –Filter sterile and anoxic stock	Culture conc. (mg/L)	Stock conc. (mg/5 mL)	Stock conc. (g/500 mL)
d-biotin	0.1	0.1	0.01
folic acid	0.1	0.1	0.01
pyridoxine hydrochloride	0.5	0.5	0.05
thiamine hydrochloride	0.25	0.25	0.025
riboflavin	0.25	0.25	0.025
nicotinic acid	0.25	0.25	0.025
DL- calcium pantothenate	0.25	0.25	0.025
Vitamin B12	0.05	0.05	0.005
p-amino benzoic acid	0.25	0.25	0.025
lipoic acid	0.25	0.25	0.025
1,4-naphthaquinone	0.2	0.2	0.02
nicotinamide	0.5	0.5	0.05
hemin	0.05	0.05	0.005

Trace salt I was added at 1 mL/L concentration, which had 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

Trace Salt II was added at 0.1 mL/L concentration which contained (in g/L):

Na_2SeO_3	0.03
Na_2WO_4	0.08

All stock solutions were filter sterilized and made anoxic by purging under a stream of argon.

METHOD:
The salts (1-6 components) in 1 L of DI water. The media was placed under a N_2 stream for 30-45 minutes. Then it was further purged with CO_2/N_2 for another 15 minutes followed by the addition of $NaHCO_3$. The bottle was sealed, autoclaved and cooled. Anoxic, sterile stocks of Trace Salts I, II, Vitamin solution, Reaszurin and Na_2S-$9H_20$ were added to the cooled minimal salts medium. pH was checked and adjusted to neutrality.

Table 4.2

Primer sequences used in this study

27F	5'-AGAGTTTGATCMTGGCTCAG-3'	Universal	IDT, Lane et al. 1991
1542 R	5′-AAGGAGGTGATCCAGCCGCA 3′-TGCGGCTGGATCACCTCCTT	Universal	Pantos,2003
704F	5′-AGATTTTCCGACGGCAGGTT-3′	Universal	Genosys
519R	GWATTACCGCGGCKGCTG	Universal	Turner et al. 1999
907R	CCGTCAATTCMTTTRAGTTT	Universal	Lane et al. 1991
1100R	AGGGTTGCGCTCGTTG	Bacteria	Genosys
1220R	TTGTAGCACGTGTGTAGCCC	Universal	

Table 4.3

Restriction enzymes used in this study

Mnl1	5'...C C T C (N)7↓...3'3'...G G A G (N)6↑...5'	Thermo scientific
HaeIII	5'...G G↓C C...3'3'...C C↑G G...5'	Thermo scientific

Fig. 4.1
PCR gel image of Ground rice soil 1(selenate), 4(arsenate).

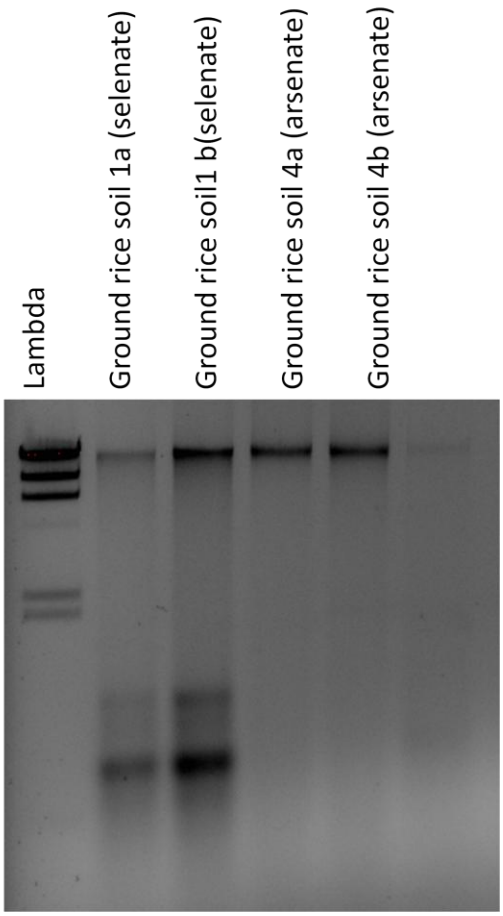


Fig. 4.2
PCR gel image of Ground rice soil 1b, 2a (selenate), 3a, 3b (arsenate).

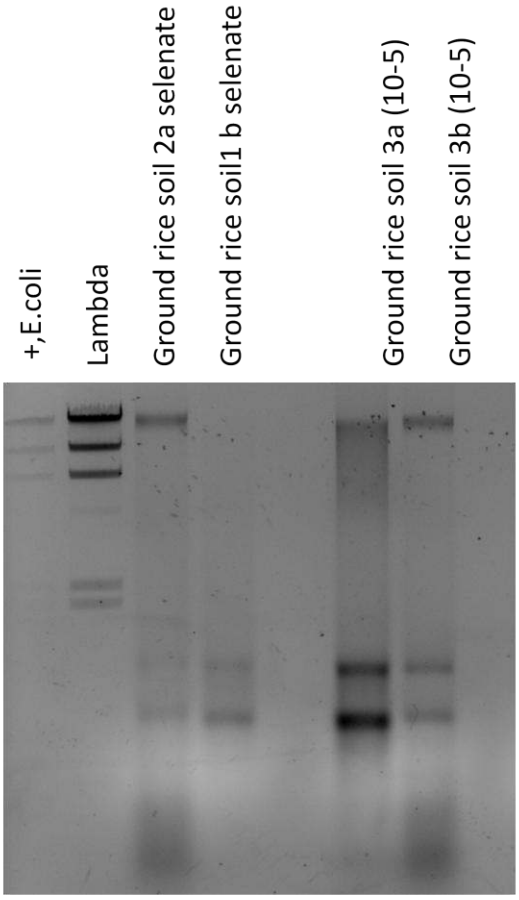


Fig. 4.3
PCR gel image of Ground rice soil 1 and Ground cabbage soil (arsenate).

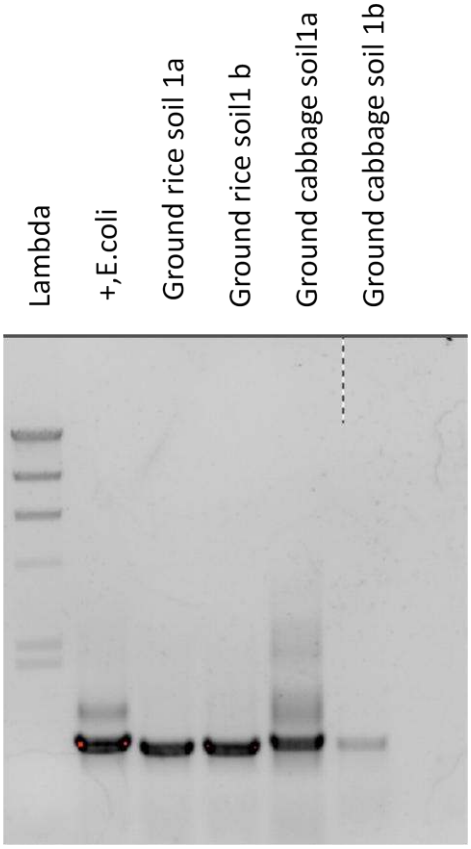


Fig. 4.4
PCR gel image of River rice soil (arsenate).

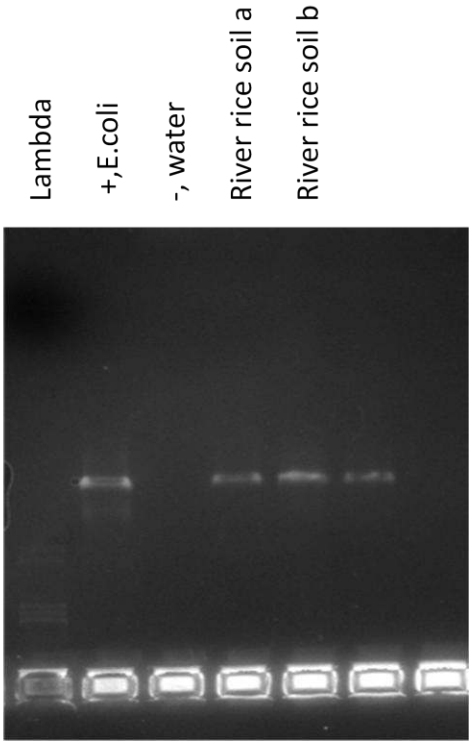


Fig. 4.5
PCR gel image of Ground rice soil 1, 3 and cabbage soil (arsenate).

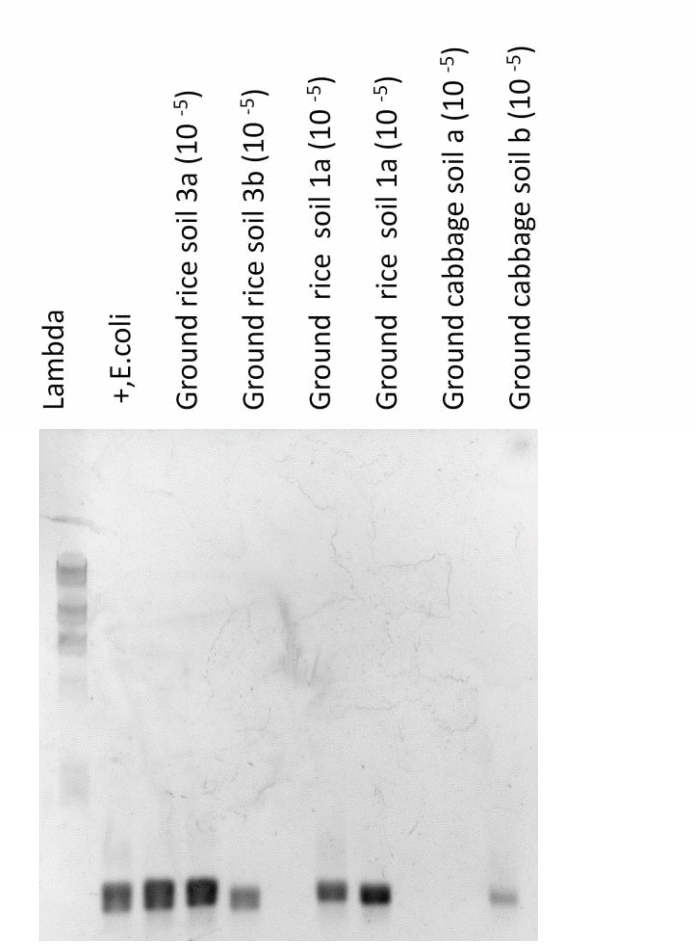


Fig 4.6

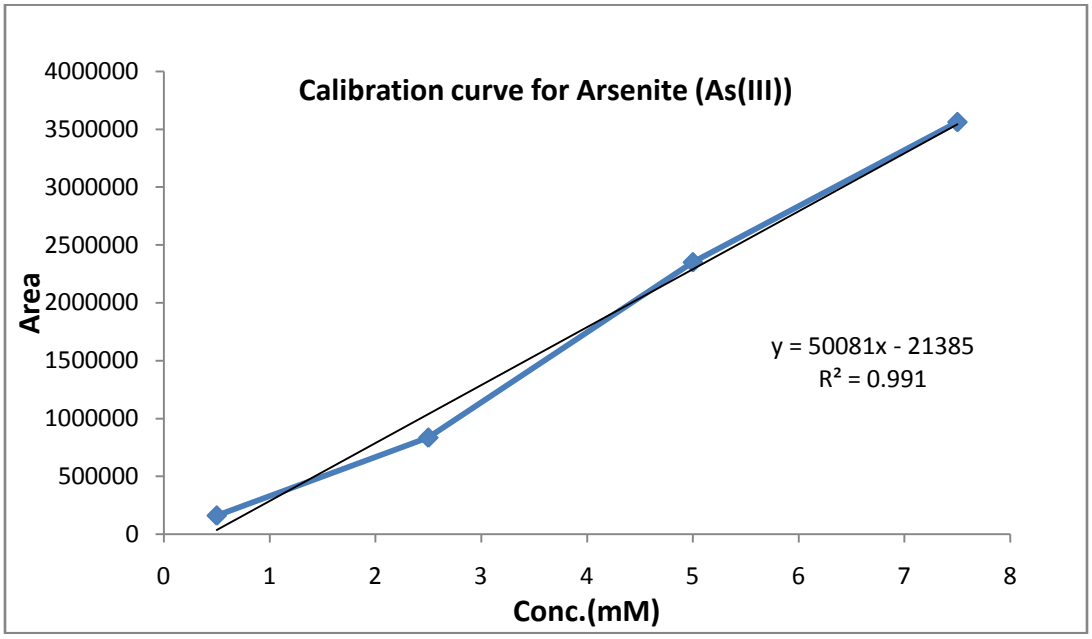


Table 4.4

	Arsenite As(III)	t=0		14 days		24 days	
		Area	Conc(mM)	Area	Conc(mM)	Area	Conc(mM)
I.1.1a	Ground Cabbage soil 1	1932091	4.28	4140663	8.6948614	4559422	9.53
I.1.1b		2119889	4.65	4901991	10.215039	3793855	8.00
II.3.1a	River Rice soil	1382291	3.18	2897189	6.2119605	2710639	5.83
II.3.1b		1508511	3.43	3191399	6.7994229	3232876	6.88
I.2.1a	Ground Rice soil 1	1567676	3.55	3438292	7.2924054	3616021	7.64
I.2.1b		1504321	3.43	3265770	6.9479229	3395606	7.20
I.2.2a	Ground Rice soil 2	2121470	4.66	3972593	8.3592684	3941225	8.29
I.2.2b		2057515	4.53	3483528	7.3827301	3848503	8.11
I.2.3a	Ground Rice soil 3	1626649	3.67	3542771	7.5010233	3788322	7.99
I.2.3b		1698413	3.81	3648729	7.7125945	3202697	6.82
I.2.4a	Ground Rice soil 4	1366616	3.15	2816738	6.0513203	2979745	6.37
I.2.4b		1521619	3.46	2897177	6.2119365	2904249	6.22

Fig. 4.7

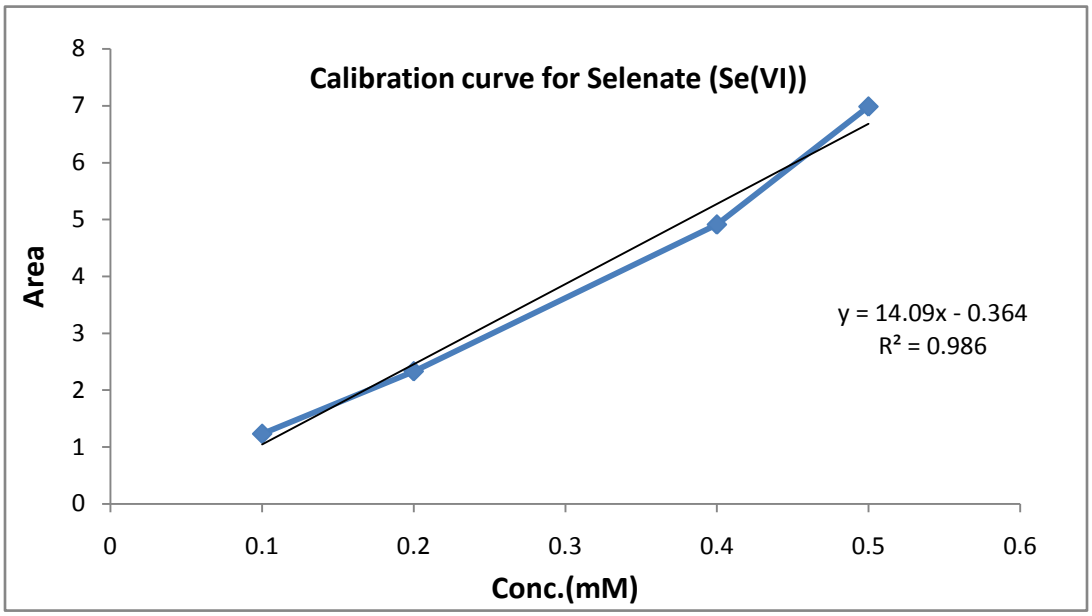


Table 4.5

	SelenateSe(VI)	t=0			t=13 days		
		Area		Conc(mM)	Area		Conc(mM)
I.1.1a	GW5	5.81	0.4378	4.37	2.026	0.16962	1.69
I.1.1b		5.34	0.4044	4.04	0.01	0.02659	0.26
I.2.1a	GW1	27.36	1.9668	19.66	0.023	0.02748	0.27
I.2.1b		6.16	0.4629	4.62	2E-04	0.02589	0.25
I.2.2a	GW2	5.27	0.3997	3.99	0.003	0.02608	0.26
I.2.2b		5.92	0.4458	4.45	0.004	0.02618	0.26
I.2.3a	GW 3	6.50	0.4873	4.87	2.104	0.17515	1.75
I.2.3b		6.33	0.475	4.74	0.939	0.09252	0.92
I.2.4a	GW4	6.50	0.4869	4.86	0.053	0.02962	0.29
I.2.4b		7.14	0.5322	5.32	0.074	0.03111	0.31
II.3.1a	RW	6.24	0.4682	4.68	0.021	0.02735	0.27
II.3.1b		6.53	0.4888	4.88	0.012	0.02669	0.26

Fig. 4.8

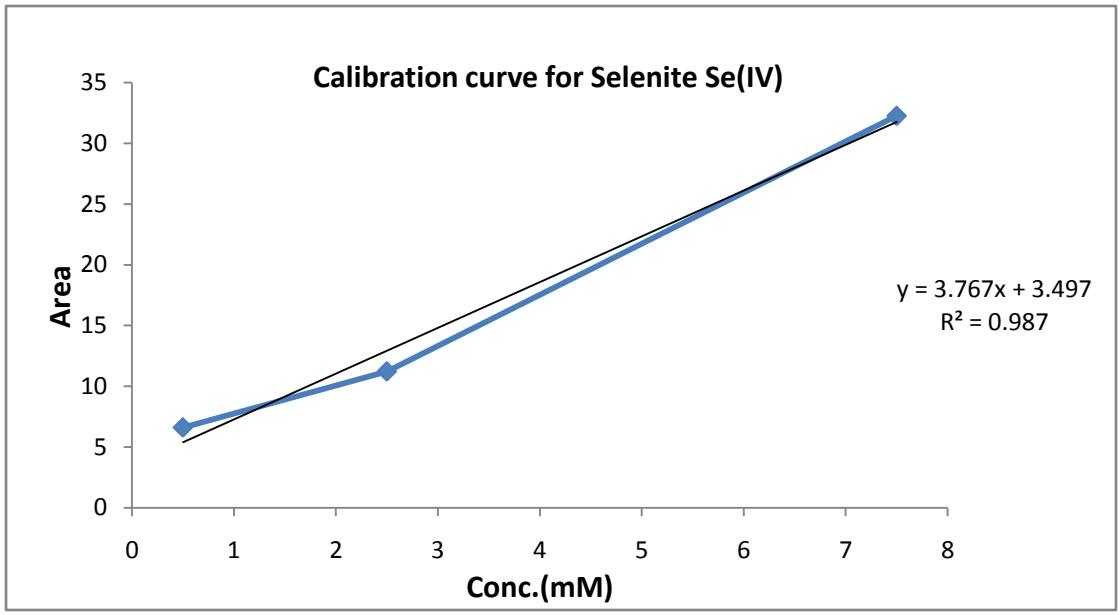


Table 4.6

	Selenite Se (IV)	t=0			t=13 days		
		Area		Conc(mM)	Area		Conc(mM)
I.1.1 a	GW5	58.43	14.5813 1819	1.45	64.98	16.3199 639	1.63
I.1.1 b		70.59	17.8090 9405	1.78	74.84	18.9372 2294	1.89
I.2.1 a	GW1	71.04	17.9285 4299	1.79	72.11	18.2125 6603	1.82
I.2.1 b		71.35	18.0108 3004	1.80	71.95	18.1700 9529	1.81
I.2.2 a	GW2	71.53	18.0586 0961	1.80	73.16	18.4912 8023	1.84
I.2.2 b		70.68	17.8329 8383	1.78	73.27	18.5204 7886	1.85
I.2.3 a	GW 3	71.5	18.0506 4635	1.80	73.46	18.5709 1286	1.85
I.2.3 b		72.24	18.2470 735	1.82	72.3	18.2630 0003	1.82
I.2.4 a	GW4	73.96	18.7036 339	1.87	71.4	18.0241 0214	1.80
I.2.4 b		71	17.9179 253	1.79	71.21	17.9736 6814	1.79
II.3. 1a	RW	76.11	19.2743 344	1.92	72.7	18.3691 7686	1.83
II.3. 1b		75.46	19.1017 9704	1.91	60.5	15.1307 8332	1.51

16S rRNA gene sequence of *Klebsiella pneumoniae AG-1*

CCGCATAATGTCGCAAGACCAAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCA
GATGGGATTAGNTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAG
AGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTCG
GGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCTTGTCGATTGACGTT
ACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAG
CGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATC
CCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAG
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CCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCT
AACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGT
CTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTA
TCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAA
GGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGC
ATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCANAAAGTATGTCGTAGTCCGGA
TTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCANAATGCTACG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTTGCAAAAGA
A

16S rRNA gene sequence of *Klebsiella pneumoniae AG-2*

ATTTGATCATGGTCAGATTGAACGCTGGCGGCAGGCTAACACATGCAAGTCGAGCGGTAGCAC
AGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGA
TGGAGGGGGATTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGACCAAAGTGGGGG
ACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGC
TCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACAC
GGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGC
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GCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAAC
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