

**Microbial Effects and Biotransformation of  
Diphenylarsinic Acid in Anaerobic Soils**

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2013

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## Summary

Chemical warfare weapons containing aromatic arsenicals, such as CLARK I (diphenylcyanoarsine), and CLARK II (diphenylchloroarsine), were produced during the World wars. Thereafter, they were discarded in several parts of Europe, China, Japan and other countries, remaining potential leakage of aromatic arsenicals to the conjunct environment. Diphenylarsinic acid (DPAA) is often found as one of the major degradation products around the dumped sites, since the compound is easily produced from CLARK I and CLARK II via hydrolysis and oxidation. Many studies have reported environmental transformation of DPAA under aerobic conditions. However, anaerobic transformation of DPAA has not yet been explored well. The objectives of this study were to investigate, 1) the effects of DPAA on anaerobic soil microbial diversity; 2) the enhancement of DPAA transformation under sulfate-reducing conditions; and 3) the DPAA-transforming microorganisms in anaerobic DPAA-contaminated soil. We conducted model experiments using anaerobic soil cultures contaminated with DPAA.

The first chapter of the thesis presents occurrence of arsenic contamination in the world, toxicity of arsenicals and impacts of arsenicals on microbial diversity based on a detailed literature survey and describes the objective of this study.

In Chapter II, the effects of DPAA contamination on soil bacterial and archaeal community structures under anaerobic conditions were examined using molecular ecological analysis after incubation of soil cultures. DPAA in the anaerobic soil cultures prepared with a paddy soil (Shindori soil) decreased due to microbiological activity. Addition of rice straw partially enhanced the extent of DPAA degradation. Inorganic arsenic acid, phenylarsonic acid and an unknown arsenical species were detected as the transformation products using LC-ICPMS. The 16S rDNA-targeted PCR-DGGE fingerprinting for bacterial community analysis revealed that anaerobic bacterial and archaeal species, including *Clostridium* and *Methanosarcina*, became abundant in the incubated soil within a week. In the soil cultures incubated with

DPAA, while a few bacterial bands found on the DGGE gels disappeared or became weaker, most of the bands showed no significant changes. Addition of DPAA had little effect on archaeal DGGE band patterns. These findings suggest that, even though DPAA may have a direct effect on some abundant bacterial species, overall bacterial and archaeal community structures under the anaerobic soil conditions tended to be stable regardless of DPAA contamination.

The next Chapter focused on identification of a major factor enhancing DPAA transformation under anaerobic conditions. As a result, the elimination of DPAA in Gleysol soils (Qiqihar and Shindori soils) was more rapid than in Mollisol and Regosol soils (Heihe and Ikarashi soils, respectively) during a 5-week incubation. No clear relationship between decreasing rates of DPAA concentrations and soil Eh values was found. The Ikarashi soil showed the slowest decrease in DPAA concentrations among the four soils, but the transformation of DPAA was notably enhanced by addition of exogenous sulfate together with acetate, cellulose or rice straw. Addition of molybdate, a specific inhibitor of sulfate reduction, resulted in the stagnation of DPAA transformation, suggesting that indigenous sulfate reducers play a role in DPAA transformation under anaerobic conditions. As metabolites of DPAA, arsenate, phenylarsonic acid, phenylmethylarsinic acid, diphenylmethylarsine oxide and three unknown arsenical compounds were detected by LC-ICPMS analysis. Subsequently, LC-TOFMS analysis revealed that the major unknown could be assigned to diphenylthioarsinic acid (DPTAA). This is the first study to reveal enhancement of DPAA transformation under sulfate-reducing conditions.

In Chapter IV, the isolations of anaerobic microorganisms capable of transforming DPAA under sulfate-reducing conditions were examined using a limiting dilution culture method and an anaerobic plate culture method. As a result, four positive microbial consortia that could transform DPAA to DPTAA were obtained. Then, bacterial 16S rRNA gene libraries were constructed and the sequences were determined. The sequencing results revealed that all the positive consortia contained *Desulfotomaculum acetoxidans* species. In contrast, absence of *dsrAB*, dissimilatory sulfite reductase genes, was confirmed in the negative consortia

showing no DPAA reduction. These findings strongly suggest that sulfate-reducing bacteria including *D. acetoxidans* take a role in the anaerobic transformation of DPAA to DPTAA.

In conclusion, this study elucidated that, 1) the presence of DPAA did not distinctly change soil bacterial and archaeal communities under submerged and anaerobic conditions, 2) the microbial transformation of DPAA in soil could be enhanced under sulfate-reducing conditions, and 3) sulfate-reducing bacteria such as *D. acetoxidans* could participate in transforming DPAA to DPTAA under sulfate-reducing conditions. Based on 2) and 3), generation of DPTAA under sulfate-reducing conditions is attributed to reaction between DPAA and hydrogen sulfide released by sulfate-reducing bacteria. To the best of our knowledge, the role of sulfate reduction in transformation of phenylarsenicals in anaerobic conditions has not been previously reported. The findings in this study can provide a novel insight for biotransformation of phenylarsenicals in contaminated soils.

## Abbreviations

As (V)	Arsenate
As (III)	Arsenite
BDPAO	Bis(diphenylarsine) oxide
CLARK I	Diphenylchloroarsine
CLARK II	Diphenylcyanoarsine
DGGE	Denaturing gradient gel electrophoresis
DMAA	Dimethylarsinic acid
DMDTAA	Dimethyldithioarsinic acid
DMMTAA	Dimethylmonothioarsinic acid
DMPAO	Dimethylphenylarsine oxide
DPMAO	Diphenylmethylarsine oxide
DPAA	Diphenylarsinic acid
DPTAA	Diphenylthioarsinic acid
FISH	Fluorescent in situ hybridization
HPLC	High-performance liquid chromatograph
LC-ICPMS	Liquid chromatograph-inductively coupled plasma mass spectrometry
LC-TOFMS	Liquid chromatograph-time of flight mass spectrometry
MDPAO	Methyldiphenylarsine oxide
MMA (III)	Monomethylarsonous acid
MMAA	Monomethylarsonic acid
MMDTAA	Monomethyldithioarsinic acid
MPAA	Methylphenylarsinic acid
PAA	Phenylarsonic acid
PAH	Polycyclic aromatic hydrocarbons
PAO	Phenylarsine oxide

PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
TMA	Trimethylarsine
TMAO	Trimethylarsine oxide
TPA	Triphenylarsine
TPAO	Triphenylarsine oxide

# **Chapter I. Introduction**

## **I-1 Occurrence of arsenic contamination**

Arsenic is a ubiquitous metalloid that ranks 20<sup>th</sup> in abundance in the earth's crust (Woolson, 1975). It exists in four oxidation states:  $\text{As}^{+5}$ ,  $\text{As}^{+3}$ ,  $\text{As}^0$  and  $\text{As}^{-3}$ . The two highest oxidation states are the most common in nature, whereas the two lowest are rare (Oremland and Stolz, 2005). Occurrence of arsenic contamination in the environment can be due to natural processes and to anthropogenic activities.

### **I-1-1 Contamination due to natural sources**

Arsenic is an undesired pollutant that naturally occurs in water, air, biota and soil. Arsenic is found in various minerals such as iron sulfides, manganese oxides, sulfides, copper, silver, iron gold and lead. Weathering, dissolution and erosion of these minerals can contribute to release arsenic to groundwater or surface water. Volcanic activity, geothermal waters, hot springs and forest fires are also natural sources of arsenic (Cortinas López, 2007).

Several typical arsenic incidents due to environmental contamination with inorganic arsenicals are described below:

- During the 1980s, the endemic arsenicosis was found successively in many areas on mainland China such as Xinjiang, Inner Mongolia, Shanxi, Liaoning, Jilin, Ningxia, Qinghai and Henan provinces, due to usage of deep-well water with geologically occurring high levels of arsenic (Hotta, 1995; Lianfang and Jianghong, 1994; Mandal and Suzuki, 2002; Xiaojuan, 1995).
- In Bangladesh, over one-half of the approximately four million wells that constitute the country's drinking water supply have levels of naturally occurring arsenic above the World Health Organization's standard of 0.01 mg L<sup>-1</sup>. About 25 million people of 2,000 villages in 178 arsenic-affected blocks of Bangladesh are in risk and 3695 (20.6%) out of 17896 people examined are suffering from arsenicosis (Jahan *et al.*, 2006; Nickson *et al.*, 2000; Tondel *et al.*, 1999).

## **I-1-2 Contamination due to anthropogenic sources**

Apart from the natural arsenic sources, accumulation of arsenic in soil and water can result from human activities due to (a) the disposal of industrial waste chemicals, (b) the smelting of arsenic bearing minerals, (c) the burning of fossil fuels, (d) the application of arsenic compounds in many products, especially insecticides and herbicides (monosodium arsenate and DMAA), used in the past few hundred years (Bissen and Frimmel, 2003).

Another potential contributing source of arsenic in soil is the previous use of harmful phenylarsenic compounds, such as chemical warfare agents. The production of chemical weapons originated from the first and second world wars and other military conflicts that occurred in the first half of the 20<sup>th</sup> century (Katkova, 2011). Later, it was partly buried over the territories of different countries and dumped in the seas, remaining potential leakage of these aromatic arsenicals from munitions to the conjunct environment (Huang *et al.*, 2011).

The most important phenylarsenic compounds, which were used as chemical warfare agents, were CLARK I, CLARK II, phenarsazine chloride, phenylarsine dichloride and arsine oil (a technical mixture of As (III) chloride, phenylarsine dichloride, diphenylarsine chloride and TPA).

The phenylarsenic compounds can be metabolized via hydrolysis and oxidation in water, soils and sediments (Jackson *et al.*, 1935; Roychowdhury and Koropchak, 1990). In Löcknitz, Germany, where chemical warfare agents were stored and attempted to eliminate by burning and reaction with sodium hypochlorite after World War II, diphenylarsine, methyldiphenylarsine, CLARK I, TPA, triphenylarsine sulfide, BDPAO, and bisdiphenylarsine were detected in soils (Köhler *et al.*, 2001). Likewise, soil contamination by phenylarsenicals has sometimes been found in Japanese paddy soil, PAA, DPAA, MPAA, DMPAO, and MDPAO were identified with concentrations up to 1.4 ppm (Baba *et al.*, 2008).

### **I-1-3 DPAA contamination in Kamisu, Japan**

In the spring of 2003, health damage was reported in Kamisu, Ibaraki Prefecture, Japan, and it was caused by drinking well water contaminated with a high concentration of arsenic, which was 450 times higher than the safety standard for drinking water in Japan (Ishizaki *et al.*, 2005). After a precise investigation, speciation of arsenic in the well water indicated the presence of BDPAO, DPAA and PAA. The predominant arsenic species in the well water was identified as DPAA, the maximum level of which in the contaminated drinking well water in the area was 15 mg of As kg<sup>-1</sup>. Groundwater contaminated by DPAA caused a poisoning incident in Kamisu-machi. The patients showed disorders such as cerebellar symptoms, tremors, myoclonus, and sleep disturbance. However, cerebellar symptoms did not recur after the patient stopped drinking water from the well.

On the other hand, in 2004, DPAA was detected in the groundwater used for the irrigation of paddy fields in Kamisu town. Furthermore, DPAA and MPAA were also detected in the paddy rice cultivated in the paddy fields that were irrigated with the contaminated groundwater (Ministry of the Environment, Government of Japan.). Ministry of the Environment in Japan investigated the origin of the contamination, and subsequently DPAA that was solidified in cement was found to be illegally buried in the ground; it was supposed to be the origin (Kinoshita *et al.*, 2008).

## I-2 Toxicity of arsenicals

Arsenic is considered to be a biologically essential element but a lot of arsenic compounds are toxic (Mayer *et al.*, 1993). It is important to note that the toxicity of a metal depends to a large extent on its speciation, which in turn influences metal bioavailability. The chemical forms and oxidation states of arsenic are more important as regards to its toxicity. Some other factors also affected arsenic toxicity, such as physical state, gas, solution, or powder particle size, the rate of absorption into cells and so on (Mandal and Suzuki, 2002). The toxicity of arsenicals decreases in the following order (Mondal *et al.*, 2006):

arsine > inorganic arsenic (III) > organic arsenic (III) > inorganic arsenic (V) > arsonium compounds and elemental arsenic

Arsenic can cause chronic and acute poisoning. Acute arsenic poisoning may cause vomiting, dryness of the mouth and throat, muscle cramps, colicky abdominal pain, tingling of the hands and feet, circulatory disorders, and nervous weakness (Bissen and Frimmel, 2003). Chronic arsenic poisoning involves non-specific symptoms such as chronic weakness, loss of reflexes, weariness, gastritis, colitis, anorexia, weight loss and hair loss. Long-term exposure through food or air results in hyperkeratosis, hyperpigmentation, cardiovascular diseases, disturbance in the peripheral (Hall, 2002; Hindmarsh *et al.*, 1986; Lu, 1990; Vahter, 1983).

In addition, the general order of toxicity for methylated arsenic species generated by the microbial activities described in section 2.1 is given as follows (Yamauchi and Fowler, 1994; Wilson *et al.*, 2010):

arsenites (As (III)) > arsenates (As (V)) > organoarsenicals (e.g. methylated species)

There is evidence that arsenic is detoxified via methylation in environmental

systems. Therefore, the arsenic biomethylation provides a potential tool for the removal of inorganic arsenic in the contaminated environment.

However, only a few toxicological data of phenylarsenicals have been available, although a poisoning incident happened in the Kizaki region, Kamisu City, Japan, as described above.

## **I-3 Environmental behavior**

### **I-3-1 Oxidation and reduction**

As (V) serves as a “nutrient” to certain anaerobes by functioning as their respiratory oxidant, the reaction of which is energetically favorable when coupled with the oxidation of organic matters (Oremland and Stolz, 2003). At least 16 species of arsenate-reducing prokaryotes represented from the  $\gamma$ -,  $\delta$ - and  $\epsilon$ -Proteobacteria, low-GC Gram-positive bacteria, thermophilic Eubacteria and Crenarchaea, were isolated from freshwater sediments, estuaries, soda lakes, hot springs and gold mines (Oremland *et al.*, 2002). This documents the widespread ability of diverse microorganisms to act on arsenate-oxidation.

Arsenite is more noxious to cells than arsenate (Pérez-Espino *et al.*, 2009). Under certain physico-chemical conditions, microbial-mediated oxidation of arsenite can be crucial for the deliberate removal of arsenic in solution: arsenite is more soluble than arsenate, and arsenate is much more effectively removed than arsenite by coagulation with Fe (III) (Lin *et al.*, 2007). In addition, arsenite oxidation can serve as an electron donor. A strain belonging to the *Agrobacterium/Rhizobium* branch of  $\alpha$ -Proteobacteria can utilize arsenite as a sole source of electrons with a doubling time of 7.6 h. This strain was isolated from a gold mine in the Northern Territory of Australia (Santini *et al.*, 2000). The process of microbiological oxidation of arsenite to arsenate has been also known for many years, and more than 30 strains representing at least nine genera have been reported to be involved, including  $\alpha$ -,  $\beta$ - and  $\gamma$ -Proteobacteria, Deinococci (i.e., *Thermus*) and Crenarchaeota.

### **I-3-2 Methylation and demethylation**

The term “biological methylation (usually contracted to biomethylation)” refers to an enzymatic transfer of a previously formed methyl group from some donor atom to some acceptor within a living organism. Biomethylation of metals and metalloids is a

widespread and relevant process in the environment, resulting in an obvious change of mobility, bioavailability and toxicity in comparison to their inorganic precursors (Sigel *et al.*, 2010). In nature, the methylation of arsenic has been attributed to the metabolic activity of bacteria, fungi, molds, mammals, or aquatic organisms (Andreae, 1986; Cullen and Reimer, 1989; Woolson, 1977). The stepwise methylation produced both non-volatile partly methylated arsenic species as well as fully methylated arsenic species, which are volatile and can therefore be determined in the gas phase (Diaz-Bone *et al.*, 2011).

The major volatile arsenic compounds formed by microorganisms are arsines, mono-, di-, tri-methylarsine (Pérez-Espino *et al.*, 2009). One example of this is *Rhodopseudomonas palustris*, which can catalyze the formation of a number of methylated intermediates from arsenite, with TMA as the end product (Qin *et al.*, 2006). After volatilization, TMA is readily oxidized to TMAO in the atmosphere, which is thought to be almost nontoxic (Bhattacharya *et al.*, 2007). Besides a wide range of bacteria (e.g. members of the genera *Enterobacter*, *Pseudomonas*, *Methanobacterium* and *Bacillus*) with the capable of arsenic methylation (Cullen and Reimer, 1989), a recent study also suggested that methanogenic Archaea are able to methylate arsenic due to side reactions between central methanogenic cofactors, methylcobalamin (Wuerful *et al.*, 2012).

In addition, strain ASV2, an unidentified Gram-negative bacterium, was found to utilize arsonoacetate or arsonochloroacetate as sole carbon and energy sources. The final product in this case is likely to be arsenite, because an inducible arsenite-oxidizing activity was found in arsonoacetate-metabolizing cells (Quinn and McMullan, 1995). Aerobic conditions can promote demethylation of monomethylarsenate and dimethylarsenate to arsenate (Shimizu *et al.*, 2011). However, anaerobic conditions can promote either methylation or demethylation of MMAA and DMAA (Gao and Burau, 1997; Sierra-Alvarez *et al.*, 2006).

Arsenic methylation by fungi and other eukaryotes has also been well-proved. As early as in 1893, Gosio had found that fungal metabolism linked to the formation of volatile arsenic (Gosio, 1893), which of this was correctly identified as TMA by

Challenger *et al.* (1933). In higher eukaryotes, glutathione reduces As (V) to As (III), which then accepts a methyl group from *S*-adenosylmethionine, producing MMAA or DMAA (National Research Council, 1999).

Arsenic methylation occurs not only in soil profiles but also in aqueous systems. Studies on hydrothermal systems have identified the evolution of arsenic gases (Planar-Friedrich *et al.*, 2006a, b). Planar-Friedrich *et al.* (2006b) identified TMA outgassing from geothermal waters at Yellowstone National Park, along with Cl and S methylated analogues ( $(\text{CH}_3)_2\text{AsCl}$ ,  $(\text{CH}_3)_2\text{AsSCH}_3$ , and  $\text{CH}_3\text{AsCl}_2$ ). The recent work from Cervini-Silva *et al.* (2010) also provides evidence to show non-enzymatic methylation of arsenic in betaine-nontronite clay-water suspensions at ambient temperature, producing MMA (III) and MMAA.

Whether higher plants are able to methylate arsenic is an important question with regard to the arsenic biogeochemical cycle, and is also highly relevant to the development of strategies to minimize arsenic contamination in the food chain (Lomax *et al.*, 2011). Lomax *et al.* (2011) reported that plants are unable to methylate inorganic arsenic, and instead take up methylated arsenic produced by microorganisms. Furthermore, Jia *et al.* (2012) also proved that addition of dried distillers grain (DDG) to the soil enhanced the uptake of different arsenic species and volatilization from the rice plants, although the rice plants lacked the arsenic methylation ability.

Although the exact mechanism of arsenic methylation is still unclear, two recognized mechanisms favor reduced conditions for methylation to occur. One is described in the early study, pentavalent species have to be reduced at first to undergo further oxidative methylation (Challenger *et al.*, 1945). Reduced species, such as As (III) or MMA (III), are more unstable than oxidized species, such as As (V) or MMA A, under normal conditions and can be easily oxidized (Gong *et al.*, 2001). Recently, the other one has been proposed by Hayakawa *et al.* (2005), who considered the methylation process requires reduced glutathione, which is more stable under reduced conditions.

### **I-3-3 Transformation of CLARKs**

The variety of organoarsenicals in the environment is wide and one group of arsenic containing compounds is characterized by a phenylic moiety originating from synthetic arsenic compounds such as chemical warfare agents, CLARK I and CLARK II. Most of them were dumped into the sea or buried in the earth in several parts of Europe, China and Japan after the World wars (Bunnett and Mikolajczyk, 1998; Daus *et al.*, 2008; Stock and Lohs, 1997) and caused environmental contamination.

These agents can be metabolized in groundwater, soils and sediments via hydrolysis and oxidation (Haas *et al.*, 1998). Consequently, the remained compounds were often detected around the dumped sites, such as PAA, PAO, DPAA and so on (**Fig. 1**).

### **I-3-4 Transformation of DPAA**

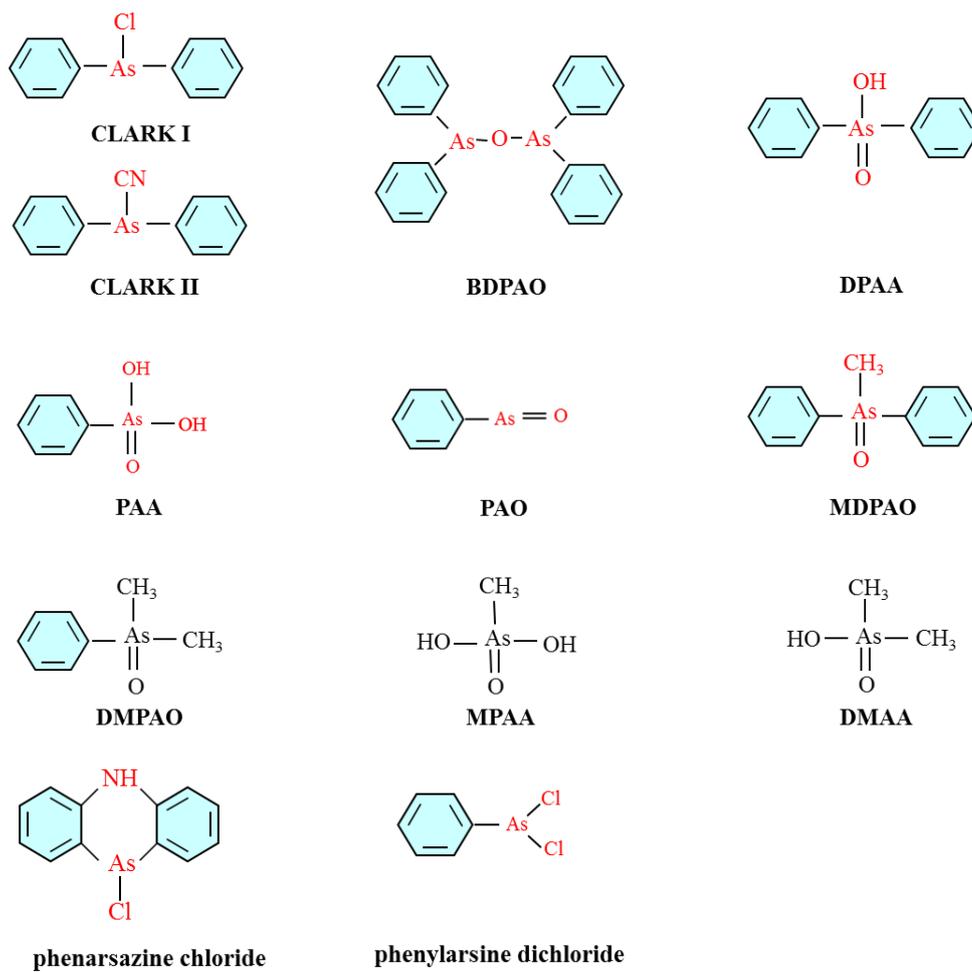
The analysis of chemical weapons and their metabolites have been gaining in scientific interests in recent years. The first case of poisoning of inhabitants by organic arsenic compounds in drinking well water in Kamisu town of Japan, were reported by Ishizaki *et al.* (2005). They demonstrated that BDPAO, DPAA and PAA were using HPLC, GC/MS and HPLC-ICPMS. According to Haas *et al.* (1998) description, it is known that BDPAO was a breakdown product of the chemical weapons, CLARK I and CLARK II. In addition, BDPAO could be further hydrolyzed into DPAA. In Germany, the groundwater polluted by warfare agents was often investigated with respect to the behavior of relevant arsenic species. Daus *et al.* (2010) analyzed the arsenic species occurring in the contaminated aquifer at depth profiles. Besides the founding of arsenate, arsenite, PAA and PAO, they have described that the occurrence of MPAA in the ground water was a clear indication of high microbial activity in the polluted environment.

The behavior of aromatic arsenicals in soils and plants has been reported. DPAA in the soil can be transformed into PAA and MPAA via dephenylation and methylation

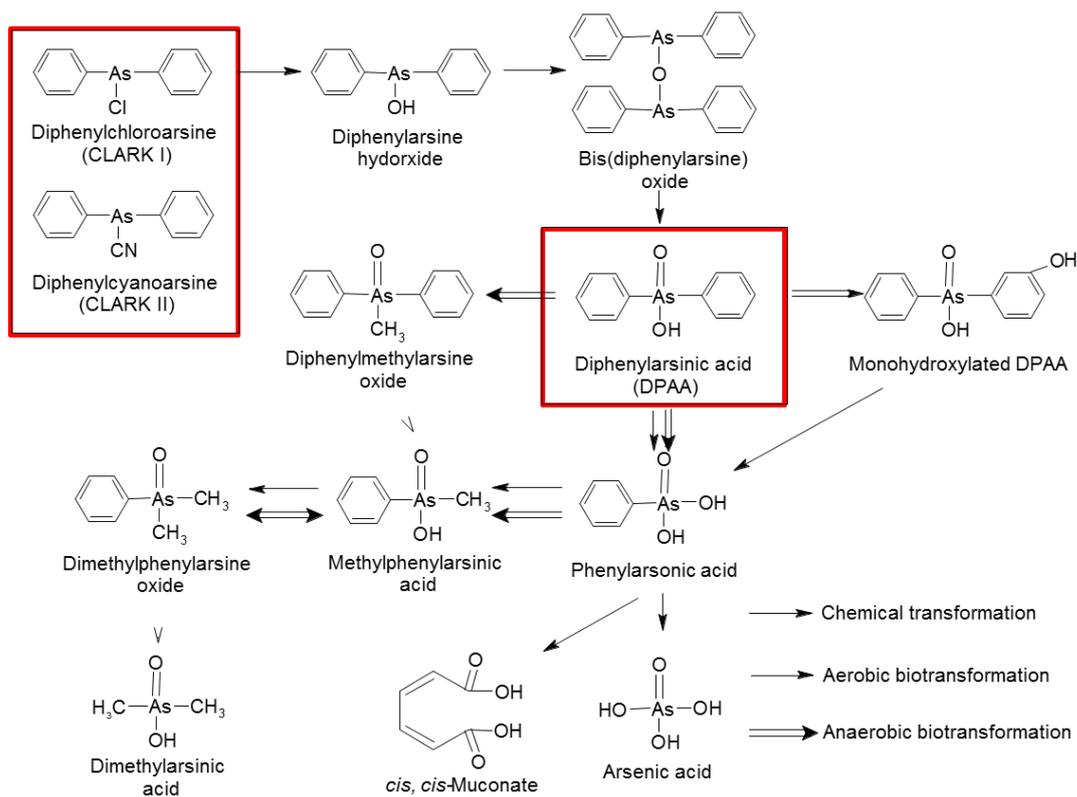
under flooded conditions. MPAA was further demethylated to DMPAO or dephenylated to MMAA. In addition, DPAA can directly methylated to MDPAO (Arao *et al.*, 2009). Maejima *et al.* (2011) reported the occurrence of PAA, MPAA and DMPAO in the soil amended with DPAA under aerobic condition. DPAA and MPAA were also detected in the paddy rice cultivated in the paddy fields that were irrigated with the DPAA-contaminated groundwater. Arao *et al.* (2009) also investigated the uptake of aromatic arsenicals in agricultural soils by rice and reported that DPAA and MPAA were not methylated in the rice plant but were methylated in the soil under anaerobic conditions.

The microbial degradation of aromatic arsenicals was first reported by Köhler *et al.* (2001), who described how bacteria play an important role in the release of arsenical compounds from the contaminated soil. These bacteria slowly degrade the various diphenyl and phenyl arsenical contaminated soil, and PAA is produced from TPAO. Nakamiya *et al.* (2007) tried to isolate DPAA-degrading microorganisms from toluene-utilizing ones and obtained *Kytococcus sedentarius* strain NK0508 that can transform DPAA to *cis, cis*-muconate and arsenic acid. Harada *et al.* (2010) reported the isolation of two novel *Ensifer-Sinorhizobium* strains (L2406 and L2413) capable of degrading DPAA using the soil-charcoal perfusion method with a mineral salt medium containing DPAA as the sole carbon source. These reports suggest an aerobic metabolic pathway of organoarsenical transformation.

According to the mentioned metabolism of DPAA, the proposed pathway for the degradation of DPAA in the environment was listed in **Fig. 2**.



**Fig. 1. Typical organoarsenic compounds from chemical weapons**



**Fig. 2. Summary of the known metabolic pathway of phenylarsenicals**

## **I-4 Impacts of arsenicals on microbial diversity**

### **I-4-1 Inorganic arsenicals**

With the acceleration of anthropogenic activities such as industrial development, heavy metal contamination has received more and more attention in the soils. Heavy metals affected the growth, morphology and metabolism of microorganisms in soils, through functional disturbance, protein denaturation or the destruction of the integrity of cell membranes (Leita *et al.*, 1995). On the other hand, many bacteria have evolved mechanisms enabling them to cope with high arsenic concentrations and some bacteria are able to use arsenic as either an electron donor or an electron acceptor, altering the redox state of arsenic, which has become one possible method in bioremediation strategies. Therefore, the relationships between soil arsenic and bacterial function and composition are important in soil microbiology.

Several attempts have been made to elucidate the effects of arsenic pollution on the composition of the soil microbial community. Maliszewska *et al.* (1985) found that arsenate stimulated the proliferation of certain groups of microorganisms in soil resulting in a shift of the community to only a few tolerant species. In recent years, molecular ecological methods, for example RFLP, FISH, DGGE etc., have been widely used for analyzing microbial community structures in various environments. Turpeinen *et al.* (2004) found a decrease in microbial diversity was observed at the wood impregnating plant with the highest level of chromated-copper-arsenate (CCA) contamination, which was closed down only two years prior to sampling. Lorenz *et al.* (2006) investigated the response of microbial activity and microbial community composition in soils affected by long-term arsenic exposure. They observed clear differences in the PCR-DGGE band patterns between a long-term As-contaminated soil and the control. More recently, Somenahally *et al.* (2011), using community quantitative-PCR and 16S rRNA gene sequencing, reported that bacterial community structure and composition in the rice rhizosphere were significantly influenced by long-term application of monosodium methylarsenate.

## **I-4-2 Phenylarsenicals**

An example investigating changes of microbial diversity caused by a phenylarsenical compound has been reported by Jiang *et al.* (2013). They found the active members of the microbial community under roxarsone (3-nitro-4-hydroxyphenylarsonic acid) stress were apparently decreased, which is almost the same as the blank sample received only distilled water without any nutrient or roxarsone. However, no report regarding microbial impacts of other phenylarsenicals has been available, as far as we know.

## **I-5 Objective of this study**

We have preliminary results that transformation of DPAA is likely to be accelerated under anaerobic conditions by flooding contaminated soil after amendment with easily-decomposable organic materials.

In this study, the following investigation was performed to determine factors, which relates to anaerobic biotransformation of phenylarsenicals in soil and identify microorganisms which participate in this process.

### *(1) Effects of DPAA on bacterial and archaeal community structures in anaerobic soil cultures*

We assessed changes in bacterial and archaeal 16S rRNA-targeted PCR-DGGE fingerprints derived from the DNA extracted from the contaminated soil. This study was conducted, since it has recently become evident that, in addition to bacteria, archaea are ubiquitous and abundant organisms in soils, especially in paddy soils (Buckley *et al.*, 1998; Hansel *et al.*, 2008; Kemnitz *et al.*, 2007).

### *(2) Enhanced transformation of DPAA in soil under sulfate-reducing condition*

We focused on environmental behavior of DPAA in a contaminated soil under anaerobic conditions. We performed experimental model studies, aiming to find the key factors affecting anaerobic transformation of DPAA.

### *(3) Identification of microorganisms participating in the transformation process of DPAA*

We attempted to identify and isolate the sulfate-reducing consortia on DPAA transformation by the limiting dilution-culture method.

**Chapter II. Effects of DPAA on bacterial  
and archaeal community structures  
in an anaerobic paddy soil**

## II-1 Introduction

Arsenic-based chemical warfare agents, such as CLARK I and CLARK II, were produced and discarded in several parts of Europe, China, Japan and other countries during the World Wars. They are known to cause water and soil contamination around the dump sites (Kurata, 1980; Haas *et al.*, 1998). It is known that CLARK I and CLARK II can be chemically transformed to DPAA via hydrolysis and oxidation in the environment (Hass *et al.*, 1998). Consequently, DPAA is often detected as one of the major organoarsenicals around contaminated sites.

Soil contamination by phenylarsenicals has sometimes been found in Japan. In 2003, prominent cerebellar symptoms attributed to DPAA-contaminated well water used for drinking were observed in several inhabitants in the Kizaki region of Kamisu City, Ibaraki, Japan (Ishii *et al.*, 2004). The environmental contamination by DPAA was attributed to the illegal dumping of DPAA itself. In this Kizaki region, DPAA and MPAA were detected in rice harvested from paddy fields that had been irrigated with the contaminated groundwater (Ministry of the Environment, Government of Japan, 2005). DPAA was also detected in the groundwater used for irrigation of the paddy fields, and PAA and BDPAO were present at much lower concentrations (Ishizaki *et al.*, 2005). According to Baba *et al.* (2008), arsenical species determined in contaminated paddy field soil samples collected in the Kizaki region were DPAA, PAA, MPAA, DMPAO and DPMAO along with inorganic arsenicals as the main species.

Currently, there is a considerable scientific interest to investigate the interaction between organic pollutants and soil microorganisms. Using signature biomarkers such as nucleic acids and fatty acids, many studies have revealed that organic contaminants can cause a shift within the soil microbial community. For example, Tu *et al.* (2011) used PCR-DGGE analyses and found that dominant bacteria in alfalfa-planted field soils contaminated with polychlorinated biphenyls were *Actinobacteria* and *Chloroflexi*, which are ubiquitously well-known biphenyl-degrading bacteria in the soil (Bedard, 2008; Correa *et al.*, 2010). Ding *et al.* (2012) described that bacterial

richness and evenness decreased in soils spiked with polycyclic aromatic hydrocarbons (PAH).

However, little information is available about the effect of organic arsenicals on soil microbial communities and microbial transformation of aromatic arsenicals. Köhler *et al.* (2001) isolated several bacterial strains capable of degrading triphenylarsine and triphenylarsine oxide from soils contaminated with organoarsenic warfare compounds. Nakamiya *et al.* (2007) tried to isolate DPAA-degrading microorganisms from toluene-utilizing ones and obtained *Kytococcus sedentarius* strain NK0508. Harada *et al.* (2010) reported the isolation of two novel *Ensifer* strains (L2406 and L2413) capable of degrading DPAA using the soil-charcoal perfusion method with a mineral salt medium containing DPAA as the sole carbon source. These reports suggest an aerobic metabolic pathway of organoarsenical transformation. However, to the best of our knowledge, the effects of contamination with organoarsenicals on whole-soil microbial communities have not yet been explored.

Therefore, the present study aimed to investigate the alteration of microbial community structures in an anaerobic paddy soil artificially contaminated with DPAA in a model experiment. We assessed changes in bacterial and archaeal 16S rRNA-targeted PCR-DGGE fingerprints derived from the DNA extracted from the contaminated soil, since it has recently become evident that, in addition to bacteria, archaea are ubiquitous and abundant organisms in soils, especially in paddy soils (Buckley *et al.*, 1998; Hansel *et al.*, 2008; Kemnitz *et al.*, 2007).

## **II-2 Materials and methods**

### **II-2-1 Chemicals**

The following chemicals were used: disodium hydrogenarsenate heptahydrate ( $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ ), PAA, DPAA, phosphoric acid and formic acid (Wako, Osaka, Japan); MPAA and DMPAO (Hayashi Pure Chemical, Osaka, Japan); methanol (Junsei, Tokyo, Japan); acetonitrile (Sigma-Aldrich, St. Louis, MO, USA).

### **II-2-2 Soil samples**

Three healthy surface soil samples (0–10 cm) were collected from the plow layer of paddy field in Niigata, Japan: Gleysol samples from the Shindori Station of the Field Center for Sustainable Agriculture and Forestry, Niigata University (N37.86, E138.96), Niigata City (collected in 2007 and 2010, Shindori soil) and Okidate (N37.16, E138.75) in Tokamachi City (collected in 2007, Okidate soil), and an Andosol sample from Noguchi (N37.21, E138.75) in Tokamachi City (collected in 2007, Noguchi soil). After air-drying, the soil samples were sieved ( $< 2$  mm) and stored in polyethylene bags at ambient temperature. The Shindori soil samples collected in 2007 and 2010 had the following characteristics: pH ( $\text{H}_2\text{O}$ ); 5.18 and 4.83, pH (KCl); 3.98 and 3.94, total carbon; 14.6 and 23.8 mg (g dry soil (gds))<sup>-1</sup>, total nitrogen; 1.35 and 2.61 mg gds<sup>-1</sup>, C/N ratio; 10.8 and 9.10, total arsenicals; 7.05 and 10.1  $\mu\text{g-As gds}^{-1}$ , respectively. The Okidate and Noguchi soil characteristics included: pH ( $\text{H}_2\text{O}$ ); 5.19 and 5.92, pH (KCl); 4.12 and 4.99, total carbon; 10.9 and 60.1 mg gds<sup>-1</sup>, total nitrogen; 1.19 and 3.60 mg gds<sup>-1</sup>, C/N ratio; 9.12 and 16.9, respectively.

### **II-2-3 Rice straw samples**

Rice straw samples were collected from the above-mentioned rice field at harvest in

2007. The samples were stored at ambient temperature after drying. The leaf sheath was used after powdering. The carbon and nitrogen contents of the rice straw were  $351 \text{ mg-C g}^{-1}$  and  $4.6 \text{ mg-N g}^{-1}$ , respectively.

#### **II-2-4 Soil cultures**

The soil cultures used were: control soil without DPAA and rice straw (treatment C); soil with addition of  $10.73 \text{ }\mu\text{g-As gds}^{-1}$  as DPAA (treatment D); soil with addition of  $10 \text{ mg gds}^{-1}$  of rice straw (treatment R); soil with addition of  $10.73 \text{ }\mu\text{g-As gds}^{-1}$  as DPAA and  $10 \text{ mg gds}^{-1}$  of rice straw (treatment DR). The basic soil culture contained 20 g of dry soil with 30 mL of deionized water in a 100 mL Erlenmeyer flask closed with a double rubber plug. The soil cultures were incubated for a total of 5 weeks at  $30 \text{ }^\circ\text{C}$  in the dark in duplicate.

#### **II-2-5 Determination of DPAA concentration**

Two ml of 16 M NaOH was added to each soil culture. After mixing with a vortex mixer for 30 s, aliquots (2 mL) of the soil suspension were centrifuged (10,000 rpm) for 5 min, and 0.5 mL of supernatant was obtained. After neutralization by the addition of 0.5 mL of 1 M HNO<sub>3</sub>, centrifugation (10,000 rpm, 5 min) was performed again. The supernatant was filtered through a 0.45  $\mu\text{m}$  membrane (Toyo Roshi Kaisha, Tokyo, Japan.) and analyzed with a HPLC (Waters, MA, USA) equipped with a reversed-phase column (CAPCELL-PAK C18 MG, 5  $\mu\text{m}$ , 4.6 mm i.d.  $\times$  250 mm, Shiseido, Tokyo, Japan) at  $40 \text{ }^\circ\text{C}$ . The mobile phase comprised 0.2% (v/v) H<sub>3</sub>PO<sub>4</sub> and CH<sub>3</sub>CN (75:25) with a flow rate of  $1 \text{ mL min}^{-1}$ . The injection volume was 10  $\mu\text{L}$ . For the UV detector, the measurement wavelength was 220 nm. The limit of detection of DPAA for HPLC was 1 ng.

## II-2-6 Estimation of metabolites

An ICPMS instrument (X series 2, Thermo Fisher Scientific, MA, USA) linked to a HPLC (Prominence series, Shimadzu, Kyoto, Japan) was used for estimation of metabolites of DPAA.

A reversed-phase column (SPELCO Discovery C18, 5  $\mu\text{m}$ , 4.6 $\times$ 250 mm, Sigma-Aldrich) was used for separation. The injection volume was 100  $\mu\text{L}$  and the mobile-phase flow rate was set at 1  $\text{mL min}^{-1}$ . A gradient program was used. Solvent A was purified water containing 0.1% formic acid, and solvent B was methanol containing 0.1% formic acid. The elution gradient was set as follows: 0–3.5 min 1% B, 3.5–6 min 1–25% B, 6–13 min 25% B, 13–17 min 25–70% B, 17–22 min 70% B, and 22–35 min 1% B. The fifth part of the eluate (0.2  $\text{mL min}^{-1}$ ) was separated by a flow splitter and directly introduced into the inert PTFE nebulizer of the ICPMS. The ICPMS was configured with the X series II organics kits, and a nickel sampler and skimmer cones. The operating parameters of the ICPMS are shown in **Table 1**. Concentrations of organoarsenicals were monitored as the  $^{75}\text{As}$  ion and logged by PlasmaLab Ver. 2.5.9.300, and the quantifications were carried out using the external standard method for each retention time and peak area using commercially available standards.

## II-2-7 PCR amplification of the 16S rRNA gene

After 1-, 3- and 5-weeks of incubation, soil DNA was extracted from the soil cultures using ISOIL for Beads Beating (Nippongene, Tokyo, Japan) according to the manufacturer's instructions. Extracted soil DNA was used for the PCR run in a TaKaRa RCR Thermal Cycler Dice (Otsu, Japan) in 0.2 mL tubes using 25  $\mu\text{L}$  reaction volumes. PCR for bacterial 16S rDNA genes was performed using the primer set, F984–968GC (5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC -3') and R1378–1401 (5'-CGG TGT GTA CAA GGC CCG GGA ACG-3') (Heuer *et al.*, 1997). Archaeal 16S rDNA

genes were amplified using the primer set, A0344f–0325 (5'ACG GGG YGC AGC AGG CGC GA–3') and A915r–934GC (5'–CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CGT GCT CCC CCG CCA ATT CCT –3') (Casamayor *et al.*, 2002). The reaction mixture for PCR contained 1  $\mu$ L of template DNA, 2.5  $\mu$ L of 10 $\times$ buffer, 2.5  $\mu$ L of 2 mM dNTPs, 1/0.8  $\mu$ L of 25 mM MgSO<sub>4</sub> (for bacteria/archaea, respectively), 0.75  $\mu$ L of each primer (10  $\mu$ M), 0.5 U of KOD–Plus–Ver.2 polymerase (Toyobo, Osaka, Japan) and 0.5  $\mu$ L of 20 mg mL<sup>–1</sup> bovine serum albumin. The amplification was carried out using 40/30 (for bacteria/archaea, respectively) cycles including denaturation at 98  $^{\circ}$ C for 10 s, renaturation at 55/58  $^{\circ}$ C (for bacteria/archaea, respectively) for 30 s, extension at 68  $^{\circ}$ C for 30 s and the final extension at 68  $^{\circ}$ C for 7 min. An aliquot (5  $\mu$ L) of PCR products was subjected to electrophoresis at 100 V for 20–30 min on 1% (w/v) agarose gel stained with ethidium bromide to confirm DNA amplification. PCR products were purified with a High Pure PCR Product purification Kit (Roche Applied Science, Mannheim, Germany).

## **II–2–8 DGGE analysis**

DGGE was performed using a DCode Universal Mutation Detection System (Bio–Rad Laboratories, Hercules, USA). Aliquots of the PCR products (100 ng) were separated on a 6% polyacrylamide gel with a linear denaturant concentration from 50 to 70%. The gels were subjected to electrophoresis for 18 h at 58  $^{\circ}$ C at 50 V, stained for 30 min with SYBR Gold nucleic acid, and then immediately photographed using a LAS–3000 lumino image analyzer (Fujifilm, Tokyo, Japan).

Selected characteristic bands appearing on the gels were excised and transferred into microtubes containing sterile water. The DNA eluted from the excised bands was then re–amplified under the PCR conditions described above and the nucleotide sequences were determined by the Fasmac DNA sequencing service (Atsugi, Japan). When needed, sequences of the DNAs were determined after cloning with pGEM–T

Easy Vector System (Promega, Madison, USA) according to the manufacturer's instructions. Plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). The sequence results were compared with those available in the GenBank database by the BLAST search engine excluding uncultured and environmental sample sequences.

### **II-2-9 Statistical analysis**

To estimate the variation of microbial communities in the soils treated with DPAA, the bacterial and archaeal DGGE band patterns obtained from the 5-week soil cultures were analyzed by cluster analysis. Each band that migrated to the same position within each gel was numbered. Band strengths were estimated by Image-J 1.44o and relative band intensities were obtained. Weak bands were assigned a value of 1, intermediate bands a value of 2 and strong bands a value of 3. Cluster analysis using Ward's method was performed using the statistical software KyPlot version 2.0 beta 15.

### **II-2-10 Nucleotide sequence accession numbers**

The sequences obtained in this study have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB700477 to AB700549.

**Table 1. Operating conditions of LC-ICPMS for analysis of organic arsenicals**

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Forward plasma power	1.4 kW
Nebulizer gas flow	0.83 L min <sup>-1</sup>
Auxiliary gas flow	0.8 L min <sup>-1</sup>
Cool gas flow	13.0 L min <sup>-1</sup>
CCT gas composition and flow	H <sub>2</sub> /He (8.16% H <sub>2</sub> ), 7 mL min <sup>-1</sup>
Ar-O <sub>2</sub> gas composition and flow	19.8% O <sub>2</sub> , 61 mL min <sup>-1</sup>
Data acquisition mode	Transient time resolved acquisition (TRA)
Dwell Times	100 ms
Timeslice duration	100 ms
Channels per AMU	1
Run duration	2,050 s

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## II-3 Results

### II-3-1 Degradation of DPAA

First, we prepared soil cultures artificially contaminated with DPAA using Noguchi, Okidate and Shindori soils and compared DPAA concentration in them after 1- and 2-week incubation. As a result, the soil cultures incubated without rice straw (treatment D) showed slow decrease of DPAA concentration (**Table 2**). When rice straw was added (treatment DR), decrease of DPAA was significantly enhanced regardless of the soil types. Among the three soil samples examined in this study, Shindori soil showed most significant response to the rice straw application regarding DPAA concentration under submerged conditions (**Table 2**). We, therefore, selected Shindori soil for further investigation.

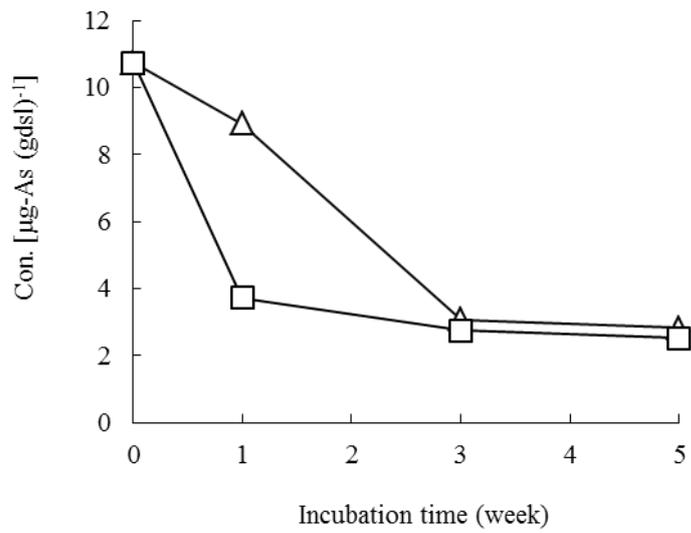
Effects of longer incubation period on a fate of DPAA were, then, examined using Shindori soil. Changes in DPAA concentrations in the soil cultures with and without rice straw (treatments DR and D, respectively) are shown in **Fig. 3**. Application of rice straw led to a rapid decrease in the DPAA concentration from 10.73 to 3.73  $\mu\text{g-As gds}^{-1}$  within the first week of the incubation period, whereas only a small reduction to 8.92  $\mu\text{g-As gds}^{-1}$  was observed in the soil cultures without rice straw. However, the degradation rates of DPAA in the soil cultures without rice straw subsequently accelerated, and the DPAA concentration in the soil cultures was similar to that in the soil cultures with rice straw after three weeks of incubation. The disappearance of DPAA in the soil cultures was suppressed by autoclaving before incubation (data not shown), suggesting that DPAA degradation in the soil cultures was associated with the presence of soil organisms.

**Table 2. Comparison of DPAA concentration in experimental soil cultures incubated under submerged conditions without and with rice straw (Treatment D and DR, respectively)<sup>¶</sup>**

Soil	DPAA concentration ( $\mu\text{g-As gds}^{-1}$ )	
	After 1 week	After 2 weeks
Noguchi (Andosol)		
-Treatment D	10.25 $\pm$ 0.27 (96)	7.89 $\pm$ 0.41 (74)
-Treatment DR	8.25 $\pm$ 0.38** (77)	3.78 $\pm$ 0.44** (35)
Okidate (Gleysol)		
-Treatment D	10.16 $\pm$ 0.38 (95)	9.14 $\pm$ 0.41 (85)
-Treatment DR	8.54 $\pm$ 0.34** (80)	3.54 $\pm$ 0.42*** (33)
Shindori (Gleysol)		
-Treatment D	10.45 $\pm$ 0.38 (98)	9.81 $\pm$ 0.28 (92)
-Treatment DR	5.87 $\pm$ 0.41*** (55)	1.17 $\pm$ 0.39*** (11)

<sup>¶</sup> Mean  $\pm$  1 S.D. (n=3), figures in parentheses are relative values to the initial concentration ( $10.73 \mu\text{g-As gds}^{-1}$ ).

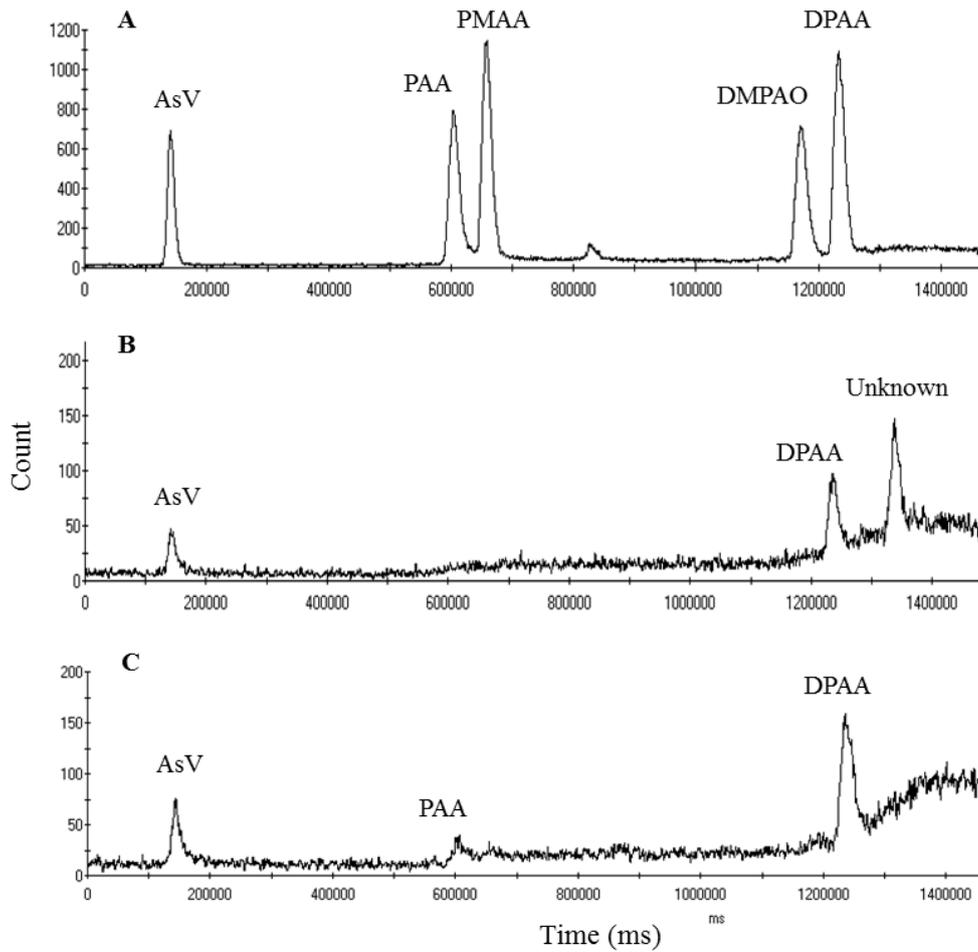
\*\*, \*\*\* Significant difference from that in the samples incubated without rice straw was found by Student's t-test at  $\alpha=0.01$  and  $0.001$ , respectively.



**Fig. 3. Residual concentration of DPAA in experimental soil cultures with (open squares) and without rice straw (open triangles).**

### **II-3-2 Metabolites of DPAA**

Arsenical metabolites of DPAA in the soil cultures incubated with rice straw were determined with LC-ICPMS (**Fig. 4**). As shown in **Fig. 4B**, inorganic arsenic acid, DPAA and one unknown peak were the main components detected in the soil culture after 1-week of incubation. After 3 weeks of incubation, the unknown component disappeared, and inorganic arsenic acid and DPAA were observed together with PAA (**Fig. 4C**). Arsenical metabolites in the soil cultures incubated without rice straw were similar (data not shown).



**Fig. 4. HPLC–ICPMS chromatograms of a standard solution containing 10  $\mu\text{g L}^{-1}$  of each of arsenic acid (As (V)), PAA, PMAA, DMPAO and DPAA (A), and sample solutions extracted from soil cultures in treatment DR after incubation for 1 (B) and 3 (C) weeks.**

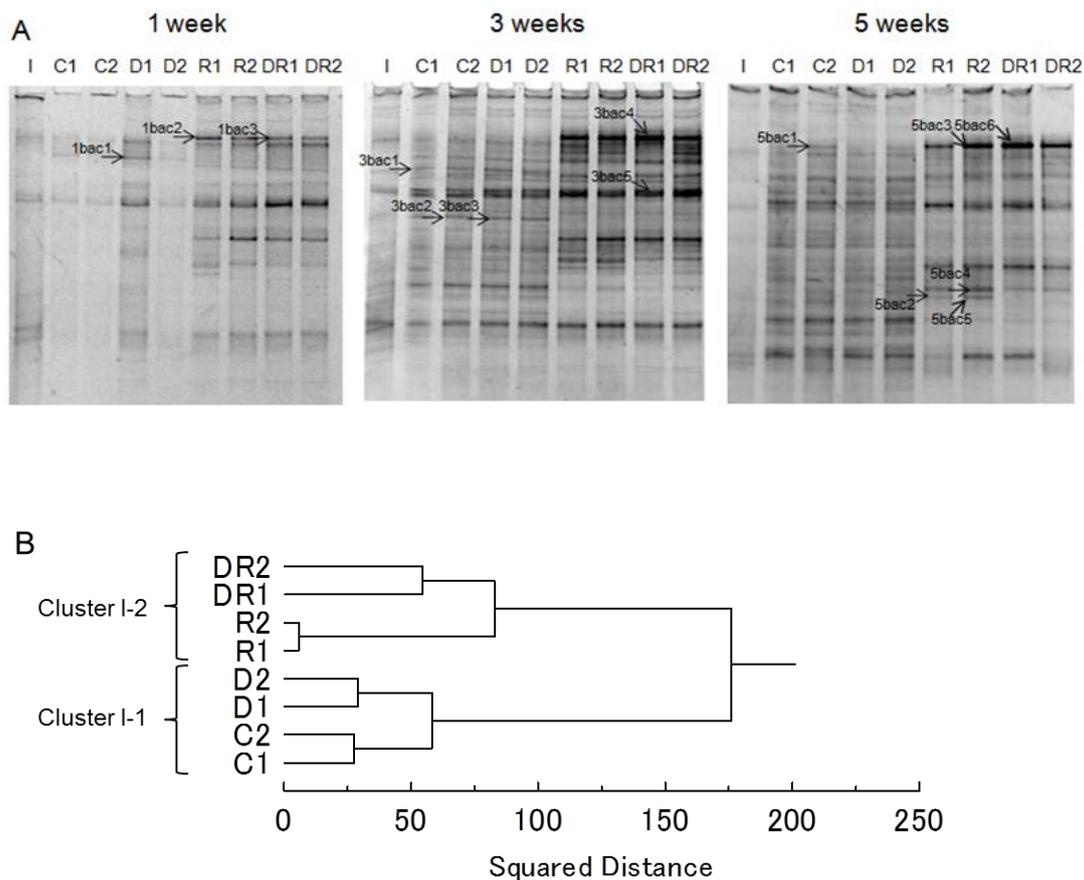
### II-3-3 Impact of DPAA on bacterial community structures

The DNA fingerprints of bacterial populations in the soil cultures incubated with and without DPAA were investigated using PCR–DGGE (**Fig. 5A**). The intensities of some major bands found in the initial soil sample were enhanced after 1 week of incubation, when rice straw was not applied. However, profiles of the PCR–DGGE fingerprints obtained from the incubated soil cultures changed in a similar way regardless of DPAA application. When rice straw was applied, differences in the PCR–DGGE fingerprints between the initial soil sample and the incubated soil cultures were more obvious. Intensities of a few bands changed due to DPAA application, as bands 5bac5 and 5bac6 disappeared and the intensity of band 5bac4 decreased in the DR treatment after 5 weeks of incubation. The other bands showed no significant changes.

Cluster analysis identified two clusters, Cluster I-1 and Cluster I-2, for bacterial DGGE band patterns in soil cultures obtained from the 5-week incubation (**Fig. 5B**). Cluster I-1 consists of the soil cultures from the control and D treatments. The other soil cultures incubated with rice straw were in Cluster I-2. Each cluster could be divided into sub-clusters, showing the effects of DPAA addition on the bacterial communities.

Sequencing results of the excised DGGE bands are shown in **Table 3**. The DGGE bands 1bac2, 1bac3, 3bac4, 5bac3 and 5bac6 were assigned to *Clostridium* species in Firmicutes. The DGGE bands 1bac1, 3bac1, 3bac5 and 5bac1 were also assigned to Firmicutes species, even though the similarities were relatively low (< 93%). The closest relatives of the DGGE bands 3bac2 and 3bac3 were the unidentified species isolated from an anoxic rice paddy soil (Hengstmann *et al.*, 1999). The other DGGE bands could not be sequenced directly due to overlapping, since a single DGGE band frequently represents several bacterial species (Sekiguchi *et al.*, 2001). Subsequently, the sequences of DGGE bands 5bac2, 5bac4 and 5bac5, which were affected by addition of DPAA, were determined after TA cloning. Six clones were obtained from each of the DGGE bands 5bac2 and 5bac4, and three clones from the DGGE band

5bac5. Plural clones of them showed the highest similarities with *bacterium Ellin7522*. The other clones had relatively high similarities with Acidobacteria, Bacteroidetes, Chloroflexi and Firmicutes.



**Fig. 5. DNA-based DGGE patterns of bacterial communities in the soil cultures during the examination period (A) and the dendrogram obtained after cluster analysis of the DGGE band patterns for the soil cultures after a 5-week incubation (B): I=initial soil, C=control without DPAA and rice straw (treatment C), D=incubated with DPAA (treatment D), R=incubated with rice straw (without DPAA, treatment R), and DR=incubated with DPAA and rice straw (treatment DR). Numbers after the abbreviations refer to duplication of the community analyses. The bands 5bac2, 5bac4 and 5bac5 were those that showed significant decreases in their intensities due to addition of DPAA.**

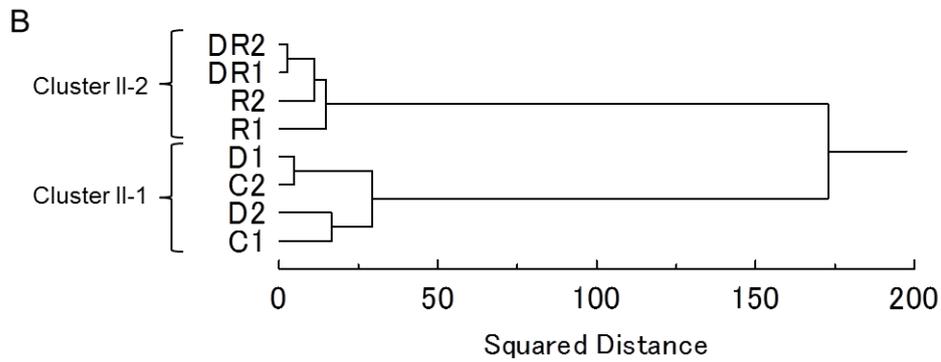
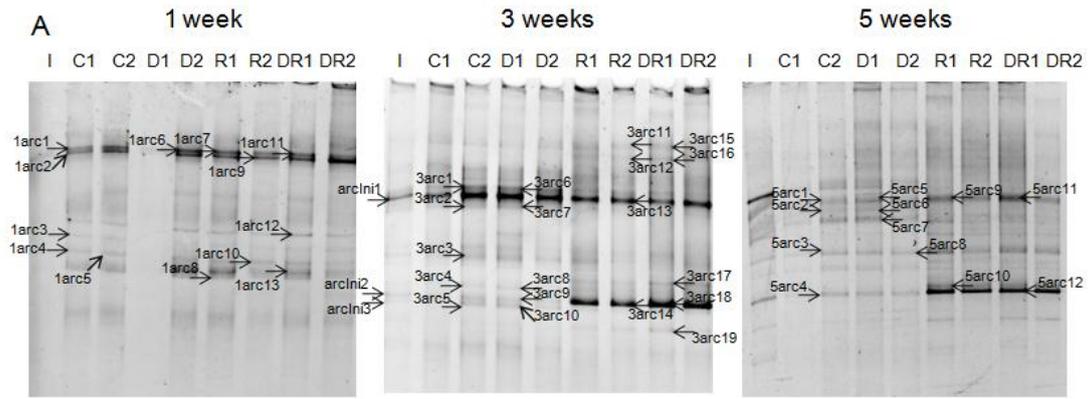
**Table 3. Closest relatives of bacterial 16S rDNA-based DGGE bands.**

Band (accession no.)	Closest relative (accession no.)	Phylum	Alignment	Homology (%)	Reference	Source
1bac1 (AB700477)	<i>Ammoniphilus oxalaticus</i> (Y14579)	Firmicutes	335/368	91	Zaitsev et al. (1998)	
1bac2 (AB700478)	<i>Clostridium</i> sp. P4-4 (FJ848367)	Firmicutes	353/362	98		peat
1bac3 (AB700479)	<i>Clostridium</i> sp. BNL1100 (CP003259)	Firmicutes	320/350	91		
3bac1 (AB700480)	<i>Ammoniphilus oxalaticus</i> RAOx-FF (Y14579)	Firmicutes	332/356	93	Zaitsev et al. (1998)	
3bac2 (AB700481)	Unidentified eubacterium from anoxic bulk soil (AJ229196)		337/358	94	Hengstmann et al. (1999)	flooded rice paddy soil
3bac3 (AB700482)	Unidentified eubacterium from anoxic bulk soil (AJ229196)		329/358	92	Hengstmann et al. (1999)	flooded rice paddy soil
3bac4 (AB700483)	<i>Clostridium</i> sp. BNL1100 (CP003259)	Firmicutes	356/357	99		
3bac5 (AB700484)	<i>Ruminococcaceae</i> bacterium HZ254R (JN656278)	Firmicutes	296/333	88		rice paddy soil
5bac1 (AB700485)	<i>Gracilibacter thermotolerans</i> JW/YJL-S1 (DQ117468)	Firmicutes	314/346	91	Lee et al. (2006)	wetland
5bac2-1* (AB700486)	Bacterium Ellin7522 (HM748732)	Acidobacteria	375/393	95	Davis et al. (2011)	soil
5bac2-2* (AB700487)	Bacterium Ellin7522 (HM748732)	Acidobacteria	374/393	95	Davis et al. (2011)	soil
5bac2-3* (AB700488)	Bacterium Ellin7522 (HM748732)	Acidobacteria	370/393	94	Davis et al. (2011)	soil
5bac2-4* (AB700489)	<i>Rhodothermus marinus</i> mm-13 (EU652067)	Bacteroidetes	342/396	86		hotspring water
5bac2-5* (AB700490)	Unidentified eubacterium clone BSV28 (AJ229190)		304/333	91	Hengstmann et al. (1999)	flooded rice paddy soil
5bac2-6* (AB700491)	<i>Streptomyces galbus</i> strain NBRC 12864 (AB184201)	Actinobacteria	400/400	100		
5bac3 (AB700492)	<i>Clostridium</i> sp. BNL1100 (CP003259)	Firmicutes	344/349	99		
5bac4-1* (AB700493)	Bacterium Ellin7522 (HM748732)	Acidobacteria	399/420	95	Davis et al. (2011)	soil
5bac4-2* (AB700494)	Bacterium Ellin7522 (HM748732)	Acidobacteria	399/420	95	Davis et al. (2011)	soil
5bac4-3* (AB700495)	<i>Anaerolinea thermolimos</i> IMO-1 (NR_040970)	Chloroflexi	358/409	88	Yamada et al. (2005)	sludge granules
5bac4-4* (AB700496)	<i>Clostridium</i> sp. 6-31 (FJ808611)	Firmicutes	377/421	90	Sizova et al. (2011)	compost
5bac4-5* (AB700497)	Firmicutes bacterium LX-B (AB669474)	Firmicutes	382/427	89		anaerobic sludge
5bac4-6* (AB700498)	<i>Bacillus</i> sp. NBK37 (GQ980246)	Firmicutes	325/346	94	Yadav et al. (2011)	soil
5bac5-1* (AB700499)	Bacterium Ellin7522 (HM748732)	Acidobacteria	397/420	95	Davis et al. (2011)	soil
5bac5-2* (AB700500)	Bacterium Ellin7544 (HM748754)	Acidobacteria	355/423	84	Davis et al. (2011)	soil
5bac5-3* (AB700501)	Bacterium Ellin5106 (AY234523)	Acidobacteria	398/431	95	Joseph et al. (2003)	soil
5bac6 (AB700502)	<i>Clostridium</i> sp. BNL1100 (CP003259)	Firmicutes	347/349	99		

### **II-3-4 Impact of DPAA on archaeal community structures**

PCR-DGGE fingerprints of archaeal communities in the soil cultures are shown in **Fig. 6A**. Compared to the bacterial DGGE profiles described above, the archaeal communities showed a lesser response to the incubation. Rice straw played an important role in shifts of the archaeal community, as shown by the intensities of several bands being enhanced by adding rice straw. For instance, strong DGGE bands such as 3arc14, 3arc18 and 5arc12 were found only in the D and DR treatments after 3 and 5 weeks of incubation. Cluster analysis for archaeal DGGE band patterns resulted in two clusters, Cluster II-1 and II-2 (**Fig. 6B**), depending on the presence or absence of rice straw. No sub-clusters based on DPAA application were observed.

Sequencing results of the archaeal 16S rDNA-DGGE bands indicate that most of the archaeal species in the soil cultures were related to methanogens in Euryarchaeota except for the DGGE bands 1arc9, 1arc12 and arcIni1, which were closest to *Nitrososphaera* species in Thaumarchaeota (**Table 4**).



**Fig. 6. DNA-based DGGE patterns of archaeal communities in the soil cultures during the examination period (A) and the dendrogram obtained after cluster analysis of the DGGE band patterns for the soil cultures after a 5-week incubation (B). Abbreviations are as shown in Fig. 5.**

**Table 4. Closest relatives of archaeal16S rDNA-based DGGE bands.**

Band (accession no.)	Closest relative (accession no.)	Regnum	Alignment	Homology (%)	Reference	Source
1arc1 (AB700503)	anaerobic methanogenic archaeon ET1-8 (AJ244284)	Euryarchaeota	459/466	98	Chin et al. (1999)	rice soil
1arc2 (AB700504)	anaerobic methanogenic archaeon ET1-8 (AJ244284)	Euryarchaeota	309/318	97	Chin et al. (1999)	rice soil
1arc3 (AB700505)	anaerobic methanogenic archaeon ET1-9 (AJ244285)	Euryarchaeota	468/482	97	Chin et al. (1999)	rice soil
1arc4 (AB700506)	anaerobic methanogenic archaeon ET1-4 (AJ244280)	Euryarchaeota	480/481	99	Chin et al. (1999)	rice soil
1arc5 (AB700507)	anaerobic methanogenic archaeon ET1-4 (AJ244280)	Euryarchaeota	478/483	99	Chin et al. (1999)	rice soil
1arc6 (AB700508)	anaerobic methanogenic archaeon ET1-8 (AJ244284)	Euryarchaeota	458/466	98	Chin et al. (1999)	rice soil
1arc7 (AB700509)	anaerobic methanogenic archaeon ET1-10 (AJ244286)	Euryarchaeota	453/462	98	Chin et al. (1999)	rice soil
1arc8 (AB700510)	<i>Methanosarcina</i> sp. HC-2 (AB288264)	Euryarchaeota	474/477	99		spring and sedimentary aquifers
1arc9 (AB700511)	<i>Nitrososphaera</i> sp. EN123 (FR773158)	Thaumarchaeota	462/482	96	Tourna et al. (2011)	garden soil
1arc10 (AB700512)	anaerobic methanogenic archaeon ET1-4 (AJ244280)	Euryarchaeota	458/487	94	Chin et al. (1999)	rice soil
1arc11 (AB700513)	anaerobic methanogenic archaeon ET1-8 (AJ244284)	Euryarchaeota	459/466	98	Chin et al. (1999)	rice soil
1arc12 (AB700514)	<i>Nitrososphaera</i> sp. EN123 (FR773158)	Thaumarchaeota	453/483	94	Tourna et al. (2011)	garden soil
1arc13 (AB700515)	<i>Methanosarcina</i> sp. HC-2 (AB288264)	Euryarchaeota	431/443	97		spring and sedimentary aquifers
arcIni1 (AB700516)	<i>Nitrososphaera viennensis</i> EN76 (FR773157)	Thaumarchaeota	463/483	96	Tourna et al. (2011)	garden soil
arcIni2 (AB700517)	archaeon LL25A6 (AJ745137)	Euryarchaeota	475/478	99	Kemnitz et al.(2005)	land and riverbank
arcIni3 (AB700518)	anaerobic methanogenic archaeon ET1-4 (AJ244280)	Euryarchaeota	478/483	99	Chin et al. (1999)	rice soil
3arc1 (AB700519)	anaerobic methanogenic archaeon ET1-8 (AJ244284)	Euryarchaeota	459/466	98	Chin et al. (1999)	rice soil
3arc2 (AB700520)	anaerobic methanogenic archaeon ET1-10 (AJ244286)	Euryarchaeota	464/469	99	Chin et al. (1999)	rice soil

3arc3 (AB700521)	anaerobic methanogenic archaeon ET1-9 (AJ244285)	Euryarchaeota	481/481	100	Chin et al. (1999)	rice soil
3arc4 (AB700522)	anaerobic methanogenic archaeon ET1-4 (AJ244280)	Euryarchaeota	479/480	99	Chin et al. (1999)	rice soil
3arc5 (AB700523)	anaerobic methanogenic archaeon ET1-4 (AJ244280)	Euryarchaeota	478/483	99	Chin et al. (1999)	rice soil
3arc6 (AB700524)	anaerobic methanogenic archaeon ET1-8 (AJ244284)	Euryarchaeota	459/466	98	Chin et al. (1999)	rice soil
3arc7 (AB700525)	anaerobic methanogenic archaeon ET1-10 (AJ244286)	Euryarchaeota	462/467	99	Chin et al. (1999)	rice soil
3arc8 (AB700526)	anaerobic methanogenic archaeon ET1-9 (AJ244285)	Euryarchaeota	481/481	100	Chin et al. (1999)	rice soil
3arc9 (AB700527)	<i>Methanosarcina</i> sp. MO-MS1 (AB598272)	Euryarchaeota	462/477	97	Imachi et al. (2011)	subseafloor sediments
3arc10 (AB700528)	anaerobic methanogenic archaeon ET1-4 (AJ244280)	Euryarchaeota	478/483	99	Chin et al. (1999)	rice soil
3arc11 (AB700529)	<i>Methanosarcina horonobensis</i> (AB288262)	Euryarchaeota	471/478	99	Shimizu et al. (2011)	groundwater
3arc12 (AB700530)	<i>Methanosarcina lacustris</i> (AY260430)	Euryarchaeota	475/479	99	Simankova et al. (2003)	waste water
3arc13 (AB700531)	anaerobic methanogenic archaeon ET1-10 (AJ244286)	Euryarchaeota	464/469	99	Chin et al. (1999)	rice soil
3arc14 (AB700532)	<i>Methanosarcina</i> sp. FR (AF020341)	Euryarchaeota	475/481	99	Cabirol et al. (1998)	sludge
3arc15 (AB700533)	<i>Methanosarcina horonobensis</i> (AB288262)	Euryarchaeota	475/479	99	Shimizu et al. (2011)	groundwater
3arc16 (AB700534)	<i>Methanosarcina</i> sp. T36 (AB288283)	Euryarchaeota	474/478	99		spring and sedimentary aifers
3arc17 (AB700535)	<i>Methanosarcina</i> sp. HC-2 (AB288264)	Euryarchaeota	477/478	99		spring and sedimentary aquifers
3arc18 (AB700536)	archaeon LL25A6 (AJ745137)	Euryarchaeota	475/478	99	Kemnitz et al. (2005)	land and riverbank
3arc19 (AB700537)	<i>Methanosarcina</i> sp. HC-2 (AB288264)	Euryarchaeota	474/479	99		spring and sedimentary aquifers
5arc1 (AB700538)	anaerobic methanogenic archaeon ET1-10 (AJ244286)	Euryarchaeota	463/468	99	Chin et al. (1999)	rice soil
5arc2 (AB700539)	anaerobic methanogenic archaeon ET1-10 (AJ244286)	Euryarchaeota	464/469	99	Chin et al. (1999)	rice soil
5arc3 (AB700540)	anaerobic methanogenic archaeon ET1-9 (AJ244285)	Euryarchaeota	481/481	100	Chin et al. (1999)	rice soil
5arc4 (AB700541)	<i>Methanosarcina</i> sp. HC-2 (AB288264)	Euryarchaeota	476/479	99		spring and sedimentary aquifers
5arc5 (AB700542)	anaerobic methanogenic archaeon ET1-10 (AJ244286)	Euryarchaeota	463/468	99	Chin et al. (1999)	rice soil

5arc6 (AB700543)	anaerobic methanogenic archaeon ET1–10 (AJ244286)	Euryarchaeota	464/468	99	Chin et al. (1999)	rice soil
5arc7 (AB700544)	anaerobic methanogenic archaeon ET1–10 (AJ244286)	Euryarchaeota	463/467	99	Chin et al. (1999)	rice soil
5arc8 (AB700545)	anaerobic methanogenic archaeon ET1–9 (AJ244285)	Euryarchaeota	481/481	100	Chin et al. (1999)	rice soil
5arc9 (AB700546)	anaerobic methanogenic archaeon ET1–10 (AJ244286)	Euryarchaeota	464/469	99	Chin et al. (1999)	rice soil
5arc10 (AB700547)	archaeon LL25A6 (AJ745137)	Euryarchaeota	476/479	99	Kemnitz et al. (2005)	land and riverbank
5arc11 (AB700548)	anaerobic methanogenic archaeon ET1–10 (AJ244286)	Euryarchaeota	461/469	98	Chin et al. (1999)	rice soil
5arc12 (AB700549)	<i>Methanosarcina</i> sp. HC–2 (AB288264)	Euryarchaeota	477/479	99		spring and sedimentary aquifers

## II-4 Discussions

As a result of sequencing the DGGE bands, anaerobic bacterial and archaeal species such as *Clostrisium*, and *Methanosarcina* appeared in the soil cultures, even after only a 1-week incubation (**Table 3 and 4**). This suggests that the soil cultures became anaerobic within a week of the incubation starting.

With respect to inorganic arsenicals, several previous reports suggest that soil organic matter may be an important factor influencing the mutual transformation between As (III) and As (V) (Balasoïu *et al.*, 2001; Redman *et al.*, 2002). On the other hand, Turpeinen *et al.* (2002) reported that the addition of a simple carbon source (0.2% glucose) had no obvious effect on methylation or reduction of soluble arsenicals in soils. As shown in **Fig. 3**, the involvement of rice straw clearly accelerated early DPAA degradation under anaerobic conditions. Although our study supplied DPAA instead of inorganic arsenicals, the result corresponds to those reported by Balasoïu *et al.* (2001) and Redman *et al.* (2002) rather than Turpeinen *et al.* (2002).

It is well known that rice straw is easily decomposed by multi-microbial activities in submerged rice soils, and that low molecular weight fatty acids are produced as the main metabolites (Watanabe, 1984; Tsutsuki and Ponnampereuma, 1987). Addition of rice straw to a submerged rice soil can, therefore, accelerate sequential reduction of electron acceptors (Patrick and Reddy, 1978; Inubushi *et al.*, 1984; Achtnich *et al.*, 1995). Substrates produced during the decomposition of rice straw in anoxic soils may become carbon sources for anaerobic soil microbes degrading DPAA.

Previous researches investigating chemical speciation and metabolites of organoarsenic compounds have reported several actual or possible metabolism pathways of DPAA degradation in polluted environmental samples. Arao *et al.* (2009) investigated changes of aromatic arsenicals under flooded and upland conditions using a contaminated soil collected from paddy fields in the Kizaki region. They

found dephenylation of DPAA into inorganic arsenic acid via PAA and methylation of PAA and MPAA into DMPAO in paddy soil under flooded conditions. Maejima *et al.* (2011) investigated transformations of DPAA during a 24-week incubation of two agricultural soils and reported that DPAA was degraded to PAA, MPAA (detected only in an Entisol soil), DMPAO and methyl diphenylarsine under anaerobic conditions. Under aerobic conditions, Nakamiya *et al.* (2007) isolated a DPAA-degrading bacterium, strain NK0508, from DPAA-contaminated soil. They proposed that DPAA could be converted into diphenylmethylarsine oxide, *cis*-muconate and arsenic acid by the strain. Harada *et al.* (2010) isolated other strains, *Ensifer* sp. L2406 and L2413, which can degrade DPAA into arsenic acid in a minimum salt medium supplemented with DPAA as a sole carbon source. They suggested an aerobic metabolic pathway of DPAA degradation via monohydroxylated DPAA and PAA.

The fact that the unknown arsenical species found in the present study was eluted after DPAA in reverse-phase LC-ICPMS, strongly suggests that the unknown species found in the anaerobic soil cultures does not correspond with any of the known metabolites described in the earlier studies discussed above. The elution behavior of the unknown arsenical species detected in the present study is similar to that of the unknown organo-arsenical compound described by Hempel *et al.* (2009). They reported microbial degradation of PAA with temporary accumulation of phenylarsine oxide and an unknown organo-arsenical compound in experimental microcosms made of anoxic groundwater/sediment mixtures taken from an anoxic, phenylarsenical-contaminated aquifer. They proposed dimeric phenyl arsenicals with S-As-bridges as possible structures of the unknown organoarsenical, since formation of thioarsenates from inorganic arsenicals in anoxic sulfidic waters is known (Stauder *et al.*, 2005). However, no evidence is available yet to support this proposal. Further studies to determine the nature and formation of these unknown arsenic species are necessary.

Only a few studies have been conducted to elucidate the effects of contamination with arsenicals on soil microbial communities. Maliszewska *et al.* (1985) found that

inorganic As (V) stimulated the proliferation of certain groups of microorganisms in soil resulting in a shift of the community to only a few tolerant species. Lorenz *et al.* (2006) investigated the response of microbial activity and microbial community composition in soils affected by long-term arsenic exposure. They observed clear differences in the PCR-DGGE band patterns between a long-term As-contaminated soil and a control. More recently, Somenahally *et al.* (2011), using community quantitative-PCR and 16S rRNA gene sequencing, reported that the bacterial community structure and composition in the rice rhizosphere were significantly influenced by long-term application of monosodium methylarsenate.

The cluster analyses in this study suggest that, while addition of DPAA has a direct effect on some abundant bacterial species as shown in the DGGE bands 5bac2, 5bac4 and 5bac5 (**Fig. 5A**), overall bacterial community structures under the anaerobic soil conditions tend to be stable after the early succession due to submergence and rice straw application, regardless of DPAA contamination (**Fig. 5B**). The archaeal community structure showed more robustness as shown in **Fig. 6**. Rice straw was more effective in altering bacterial and archaeal communities than DPAA. As far as we know, this is the first report to describe the impacts of DPAA on soil microbes under flooded conditions.

In conclusion, DNA based PCR-DGGE fingerprinting targeting bacterial and archaeal 16S rRNA genes revealed that the presence of DPAA did not distinctly change soil bacterial and archaeal communities under submerged and anaerobic conditions.

**Chapter III. Enhanced transformation of  
DPAA in soil under sulfate-reducing  
conditions**

### III-1 Introduction

Chemical warfare weapons containing aromatic arsenicals, such as CLARK I and CLARK II, were mainly produced as vomiting and sneezing agents during World Wars I and II. They were abandoned in Europe, China, Japan, and other countries by sea-dumping or earth-burial after the Wars. Many studies have reported the presence of these chemical warfare agents as well as their metabolites in polluted environmental samples (Arao *et al.*, 2009, 2011; Baba *et al.*, 2008; Daus *et al.*, 2008; Ishizaki *et al.*, 2005; Maejima *et al.*, 2011). Haas *et al.* (1998) noted that these agents can be metabolized in groundwater, soils, and sediments via hydrolysis and oxidation, resulting in the formation of DPAA. In the Kizaki region of the city of Kamisu (Ibaraki, Japan), groundwater contaminated by DPAA led to prominent cerebellar symptoms in several inhabitants (Ishii *et al.*, 2004). How to remedy soil contaminated with phenylarsenical compounds such as DPAA remains an unsolved problem.

The process of arsenic transformation made by various microorganisms in the environment has been extensively studied and detailed as it involves various processes including oxidation, reduction and methylation (Daus *et al.*, 2010; Oremland and Stolz, 2003). For instance, *Agrobacterium albertimagni* strains can rapidly oxidize arsenite using a mechanism consistent with arsenic detoxification (Salmasi *et al.*, 2002), while bacterium *Pseudomonas fluorescens* microbially reduces arsenate to arsenite (Cullen and Reimer, 1989). Some aerobic and anaerobic microorganisms are contributed to inorganic arsenic methylation, producing methyl arsenic, such as monomethylarsonic acid, dimethylarsinic acid, and trimethylarsine oxide (Cullen and Reimer, 1989).

According to the described biotransformation of inorganic arsenic, one possible solution to removing phenylarsenicals from contaminated environments is bioremediation using microorganisms that can transform phenylarsenicals into inorganic forms, followed by phytoremediation using hyperaccumulating plants such as *Pteris vittata* L. that can absorb inorganic arsenical compounds (Ma *et al.*, 2001). Generally, bioremediation of contaminants can be accomplished by two methods:

bioaugmentation and biostimulation (Elgh-Dalgren *et al.*, 2011; Sarkar *et al.*, 2005). Bioaugmentation utilizes specially adapted or isolated bacterial strains, which are added to the contaminated soil. Although several researchers have successfully isolated several strains capable of degrading phenylarsenical compounds (Köhler *et al.*, 2001; Nakamiya *et al.*, 2007; Harada *et al.*, 2010), their effectiveness in the soil environment has not yet been reported.

The process of biostimulation utilizes indigenous microorganisms in the contaminated environments, which are stimulated by addition of easily available carbon sources, nutrients, water or air. Cortinas *et al.* (2006) reported the rapid bioconversion of roxarsone (3-nitro-4-hydroxyphenylarsonic acid), an organoarsenical feed additive, to the corresponding aromatic amine under anaerobic conditions. They also showed that the metabolite was slowly eliminated under methanogenic and sulfate-reducing conditions, whereas little or no removal occurred in heat-killed inoculum controls. Nakamiya *et al.* (2007) isolated *Kytococcus sedentarius* strain NK0508, which was capable of degrading DPAA when supplemented by toluene as a carbon source. Their results suggest the possibility that addition of toluene to a contaminated soil may be effective in decreasing DPAA. However, we are not aware of any useful biostimulation method having been developed yet for amelioration of soils contaminated with DPAA.

In this study, we focused on a biostimulation method to decrease DPAA concentrations in a contaminated soil under anaerobic conditions. We performed experimental model studies, aiming to find the key factors affecting DPAA transformation in the contaminated soil.

## III-2 Materials and methods

### III-2-1 Reagents

HPLC grade-acetonitrile and 85% phosphoric acid were purchased from Sigma-Aldrich (MO, USA) and Wako Pure Chemical Industries (Osaka, Japan), respectively. The following arsenic compounds were used as calibration standards for liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICPMS): 60% arsenic acid, DPAA and PAA were obtained from Wako Pure Chemical Industries; PMAA and DPMAO from Hayashi Pure Chemical Industry (Osaka, Japan). Cellulose, sodium acetate and potassium sulfate were purchased from Wako Pure Chemical Industries, and sodium molybdate dihydrate from Kanto Chemical (Tokyo, Japan). Purelab Ultra water (Organo, Tokyo, Japan) was used for analysis of DPAA metabolites with LC-ICPMS.

### III-2-2 Soil and rice straw samples

Two samples of Japanese soils were collected from the surface layer (0-10cm in depth) of a fallow upland soil on the Ikarashi campus of Niigata University (Ikarashi soil) and a rice field soil from the Shindori Station of the Field Center for Sustainable Agriculture and Forestry, Niigata University (Shindori soil) in April and June 2010, respectively. The Ikarashi soil was a sand-dune Regosol and the Shindori soil was a Gleysol. An additional two soil samples were collected in Heilongjiang, China, in September 2010: a Mollisol (Chernozem) from a soybean field in Heihe (Heihe soil) and a Gleysol from a rice field in Qiqihar (Qiqihar soil). After air-drying, the soil samples were passed through a 2-mm sieve and stored in polyethylene bags at ambient temperature. The soil physico-chemical properties are summarized in **Table 5**.

Rice straw samples were collected from the Shindori Station of the Field Center for Sustainable Agriculture and Forestry, Niigata University at harvest in 2007. The

samples were stored at ambient temperature after drying. The leaf sheath was used after being ground to a powder. The carbon and nitrogen contents of the rice straw were  $350.7 \text{ mg g}^{-1}$  and  $4.6 \text{ mg g}^{-1}$ , respectively.

### **III-2-3 Anaerobic transformation of DPAA in the four soils**

Soil cultures containing 20 g of the air-dried Ikarashi, Heihe, Qiqihar and Shindori soils with 30 mL of deionized water in a 100 mL Erlenmeyer flask were prepared. Each soil culture was artificially contaminated with DPAA at a level of ca.  $10 \text{ } \mu\text{g-As gds}^{-1}$  and was closed with a double rubber plug. Rice straw ( $10 \text{ mg gds}^{-1}$ , equivalent to  $3.5 \text{ mg-C gds}^{-1}$ ) was also added to selected soil cultures. The soil cultures were incubated for a total of 5 weeks at  $30 \text{ } ^\circ\text{C}$  in the dark in duplicate.

### **III-2-4 Effects of exogenous C and S sources**

The Ikarashi soil was used to examine the effect of adding exogenous sulfate as potassium sulfate at rates of 20, 180 and  $425 \text{ } \mu\text{g-S gds}^{-1}$  to the soil cultures modified with  $3.5 \text{ mg-C gds}^{-1}$  of rice straw, sodium acetate or cellulose. Soil cultures, in which sulfate reduction was suppressed, were also incubated. To suppress sulfate reduction, sodium molybdate, which is known to be an analog of sulfate and which acts as an inhibitor of ATP sulfurylase, was used at a final concentration of 5 mM in the solution (Harada *et al.*, 2001; Taylor and Oremland, 1979).

### **III-2-5 Determination of DPAA concentrations using HPLC**

The determination of DPAA concentration in soil culture was described in **Chapter II**.

### **III-2-6 Estimation of metabolites using LC-ICPMS**

DCAA metabolites in soil cultures were estimated with LC-ICPMS, which was described in details in **Chapter II**.

### **III-2-7 Ion chromatography**

Sulfate and acetate were extracted from soil cultures by shaking at 200 rpm for 1 h, after a 5-fold volume of deionized water against the dry soil weight was added. The soil/water samples were centrifuged (3,000 rpm, 10 min) and the supernatant was filtered through a 0.45 µm membrane (Toyo Roshi kaisha). An aliquot (20 µL) of each sample was then analyzed with an ion chromatograph (LC-10AD, Shimadzu, Kyoto, Japan) equipped with a conductivity detector (CDD-6AD, Shimadzu). Separation was performed with a Shim-pack IC-A1 column (12.5 µm, 4.6 mm i.d.×100 mm, Shimadzu) with a Shim-pack IC-GA1 guard column (4.6 mm i.d.×10 mm, Shimadzu) at 40 °C. The mobile phase was 1.2 mM potassium hydrogen phthalate (pH 4.2) and the flow rate was 1.5 mL min<sup>-1</sup>.

### **III-2-8 Statistical analysis**

Tukey's test ( $P < 0.05$ ) was performed to analyze statistical differences between treatments using the statistical software KyPlot version 2.0 beta 15. Significant differences between treatments at each week ( $P < 0.05$ ) are indicated as different letters in the figures.

**Table 5. Characteristics of the sampling sites and essential soil properties in the surface 0–10 cm.**

Site	Location	Soil order	Crop	EC <sup>a</sup> ( $\mu\text{s cm}^{-1}$ )	pH (H <sub>2</sub> O) <sup>a</sup>	pH (KCl) <sup>a</sup>	Total C <sup>a</sup> (mg-C gds <sup>-1</sup> )	Total N <sup>a</sup> (mg-N gds <sup>-1</sup> )	CN ratio	SO <sub>4</sub> <sup>2-a</sup> ( $\mu\text{g-S gds}^{-1}$ )
Heihe	Heilongjiang, China	Mollisol	Soybean	157.43 $\pm$ 4.09	5.38 $\pm$ 0.03	4.51 $\pm$ 0.01	29.74 $\pm$ 0.87	3.12 $\pm$ 0.09	9.53	5.89 $\pm$ 0.36
Ikarashi	Niigata, Japan	Regosol	Fallow	34.1 $\pm$ 1.50	5.92 $\pm$ 0.02	4.43 $\pm$ 0.02	3.46 $\pm$ 0.09	0.40 $\pm$ 0.00	8.65	0.49 $\pm$ 0.69
Qiqihar	Heilongjiang, China	Gleysol	Rice	126.83 $\pm$ 1.31	6.11 $\pm$ 0.00	4.88 $\pm$ 0.01	18.39 $\pm$ 0.25	1.79 $\pm$ 0.04	10.27	N.D. <sup>b</sup>
Shindori	Niigata, Japan	Gleysol	Rice	182.17 $\pm$ 4.90	4.83 $\pm$ 0.00	3.80 $\pm$ 0.01	23.77 $\pm$ 0.92	2.61 $\pm$ 0.06	9.11	60.75 $\pm$ 3.37

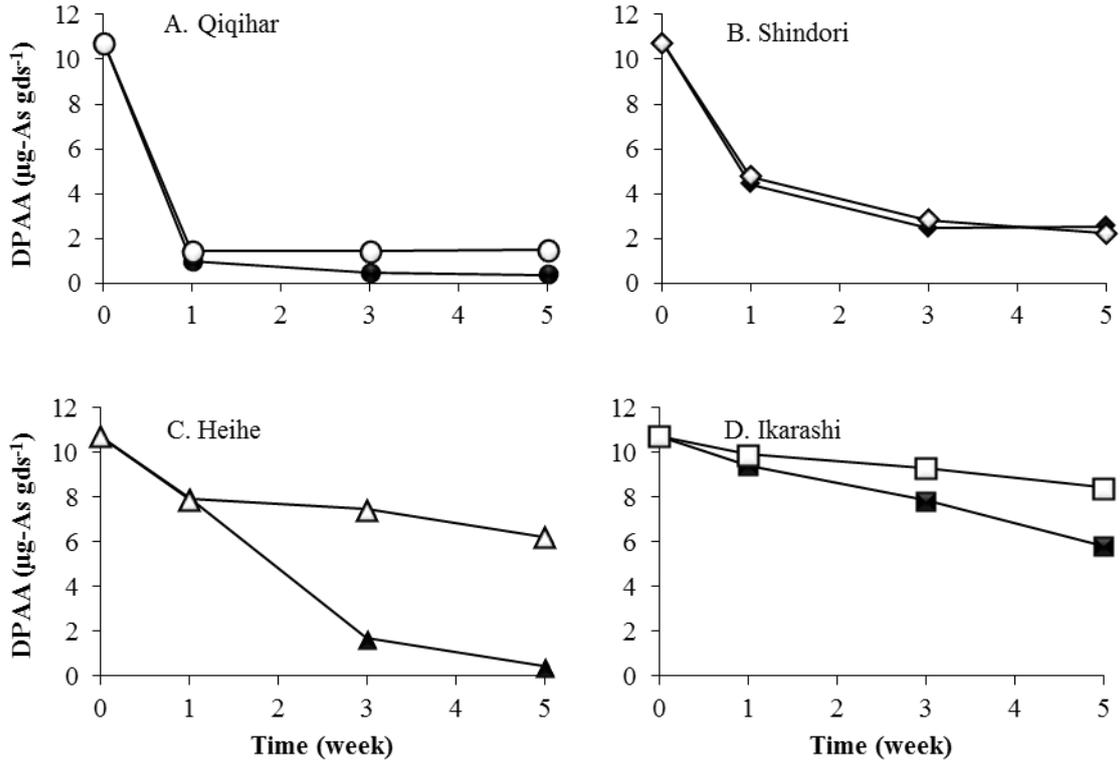
<sup>a</sup>Average  $\pm$ S.D., <sup>b</sup>no data

### III-3 Results

#### III-3-1 DPAA concentrations

**Figure 7** shows changes in DPAA concentrations determined using HPLC in the soil cultures with the four different soil samples under submerged conditions. A rapid decrease in DPAA concentrations was observed in the soil cultures from the two rice soils, Qiqihar and Shindori (**Fig. 7A and B**). The Qiqihar soil had the most rapid decrease in DPAA concentrations from the initial 10.73 to 0.97  $\mu\text{g-As gds}^{-1}$  within a week (**Fig. 7A**). It was followed by the Shindori soil, where the DPAA concentration decreased to 4.42  $\mu\text{g-As gds}^{-1}$  within a week (**Fig. 7B**). Such rapid decrease of DPAA in the two soil cultures was achieved regardless of the addition of rice straw.

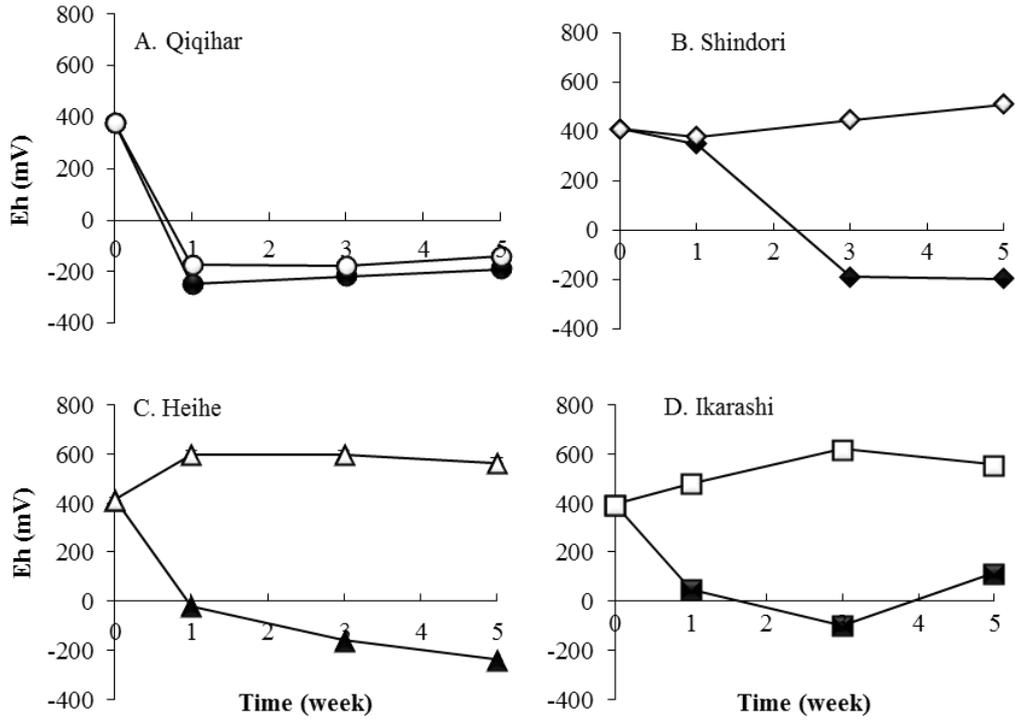
The soil cultures using the Heihe and Ikarashi upland soils showed slower decreases in DPAA concentrations (**Fig. 7C and D**). In the 5-week incubation without rice straw, DPAA concentrations of 6.24 and 8.44  $\mu\text{g-As gds}^{-1}$  of DPAA remained in the Heihe and Ikarashi soil cultures, respectively. Addition of rice straw resulted in different responses in the two soils. Elimination of DPAA was greatly enhanced by rice straw addition in the Heihe soil, as the residual DPAA concentrations after 3 and 5 weeks of incubation were similar to those in the Qiqihar soil. In contrast, the effect of rice straw addition on DPAA elimination was only slight in the Ikarashi soil cultures.



**Fig. 7. Changes in DPAA concentrations during static incubation of the soil cultures with the four soil samples. Filled and open symbols are for the soil cultures incubated with and without rice straw, respectively. The initial DPAA concentration in the soil cultures was adjusted to be  $10.73 \mu\text{g-As gds}^{-1}$ . Each point corresponds to the average of duplicate analyses.**

### III-3-2 Eh values

Changes in redox potentials during the incubation are shown in **Fig. 8**. Reducing conditions were quickly induced in the Qiqihar soil regardless of rice straw application, as the Eh values decreased to approximately  $-200$  mV within a week and this low Eh value was maintained until the end of the incubation (**Fig. 8A**). This indicates strong-gley soil characteristics in the Qiqihar soil which led to anaerobic conditions immediately after the incubation started. The Shindori soil is also a Gleysol but the redox potentials remained high and stable in the soil cultures without rice straw addition (**Fig. 8B**). When rice straw was added, the Eh value at 1 week did not differ from that without rice straw addition, but strongly reducing conditions were established thereafter. As shown in **Fig. 8C and D**, the patterns of Eh changes during incubation of the other two soil cultures, Heihe and Ikarashi, were somewhat similar to that in the Shindori soil in that Eh values showed positive values without rice straw addition, while rice straw application led to reducing conditions. No clear relationship between the rates of decrease in DPAA concentrations (**Fig. 7**) and soil Eh values (**Fig. 8**) was found.



**Fig. 8.** Changes in soil redox potential (Eh) during static incubation of the soil cultures with the four soil samples. Filled and open symbols are for the soil cultures incubated with and without rice straw, respectively. Each point corresponds to the average of duplicate analyses.

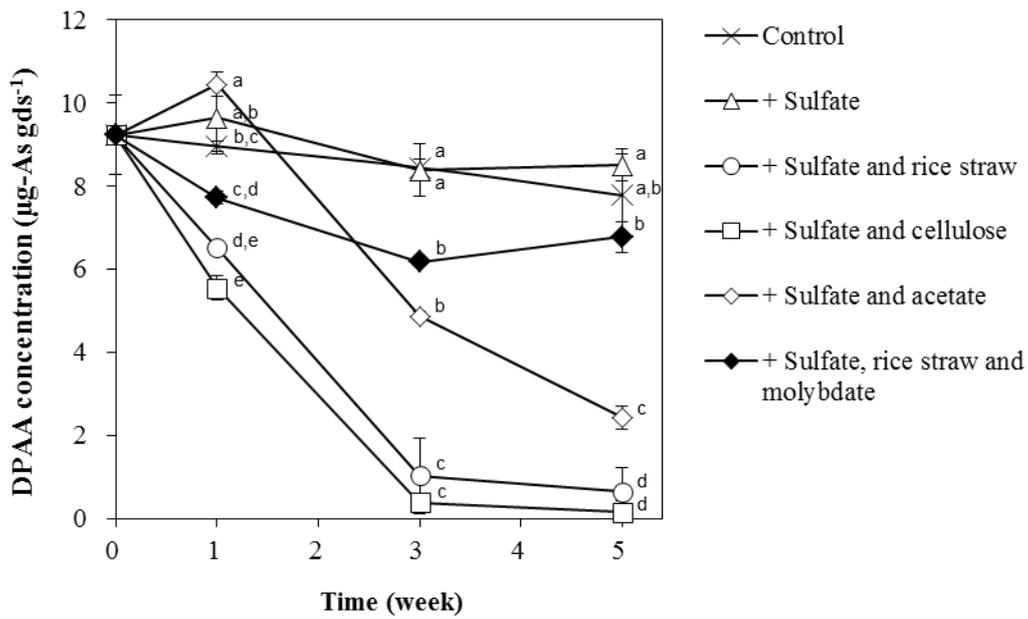
### III-3-3 DPAA transformation with exogenous sulfate

The Ikarashi soil contains much smaller amounts of total carbon and sulfate compared with the other soils (**Table 5**). Therefore, it is probable that little sulfate reduction could occur in the Ikarashi soil cultures incubated without exogenous sulfate and carbon sources. We, then, examined effects of adding exogenous sulfate in combination with several carbon sources to the soil cultures on the fate of DPAA.

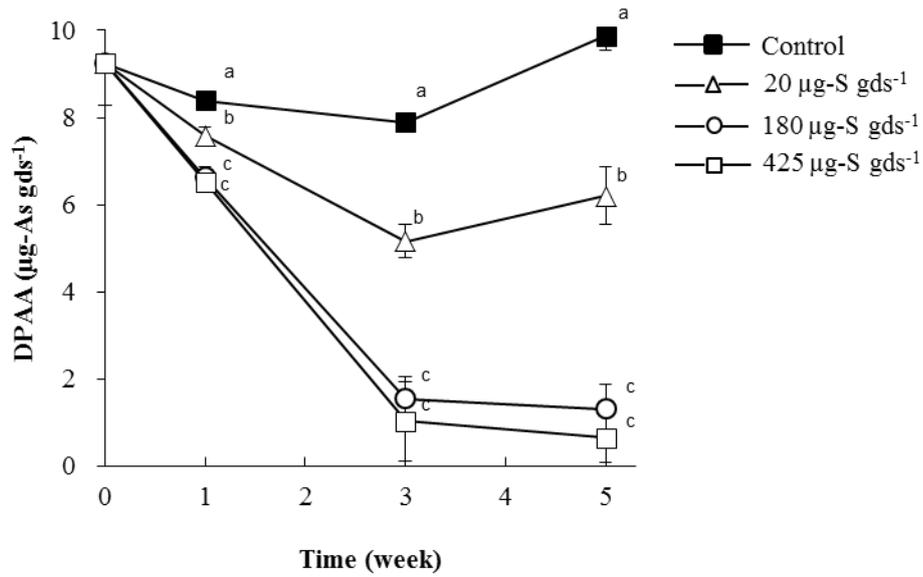
**Figure 9** shows changes in DPAA concentrations in the Ikarashi soil cultures modified with  $425 \mu\text{g-S gds}^{-1}$  of sulfate. When no additional exogenous carbon source was added, the DPAA concentration gradually changed from an initial  $9.24$  to  $8.51 \mu\text{g-As gds}^{-1}$  at the end of the five-week incubation, which was consistent with those in the control soil without exogenous sulfate.

When sulfate was added together with exogenous carbon sources, a rapid decrease in DPAA concentrations was observed and typical smell of hydrogen sulfide was detected. Supplementation with rice straw and cellulose accelerated elimination of DPAA at similar rates, and DPAA concentrations finally decreased to  $0.65$  and  $0.16 \mu\text{g-As gds}^{-1}$ , respectively. Supplementation with acetate had no effect on the DPAA concentration during the first-week of incubation but a steep decrease in DPAA concentrations was detected subsequently.

The effect of adding sulfate at two lower concentrations of  $20$  and  $180 \mu\text{g-S gds}^{-1}$ , together with rice straw, was then examined. Addition of sulfate at  $180 \mu\text{g-S gds}^{-1}$  was similarly effective in decreasing the DPAA concentration as  $425 \mu\text{g-S gds}^{-1}$  as sulfate (**Fig. 10**). However, when  $20 \mu\text{g-S gds}^{-1}$  of sulfate was applied, the decrease in DPAA concentrations was slower.



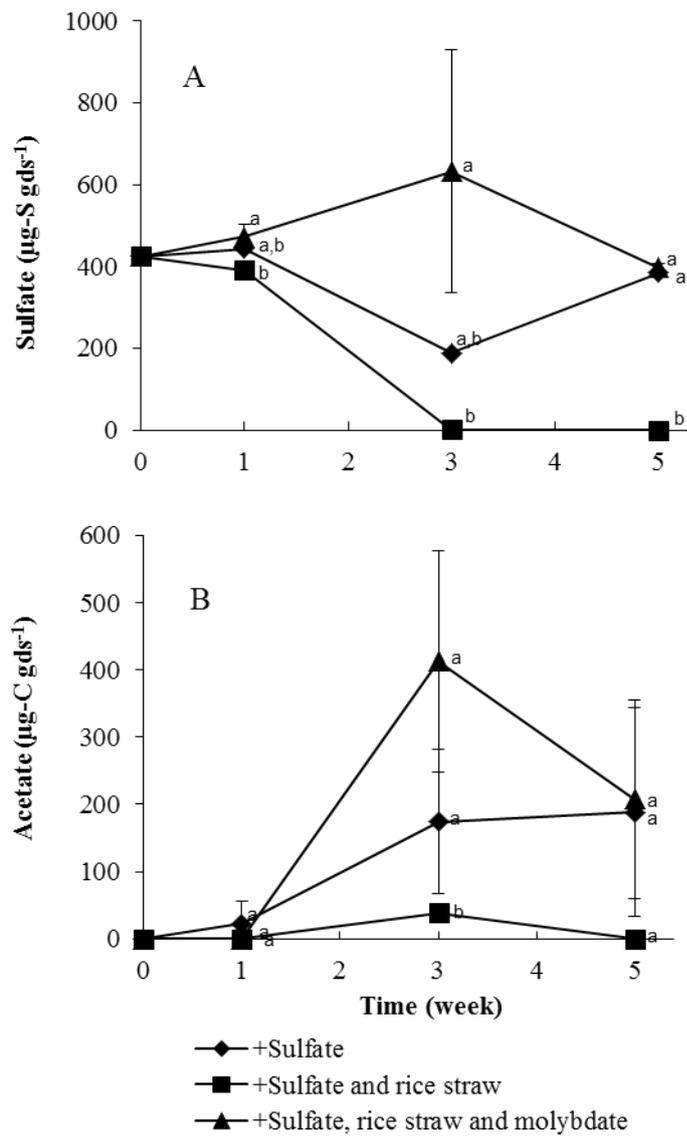
**Fig. 9.** Effects of addition of sulfate at  $425 \mu\text{g-S gds}^{-1}$  and a carbon source at  $3.5 \text{ mg-C gds}^{-1}$  on the DPAA concentrations in the Ikarashi soil cultures incubated under static conditions.  $\text{K}_2\text{SO}_4$  was used as the sulfate source. Molybdate ( $\text{Na}_2\text{MoO}_4$ , 5 mM) was used to suppress sulfate reduction. Each point and bar corresponds to the average  $\pm$  S.D. (n=3)



**Fig. 10.** Effects of sulfate addition at levels of 20, 180 and 425  $\mu\text{g-S gds}^{-1}$  on DPAA concentrations in the Ikarashi soil cultures with rice straw amendment, incubated under static conditions. Each point and bar corresponds to the average  $\pm$ S.D. (n=3)

### III-3-4 Occurrence of sulfate reduction in the Ikarashi soil

Since the Ikarashi soil was collected from a well-drained sand-dune upland field, it was assumed that it was unlikely to have been exposed to anaerobic conditions. Therefore, the occurrence (or not) of sulfate reduction in the soil cultures incubated with  $425 \mu\text{g-S gds}^{-1}$  of exogenous sulfate were investigated by measuring sulfate and acetate concentrations in solution. The suppressing effects of molybdate on sulfate reduction were also examined. As a result, measured sulfate concentrations fluctuated between  $188$  and  $443 \mu\text{g-S gds}^{-1}$  and only small amounts of acetate were detected in the soil cultures incubated without rice straw (**Fig. 11**). This suggests that sulfate reduction was partially initiated but subsequently stagnated due to the absence of carbon sources in the Ikarashi soil. On the other hand, when rice straw was added, sulfate concentrations decreased dramatically and disappeared by week three of the incubation. Also, constant accumulation of acetate was found in the solution. This supports the suggestion that sulfate reduction can occur in the Ikarashi soil cultures, if sufficient amounts of sulfate and carbon sources are supplied.



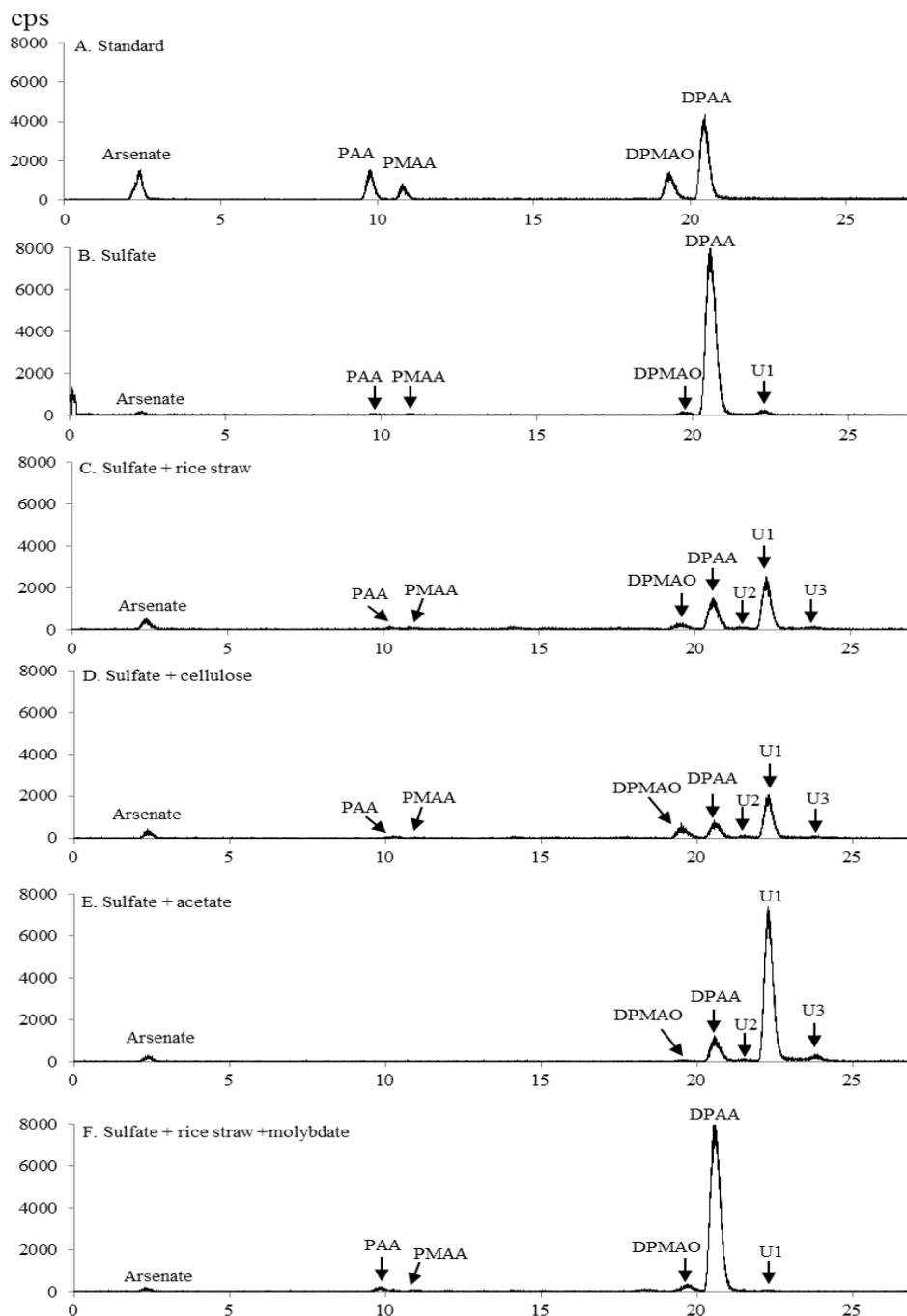
**Fig. 11. Changes in sulfate (A) and acetate (B) concentrations in the Ikarashi soil cultures amended with sulfate and with the suppressing effects of molybdate addition ( $\text{Na}_2\text{MoO}_4$ , 5 mM) on sulfate reduction. Each point and bar corresponds to the average  $\pm$  S.D. (n=3)**

### III-3-5 Metabolites of DPAA

Arsenical metabolite products from the transformation of DPAA were investigated using LC-ICPMS in the Ikarashi soil cultures after the 5-week incubation under sulfate-reducing conditions. As shown in **Fig. 12**, unidentified As-containing intermediates, designated unknown 1 (U1), unknown 2 (U2) and unknown 3 (U3), appeared on the chromatograms as well as arsenate, DPMAO, PMAA and PAA.

When the soil cultures were incubated with exogenous sulfate only (**Fig. 12B**), small peaks ( $< 0.3 \mu\text{g-As gds}^{-1}$ ) of arsenate, PAA, PMAA, DPMAO and U1 were detected as the metabolites of DPAA. When rice straw or cellulose was added to the soil cultures as the extra carbon source together with sulfate (**Fig. 12C and D**), U1 was observed as the main metabolite, followed by DPMAO, arsenate, PAA, PMAA, U2 and U3. The relative concentrations of U1 were 1.75 and 1.21  $\mu\text{g-As gds}^{-1}$  in the soil cultures incubated with rice straw and cellulose, respectively, and the others were all less than 1  $\mu\text{g-As gds}^{-1}$ . In addition, a small peak of PMAA was also found in the cultures incubated with rice straw. In the soil cultures amended with acetate and sulfate (**Fig. 12E**), U1 at a relative concentration of 4.37  $\mu\text{g-As gds}^{-1}$  was detected as the main metabolite, followed by arsenate, DPMAO, U2 and U3. Concentrations not exceeding 0.1  $\mu\text{g-As gds}^{-1}$  of PAA, DPMAO and PMAA were also detected. High recovery rates of 99.1 and 78.8% were achieved for the soil cultures incubated with sulfate and sulfate+acetate, respectively. On the other hand, the recovery rates for the soil cultures incubated with sulfate+rice straw and sulfate+cellulose were 37.4 and 32.1%, respectively, suggesting subsequent transformation of the metabolites into other unknown arsenical species that could not be detected under the analytical conditions used in this study.

As described above, the presence and concentrations of sulfur and carbon sources were major factors regulating DPAA transformation in the Ikarashi soil (**Fig. 9**). When molybdate was added to suppress sulfate reduction (**Fig. 12F**), only small peaks of arsenate, PAA, PMAA, DPMAO and U1 were found in the soil cultures.



**Fig. 12. LC-ICPMS chromatograms obtained from: (A) the standard solution containing each of  $5 \mu\text{g-As L}^{-1}$  arsenate,  $2 \mu\text{g-As L}^{-1}$  phenylmethylarsinic acid (PMAA),  $5 \mu\text{g-As L}^{-1}$  PAA,  $4 \mu\text{g-As L}^{-1}$  diphenylmethylarsine oxide (DPMAO) and  $5 \mu\text{g-As L}^{-1}$  DPAA; the 5-week soil cultures incubated with (B) sulfate, (C) sulfate and rice straw, (D) sulfate and cellulose, (E) sulfate and acetate and (F) sulfate, rice straw and molybdate. All experiments were conducted at concentrations of  $3.5 \text{ mg-C gds}^{-1}$  and  $425 \mu\text{g-S gds}^{-1}$ .**

### III-4 Discussions

As shown in **Fig. 7**, decreases in DPAA concentrations and responses to rice straw addition varied among the soils examined. It is notable that, unlike the Heihe soil, addition of rice straw did not decrease DPAA concentration in the Ikarashi soil cultures, although both were upland soils. Therefore the question arises as to what the major factors are that limited the transformation of DPAA in the Ikarashi soil.

There are some clear differences in soil physicochemical properties between the Ikarashi and Heihe soils (**Table 5**). The Ikarashi soil had significantly lower amounts of total carbon, total nitrogen and sulfate than the Heihe soil. Addition of organic and inorganic nutrients has been found to increase microbial metabolism of some target pollutants (Swindoll *et al.*, 1988; Manilal and Alexander, 1991). However, addition of cellulose, polyacetate, glucose, sucrose, formate, acetate and lactate were not effective in decreasing DPAA concentrations in the Ikarashi soil cultures, as also was addition of ammonium nitrate with one of the carbon sources (data not shown). This indicates that factors other than the availability of carbon and nitrogen from different sources are affecting the fate of DPAA in the Ikarashi soil.

However, when sufficient metabolizable carbon sources are supplied, sulfate concentrations can play a key role in the transformation of DPAA in the Ikarashi soil cultures (**Fig. 9**) and participation of sulfate-reducing bacteria is expected.

Hempel *et al.* (2009) investigated the microbial degradation of PAA and DPAA in microcosms comprising anoxic groundwater-sediment mixtures. They found that PAA transformation occurred under sulfate-reducing conditions, but DPAA transformation did not. According to Hempel *et al.* (2009), a pure culture of *Desulfovibrio gigas* spiked with PAA showed that the elimination process was linked to the presence of sulfide formed through bacterial activity. Stauder *et al.* (2005) also reported that thio-arsenates were formed as the dominant forms of As-S complexes in an anoxic sulfidic environment. Therefore, it is speculated that sulfate-reducing

bacteria may participate in transformation of the DPAA in the soil cultures examined in this study.

In the soil cultures modified with molybdate, sulfate concentrations increased during the first three weeks of the incubation and then decreased slightly. Compared with the unsuppressed soil cultures incubated with sulfate and rice straw, greater accumulation of acetate was observed. Harada *et al.* (2001) also reported a similar temporary increase in sulfate concentrations and accumulation of acetate in paddy soil slurries incubated under sulfate reduction–suppressed conditions. This is probably caused by inhibition of sulfate reduction by molybdate and generation of sulfate from elemental sulfur and sulfide by sulfur oxidizing bacteria (Stubner *et al.*, 1998).

Since rice straw contains a certain amount of easily decomposable polysaccharides, acetate can be produced by degradation of rice straw in soil cultures (Glissmann and Conrad, 2000; Gotoh and Onikura, 1971; Tsutsuki and Ponnampuruma, 1987). It is known that acetate is a favorable carbon source for sulfur reducing microorganisms (Maier *et al.*, 2009) and sulfate reduction can be activated in the presence of sufficient amounts of sulfate and carbon sources (Herlihy and Mills, 1985; Howarth and Teal, 1979; Westrich and Berner, 1984)

In this study, elimination of sulfate and accumulation of acetate were found in the straw–amended soil cultures incubated with exogenous sulfate (**Fig. 11**). This indicates that indigenous sulfate–reducing microorganisms in the Ikarashi soil were activated by incubation with exogenous sulfate and acetate derived from rice straw decomposition. Greater accumulation of acetate after the addition of molybdate can be explained by decreased acetate consumption due to the suppression of sulfate reduction. The large difference in acetate accumulation between the soil cultures incubated with and without molybdate may mean that sulfate–reducing microorganisms are a major consumer of acetate in the soil cultures.

Under anaerobic conditions, sulfate–reducing bacteria oxidize simple organic compounds, including acetate, by utilizing sulfate as an electron acceptor (Jong and Parry, 2003). Uhrig *et al.* (1996), using sulfate–reducing bacteria, observed a rapid removal of arsenic from aqueous solution with an additional carbon source. With

respect to organoarsenical compounds, Cortinas *et al.* (2006) reported that the biotransformation of roxarsone and elimination of its metabolites was stimulated under sulfate-reducing and methanogenic conditions. Although our results did not clearly confirm that sulfate-reducing bacteria directly participate in DPAA transformation, they demonstrate that sulfate reduction play a significant role in DPAA removal.

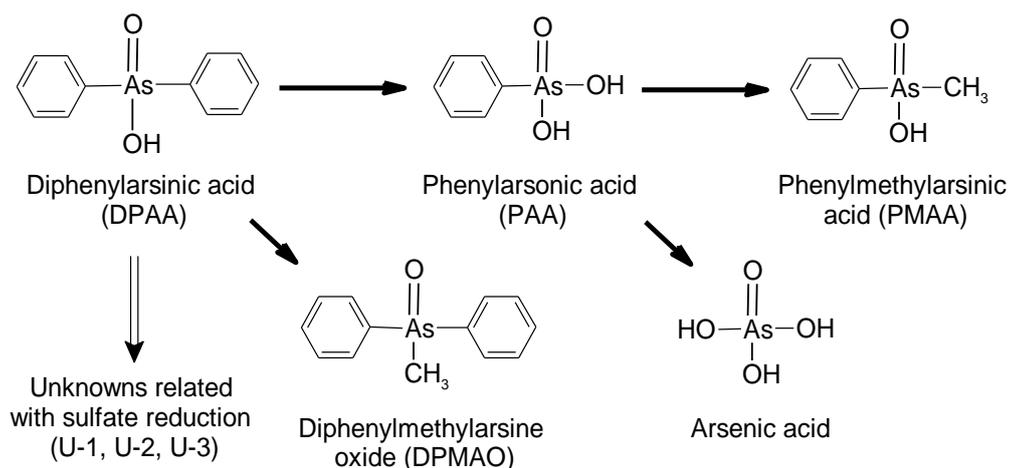
The proposed transformation pathway of DPAA under anaerobic soil conditions was shown in **Fig. 13**. One pathway may include the transformation of DPAA to U1, U2 and U3, and be strongly related to sulfate reduction. It is the main transformation pathway of DPAA in the Ikarashi soil cultures incubated with exogenous sulfate and carbon sources as shown in **Fig. 12**. Since the three unknown arsenical species were found only in the soils amended with sulfate, sulfur atoms are probably involved in the formation of the unknown species. Hempel *et al.* (2009) detected an unknown arsenical species transformed from PAA generated from the living microcosms using sulfate as the electron acceptor. The unknown species was eluted after DPAA in reverse-phase LC-ICPMS as were U1, U2 and U3 in this study. Hempel *et al.* (2009) hypothesized that the unknown species had a dimeric structure with two PAA molecules possibly linked by a sulfur bridge. Formation of similar arsenical compounds from DPAA was speculated in this study.

Recently, additional LC-ICP analyses carried out by our group showed that the elution pattern of U1 was similar to that of the chemical reaction product obtained by reaction between DPAA with hydrogen sulfide (**Fig. 14**) (Hisatomi *et al.*, personal communication). The mass spectrum of U1 determined using LC/TOF-MS also corresponded to that of the chemical reaction product (**Fig. 14**) and high resolution mass spectral analyses revealed that the chemical composition of the unknown arsenical was estimated to be C<sub>12</sub>H<sub>12</sub>AsSO. Consequently, the chemical structure of the main unknown U1 was elucidated as diphenylthioarsinic acid (DPTAA).

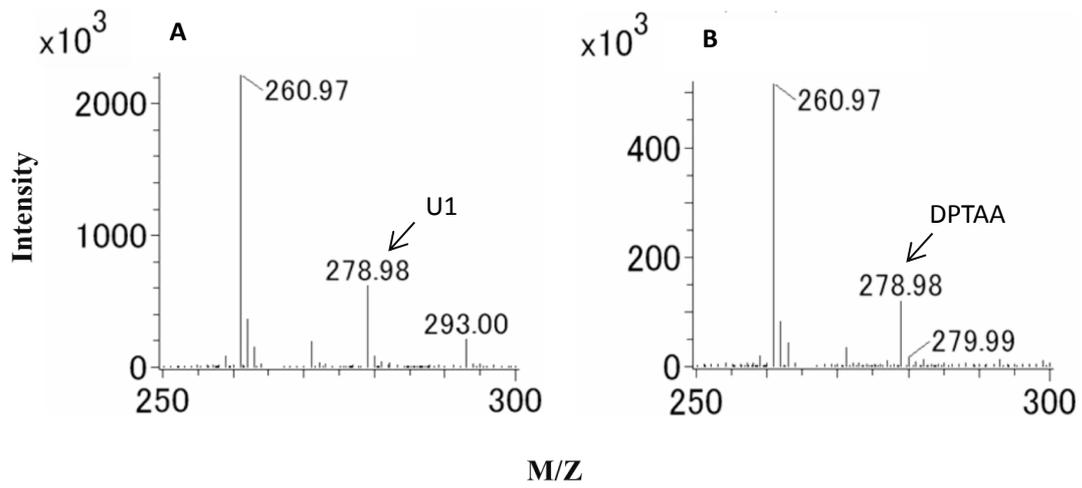
The other pathway may be dephenylation and methylation from DPAA to DPMAO and PAA that can occur without sulfate being present, as suggested by Arao *et al.* (2009) and Maejima *et al.* (2011). Both these studies reported that DPAA can be

transformed into PAA and subsequently methylated into DPMAO via PMAA under anaerobic conditions.

In this study, DPAA transformation rates were compared in four different soils and the effects of adding exogenous sulfate and carbon sources were examined. We found that transformation of DPAA can be enhanced under sulfate-reducing conditions. To the best of our knowledge, the role of sulfate reduction in transformation of DPAA in anaerobic conditions has not been previously reported. The findings in this study can provide a novel insight for biotransformation of DPAA in contaminated soils.



**Fig. 13. Transformation pathway of DPAA under anaerobic soil conditions proposed in this study. A double-lined arrow shows the transformation strongly related to sulfate reduction. Bold arrows show dephenylation and methylation of DPAA that can occur without sulfate being present.**



**Fig. 14. Determination of the main unknown (U1) using LC-TOFMS. (A) soil culture and (B) diphenylthioarsenic acid chemically synthesized, DPTAA.**

**Chapter IV. Sulfate–reducing bacteria as  
mediator of transformation of DPAA  
under anaerobic soil conditions**

## IV-1 Introduction

Arsenic is a ubiquitous and toxic metalloid that ranks twentieth in abundance in the earth's crust (Woolson, 1975). Besides geogenic arsenic contamination, anthropogenic inputs of arsenic may lead to environmental pollution, remaining potential risk for human health.

Phenylarsenic compounds, CLARK I and CLARK II, are best known as chemical warfare agents produced in the First and Second World wars. Even today these chemical weapons can be found around the dumped sites, especially in Belgium, Germany, China, Japan, and the Baltic Sea (Bausinger *et al.*, 2005; Hanaoka *et al.*, 2005; Ishizaki *et al.*, 2005; Garnaga *et al.*, 2006; Wada *et al.*, 2006; Daus *et al.*, 2010). These agents can be chemically transformed to diphenylarsinic acid (DPAA) in groundwater, soils and sediments via hydrolysis and oxidation (Haas *et al.*, 1998). In 2003, prominent cerebellar symptoms attributed to DPAA-contaminated well water used for drinking were observed in several inhabitants in the Kizaki region of Kamisu City, Ibaraki, Japan (Ishii *et al.*, 2004). Although the contaminant source was excavated, it still remains an unsolved problem how to eliminate low-level arsenicals around the contaminated fields.

Dynamic metabolism of aromatic arsenic has extensively been studied in this decade (Ishizaki *et al.*, 2005; Arao *et al.*, 2009; Baba *et al.*, 2008; Maejima *et al.*, 2011). Several reports have been described so far regarding microorganisms participating in aerobic transformation of aromatic arsenicals. Köhler *et al.* (2001) described bacteria play an important role in the release of arsenical compounds from organoarsenic warfare agent contaminated soil. These bacteria slowly degrade the various diphenyl and phenyl arsenical compounds, and release PAA from triphenylarsine oxide. Nakamiya *et al.* (2007) tried to isolate DPAA-degrading microorganisms from toluene-utilizing ones and obtained *Kytococcus sedentarius* strain NK0508 that can transform DPAA to *cis*, *cis*-muconate and arsenic acid. Harada *et al.* (2010) reported the biodegradation of DPAA to arsenic acid by two novel *Ensifer* strains (L2406 and L2413) isolated from the contaminated soil, using

the soil–charcoal perfusion method with a mineral salt medium containing DPAA as the sole carbon source.

On the other hand, only a few reports have described anaerobic biotransformation of phenylarsenicals. Recently, we reported biotransformation of DPAA to arsenate, phenylarsonic acid, phenylmethylarsinic acid, diphenylmethylarsine oxide and three unknown arsenical compounds, when a carbon source was added to submerged DPAA–contaminated soil cultures together with sulfate (in **Chapter III**). The chemical structure of the main arsenic unknown was then determined as diphenylthioarsinic acid (DPTAA) (Hisatomi *et al.*, personal communication). Addition of molybdate, a specific inhibitor of sulfate reduction (Taylor and Oremland, 1979), resulted in the stagnation of DPAA transformation (in **Chapter III**). According to the results, it was hypothesized that indigenous sulfate reducers play a role in transformation of DPAA under anaerobic conditions.

In this study, we incubated anaerobic soil microcosms contaminated with DPAA under sulfate–reducing conditions and tried to obtain microorganisms mediating DPAA–transformation from them. The objective of this report is to document isolation and characterization of several anaerobic sulfate–reducing consortia that could transform DPAA to DPTAA.

## **IV-2 Materials and methods**

### **IV-2-1 Reagents**

All chemicals used in this study were of analytical-grade or higher. DPAA used for isolation of anaerobic consortia capable of transforming DPAA under sulfate-reducing conditions was purchased from Wako (Osaka, Japan). Analytical standards used in LC-ICPMS analysis were described in **Chapter III**.

### **IV-2-2 Soil and rice straw**

The healthy soil sample was taken from the surface layer (0–10cm in depth) of a fallow upland soil in the Ikarashi Campus of Niigata University in 2011. The soil samples were passed through a 2-mm sieve and stored in polyethylene bags at ambient temperature. Rice straw was collected from the Shindori Station of the Field Center for Sustainable Agriculture and Forestry, Niigata University at harvest in 2007. The samples were stored at ambient temperature after drying. The leaf sheath was used after being ground to a powder. Characteristics of the soil and the rice straw were described in **Chapter III**.

### **IV-2-3 Incubation of anaerobic soil microcosms**

To prepare the indigenous bacterial inoculum, 20 g of the air-dried soils, 30 mL of deionized water and potassium sulfate at a rate of 425  $\mu\text{g-S gds}^{-1}$  to the soil culture were added in a 100 mL Erlenmeyer flask. Rice straw powder (10  $\text{mg gds}^{-1}$ , equivalent to 3.5  $\text{mg-C gds}^{-1}$ ) was added as an exogenous carbon source. The soil culture was artificially contaminated with DPAA at a level of ca. 10  $\mu\text{g-As gds}^{-1}$  and was sealed with a double rubber plug. After 3-week incubation, the soil culture showing a significant decrease in DPAA concentration was determined using high performance liquid chromatography (HPLC) as described in **Chapter III** and was

used as the inoculum for isolation of microorganisms with the capability of DPAA transformation.

#### **IV-2-4 Isolation of DPAA-transforming consortia**

Isolation of microorganisms with the capability of DPAA transformation from the anaerobic soil cultures was performed by a limiting dilution-culture method and an anaerobic plate culture method.

An aliquot (1 mL) of the inoculum described above was adequately diluted with sterile saline and transferred into glass tubes containing 9 mL of pH 7.0 mineral salt medium (MM) consisting of 1.2 g  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 0.5 g  $\text{KH}_2\text{PO}_4$ , 0.5 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 10 mL trace element solution (Yamazaki et al. 2008) and 10 mL vitamin solution (Harada *et al.*, 2010) per liter. The media was supplemented with  $0.3 \text{ g-C L}^{-1}$   $\text{CH}_3\text{COONa}$  and  $1431 \text{ }\mu\text{g-As L}^{-1}$  DPAA (final concentrations), and autoclaved at  $121 \text{ }^\circ\text{C}$  for 20 min. The trace element solution and the vitamin solution were filter-sterilized and added into MM after autoclaving. After 1 mL of paraffin was layered on the medium, the tube was sealed with a screw cap and anaerobically incubated under static and dark conditions at  $30 \text{ }^\circ\text{C}$ . DPAA concentrations in the tubes were then determined periodically as follows: an aliquot (1 mL) of each enrichment cultures was centrifuged (10,000 rpm) for 5 min and the supernatant was subsequently filtered through a  $0.45 \text{ }\mu\text{m}$  membrane (Toyo Roshi Kaisha, Tokyo, Japan). The tubes showing a reduction in the DPAA concentrations were determined as “positive” by HPLC (in **Chapter III**). This procedure was repeated several times.

The culture medium in the positive tubes was adequately diluted with sterile saline and was layered on MM agar supplemented with supplemented with  $0.3 \text{ g-C L}^{-1}$   $\text{CH}_3\text{COONa}$  and  $1431 \text{ }\mu\text{g-As L}^{-1}$  DPAA. Anaerobic cultivation was performed in a closed glass jar containing AnaeroPack-Anaero (Mitsubishi Gas Chemical Co., Tokyo, Japan) at  $30 \text{ }^\circ\text{C}$  in the dark for 3 weeks. Capabilities of the colonies found on the plates to transform DPAA were examined in glass tubes containing MM

supplemented with  $1422 \mu\text{g-As L}^{-1}$  DPAA using the method described above. The metabolites in the culture fluids were, then, analyzed using LC-ICPMS, according to **Chapter III**.

#### **IV-2-5 Characterizations of isolated consortia**

Characterizations of the isolated consortia were performed based on comparison of the partial 16S rRNA sequences. Total DNA was extracted from the culture fluids of the positive tubes using DNeasy Blood & Tissue Kit (QIAGEN, Venlo, Netherlands). The amplification of 16S rRNA genes were performed with a primer pair of E27f and E1540r (Weisburg *et al.*, 1991) using the following program: 95 °C, 10 min; 35 cycles of 95 °C 30 s, 60 °C 30 s, and 72 °C 60 s; and 72 °C 10 min; final hold at 4 °C. The reaction mixture for PCR contained 1  $\mu\text{L}$  of template DNA, 2.5  $\mu\text{L}$  of 10 $\times$ buffer, 2.5  $\mu\text{L}$  of 2 mM dNTPs, 2.0  $\mu\text{L}$  of 25 mM  $\text{MgSO}_4$ , 0.25  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ), 0.1 U of rTaq polymerase (Toyobo, Osaka, Japan). An aliquot (5  $\mu\text{L}$ ) of PCR products was subjected to electrophoresis at 100 V for 20–30 min on 1% (w/v) agarose gel stained with ethidium bromide to confirm DNA amplification.

The sequences of the amplicons were determined after cloning with pGEM-T Easy Vector System (Promega, Madison, USA) according to the manufacturer's instructions. Plasmids were purified using High Pure Plasmid Isolation Kit (Roche Applied Science, Mannheim, Germany) and the nucleotide was sequenced by the Fasmac DNA sequencing service (Atsugi, Japan). The sequence results were compared with those available in the GenBank database by the BLAST search.

#### **IV-2-6 Detection of *dsrAB***

DNA extracts from the positive and negative tubes were prepared as above and were used as the templates for PCR detection of *dsrAB* gene coding for dissimilatory sulfite reductase, which is unique to sulfate-reducing bacteria (Wanger *et al.*, 1998). The

amplification of 16S rRNA genes were performed with a primer pair of *dsr1F* (5'-ACSCACTGGAAGCACG-3') of 16S rRNA and *dsr4R* (5'-GTGTAGCAGTTACCGCA-3') (Wanger *et al.*, 1998). The other PCR conditions were same as those for 16S rRNA genes as described above.

#### **IV-2-7 Inoculation study using known sulfate-reducing bacterial strains**

Pure strains of sulfate-reducing bacteria were examined to confirm whether or not they have ability to transform DPAA. The type strain of *Desulfovibrio aerotolerans* (JCM 12613<sup>T</sup>) was purchased from Japan Collection of Microorganisms, RIKEN BioResource Center (Wako, Japan), and was cultivated in 9 mL DvO5 medium (Mogensen *et al.*, 2005). After layered with 1 mL paraffin, the tubes were sealed with screw caps and were anaerobically incubated at 30 °C. The ability of the strains to transform DPAA was examined using HPLC and LC-ICPMS as described above.

#### **IV-2-8 Nucleotide sequence accession numbers**

The sequences obtained in this study have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB779717 to AB779747.

## IV-3 Results

### IV-3-1 Isolation and characterization of DPAA-transforming consortia

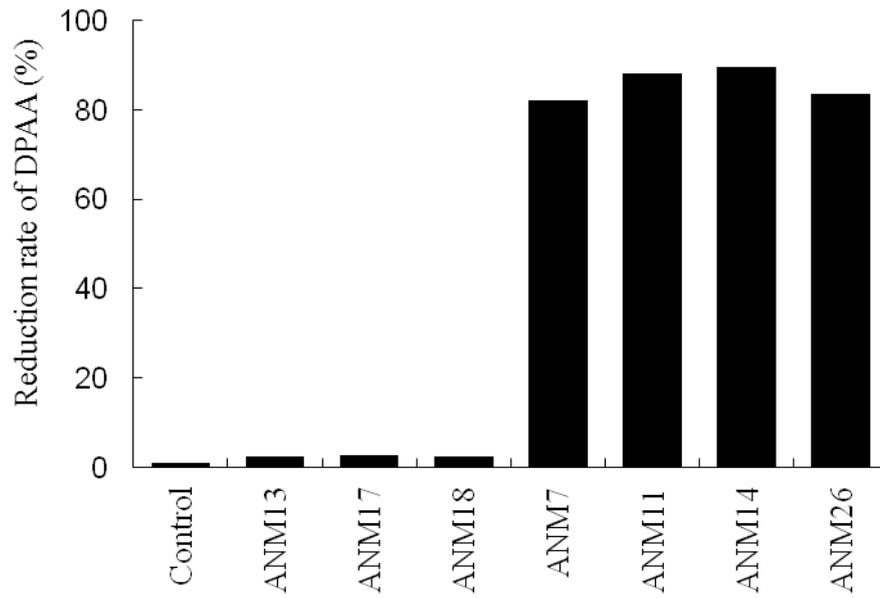
After 3-weeks incubation, DPAA concentration in the soil microcosms decreased from the initial 9.24 to 1.39  $\mu\text{g-As gds}^{-1}$ . After isolation of anaerobic microorganisms capable of transforming DPAA from the soil microcosms, four positive tube cultures showing significant decrease of DPAA concentration in MM supplemented with 0.3  $\text{g-C L}^{-1}$   $\text{CH}_3\text{COONa}$  (ANM7, ANM11, ANM14 and ANM26) were found. The transforming rates of DPAA in the positive tube cultures were 80–89% after 4-week incubation, while those of the negative tube cultures were not more than 3% (**Fig. 15**). The typical smell of hydrogen sulfide and black precipitation were found only in the positive tubes, meaning sulfide production and subsequent precipitation of FeS.

To characterize the DPAA-transforming consortia, 16S rRNA gene clone libraries were constructed from the four positive tube cultures and each 7 or 8 sequences were determined as shown in **Table 6**. Homology search of the sequence data against the Genbank database revealed that ANM7 and ANM26 mainly composed of *Acinetobacteria*, *Bacillus* and *Desulfotomaculum* species. ANM11 and ANM14 contained *Desulfotomaculum* and *Bacillus* species. Among the 31 sequences determined in this study, 16 sequences were assigned to *Desulfotomaculum acetoxidans*, which grow with acetate as an electron donor and a carbon source (Widdel and Pfennig, 1977). The sulfate-reducing bacterial species were commonly found in the four positive consortia. It is interesting that *Desulfotomaculum acetoxidans* were determined in the microbial consortia originated from the sand-dune upland soil, while known *Desulfotomaculum acetoxidans* species have been isolated only from anaerobic environments such as piggery waste, animal manure, rumen content, dung-contaminated freshwater, rice field soil and freshwater sediment (Widdel and Pfennig, 1977; Scholten and Stams, 2000).

As above, it is suggested that sulfate-reducing bacteria were most consistent in the DPAA-transforming consortia. We then examined whether or not the negative

consortia showing no DPAA reduction contained sulfate-reducing bacteria using the PCR detection method targeting *dsrAB*. As a result, while *dsrAB* was detected in all the positive consortia, no PCR product was found in the negative ones (**Fig. 16**). These results indicate strong relationship between sulfate-reducing bacteria and DPAA-transformation under anaerobic conditions.

To confirm participation of sulfate-reducing bacteria in the DPAA-transformation process, ability of a sulfate-reducing bacterial strain, *Desulfovibrio aerotolerans* JCM 12613<sup>T</sup>, to transform DPAA was examined in pure culture. After 4-week incubation, 95% decrease of DPAA was found in the culture medium, while little change in DPAA concentration was observed in the uninoculated control. This fact gave further support to the hypothesis that sulfate-reducing bacteria have a function to transform DPAA.

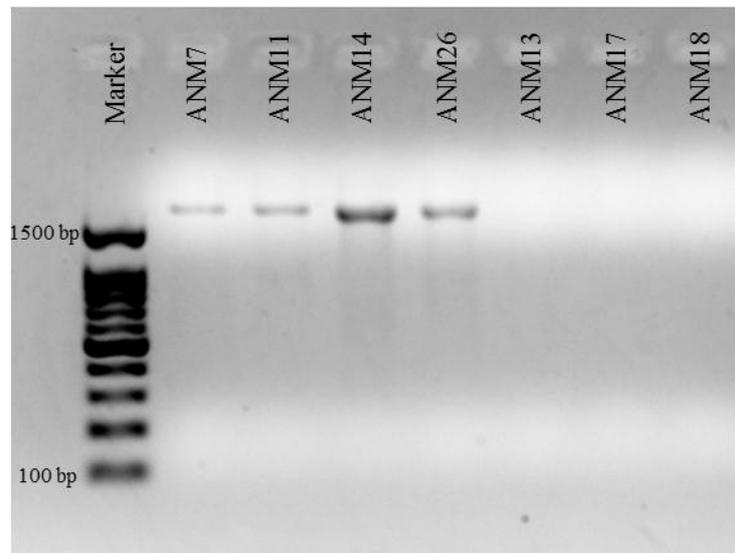


**Fig. 15. Transforming rates of DPAA in the four positive tube cultures (ANM7, ANM11, ANM14 and ANM26) after 4-week incubation as well as those in randomly-selected three negative tube cultures (ANM13, ANM17 and ANM18) and the uninoculated culture.**

**Table 6** Closest relatives of bacterial 16S rDNA-based clone sequence

Clone (accession no.)	Closest relative (accession no.)	Order	Alignment	Homology (%)	Reference	Source
ANM7-1 (AB779717)	<i>Acinetobacter sp.</i> (JQ917834)	Pseudomonadales	737/739	99		tallgrass prairie soil
ANM7-2 (AB779718)	<i>Acinetobacter sp.</i> (EF520734)	Pseudomonadales	738/739	99	Song <i>et al.</i> (2008)	waste vegetable oil
ANM7-3 (AB779719)	<i>Desulfotomaculum acetoxidans</i> (CP001720)	Clostridiales	739/740	99	Spring <i>et al.</i> (2009)	
ANM7-4 (AB779720)	<i>Acinetobacter sp.</i> (JQ917834)	Pseudomonadales	704/705	99		tallgrass prairie soil
ANM7-5 (AB779721)	<i>Acinetobacter sp.</i> (JQ917834)	Pseudomonadales	705/705	100		tallgrass prairie soil
ANM7-6 (AB779722)	<i>Acinetobacter sp.</i> (JQ917834)	Pseudomonadales	705/705	100		tallgrass prairie soil
ANM7-7 (AB779723)	<i>Bacillus sp.</i> MBEA31 (AB733526)	Bacillales	718/718	100		deep-sea
ANM7-8 (AB779724)	<i>Acinetobacter sp.</i> (JQ917834)	Pseudomonadales	705/705	100		tallgrass prairie soil
ANM11-1 (AB779725)	<i>Desulfotomaculum acetoxidans</i> (CP001720)	Clostridiales	584/622	94	Spring <i>et al.</i> (2009)	
ANM11-2 (AB779726)	<i>Bacillus sonorensis</i> (JX518264)	Bacillales	717/718	99		salt crystallizers
ANM11-3 (AB779727)	<i>Bacillus licheniformis</i> (DQ295037)	Bacillales	718/718	100		probiotic products
ANM11-4 (AB779728)	<i>Bacillus licheniformis</i> (JN998728)	Bacillales	716/718	99		fermented soybean paste
ANM11-5 (AB779729)	<i>Bacillus licheniformis</i> (JX203259)	Bacillales	717/719	99		
ANM11-6 (AB779730)	<i>Desulfotomaculum acetoxidans</i> (CP001720)	Clostridiales	730/731	99	Spring <i>et al.</i> (2009)	
ANM11-7 (AB779731)	<i>Desulfotomaculum acetoxidans</i> (CP001720)	Clostridiales	662/668	99	Spring <i>et al.</i> (2009)	
ANM11-8 (AB779732)	<i>Bacillus licheniformis</i> (DQ295037)	Bacillales	717/718	99		probiotic products
ANM14-1 (AB779733)	<i>Desulfotomaculum acetoxidans</i> (NR_027608)	Clostridiales	687/695	99	Stackebrandt <i>et al.</i> (1997)	
ANM14-2 (AB779734)	<i>Desulfotomaculum acetoxidans</i> (CP001720)	Clostridiales	705/717	98	Spring <i>et al.</i> (2009)	
ANM14-3 (AB779735)	<i>Desulfotomaculum acetoxidans</i> (CP001720)	Clostridiales	624/629	99	Spring <i>et al.</i> (2009)	
ANM14-4 (AB779736)	<i>Desulfotomaculum acetoxidans</i> (CP001720)	Clostridiales	720/731	98	Spring <i>et al.</i> (2009)	

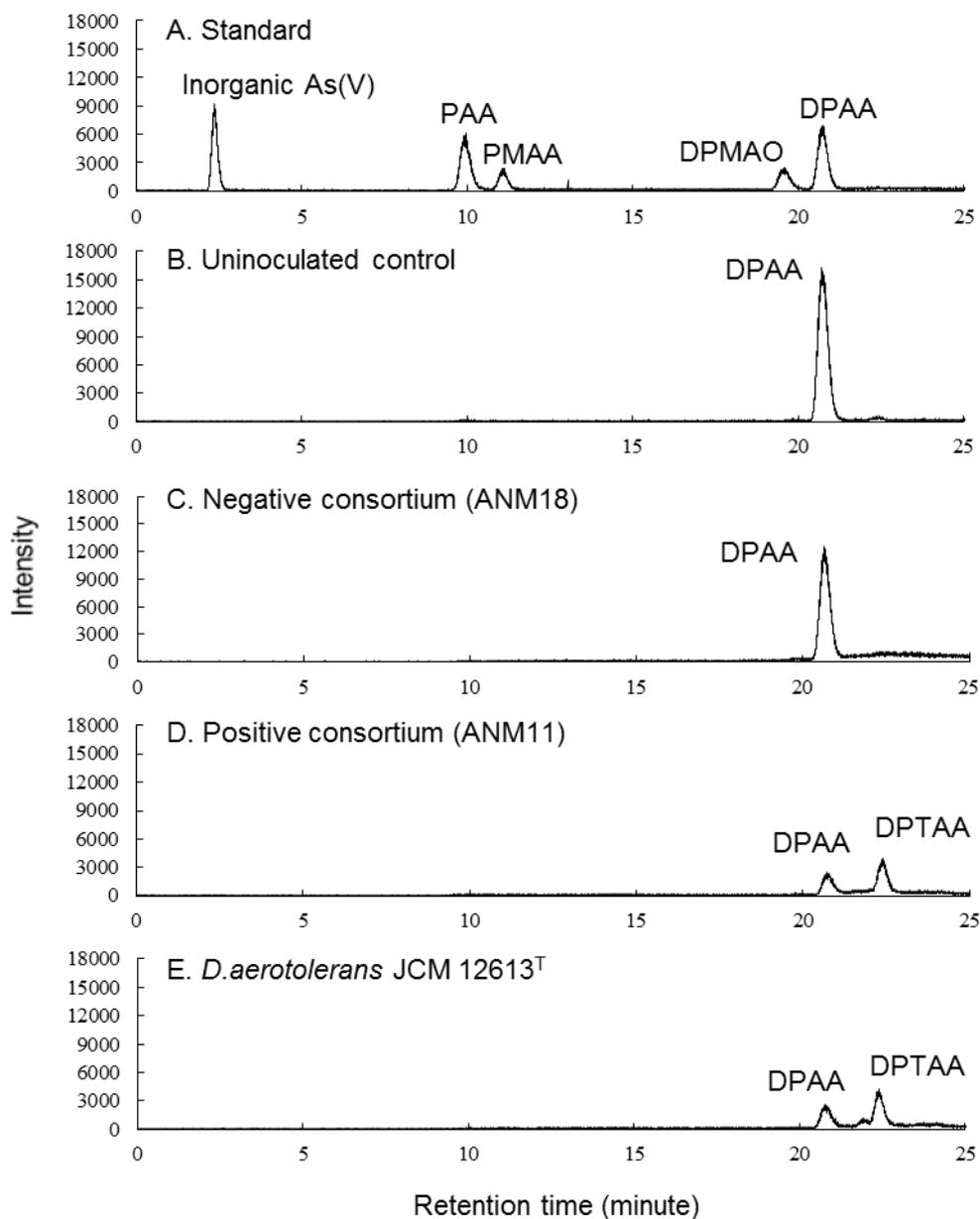
ANM14-5 (AB779737)	<i>Desulfotomaculum acetoxidans</i> (CP001720)	Clostridiales	619/622	99	Spring <i>et al.</i> (2009)	
ANM14-6 (AB779738)	<i>Bacillus licheniformis</i> (GU323372)	Bacillales	729/734	99		
ANM14-7 (AB779739)	<i>Desulfotomaculum acetoxidans</i> (CP001720)	Clostridiales	691/695	99	Spring <i>et al.</i> (2009)	
ANM14-8 (AB779740)	<i>Desulfotomaculum acetoxidans</i> (CP001720)	Clostridiales	724/731	99	Spring <i>et al.</i> (2009)	
ANM26-1 (AB779741)	<i>Acinetobacter</i> sp. (JQ917834)	Pseudomonadales	711/712	99		tallgrass prairie soil
ANM26-2 (AB779742)	<i>Desulfotomaculum acetoxidans</i> (CP001720)	Clostridiales	725/731	99	Spring <i>et al.</i> (2009)	
ANM26-3 (AB779743)	<i>Desulfotomaculum acetoxidans</i> (NR_027608)	Clostridiales	723/731	99	Stackebrandt <i>et al.</i> (1997)	
ANM26-4 (AB779744)	<i>Bacillus</i> sp. MBEA31(AB733526)	Bacillales	751/753	99		deep sea
ANM26-5 (AB779745)	<i>Acinetobacter oleivorans</i> DRI(EF520734)	Pseudomonadales	738/738	100	Song <i>et al.</i> (2008)	waste vegetable oil
ANM26-6 (AB779746)	<i>Desulfotomaculum acetoxidans</i> (NR_027608)	Clostridiales	687/697	99	Stackebrandt <i>et al.</i> (1997)	
ANM26-7 (AB779747)	<i>Desulfotomaculum acetoxidans</i> (CP001720)	Clostridiales	652/658	99	Spring <i>et al.</i> (2009)	



**Fig. 16. PCR amplification of *dsrAB* gene coding for dissimilartory sulfate reductase.**

#### IV-3-2 DPAA metabolites

LC-ICPMS analysis was performed to identify the metabolites of DPAA in the negative and positive tube culture fluids after 4-week incubation under sulfate-reducing conditions. Both in the uninoculated control and the negative consortium, ANM18, no DPAA metabolites were detected during the whole cultivation (**Fig. 17 B and C**). High As recovery rates of 93.9% and 101.8% were achieved for the uninoculated control and ANM18, respectively. In contrast, DPTAA was observed as the main metabolite in the positive consortium (ANM11) and the *Desulfovibrio aerotolerans* JCM 12613<sup>T</sup> culture at the concentrations of 223.5 and 224.3  $\mu\text{g-As L}^{-1}$  estimated using the DPAA peak as the standard for calculation, respectively. The metabolic pattern of DPAA is similar with that found in the anaerobic soil cultures incubated under sulfate-reducing conditions (in **Chapter III**). Unlike the uninoculated control and the negative consortium, the positive consortium and *Desulfovibrio aerotolerans* JCM 12613<sup>T</sup> culture showed a low recovery rate at 28.7 and 31.3%, respectively. This suggests subsequent transformation of the metabolites into other unknown arsenical species that could not be detected under the analytical conditions used in this study. Further investigations are strongly required to elucidate behavior of thionated phenylarsenicals in environment.



**Fig. 17.** LC-ICPMS chromatograms obtained from (A) a standard solution containing each of  $5 \mu\text{g-As L}^{-1}$  arsenate,  $2 \mu\text{g-As L}^{-1}$  phenylmethylarsinic acid (PMAA),  $5 \mu\text{g-As L}^{-1}$  PAA,  $4 \mu\text{g-As L}^{-1}$  diphenylmethylarsine oxide (DPMAO) and  $5 \mu\text{g-As L}^{-1}$  DPAA; (B) MS control; (C) negative culture (ANM18); (D) positive culture ANM11; (E) *Desulfovibrio aerotolerans* strain.

#### IV-4 Discussions

Under reducing environments, arsenic can form insoluble sulfide compounds in the presence of S and Fe (Buddhawong *et al.*, 2005; Singhakant *et al.*, 2009), such as  $As_2S_3$  and  $AsFeS$ . Moreover, sulfate-reducing bacteria can mediate the precipitation of arsenosulfide minerals. Newman *et al.* (1997) pointed out that *Desulfotomaculum auripigmentum* precipitate arsenic trisulfide ( $As_2S_3$ ) through the reduction of As (V) to As (III). Groudev *et al.* (2008) also described that the precipitation of arsenic as sulfides in sediments was due to active sulfate-reducing bacteria, including the genera of *Desulfovibrio*, *Desulfobulbus*, *Desulfococcus*, *Desulfobacter* and *Desulfosarcina*. Similar publication was also reported by Duncan *et al.* (2004). Additionally, sulfate-reducing bacteria can contribute to the anaerobic biotransformation of organoarsenical pesticides monomethylarsinic acid (MMAA) and dimethylarsinic acid (DMAA) (Sierra-Alvaerz *et al.*, 2006). A portion of DMAA and MMAA in municipal landfill leachate was demonstrated to transform into thiol-organoarsenicals, identified as dimethyldithioarsinic acid (DMDTAA), dimethylmonothioarsinic acid (DMMTAA) and monomethyldithioarsinic acid (MMDTAA) by HPLC-ICPMS and LC-ESI-MS/MS (Li *et al.*, 2011). Although several studies have shown that microbial sulfate reduction mediates solidification of inorganic arsenicals and thionation of methylarsenicals, this is the first report regarding transformation of phenylarsenicals mediated by sulfate-reducing bacteria, to the best of our knowledge.

In conclusion, we obtained several anaerobic consortia mediating transformation of DPAA to DPTAA from the soil cultures and found that the main constituent bacteria in the consortia were sulfate-reducing bacteria such as *Desulfotomaculum acetoxidans*. The transformation could also be carried out in a pure culture of *Desulfovibrio aerotolerans* JCM 12613<sup>T</sup>. These facts indicate that sulfate-reducing bacteria can generally participate in transformation of DPAA to DPTAA under anaerobic conditions, when sufficient metabolizable carbon sources were supplied.

## **Chapter V. Conclusions and future work**

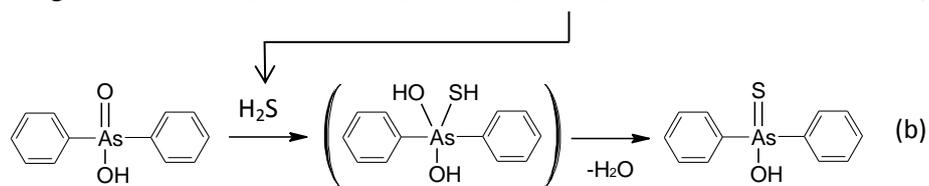
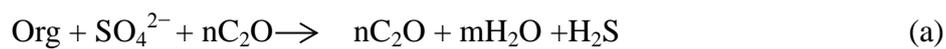
## V-1 Conclusions

The following conclusions can be drawn from this study:

- 1) DNA based PCR–DGGE fingerprinting targeting bacterial and archaeal 16S rRNA genes revealed DPAA and/or its metabolites have a direct effect on some abundant bacterial species, overall bacterial and archaeal community structures under the anaerobic soil conditions tend to be stable after early successional changes due to submergence and rice straw application, regardless of DPAA contamination
- 2) DPAA transformation rates were compared in four different soils and the effects of adding exogenous sulfate and carbon sources were examined. We found that DPAA degradation can be improved by addition of exogenous sulfate and carbon source. As the main unknown metabolite, the chemical structure of U1 was elucidated as diphenylthioarsinic acid (DPTAA).
- 3) The sequencing results revealed that four positive microbial consortia that could transform DPAA to DPTAA contained *Desulfotomaculum acetoxidans* species. These findings suggest that *D. acetoxidans* may take a role in the anaerobic transformation of DPAA to DPTAA.

Basing on 2) and 3), we estimated the new transformation pathway–DPAA thionation in submerged soil as follows:

- 1) Sulfate–reducing bacteria oxidize simple organic compound (e.g. acetate) by utilizing sulfate as an electron acceptor and generating hydrogen sulfide under anaerobic conditions (Eq. (a)),
- 2) The biologically produced hydrogen sulfide reacts with dissolved DPAA to form DPTAA (Eq. (b))



To the best of our knowledge, the role of sulfate reduction in transformation of DPAA in anaerobic conditions has not been reported. The findings in this study can provide a novel insight for biotransformation of DPAA in contaminated soils.

## V-2 Future work

We have reported anaerobic DPAA transformation can be enhanced under sulfate-reducing condition and identified DPTAA as a metabolite of DPAA. As several unknown arsenicals were also found during anaerobic DPAA-transformation, further studies should be carried out to investigate the presence of any other S-constituted phenylarsenicals and their behavior in the experimental model, using LC-TOF-MS or LC-ESI-MS method. Meanwhile, the relevant sulfate-reducing bacteria capable of DPAA-thionating will be isolated by a limiting dilution culture method or identified by 16S rRNA-targeted PCR.

In the last decades, a wide range of bacteria (e.g. members of the genera *Enterobacter*, *Pseudomonas*, *Methanobacterium* and *Bacillus*) with the capability of inorganic arsenic methylation has been extensively discussed. However, no information on microorganisms associated with methylation of phenylarsenicals has been available yet. Therefore, investigations with the aim to isolate microorganisms contributing to methylation of phenylarsenicals in anoxic soils contaminated with DPAA should be performed.

For bioremediation of phenylarsenicals, we considered that the combination of phenylarsenical-thionation/methylation will be favorable for the transformation of DPAA to inorganic arsenicals. In case, the phytoremediation method will be tested for the further arsenical removal, etc. the absorption by plants.

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## **Acknowledgements**

This research was financed in part by the Grants-in-Aid for Scientific Research (no. 22510083) from the Japan Society for the Promotion of Science and Iijima Memorial Foundation for the Promotion of Food Science and Technology. We thank the Field Center for Sustainable Agriculture and Forestry, Niigata University, for providing paddy soil samples. We thank the Field Center for Sustainable Agriculture and Forestry, Niigata University, for providing paddy soil samples.