Detection of Arsenite-oxidizing bacteria in groundwater from a gold mine under different geochemical environments

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การตรวจพบอาซีไนท์-ออกซิไดซิ่งแบคทีเรียในน้ำใต้ดินบริเวณเหมืองทองภายใต้สภาพแวดล้อมทางธรณีเคมีที่แตกต่างกัน

นายสุพีรภัทร ไกรเดช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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สุพีรภัทร ไกรเดช : การตรวจพบอาซีไนท์-ออกซิไดซิ่งแบคทีเรียในน้ำใต้ดินบริเวณเหมืองทองภายใต้สภาพแวดล้อมทางธรณีเคมีที่แตกต่างกัน (Detection of Arsenite-oxidizing bacteria in groundwater from a gold mine under different geochemical environments) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ศรีเลิศ โชติพันธรัตน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร. พรินท์พิดา สนธิพันธ์, 80 หน้า.

เนื่องด้วยประชากรโลกกว่าหลายล้านคนรวมถึงประเทศไทยมีความเสี่ยงในการบริโภคน้ำบาดาลปนเปื้อนสารหนูสูงกว่ามาตรฐาน ยิ่งไปกว่านั้นสารหนูในน้ำบาดาลมักปรากฏอยู่ในรูปที่เป็นพิษสูง (อาซิไนท์) มากกว่ารูปที่เป็นพิษน้อย (อาร์ซิเนต) ดังนั้นงานวิจัยนี้ต้องการนำศาสตร์ทางด้านจุลชีววิทยา ธรณีวิทยา และวิเคราะห์ทางสถิติมาศึกษากลุ่มประชากรของอาร์ซิไนต์-ออกซิไดซิ่งแบคทีเรียในน้ำบาดาลปนเปื้อนสารหนูและตรวจหาความสัมพันธ์ของกลุ่มประชากรนี้ต่อสภาพแวดล้อมเพื่อความเข้าใจและนำไปต่อยอดในการพัฒนาเทคโนโลยีการบำบัดสารหนูทางชีวภาพต่อไปในอนาคต โดยทางด้านจุลชีววิทยานั้นจะมุ่งเน้นการตรวจพบและระบุสายพันธ์ของกลุ่มแบคทีเรียนี้โดยใช้วิธี PCR-DGGE การโคลนนิ่ง และการอ่านลำดับดีเอ็นเอสายยีนอาซีไทน์-ออกซิเดส (*aoxB*) และทำการเปรียบเทียบกลุ่มประชากรที่ได้จากน้ำบาดาลจำนวน 9 แหล่งจากเหมืองทองและชุมชนโดยรอบรวมถึงน้ำผิวดิน และดิน (เพื่อเป็นตัวแทนของจุดเหนือน้ำ ภายใน และทางน้ำของพื้นที่เหมือง) ผลการทดลองแสดงให้เห็นว่ากลุ่มประชากรของอาซีไนท์-ออกซิไดซิ่งแบคทีเรียมีความคล้ายคลึงกับ กลุ่ม แอลฟา (α)- และเบต้า(β)-โปรทีโอแบคทีเรีย โดยแต่ละกลุ่มที่มาจากน้ำบาดาล น้ำผิวดินและดินมีความแตกต่างกัน ซึ่งในน้ำบาดาลนั้นกลุ่มประชากรส่วนใหญ่จะคล้ายคลึงกับกลุ่มเบต้า(β)-โปรทีโอแบคทีเรีย ซึ่งประกอบไปด้วยจีนัส *Hydrogenophaga Burkholderia Alcaligenes Variovorax Thiomonas* และ *Cupriavidus* ผลการทดลองนี้สื่อให้เห็นว่ากลุ่มประชากรอาซีไนท์-ออกซิไดซิ่งแบคทีเรียท้องถิ่นเหล่านี้อาจจะมีความเกี่ยวข้องในการควบคุมวัฏจักรของสารหนูในสิ่งแวดล้อมของน้ำบาดาลโดยการเปลี่ยนรูปสารหนูให้มีความเป็นพิษน้อยลงและลดความสามารถในการแพร่กระจายตามธรรมชาติ ยิ่งไปกว่านั้นการทำโมเดลทางธรณี-เคมีของสารหนูด้วยโปรแกรม PHREEQC และการวิเคราะห์ทางสถิติแสดงให้เห็นว่าอาซีเนทเป็นรูปของสารหนูที่ปรากฏในน้ำบาดาลส่วนมากในการศึกษานี้ แต่รูปแบบของสารหนูไม่พบว่าเป็นปัจจัยที่ส่งผมต่อกลุ่มประชากรของอาซีไนท์-ออกซิไดซิ่งแบคทีเรียนี้ แต่พบว่าค่าความเป็นกรด-ด่าง (pH) ดัชนีที่แสดงถึงระดับความสามารถในการเกิดออกซิเดชันและการศักยภาพการเกิดออกซิเดชัน-รีดักชัน (ORP) และค่าออกซิเจนละลายน้ำ (DO) แสดงให้เห็นถึงความสัมพันธ์โดยตรงอย่างมีนัยสำคัญ อีกทั้งความเข้มข้นของสารหนูและเหล็กยังพบว่าเป็นปัจจัยรองที่อาจส่งผลต่อกลุ่มประชากรของอาซีไนท์-ออกซิไดซิ่งแบคทีเรียนี้

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Millions of people around the world potentially expose to arsenic (As) contaminated groundwater. Besides, the dominant form of As in groundwater is arsenite (As^{+3}) and is more toxic than arsenate (As^{+5}). This study integrated the microbial investigation, geochemical modeling, and multivariate statistical analysis to investigate the arsenite-oxidizing bacteria community from As-contaminated groundwater and its environmental influencing factors in order to understand and further develop the *in-situ* arsenic bioremediation technology. Microbial investigation was focusing on the detection and identification of native arsenite-oxidizing bacterial community using PCR-DGGE, cloning and sequencing of arsenite oxidase (*aoxB*) gene amplicons. Nine groundwater samples were collected from the gold mine and residential areas. Surface water and soil samples were also collected from upstream, within, and downstream of the gold mining area and then were compared each other. The results showed that the majority of arsenite-oxidizing bacteria was related to *α*-, *β*-proteobacteria-like clusters in which the environmental media divided them into separated clusters. Many of groundwater clones revealed affiliated to the member of *β*-proteobacteria class where it was contributed by *Hydrogenophaga, Burkholderia, Alcaligenes, Variovorax, Thiomonas, and Cupriavidus* genera. This finding implied that these native arsenite-oxidizing bacteria might play a key role in controlling an As geochemistry in As-contaminated groundwater. Moreover, PHREEQC geochemical modeling of As and multivariate statistical analysis revealed that As presented as As^{5+} in most groundwater samples. However, the speciations seemed to be not driven by the arsenite-oxidizing bacterial community, but geochemical charateristics of groundwater, which were pH, ORP, and DO, influence on the shape their communities significantly, while As, and Fe concentrations play a minor role.

Field of Study:	Hazardous Substance and Environmental Management	Student's Signature	.....
		Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....

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Chapter 1
INTRODUCTION

1.1 Background

Arsenic is a ubiquitous toxic metalloid that is ranked 20th the abundant element on earth's crust (Bahar et al., 2013) . It is known as a carcinogen that has a potential of developing cancer when expose in long term (Lievremont et al., 2009). In addition to its natural origins, the anthropological sources of arsenic (As) from both industrial and household activities have been increasing over the past several decades (Satyanarayana, 2012). Consequently, arsenic contamination has become a great concern throughout the world. Vast areas are contaminated with arsenic, besides, over 100 millions of people worldwide potentially expose to As-contaminated groundwater. Unfortunately, this long-term exposure could lead to several diseases such as hyperkeratosis, hyperpigmentation, cardiovascular disease, circulatory disorders, etc. (Satyanarayana, 2012). One of the major sources that involved As contamination from human activity is a gold mining where lots of As-containing ores are disrupted (e.g. pyrite and arsenopyrite) (Hudson-Edwards and Santini, 2013). As a result, some mining areas might have higher As concentration in their surrounding area that brings about the uncertainty of land use and consumption behavior. This could affect to the several adverse health effect developments on local people, lack of land utilization, lack of trade and investment, and unreliability of mining organization. Hence, some potentially high arsenic contaminated areas might pay attention to control this particular contaminant effectively. Various types of technology (e.g. chemical oxidation reduction, adsorption, and ion exchange) could be considered based on many factors (e.g. cost of investment and effectiveness), but some limitations still need to be accepted. However, one alternative that can be applied in broad range of situations, bioremediation, have been being studied and developed for decades and still need more in-depth research in order to improve its capability continuously.

Microbial activities play a key role in controlling arsenic in environment, microbial transformation of arsenic still be a good practical alternative way of treating arsenic species in natural environment that comes with a low-cost investment, an

effectiveness, and an ecofriendly mean to nature. One way to remediate groundwater contaminated with arsenic is to transform arsenite (As^{3+}), which is usually found in natural environment, into arsenate (As^{5+}) by biological oxidation reaction (Bachate et al., 2012). As a result, arsenate (As^{5+}) will be adsorbed easier and become much less toxic (Bahar et al., 2013).

Since the arsenite-oxidation bacteria were first introduced in 1918, lots of related bacteria have been isolated from various types of environment and studied about their abilities such as soil, industrial wastewater, water sediment, and surface water (Bahar et al., 2013). However, only a few studies have focused on these bacteria in groundwater environment where a number of groundwater have also been contaminated worldwide. For example, *Comamonadaceae*, *Acidovorax*, *Acinetobacter*, and *Hydrogenophaga* were detected in Bangladesh groundwater tube wells (Sutton et al., 2009); *Psedomonadaceae, Moraxellaceae, Rhizobiaceae*, and *Microbacteriaceae* were detected in West Bangal arsenic contaminated groundwater (Paul et al., 2014). This current study is contributed to the first few research of Thailand aiming to survey the communities of arsenite-oxidizing bacteria in groundwater. This research focuses on investigating the presence of arsenite-oxidizing bacteria in potentially contaminated groundwater in Thailand and explaining the presence of As speciation, including As^{3+} and As^{5+} in groundwater using the geochemical modeling program, PHREEQC. In addition, one of the directed sources of As, a gold mine and its surrounding areas will be specified as the study area as it is associated with lots of As-containing ores. Therefore, the detection of these arsenite-oxidizing bacteria in the particular area would exist generally in nature; for this reason, it could have potential of using as the bioremediation approach in natural groundwater effectively.

1.2 Objectives

1.2.1 To investigate the communities of arsenite-oxidizing bacteria in arsenic-contaminated groundwater collected around/within a gold mine area

1.2.2 To theoretically explain the arsenic speciation in groundwater by using PHREEQC modeling program with on-site geochemical parameters

1.2.3 To understand the relationship between the community of arsenite-oxidizing bacteria and geochemical conditions including arsenic speciations of groundwater samples

1.3 Hypothesis

1.3.1 The majority of the detected arsenite-oxidizing bacteria might belong to the class *Proteobacteria* which is highly dominated in As-contaminated groundwater.

1.3.2 Physiochemical properties of groundwater could affect the presence of As species in groundwater environment.

1.3.3 Arsenite-oxidizing bacteria may be detected in groundwater environments with the presence of both As^{3+} and As^{5+} species

1.4 Scopes of the study

1.4.1 The samples, including groundwater, surface water, and soils were collected from a gold mine which potentially contaminated by As.

1.4.2 Geochemical parameters that were measured on site were pH, temperature, total dissolved solid (TDS), conductivity, dissolved oxygen (DO), and oxidation reduction potential (ORP).

1.4.3 Geochemical parameters, including arsenic concentration, Total Organic Carbon (TOC), Total Kjeldahl Nitrogen (TKN), nitrate, total phosphorus, and hardness were analyzed using Atomic Absorption Spectroscopy (Gaseous hydride), TOC VCPH, Macro-Kjegahl, cadmium reduction, Ascorbic acid, and EDTA tritration, respectively. Manganese (Mn), iron (Fe) were analyzed by Atomic Absorption Spectroscopy (AAS). Lead (Pb) was analyzed by Atomic Absorption Spectroscopy (Graphite furnace). Turbidity, cyanide (CN^-), and sulfate were measured by spectrophotometer.

1.4.4 The communities of arsenite-oxidizing bacteria were analyzed by cloning and Denaturation Gradient Gel Electrophoresis (DGGE) approaches

1.4.5 The arsenite oxidase gene (*aoxB*) was used as a gene marker for investigating arsenite-oxidizing bacteria.

1.4.6 PHREEQC modeling program was used to explain the presence of As speciation in groundwater.

1.4.7 The Pearson's coefficient correlation and redundancy analysis were used to analyze the influencing environmental factors on the arsenite oxidizing bacterial communities.

Chapter 2
BACKGROUND AND LITERATURE REVIEW

2.1 Sources of As contamination

2.1.1 Natural sources

Arsenic (As) is ranked 20th the most abundant element on earth's crust with an average abundant concentration ranges between 2 to 5 mg/kg (Ahuja, 2008). It is widely distributed in nature that can be found in rocks, soil, water, sediments, and air showing that arsenic concentration is detected in a wide-range concentration in some environments which its natural abundance is one of the factors (Satyanarayana, 2012). However, there are many of natural factors that influence the concentration of arsenic in environments such as organic and inorganic components of the soil, redox potential, pH, and microbial activities. (Ahuja, 2008; Pal, 2015). In natural, the most abundant arsenic ore mineral is pyrite (FeS_2), chalcopyrite ($CuFeS_2$), galena (PbS), and marcasite (FeS_2), respectively (Pal, 2015). As a results of these weathering rocks and minerals, the subsequent leaching and runoff, As is mainly introduced into soil and water naturally (Satyanarayana, 2012). For example, the weathering of As-containing pyrite by the oxidation reaction occurring naturally resulted in the releasing of arsenic in environment are shown in Eq. 2.1, 2.2, and 2.3 indicating that arsenic can come from several minerals and reactions (Ahuja, 2008; Satyanarayana, 2012; Pal, 2015).

$$4FeAsS + 13O_2 + 6H_2 \longleftrightarrow 4SO_4^{2-} + 4AsO_4^{3-} + 4Fe^{3+} + 12H^+ \quad \textbf{Eq 2.1}$$

(Ahuja, 2008)

$$FeAsS + 13\ Fe^{3+} + 8H_2O \longrightarrow 14Fe^{2+} + SO_4^{2-} + 13H^+ + H_3AsO_{4(aq.)} \quad \textbf{Eq 2.2}$$

(Satyanarayana, 2012)

$$FeS_2 + \frac{15}{4}O_2 + \frac{7}{2}H_2 \longrightarrow Fe(OH)_3 + 2SO_4^{2-} + 4H^+ \quad \textbf{Eq 2.3}$$

(Pal, 2015)

2.1.2 Anthropological sources

The anthropological sources of As are also the major sources of As contamination. Its proportion can be ranked from commercial wastes 40%, coal ash

22%, mining industry 16%, and the atmospheric fallout from the steel industry 13% (Satyanarayana, 2012). Arsenic is widely used in the composition for several purposes from household uses such as wood preservatives, paints, drugs, dyes, metals, and semiconductors. In agricultural and industrial uses such as pesticides, fertilizers, mining smelting, and landfilling (Pal, 2015).

According to both natural and anthropological sources, the As concentration may high in some areas due to their geological characteristic, local activities, drainage system, and regulations associated with each areas (e.g. US is 10 μg/l; (WHO, 2011); therefore, some people might expose to high As concentration in some areas. Fig 2.1 illustrates the world map of population whose daily water consumption contains high arsenic level (>10 μg/l);(Lievremont et al., 2009).

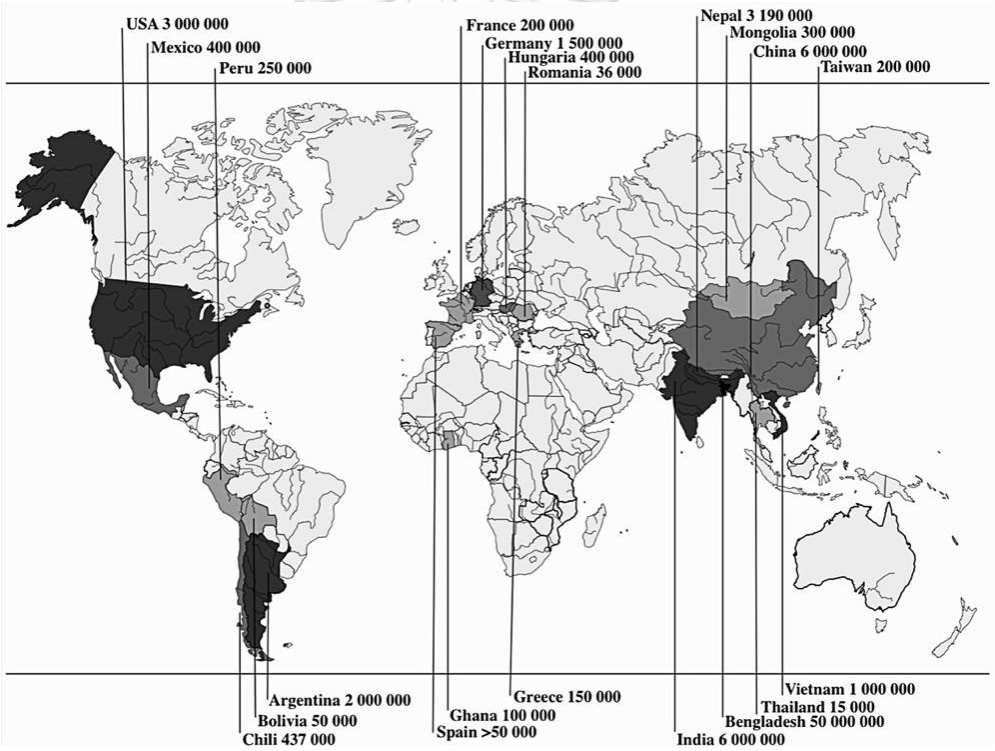


Figure 2.1 World map of population at risk whose daily water consumption contains As level above 10 μg/l. The shade grey indicate the number of persons contaminated, working from the palest (the lowest numbers) to the darkest shade (the highest number); (Lievremont et al., 2009).

2.1.2.1 Gold mining process as an association with arsenic releasing

Arsenic usually found as As-bearing mineral forms such as the component in sulfur-containing minerals; thus, it is rarely found as a pure metal (Hudson-Edwards and Santini, 2013). However, arsenopyrite (FeAsS) introduces one of the most common arsenic-containing ores; besides, it is usually affiliated with gold mining activities since significant amount of gold can be associated in arsenopyrite. Gold can present in arsenopyrite as liberated particles, attachments, and submocroscopic as can be detected mostly in Giant Yellowknife, Campbell Mine (Canada), and Sao Bento (Brazil) (Zhou et al., 2004).

One of the As releasing causes is the excavation process, the rate of arsenic releasing from As-containing sulfide minerals can be promoted in which the minerals are exposed to the weathering process. The example of As releasing from arsenopyrite can be demonstrated in the Eq.2.4

$$FeAsS + 7H_2O \longleftrightarrow Fe^{2+}_{(aq)} + H_3AsO_{3\,(aq)} + 11H^+ + 11e^- + SO_4^{2-} \quad \textbf{Eq. 2.4}$$

(Drahota et al., 2009)

In case of these gold-associated with pyrite or arsenopyrite, the gold extraction processes, such as roasting, are used in some mining. This gold extraction process can convert arsenic into an arsenic trioxide gas (As_2O_3) together with sulfur content by heating it up in the presence of high temperature air as shown in Eq.2.5 (Sturm, 2009). Nevertheless, converting these arsenic-containing ores into arsenic trioxide is likely to make it more soluble and bioaccessible than the original (Fraser et al., 1991).

$$2FeAsS + 5O_2 \xrightarrow{\Delta H = -1987\ KJ/mol} Fe_2O_3 + 2SO_2 + As_2O_3 \quad \textbf{Eq 2.5}$$

(Sturm, 2009)

2.2 Chemical properties of As

Arsenic has atomic number of 33. It is ranked 20th most abundant element in the earth's crust, 14th in seawater, and 12th most abundant in human body (Pal, 2015).

Arsenic exists in various forms of oxidation states: Arsenide (As^{-3}), Arsenic element (As^{0}), Arsenite (As^{3+}), and Arsenate (As^{5+}) (Bahar et al., 2013). However, only arsenite and arsenate are the most common forms in natural water such as H_3AsO_3, and H_3AsO_4. These two oxidation states depend on the oxidation-reduction potential (Eh) and pH of the water as shown in Fig 2.2. In general surface and groundwater pH conditions, H_2AsO^{4-} (As^{5+}), $HAsO_4^{2-}$(As^{5+}), and H_3AsO_3 (As^{3+}) are the most predominant arsenic species.

Arsenite tend to be stable in reducing condition or an anaerobic environment, while arsenate are stable in oxidizing condition or aerobic environment. Since arsenite are more soluble and less adsorb than arsenate, therefore, arsenic concentration in water will be detected as higher concentration in reducing environment than in oxidizing environment.

The role of redox condition also be a part of controlling the presence of As in environment. Organic matters have been suggested on influencing the reducing condition in aquifer which is favorable to the formation of arsenic-containing sulfide minerals (Pal, 2015). The addition of ferric irons (Fe^{3+}) into water with the presence of oxygen could increase the oxidation rate of arsenite under acid condition. In addition, manganese oxide (MnO_2), the common oxidant found in an aquifer also induce the oxidation of arsenite in wide range of pH.

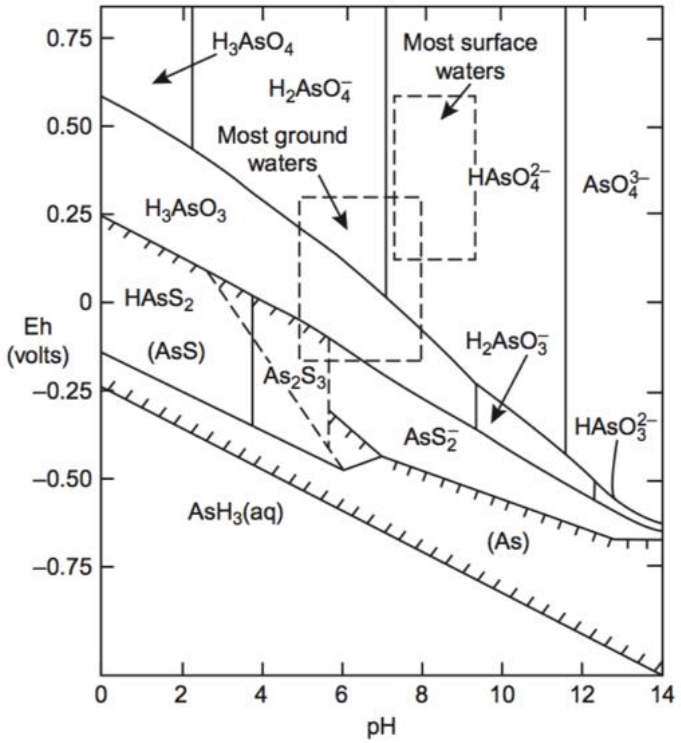


Figure 2.2 Eh-pH diagram for As at 25℃ and 1 bar total pressure, with total arsenic 10^{-5} mol/l; symbols for solid species are enclosed in parentheses in crosshatched area, which indicates solubility less than 10-5 mol/l (Pal, 2015).

2.3 PHREEQC Geochemical modeling

PHREEQC is a modeling program developed by US Geological Survey using *C* Language for coding. The model is based on equilibrium chemistry of aqueous solution that capable of performing speciation and solubility, reaction path, inverse mass balance modeling, and one-dimensional advective-dispersive-reactive transport calculations (Zhu and Anderson, 2002). PHREEQC can be used for calculating speciation, saturation indices, the distribution species of a specified solution composition (Parkhurst and Appelo, 2013). For calculating speciations, PHREEQC will perform an aqueous modeling using total concentration of elements in solution by calculating the activities of all aqueous species, additionally, these activities can be used for saturation indices for minerals.

PHREEQC is applicable for modeling in various hydrogeochemical environments; however, some limitations are needed to be considered. Phreeqc uses internal thermodynamic database that providing from many sources and literatures. It

should be careful about selecting the suitable database that consistent with the actual data (Parkhurst and Appelo, 2013).

Phreeplot was used to generate the predominant diagram as it contains an embedded version of PHREEQC. Accordingly, it can calculate predominance and mineral stability diagrams which are generally known as pe-pH diagram. The diagram uses the Dzobak & Morel (1990) Dl model for Hfo to estimate As adsorption by Hfo (Dzombak and Morel, 1990). The adsorbed species were included by using hfo.inc database which links to the precipitation of $Fe(OH)_3$ (a) to the Hfo surface (Kinniburgh and Cooper, 2011).

PHREEQC have been used to calculate speciation of heavy metal including As in many studies. As species was model by PHREEQC model to investigate the influence of PO_4^{3-} on As bioavailability to *Lemna gibba*. The results were ensured by comparing to the natural freshwater condition as well as modifying the organic ligands and compounds through the published databases (Mkandawire et al., 2003). Couture and Cappellen (2011) used PHREQC model to calculate As speciation to investigate the oxidation of As^{3+} by zero-valent sulfur under highly reducing condition. The thermodynamic database WATEQ4F imbedded within PHREEQC was used for modeling As speciation. Moreover, the Eh-pH diagram were generated using Phreeplot computer code (Couture and Cappellen, 2011). Commonly, PHREEQC coupled with WATEQ4F was used for theoretical calculate the As speciation; for example, in the study of Daus et al (2006) and Drahota et al. (2009) for predict the change of As specie during storage using phosphoric acid, and the mineralogical and geochemical controls of arsenic speciation, respectively (Daus et al., 2006; Drahota et al., 2009).

2.4 Microbial As Transformation

Even though various of arsenic treatment technologies have been developed through physical and chemical methods such as chemical oxidation reduction, adsorption, ion exchange, and membrane filtration, some of them still have limitations such as impact of microbial and geochemical processes, the interference from oxides, hydroxides, carbonates, and sulfides, high operational cost and maintenance and toxic waste generation (Bahar et al., 2013). Alternatively, bioremediation of As still be a

good choice for consideration, because of its cost effectiveness and environmental compatibility.

This heavy metal bioremediation involves biotransformation, bioaccumulation, biosorption, and biovolatilizations (Bahar et al., 2013). These processes are performed by microbial activities to detoxify, mobilize or immobilize through oxidation-reduction, biomethylation, sorption, and complexation processes (Bahar et al., 2013). However, arsenic bioremediation usually relies on the oxidation-reduction process by converting arsenite into arsenate; thus, make them to be less toxic and less mobile (Bahar et al., 2013).

In 1918, Green isolated arsenic transforming bacteria for the first time including arsenite oxidizing bacteria, *Bacillus arsenoxydans*, and arsenate reducing bacteria, *Bacterium arenreducens* (Satyanarayana, 2012). After that, many As transforming bacteria have been isolated from various types of environments including surface and groundwater, soil and sediment, wastewater, coastal and seawater.

2.4.1 Microbial Arsenite oxidation

The arsenite-oxidizing bacteria have been known for many years that this group of bacteria have ability to oxidize arsenite into arsenate. Over than 30 strains have been studied indicating at least nine genera which α, β, γ-Protecbacteria are the most dominant groups (Oremland and Stolz, 2003). The arsenite-oxidizing bacteria can be divided in two types of mechanisms encountering arsenite.

Heterotrophic arsenite oxidizers (HAOs) is considered as a detoxification mechanism which transforming arsenite that encountered on the cellular outer membrane into less toxic form, arsenate. However, this mechanism still needs organic carbon as electron donor for their source of energy and growth (Islam, 2008; Satyanarayana, 2012). This detoxification mechanism of HAOs can be shown in an exergonic reaction in Eq. 2.6

$$2H_3AsO_3 + O_2 \xrightarrow{\Delta G^\circ = -256 KJ/mol} HAsO_4^{2-} + 3H^+ \qquad \textbf{Eq. 2.6}$$

(Santini et al., 2000)

Chemolithoautotrophic arsenite oxidizers (CAOs), on the other hand, gain energy from oxidizing arsenite in which arsenite is electron donor, while oxygen or

nitrate are reduced as electron acceptors to fix CO_2 into cell for their growth (Islam, 2008). Therefore, microbial arsenite oxidation to arsenate can be performed by both for detoxification and energy generation (Islam, 2008).

The arsenite oxidase (*aox*) or in some studies called *aro*/*aso* is the key enzyme of these arsenite oxidizing microorganisms. They are also divided into large subunits *aoxB* or *aroA* or *asoA*. Studies of this enzyme have suggested that it is linked to the catalytic activity of a membrane-bound periplasmic enzyme (Bahar et al., 2013). Recently, some researchers have proposed a new nomenclature for arsenite oxidase, *aio* by purifying from *Alcaligenes faecalis* and the two genes encoding the large and small subunits as *aioA* and *aioB* (Bahar et al., 2013).

However, the biological oxidation of arsenite to arsenate is crucial to study in term of using as a bioremediation approach for arsenic contamination because if arsenite, as a pollutant, is transformed into arsenate, it will be easier to immobilize on to strong adsorbents; thus, make it easy to be remediated (Oremland and Stolz, 2003). For some examples, the well-known arsenite oxidizing bacteria strain NT-26, a fast growing aerobic bacteria from gold mine soil, belongs to α-*Proteobacteria* and have shown an ability to grow either by chemoautotrophic or conventional heterotrophic (Oremland and Stolz, 2003). Another isolated arsenite oxidizer from different environments were also studied such as strain ULPAs1 from arsenic-contaminated water, strain M14 from bottom sediment, and strain HR13 from hot spring. Other than aerobic oxidation of arsenite, there are some bacteria are able to grow in anoxic condition with nitrate as electron acceptor. A study of bacterium strain MLHE-1 showed that it can oxidize arsenite under anaerobic condition as the following condition (Oremland and Stolz, 2003):

$$H_2AsO_3^- + NO_3^- \xrightarrow{\Delta G^\circ = -56.5\ KJ/mol} HAsO_4^{2-} + NO_2^- + H^+ \quad \textbf{Eq. 2.7}$$

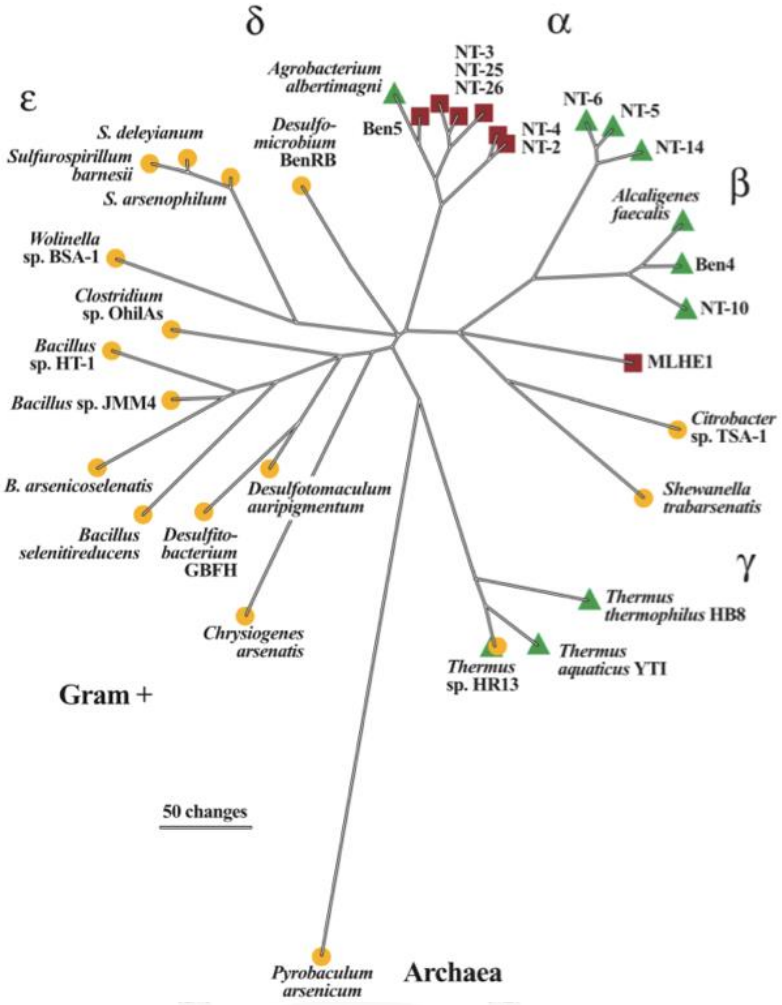


Figure 2.3 Phylogenetic diversity tree of arsenic-metabolizing prokaryotics. Yellow circles indicate Dissimiatory arsenite-respiring prokaryotes (DARPs), green triangles indicate heterotrophic arsenite oxidizers (HAOs), red squares indicate chemoautotrophic arsenite oxidizers (CAOs). In some cases, the microbe has been found the relation into more than one group (Oremland and Stolz, 2003)**.**

2.4.2 Microbial Arsenate Reduction

In contrast to arsenite oxidation, many researchers have also studied another biotransformation of As species by focusing the reduction of arsenate. These arsenate reduction bacteria are divided into two types of mechanisms as same as the arsenite oxidation bacteria (Lievremont et al., 2009).

The first arsenate reducer relates to detoxification process. Because arsenate has similar structure with phosphate; thus, they need to prevent arsenate from entering into

their cell by up taking it as a nutrient by phosphate transporter (Lloyd and Oremland, 2006).This process is followed by the excretion of the transformed arsenite from the bacteria cell (Lievremont et al., 2009). This process involves *ars* enzyme which composes of at least three genes (*ars*R: a transcriptional repressor, *ars*B: a transmembrane efflux pump, and *ars*C: an arsenate reductase) (Lievremont et al., 2009).

The second arsenate reducer is considered as dissimilatory arsenate-reducing prokaryotes (DARPs) (Oremland and Stolz, 2003). It was first identified in the mid 1990s with the two bacteria, *Sulfurospirillum arsenophilum and Sulfurospirillum barnesii*, that belong into ε-*Proteobacteria* group, and many groups have also been identified afterward including γ - and δ-*Proteobacteria* (Lloyd and Oremland, 2006). These arsenate reducer breath arsenate and gain energy by utilizing arsenate as electron acceptor (Lievremont et al., 2009). The arsenate respiration mechanism involved with the respiratory arsenate reductase (*arr*) enzyme which consists of two subunits, *arr*A and *arr*B and has been identified as a membrane bound heterodimer protein (Lievremont et. al., 2009). This enzyme is derived from *Chrysiogenes arsenatis*, the bacteria isolated from goldmine wastewater, and found the relation with dimethylsufoxide (DMSO) family of mononuclear molybdenum-containing enzymes (Lloyd and Oremland, 2006).

To summarize, the biotransformation of arsenic by bacteria can be done by 3 main enzymes including *aox*, *arr* and *ars* which are illustrated by Jonathan R. Lloyd and Ronald S. Oremland in Fig 2.4.

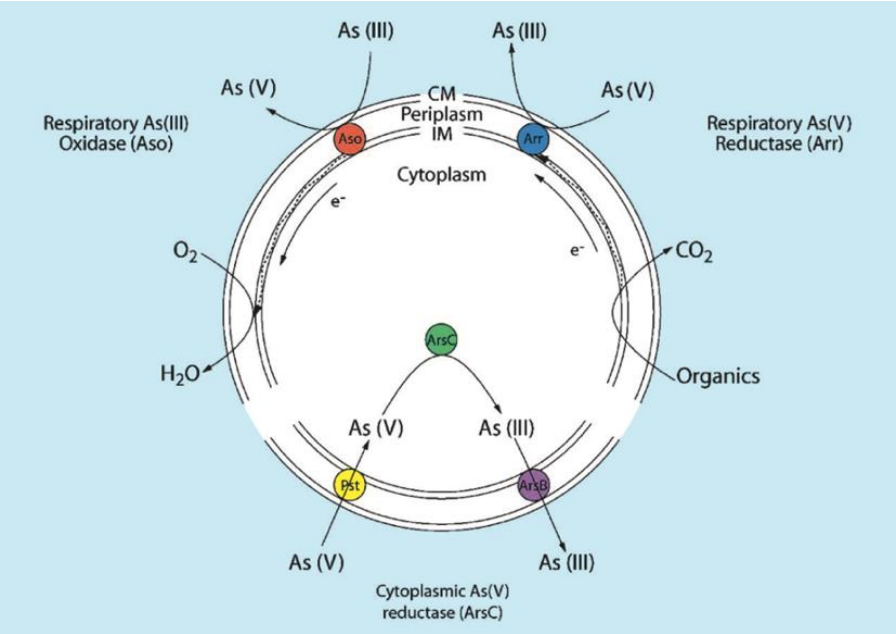


Figure 2.4 The three main enzymes *(aso* or *aox*, *arr*, and *arsC*) of microbial transformation of arsenic in the environment (Lloyd and Oremland, 2006).

2.5 *Proteobacteria* group: arsenite-resistant bacteria

Proteobacteria is a group of microorganisms. They are Gram-negative which have lipopolysaccharides composed on their outer membranes. Their metabolisms are diverse including chemoautotrophic, chemoorganotrophic, and phototrophic metabolisms. The *Proteobacteria* are phylogenetically defined based on sequence of 16S rRNA into five groups: α-*Proteobacteria*, β-*Proteobacteria*, γ-*Protecbacteria*, *δ* – *Proteobacteria*, and ε-*Proteobacteria* (Marin, 2011). Commonly, the arsenite-resistant bacteria are found the affiliation with *Proteobacteria* group in various classes. Short reviews of some interesting genus of *Proteobacteria* are described below:

2.5.1 Alcaligenes

The Alcaligenes genus belongs to β-*Proterobacteria* class, *Alcaligenaceae* family. It is gram negative which has rods or coccobacilii shape with 0.5-1.2 X 1.0-3.0 μm on size. This bacteria is a strictly aerobic bacteria that use oxygen as the terminal

electron acceptor in respiratory metabolism. Alcaligenes faecalis (*Achromobacter arsenoxydans-tres*, *Pseudomonas odorans*) are the species belongs into this genus. In some strains are well known that are able to oxidize arsenite as electron donor. It is found in several types of environment such as soil, water, and wastewater treatment plants (Garrity, 2005).

2.5.2 Achromobacter

These are strictly aerobic bacteria with rod-shaped. They also belong to β-proterobacteria, *alcaligenaceae* family. There has genomic analysis of *Achromobacter arsenitoxydans,* isolated from arsenic-contaminated pig farm soil, showed that it contains arsenic resistance operons (*ars*) and arsenite oxidation operons (*aox*) and be able to oxidize arsenite to arsenate effectively (Li et al., 2012).

2.5.3Acidiphilium

The Acidiphilium genus belongs to α-*Proteobacteria* class, *Acetobacteraseae* family. The cells are straight rods shape with 0.3-1.2 X 4.2 μm on size. It is strictly aerobic bacteria which can grow either chemoorganotrophic or chemolithotrophic. It is known that *Acidiphilium multivorum* has the ability to oxidize arsenite into arsenate, while other similar species does not. Its cell contains arsenic resistance (*ars*) operon which consists of five genes: *arsR*, *arsD, arsA*, *arsB,* and *arsC (Garrity, 2005)*.

2.6.4 Arcobacter

Only few arsenite resistant bacteria belong to ε-*Proteobacteria*, Arcobacter genus which belongs to Campylobacteraceae family are one of the arsenite oxidizers. They are slender or curved rods shapes which has 0.2-0.9 X 0.5-3 μm on size. *Arcobacter Butzleri*, *arsobacter cryaerophilus*, and *Arcobacter skirrowii* were able to grow in 0.001% sodium arsenite medium (Garrity, 2005).

2.6 Arsenite-oxidizing bacteria detected from natural environments

The community of arsenite-oxidizing bacteria were studied in many research worldwide with an aim of identifying the native bacteria involve arsenite oxidation in several environments. Marianne Quemeneur et al. (2010) have collected eight arsenic-contaminated water including surface and groundwater from upstream and downstream of mining areas and have identified the abundance of arsenite-oxidizing bacteria. The

Real time PCR (qPCR) and Denaturing Gradient Gel Electrophoresis (DGGE) were used in the analytical processes by using *aoxB* enzymes targeting primers. Results showed that various of arsenite-oxidizing bacteria species belong to α-, β-, and γ-*Proteobacteria* such as *Thiomonas arsenuvorans* DSM 16361, *Acidovorax* sp.75, *Acinetobacter* sp.33, *Alcaligenes* sp.T12RB, *Aminobacter* sp.86, and *Pseudomonas* sp.46 (Quemeneur et al., 2010). Chen and Shao (2009) have studied the diversity of arsenite-resistant bacteria in the varied arsenite concentration (2 to 100 mM) enriched samples from deep-sea sediments using PCR and DGGE as analytical instruments with universal and arsenite transporter genes-targeting primers. Results showed that the γ-*Proteobacteria* are the most dominant group followed by *Actinobacteria*, α-*Proteobacteria*, and *cytophaga-Flavobacterium* (CFB), respectively. In addition, the denaturing gradient gel electrophoresis revealed that *Microbacterium esteraomaticum* was the dominant member that showed the highest arsenite resistant in the enriched communities (Chen and Shao, 2009). Similary, Paul et al. (2014) have characterized nine bacteria strains from arsenic-contaminated groundwater from West Bengal by using 16S rRNA gene targeting primers in PCR amplification process. Results showed that strains belong various of generas: *Psedomonadaceae, Moraxellaceae* (γ-*Proteobacteria*), *Rhizobiaceae* (α-*Proteobacteria*), and *Microbacteriaceae* (*Actinobacteria*). The minimum inhibitory comcentration (MIC) of arsenite was also tested in which the hightest MIC was detected to be 40mM indicating high arsenic resistant and have a potential on bioremediation (Paul et al., 2014). Moreover, Santini et al. (2000) have isolated arsenite-oxidizing bacteria strain NT-26 from arsenopyrite (FeAsS) rock of a gold mine. The identification was done by PCR amplification using 16S rDNA primers. The results showed that strain NT-26 belongs to the *Agrobacterium/Rhizobium* branch of α-*Proteobacteria*. They found the chemolithoautotrophic characteristic that growing rapidly under the presence of oxygen. NT-26 is able to grow in the enrich medium containing 5mM of arsenite which has optimum pH of 5.5, double time of 7.6hr. (Santini et al., 2000). In the same trend, Sutton et al. (2009) have characterized the population of As-mobilization bacterial in the deep and shallow tube wells in Bangladech using PCR, DGGE, and clone library as the characterizing tools and found the majority of the sequencing belonged to β -*Proteobacteria* (75% from clone library, and 84% from DGGE). High arsenic tolerance

bacteria were identified as different genera such as *Comamonadaceae*, *Acidovorax*, *Acinetobacter*, and *Hydrogenophaga* (Sutton et al., 2009) Interestingly, Oremland et al. (2002) have enriched a bacteria strain MLHE-1, a sample from the Mono lake's anoxic bottom waters, with 5mM arsenite concentration and 5 mM NaNO3 concentration. Results indicated the chemoautotrophic growth as shown in the eq. 2.8 with arsenite and nitrate as electron donor and accecptor, respectively. The phylogenetic analysis suggested that this bacteria strain was affiliated with *Ectothiorhodospiraceae* of the γ-*Proteobacteria* group (Oremland et al., 2002).

$$H_2AsO_3^- + NO_3^- \xrightarrow{(\Delta G^\circ = -87.16\ KJ/mol)} H_2AsO_4^- + NO_2^- \quad \textbf{Eq.2.8}$$

(Oremland et al., 2002)

On the other hand, Drahota et al. (2009) have investigated factors that influences the speciation and mobilization of arsenic in natural soil, sediment, and water. By PHREEQC modeling, it indicated the $HAsO_4^{2-}$ was the predominate form of arsenate, while H_3AsO_3 was the predominate form of arsenite. The higher arsenic concentration was detected under redox transition zone that As-bearing ores were dissolute such as scorodite, pharmacosiderite, and arseniosiderite. They suggested the microbial activity might play an important role on controlling in the redox state in water that affected on arsenic speciation and have correlation with dissolved organic carbon concentration in water (Drahota et al., 2009).

Summary of detected arsenite-oxidizing bacteria is shown in Table 2.1

Table 2.1 Summary of arsenite-oxidizing bacteria detected from environments

Arsenite-oxidizing bacteria	Isolated environments	Detection methodology	Reference
Thiomonas arsenuvorans DSM 16361, *Acidovorax* sp.75, *Acinetobacter* sp.33, *Alcaligenes* sp.T12RB, *Aminobacter* sp.86, and *Pseudomonas* sp.46	As-contaminated surface and groundwater	qPCR, DGGE	(Quemeneur et al., 2010)
Microbacterium esteraomaticum	Deep sea sediment	PCR-DGGE	(Chen and Shao, 2009)
Psedomonadaceae, *Moraxellaceae*, *Rhizobiaceae*, *Microbacteriaceae*	As-contaminated groundwater	PCR, cloning	(Paul et al., 2014)
Agrobacterium, *Rhizobium*	Arsenopyrite rock from a gold mine	Enrichment, PCR	(Santini et al., 2000)
Bacteria strain MLHE-1, *Ectothiorhodospiraceae*	Lake's bottom water	PCR-DGGE	(Oremland et al., 2002)
Comamonadaceae, *Acidovorax*, *Acinetobacter*, *Hydrogenophaga*	Deep and shallow tube wells	PCR-DGGE, Cloning	(Sutton et al., 2009)
Bacillus, Aneurinibacillus aneurinilyticus	As-contaminated groundwater	Serial dilution, PCR	(Dey et al., 2016)
Pseudomonas, Bacillus, Psychrobacter, Vibrio, Citrobacter, Enterobacter, and *Bosea*	As-contaminated groundwater	Enrichment, PCR	(Liao et al., 2011)

Arsenite-oxidizing bacteria	Isolated environments	Detection methodology	Reference
Pseudomonas, Bacillus, Brevibacillus, Delftia, Wohlfahrtiimonas and *Dietzia*	As-contaminated soil	PCR, cloning	(Sanyal et al., 2016)
A. tumefaciens, Buttianuxella agrestis, A. tumefaciens, A. faecalis, Citrobacter sp., Acinetobacter, and *A. xylosoxidans*	As-contaminated drinking water	Enrichment, PCR	(Chang, 2015)
Acinetobacter, Aeromonas, Agrobacterium, Comamonas, Enterobacter, Pantoea, Pseudomonas sp.	Natural and constructed wetlands	Enrichment, PCR	(Chang et al., 2010)

Chapter 3
METHODOLOGY

3.1 Experimental Framework

The overall experimental framework of this study is shown in Fig 3.1. The experiments were planned to start from collecting the samples. Then, the experiments were divided into 3 main sections. First, the chemical analysis of both ground and surface water was conducted and followed by modeling the speciation of As using the obtained water parameters in the second step. On the other hands, the biological analysis was performed in the following part. The obtained As speciation results and the community of arsenite-oxidizing bacteria were used to investigate the environmental influence on bacterial community.

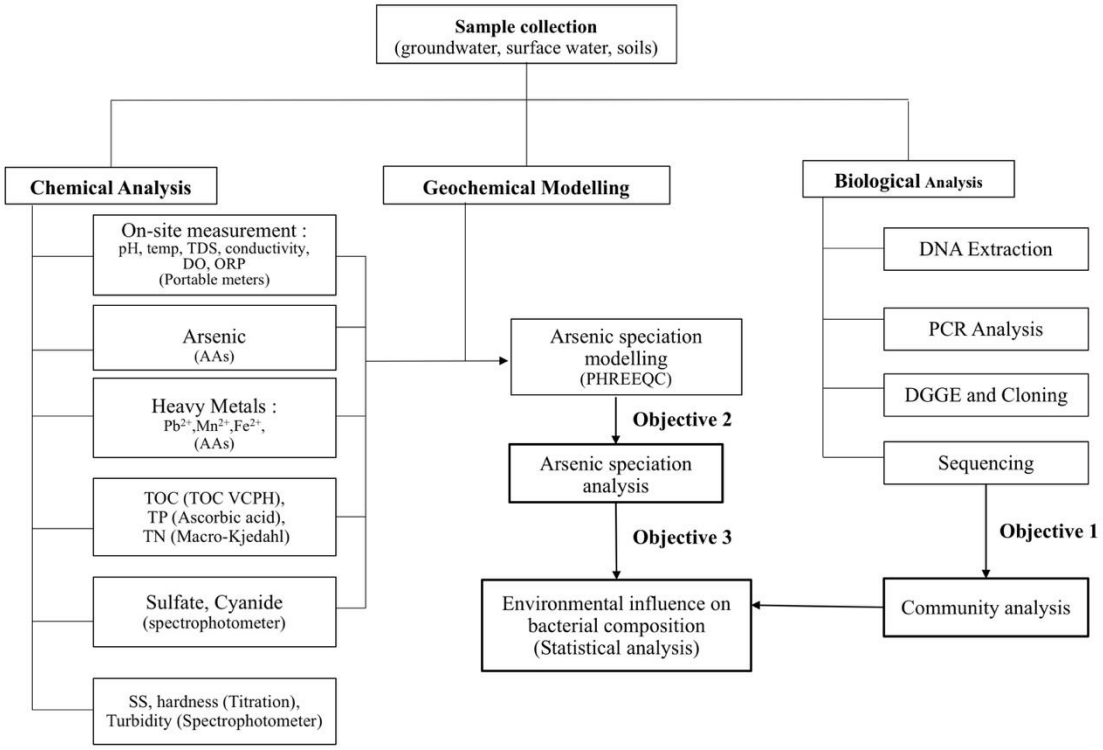


Figure 3.1 Overall experimental framework of the study

3.2 Sample collection

3.2.1 Study area

A gold mining in Pichit province, Thailand was defined as the study area as shown in Fig.3.2. Nine groundwater sampling locations was selected, including four mining stations (MA stations; MA-1, MA-2, MA-3 and MA-4) and five stations surrounding of a mining area, representing a residential area (RA stations; RA-1, RA-2, RA-3, RA-4 and RA-5). These sampling locations were grouped based on groundwater flow from upstream to downstream of the mining location and the previous As concentrations in groundwater. The RA-1 station was presumed to be the control station as it is located in the upstream from the mining area. Groundwater samples were collected from all sampling stations. However, surface water and soil samples were additionally collected from sampling sites RA-1, MA-1 and RA-4, representing upstream, mining and downstream locations, respectively. All sampling stations are listed in Table 3.1.

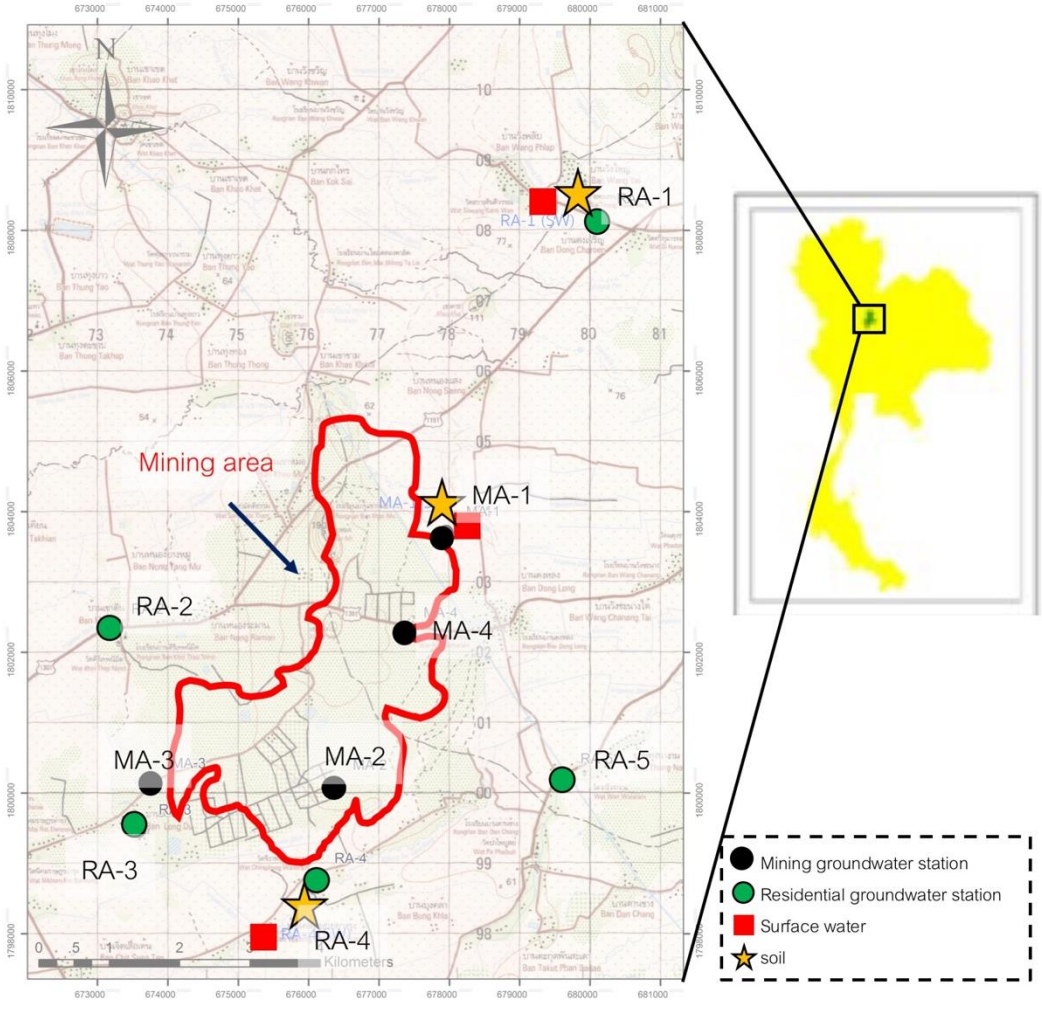


Figure 3.2 The sampling locations around and within the mining area (the red line boundary).

Table 3.1 The Sampling Stations in the study

Station*	Latitude	Longitude	Location	Represented Location
MA-1, MA-1$_{soil}$	16°18'27.9"N	100°39'56.3"E	Tai Dong Sub-district, Wang Pong district, Pechchaboon Province	Mining area (represented all media)
MA-2	16°16'31.0"N	100°39'01.1"E	Kao Jet Look Sub-district, Thap Klor district, Pichit Province	Mining area
MA-3	16°16'32.5"N	100°37'34.4"E	Kao Jet Look Sub-district, Thap Klor district, Pichit Province	Mining area
MA-4	16°17'41.2"N	100°39'35.5"E	Kao Jet Look Sub-district, Thap Klor district, Pichit Province	Mining area
RA-1, RA-1$_{soil}$	16°20'52.0"N	100°41'09.7"E	Wang Hin Sub-district, Wang Pong district, Pichit Province	Upstream mining area
RA-2	16°17'44.1"N	100°37'15.8"E	Kao Jet Look Sub-district, Thap Klor district, Pichit Province	Residential area
RA-3	16°16'11.1"N	100°37'28.1"E	Kao Jet Look Sub-district, Thap Klor district, Pichit Province	Residential area
RA-4, RA-4$_{soil}$	16°15'47.9"N	100°38'52.0"E	Kao Jet Look Sub-district, Thap Klor district, Pichit Province	Downstream of mining area
RA-5	16°16'33.1"N	100°40'50.1"E	Tai Dong Sub-district, Wang Pong district, Pichit Province	Residential area
MA -1$_{SW}$	16°18'39.9"N	100°39'53.6"E	Ban Kao Din ditch, 5 km. western area from the site, Kao Jet Look Sub-district, Thap Klor district, Pichit Province	Surface water from mining area
RA-1$_{SW}$	16°20'59.5"N	100°40'42.9"E	Sai Yang Rung weir, 5 km. southern area from the site, Kao Jet Look Sub-district, Thap Klor district, Pichit Province	Surface water from the upstream of mining area
RA-4$_{SW}$	16°15'22.4"N	100°38'26.7"E	Emergency pond of the 1st metal slug pond (TSF-1), southern area of the site, Kao Jet Look Sub-district, Thap Klor district, Pichit Province	Surface water from the downstream of mining area

**MA = Mining area, RA = Residential area and SW =surface water*

3.2.2 Sampling method

The samples were collected on 8-9 June 2016. Groundwater was taken from monitoring wells using a sampling bailer with the attaching rope at the end. Bailer was slowly dropped by lowering the rope until it reached the groundwater surface by avoiding the disturbance of bottom sediment that could resulted in the re-suspended of particles in water. In this step, the length of rope was marked in order to measure the groundwater depth. After water fully fill into the bailer, the bailer was withdrawn from the groundwater well and then was slowly pour into the received bucket. After that, groundwater was collected and preserved, depending to each parameter immediately, which was done while measuring on-site parameters.

Surface water were collected from the nearest location from the groundwater well. The bucket attached a rope was thrown out onto surface water, then draw the bucket back to collect surface water. Soils were collected from groundwater well area by using shovel from the soil surface about 300 g. Heavy metals were filtered through an 0.45 μm filter paper then collected into HDPE bottles followed by the acidification with HNO_3 to lower pH than 2. Nitrogen species (NO_3^-, NO_2^-) was collected into HDPE bottles and then acidified with H_2SO_4 to lower pH than 2. Total organic carbon in groundwater was collected into amber bottle and then lower pH than 2 using H_2SO_4 to avoid microbial activities. Three liters of water for microorganism analysis were collected using two of 1.5-liter plastic bottles. All samples were kept in an ice box during transportation prior to analysis.

3.3 Physicochemical analysis

Field measurements were done on-site in order to measure basic parameters including pH, temperature, conductivity, dissolved oxygen (DO), and oxidation-reduction potential (ORP) using portable meters.

Total organic carbon (TOC) were analyzed using TOC-VCPH which the detection limit of 4 μg/l. The standard solution was prepared by weighing accurately 0.2125 g. of $C_8H_5KO_4$ and dissolved in 100ml DI water. The standard curve was prepared from 5 concentration levels of standard solution: 0.1, 1, 5, 10, 50 ppm. The measurement was done from 5-10 ml of water samples.

For nitrogen species, total kjeldal nitrogen (TKN) were analyzed by Macro-Kjeldahl method with 0.1 mg/l detection limit. Briefly, ground and surface water samples with volume 250 ml. were added into kjeldahl flask. Then, 25 ml of borate buffer and 6N NaOH were added until pH 9.5 was reached. The digestion reagent of 50 ml was added then boiled until volume decreased to 25-50 ml in the fume hood. After cooling, it was diluted to 300 ml with DI water and 50 ml of sodium hydroxide-thiosulfate reagent was added to adjust pH >11. Samples were distilled and 200 ml were collected. The 50 ml of boric acid solution was added prior to titration step. The 0.04N of H_2SO_4 was used as an absorbent solution (WEF, 1999). On the other hands, nitrate (NO_3^-) was analyzed by cadmium reduction procedure with the detection limit of 0.05 mg/l. In this procedure, Cd 40-60 mesh was used to reduce nitrate to nitrite. Then, it was reacted in an acidic medium with sulfanilic acid to form an intermediate diazonium salt. The salt couples with chromotropic acid to form pink-colored product. The 507 nm wavelength of spectrophotometers or 520 nm of colorimeter was used to measure the products (EPA, 1993).

Total phosphorus was analyzed by ascorbic acid methods with the detection limits 0.01 mg/l. The analysis was started with adding 5 ml of sample into Erlenmeyer flask. Then, 800 µl of ascorbic solution (prepared by dissolving 0.88g of ascorbic acid in 50 ml DI water) were added. The flask was cap and mix several times. The solution was measured in the colorimeter at 625 nm wavelength red led.

Arsenic concentration in water was measured by Atomic Absorption Hydride (continuous) ZEEnit 700P with the detection limit of 2.78 µg/l. The analytical procedure was performed following the determination of arsenic by atomic adsorption spectroscopy guideline (USDA, 2016).

3.4 Biological analysis: Detection of arsenite-oxidizing bacteria

3.4.1 Sample preparation and DNA extraction

The collected water was filtered through a 0.2 µm Cellulose Nitrate Membrane filter in total volume of 100-1000 ml, depending on the suspended solid in water. The filters were stored at -20°C until analysis. Soil sample of 0.5 g was used for DNA extraction. The DNA extraction was done by using the FastDNA® SPIN KIT for Soil, (MP Biomedicals, LLC). The procedure was follow from the supplemental guideline

document from the manufacturer. The quality and quantity of extracted DNA were analyzed by agarose gel electrophoresis and spectrophotometer using nanadrop, respectively. The extracted DNA was diluted to 5 ng/µl for using as a template for PCR approach.

3.4.2 Polymerase Chain Reaction (PCR)

The PCR amplification was performed using T100™ Thermal Cycler, Biorad using *aox*-targeting primers: *aox*BM1-2F-ND and *aox*BM2-1R-ND (Table 3.2). Both primers have a specific fragment of *ca.* 550bp. The GC clamp added at the end of primer *aox*BM2-1R-ND-GC (5'-CCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGC-3') was also used for DGGE analysis (Quemeneur et. al., 2010). These primers were used to amplify the functional arsenite oxidase gene fragment. The PCR master mix in total volume of 50 µl was prepared followed by a guideline from the Taq DNA Polymerase manufacturer (Thermo Scientific) starting from 5 µl of 10X Taq Buffer, 2mM of dNTP mix, 0.2 µM of forward and reverse primers, 2.5 mM of $MgCl_2$, 1.25U of Taq DNA Polymerase, 1.5 µl of BSA(additional), and 0.1µl of template DNA. The PCR process was performed at conditions from an initial denaturation step 95°C for 2 minutes, 34 cycles of denaturation 95°C or 30 seconds, annealing 50, 52, 55 or 60°C for 30 seconds, extension 72°C, 45 seconds, followed by a Final extension 72°C for 10 minutes.

Table 3.2 Primers used in the study

Primer	Target gene	Sequence [5'-3']	Reference
***aox*BM1-2F-ND**	Arsenite oxidase	CCACTTCTGCAT CGTGGGCTGTGGCTA	(Quemeneur et. al, 2010)
***aox*BM2-1R-ND**	Arsenite oxidase	GGAGTTGTAGGCGGGCCGGTTGTGGAT	(Quemeneur et. al, 2010)
***aox*BM2-1R-ND-GC**	Arsenite oxidase	CCGCCGCGCGGCGGGCGGGGCGGGGGCACGG GCGGAGTTGTAGGCGGGCCGGTTGTGGAT	(Quemeneur et. al, 2010)

3.4.3 Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products were used for DGGE approach using D-Code™ Universal Mutation Detection System, BioRad. The gel contains with 6% (v/v) polyacrylamide (made with 37.5:1 bis-acrylamide solution) with the varying 30/70, 10/90, 30, 90 urea and formamide denaturing gradients. Samples were loaded at 20 μl, and run at 85V at 840 minutes (14 hrs.) running time. The electrophoresis was applied at a constant temperature of 60°C in a 1X Tris-acetate EDTA (TAE). Then, the gels were strained with ethidium bromide for 30 minutes, then de-strained and analyzed in an UV transilluminator (WEALTEC). The dominant band was cut and re-amplified to check for its purity before being sequenced.

3.4.4 Clone library

Purified PCR products (from the non-GC clamp primer) were used for cloning using pGEM®-T and pGEM® T-Easy Vector systems for ligation and XL1-Blue Supercompetent cells (Agilent), following the manufacturer's protocols. Briefly, the ligation process was performed as the providing recommendation with an overnight incubation at 4°C. The ligated solution was transformed into the competent cell with β-mercaptoethanol added. The heat shocking process was performed at 42°C for 45 seconds as recommendation protocols. The vector ligated cells were spread on LB agar plate adding Ampicillin, X-Gal, and IPTG with total concentration 10mg/ml, 80 μg/ml, and 2 mM, respectively. After incubating overnight, the remain white cells were picked

up and checked by PCR process using *aox* targeting primer. Approximately 20-25 colonies of each library were randomly selected for sequencing.

3.4.5 DNA Sequencing and analysis

PCR product with the amount of 50 µl was sent to analyze the sequence at Macrogen company, south Korea, performing standard sequencing by using ABI3730XL DNA analyzer. The Basic Local Alignment Search Tool (BLAST) was used in the National Center for Biotechnology Information (NCBI). All obtained DNA sequences were clustered by the operational taxonomic units (OTUs) based on >97% sharing similarity using a CD-HIT suit: Biological sequence clustering and comparison (Li and Godzik, 2006). Each OTUs then was selected for constructing phylogenetic tree by Mega version 7.0.21 (Kumar et al., 2016), using Neighbor-Joining method with 1000 bootstrap tests (Saitou and Nei, 1987).

3.5 PHREEQC geochemical Modeling of As in groundwater

The As speciations in groundwater were calculated by PHREEQC program (U.S. Geological Survey). Data collected from the site were added into PHREEQC program. The analyzed parameters were total As concentration, pH, ORP, temperature, dissolved oxygen (DO), cations, and anions Fe^{2+}, Mn^{2+}, SO_4^{2-}. The thermodynamic data including dissolution values, enthalpy, and dissociation equation were used from WATEQ4F.dat database as it is provided by the program because it is suitable for modeling various types of heavy metal in water and included lots of arsenic thermodynamic data. This databased refers to arsenic thermodynamic data from (Nordstrom and Archer, 2003). The modeling output that was used in the discussion regarding the As speciation (arsenite and arsenate concentrations) (Parkhurst and Appelo, 2013). Moreover, the As-Hfo adsorption was modeled in order to determine the effect of Fe on As sorption in the natural environment. In this part, the As-Hfo sorption diagram was modeled using input parameters including Total As, Mn, Fe, and SO_4^{2-}. These four parameters were grouped based on actual values from site measurements.

3.6 Multivariate analysis

Water parameters and bacterial compositions were analyzed for Pearson's correlation coefficients and RDA using XLSTAT 2017 (Adionsoft). Pearson's correlations were analyzed where the difference was considered the significantly difference when *p*-value is less than 0.05 ($p < 0.05$) following the one-way ANOVA. RDA is often used in the field of ecology to explain matrix of sites/ species abundances by a set of environmental variables. The sets of X and Y were assumed to be linear. All set of variables were analyzed in 1000 numbers of permutations.

3.7 Experimental Timeline

The experimental timeline is shown in the Table 3.3 starting from May 2016 to December 2017

Table 3.3 Experimental timeline in this study

Procedures	**Time Period**													
	2016								**2017**					
	5	6	7	8	9	10	11	12	1	2-8	9	10	11	12
Literature review	■	■	■	■	■	■								
Sample collection		■												
Chemical Analysis		■	■											
DNA Extraction				■										
PCR analysis				■	■									
DGGE analysis						■	■							
Bacterial Cloning							■	■	■					
Proposal defense									■					
Biological analysis										■				
PHREEQC model										■				
discussion											■	■		
Conference													■	
Thesis defense														■

Chapter 4
RESULTS AND DISCUSSION

4.1 Site characteristic and water quality

All samples were collected from nine locations, including mining and residential areas, as shown in Fig. 3.2 within consecutive two days. The groundwater wells, which were collected the samples, of both mining and residential areas were different. The mining area collecting wells were static wells, whereas all residential area wells, except for station no. RA-1, were pumping wells because the pumping system were installed and continuously pumped groundwater into the storage units to support water consumption as shown in Fig 4.1.

Figure 4.1 Groundwater sampling wells of the mining area (static wells; left), and the residential area (pumping wells; right)

Groundwater and surface water (represented upstream, within, and downstream of the mining area) are shown in table 4.1. Across all samples, pH values showed slightly acidic to neutral in the range of 4.54 to 8.22. Temperatures were relatively constant, in the range of 29.20 to 34.00 ℃, across all sampling stations (Table 4.1). DO and ORP varied in a broad range of 2.84 to 7.63 mg/l and -12.30 to 390.30 mV, respectively. Although TOC levels were relatively constant, in the range of 1.19 to 4.19 mg/l, in most of groundwater and surface water analyzed in this study, the value was quite high in sample RA-4_{SW} representing 16.62 mg/l. The concentrations of SO_4^{2-} varied, in the range of 0.45 to 792.30 mg/l, across all samples. As for other ion species which include TP, As, Mn and Fe, and the concentrations of each ion were relatively

similar across all samples; they were in the range of 0.01 to 0.06 mg/l, 2.05 to 6.63 μg/l, 0.10 to 1.07, and 0.11 to 1.19 mg/l, for TP, As, Mn, Fe, respectively (Table 4.1). We can see similar levels across all sample except for SO_4^{2-} that has higher values in some stations.

Table 4.1 Geochemical characteristics in groundwater and surface water within and outside of the gold mine area

Station	pH	Temp. ℃	DO mg/l	ORP mV	TOC mg/l	TP mg/l	TAs μg/l	Mn mg/l	Fe mg/l	SO_4^{2-} mg/l
MA-1	6.87	32.30	2.84	59.40	1.34	0.02	3.52	0.16	0.18	5.00
MA-2	6.70	31.80	2.95	88.90	1.40	0.06	3.50	1.07	0.21	4.25
MA-3	4.90	31.00	2.92	296.90	1.22	0.01	3.12	0.04	0.14	1.53
MA-4	6.39	30.50	3.29	115.40	3.24	0.05	4.45	0.69	0.18	6.34
RA-1	6.50	29.20	3.87	212.20	1.24	0.02	3.97	0.12	0.11	39.20
RA-2	4.54	30.30	4.73	152.20	1.87	0.04	2.61	0.10	0.13	0.45
RA-3	6.80	30.60	2.38	-12.30	1.19	0.02	4.30	0.65	0.92	1.24
RA-4	7.21	32.30	4.79	149.50	1.43	0.04	2.05	0.40	0.57	700.56
RA-5	7.16	29.80	5.73	171.90	1.34	0.01	6.63	0.14	0.11	29.75
MA-1$_{SW}$	6.98	33.40	7.14	308.10	2.92	0.02	2.31	1.04	1.19	1.92
RA-1$_{SW}$	6.34	31.00	7.63	390.30	4.19	0.05	2.29	0.21	0.19	29.19
RA-4$_{SW}$	8.22	34.00	7.19	269.10	16.62	0.03	2.66	0.16	0.26	792.30

Based on water qualities, considering pH and ORP values, As was potentially presented in both trivalent and pentavalent forms, as shown in Fig 2.2, which mainly consisted of H_3AsO_3 (As^{3+}), $H_2AsO_4^-$(As^{5+}), and $HAsO_4^{2-}$(As^{5+}). On the contrary, the level of DO in groundwater appeared to be low as compared those of surface water, which might be affected by two reasons. First, groundwater contained low organic contents; thus, DO remained residue and still was available to microbes. Second, the groundwater depths were shallow (approx. 3-6 m. depth); therefore, groundwater was not in completely anaerobic condition. However, surface water, on the other hands, presented in very high levels of dissolved oxygen (DO) in all samples. These physicochemical parameters indicated that aerobic bacteria possibly survived in water samples especially in surface water by utilizing organic matters, and oxygen as an electron donor and acceptor for their growth and respiration, respectively. In this study,

the As levels in all samples, including those collected from within and outside the gold mining area, did not exceed the WHO drinking water standard (<10 μg/l) and Thailand groundwater standard (<50 μg/l) (WHO, 2011; DGR, 2015). The results demonstrated that anthropological disturbance likely had no effect on the As level in groundwater analyzed in this study. However, the presence of As may affect the existence of native bacteria that required As as an essential substance for their growth.

4.2 The presence of As by the geochemical modeling PHREEQC

Groundwater and surface water geochemical parameters from Table 4.1 including total As concentration, pH, ORP, temperature, DO, Mn, Fe, and So_4^{2-} were added into PHREEQC geochemical model using WATEQ4F.dat database. As a result, the modeling outputs including concentration of As species (trivalent and pentavalent), and the first three dominant species are showed in Table 4.2. From all 12 simulations, the arsenite (As^{3+}) dominated in only 2 samples, while arsenate (As^{5+}) dominated the rest of the samples. From these results, the most found the 1st dominant specie of As^{5+} were $H_2AsO_4^-$ followed by $HAsO_4^{2-}$, whereas, H_3AsO_3 represented the most found As^{3+} dominant specie because it thermodynamically stable in the certain oxic level and pH conditions (Smedley, 2008). These species are the normal As forms which could be found in natural surface and groundwater environments (Pal, 2015). As compared these results from Fig 2.2, these species were well matched with the pe-pH diagram, perhaps, the major factor controlling As species could be contributed to pH and oxidation-reduction potential (Pal, 2015), in which dissolved anions and cations might only have minor controllable roles. However, most groundwater showed slightly oxic conditions rather than anaerobic or reducing condition, which were resulted in As^{5+} domination condition in this study. Nevertheless, the low pH value in RA-2 station and the negative redox state in RA-3 station could be the major reasons supporting the As^{3+} dominations in those stations that made them differed from the rest.

Table 4.2 As speciation by the geochemical model PHREEQC showing arsenite and arsenate concentrations, and the first three dominant species in the systems.

Station	Obs.			model			
	TAs µg/l	As^{3+} µg/l	As^{5+} µg/l	Dominant form	1st Dominant specie	2nd Dominant specie	3rd Dominant specie
MA-1	3.52	0.08	3.45	5+	$H_2AsO_4^-$	$HAsO_4^{2-}$	H_3AsO_3
MA-2	3.50	0.03	3.47	5+	$H_2AsO_4^-$	$HAsO_4^{2-}$	H_3AsO_3
MA-3	3.12	0.00	3.12	5+	$H_2AsO_4^-$	$HAsO_4^{2-}$	H_3AsO_4
MA-4	4.45	0.06	4.40	5+	$H_2AsO_4^-$	$HAsO_4^{2-}$	H_3AsO_3
RA-1	3.97	0.00	3.97	5+	$H_2AsO_4^-$	$HAsO_4^{2-}$	H_3AsO_4
RA-2	2.61	2.61	0.00	3+	H_3AsO_3	$H_2AsO_4^-$	H_2AsO^{3-}
RA-3	4.30	4.00	0.30	3+	H_3AsO_3	$H_2AsO_4^-$	$HAsO_4^{2-}$
RA-4	2.05	0.00	2.05	5+	$HAsO_4^{2-}$	$H_2AsO_4^-$	AsO_4^{3-}
RA-5	6.63	0.00	6.64	5+	$H_2AsO_4^-$	$HAsO_4^{2-}$	H_3AsO_4
$MA\text{-}1_{SW}$	2.31	0.00	2.31	5+	H_2AsO4^-	$HAsO_4^{2-}$	H_3AsO_4
$RA\text{-}1_{SW}$	2.29	0.00	2.29	5+	H_2AsO4^-	$HAsO_4^{2-}$	H_3AsO_4
$RA\text{-}4_{SW}$	2.66	0.00	2.66	5+	$HAsO_4^{2-}$	$H_2AsO_4^-$	AsO_4^{3-}

Obs. = Observation values from site measurements

Moreover, the sorption model was included in this study as sorbtion plays an important role in As speciation (Smedley, 2008). Specifically, Hfo-As sorption model was considered in the study due to the strong sorption capacities for As of amorphous iron oxides which could influent the As mobility in the system (Dzombak and Morel, 1990). The models used Dzombak and Morel (1990) diffuse layer (DL) model for Hfo to estimate As adsorption by Hfo. It was done in Phreeplot extension in PHREEQC model which can generate predominant diagram from the input conditions. Prior to modeling, each sample were grouped, based on the major ions (As, Fe, Mn, and SO_4^{2-}) and then averaged these values into 2 ranges, resulting in 6 conditions as shown in Table 4.3. All predominant diagrams were generated from 6 environmental conditions illustrated in Fig 4.2, which shows each sample falling in the different regions. The effects on these ions on As can change the arsenic forms in which Fe and S species seemed to have the major role on this model. The increasing of Fe and SO_4^{2-} levels presented in the wider range of $Hfo\_wHAsO_4^{3-}$, and $AsS(OH)(HS)^-$, respectively; thus,

increasing the adsorbed ions in the system. For example, the increase Fe concentration from 0.003 to 0.013 mmol resulted in a large Hfo_w$HAsO_4^{3-}$ region in the condition 3, and the increase SO_4^{2-} concentration from 0.1 to 6 mmol also resulted in a wider $AsS(OH)(HS)^-$ region in condition 4. However, most samples can be represented by condition no.1 as all ions in water were relatively low, and some other unique samples were represented by the following conditions. Comparing the dominant species to the speciation model, most of the dominant species were not much different except some conditions that fall into the Hfo_w$HAsO_4^{3-}$ region in which Hfo dissoluted in either highly oxidative or high pH value and absorb As species presented in As-associated with Fe species.

Furthermore, the As forms under oxic condition are suggested to relate with electrostatic repulsion from negative charge of oxide surface resulted in the Hfo instability and dissolution (Smedley, 2008). The strong sorption tendency of iron oxides at near to neutral pH range in the oxic condition is one of the major reasons causing the low As concentration in most natural groundwater (Smedley, 2008). However, Fe concentrations in this study were relatively similar; thus, the Hfo sorption might not be the major factor controlling As concentration in groundwater. On the other hand, the dissolved As associated with S-species occurs in highly reducing condition where the As-S mineral limits dissolved As concentration in high sulphide concentration (Smedley, 2008). Similar case with Fe, the As binding with S minerals are also rarely occurred in this study as it requires an extremely reducing redox condition.

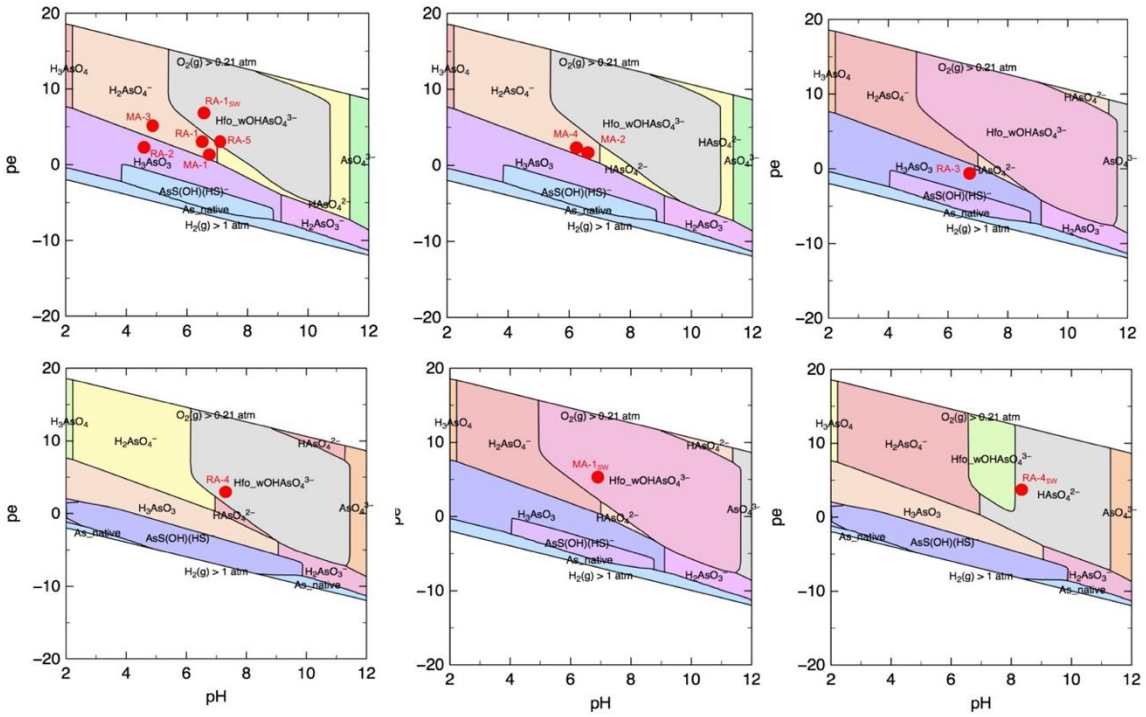


Figure 4.2 Predominant diagrams of arsenic in Hfo system generated by Phreeplot in PHREEQC model showing all possible cases of groundwater conditions in this study

Table 4.3 Arsenic-Hfo adsorbtion modeling by PHREEQC geochemical model using two-average levels for each input parameters (TAs, Fe, SO_4^{2-}, and Mn)

Station	TAs (µmol)	Fe (mmol)	SO_4^2 (mmo)	Mn (mmol)	Dominant Species	Condition type
MA-1	0.05	0.0030	0.1	0.003	$H_2AsO_4^-$	1
MA-2	0.05	0.0030	0.1	0.015	$H_2AsO_4^-$	2
MA-3	0.05	0.0030	0.1	0.003	$H_2AsO_4^-$	1
MA-4	0.05	0.0030	0.1	0.015	$H_2AsO_4^-$	2
RA-1	0.05	0.0030	0.1	0.003	$H_2AsO_4^-$	1
RA-2	0.05	0.0030	0.1	0.003	H_3AsO_3	1
RA-3	0.05	0.0130	0.1	0.015	H_3AsO_3	3
RA-4	0.05	0.0130	6.0	0.003	$hfo\_wOHAsO_4^{3-}$	4
RA-5	0.05	0.0030	0.1	0.003	$hfo\_wOHAsO_4^{3-}$	1
MA-1$_{SW}$	0.09	0.0130	0.1	0.015	$hfo\_wOHAsO_4^{3-}$	5
RA-1$_{SW}$	0.05	0.0030	0.1	0.003	$hfo\_wOHAsO_4^{3-}$	1
RA-4$_{SW}$	0.05	0.0030	6.0	0.003	HAsO42-	6

4.3 The presence of arsenite-oxidizing bacteria in groundwater, surface water and soil samples

All extracted DNA from groundwater, surface water and soil were screened for the presence of arsenite-oxidizing bacteria using PCR-based approach, targeting *aoxB* genes. The results demonstrated that 12 out of 15 samples showed positive amplifications with the *aoxB*-targeting primer pair with non-GC clamp (Fig 4.3). The three negative results were RA-2, RA-3, and RA-5. These three samples with negative PCR signals were collected from the residential area. However, the reason for negative amplification was not clear. One possible reason was groundwater movement which corresponded to pumping installation. All these three samples, RA-2, RA-3, and RA-5, were collected from sampling stations with pumping installation, resulting in short hydraulic retention time and high flow. Therefore, short hydraulic retention time and high flow could affect the detection of arsenite-oxidizing bacteria in groundwater samples analyzed in this study. It was reported that the theoretical hydraulic retention time of freshwater was an important variable affecting distribution of bacterial taxa (Lindstrom et al., 2005). Water flow could be related to bacterioplankton community composition and the import of bacterial cells from drainage area (Lindstrom et al., 2006). However, 12 positive samples were further investigated for arsenite-oxidizing bacterial community using DGGE, cloning and sequencing.

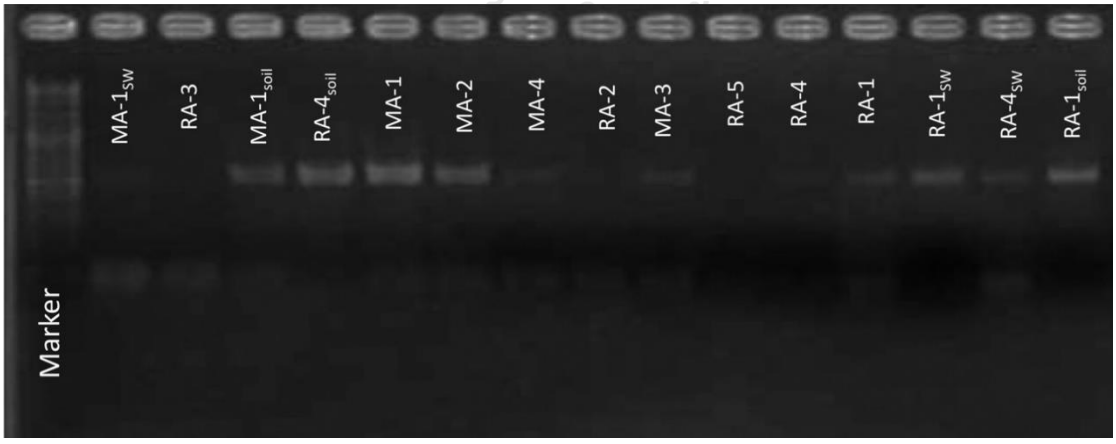


Figure 4.3 Agarose gel electrophoresis showing the PCR products of 15 samples amplified by primers aox-BM1-2F-ND and aox-BM2-1R-ND.

Figure 4.4 Agarose gel electrophoresis showing the PCR products of 15 samples amplified by primers aox-BM1-2F-ND and aox-BM2-1R-ND-GC

4.3.1 The arsenite-oxidizing bacterial community analyzed by DGGE

The positive PCR products amplified with the GC clamp primer (Fig 4.4) were subsequently run on DGGE. The DGGE results revealed that among all samples, only one dominant band from samples MA-1, and MA-2 appeared on DGGE gel (Fig 4.5). The PCR products of *aoxB* gene fragments retrieved from samples MA-1 and MA-2 were relatively clear and sharp, compared to other samples (Fig 4.4). This could be the reasons for the positive DGGE bands. The results indicated that the detected *aoxB*-carrying bacteria in MA-1, and MA-2 could survive and highly dominate in groundwater samples collected from the gold mine area but their diversity was low. Perhaps, the activities of a gold mine (e.g. As-containing mineral disturbance) might impact the presence of these bacteria (Hudson-Edwards and Santini, 2013). Physicochemical parameters of groundwater samples MA-1 and MA-2 were rather similar (Table 4.1); this certain condition may favor the existence of arsenite-oxidizing bacteria in these two samples. The obtained nucleotide sequences from the dominant DGGE bands were blasted against the Genbank nucleotide database. The results revealed that they showed 99% identity to uncultured bacterium clone K1-70r-GW isolated from groundwater tube well (KP072498). Phylogenetic analysis revealed revealed that the analyzed sequence belonged to *Hydrogenophaga* genus (88% identity) (BAK39656), a member of β-*Proteobacteria* group (Hassan et al., 2015) (Fig 4.6). The *Hydrogenophaga* genus is known as "knallgas" bacteria that are capable of fixing CO_2 while utilizing molecular hydrogen and oxygen as an electron donor and an accepter,

respectively (Aragno et al., 1999). It has been reported that the *Hydrogenophaga* genus was present in contaminated groundwater with a wide range of As concentrations (Sutton et al., 2009; Quemeneur et al., 2010; Ghosh et al., 2014; Li et al., 2015). The *Hydrogenophaga* genus was detected in groundwater contaminated by As in the range of 35-117 μg/l (Ghosh et al., 2014). Groundwater from shallow tube wells with As concentration of 332 μg/l and groundwater from a gold mine with As concentration of 1846 μg/l also harbored the *Hydrogenophaga* genus (Sutton et al., 2009; Quemeneur et al., 2010). The *Hydrogenophaga* genus was present in groundwater with a board range of As concentrations of 1-763 μg/l (Li et al., 2015). Moreover, the *Hydrogenophaga* genus were also found in other As-contaminated environment such as soils (As = 17.6 – 246.6 mg/kg), sediments (As = 1.5 – 77.7 mg/kg) and hot creek (As ≈ 200 μg/l) (Salmassi et al., 2006; Hu et al., 2015; Li et al., 2015). This indicated that arsenite-oxidizing bacteria associated to the *Hydrogenophaga* genus may survive in a broad range of As levels in natural environment, including groundwater. These findings imply that the *Hydrogenophaga* genus potentially plays a key role in controlling As mobility and toxicity in groundwater environment. However, the other procedure, DNA cloning, was also performed in this study to avoid the limitations of DGGE technique that might not be able to reveal some minor bacterial species.

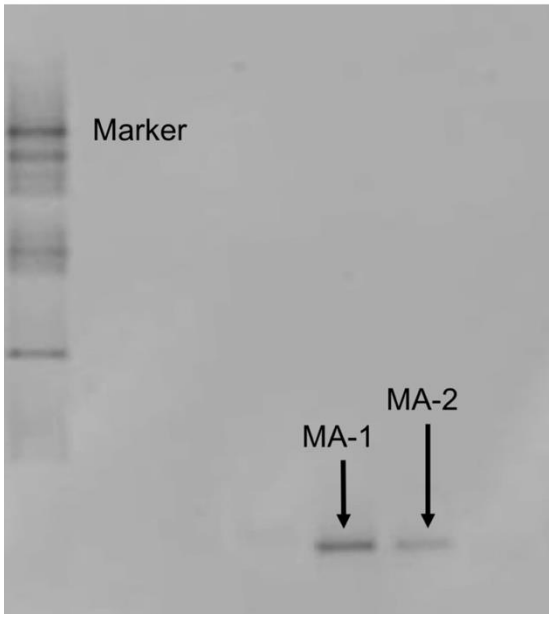


Figure 4.5 DGGE profile of detected *aoxB* gene recovered from MA-1, and MA-2 stations

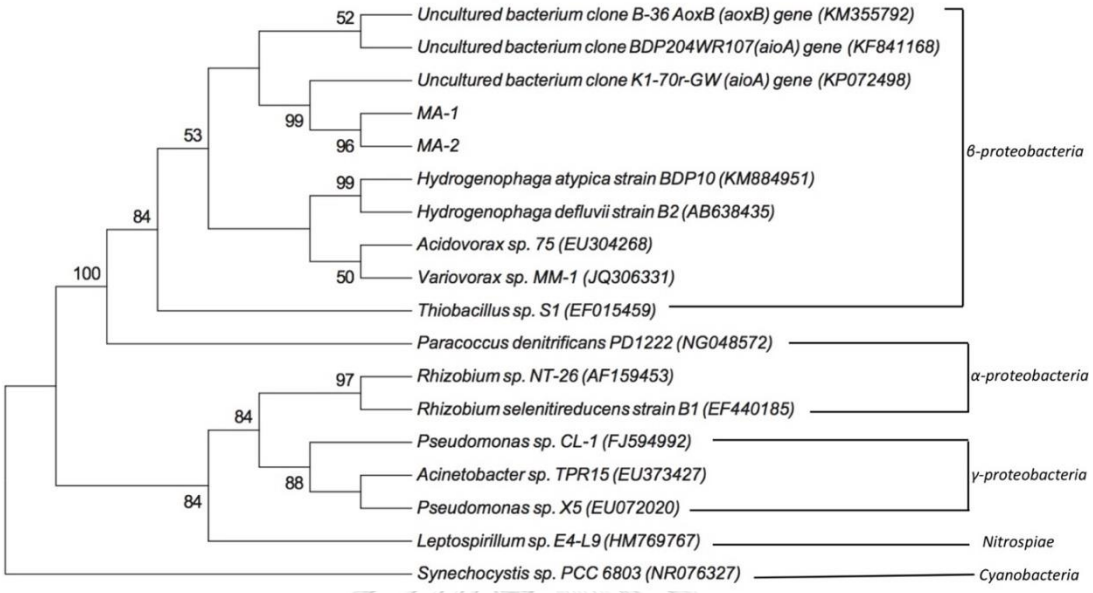


Figure 4.6 Phylogenetic tree of the DGGE band isolates from sample MA-1, and MA-2

4.3.2 The arsenite oxidizing bacterial community analyzed by cloning

Purified PCR products of 12 positive arsenite-oxidizing bacterial samples (MA-1, MA-2, MA-3, MA-4, RA-1, RA-4, MA-1_{soil}, RA-1_{soil}, RA-4_{soil}, MA-1_{SW}, RA-1_{SW}, and RA-4_{SW}) were ligated and transformed into *E.coli* (XL-1 blue strain) competent cell to disclose community compositions in each sample. Summary of the number of clones in each library, the number of OTUs classified based on >97% nucleotide sequence similarity, and the number of assigned clones are shown in Table 4.4. Each sample contained 16 to 25 representative clones which were assigned into 2 to 18 OTUs. The results showed that groundwater samples. MA-1, MA-2, MA-4, and RA-1 harbored low OTUs numbers, in the range of 2-4 OTUs, while MA-3 was represented by 11 OTUs. All surface water samples, MA-1_{SW}, RA-1_{SW}, and RA-4_{SW} contained 6 OTUs. The numbers of OTUs were relatively high, in the range of 11-18 OTUs, in soil samples. This finding indicated that the community of arsenite-oxidizing bacteria in soil was the most diverse, compared to those in surface water and groundwater. It could be inferred that groundwater are flavored to only some specific bacterial assemblages, whereas surface water and soil seemed promoting a more diverse community of arsenite-oxidizing bacteria. The results also demonstrated that the numbers of sequences in OTUs MA-1(0), and MA-2(0) were relatively high, in total of 21 and 17 sequences, respectively (Table 4.4). This finding implied that particular arsenite-oxidizing bacterial species were dominant in these two samples and they were associated with the bacterial genus *Hydrogenophaga*. Consequently, the cloning and DGGE results revealed that the genus *Hydrogenophaga* were highly detected in samples MA-1 and MA-2.

Table 4.4 Summary of the number of clones and OTUs in 12 samples

Sample	No. of clone	No. of OTU	OTU number and number of assigned clones																	
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
MA-1	24	3	21	2	1															
MA-2	23	2	17	6																
MA-3	25	11	12	4	1	1	1	1	1	1	1	1	1							
MA-4	23	4	17	3	2	1														
RA-1	24	6	19	1	1	1	1	1												
RA-4	19	3	14	4	1															
$MA\text{-}1_{soil}$	23	18	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1
$RA\text{-}1_{soil}$	16	14	2	2	1	1	1	1	1	1	1	1	1	1	1	1				
$RA\text{-}4_{soil}$	24	11	8	4	3	2	1	1	1	1	1	1	1							
$MA\text{-}1_{SW}$	22	6	14	4	1	1	1	1												
$RA\text{-}1_{SW}$	19	6	9	3	3	2	1	1												
$RA\text{-}4_{SW}$	19	6	12	3	1	1	1	1												

The nucleotide sequences of each representative OTU were blast against the Genbank database. The results demonstrated that most of sequences were matched with sequences from uncultured bacteria isolated from various environments in several studies (Quemeneur et al., 2010; Jia et al., 2014; Han et al., 2015; Zhang et al., 2015; Zeng et al., 2016). Those environments were likely appeared contaminated with broad range of arsenic levels, for example, 15-1846 µg/l in freshwater, and 0.06-117.47 g/kg in soil (Quemeneur et al., 2010; Zeng et al., 2016). This finding imply that arsenite-oxidizing bacteria are ubiquitous in As-contaminated environments. Nonetheless, the *aoxB* clones could be identified in similar ranges of identities with the Genbank reference sequences (lowest = 74%, highest = 99% identity). The mining groundwater clones showed 84 – 99% identity, 79 - 98% identity for residential groundwater clones, 74 – 99% identity for soils, and 76 – 99% identity for surface water clones (Appendix; Table A2-A5). The majority of arsenite-oxidizing bacteria recovered from this study belonged to the phylum *Proteobacteria*, associated with the classes *α*- and *β*-*Proteobacteria*. Others were closely related to *γ-Proteobacteria* and *Firmicutes* (Appendix; Table A5)

The phylogenetic tree was constructed based on Neighbor-Joining method with 1000 bootstrap tests (Fig. 4.9) All representative OTUs recovered from this study were included in the phylogenetic analysis. The reference sequences were cultured and uncultured arsenite-oxidizing bacterial sequences retrieved from the Genbank database.

Synechocystis sp. (Cyanobacteria) (Genbank acc. No. NR076327) was used as an outgroup (Fig. 4.9).

There were in total of 90 OTUs from 12 clone libraries. Phylogenetic analysis revealed that RA-1_{soil}(5), RA-1_{soil}(13), MA-1_{soil}(6), MA-3(5), MA-1_{soil}(8), and RA-4_{soil}(0) showed strong bootstrap values as associated with uncultured bacterium clone ZJ-aroA arsenite oxidase (*aroA*) (KP060393, KP060370, KP060194, KP060185 and KP060302) isolated from paddy soils, with As concentrations in the range of 0.4 to 3.6 mg/kg , in China (Zhang et al., 2015). Furthermore, MA-3(0) and RA-4_{soil}(10) were closely related to uncultured bacterium clone T12TOA3 arsenite oxidase large subunit (*aoxB*) gene isolated from As-contaminated river basin in France (EU304319 and EU304296; Fig 4.9). As concentrations in river basin varied in the range of 15 to 1,846 ug/l and arsinite-oxidizing bacteria were recovered by DGGE technique (Quemeneur et al., 2010). RA-1_{soil}(9) and RA-4_{soil}(3) sequences were closely related to uncultured bacterium clone C11 arsenite oxidase large subunit (*aioA*) gene isolated from mine tailing with As concentrations in the range of = 0.06-117.47 g/kg (KT992315 and KT992237; (Zeng et al., 2016). RA-1(2) was associated with uncultured bacterium clone Q6797-2-6 arsenite oxidase (*aroA*) gene retrieved from As-contaminated soils with As concentrations in the range of 8.7-81.2 mg/kg) (KP726568;(Gu et al., 2017). RA-1_{soil}(0) were also related to uncultured bacterium clone aroA-rhizosoil-7 arsenite oxidase (*aroA*) gene from rhizosoil (JX489088) with As-contamination in the range of 0.43-0.47 g/kg (Jia et al., 2014). RA-1_{SW}(1), RA-1_{soil}(7), and MA-3(10) were similar to uncultured bacterium aioA gene, clone N-4d5 isolated from sediments in Japan (AB730976, AB731084 and AB838863;(Yamamura et al., 2014). MA-3(7) were closely related to uncultured bacterium *aioA* gene, clone Aio_aoxBM1-2F/3-2R_L-32 retrieved from soils (LC012221;(Dong et al., 2016). Phylogenetic analysis also showed that arsenite-oxidizing bacteria recovered from samples MA-1_{soil}(2), MA-1_{soil}(17), RA1-1, and MA-1_{soil}(10) were related to uncultured bacterium clone Q6429-2-15 arsenite oxidase (*aroA*) gene from rhizophere (KP726747, KP726558 and KP726744) ((Han et al., 2015). RA-4_{soil}(6) was closely related to cutured bacteria *Alcaligenes faecalis* strain 17S (KC282374; (Tang, 2012). These findings lead to the fact that the major bacteria found in this study commonly found in solid media (soils, sediments) rather than aqueous media (water) which could support the diverse OTUs that were

discussed earlier. Some arsenite-oxidizing bacteria species found in soil media could also exist in aqueous media.

For further investigation, the phylogenetic tree showed that the two main clusters were α-*Proteobacteria*-like and β- *Proteobacteria*-like clusters (Fig. 4.9). However, *γ-Proteobacteria*-like cluster appeared to be a minor cluster. The total of 12 clone libraries, containing 90 representative OTUs, could be contributed to α-*Proteobacteria*-like cluster 62 OTUs, and 28 OTUs to β-*Proteobacteria*-like cluster. Since α-*Proteobacteria*-like cluster was very broad, it was divided into three sub-clusters, including cluster α_1 (28 OTUs), α_2 (26 OTUs), and α_3 (8 OTUs). While the β-*Proteobacteria*-like cluster remained one main cluster (28 OTUs).

Sub-cluster α_1 was represented by *Chelatociccus* (KX432183), *Bradyrhizobiaceae* (AB974343), *Ancylobacter* (EF015461) and *Microvirga* (KT388112) as cultured bacteria (Fig 4.9). Sub-cluster α_1 also contained 4 OTUs from mining groundwater samples, 1 OTU from residential groundwater and 23 OTUs from soil samples. There was no representative from surface water in Sub-cluster α_1. Sub-cluster α_2 contained *Aminobacter* (EU304278), *Ensifer* (KX274403), *Rhizobium* (KT992344) and *Gemmobacter* (KX274407) as cultured bacteria. Sub-cluster α_2 also contained 5 OTUs from mining groundwater, 1 OTUs from residential groundwater, 5 OTUs from soil, 15 OTUs from surface water. Sub-cluster α_3 was the smallest sub cluster. This cluster contained neither cultured bacteria nor analyzed sequences from residential groundwater. Sub-cluster α_3 comprised of 1 OTUs from mining groundwater, 6 OTUs from soil and 1 OTUs from surface water. Another major arsenite-oxidizing bacterial cluster found in this study was β- *Proteobacteria*-like clusters. Cluster β–*Proteobacteria* comprised of in total of 28 OTUs, including 10 OTUs from mining groundwater, 7 OTUs from residential groundwater, 9 OTUs from soil and 2 OTUs from surface water. In addition, cultured bacteria, including the *Acinetobacter* (EU304275), *Pseudomonas* (EU304277), *Variovorax* (KM199763 and DQ380569), *Alcaligenes* (KC282374), *Burkholderia* (GU731249), *Cupriavidus* (AB974345), *Thiomonas* (EU304261), *Hydrogenophaga* (KM884951), were the member of this cluster. It seemed that bacteria in this study have potential on oxidizing arsenite in different levels and their existence in arsenic contaminated environments were confirmed in numbers of studies. Bacterial genera *Pseudomonas* and *Rhizobium*

were isolated from As-rich groundwater. Both were able to oxidize high concentration of arsenite as observed by growth pattern and transformation kinetics (Paul et al., 2014). Bacterial strain SDB1 isolated from mine tailing, belonging to *Ensifer* (α-proeobacteria-like cluster), were able to use arsenite and CO_2 as the respective electron and carbon sources (Lugtu et al., 2009). *Acinetobacter* was the predominant genus in the groundwater that contributed to 62.41%of total microbial species, also was one of the four main species found in high arsenic contaminated groundwater (Li et al., 2015). *Acidovarax*, *Acinetobacter* and *Hydrogenophaga*, the identified bacteria from shallow tube wells, were associated with tolerance of high arsenic concentration (Sutton et al., 2009). *Thiomonas* isolated from acid mine drainage waters was able to gain energy from oxidizing arsenite autotrophically (Duquesne et al., 2008). The identified bacteria, *Alcaligenes,* had the highest arsenite oxidizing activity among the five bacterial genera. *Alcaligenes* oxidized 1 mM of arsenite within 40 hours during heterotrophic growth (Yoon et al., 2009).

The bacterial composition analysis (Table A1) demonstrated that sub-clusters α_1 and α_3 were likely to represent arsenite-oxidizing bacteria species found in soils. Sub-clusters α_1 and α_3 approximately 53.48%, and 13.95%, respectively, were associated with those recovered from soil. Sub-cluster α_2, on the other hands, seemed to be affiliated with arsenite-oxidizing bacterial species found in surface water, accounting for 83.33% of total surface water OTUs. Cluster β–*Proteobacteria* seemed to represent arsenite-oxidizing bacterial species retrieved from groundwater. The analyzed sequences recovered from mining groundwater and residential groundwater approximately 50% and 77.77% of total OTUs, respectively, belonged to this β-*Proteobacteria*-like cluster. The redundancy analysis (RDA) of these 4 main clusters (α_1-, α_2-, α_3-, and β- *Proteobacteria*-like cluster) and the environmental types (groundwater, surface water, and soil) confirmed this distribution pattern (Fig 4.7). The RDA axes carried the cumulative variance of 99.61% (F1 = 55.44%, and F2 = 44.77%). It showed that three environmental types, including groundwater, surface water and soil, were related to each cluster as discussed above and placed in different quadrant indicating that dominant arsenite-oxidizing bacteria species might differ among environmental types.

Specific cluster of arsenite-oxidizing bacteria may play important roles depending on the environmental types. Considering the arsenite-oxidizing bacteria community trend in this study, many studies could be supported. Ghosh et al. (2014) have reported that *Proteobacteria* were the dominant phylum detected in As-contaminated groundwater. The phylogenetic analysis of *aioA* gene (which also called *aoxB* gene) demonstrated two major clusters represented by α-and β-*Proteobacteria* with strong bootstrap support (Ghosh et al., 2014). The NJ phylogenetic tree of *aoxB* sequences from the study of Quemeneur et al. (2010) consisted of β–*Proteobacteria* (55%), and those from α-*Proteobacteria* and *γ-Proteobacteria* were found in their study. The study also reported that the large majority of arsenite-oxidizing bacteria belonged to *Proteobacteria* phylum which lead to the fact that most of them were originated from mesophilic environments which is alike the environmental conditions in this study (Quemeneur et al., 2008; Quemeneur et al., 2010). The arsenite-oxidizing bacteria isolated from groundwater mining area were associated with two separate subdivisions of the *Proteobacteria* within the chemolithoautotrophic arsenite oxiders belonged to the α-*Proteobacteria*, whereas the heterotrophic arsenite oxidizer belong to the β–*Proteobacteria* (Santini et al., 2002). Meanwhile in As-contaminated soil, β–*Proteobacteria* cluster was dominant, accounting for 56.67%, across all four microbial genera, including α–, β–, *γ–Proteobacteria*, and *Archea* (Sanyal et al., 2016). Other factors, potentially affecting the arsenite-oxidizing bacterial cluster, were geochemical and methodology of analysis. For example, Chen and Shao, (2009) found that the arsenite-resistant bacteria isolated from ocean sediments were contributed to *γ-proteobacteria*, *Actinobacteria*, α-*Proteobacteri*a, and *Cytophaga-Flavobacterium* (CFB) based on 16s rRNA phylogenic analysis (Chen and Shao, 2009). Therefore, this should be noted that the community of arsenite-oxidizing bacteria were diverse and might be similar or different from other studies. However, further investigating of environmental influence on arsenite-oxidizing bacteria were shown in pearson's correlation (Table 4.5), and RDA plot (Fig 4.8) in which the set of bacterial composition in each sampling stations and environmental parameters were included in the analysis.

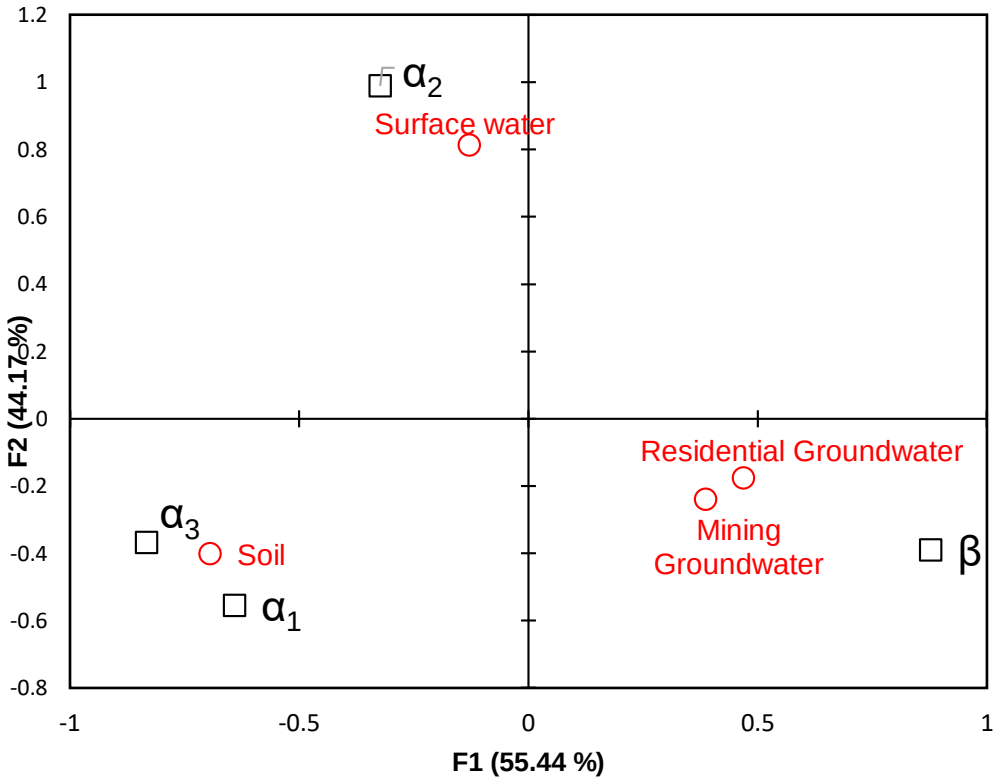


Figure 4.7 RDA plot of the 4 main clusters from phylogenetic tree and sample types (groundwater, surface water and soil). The squares represent the 4 main arsenite-oxidizing bacterial clusters and the circles represent environmental media types

4.4 Geochemical factors affecting arsenite-oxidizing bacteria in groundwater

Pearson's correlation coefficients were calculated to investigate the influence of environmental parameters on arsenite-oxidizing bacterial clusters (Table 4.5) The pearson's correlation analysis included 9 environmental parameters (pH, temperature, DO, ORP, TOC, As, Mn, Fe, SO_4^{2-}) and 2 arsenic species ($H_2AsO_4^-$, and $HAsO_4^{2-}$). The results showed that only 3 environmental factors, pH, DO and ORP, likely impacted the existence of arsenite-oxidizing bacterial clusters found in this study. pH showed the significantly negative correlation to **α1** cluster. This result was influent by the lowest pH value of water sample in this study (station MA-3, pH = 4.90) that was contributed by **α1** cluster with proportion of 60%. Dissolved oxygen (DO) and oxidation-reduction potential (ORP) showed positive correlation to α2 cluster. However, DO and ORP were negatively correlated to β cluster (Table 4.5). Compared with other studies, the RDA

was also conducted to illustrate the overall correlation among environmental variables and bacterial composition (Fig 4.8). The RDA axes explained 59.1% and 37.77% of F1, and F2, respectively. The 4 clusters were still distributed on the different quadrants which were the same pattern as described in Fig 4.7. Environmental variables showed different positive and negative correlations to bacterial clusters (e.g. ORP and DO showed positive correlation to α_2-*Proteobacteria* cluster, whreras they were negatively correlated to β-*Proteobacteria* cluster). The results showed that pH, DO and ORP exhibited the strongest axes scores (F1 and F2 scores are 0.416, -0.572 for pH, -0.598, -0.609 for DO, and -0.782, -0.151 for ORP). It was found that these 3 parameters could correlate with the same clusters as discussed in Pearson's correlation. In addition, As and Fe were the major ions that seemed to influent the presence of arsenite-oxidizing bacterial clusters in this study (Fig 4.8). These findings were supported by Gu et al. (2017). They found that the most important factors in controlling the bacterial community structure and As transformation genes were soil pH, phosphate-extractable As, and amorphous Fe content which were significantly correlated on PCoA and the Mantel test analysis. (Gu et al., 2017). Similarly, by conducting RDA analysis, Lindstorm et al. (2005) found that pH was the most strongly environmental variable that related to the distribution of bacterial group in freshwater. Moreover, pH was suggested on selected bacterial taxa in acidic and alkaline environments (Lindstrom et al., 2005). Hartman et al. (2008) found that bacterial community composition and diversity strongly responded to soil pH across all wetland sites. Soil pH affected the diversity of bacterial phyla and species based on 97% sequence similarity (Hartman et al., 2008). Similarly, the presence of dominant bacterial taxa in both shallow and deeper aquifer were suggested to be influenced by the presence of nutrients and favorable redox conditions (ORP) (Sultana et al., 2011). Dissolved As, ORP, and oxygen levels were highlighted on influencing arsenite-oxidizing bacterial diversity (Quemeneur et al., 2010). On the other hand, Li et al. (2015) found that high As in groundwater and sediments may challenge certain microbial populations, and favor As-resistant assemblages. This might be the reasons for the difference of microbial populations in high and low arsenic environments (Li et al., 2015). Also, Sheik et al. (2012) found that the structure, diversity, and abundance of microbial communities were highly influenced by the concentration of As. Iron (hydr) oxide was suggested to be an

important factor in shaping the microbial community in As-contaminated soils as it provided the most important sorption phase for As (Yang et al., 2015). Guo et al. (2017), by performing the RDA and Pearson's correlation analyzes, found that salinity, DOC, TN, TP, DO, and pH were the major environmental factors affecting bacterial community structure in the intertidal biofilm. The bacterial diversity was significant correlated with DO and pH; the bacterial abundance was significantly correlated with DO, DOC, and TP (Guo et al., 2017). From PCA analysis by Ghosh et al. (2014), the major ions influencing the distribution of arsenite-oxidizing bacteria were Na, Mg, Fe, K, and As, whereas, Na, Fe, and As had negative regulatory roles (Ghosh et al., 2014). Therefore, the findings in this study could be also confirmed by many research.

The major found could be accounted to the strong correlations of pH, DO, and ORP. These crucial water parameters, representing water quality, seemed shaping the arsenite oxidizing bacterial community in this study. The reasons supported these correlations could be hypothesized on the specific bacterial ability on tolerant and favorable on the environmental conditions. For example, Lauber et al. (2009) have suggested that soil pH may not alter bacterial community but may instead function as an integrating variable that provides an integrated index of soil conditions; in other words, soil pH often directly or indirectly related to soil characteristics (e.g. nutrient availability, cationic metal solubility, organic C characteristics, soil moisture regiment, and salinity) (Weil and Brady, 2007; Lauber et al., 2009) which could result in unique environmental condition favored by a certain bacterial group. Another reason could be the hypothesis that pH directly can determine a physiological constraint on bacteria which can alter the competitive outcomes or reduce the net growth of individual taxa that are unable to survive if pH changes to a certain range. Therefore, the extreme pH may impose a significant stress that certain bacteria may tolerate better than others (Madigan and Martinko, 2006; Lauber et al., 2009). Moreover, pH was predicted to influent the hydrolysis reactions of dissolved organic matter such as organic acids, proteins, and humic materials (SC et al., 2009). Lower pH was shown to lead to an increased activity of total protease in microcosms and extracellular α- and β-glucosidase in laboratory experiments of the direct pH effect investigation (Grossart et al., 2006; Piontek et al., 2009). Potter et al. (2000) have suggested that in variable redox environments, microbes may have developed defense mechanisms for withstanding the

redox stress due to both the presence and absence of O_2 including antioxidant enzymes such as superoxide reductase (Potter et al., 2000). Also, the fluctuation-adapted of bacterial community in the redox fluctuation environment was suggested to be likely dominated by organisms that retain physiological tolerance mechanisms which allow them to withstand energetically unfavorable redox periods (Ridge and Firestone, 2005). On the other hands, Lorenz et al. (2006) found that *Proteobacteria* appear to be more tolerant to As than several other groups of bacteria in which the changes of microbial community composition were found accompanied by changes in enzyme activities (e.g. xylanase activities) (Lorenz et al., 2006). The change of microbial community structure and selection for resistance were suggested to be responded from soil metal contaminated and maintaining metabolic activity (Turpeinen et al., 2004). Lastly, Maliszawska et al. (1985) have found that arsenate stimulated the proliferation of certain groups of microorganisms in soil resulted in a shift of the community to comprise only a few tolerant species (Maliszewska et al., 1985).

Table 4.5 Pearson's correlation coefficient and significant values of the 4 arsenite oxidizing bacterial clusters and environmental variables

Variables	α_1		α_2		α_3		β	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
pH	**-0.769**	**0.015**	0.045	0.909	-0.432	0.245	0.343	0.366
Temp.	-0.229	0.554	0.194	0.617	0.212	0.584	-0.089	0.820
DO	-0.326	0.392	**0.838**	**0.005**	0.331	0.384	**-0.672**	**0.048**
ORP	0.289	0.450	**0.706**	**0.034**	0.463	0.210	**-0.843**	**0.004**
TOC	-0.205	0.597	0.122	0.754	-0.184	0.636	-0.012	0.975
As	0.039	0.921	-0.463	0.209	-0.279	0.467	0.443	0.232
$H_2AsO_4^{-*}$	0.338	0.374	-0.204	0.599	0.028	0.944	0.035	0.929
$HAsO_4^{2-*}$	-0.516	0.155	-0.192	0.622	-0.363	0.338	0.448	0.227
Mn	-0.391	0.298	0.252	0.513	0.196	0.613	-0.067	0.864
Fe	-0.230	0.552	0.520	0.151	0.589	0.095	-0.421	0.259
SO_4^{2-}	-0.213	0.582	-0.235	0.543	-0.303	0.428	0.343	0.367

Note: ** = Arsenic species from PHREEQC modeling*
The bold indicate significant values at the 0.05 level (P<0.05)

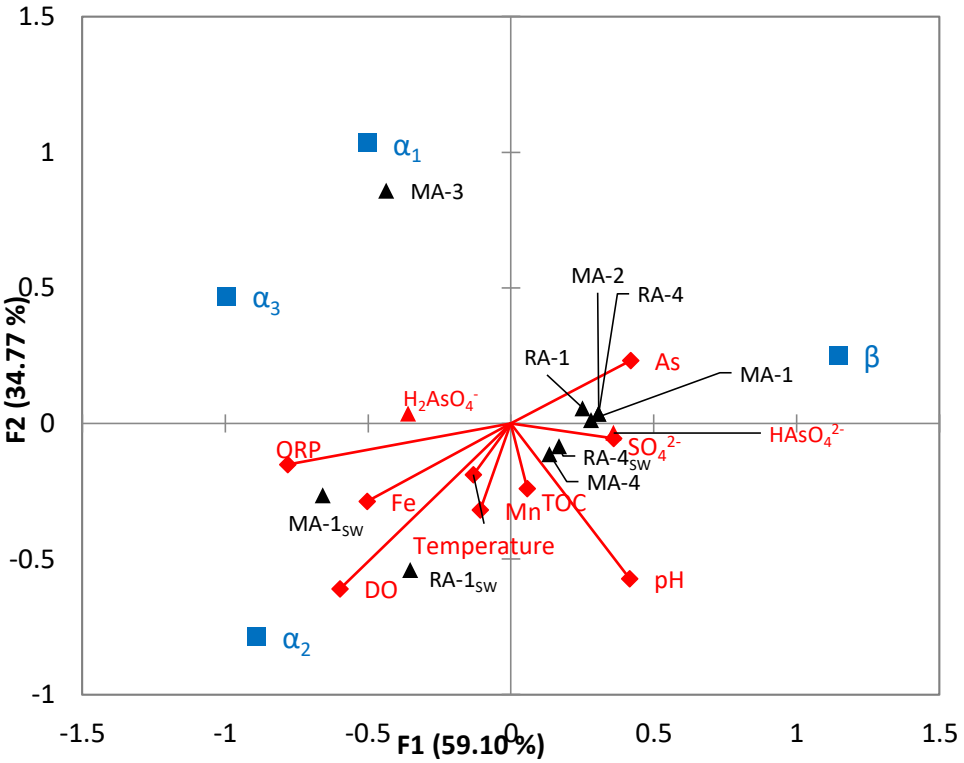
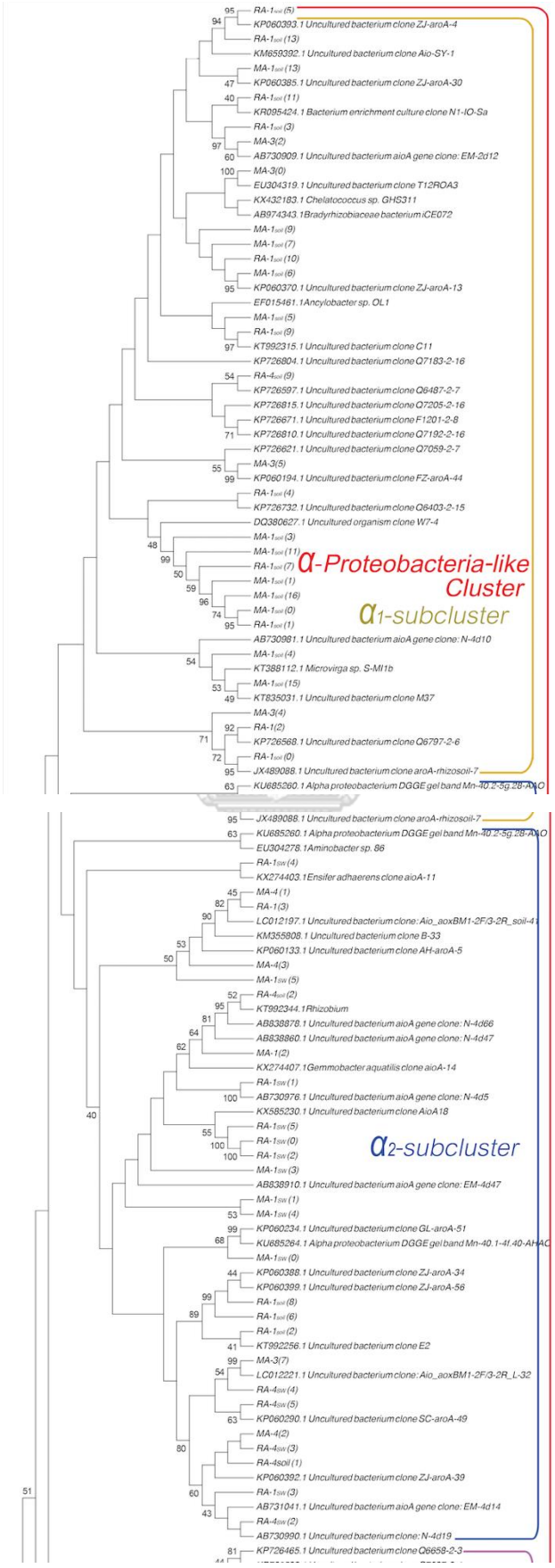


Figure 4.8 RDA plot of the 4 main clusters, environmental factors, and sampling stations. The blue squares represent the 4 main arsenite oxidizing bacterial clusters (response variables), the red diamond and black triangle represent environmental media types and sampling points as explanatory variables, respectively


α-Proteobacteria-like Cluster
α1-subcluster
α2-subcluster

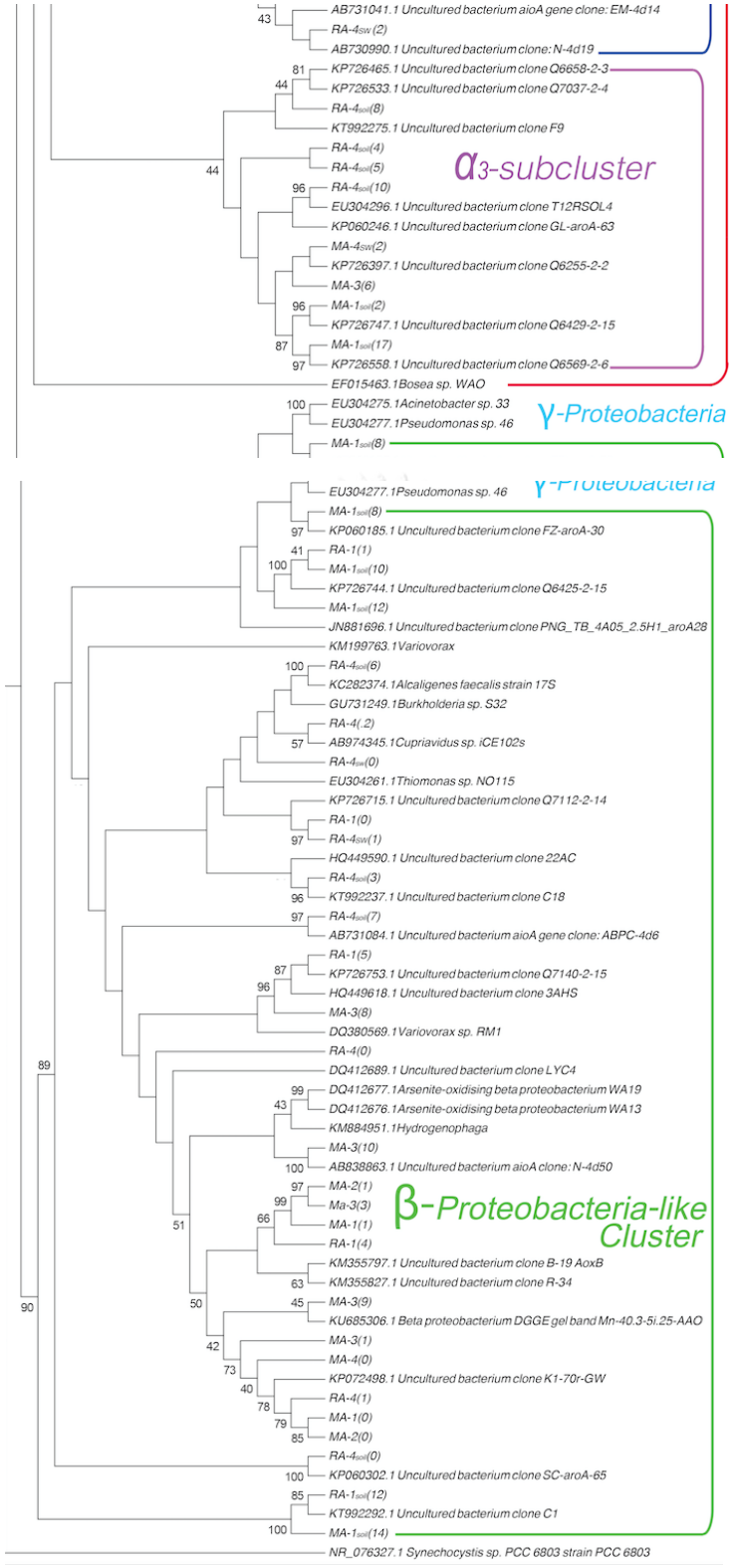


Figure 4.9 Phylogenetic tree of evolutionary relationships of *aoxB* taxa. Clusters were divided in to 4 main clusters: α_1-, α_2-, α_3-, β-*Proteobacteria*-like clusters. The phylogenetic tree refers to the original version in Fig A1.

Chapter 5
CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In this study, the major arsenic specie found in groundwater sampling stations was arsenate (As^{5+}) which was mainly influent by pH and ORP. However, Fe and SO_4^{2-} could also be minor environmental factors governing the presence of As in groundwater as revealing from PHREEQC modeling. On the other hands, the presence of arsenite-oxidizing bacterial community mainly consisted of α-and β-*Proteobacteria*-like clusters. The dominant clusters found in groundwater, surface water and soil were β-, α_2- and α_1-/ α_3-*Proteobacterial* clusters, respectively. The distributions of arsenite-oxidizing bacteria in mining and residential areas were not different, but their isolated environments (groundwater, surface water, and soil) seemed to be the key factor affecting the distribution pattern of arsenite-oxidizing bacteria analyzed in this study. This finding indicated that the detected arsenite-oxidizing bacteria in groundwater, surface water and soil may have their specific roles and abilities in each environment. Moreover, the arsenite-oxidizing bacterial diversity was lower in groundwater compared to those in surface water and soil. However, the majority of arsenite-oxidizing bacteria found in groundwater collected from mining and residential areas, approximately 50% and 78%, respectively, were affiliated to β-*Proteobacteria*-like cluster. The members of β-*Proteobacteria*-like cluster, including *Hydrogenophaga, Burkholderia, Alcaligenes, Variovorax, Thiomonas, and Cupriavidus,* seemed having a potential for further development of *in-situ* As-bioremediation. In addition, the statistical analysis revealed that the main environmental variables influencing the distribution of arsenite-oxidizing bacterial clusters were pH, ORP, and DO, while As and Fe concentrations also appeared to impact the distribution of arsenite- oxidizing bacterial clusters detected in this study. This study showed the detection of arsenite-oxidizing bacteria in groundwater with low As concentration and revealed environmental factors affecting their distribution pattern. This knowledge is useful for further investigation on the enrichment of arsenite-oxidizing bacteria from groundwater. Although arsenite-oxidizing bacteria were detected in groundwater

analyzed in this study, the activity in oxidizing arsenite was not yet verified. To implement As bioremediation technology, future work should be conducted on the enrichment and isolation of arsenite-oxidizing bacteria in order to further study on their physiology, their ability in arsenite oxidation and their tolerance on As levels.

Another concern was that

the similar As concentrations detected in groundwater samples could lead to the similar geochemical modeling which might not represent groundwater contaminated with high As levels. In addition, in the modeling section, many ions were needed as many as possible in order to mimic natural conditions; however, there were only 4 major ions (As, Fe, Mn, and SO_4^{2-}) that were used as the input parameters due to the undetected of the other ions such as TP and TN. Therefore, other ions such as Ca^{2+}, Mg^{2+}, HCO_3^- and Cl^- should be analyzed and included as the input parameter in the model in order to increase accuracy in mimicking natural conditions.

Nevertheless, the surveying of arsenite-oxidizing bacterial community in this study is one of the first few research in Thailand aiming to developing the *in-situ* As bioremediation technology. The results from this study revealed that the majority of arsenite-oxidizing bacteria detected from both mining and residential groundwater samples belonged to *β-Proteobacteria* class. Also, it has been reported that *β-Proteobacteria* class was capable of oxidizing arsenite in various concentrations. Thus, the future work might be focusing on the abundance, enrichment and isolation of individual bacterial species and investigation of arsenite oxidizing activities.

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APPENDIX

Table A 1 Bacterial composition based on the 4 clusters of each library

Station	α_1	α_2	α_3	β
MA-1	0.00	4.17	0.00	95.83
MA-2	0.00	0.00	0.00	100.00
MA-3	60.00	4.00	4.00	32.00
MA-4	0.00	26.09	0.00	73.91
Mining groundwater	20.00	25.00	5.00	50.00
RA-1	4.17	4.17	0.00	91.67
RA-4	0.00	0.00	0.00	100.00
residential groundwater	11.11	11.11	0.00	77.77
$MA\text{-}1_{SW}$	0.00	95.45	4.55	0.00
$RA\text{-}1S_W$	0.00	100.00	0.00	0.00
$RA\text{-}4_{SW}$	0.00	21.05	0.00	78.95
Surface water	0.00	83.33	5.55	11.11
$MA\text{-}1_{soil}$	69.57	0.00	13.04	17.39
$RA\text{-}1_{soil}$	75.00	18.75	0.00	6.25
$RA\text{-}4_{soil}$	4.17	29.17	16.67	50.00
Soil	53.48	11.62	13.95	20.93

Cluster α_1

Table A 2 The Cluster α_1 OTU blast results showing the 1st matched uncultured and cultured bacteria with their affiliated class/order found in each library

OTU	Uncultured bacteria		Cultured bacteria		Affiliation (class/order)
	Species	identity	Species	identity	
MA-3(0)	Uncultured bacterium clone T12ROA3	99	Chelatococcus sp. CO-6	82	*α-proteobacteria/ Rhizobiales*
MA-3(2)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: EM-2d12	92	Chelatococcus sp. CO-6	84	*α-proteobacteria/ Rhizobiales*
MA-3(4)	Uncultured bacterium clone Q6895-2-2	89	Microvirga ossetica strain V5/3M	82	*α-proteobacteria/ Rhizobiales*
MA-3(5)	Uncultured bacterium clone FZ-aroA-44	95	Microvirga ossetica strain V5/3M	83	*α-proteobacteria/ Rhizobiales*
RA-1(2)	Uncultured bacterium clone Q6797-2-6	91	Microvirga sp. S-MI1b	81	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(0)	Uncultured bacterium clone Q6487-2-7	83	Chelatococcus sp. CO-6	81	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(1)	Uncultured bacterium clone Q7205-2-16	84	Chelatococcus sp. CO-6	81	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(3)	Uncultured bacterium clone Q7059-2-7	86	Chelatococcus sp. CO-6	83	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(4)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: N-4d10	88	Microvirga ossetica strain V5/3M	87	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(5)	Bacterium enrichment culture clone N1-IO-Sa	87	Chelatococcus sp. CO-6	86	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(6)	Uncultured bacterium clone ZJ-aroA-13	94	Xanthobacter autotrophicus Py2	82	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(7)	Uncultured bacterium clone Aio-SY-1	86	Microvirga sp. S-MI1b	81	*α-proteobacteria/ Rhizobiales*

MA-1_{soil}(9)	Uncultured bacterium clone Aio-SY-1	89	Chelatococcus sp. CO-6	87	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(11)	Uncultured bacterium clone Q6403-2-15	84	Nitrobacter hamburgensis X14	78	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(13)	Uncultured bacterium clone ZJ-aroA-30	88	Chelatococcus sp. CO-6	85	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(15)	Uncultured bacterium clone M37	87	Microvirga ossetica strain V5/3M	86	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(16)	Uncultured bacterium clone Q7183-2-16	84	Ancylobacter sp. OL1	82	*α-proteobacteria/ Rhizobiales*
RA-1_{soil}(0)	Uncultured bacterium clone aroA-rhizosoil-7	91	Microvirga ossetica strain V5/3M	82	*α-proteobacteria/ Rhizobiales*
RA-1_{soil}(1)	Uncultured bacterium clone F1201-2-8	84	Chelatococcus sp. CO-6	82	*α-proteobacteria/ Rhizobiales*
RA-1_{soil}(3)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: EM-2d12	91	Chelatococcus sp. CO-6	85	*α-proteobacteria/ Rhizobiales*
RA-1_{soil}(4)	Uncultured bacterium clone Q7192-2-16	88	Chelatococcus sp. CO-6	85	*α-proteobacteria/ Rhizobiales*
RA-1_{soil}(5)	Uncultured bacterium clone ZJ-aroA-4	99	Microvirga ossetica strain V5/3M	86	*α-proteobacteria/ Rhizobiales*
RA-1_{soil}(7)	Uncultured organism clone W7-4	85	Microvirga sp. S-MI1b	80	*α-proteobacteria/ Rhizobiales*
RA-1_{soil}(9)	Uncultured bacterium clone C11	87	Chelatococcus sp. CO-6	84	*α-proteobacteria/ Rhizobiales*
RA-1_{soil}(10)	Uncultured bacterium clone Aio-SY-1	89	Chelatococcus sp. CO-6	86	*α-proteobacteria/ Rhizobiales*
RA-1_{soil}(11)	Uncultured bacterium clone Aio-SY-1	90	Chelatococcus sp. CO-6	85	*α-proteobacteria/ Rhizobiales*
RA-1_{soil}(13)	Uncultured bacterium clone ZJ-aroA-4	96	Microvirga ossetica strain V5/3M	86	*α-proteobacteria/ Rhizobiales*

RA-4_{soil}(9)	Uncultured bacterium clone Q6487-2-7	89	Microvirga ossetica strain V5/3M	88	*α-proteobacteria/ Rhizobiales*

Cluster α_2

Table A 3 The Cluster α_2 OTU blast results showing the 1st matched uncultured and cultured bacteria with their affiliated class/order found in each library

OTU	Uncultured bacteria		Cultured bacteria		Affiliation (class/order)
	Species	identity	Species	identity	
MA-1(2)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: N-4d47	92	Defluviimonas alba strain cai42	91	*α-proteobacteria/ Rhodobacterales*
MA-3(7)	Uncultured bacterium aioA gene for arsenite oxidase, partial cds, clone: Aio_aoxBM1-2F/3-2R_L-32	94	Aminobacter aminovorans strain KCTC 2477	83	*α-proteobacteria/ Rhizobiales*
MA-4(1)	Uncultured bacterium aioA gene for arsenite oxidase, partial cds, clone: Aio_aoxBM1-2F/3-2R_soil-41	91	Bradyrhizobiaceae bacterium iCE072	81	*α-proteobacteria/ Rhizobiales*
MA-4(2)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: N-4d19	89	Aminobacter aminovorans strain KCTC 2477	86	*α-proteobacteria/ Rhizobiales*
MA-4(3)	Uncultured bacterium clone AH-aroA-5	86	Aminobacter sp. 86	82	*α-proteobacteria/ Rhizobiales*
RA-1(3)	Uncultured bacterium aioA gene for arsenite oxidase, partial cds, clone: Aio_aoxBM1-2F/3-2R_soil-41	91	Bradyrhizobiaceae bacterium iCE072	81	*α-proteobacteria/ Rhizobiales*
RA-1_{soil}(2)	Uncultured bacterium clone E2	89	Defluviimonas alba strain cai42	82	*α-proteobacteria/ Rhodobacterales*
RA-1_{soil}(6)	Uncultured bacterium clone ZJ-aroA-34	94	Aminobacter aminovorans strain KCTC 2477	82	*α-proteobacteria/ Rhizobiales*
RA-1_{soil}(8)	Uncultured bacterium clone ZJ-aroA-56	93	Aminobacter aminovorans strain KCTC 2477	85	*α-proteobacteria/ Rhizobiales*

RA-4_{soil}(1)	Uncultured bacterium clone ZJ-aroA-39	89	Aminobacter aminovorans strain KCTC 2477	84	*α-proteobacteria/ Rhizobiales*
RA-4_{soil}(2)	Uncultured bacterium clone aioA-17	99	Rhizobium sp. strain CM7	96	*α-proteobacteria/ Rhizobiales*
MA-1_{sw}(0)	Alpha proteobacterium enrichment culture isolate DGGE gel band Mn-40.1-4f.40-AHAO	88	Aminobacter aminovorans strain KCTC 2477	86	*α-proteobacteria/ Rhizobiales*
MA-1_{sw}(1)	Uncultured bacterium clone GL-aroA-51	82	Defluviimonas alba strain cai42	81	*α-proteobacteria/ Rhodobacterales*
MA-1_{sw}(3)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: N-4d66	89	Defluviimonas alba strain cai42	88	*α-proteobacteria/ Rhodobacterales*
MA-1_{sw}(4)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: N-4d5	81	Aminobacter sp. 86	77	*α-proteobacteria/ Rhizobiales*
MA-1_{sw}(5)	Alpha proteobacterium enrichment culture isolate DGGE gel band Mn-40.2-5g.28-AAO	86	Aminobacter aminovorans strain KCTC 2477	84	*α-proteobacteria/ Rhizobiales*
RA-1_{sw}(0)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: EM-4d47	83	Gemmobacter aquatilis clone aioA-14	83	*α-proteobacteria/ Rhodobacterales*
RA-1_{sw}(1)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: N-4d5	99	Rhizobium sp. strain CM7	85	*α-proteobacteria/ Rhizobiales*
RA-1_{sw}(2)	Uncultured bacterium clone C6	84	Gemmobacter aquatilis clone aioA-14	83	*α-proteobacteria/ Rhodobacterales*
RA-1_{sw}(3)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: EM-4d14	93	Aminobacter aminovorans strain KCTC 2477	87	*α-proteobacteria/ Rhizobiales*
RA-1_{sw}(4)	Thauera sp. MZ1T	82	Thauera sp. MZ1T	82	*β-proteobacteria/ Rhodocyclales*

RA-1_{sw}(5)	Uncultured bacterium clone AioA18	85	Rhizobium sp. strain CM7	81	*α-proteobacteria/ Rhizobiales*
RA-4_{sw}(2)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: EM-4d14	93	Ensifer sp. strain CM6	76	*α-proteobacteria/ Rhizobiales*
RA-4_{sw}(3)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: EM-4d14	91	Agrobacterium tumefaciens strain 5A	76	*α-proteobacteria/ Rhizobiales*
RA-4_{sw}(4)	Uncultured bacterium clone SC-aroA-49	88	Aminobacter aminovorans strain KCTC 2477	86	*α-proteobacteria/ Rhizobiales*
RA-4_{sw}(5)	Uncultured bacterium clone SC-aroA-49	89	Rhizobium sp. strain CM7	82	*α-proteobacteria/ Rhizobiales*

Cluster α_3

Table A 4 The Cluster α_3's OTU blast results showing the 1st matched uncultured and cultured bacteria with their affiliated class/order found in each library

OTU	Uncultured bacteria		Cultured bacteria		Affiliation (class/order)
	Species	identity	Species	identity	
MA-3(6)	Uncultured bacterium clone Q6658-2-3	84	Bosea sp. S41RM2	77%	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(2)	Uncultured bacterium clone Q6429-2-15	89	Microvirga ossetica strain V5/3M	77%	*α-proteobacteria/ Rhizobiales*
MA-1_{soil}(17)	Uncultured bacterium clone Q6569-2-6	88	Bradyrhizobiaceae bacterium iCE072	78%	*α-proteobacteria/ Rhizobiales*
RA-4_{soil}(4)	Uncultured bacterium clone Q6255-2-2	83	Aminobacter sp. 86	79%	*α-proteobacteria/ Rhizobiales*
RA-4_{soil}(5)	Uncultured bacterium clone GL-aroA-63	86	Starkeya novella DSM 506	79%	*α-proteobacteria/ Rhizobiales*
RA-4_{soil}(8)	Uncultured bacterium clone F9	88	Ancylobacter sp. OL1	80%	*α-proteobacteria/ Rhizobiales*
RA-4_{soil}(10)	Uncultured bacterium clone T12RSOL4	87	Bradyrhizobium sp. S452	78%	*α-proteobacteria/ Rhizobiales*

MA-1_{sw}(2)	Uncultured bacterium clone Q7037-2-4	82	Defluviimonas alba strain cai42	79%	*α-proteobacteria/ Rhodobacterales*

Cluster β

Table A 5 The Cluster β's OTU blast results showing the 1st matched uncultured and cultured bacteria with their affiliated class/order found in each library

OTU	Uncultured bacteria		Cultured bacteria		Affiliation (class/order)
	Species	identity	Species	identity	
MA-1(0)	Uncultured bacterium clone K1-70r-GW	98	Hydrogenophaga atypica strain BDP10	88%	*β-proteobacteria/ Burkholderiales*
MA-1(1)	Beta proteobacterium enrichment culture isolate DGGE gel band Mn-40.3-5i.25-AAO	88	Hydrogenophaga bisanensis strain BDP20	84%	*β-proteobacteria/ Burkholderiales*
MA-2(0)	Uncultured bacterium clone K1-70r-GW	98	Hydrogenophaga atypica strain BDP10	88%	*β-proteobacteria/ Burkholderiales*
MA-2(1)	Uncultured bacterium clone R-34 AoxB	83	Arsenite-oxidising beta proteobacterium WA19	81%	*β-proteobacteria/ Burkholderiales*
MA-3(1)	Uncultured bacterium clone K1-70r-GW	97	Hydrogenophaga atypica strain BDP10	89%	*β-proteobacteria/ Burkholderiales*
MA-3(3)	Uncultured bacterium clone B-19 AoxB	85	Arsenite-oxidising beta proteobacterium WA13	81%	*β-proteobacteria/ Burkholderiales*
MA-3(8)	Uncultured bacterium clone 3AHS	90	Hydrogenophaga bisanensis strain BDP20	84%	*β-proteobacteria/ Burkholderiales*
MA-3(9)	Beta proteobacterium enrichment culture isolate DGGE gel band Mn-40.3-5i.25-AAO	94	Hydrogenophaga atypica strain BDP10	88%	*β-proteobacteria/ Burkholderiales*
MA-3(10)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: N-4d50	99	Hydrogenophaga atypica strain BDP10	87%	*β-proteobacteria/ Burkholderiales*

MA-4(0)	Uncultured bacterium clone K1-70r-GW	96	Hydrogenophaga atypica strain BDP10	89%	*β-proteobacteria/ Burkholderiales*
RA-1(0)	Uncultured bacterium clone Q7112-2-14	81	Thiomonas arsenivorans strain DSM 16361	76%	*β-proteobacteria/ Burkholderiales*
RA-1(1)	Uncultured bacterium clone Q6425-2-15	84	Burkholderia vietnamiensis LMG 10929	81%	*β-proteobacteria/ Burkholderiales*
RA-1(4)	Uncultured bacterium clone K1-70r-GW	92	Hydrogenophaga atypica strain BDP10	88%	*β-proteobacteria/ Burkholderiales*
RA-1(5)	Uncultured bacterium clone Q7140-2-15	95	Hydrogenophaga bisanensis strain BDP20	87%	*β-proteobacteria/ Burkholderiales*
RA-4(0)	Uncultured bacterium clone LYC4	90	Acinetobacter lwoffii strain BDP2	85%	*γ-proteobacteria/Pseudomonadales*
RA-4(1)	Uncultured bacterium clone K1-70r-GW	98	Hydrogenophaga atypica strain BDP10	88%	*β-proteobacteria/ Burkholderiales*
RA-4(2)	Cupriavidus sp. iCE102s	79	Cupriavidus sp. iCE102s	79%	*β-proteobacteria/ Burkholderiales*
MA-1_{soil}(8)	Uncultured bacterium clone FZ-aroA-30	84	Ralstonia solanacearum strain KACC 10722	78%	*β-proteobacteria/ Burkholderiales*
MA-1_{soil}(10)	Uncultured bacterium clone PNG_TB_4A05_2.5 H1_aroA28	87	Variovorax sp. IDSBO-4	92%	*β-proteobacteria/ Burkholderiales*
MA-1_{soil}(12)	Uncultured bacterium clone 22AC	74	Burkholderia pseudomallei PB08298010	85%	*β-proteobacteria/ Burkholderiales*
MA-1_{soil}(14)	Tumebacillus sp. AR23208	94	Tumebacillus sp. AR23208	94%	*Bacilli/Bacillales (Firmucutes)*
RA-1_{soil}(12)	Uncultured bacterium clone C1	83	Pseudogulbenkia nia sp. NH8B	95%	*β-proteobacteria/ Neisseriales*
RA-4_{soil}(0)	Uncultured bacterium clone SC-aroA-65	90	Variovorax sp. RM1	82%	*β-proteobacteria/ Burkholderiales*
RA-4_{soil}(3)	Uncultured bacterium clone C18	88	Cupriavidus sp. USMAHM13	75%	*β-proteobacteria/ Burkholderiales*

RA-4_{soil}(6)	Alcaligenes faecalis strain NCIB 8687	88	Alcaligenes faecalis strain NCIB 8687	88%	*β-proteobacteria/ Burkholderiales*
RA-4_{soil}(7)	Uncultured bacterium aioA gene for arsenite oxidase large subunit, partial cds, clone: ABPC-4d6	88	Hydrogenophaga atypica strain BDP10	77%	*β-proteobacteria/ Burkholderiales*
RA-4_{sw}(0)	Uncultured bacterium clone LYB4	76	Arsenite-oxidising beta proteobacterium WA19	76%	*β-proteobacteria/ Burkholderiales*
RA-4_{sw}(1)	Uncultured bacterium clone Q7112-2-14	79	Thiomonas sp. X19	72%	*β-proteobacteria/ Burkholderiales*

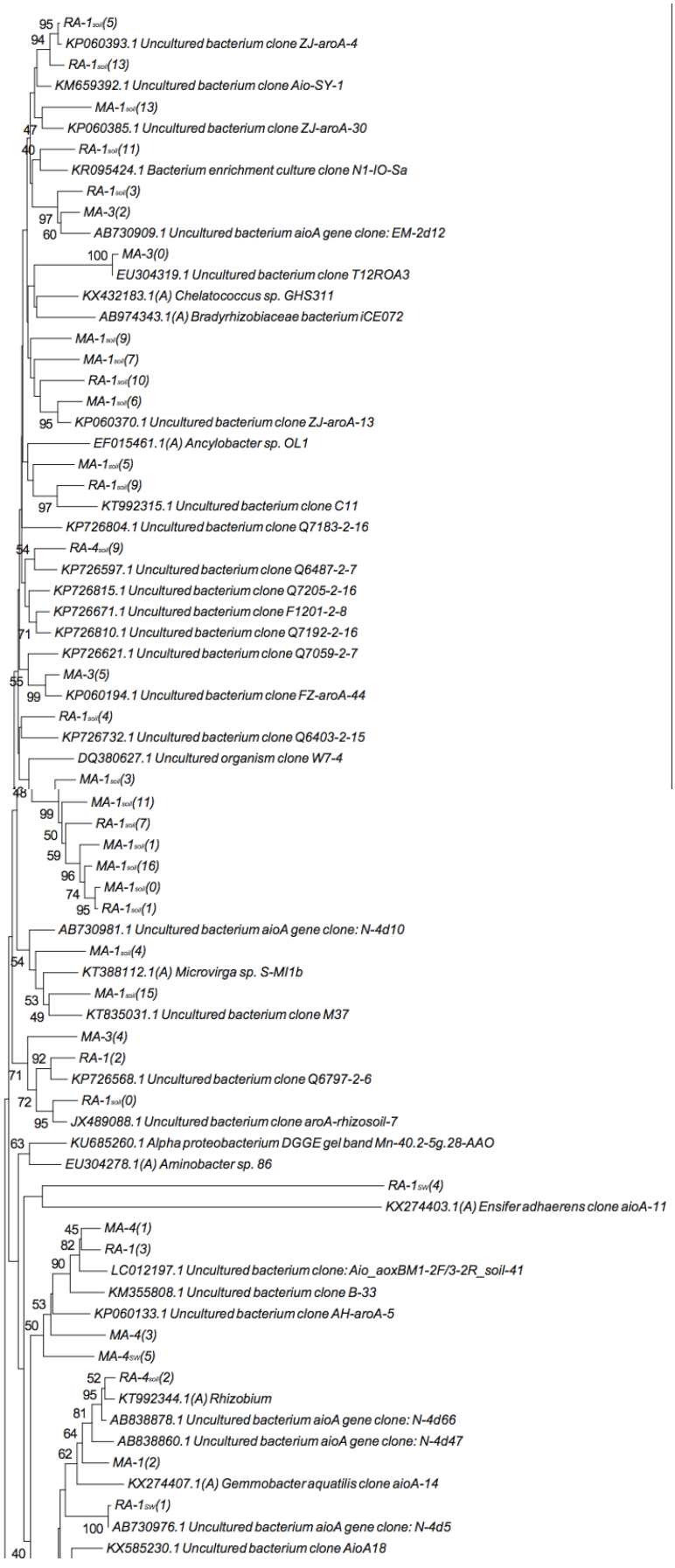
Table A 6 RDA results supporting Fig 4.5

Eigenvalues and percentages of inertia (RDA)			
	F1	**F2**	**F3**
Eigenvalue	1.193	0.951	0.008
Constrained inertia (%)	55.437	44.173	0.390
Cumulative %	55.437	99.610	100.000
Total inertia	29.832	23.770	0.210
Cumulative % (%)	29.832	53.601	53.811
Scores (Response variables)			
	F1	**F2**	**F3**
α_1-cluster	-0.641	-0.556	-0.070
α_2-cluster	-0.323	0.990	0.007
α_3-cluster	-0.833	-0.369	0.088
β-cluster	0.878	-0.391	0.035
Scores (Explanatory variables)			
	F1	**F2**	**F3**
Mining groundwater	0.386	-0.240	-0.086
Residential Groundwater	0.469	-0.176	0.081
soil	-0.695	-0.401	0.018
Surface water	-0.129	0.814	0.006

Table A 7 RDA results supporting Fig 4.6

Eigenvalues and percentages of inertia (RDA)				
	F1	**F2**	**F3**	**F4**
Eigenvalue	2.364	1.391	0.245	0.000
Constrained inertia (%)	59.104	34.769	6.126	0.000
Cumulative %	59.104	93.874	100.000	100.000
Total inertia	59.104	34.769	6.126	0.000
Cumulative % (%)	59.104	93.874	100.000	100.000
Scores (Response variables)				
	F1	**F2**	**F3**	**F4**
α_1-cluster	-0.502	1.033	-0.310	0.000
α_2-cluster	-0.889	-0.787	-0.074	0.000
α_3-cluster	-0.995	0.469	0.453	0.000
β-cluster	1.146	0.249	0.200	0.000
Contributions (Response variables)				
	F1	**F2**	**F3**	**F4**
α_1-cluster	0.075	0.542	0.278	0.105
α_2-cluster	0.236	0.315	0.016	0.433
α_3-cluster	0.296	0.112	0.591	0.001
β-cluster	0.393	0.031	0.115	0.461
Scores (Explanatory variables)				
	F1	**F2**	**F3**	**F4**
pH	0.416	-0.572	0.371	-0.152
Temperature	-0.132	-0.188	0.462	-0.614
DO	-0.598	-0.609	0.188	0.015
ORP	-0.782	-0.151	-0.347	0.218
TOC	0.058	-0.240	-0.108	-0.197
As	0.419	0.232	-0.053	0.285
Mn	-0.107	-0.318	0.611	-0.255
Fe	-0.503	-0.287	0.739	-0.104
SO_4^{2-}	0.359	-0.056	0.051	-0.253
$H_2AsO_4^-$	-0.359	0.036	-0.079	0.286
$HAsO_4^{2-}$	0.359	-0.036	0.079	-0.286
RA-1	0.250	0.056	-0.003	0.966
RA-4	0.307	0.037	0.142	-0.211
MA-1	0.280	0.013	0.106	-0.202
MA-2	0.307	0.037	0.142	-0.211
MA-3	-0.436	0.859	-0.259	-0.067
MA-4	0.135	-0.114	-0.081	-0.158
$MA\text{-}1_{SW}$	-0.659	-0.264	0.702	0.059

RA-1_{SW}	-0.353	-0.540	-0.711	-0.009
RA-4_{SW}	0.168	-0.085	-0.038	-0.168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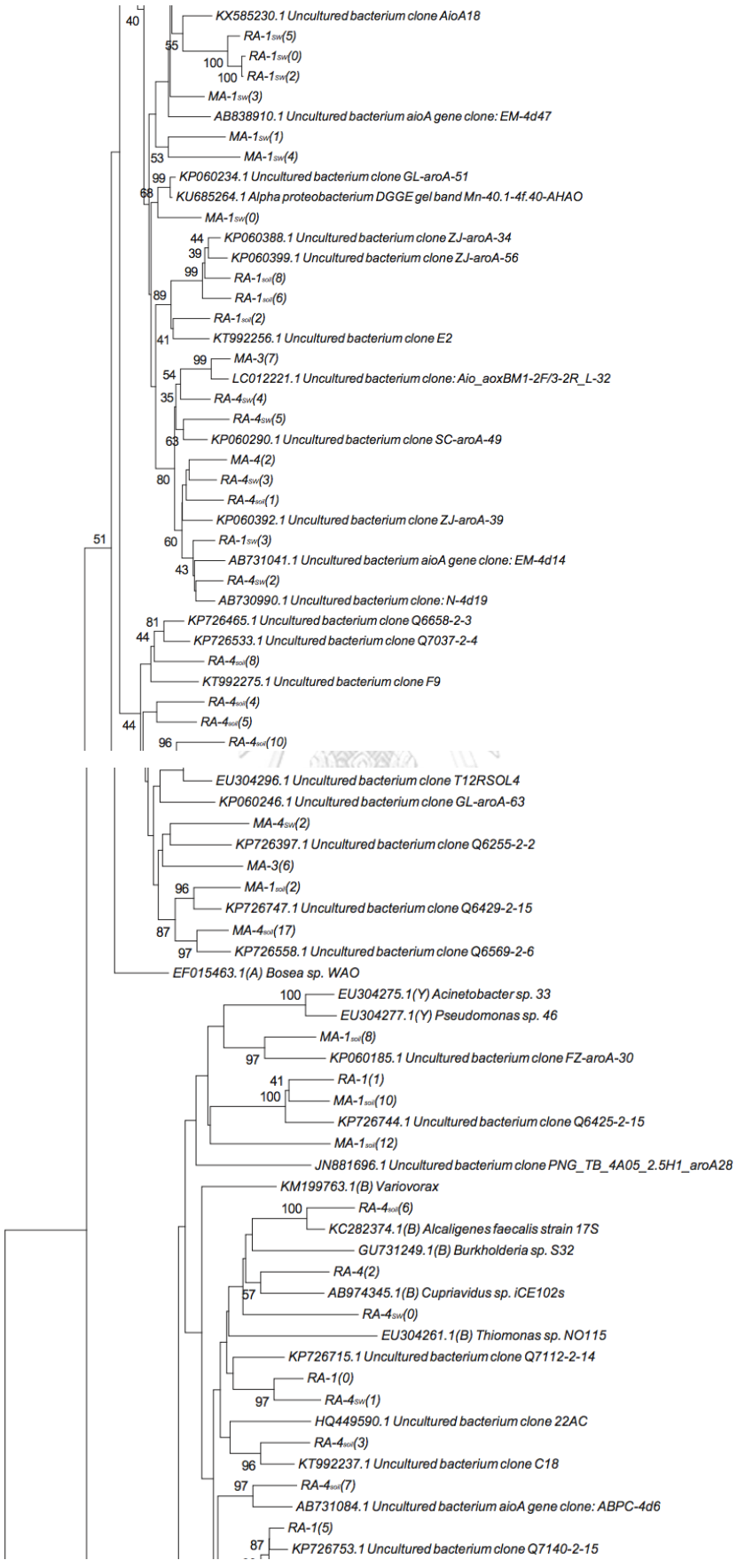
KM659392.1 Uncultured bacterium clone Aio-SY-1
MA-1soil(13)
KP060385.1 Uncultured bacterium clone ZJ-aroA-30
RA-1soil(11)
KR095424.1 Bacterium enrichment culture clone N1-IO-Sa
RA-1soil(3)
MA-3(2)
AB730909.1 Uncultured bacterium aioA gene clone: EM-2d12
MA-3(0)
EU304319.1 Uncultured bacterium clone T12ROA3
KX432183.1(A) Chelatococcus sp. GHS311
AB974343.1(A) Bradyrhizobiaceae bacterium iCE072
MA-1soil(9)
MA-1soil(7)
RA-1soil(10)
MA-1soil(6)
KP060370.1 Uncultured bacterium clone ZJ-aroA-13
EF015461.1(A) Ancylobacter sp. OL1
MA-1soil(5)
RA-1soil(9)
KT992315.1 Uncultured bacterium clone C11
KP726804.1 Uncultured bacterium clone Q7183-2-16
RA-4soil(9)
KP726597.1 Uncultured bacterium clone Q6487-2-7
KP726815.1 Uncultured bacterium clone Q7205-2-16
KP726671.1 Uncultured bacterium clone F1201-2-8
KP726810.1 Uncultured bacterium clone Q7192-2-16
KP726621.1 Uncultured bacterium clone Q7059-2-7
MA-3(5)
KP060194.1 Uncultured bacterium clone FZ-aroA-44
RA-1soil(4)
KP726732.1 Uncultured bacterium clone Q6403-2-15
DQ380627.1 Uncultured organism clone W7-4
MA-1soil(3)
MA-1soil(11)
RA-1soil(7)
MA-1soil(1)
MA-1soil(16)
MA-1soil(0)
RA-1soil(1)
AB730981.1 Uncultured bacterium aioA gene clone: N-4d10
MA-1soil(4)
KT388112.1(A) Microvirga sp. S-MI1b
MA-1soil(15)
KT835031.1 Uncultured bacterium clone M37
MA-3(4)
RA-1(2)
KP726568.1 Uncultured bacterium clone Q6797-2-6
RA-1soil(0)
JX489088.1 Uncultured bacterium clone aroA-rhizosoil-7
KU685260.1 Alpha proteobacterium DGGE gel band Mn-40.2-5g.28-AAO
EU304278.1(A) Aminobacter sp. 86
RA-1SW(4)
KX274403.1(A) Ensifer adhaerens clone aioA-11
MA-4(1)
RA-1(3)
LC012197.1 Uncultured bacterium clone: Aio_aoxBM1-2F/3-2R_soil-41
KM355808.1 Uncultured bacterium clone B-33
KP060133.1 Uncultured bacterium clone AH-aroA-5
MA-4(3)
MA-4SW(5)
RA-4soil(2)
KT992344.1(A) Rhizobium
AB838878.1 Uncultured bacterium aioA gene clone: N-4d66
AB838860.1 Uncultured bacterium aioA gene clone: N-4d47
MA-1(2)
KX274407.1(A) Gemmobacter aquatilis clone aioA-14
RA-1SW(1)
AB730976.1 Uncultured bacterium aioA gene clone: N-4d5
KX585230.1 Uncultured bacterium clone AioA18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MA-1SW(4)
KP060234.1 Uncultured bacterium clone GL-aroA-51
KU685264.1 Alpha proteobacterium DGGE gel band Mn-40.1-4f.40-AHAO
MA-1SW(0)
KP060388.1 Uncultured bacterium clone ZJ-aroA-34
KP060399.1 Uncultured bacterium clone ZJ-aroA-56
RA-1SOIL(8)
RA-1SOIL(6)
RA-1SOIL(2)
KT992256.1 Uncultured bacterium clone E2
MA-3(7)
LC012221.1 Uncultured bacterium clone: Aio_aoxBM1-2F/3-2R_L-32
RA-4SW(4)
RA-4SW(5)
KP060290.1 Uncultured bacterium clone SC-aroA-49
MA-4(2)
RA-4SW(3)
RA-4SOIL(1)
KP060392.1 Uncultured bacterium clone ZJ-aroA-39
RA-1SW(3)
AB731041.1 Uncultured bacterium aioA gene clone: EM-4d14
RA-4SW(2)
AB730990.1 Uncultured bacterium clone: N-4d19
KP726465.1 Uncultured bacterium clone Q6658-2-3
KP726533.1 Uncultured bacterium clone Q7037-2-4
RA-4SOIL(8)
KT992275.1 Uncultured bacterium clone F9
RA-4SOIL(4)
RA-4SOIL(5)
RA-4SOIL(10)
EU304296.1 Uncultured bacterium clone T12RSOL4
KP060246.1 Uncultured bacterium clone GL-aroA-63
MA-4SW(2)
KP726397.1 Uncultured bacterium clone Q6255-2-2
MA-3(6)
MA-1SOIL(2)
KP726747.1 Uncultured bacterium clone Q6429-2-15
MA-4SOIL(17)
KP726558.1 Uncultured bacterium clone Q6569-2-6
EF015463.1(A) Bosea sp. WAO
EU304275.1(Y) Acinetobacter sp. 33
EU304277.1(Y) Pseudomonas sp. 46
MA-1SOIL(8)
KP060185.1 Uncultured bacterium clone FZ-aroA-30
RA-1(1)
MA-1SOIL(10)
KP726744.1 Uncultured bacterium clone Q6425-2-15
MA-1SOIL(12)
JN881696.1 Uncultured bacterium clone PNG_TB_4A05_2.5H1_aroA28
KM199763.1(B) Variovorax
RA-4SOIL(6)
KC282374.1(B) Alcaligenes faecalis strain 17S
GU731249.1(B) Burkholderia sp. S32
RA-4(2)
AB974345.1(B) Cupriavidus sp. iCE102s
RA-4SW(0)
EU304261.1(B) Thiomonas sp. NO115
KP726715.1 Uncultured bacterium clone Q7112-2-14
RA-1(0)
RA-4SW(1)
HQ449590.1 Uncultured bacterium clone 22AC
RA-4SOIL(3)
KT992237.1 Uncultured bacterium clone C18
RA-4SOIL(7)
AB731084.1 Uncultured bacterium aioA gene clone: ABPC-4d6
RA-1(5)
KP726753.1 Uncultured bacterium clone Q7140-2-15

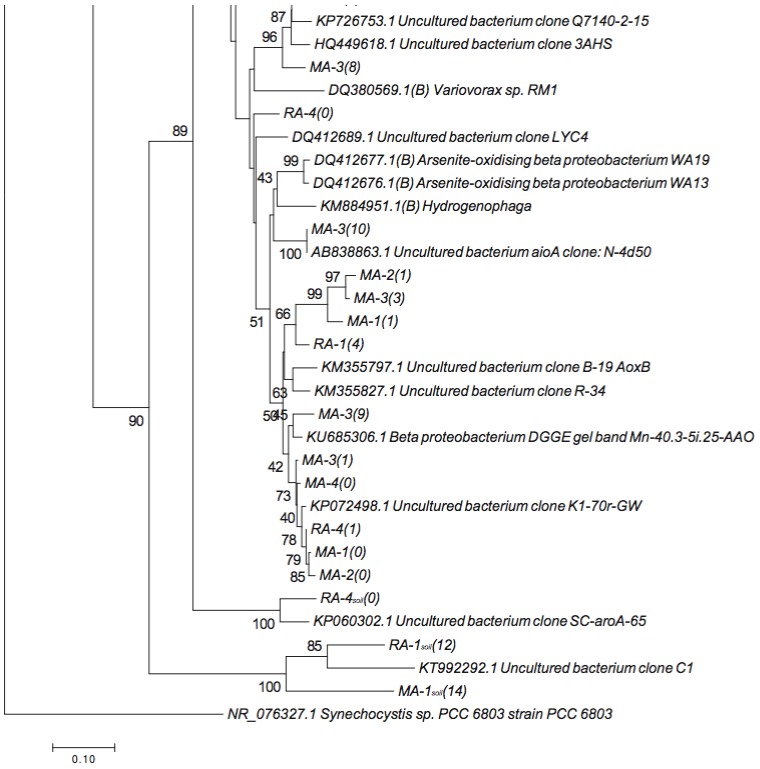

Figure A 1 Evolutionary relationships of taxa of *aoxB*-carrying bacteria in this study

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 15.60789115 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 176 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 236 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

VITA

Mr. Supeerapat Kraidech was born in August 10, 1992 in Chonburi, Thailand. He graduated a Bachelor's Degree in 2014 from Department of Environmental Engineering, Faculty of Engineering, King Mongut's University of Technology Thonburi. Then he started a Master's Degree of Science at International Program in Hazardous Substance and Environmental Management, graduate School. Chulalongkorn University in May 2015.