Arsenic removal potential of temperate zone fern at low temperature and its application to an arsenic contaminated leachate.

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Doctoral Dissertation

Arsenic removal potential of temperate zone fern at low temperature and its application to an arsenic contaminated leachate

(低温における温帯性シダのヒ素除去能力の検討とそのヒ素含有浸出水浄化への応用)

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List of Contents

CHAPTER 1

General introduction

1-1 Hazards of As contamination in water

The general collective term “heavy metal” means the group of metals and metalloids with atomic density greater than 4000 kg/m^3 or 4 times more than water (Garbarino et al., 1995) and they are natural components of the earth’s crust. The main threats to human health from heavy metals are associated with exposure to mercury (Hg), lead (Pb), cadmium (Cd) and arsenic (As). The heavy metals are generally more persistent than organic contaminants in the environment, such as pesticides or petroleum byproducts. The heavy metal compounds in soil may become mobile depends on their speciation and the pH of soil, which results in a fraction of them may leach to aquifer or become bio-available to living organisms (Alloway, 1990; Santona et al., 2006).

Heavy metals have been used in many different areas for thousands of years. The basic use of metallic arsenic is in alloys of lead (for example, in car batteries and ammunition). Arsenic is a common n-type dopant in semiconductor electronic devices, and after doped silicon the second most commonly used semiconductor is the onto electronic compound gallium arsenide. Lead has been used for at least 5000 years, recent applications includes building materials, pigments for glazing ceramics, and pipes for transporting water etc. Cadmium pigments were used extensively in the mid-1800s, but finally the scarcity of the metal limited the use in artists’ materials until the early 1900s.

Although adverse health effects of heavy metals have been known to all for a long time, exposure to heavy metals continues and is even increasing in some areas. For example, arsenic is still common in wood preservatives, and tetra-ethyl lead remains a common additive to petrol. Although this use has decreased dramatically in the developed countries, but developing countries still has been using in most cases. Actually, in the middle of the 19th century, production of heavy metals increased steeply for more than 100 years with concomitant emissions to the environment.

Contamination in the food chain is the major pathway of heavy metal exposure for humans (Khan et al. 2008). Industrial or municipal wastewater that used for irrigation, is a common reality for heavy metal pollution in three fourth of the cities in Asia, Africa, and Latin America (Gupta et al. 2008). Industries wastewater or contaminated water from other sources carries an appreciable amount of toxic heavy metals which create a problem for safe rational utilization of agricultural soil (Yadav et al. 2002; Chen et al. 2005; Singh et al. 2004).

Comparing with other heavy metals, the adverse effect of arsenic pollution is more severe. As toxicity can be classified into two categories, such as acute and chronic: acute toxicity may cause digestive disturbance and fast pulse. More serious diseases like cancer or nerve disturbance might be caused by chronic toxicity. Moreover, it was reported that 42.7 million of people in West Bengal, India and 79.9 million of people in Bangladesh are suffered from groundwater contaminated by As since the As levels in groundwater in these areas are above the World Health Organization maximum permissible limit of 50 µg/L (Chowdhury *et al.* 2000). Considering the aforementioned situation, it is high time to control heavy metal pollution, especially As pollution from contaminated site.

1-2 Recent remediation technologies for As removal from water

Several technologies are available for the remediation of heavy metals contaminated water with some definite outcomes such as: (i) complete or substantial destruction/degradation of the pollutants, (ii) extraction of pollutants for further treatment or disposal, (iii) stabilization of pollutants in forms less mobile or toxic, (iv) separation of non-contaminated materials and their recycling from polluted materials that require further treatment, and (v) containment of the polluted material to restrict exposure of the wider environment (Nathanail and Bardos, 2004; Scullion, 2006; M.A. Hashim et al. 2011). The treatment technologies can be divided into the following classes: i. Chemical Treatment Technologies, ii. Biological/Biochemical/Biosorptive Treatment Technologies, iii. Physico-Chemical Treatment Technologies. The use of biological methods has been recommended because they can be cheap, effective and environmental-friendly compared with other conventional (physical and chemical) methods (Hanif, Bhatti and Hanif 2009). The biological processes such as phytoremediation, phytoextraction and hyperaccumulation have been used for long term remediation purposes in conjugation with some other more intense remediation process. In-situ arsenic removal by microorganisms, plants and ferrous oxides has been proved to be a very effective and sustainable technology in practice. Although it is a long term process but it has long lasting effect on aquifer. No waste is generated and practically no chemical is required to create an oxygenation zone in the aquifer which makes this process cost effective and environmental friendly. It maintains a very fine balance between coprecipitation of As(V) with iron(III) and adsorption of the former into the later (M.A. Hashim et al. 2011). Adsorption is more acceptable than coprecipitation and can be achieved by calculated oxygenation process. Biosorption is a highly practical solution for heavy metal remediation from water or soil and is a much researched field of study. Biosorption is cost effective, environment friendly and has possibility of metal recovery and also generates minimum sludge. Biosurfactants are biodegradable and they can solubilize metals by reducing surface tension and increasing their wettability, thus bringing them out of soil or aquifer matrix. However, the practical field application for heavy metal removal is still limited. Metal uptake by various plants and organisms is principally a slower natural process that can be used in field for long term remediation measures. However, the immobilized metals can be leach back in the solution under influence of acidic pH. Agricultural wastes and cellulosic materials have huge potential to be used again for biosorption of heavy metals through ion exchange process, surface complexation and electrostatic interactions.

The technologies that have been used recently and are undergoing further tests in laboratory are also discussed. The summary of some important heavy metal remediation methods for treatment of contaminated water are given at Table 1-1.

Contaminated water treatment technologies have come a long way since the days of their inception. Much research has been done on various technologies ranging from simple ex-situ physical separation techniques to complex in-situ microbiological and adsorption techniques. In modern days, sustainability is the keyword to apply any process. Instant remediation may provide a temporary solution to a problem but it may not be a permanent one. Therefore, natural processes and biogeochemistry of the water should be given due consideration before planning remediation processes. Considering sustainability of method, cost and the effect on the environment, biological methods like phytoremediation might be one suitable solution for removal of heavy metals from contaminated water.

Table 1-1 Heavy metal remediation methods for contaminated water (Hashim et al. 2011. Partially modified)

Remediation method	**Application and Advantage**
Reduction by dithionites	Injected in aquifer for Redox sensitive elements (Cr, U, Th). Active over larger area; Long lasting effect.
Reduction By H_2S (g)	Application by carrier gas medium, no secondary waste generation
Bioremediation	To digest heavy metals by biological activity
Phytoremediation	To remove toxic heavy metals by using plant, low cost and environment friendly method for widely spread pollution
ISBP process	immobilizes the heavy metals as sulphide precipitate through BSR process
Chelate Flushing	In-situ injection of chelates e.g. EDTA, NTA, DTPA, SDTC, STC, K_2BDET . Ligands act at very low dose; Stable complexes formed; Chelates can be regenerated
Remediation by selective ion exchange	In-situ use of synthetically prepared type II SIRs and ion exchange resins in PRBs Selectively remove low level of metal ions from contaminated aquifer, despite high concentration
Chemical fixation	Using red mud and mixture of $FeSO_4$, $CaCO_3$, $KMnO_4$ and $Ca(H_2PO_4)_2$; Stabilization of metals like As, Pb etc. by oxidizing and trapping in the structure
Immobilization of radionuclides	Removal of U, Tc and Ra by micro-organisms of geobacter species

1-3 Arsenic hyperaccumulating tropical and temperate zone ferns

The term “hyperaccumulator” describes a number of plants that belong to distantly related families and to accumulate extraordinarily high amounts of heavy metals in the aerial organs, far in excess of the levels found in the majority of species, without suffering phytotoxic effects (Nicoletta R. et al., 2011). Although hyperaccumulation of heavy metals or metalloids is a rare phenomenon in terrestrial higher plants, more than 400 taxa of hyperaccumulator species have been identified, in those about three-quarters are nickel (Ni) hyperaccumulators (Baker *et al*., 2000). A wide range of plant species have been identified as being As resistant (Meharg & Hartley-Whitaker, 2002), and As hyperaccumulation was discovered initially in the brake fern *Pteris vittata* (Ma *et al*., 2001). Table 1-2 showed some naturally grown As hyperaccumulating plants. Though *P. vittata* is the mostly studied arsenic hyperaccumulator fern, it is limited by its cold tolerance. Being a tropical zone fern, it cannot accumulate As at low temperature ($\leq 5^0$C) instead of its highest efficiency at 25^0C. *P. vittata* naturally grows in Florida (USA), South east part of Asia and very south part of Japan, belongs to sub-tropical zone (average temperature: 7-15°C in winter; 25-30°C in summer). The germination and growth rates of P. vit*tata* are also limited at around 25°C (Wan et al. 2009).

Table 1-2 Some naturally grown As hyperaccumulating plants.

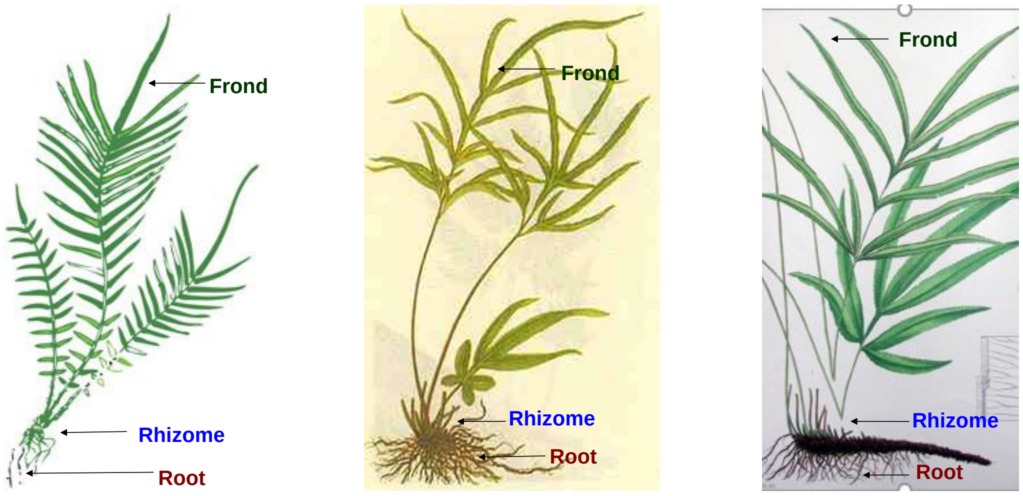
Plant species	Quantity of As (mg/Kg)	Referances
P. vittata	22,630 (in shoot)	Ma et al., 2001
P. multifida	5,510 (in frond)	Terrence S. et al., 2009
P. cretica	5,584 (in frond)	Terrence S. et al., 2009
Lemna gibba	1,021 (in shoot)	Mkandawire and Dudel, 2005
Ceratophyllum demersum	963 (in shoot)	Saygideger et al., 2004
Typha latifolia	1,120 (in shoot)	Ye et al., 1997
Pteris longifolia	5,000 (in frond)	Zhao et al., 2002
Pteris umbrosa	5,000 (in frond)	Zhao et al., 2002
Pityrogramma calomelanos	5,000-8350 (in frond)	Francesconi et al., 2002

Aiming at searching for cold tolerant As hyperaccumulating fern, two renowned temperate zone fern named *P. cretica* and *P. multifida* was considered in this study. In order to compare As accumulating potential and cold tolerance ability of the above temperate ferns, tropical zone fern *P.vittata* was considered as the most studied As hypreaccumulator. *Pteris multifida* (spider brake fern) and *Pteris cretica* (Cretan brake) are two common plants native to Europe, Asia and Africa. Both of them are also distributed almost all areas in Japan and China including temperate areas. *P. cretica* can grow in a minimum temperature of 2 °C (RHS et al., 2008). Similarly, *P. multifida* has already shown some ability to tolerate low temperatures from 5 °C to -4.6°C (measured by thermometer in the field) during field experiments (Sugawara, Chien and Inoue 2015), which is an essential property for large scale phytoremediation in temperate zones such as the northern part of Japan. Among the above As accumulating plants, *Pteris* is the largest genus that has the significant potential to hyperaccumulate As. From the *Pteris* genus, *P. multifida* as well as *Pteris cretica* draw our attention because they naturally grow at temperate zone thus have potential to accumulate As at low temperature.

1-4 Phytofiltration by temperate zone ferns at low temperature

Phytoremediation is a plant-based green technology, a promising technology for environmental pollution caused by unavoidable limitations of traditional technologies (Rahman et al., 2008a). Nowadays, heavy metal accumulation by plants, such as *Cynodon dactylon* (Wu *et al.* 2010), *Salvinia natans* (Dhir and Srivastava 2011), *Melastoma malabathricum* L. (Selamat, Abdullah and Idris 2014), Switchgrass (Jeke, Zvomuya and Ross 2016) and *Pteris vittata* (Ronzan et al. 2017) have been reported as successful phytoremediation strategies. The use of plants in the process of phytoremediation from aquatic medium is more commonly known as phytofiltration. In this study, I have tried to introduce phytofiltration potential of two temperate zone ferns (*P. multifida* and *P. cretica*) at low temperature compared with most studied tropical zone fern *P. vittata* for the first time.

Temperature has a deep effect on plant growth since low temperature affects transpiration, growth and metabolism of plants and therefore both uptake and elimination efficiencies of pollutants (Yu et al. 2005). But the plants that grow naturally in temperate zone can survive and accumulate heavy metals even at low temperature. Temperate zone plants may have temperature sensing systems which help them to adapt adverse environment. In this study, effects of low temperature on temperate zone ferns (*P. multifida* and *P. cretica*) were compared with a tropical zone fern (*P. vittata*) which distinguished the morphological and heavy metal removal potential of temperate and tropical zone ferns. Temperate zone ferns *P. multifida* and *P. cretica* are confirmed as hyperaccumulators of inorganic arsenic which *P. multifida* as well as *P. cretica* are efficient at arsenic extraction with maximum frond arsenic concentrations of 5,510 ppm and 5,584 ppm respectively (Terrence S. et al., 2009). *P. multifida* was considered more tolerant to arsenic than *P. cretica* was since the biomass of *P. multifida* was bigger than *P. cretica*. Another study showed that *P. multifida* can hyperaccumulate As in their fronds with high concentrations in addition to *P. vittata* and *P. cretica* which have been previously identified as As hyperaccumulators (Wang HB1 et al., 2006). On the other hand, tropical fern *P. vittata* is the first discovered As hyperaccumulator, able to accumulate 22,630 mg/kg of As in the shoots from soil contained 15,00 mg/kg of As (Ma et al., 2001). The appearance and name of each parts of *P. vittata*, *P. multifida* and *P. cretica* are showing Fig: 1-1.



Pteris vittata *Pteris multifida* *Pteris cretica*

Figure 1-1 shows the appearance and name of each parts of three ferns.

1-5 As accumulation mechanism of *P. vittata*

Pteris vittata, the first discovered and mostly studied As hyperaccumulator, can accumulate 22,630 mg/kg of As in the shoots when the fern was cultivated in soil contained 1,500 mg/kg of As (Ma et al., 2001). Fig 1-2 shows the appearance and parts of *P. vittata*.

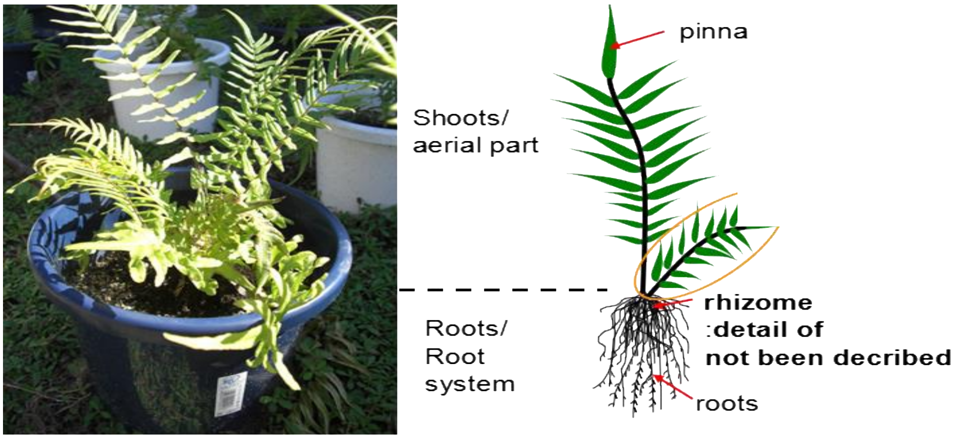
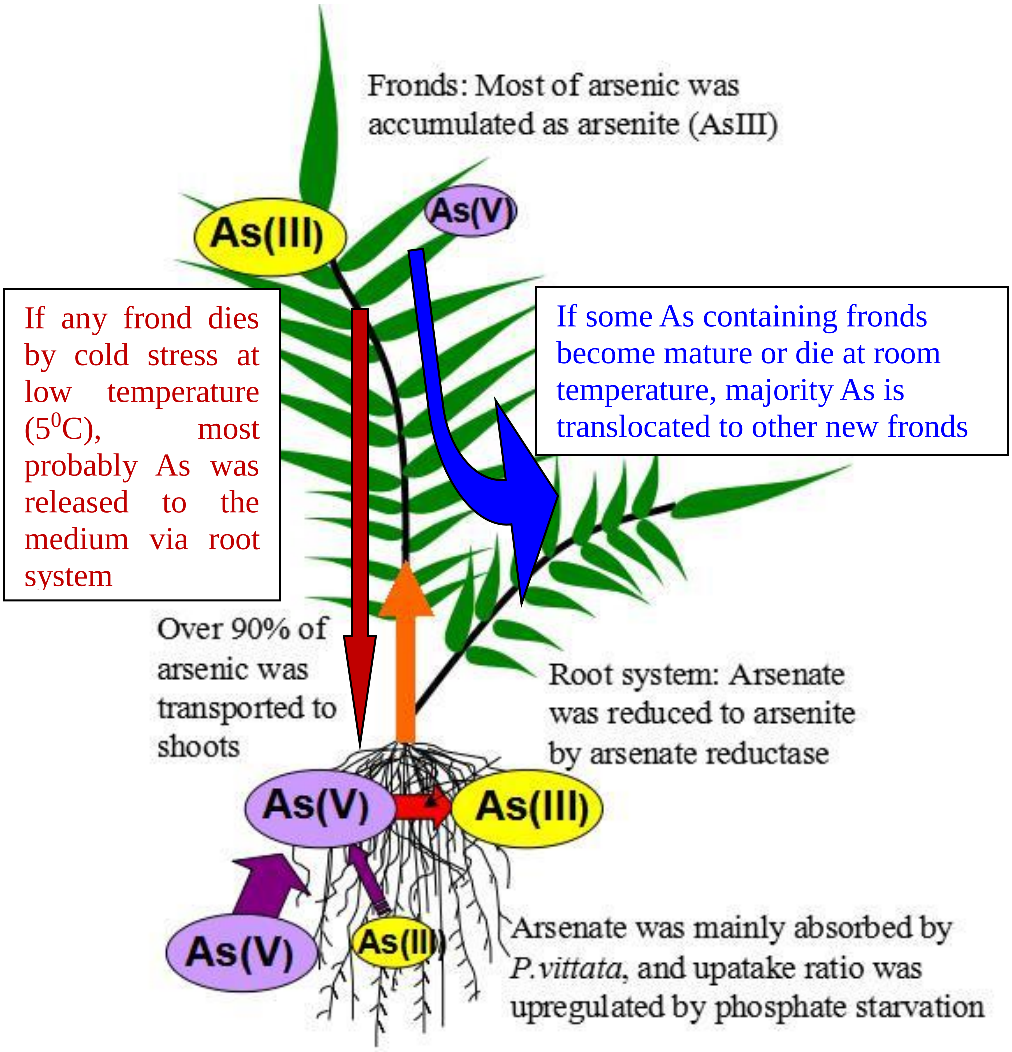


Fig. 1-2 Appearance of *P. vittata* and name of each part. (Sugawara K., PhD thesis, 2015)

Arsenate (As(V)) is mainly absorbed by *P. vittata* from the environment, then As(V) was reduced to arsenite (As(III)) within root system. More than 90% of As(III) was transported to shoots via xylem and accumulate as free As(III) in the fronds (Ma et al., 2001; Su et al., 2008). Actually As(V) is absorbed via phosphate transporter due to the chemical analog between As(V) and phosphate (Meharg and Macnair, 1992). In case of As(III), it is transported via the aquaporin, which was one of water transporter (Bienert et al., 2008). Elemental As accumulation mechanism was described like above, but detail of each accumulation and transportation has not been described. For example, *P. vittata* reduces As(V) to As(III) which is more toxic form. To describe this phenomenon, As(III) specifically was bound to detoxification with low molecular thiols such as phytochelatin, glutathione. Then, Arsenite-thiol complex was transported into the cell vacuole (Zhang et al., 2004; Pickering et al., 2006). However, it was reported that the material balance of sulfur and arsenic did not match in pinna of *P. vittata* (Sakai et al., 2010). Furthermore, Moore et al. (2014) reported that As was not only accumulated in vacuolar of cell but also the cell wall in *Oryza sativa*. These reports were suggested that *P. vittata* may has other As resistance and hyperaccumulation system besides As-thiol conjugation and accumulation of As in the vacuolar. K. Sugawara (2015) has conducted As translocation and efflux test at room temperature (25°C) in hydroponics system to ensure the translocation of As from As-exposed fronds to other organs. During 24h exposure, majority of As was translocated to other organs with very little As excretion from roots to medium. If some As containing fronds become mature or die at 25°C, most of the As was translocated to other new fronds but if any frond dies by cold stress at low temperature (5°C), most probably As was released to the medium via root system. Thus, it is important to understand As hyperaccumulation and translocation system in *P. vittata*. As accumulation and transportation mechanism of *P. vittata* shows in Fig. 1-3.



Fronds: Most of arsenic was accumulated as arsenite (AsIII)

If any frond dies by cold stress at low temperature (5^{0}C), most probably As was released to the medium via root system

If some As containing fronds become mature or die at room temperature, majority As is translocated to other new fronds

Over 90% of arsenic was transported to shoots

Root system: Arsenate was reduced to arsenite by arsenate reductase

Arsenate was mainly absorbed by *P.vittata*, and upatake ratio was upregulated by phosphate starvation

Fig. 1-3 Arsenic accumulation and translocation scheme of *P. vittata*. (K. Sugawara, PhD thesis, 2015)

1-6 Application to leachate

Final target is to apply temperate zone ferns as well as tropical zone ferns in a As contaminated leachate at the temperate zone of northern part of Japan. Leachate that produced from precipitation and ground water seeping through municipal waste in landfills is contaminated with various organic and inorganic substances from the landfill waste (Baedecker and Back, 1979; Christensen and others, 2001). Leachate seeping from a landfill contaminates the water beneath the landfill. Water contamination also extends away from the landfill in the direction of water flow, forming a leachate plume that can result in the release of naturally occurring arsenic.

A significant source of metals released into the environment is the solid waste disposals (open dumps, landfills, sanitary landfills or incinerators). (Yarlagadda et al. 1995; Waheed et al. 2010; Iwegbue et al. 2010; Bretzel and Calderisi 2011; Rizo et al. 2012). Primarily leachate is produced in association with precipitation that infiltrates through the refuse and normally results in the migration of leachate into the groundwater zone and pollutes it (Samuding 2009). Waters and soils have been contaminated with heavy metals such as lead, arsenic, zinc, iron, manganese, chromium, and cadmium due to migration of leachate, and these heavy metals in solid wastes lead to serious problems because they cannot be biodegraded (Hong et al. 2002). Co-disposed industrial wastes, incinerator ashes, mine wastes and household hazardous substances such as batteries, paints, dyes, inks, etc. are the major sources of heavy metals in landfills (Erses and Onay 2003). Water and soil contamination by heavy metals from waste disposal sites is a serious problem in industrial and urban areas (Mandal and Sengupta 2006). Actually soil and water are regarded as the ultimate sink for heavy metals discharged into the environment, as many heavy metals are bound to soils and sediments (Obiajunwa et al. 2002).

Although it is not so easy to keep the healthy condition of plants at any temperature during practical application to As contaminated leachate, we approached for adaptation of ferns at low temperature and considerable removal of As from contaminated leachate.

1-7 Critical points of As removal by temperate zone ferns at low temperature

As is extremely toxic for living beings and it is highly persistent pollutants. Once they get into groundwater, it becomes extremely difficult to handle them due to the complex speciation chemistry coming into play. However, many techniques have been devised over the past few decades to remediate As from water. Phytoremediation is one of the most promising technologies have been served as a cost effective, environment friendly technology for widely spread low concentration contamination. Anyway, Plants may develop physiological disorders when exposed to low temperature. Low but non-freezing temperatures is called chilling injury refers to an injury that is caused by a temperature drop to below to 10°C to 15°C but above the freezing point. Chilling injury occurs in tropical and subtropical plants at10°C to 25° C and temperate plants at 0 to 15°C. Being temperate zone ferns, *P. multifida* as well *P. cretica* showed usual efficiency for As removal at 10°C, 15°C and 25°C but chilling effect was noticed in tropical zone fern *P. vittata* at 10°C, 15°C. This effect is manifested by physiological and cytological changes when cytological changes may be reversible or irreversible depending upon time of exposure to low temperature. The most common site implicated for chilling injury is the plasma membrane. The consequences of this change may lead to cell leakage or disruption. Most common symptoms of chilling stress is rapid wilting followed by water soaked patches which develop into sunken pits that reflect cells tissue collapse. Following warming, the sunken pits usually dry up, leaving necrotic patches of tissues on the leaf surface.

During hydroponic cultivation and application to leachate, lowest considered temperature (5°C or ≤5°C) was always critical for all temperate and tropical zone ferns. Severe cold stress was observed when they are exposed to a low temperature like 0^0C. In case of tropical zone fern *P. vittata*, Symptoms like desiccation or burning of foliage was noticed and As accumulation was almost stopped, even accumulated As was released to the medium. Water-soaked areas that progress to necrotic spots on leaves and death of sections of the plant or the entire plant. Close examination of plants several days or weeks at ≤5°C may reveal a dead or weakened root system or split bark on stems or branches. Although temperate zone fern like *P. multifida* or *P. cretica* was still green in appearance at 5°C, but their As accumulation efficiency was decreased significantly, sometimes released some amount of accumulated As to the media at ≤5°C.

Moreover, the growth rate of temperate zone ferns (*P. multifida* or *P. cretica*) are considerably slower than tropical fern *P. vittata*. Further investigations are recommended to overcome the above difficulties for more successful phytoremediation.

1-8 Objectives of present research

Ferns are promising for phytoremediation of As contaminated site. Although *P. vittata* is the most studied As hyperaccumulator fern, it is limited to its cold tolerance. In this research, temperate zone ferns *P. multifida* and *P. cretica* were considered for investigating their heavy metal accumulating potential at four different temperature. Comparative potential of temperate zone fern *P. vittata* was also determined to find out the suitable fern for successful phytoremediation at low temperature. However, release tendency of those three ferns under cold stress has also been investigated but these results are not enough for searching the most suitable fern for practical application to arsenic contaminated water. In addition, temperate zone ferns as well as tropical zone ferns were applied in a arsenic contaminated dumping site in winter for the first time. However, survival of the ferns under cold stress and continuous arsenic removal was a big challenge at this application. The objectives of the present study were 1: Evaluation of basic potential of temperate zone ferns *p. multifida* and *P. cretica* to remove arsenic, cadmium and lead from contaminated water; 2: Determination of the effect of low temperature on arsenic removal potential of temperate zone and tropical zone ferns; 3: Investigation of the efficiency of the practically applied temperate zone and tropical zone ferns in a arsenic contaminated dumping site leachate.

1-9 Contents of this thesis

This doctoral thesis consists of 5 chapters.

Chapter 1 “General introduction” was written about back ground information of As contamination and phytoremediation of As from water, temperate and tropical zone ferns, and summarized issues of phytoremediation by temperate zone ferns at low temperature.

Chapter 2 “Arsenic, lead and cadmium removal potential of *Pteris vittata, Pteris multifida* and *Pteris cretica* from contaminated water” was investigated the basic potential of temperate zone fern *P. multifida* and *P. cretica* for removal of three top toxic metals (arsenic, lead and cadmium) and tropical zone fern *P. vittata* was considered for comparative analysis. Translocation of accumulated metals in different parts of *P.multifida, P. cretica* and *P. vittata* were also observed.

Chapter 3 “Influence of temperature on comparative arsenic accumulation and release by *P. multifida*, *P. vittata* and *P. cretica*” was highlighted the effect of different temperature on arsenic accumulating potential of temperate zone and tropical zone ferns. Additionally, accumulated arsenic release tendency of those three ferns was also observed for investigating comparatively higher cold tolerant fern for successful application of phytoremediation.

Chapter 4 “Practical application of temperate zone ferns to an arsenic contaminated leachate” was discussed about first time application of temperate zone fern *P. cretica* and tropical zone fern *P. vittata* to an arsenic contaminated dump site leachate for removal of As all over the year where adaptation of the plants with fluctuating temperature was a big Challenge.

Chapter 5 “Conclusion” was summarized all of results that have done in this thesis and also dedicated some unsolved issues to the future researchers.

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CHAPTER 2

Arsenic, Lead and Cadmium Removal Potential of *Pteris vittata, Pteris multifida* and *Pteris cretica* from Contaminated Water

2.1 Introduction

Heavy metal pollution is a great threat worldwide. This type of pollution has accelerated rapidly since the onset of the industrial revolution (Gisbert *et al.* 2003). Retention of heavy metals at high concentrations in the environment exerts toxic effects on fauna and flora (Xue *et al.* 2010). Increased metal concentrations in the soil or water up to toxic levels is also a severe environmental problem (Briat et al. 1999).

Arsenic (As), lead (Pb) and cadmium (Cd) are heavy metals of particular interest because of their unique toxic and carcinogenic effects. It was reported that 42.7 million people in West Bengal, India and 79.9 million people in Bangladesh suffered from groundwater contamination by As, with As levels in groundwater above the World Health Organization maximum permissible limit of 50 µg/L (Chowdhury *et al.* 2000). Most of the previous studies of As focused on As(V) remediation (Mandal *et al.* 2012; Natarajan *et al.* 2011; Tu *et al.* 2002; Ma et al. 2001), but in this research, we have used As(III) for hydroponic culture which is almost 2 to 10 times more toxic than As(V) (Goyer *et al.* 2001). In case of Cd poisoning, itai–itai disease is the most severe form of chronic poisoning caused by prolonged oral Cd ingestion which developed in numerous inhabitants of the Jinzu River basin in Toyama Prefecture of Japan (Inaba et al. 2005). Severe exposure to Pb can induce badly damage of the central nervous system (Kaul *et al.* 1999; Hertz-Picciotto 2000). According to Cui *et al.* (2004), soil and vegetables were heavily polluted by Pb and Cd near a smelter producing Pb in a suburb of Nanning, the capital of Guangxi Province in southern China.

For removal and recovery of toxic heavy metals, the use of biological methods has been recommended because they can be cheap, effective and environmentally-friendly compared with other conventional (physical and chemical) methods (Hanif, Bhatti and Hanif 2009). Phytoremediation is one of the most promising biological technologies for the removal of environmental pollution caused by the limitations of traditional technologies (Rahman *et al.* 2008). Recently, heavy metal accumulation by plants, such as *Cynodon dactylon* (Wu *et al.* 2010), *Salvinia natans* (Dhir and Srivastava 2011), *Melastoma malabathricum* L. (Selamat, Abdullah and Idris 2014), Switchgrass (Jeke, Zvomuya and Ross 2016) and *Pteris vittata* (Ronzan et al. 2017) has been tested and the effectiveness of phytoremediation by these plants has been demonstrated. The use of fern for this removal technique has developed considerable interest since the initial report of a Chinese brake fern *(Pteris vittata)* as an arsenic hyperaccumulator (Ma et al. 2001). Although *P. vittata* is the most studied fern, it is limited in its cold-tolerance. In case of Japan, *P. vittata* naturally grows in very south part of Japan, belongs to sub-tropical zone (average temperature: 7-15°C in winter; 25-30°C in summer). Being a tropical zone fern, germination and growth rates of *P. vittata* were also limited at around 25°C (Wan et al. 2009). Thus, it is of interest to find an alternative cold-tolerant heavy metal-accumulating fern.

Pteris multifida as well as *Pteris cretica* are two As hyperaccumulating temperate zone fern that can accumulate 5,510 and 5,584 mg/kg As respectively (Terrence S. et al., 2009) in the fronds from soil. This is one of the world's common plants, distributed almost all areas in Japan and China including temperate areas. *P. multifida* has already shown some ability to tolerate low temperatures from 5 °C to -4.6°C (measured by thermometer in the field) during field experiments (Sugawara, Chein and Inoue 2015), which is an essential property for large scale phytoremediation in temperate zones such as the northern part of Japan. There is currently limited knowledge on the accumulation and translocation of heavy metals by *P. multifida* and *P. cretica* to date there is no published information on the potential for accumulation of As, Pb and Cd by those two temperate zone ferns from solution. Therefore, the aims of the present study were: (I) to determine the potential of temperate ferns for heavy metal accumulation from both water and soil media and to compare that with tropical fern *P. vittata*; (II) to compare the long-term accumulation of heavy metals by temperate fern (*P. multifida)* and tropical fern (*P. vittata*); and (III) to investigate the translocation of heavy metals from roots to aerial tissues. Hopefully the results of this study will provide novel information on the ability of temperate ferns to accumulate and translocate As, Pb and Cd from both water and soil. These results will contribute to the application of *P. multifida* and *P. cretica* and for phytofiltration or phytoremediation of multi-metal-contaminated soil and contaminated water.

2.2 Materials and methods

2.2.1 Plant materials

P. multifida, *P. cretica* and *P. vittata* were obtained from Fujita Co. (Tokyo, Japan). Fujita company prepared those *Pteris* from spore. Almost 7 or 8 months had passed to prepare *Pteris* sporophytes from spores. We received 4 or 5 frond containing fern at the age of 7 or 8 months. The average height and fresh weight was around 23cm and 3.5g respectively.

Efforts were made to ensure the uniformity and similarity of the size of plants used in the experiments. The ferns were used without sterilization for hydroponic cultivation, but sand was washed with tap water to remove the adhering compost from the roots, which is relevant to real treatment conditions (Natarajan et al 2008; Huang et al. 2004). Then plants were transplanted to 500 mL opaque containers where they were acclimatized in 400 mL 5 times diluted Hoagland's solution (Hoagland and Arnon 1950) for two weeks in a growth chamber with the following conditions: 16-h light period with a light intensity of around 280 µmol / m^2s ; 25 °C daytime; 20 °C night; and 70% relative humidity. All of the experiments of this study were done with the same growth conditions as mentioned above. After adapting to the hydroponic environment, the plants were used for heavy metal accumulation experiments. The experiments were set up with five plants of approximately equal size and after analysis, the average of best three plants was selected for results.

2.2.2 Comparative hydroponic accumulation trial of *P. multifida, P. cretica* and *P. vittata*

To determine the As(III), Pb and Cd accumulation potential of *P. multifida* and to establish its efficiency compared with *P. vittata,* 4-day hydroponic trials were conducted with both *P. multifida, P. cretica* and *P. vittata*. In this study, the more toxic form of As, As(III), was used during the hydroponic experiments to obtain the responses of those three ferns to As(III). Plants were pre-cultivated in 5 times diluted

Hoagland's nutrient solution. The nutrient solution was composed of: 8 mM of KNO_3; 4 mM $Ca(NO_3)_2$; 2 mM $MgSO_4$; 1 mM $NH_4H_2PO_4$; 50 µM H_3BO_3; 9 µM $MnSO_4$; 1 µM $ZnSO_4$; 0.2 µM $CuSO_4$; 0.1 µM Na_2MoO_4; and 60 µM Fe(III)-EDTA. The acclimatized plants were incubated in 0.1 M 2-morpholinoethanesulfonic acid monohydrate (MES) buffer solution for three days to allow them to adjust to the buffer solution. Then *P. multifida, P. cretica* and *P. vittata* plants were transplanted to mixed metal solution where they have been growing for 4 days. Metal solutions were prepared by using 0.1 M MES buffer. Every day, 1 mL of sample solution was collected at a fixed time for analysis and initial solution level (400mL) was supplemented very carefully by adding Milli-Q water. pH was kept at around 6.0 by adding 0.1M HNO_3 or 0.1M NaOH initially and was not adjusted again during the experiment. The buffer solution maintained the pH within the range of 5.98 to 6.04 during the 4 days' experiment.

To investigate the long-term accumulation and translocation ability of *P. multifida and P. vittata,* a separate long-term hydroponic experiment was conducted over 24 days. Initial concentrations were measured before plantation when concentrations of Pb ($Pb(NO_3)_2$), Cd ($Cd(NO_3)_2 . 4H_2O$) and As(III) ($NaAsO_2$) in mixed metal solution were 27µg/L, 30µg/L and 33µg/L respectively. All methods were the same as described above for the 4-day experiment but this time, 5 times diluted Hoagland's solution was used initially and solution was continuously aerated to ensure survival and health of the plant during the longer incubation period (24 days). All experiments were kept in the growth chamber.

2.2.3 Pot soil experiment for As(V), Pb and Cd accumulation and tranportation

Pot soil experiments were conducted to investigate As(V), Pb and Cd uptake and transportation by *P. multifida* and *P. vittata* from pot soil medium from July to October, 2015. During the 3-month pot soil experiment, it was not possible to prevent the oxidation of As, so As(V) was spiked into the soil instead of As(III). During pre-cultivation, plants were grown in pot soil for 2 weeks to allow them to acclimatize to the pot conditions, where each pot contained about 3 kg mixture of sand and peat moss (4:1, v/v). According to Zheng et al. (2003), high concentration Pb reacted with As in soil solution to form a stable mineral. To avoid those possibilities, individual metal solutions of As(V) ($Na_2HAsO_4 \cdot 7H_2O$), Pb ($Pb(NO_3)_2$) and Cd ($Cd(NO_3)_2 \cdot 4H_2O$) were injected into separate pots so that in each pot metal concentration was maintained at 0.5 mg heavy metal/kg soil. Initial metal concentration of pot soil (0.5 mg/kg) was higher than that of hydroponic solution (about 30 µg/L) because usually, in soil most of the metals are absorbed as soon as possible and would like to form some insoluble compounds like minerals or hydroxides. Thus, the availability of metals in soil is always comparatively lower than that of metal solution (containing metals as ions). Plants were watered every day and changes in the physical appearance of plants were recorded. Each metal was injected into triplicate separate pots for both *P. multifida* and *P. vittata* where each pot contained one plant only. After 3 months' incubation, plant samples were digested for inductive coupled plasma mass spectroscopy (ICP-MS) analysis.

2.2.4 Sample preparation and chemical treatment

After the incubation period was completed, plant roots and rhizomes were immersed in a solution containing 1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$, and 5 mM MES (pH 6.0) for 10 min and then briefly rinsed in distilled water. Fronds were also washed three times with distilled water and then dried in an oven for 3 days. The dried plant samples (10 mg) were digested in 3 mL of concentrated HNO_3 on a heating block (ALB-121, Acinics, Japan) at 130 °C for 90 min. The digests were subsequently cooled and diluted 10-fold using Milli-Q water (Merck Millipore Corporation, Darmstadt, Germany), filtered through a PTFE 0.45 µm filter membrane (Merck Millipore Corporation) and then stored for analysis in 15 mL polypropylene tubes. As(III) and As(V) were separated by using an As speciation cartridge (Metal Soft Center, Highland Park, NJ, USA) that retains As(III) (Meng *et al.* 2001). As species in the solution were analyzed by the process described previously (Huang, Hatayama and Inoue 2011)

Inductively coupled plasma mass spectrometry (ICP-MS; ELAN 9000, Perkin Elmer, SCIEX) was used for quantitative analysis of As, Pb, and Cd. The internal standard used during analysis was 10 µg/L indium (In). Standard reference materials were standard solution of As, Pb, and Cd (Wako chemicals, Japan) and recovery rates were more than 100%. All glassware was washed five times with detergent solution, 3% HNO_3 and Milli-Q water. All reagents were of analytical grade.

2.2.5 Data analysis

The values reported in both text and figures are the mean ± SE (standard error of the mean). The statistical significance (at 95% confidence) was tested using analysis of variance, where appropriate, and the numbers of replicates are included in the figure legends.

2.3 Results and discussion

2.3.1 As, Pb and Cd uptake from hydroponic mixed metal solution

Temperate zone fern has the potential to accumulate and store As, Pb and Cd from multi-metal solution, although the accumulation efficiency varied for each individual metal. The concentrations of As, Pb and Cd in the mixed metal solution decreased with increasing exposure time. To compare the efficacy of *P. multifida, P. cretica* and *P. vittata* for accumulating As(III), Pb and Cd, a short-term (4 days) hydroponic experiment was conducted. A long-term (24 day) hydroponic experiment was also conducted to clarify the long-term accumulation ability of those two ferns.

2.3.1.1 As(III) was removed by temperate zone fern (*P. multifida* and *P. cretica)*, but not by tropical fern *P. vittata* during short-term incubation

During the short-term (4 day) hydroponic experiment, a mixed metal solution was used where the form of As was As(III). To the best of our knowledge, the As(III) removal potential of *P. multifida* has not yet been tested, even though As(III) is almost 2 to 10 times more toxic than As(V) (Goyer 2001). During this experiment, the hydroponic conditions were strictly maintained to prevent the oxidation of As(III). Nutrients were not added to the metal stock solution to avoid the interactions between nutrients and metals. According to Su et al. (2008), in hydroponic solutions without nutrient, the possibility of oxidation from As(III) to As(V) is very low, which agrees with our results. Speciation tests for As in the mixed metal solution showed that even on the last day of experiment, almost 93% of As was measured as As(III) (Fig. 2-1).

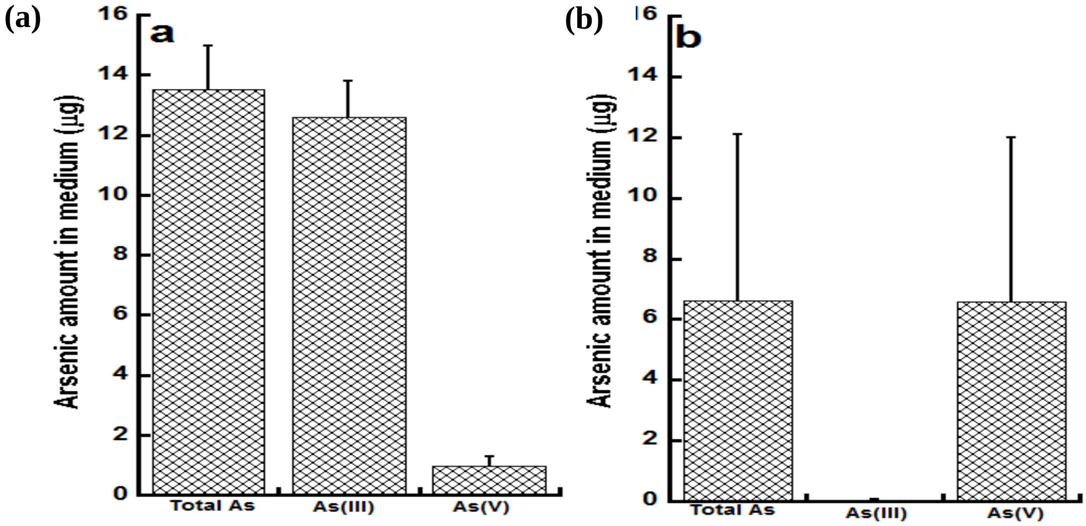


Fig. 2-1 Amount of As(III) and As(V) in the hydroponic solution after 4 days' experiment of *Pteris vittata* (n = 3) (a) and Amount of As(III) and As(V) in the hydroponic solution after 24 days' experiment of *Pteris multifida* (n = 3) (b)

During this 4 days' experiment, concentrations of As, Cd and Pb were removed from hydroponic solution by *P. vittata*, *P. multifida* and *P. cretica* although initial concentrations of three target metals were not same in the mixed metal solution as the set value was 30 µg/L [Fig. 2-2 (a)(b)(c)]. Tropical fern *P. vittata* accumulated 79% Pb and 45% Cd respectively, but no As(III) at all from the mixed metal solution [Fig. 2-3 (a)] where 63%, 44% and 33% of Pb, Cd and As(III), respectively, were accumulated by *P. multifida* within 4 days' [Fig. 2-3 (b)]. As(III) accumulation by *P. multifida* was significantly higher than that of *P. vittata* (*P* = 0.005). Cd and Pb accumulation by *P. multifida* and *P. vittata* were almost similar (*P* = 0.41 and P = 0.94 respectively) during short term. Another temperate fern *P. cretica* accumulated 90%, 71% and 43% of Pb, Cd and As(III), respectively from hydroponic mixed metal solution [Fig. 2-3 (c)].

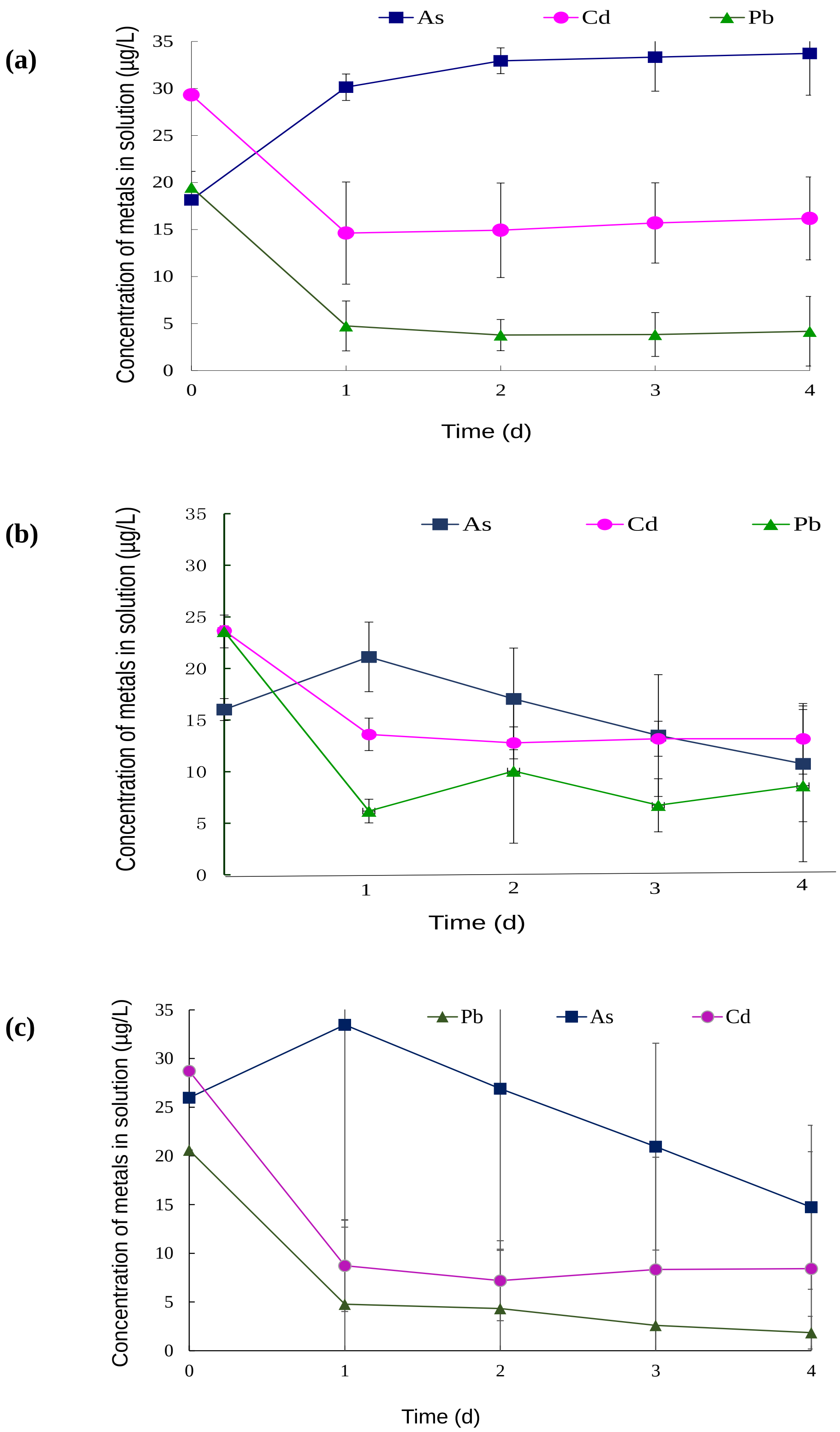
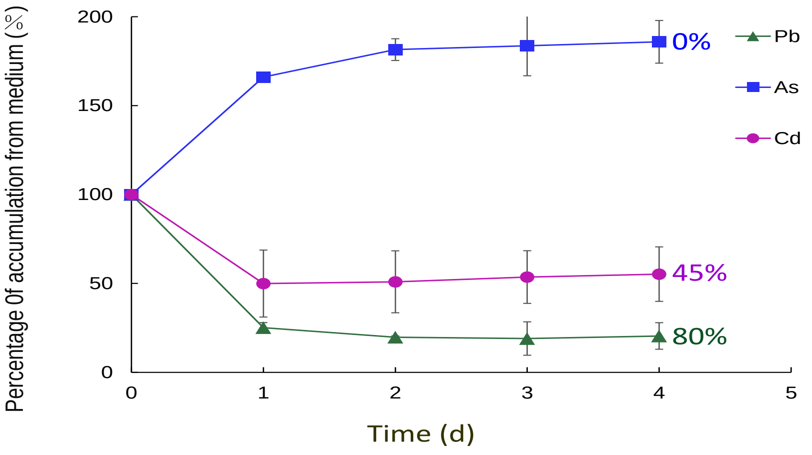


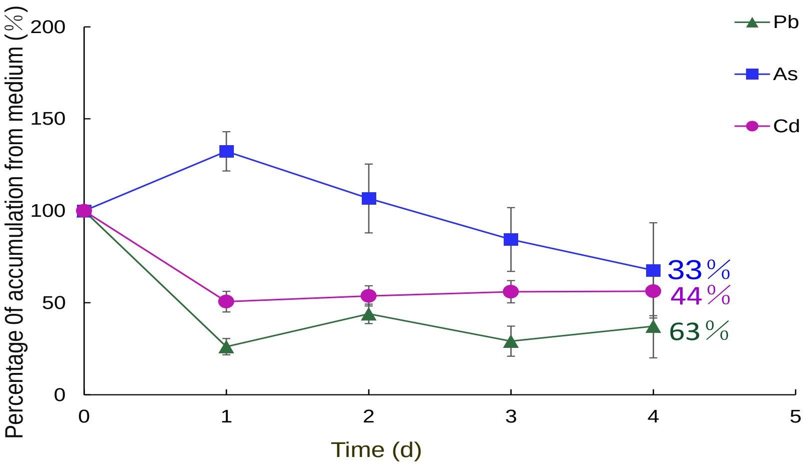
Fig. 2-2 Concentrations of arsenic (III) [As(III)], cadmium (Cd) and lead (Pb) in hydroponic solution during 4 days' incubation with (a) *Pteris vittata (n* = 3); (b) *Pteris multifida (n* = 3); (c) *Pteris cretica (n* = 3)

(a)



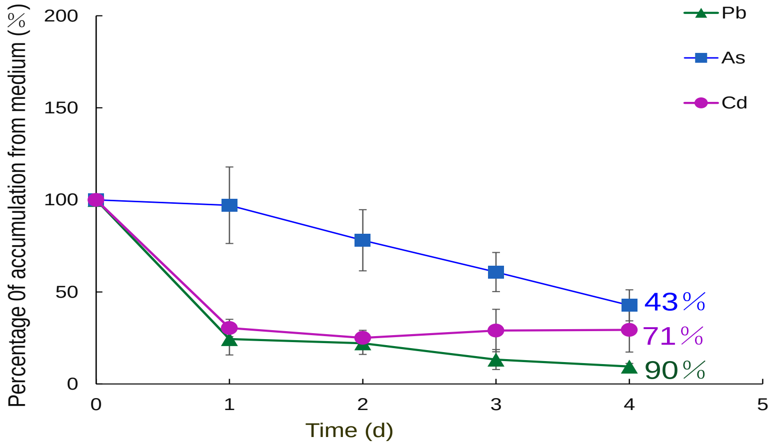
4 days accumulation

(b)



4 days accumulation

(c)



4 days accumulation

Fig. 2-3 Percentage of accumulation of arsenic (III) [As(III)], cadmium (Cd) and lead (Pb) by (a) *Pteris vittata ;* (b) *Pteris multifida*; (c) *Pteris cretica* from hydroponic solution during 4 days' incubation *(n* = 3)

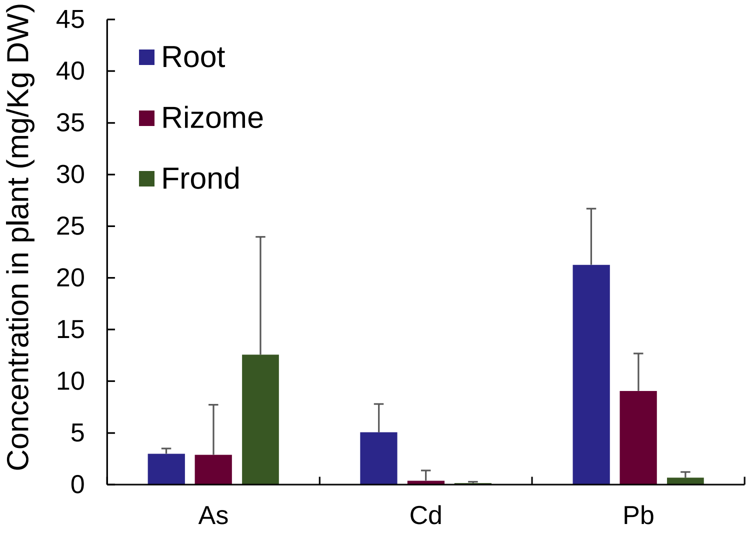
Comparative result between two temperate ferns showed that Pb and Cd accumulation capacity of *P. cretica* was higher than *P. multifida*. The first sampling (0 h) in this experiment was done after transplantation of the ferns. It is likely that at first, some of the As(III) was adsorbed by the root surface but was released back into solution within day 1 of the experiment; *P. multifida* then started to absorb up to 33% of the As(III) within 4 days' but *P. vittata* could not accumulate As(III) at all.

This unique result suggests that temperate fern (*P. multifida* and *P. cretica)* are better than tropical fern *P. vittata* for As(III) removal. In some plants, As(III) can be transported by aquaglyceroporin channels (Hatayama et al. 2011; Bienert *et al.* 2008). Further investigations are needed to clarify the mechanism of As(III) accumulation and transportation by *Pteris* species.

2.3.1.2 Transportation of heavy metals during hydroponic experiments

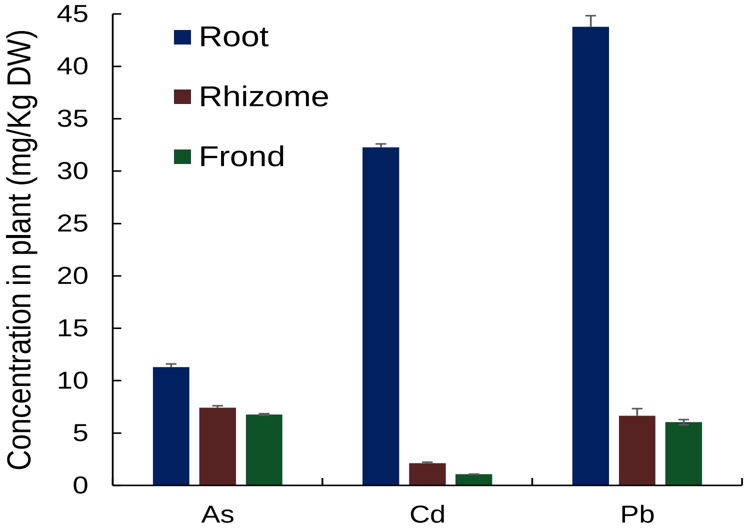
For successful application of phytoextraction or phytofiltration, accumulation and transportation of heavy metals is very important (Rozas, Alkorta and Garbisu 2006). If the metals are not transported to aerial plant parts but stay attached to the root then there is a high possibility that they can be released again to the media. To investigate the transportation of As, Pb and Cd from the root to other plant organs in temperate fern *P. multifida*, and *P. cretica,* concentrations of As, Pb and Cd in fronds, rhizomes and roots after 4 days' incubation were analyzed. As shown in [Fig. 2-4 (a)], concentration of As in the frond of *P. multifida* was comparatively higher than root or rhizome but not significant (*P* = 0.08 and 0.13 respectively). In case of *P. cretica*, As was distributed to root, rhizome and frond within 4 days' [Fig. 2-4 (b)].

(a)



Pteris multifida (after 4 days' accumulation)

(b)



Pteris cretica (after 4 days' accumulation)

Fig. 2-4 Transportation of arsenic (As), cadmium (Cd) and lead (Pb) in different parts (fronds, rhizome and roots) of (a) *Pteris multifida*; (b) *Pteris cretica* after the 4-day hydroponic experiment (*n* = 3)

Significantly higher concentration of Cd and Pb were stored in the root and rhizome ($P \leq 0.01$) rather than frond in both temperate ferns. In case of *P. multifida*, although 44% Cd was removed from the solution, but concentration in plant was lower. Thus, Cd might be just attached with the root surface and washed away from root during washing time. But most of the removed Pb was detected in the root of *P. multifida*. Transportation pattern of *P. multifida* can be compared with *P. vittata* which was incubated in mixed metal solution (100µM As +60µM Cd) for 15 days (Ronzan et al. 2017). Significantly higher concentration of As was detected in frond ($P < 0.05$) rather than root of *P. vittata* and significantly higher Cd was detected in root and rhizome than fronds ($P < 0.01$) which was similar as *P. multifida* of this research. According to Wu et al 2009 *P. vittata* could accumulate and store Pb mainly in root but some concentration was also detected in frond which consistent with our result of Pb accumulation by *P. multifida.* Concentration of Pb and Cd that removed by *P. cretica* from the hydroponic solution were mostly detected in the root of *P. cretica*.

2.3.1.3 Comparative long-term accumulation by temperate fern (*P. multifida*) and tropical fern (*P. vittata*) from hydroponic solution

During the long-term experiment, concentrations of As, Cd and Pb were decreased with time from hydroponic solution by *P. vittata* and *P. multifida* [Figs. 2-5 (a)(b)]. *P. vittata* accumulated 98% As, 43% Cd and 47% Pb during 24days [Fig 2-6 (a)]. In case of long term incubation, significantly higher amount of As (12.3 ± 0.2 µg) was removed by *P. vittata* than that of Cd (5.5 ± 0.6 µg) and Pb (5.9 ± 2.0 µg) ($P < 0.0001$ and $P = 0.0003$ respectively). *P. multifida* accumulated 90%, 36% and 50% of Pb, Cd and As, respectively, from mixed metal solution, as shown in [Fig. 2-6 (b)]. Removal potential of *P. multifida* was compared in case of three target metals. Within 24 days, amount of Pb (9.5 ± 1.2 µg) that removed from hydroponic solution, was significantly higher than that of Cd (4.2 ± 0.1 µg) and As (6.6 ± 2.0 µg) ($P = 0.007$ and $P = 0.04$ respectively). To avoid the initial adsorption and release observed in the short-term experiment, the first sampling (0 h) was undertaken before transplantation. The major difference between the long-term and short-term experiments was that essential nutrients were added initially to the mixed metal solution in the long-term experiment to ensure the health and survival of the plant, which might have caused some interaction between metals and nutrients. According to our hypothesis, the interaction between metals and nutrients during the long-term experiment resulted in slow accumulation of heavy metals during the first few days. In the case of the long-term (24 days) experiment with nutrient, it is most likely that As (III) was gradually oxidized to As (V) and finally almost all As (III) was converted to As (V) (99.7%) (Fig. 2-1). According to Hatayama et al. 2011 in hydroponic condition, most of the arsenite was almost completely oxidized to arsenate (96%) within 48 h. After oxidation As (V) might be transported by the phosphate transporter (Wang *et al.* 2002; Hatayama et al. 2011). According to Hughes (2002), phosphate uptake can be replaced by As (V), which would interrupt many biochemical pathways. But as the initial phosphate concentration in the solution was high, that time phosphate might be transported mainly by the phosphate transporter for the first few days. As the phosphate concentration decreased within first few days, ferns might have started to accumulate As (V) by using the phosphate transporter. The decrease in phosphate concentration from the solution was analyzed to support the above possibilities.

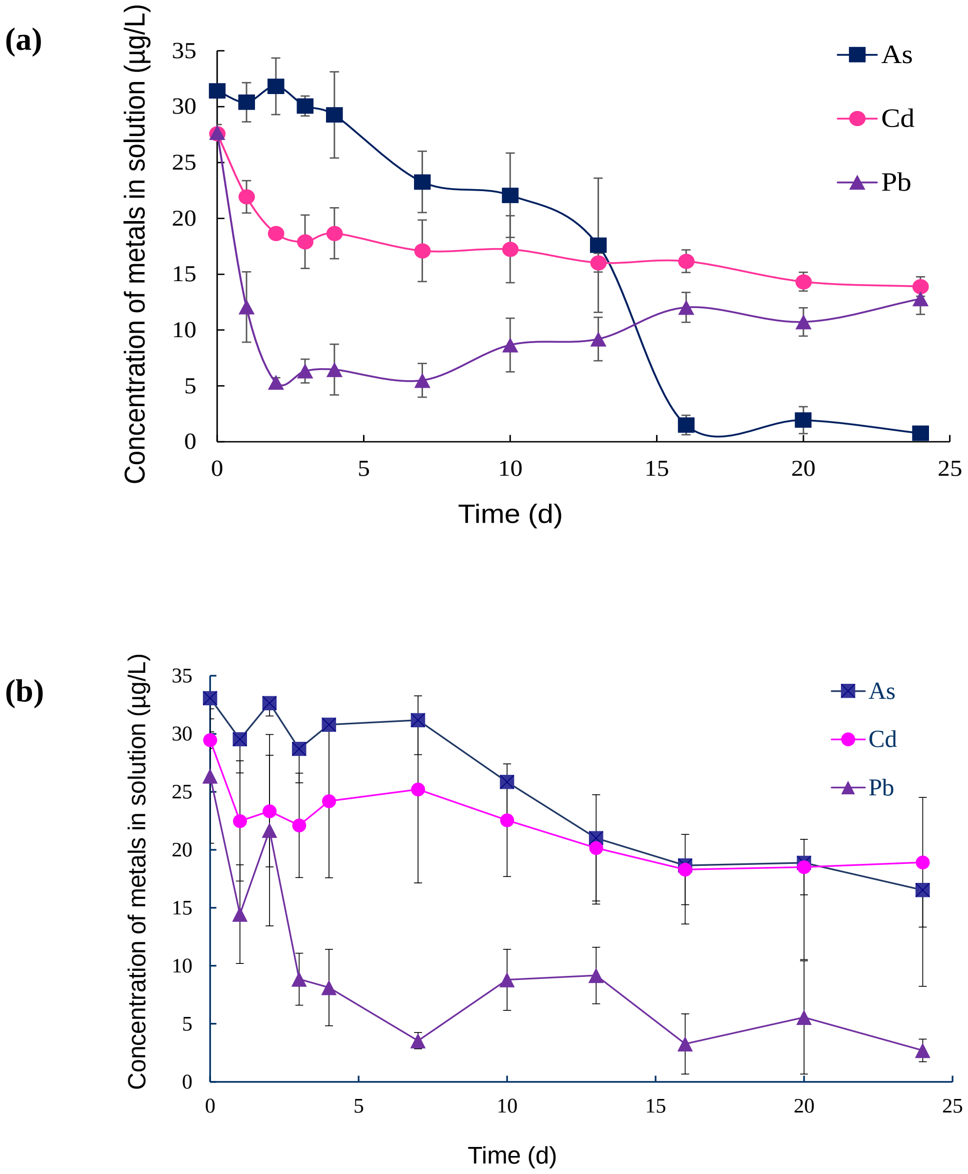
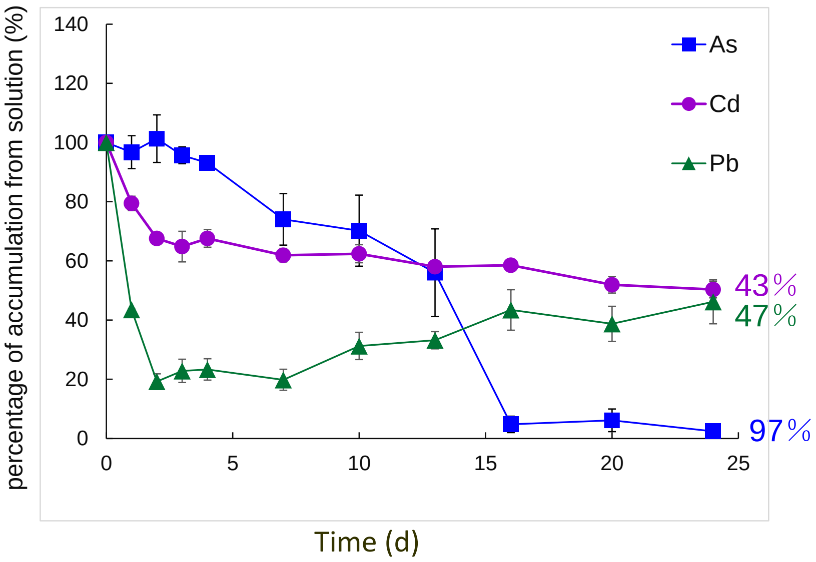


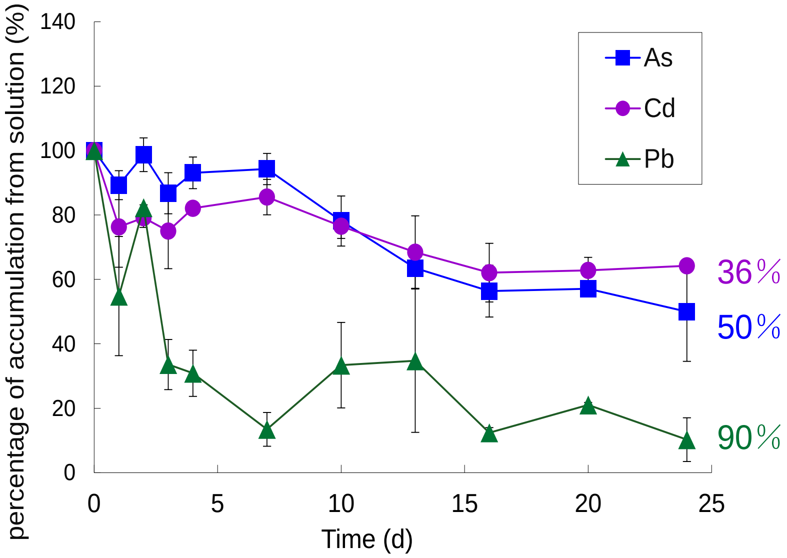
Fig. 2-5 Concentrations of arsenic(III) [As(III)], cadmium (Cd) and lead (Pb) in hydroponic solution during 24 days' incubation with (a) *Pteris vittata* (n=3); (b) *Pteris multifida* (n=3)

(a)



24 days accumulation (*P. vittata*)

(b)

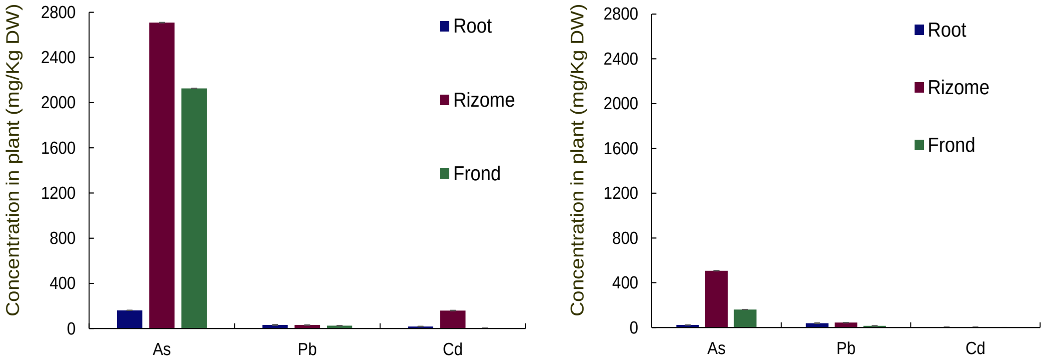


24 days accumulation (*P. multifida*)

Fig. 2-6 Percentage of arsenic(III) [As(III)], cadmium (Cd) and lead (Pb) accumulation from hydroponic solution during 24 days' incubation with (a) *Pteris vittata* (n=3); (b) *Pteris multifida* (n=3)

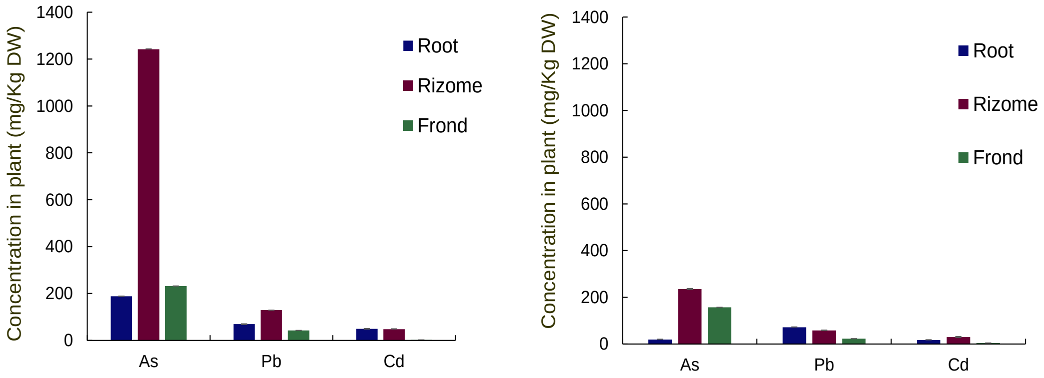
2.3.2 As(V), Pb and Cd accumulation and transportation by temperate fern (*P. multifida*) and tropical fern (*P. vittata*) from pot soil

A long-term pot soil experiment confirmed the uptake and transportation of As(V), Pb and Cd from soil by *P. vittata* and *P. multifida*. After 3 months' assimilation in metal-injected pot soil at 0.5 mg/kg metals concentration, *P. vittata* accumulated significantly higher concentrations of As (5.0×10^3 mg/kg) ($P < 0.01$) and Cd (1.8×10^2 mg/kg) ($P < 0.01$) but lower Pb (88 mg/kg) ($P < 0.05$) than *P. multifida* (Fig. 2-10). The transportation pattern was also different for *P. vittata* than for *P. multifida*. After the 3-month experiment, rhizomes and fronds of *P. vittata* showed significantly higher concentrations of As than root ($P < 0.05$). Concentration of Pb was not significantly varied in root and rhizome ($P = 0.37$) but Cd was transported to the rhizome significantly ($P < 0.01$). Transportation of As, Pb and Cd in *P. vittata* compared with the control plants was shown in Fig. 2-7 (a).



Pteris vittata **Pteris vittata (control)**

Fig. 2-7 (a) Concentrations of arsenic (As), cadmium (Cd) and lead (Pb) in different parts (fronds, rhizome and roots) of *Pteris vittata* after the 3-month pot soil experiment ($n = 3$)



Pteris multifida

***Pteris multifida* (control)**

Fig. 2-7 (b) Concentrations of arsenic (As), cadmium (Cd) and lead (Pb) in different parts (fronds, rhizome and roots) of *Pteris multifida* after the 3-month pot soil experiment *(n* = 3)

Comparative result showed that temperate fern *P. multifida* contained 1.6×10^{3}, 2.4×10^{2} and 99 mg/kg dw of As, Pb and Cd, respectively [Fig. 2-7 (b)]. Significantly higher concentration of As was stored in the rhizome (1.2×10^{3} mg/kg) ($P < 0.05$) than other parts, similar to the results of Sugawara, Chein and Inoue (2015). The results of this study and those of Sugawara, Chien and Inoue (2015) indicated that, during long-term exposure to As-contaminated soil, *P. multifida* might have stored the maximum concentration of As in the rhizome, although the pathway of accumulation started with the root and was then transported to the frond before finally ending up in the rhizome. The morphology of *P. multifida* is suited to the storage of nutrients and heavy metals for long time periods, as the rhizomes are relatively large in size. Control *P. multifida* plants also showed transportation of metals even at very low metal concentrations. Pb-infused *P. multifida* stored almost two times higher concentrations of Pb in the rhizome than in the root ($P < 0.05$). Concentration of Pb that accumulated by *P. multifida* from pot soil was higher than the accumulated concentration by *P. vittata* which matches with the previous results of those two ferns from hydroponic solution. Rhizome and root concentrations of Cd were almost the same (P = 0.93) in *P. multifida*. Further investigations are needed to clarify the mechanism of accumulation and storage of heavy metals over long time periods.

2.3.3 Responses of temperate fern after three months' exposure to As, Pb and Cd

Even after 3 months' growth in As, Pb and Cd injected soil, *P. multifida* plants still looked healthy. Natural spontaneous changes in plant condition, like the formation of new fronds, was also noticed which can be compared with *P. vittata* that grew healthily at sites highly contaminated by As and Pb (An et al., 2006; Wu et al., 2007). Kachenko et al. 2007 also demonstrated that pot trials Of *P. vittata* possesses a relatively high tolerance to Cd.

With respect to the control, the appearance of As spiked plants were not damaged by metal toxicity after three months exposure which is similar with the findings of Ronzon et al. 2017 who have done the details histological analysis of *P. vittata* on the apical, median and basal part after As exposure. The exposure of *P. multifida* to 0.5 mg Cd/kg soil for three months did not cause any visual modification in comparison with the control. But according to Ronzon et al 2017, Cd caused a strong cell wall thickening in all the tissues around the midrib and epidermis when exposed to 60 µM Cd. Wu et al. 2009 also stated that besides having a high level of As tolerance, *P. vittata* also possesses considerable tolerance to Pb which is similar as the Pb tolerance of *P. multifida* in this experiment. Comparative dry biomass of metal injected plants and control plants confirmed that the growth and vitality of *P. multifida* had not been affected by the toxic metals (Table 2-1). The tolerance of *P. multifida* to 0.5 mg As, Pb and Cd/kg soil for three months provides evidence that this plant could be practically applied to toxic metal contaminated environments.

Table. 2-1 Dry biomass of *Pteris multifida* and *Pteris vittata* after pot soil experiment.

Pteris multifida	**Root (g)**	**Rhizome (g)**	**Frond (g)**
As treatment	1.09	0.98	0.48
Pb treatment	0.31	0.74	1.03
Cd treatment	0.84	1.39	1.66
Control treatment	0.39	0.62	1.03
ANOVA	*	*	NS
Pteris vittata	**Root (g)**	**Rizome (g)**	**Frond (g)**
As treatment	1.50	0.73	1.15
Pb treatment	1.46	0.96	2.03
Cd treatment	2.24	2.02	1.08
Control treatment	1.57	0.47	1.01
ANOVA	*	*	*

Significance determined by ANOVA : NS not significant, * $P < 0.05$,

2.4 Conclusion

In the present study, temperate ferns (*P. multifida* and *P. vittata*) were chosen because of its high cold tolerance in the field. The potential of temperate ferns to remove As(III), Pb and Cd from solution and comparison with *P. vittata* was reported here for the first time. Our results showed that during the hydroponic experiment, heavy metal concentrations in the solution decreased continuously with time. *P. multifida* accumulated 90%, 36% and 50% of Pb, Cd and As, respectively, from the mixed metal solution within 24 days. The hydroponic experiment comparing *P. multifida* and *P. cretica* with *p. vittata* revealed that 33% and 43% of As(III) was removed by *P. multifida* and *P. cretica* respectively, whereas *P. vittata* could not accumulate As(III) at all within 4 days'. As was translocated to the frond and some Pb and Cd was also translocated to the rhizome from the root during the hydroponic experiment. The results of the long-term (3 months) pot soil experiment provided confirmation of the As(V), Pb and Cd uptake and translocation potential of temperate fern (*P. multifida).* After 3 months, *P. multifida* grown in metal-spiked soil stored significantly higher concentrations of As, Pb and Cd compared with the control plants ($P < 0.01$). The heavy metal (As, Pb and Cd) accumulation capacity and translocation ability of temperate ferns were demonstrated in this study, providing evidence that it could be applied for the treatment of water and soil that are not only contaminated with As but also co-contaminated with Pb and/or Cd.

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CHAPTER 3

Arsenic accumulating potential of temperate zone ferns at low temperature

3.1 Introduction

The metalloid Arsenic (As) is a carcinogenic element and persistent contaminant in water and soil. There is a practical need to control As because of its demonstrated toxicity and its mobility in the environment (Fowler et al., 2013). As presents simultaneously with other metals in the environment (Groudev et al., 2001; Kim et al., 2003), and can be readily absorbed by the plants, with negative effects on the growth. Moreover, the presence of As in the environment affects the plant uptake of essential metals due to their chemical similarity with these elements, and to competition for the same cellular transporters/channels (Verbruggen et al., 2009). It was reported that 42.7 million people in West Bengal, India and 79.9 million people in Bangladesh suffered from groundwater contamination by As, with As levels in groundwater above the World Health Organization maximum permissible limit of 50 µg/L (Chowdhury *et al.* 2000).

For removal and recovery of toxic heavy metals, biological methods have been recommended as cheap, effective and environmentally-friendly compared with other conventional (physical and chemical) methods (Hanif, Bhatti and Hanif 2009). Phytoremediation is one of the most promising biological technologies for the removal of environmental pollution (Rahman *et al.* 2008). Recently, heavy metal accumulation by plants, such as *Cynodon dactylon* (Wu *et al.* 2010), *Salvinia natans* (Dhir and Srivastava 2011), *Melastoma malabathricum* L. (Selamat, Abdullah and

Idris 2014), Switchgrass (Jeke, Zvomuya and Ross 2016) and *Pteris vittata* (Ronzan et al. 2017) has been tested and the effectiveness of phytoremediation by these plants has been established. Since the initial report of a Chinese brake fern *(P. vittata)* as an arsenic hyperaccumulator (Ma et al. 2001), the use of fern for this removal technique has developed considerable interest. Usually, *P. vittata* naturally grows in sub-tropical zone (average temperature: 7-15°C in winter; 25-30°C in summer). Although *P. vittata* is the most studied fern, it is limited in its cold-tolerance for being a tropical zone fern, even germination and growth rates of *P. vittata* were also limited at around 25°C (Wan et al. 2009).

Actually, for tropical zone plants, lower temperature affects transpiration, growth and metabolism of plants and therefore both uptake and elimination efficiency of pollutants (Yu et al. 2005). In addition, temperature has a profound effect on plant growth rates. Usually higher temperatures will result in greater biomass production and distribution of submersed macro phyte communities (Marschner, 1995 and Rooney and Kalff, 2000). Water chemistry may also be influenced by the effect of temperature (Fritioff et al. 2005). As cool water contains more dissolved oxygen than warm water, metal concentration in the interstitial water of the sediment may decrease with decreasing temperature because of low redox potentials (Förstner, 1979). Moreover, there is a positive relationship between temperature and metal accumulation has been identified (Almas and Singh 2001; Mander and Jessen 2002; Fritioff et al. 2005).

As successful phytoremediation requires the treatment of contaminated sites for all over the year, so it is of interest to find an alternative cold-tolerant heavy metal-accumulating fern. *P. multifida* (spider brake fern) as well as *P. cretica (*Cretan brake fern) are two As hyperaccumulators which can accumulates 5510 mg/kg and 5584 mg/kg of As from 200 mg/kg and 100 mg/kg As contaminated soil (Slonecker et al., 2009). Actually these are very common plants native to Europe, Asia and Africa. Both of them are also distributed almost all areas in Japan and China including temperate areas. *P. cretica* can grow in a minimum temperature of 2 °C (RHL et al., 2008). Similarly, *P. multifida* has already shown some ability to tolerate low temperatures from 5 °C to -4.6°C (measured by thermometer in the field) during field experiments (Sugawara, Chien and Inoue 2015), which is an essential property for large scale phytoremediation in temperate zones such as the northern part of Japan.

There is currently limited knowledge of effect of temperature on the accumulation of heavy metals by ferns and to date there is no published information of effect of temperature on As accumulating potential of three different ferns from hydroponic solution. Therefore, the aims of the present study were: (I) to determine the effect of temperature on comparative As accumulating potential of *P. vittata, P. cretica* and *P. multifida* from water; (II) to investigate the comparative tendency to release accumulated As to the water media by *P. multifida, P. cretica* and *P. vittata*; and (III) to suggest the comparatively better cold tolerant As hyperaccumulating fern for the practical application of As contaminated water site. Hopefully the results of this study will provide novel information on the ability of the above three ferns to accumulate As from water. These results may contribute to the future research to select the most effective fern for the applied phytofiltration of As-contaminated water.

3.2 Materials and Methods

3.2.1 Plant materials

P. multifida, P. cretica and P. vittata were obtained from Fujita Co. (Tokyo, Japan). At Fujita company those *Pteris* were prepared from spore [Fig 3.1 (a)]. To prepare *Pteris* sporophytes from spores, almost 7 or 8 months had passed. 4 or 5 frond containing ferns were received at the age of 7 or 8 months. The average height and fresh weight was around 23 cm and 3.50 g respectively. Efforts were made as much as possible to ensure the uniformity and similarity of the size of plants used in the experiments [Fig 3.1 (b)]. Although sand was washed with tap water to remove the adhering compost from the roots, which is relevant to real treatment conditions (Natarajan et al 2008; Huang et al. 2004) but the ferns were used without sterilization for hydroponic cultivation. For each set-up, five plants of approximately equal size were used and after analysis, the average value of best three plants was selected for results.

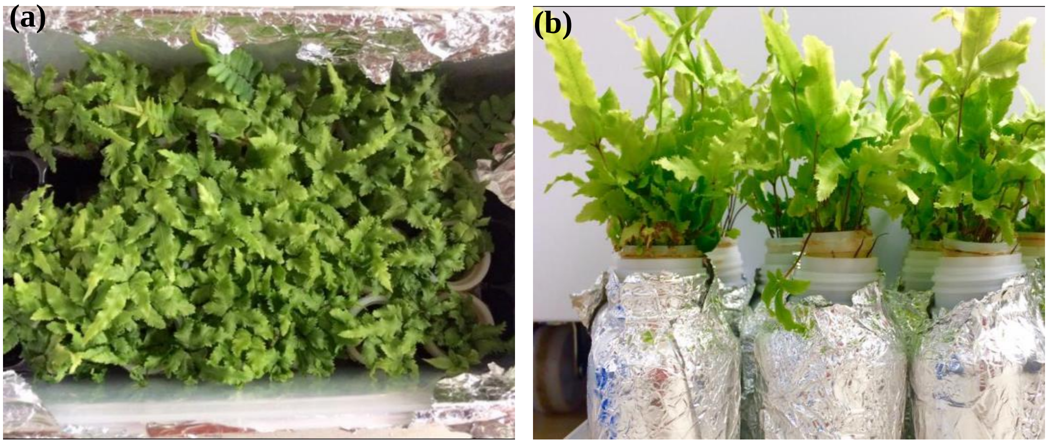


Fig 3.1(a) *Pteris* sporophytes produced from spores; (b) Uniformly sized plants that used for experiments

3.2.2 Growth condition:

Then plants were transplanted to opaque containers where they were acclimatized in 250 mL 5 times diluted Hoagland's solution (Hoagland and Arnon 1950) for three weeks in order to ensure their recovery. The Hoagland's solution was composed of: 8 mM of KNO_3; 4 mM $Ca(NO_3)_2$; 2 mM $MgSO_4$; 1 mM $NH_4H_2PO_4$; 50 µM H_3BO_3; 9 µM $MnSO_4$; 1 µM $ZnSO_4$; 0.2 µM $CuSO_4$; 0.1 µM Na_2MoO_4; and 60 µM Fe(III)-EDTA. For pre-cultivation of ferns, growth conditions were as the following: 16-h light period with a light intensity of around 280 µmol /m^2s; 25 °C daytime; 20 °C night. Humidity for the pre-cultivation of *P. vittata* was 70% but in case of *P. multifida* and *P. cretica* it was increased to 80%. Because those temperate ferns were growing faster at higher humidity. After adapting to the hydroponic environment, the plants were used for temperature controlled As accumulation experiments.

3.2.3 Comparative As accumulation trial of three ferns at 5^0C, 10^0C, 15^0C and 25^0C

In this study, temperature controlled accumulation test has done for ensuring the effect of different temperature on accumulation efficiency. After adapting with the hydroponic condition, 4 sets of each fern (*P. vittata, p. cretica and P. multifida*) has transplanted to 5 mg/L As(V) solution which was made by dissolving sodium arsenate into milli-Q water (Millipore, USA). Initial sampling has done before transplantation of plants to the As solution. Then four sets of plant (each set contains 5 plants) were decided to move into 4 individual incubators kept at 5^0C, 10^0C, 15^0C and 25^0C. For maintaining the temperature at 5^0C, 10^0C and 15^0C and other conditions, incubators were preset before 24 hours. The plant set for 25^0C was kept at the controlled growth chamber with maintaining the conditions explained above including strictly complied 25^0C temperature by air condition. Until 5th day of experiment, sampling was done every day and then alternate day sampling was done up to the end of the experiment (15 days).

3.2.4 Temperature dependent release tendency of three ferns

Probability the tendency to release accumulated metal to the medium by the plant is one of the great concern in case of phytoremediation. Release of accumulated As may inhibit successful phytoremediation by ferns even at the slightly contaminated site. Release experiments were also done for ensuring the release property of the plant. In the first step, the 4 set of plants that were selected for release experiment, were assimilated to 5 mg/L arsenic solution for two weeks at room temperature (25^0C) so that they can accumulate arsenic as much as possible. Then arsenic containing ferns were washed away three times and wiped away. Then in the second step, that arsenic containing ferns were transplanted to the Milli-Q water and send each set to different incubator at 5^0C, 10^0C, 15^0C to observe the effect of different temperatures to the release property of three ferns. The plant set for 25^0C was also kept in the temperature controlled (25^0C) growth chamber together with accumulation set. For this release test, sampling schedule of water was same as the above accumulation experiment from first to last.

Photograph of the plants were taken each and every day for observing the gradual change of physical appearance by the effect of temperature. Solution pH was also recorded initially at every temperature for both accumulation and release experiment.

3.2.5 Sample preparation and chemical treatment

After completion of 15 days' experiment, plant samples from accumulation and release test were harvested to wash it 3 times with Milli-Q water, dried in oven for 3 days and about 10 mg of dried plant samples were digested with 4 ml of concentrated HNO_3 on a heating block (ALB-121, Acinics, Japan) at 130°C for 90 min. The digested plant samples were cooled at room temperature and diluted using Milli-Q water (Millipore, USA), filtered with a PTFE 0.45µm filter membrane (Millipore, USA) and stored for analysis in polypropyline tubes.

Quantitative analysis of As was done by using inductively coupled plasma mass spectrometry (ICP-MS) (ELAN 9000, Perkin Elmer, SCIEX). Internal standard was 10 ng/ml indium (In). Detergent solution, 3% HNO_3 and Milli-Q water were used for proper washing of glass wares. All reagents were of analytic grade.

3.2.6 Data analysis

The values reported in both text and figures are the mean ± SE (standard error of the mean). The statistical significance (at 95% confidence) was tested using analysis of variance, where appropriate, and the numbers of replicates are included in the figure legends.

3.3 Results and Discussion

3.3.1 Temperature dependent As accumulation of three ferns at 5^0C, 10^0C, 15^0C and 25^0C

Change of temperature significantly affects the As removal potential of *P. vittata*, *P. multifida*, and *P. cretica*. During 15 days experiment, tropical zone fern *P. vittata* showed that 95%, 53%, 37% and 7% As was up taken at 25^0C, 15^0C, 10^0C and 5^0C respectively Fig 3-2(a). Higher accumulation capacity of *P. vittata* at room temperature (25^0C) supports the hydroponic accumulation results of Wang et al. (2002). But being a tropical fern, accumulation potential of this fern was clearly affected by low temperature which was not reported yet. Accumulation efficiency of *P. vittata* at 10^0C or 5^0C was significantly lower than the efficiency at 25^0C ($P<0.05$). At the same time, temperate fern *P. multifida* accumulated 100%, 99%, 93% and 30% of As at 25^0C, 15^0C, 10^0C and 5^0C respectively from 5 mg/L sodium arsenate solution, as shown in Fig 3-2(b). Removal potential of *P. multifida* was compared in case of four different target temperature. Comparative study between *P. vittata* and *P. multifida* showed that although maximum accumulation capacity at 25^0C was almost same for both of them, being a temperate zone fern, As accumulating potential of *P. multifida* was significantly higher at 10°C ($P<0.05$) compared with *P. vittata*. Accumulation capacity of *P. multifida* at 5°C was also higher than *P. vittata* but not at a level of significance ($P>0.05$). Another temperate zone fern *P. cretica* accumulated 57% As at 25^0C, 52% As at both 15^0C and 10^0C and 27% at 5^0C during 15 days (Fig. 3-2(c)). Although maximum accumulation capacity of *P. cretica* at 25^0C was almost 50% lower than other two ferns, at low temperature like 10^0C, As uptake capacity of *P. cretica* was significantly higher ($P<0.01$) than *P. vittata* where at 5^0C it was also higher but not significant (*P*>0.05) compared with *P. vittata*. Absorption accumulation might be influenced by temperature than adsorption (Garnham at al. 1992; Harris 1999) which supports the above results about As absorption by *P. vittata*. However, the chemistry of the root zone is responsible for the degree to which temperature can affect the mechanism of accumulation (Peer et al. 2006), biomass and plant growth (Almas and Singh 2001), and other metal and nonmetal constituents (Fritioff et al. 2005; Peer et al. 2006). A number of studies, specially Fritioff et al. (2005), have shown that temperature has a positive effect on metal accumulation in various species. In agreement with these studies, our study noted that although not significant, both species tended to accumulate more As under higher versus lower temperatures. Different patterns of accumulation and growth of tropical and temperate ferns at low temperature indicated that tropical fern *P. vittata* might not belongs to the active temperature sensing system, where temperate ferns were changing their morphology in winter to adapt with low temperature by using their temperature sensing system.

If accumulation capability at room temperature (25 °C) was considered as standard (100%) for all three target ferns, at low temperature like 15^0C and 10^0C, temperate fern *P. multifida* as well as *P. cretica* has accumulated almost 100% As but only 67% and 31% As was up-taken by tropical fern *P. vittata* at 15^0C and 10^0C respectively [Figs. 3-3 (a)(b)(c)]

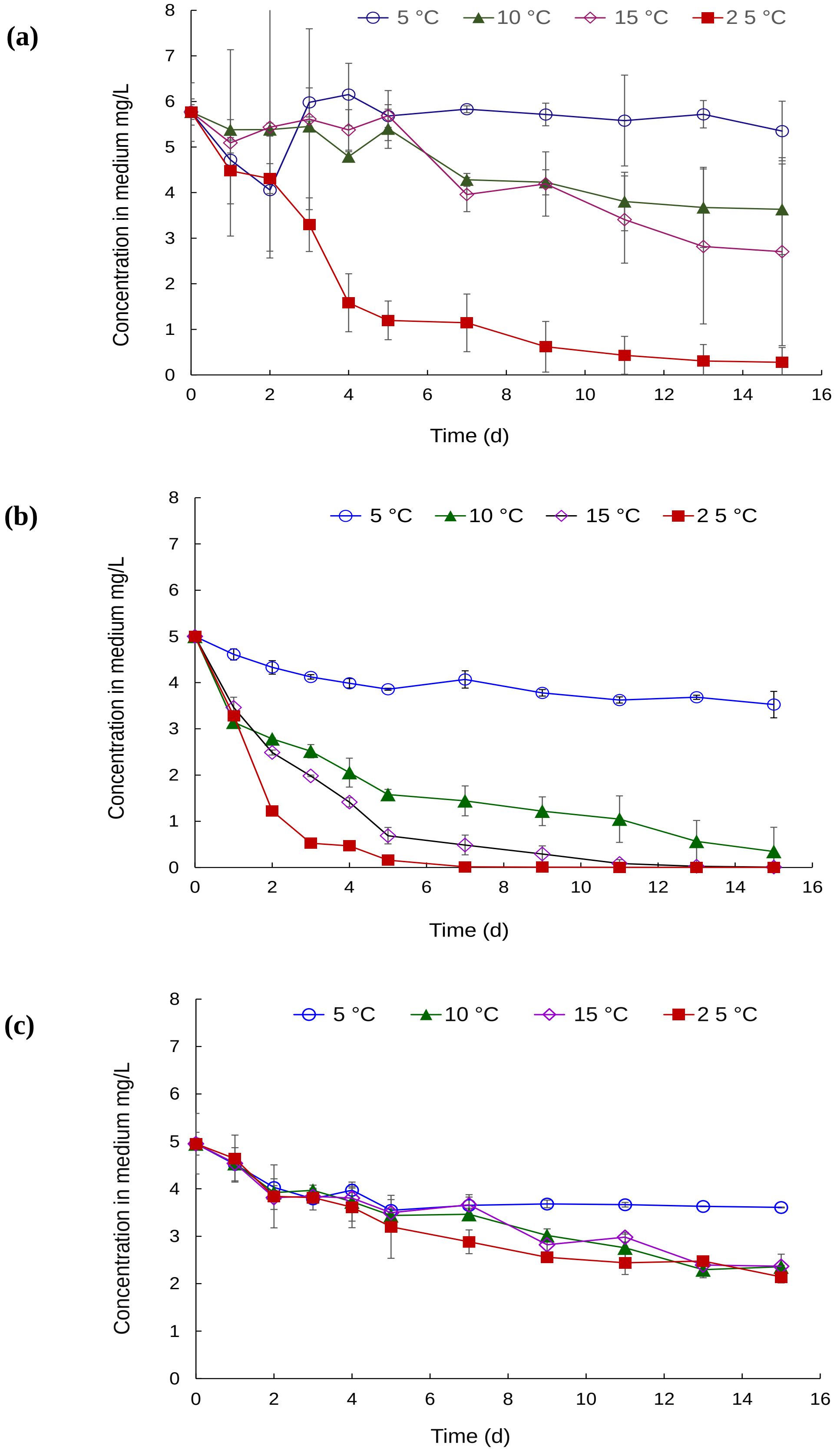


Fig 3-2 Concentrations of arsenic (As) removed from hydroponic solution during 15 days' incubation at 25^0C, 15^0C, 10^0C and 5^0C with (a) *Pteris vittata* (n=3); (b) *Pteris multifida* (n=3); (c) *Pteris cretica* (n=3)

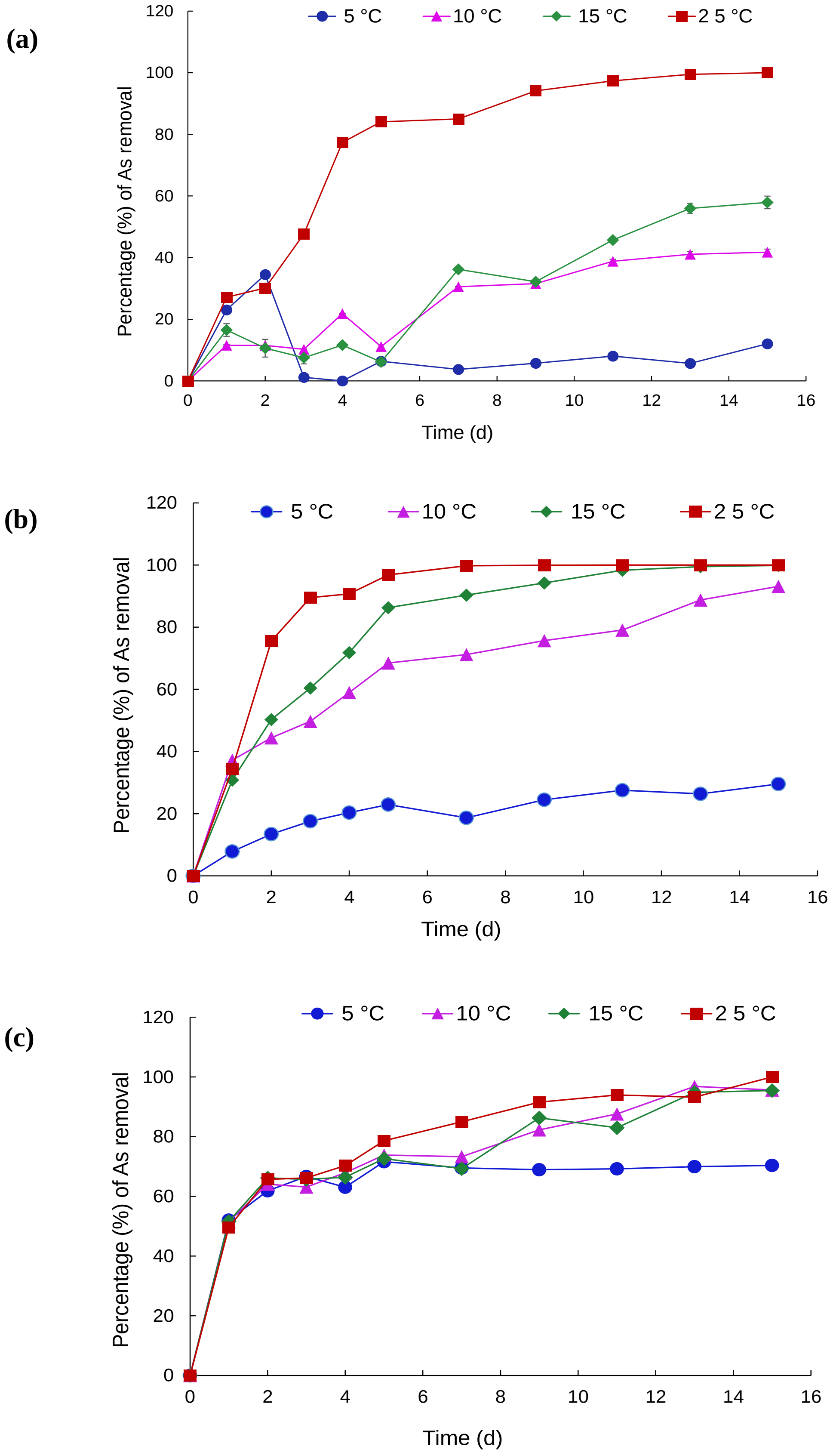


Fig 3-3 Percentage of arsenic (As) removed by (a) *Pteris vittata;* (a) *Pteris multifida;* (a) *Pteris cretica* from hydroponic solution during 15 days' incubation at 15^0C, 10^0C and 5^0 C (n=3) when removal at 25^0C was considered as standard (100% removal)

3.3.2 Accumulation of As in root, rhizome and frond of three ferns

The metal concentrations in plants increased with increasing temperature (Fig 3-4 (a)(b)(c)) after 15 days' assimilation in 5 mg/L arsenic injected hydroponic solution, in case of tropical zone fern *P. vittata*, 2.6×10^3, 2.3×10^3, 1.0×10^3 and 2.0×10^2 mg/kg dw of As was up-taken by the plant at 25^0C, 15^0C, 10^0C and 5^0C respectively. After 15 days' hydroponic experiment, comparative concentration of accumulated As in *P. vittata* was lower than *P. multifida* at all temperature but not at the level of significance ($P > 0.05$) as *P. multifida* contained 4.4×10^3, 2.7×10^3, 1.9×10^3 and 4.6×10^2 mg/kg dw of As at 25^0C,15^0C, 10^0C and 5^0C respectively. Significantly higher concentration of As was stored at 25^0C (4.4×10^3 mg/kg) ($P < 0.05$) than at lower temperature like 5^0C or 10^0C, similar to the results of Xiao-Zhang Y. et al. (2010) about Cr(VI) accumulation by hybrid willows. Their results indicated that, At the low temperature (11^0C), 12.53% of the applied Cr(VI) were removed from the hydroponic solution but at high temperature like 32^0C, 61.30% of the applied mass was removed by plants. At 25^0C,15^0C, 10^0C accumulated As was clearly transported to frond by *P. vittata* within 15 days' where in *P. multifida* or *P. cretica*, As was distributed into root, rhizome and frond. After 15 days' accumulation, almost 40% accumulated As was remained in the root of *P. multifida*. No significant difference was found between the concentrations of As in three different parts of *P. multifida* or *P. cretica* ($P > 0.05$). This result indicated that As transportation capacity of *P. vittata* from root to frond might be faster than other two ferns.

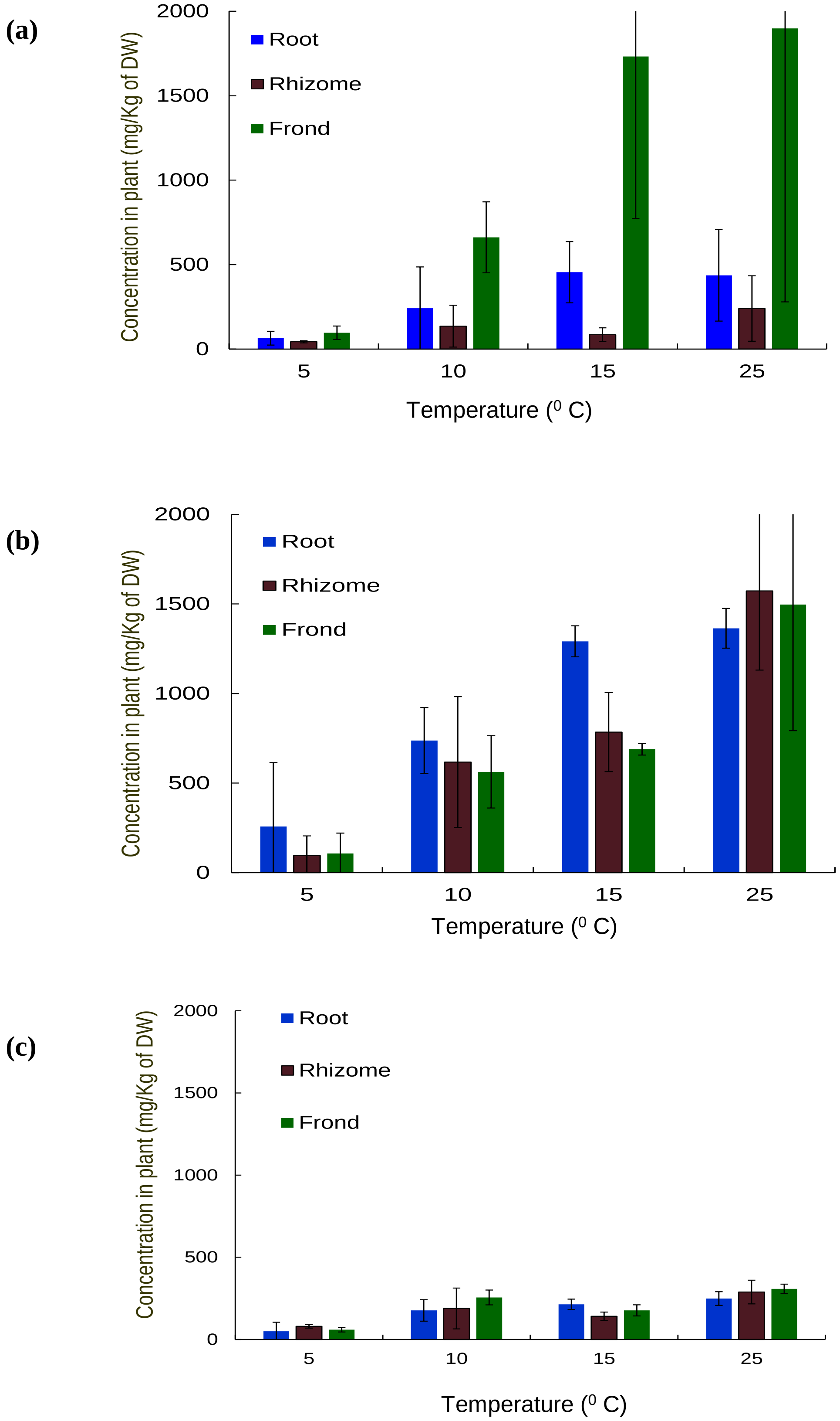


Fig. 3-4 Transportation of arsenic (As) in different parts (fronds, rhizome and roots) of (a) *Pteris vittata;* (b) *Pteris multifida;* (c) *Pteris cretica* after the 15-days' hydroponic experiment *(n* = 3)

Comparison between the dry biomass of three target ferns showed that biomass of *P. cretica* was higher than other two ferns. As the dry biomass of frond was significantly higher ($P < 0.05$) than root (Table 3-1) in all ferns at 25^0C, 15^0C, 10^0C, the amount of As stored at frond was also significantly higher ($P < 0.05$) compared with root (Fig. 3-5 (a)(b)(c)).

Table 3-1: Dry biomass of tropical (*P. vittata*) and temperate (*P. multifida* and *P. cretica*) zone ferns at four different temperature

		Dry biomass (g) of three ferns		
		P. vittata	*P. multifida*	*P. cretica*
	Root	**0.2**±0.05	**0.3**±0.08	**0.2**± 0.04
5 ° C	Rhizome	**0.2**±0.01	**0.1**±0.04	**0.2**±0.02
	Frond	**0.5**±0.01	**0.4**± 0.03	**0.9**± 0.10
	Root	**0.2**±0.10	**0.3**± 0.04	**0.3**± 0.02
10 ° C	Rhizome	**0.3**±0.10	**0.2**± 0.01	**0.2**± 0.03
	Frond	**0.5**±0.10	**0.7**± 0.2	**1.9**± 0.00
	Root	**0.2**±0.07	**0.3**± 0.02	**0.2**± 0.02
15 ° C	Rhizome	**0.3**±0.10	**0.1**± 00	**0.2** ±0.09
	Frond	**0.6**±0.20	**0.8**± 0.06	**1.6**± 0.30
	Root	**0.3**±0.08	**0.2**± 0.06	**0.3**± 0.07
25 ° C	Rhizome	**0.3**±0.10	**0.1**± 0.02	**0.2**± .0.08
	Frond	**0.8**±0.30	**0.5**± 0.20	**1.4**± 0.50

Maximum amount of As was found in the frond of *P. vittata*, *P. multifida* and *P. cretica*. Comparative transportation between three ferns indicated that the majority of the As was accumulated in the fronds of *P. vittata* and *P. cretica* and small amounts were remained in roots and rhizomes. At 25^0C,15^0C, 10^0C accumulated amount of As in the frond was significantly higher ($P \leq 0.05$) than root (Fig. 3-5(a)) in *P. vittata*. But in *P. multifida*, almost 35% As of total accumulated amount was remained in the root.

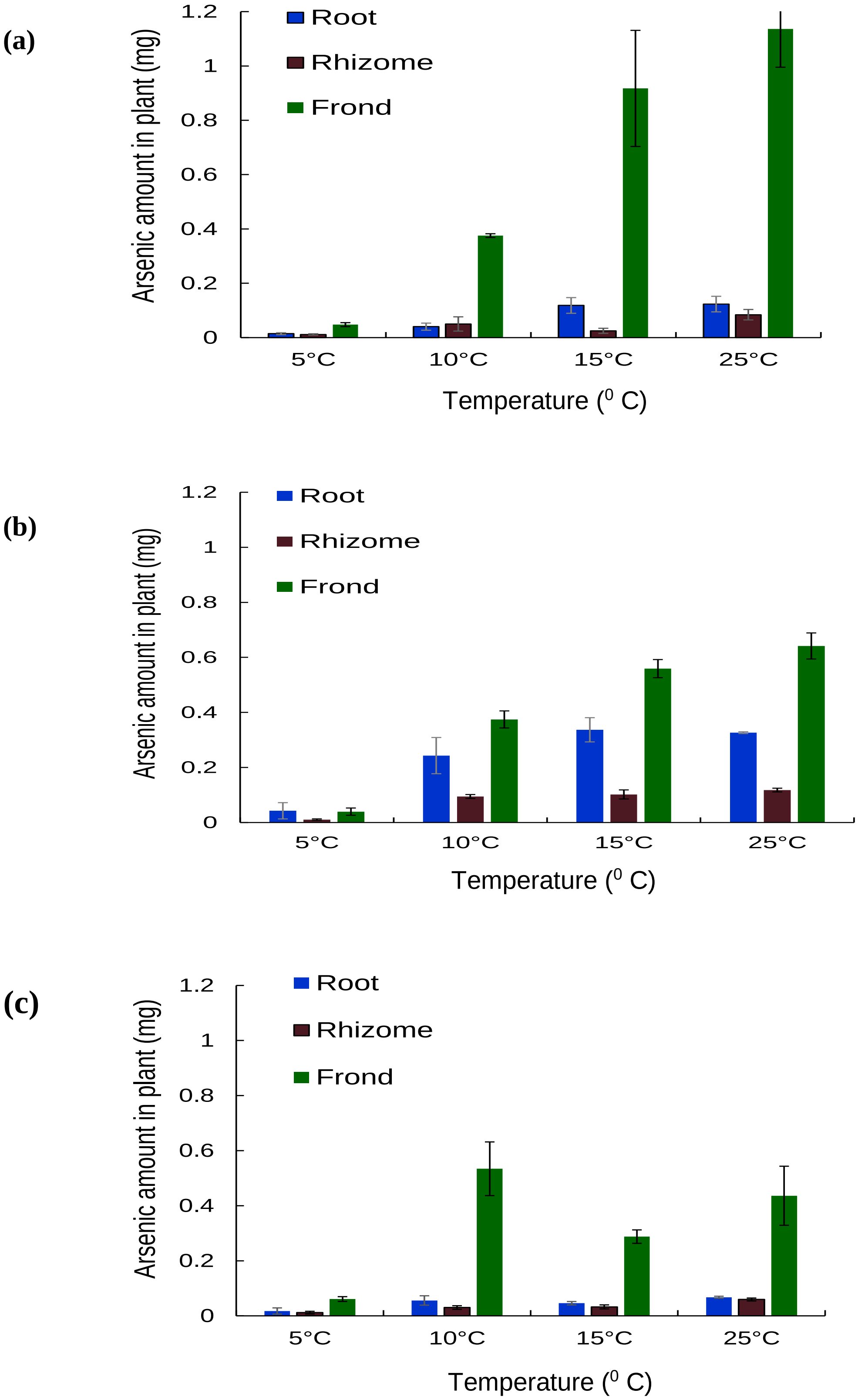


Fig. 3-5 Transported amount of arsenic (As) in different parts (fronds, rhizome and roots) of (a) *Pteris vittata;* (b) *Pteris multifida;* (c) *Pteris cretica* after the 15-days' hydroponic experiment *(n* = 3)

So, the transportation rate of *P. multifida* from root to frond might be slower than other two ferns. Another temperate zone fern *P. cretica* up-taken 8.5×10^{2}, 5.3×10^{2}, 6.2×10^{3} and 1.9×10^{2} mg/kg dw of As at 25^{0}C, 15^{0}C, 10^{0}C and 5^{0}C respectively. Total amount of As accumulated by *P. cretica* was lower than *P. multifida* and *P. vittata* but not significance level ($P = 0.07$). Amount of As in frond was significantly higher ($P < 0.05$) than root because of high dry biomass of frond of *P. cretica* (Fig. 3-5(c)). Mass balance calculation between accumulated As amount in plant (*P. vittata*, *P. multifida* and *P. cretica*) and amount of As removed from solution showed that at 25^{0}C, balance was good (92%) but at low temperature amount of As in plant was lower than the amount removed from solution. Low temperature might be damaged the plants or made them weak, thus during washing some amount of As might be washed away from the plant.

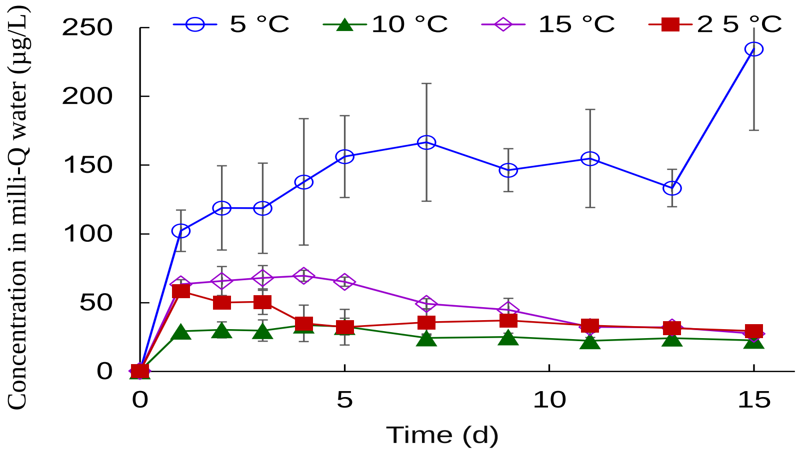
3.3.3 Effect of temperature on As release tendency of three ferns

Now-a-days release of accumulated metal by plant to the media is one of the great concern for successful phytoremediation. Low temperature is one of the vital issue that makes plants too weak to retain the accumulated metals. In this research, release experiments have done at four different temperatures to ensure the release tendency of plants by the effect of low temperature. After 15 days' pre-cultivation to 5 mg/L As solution at 25^{0}C, *P. vittata*, P. *multifida* and *P. cretica* has accumulated almost 100% As. Then they have transplanted to Milli-Q water kept at four temperature controlled incubators at 25^{0}C,15^{0}C, 10^{0}C and 5^{0}C. Tropical fern *P. vittata* showed that 3.5 µg/L, 27 µg/L, 22 µg/L and 234.5 µg/L As was released to the medium at 25^{0}C,15^{0}C, 10^{0}C and 5^{0}C respectively (Fig. 3-6(a)). At 25^{0}C 0.9 ± .03 µg amount of As was released to

Milli-Q water but at 15^0C and 10^0C 6.7±0.7 µg and 5.5±0.8 µg As was released respectively. As *P. vittata* is a tropical zone fern, at 5^0C it has almost died and 10% of total accumulated As 59±2.2 µg was released to Milli-Q water which was not accumulated again by *P. vittata*. Temperate fern *P. multifida* released 1.9 µg/L, 1.2 µg/L, 4.9 µg/L and 83.2 µg/L As to the Milli-Q water at 25^0C, 15^0C, 10^0C and 5^0C respectively within 15 days' (Fig. 3-6(b)). If the released amount was considered, 0.4 ± 0.05 µg As at 25^0C and 15^0C and 1 ± 0.09 µg at 10^0C was released when all released concentrations were again up-taken by this fern *P. multifida.* Although *P. multifida* is also sensitive to 5^0C, but only 3.7% (21 ± 0.07 µg) of total accumulated As was released to the Milli-Q water at this low temperature. On the other hand, *P. cretica* released 1.0 µg/L, 1.9 µg/L, 1.6 µg/L and 34.1 µg/L As at 25^0C, 15^0C, 10^0C and 5^0C respectively (Fig. 3-6(c)). Released amount was also calculated. 0.2±0.09 µg, 0.4±0.08 µg and 0.4±0.05 µg As was released at 25^0C, 15^0C, 10^0C temperature respectively when that amounts were accumulated again within 15 days'. Although it is also a temperate zone fern, it might be sensitive to low temperature like 5^0C as it has released 10% (34±1.4 µg) of total accumulated As to the medium.

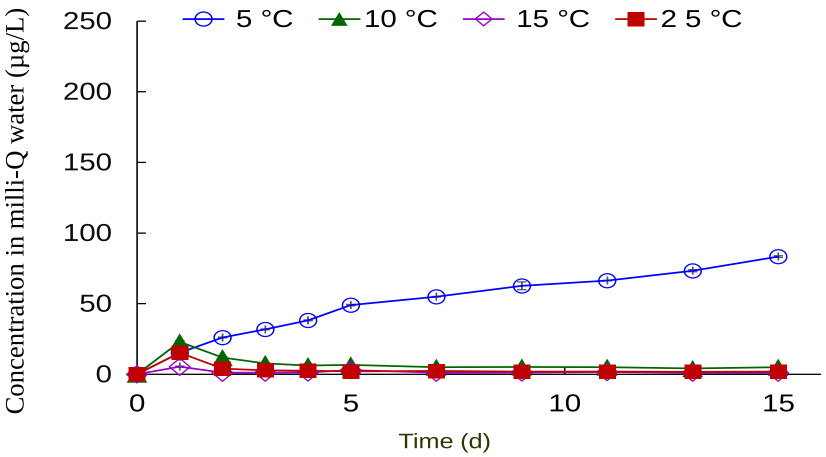
These results clearly showed that being a temperate zone fern, *P. mutifida* can retain almost all accumulated As at 25^0C, 15^0C and even at 10^0C although its transportation rate of As from root to frond was slower than other two ferns. 5^0C temperature is still critical for all plants to survive in a healthy state. Each and every results are the average of three similar ferns where SD values were put at each point. In case of *P. multifida* the results from three ferns were almost same and SD values were too small to appear.

(a)



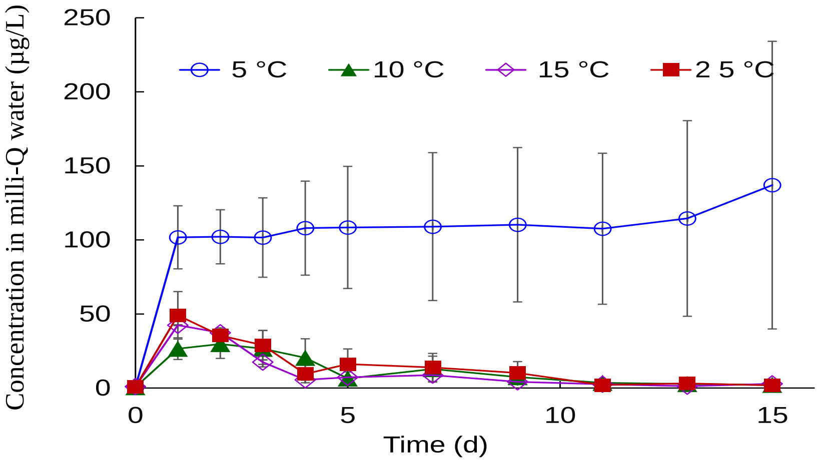
Pteris vittata

(b)



Pteris multifida

(c)



Pteris cretica

Fig 3-6 Concentrations of released arsenic (As) in milli-Q water during 15 days' incubation at 25^0C, 15^0C, 10^0C and 5^0C with (a) *Pteris vittata* (n=3); (b) *Pteris multifida* (n=3); (c) *Pteris cretica* (n=3)

3.4 Conclusion

Results from this study demonstrated that *P. multifida* removed almost same concentration of As as *P. vittata* from hydroponic solution at 25^0C but at low temperature, accumulation potential of this temperate fern was faster and more effectively than *P. vittata* and *P. cretica*. For being a temperate zone fern, at low temperature like 10^0C or 15^0C, *P. multifida* accumulated significantly higher concentration of As ($P<0.05$) compared with most studied tropical zone fern *P. vittata* . At 5°C or 15°C accumulation capacity of *P. multifida* was also higher than *P. vittata* but not at a level of significance ($P>0.05$). Another temperate zone fern *P. cretica*, although it was not so sensitive to change in temperature but the maximum accumulated As by this fern was significantly lower ($P=0.005$) that *P. multifida*. Maximum amount of As was stored in the frond at all temperature for all three ferns. Analysis of comparative release properties showed that except 5^0C, initially released small concentration of As in Milli-Q water was accumulated again by *P. multifida* as well as *P. cretica* but tropical zone fern *P. vittata* could not accumulate total concentration of initially released As. Though temperate zone fern *P. multifida* and *P. cretica* released lower concentration than *P. vittata* at 5^0C but 5^0C temperature is still critical for all plants. Therefore, more investigations are needed to make plants sustainable even at $\leq 5^0$C. Comparative potential of As accumulation, translocation and release of *P. multifida, P. cretica and P. vitata* in this study providing evidence that *P. multifida* could be the most suitable fern for the treatment of As contaminated water at temperate zone area.

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CHAPTER 4

Application of tropical and temperate zone ferns to an arsenic contaminated leachate at low temperature

4.1 Introduction

In Japan, arsenic contaminated leachate might be produced by mining activities and dumped excavated soil from various purpose such as tunnel drilling, construction of roads and railroads, construction of subway etc. Extreme care about arsenic contamination is necessary for reuse and landfill disposal. In addition, environmental standard in soil (leaching) for arsenic is 10 µg/L which should be maintained strictly to ensure safe disposal of leachate. So, proper management is required.

A significant source of metals released into the environment is the solid waste disposals (open dumps, landfills, sanitary landfills or incinerators). (Yarlagadda et al. 1995; Waheed et al. 2010; Iwegbue et al. 2010; Bretzel and Calderisi 2011; Rizo et al. 2012). Primarily leachate is produced in association with precipitation that infiltrates through the refuse and normally results in the migration of leachate into the groundwater zone and pollutes it (Samuding 2009). Waters and soils have been contaminated with heavy metals such as lead, arsenic, zinc, iron, manganese, chromium, and cadmium due to migration of leachate ,and these heavy metals in solid wastes lead to serious problems because they cannot be biodegraded (Hong et al. 2002).

During the construction of Sendai City Subway Tozai Line which opened on 6th December, 2015, about 600,000 m^3 excavated soil containing small amount of As was dumped at Akaishi area from 2008 to 2014 that may dissolve arsenic and produce arsenic contaminated leachate. The dumping site generates about 30 L leachate water/

min which contains 20 to 30 µg/L As that exceed the arsenic concentration of Japanese water quality standard (10 µg/L). Therefore, a process for removing arsenic was required. Currently, leachate water containing arsenic is purified at the site by adsorption method. However, high processing cost and generation of secondary waste is a problem for long term removal of widely spread arsenic contamination.

For removal of toxic heavy metals like arsenic from water, some traditional methods such as adsorption method, ion exchange method, coagulation sedimentation method etc. has been applying. Although those methods are effective, but proper application requires high cost. Another problem is the production of secondary waste. the use of biological methods like phytoremediation has been recommended for pre-treatment because they can be cheap, effective and environmentally-friendly compared with other conventional (physical and chemical) methods (Hanif, Bhatti and Hanif 2009). By applying phytoremediation, amount of sludge can be reduced significantly. This is one of the most promising biological technologies for the removal of environmental pollution caused by the limitations of traditional technologies (Rahman *et al.* 2008). However, successful application of phytoremediation takes a long time to process and depends on natural conditions.

In Japan, although adsorption method is used in preference, a high performance adsorbent is required to lower the arsenic concentration. There is a problem that the cost is particularly high. One of the successful environmental remediation is phytoextraction. Plants have the ability to take moisture from roots and to absorb nutrients as well as pollutants from the soil. Some plants have the ability to absorb pollutant and concentrate in the body. The merit of this method is low cost, effective, wide range of pollutant can also be treated.

To the best of my knowledge, hydroponic method by using *P. vittata* has not yet been applied for the removal of arsenic from contaminated leachate at low temperature, even though *P. vittata* has already applied for the phytoremediation of arsenic from contaminated soil (XIAO X. et al., 2008). Hydroponic culture system with no aeration was developed to apply tropical/sub-tropical fern *P. vittata* by Huang et al. (2015). The feature of this hydroponic culture method is the provision of nutrient salts. This system promotes root growth in hydroponic cultivation at underwater. *P. vittata* also applied to treat the arsenic contaminated leachate at Akaishi site. Actually treatment by *P. vittata* had started from July 2015 without any protection from cold stress but in winter *P. vittata* had started to damage by severe cold stress when outside air temperature decreased to 0^0C or below 0^0C. According to their results, from August 2015 to November 2015 *P. vittata* has gained biomass and accumulated As from Akaishi landfill leachate (Huang et al. 2018), but from November 2015 tropical fern *P. vittata* has started to damage by cold stress and released As to the hydroponic media (Huang, personal communication). Usually, *P. vittata* naturally grows in sub-tropical zone (average temperature: 7-15°C in winter; 25-30°C in summer). Although *P. vittata* is the most studied fern, it is limited in its cold-tolerance for being a tropical zone fern, even germination and growth rates of *P. vittata* were also limited at around 25°C (Wan et al. 2009).

On the other hand, *P. cretica (*Cretan brake fern) is also an As hyperaccumulators which can accumulates 5584 mg/kg of As from 100 mg/kg As contaminated soil (Slonecker et al., 2009). *P. cretica* can grow in a minimum temperature of 2 °C (RHL et al., 2008). These two ferns are very common plants native to Europe, Asia and Africa. *P. cretica* distributed almost all temperate areas in Japan and China where *P. vittata* likes to grow at tropical/sub-tropical areas. Effect of low temperature on As accumulation by *P. vittata* as well as *P. cretica* was determined in chapter-3 which showed that As accumulating potential of *P. vittata* was decreased at low temperature (10^0C) where *P. cretica* showed almost same potential at 10^0C and 25^0C.

In this chapter, target is to apply temperate zone ferns as well as tropical zone ferns in As contaminated leachate at the temperate zone of northern part of Japan. I have approached for adaptation of ferns at low temperature and considerable removal of As from contaminated leachate although it is not so easy to keep the healthy condition of plants at any temperature during practical application to As contaminated leachate.

To protect the plants from severe cold temperature in winter season, very simple type greenhouse was used. Greenhouse plastic sheets were used above still frame to make this simple protection system. In order to treat by two different ferns, total leachate water coming from the entrance has divided into two tanks. Arsenic removal efficiency of those two ferns has examined regularly by collecting water and plant samples from tanks and analyzed arsenic concentration in the laboratory by ICP-MS.

4.2. Materials and methods

4.2.1 Plant materials

P. cretica and *P. vittata* were bought from Fujita Co. (Tokyo, Japan). Those *Pteris* were prepared from spore. It takes almost 7 or 8 months to prepare *Pteris* sporophytes from spores. We received 4 or 5 frond containing fern at the age of 7 or 8 months. The average height of those two ferns were around 20.0 cm. To ensure the uniformity and similarity of the size of plants used in the experiments, efforts have done. The ferns were used without sterilization for hydroponic cultivation, but sand was washed with tap water to remove the adhering compost from the roots, which is relevant to real treatment conditions (Natarajan et al 2008; Huang et al. 2004). Plants were treated with Hoagland's solution (Hoagland and Arnon 1950) in 250 mL opaque bottle in a growth chamber with the following conditions: 16-h light period with a light intensity of around 280 µmol /m^2s ; 25 °C daytime; 20 °C night; and 70% relative humidity.

4.2.2 Hydroponic trial with various concentrations of nutrient (Hoagland's solution)

To determine the lowest concentration of nutrient that is enough for natural plant growth, 5-months hydroponic trials were conducted with both *P. cretica* and *P. vittata*. In this study, plants were treated with 5, 10, 50, 100, 500, 1000 times diluted Hoagland's solution and tap water where Hoagland's solution was composed of: 8 mM of KNO_3; 4 mM $Ca(NO_3)_2$; 2 mM $MgSO_4$; 1 mM $NH_4H_2PO_4$; 50 µM H_3BO_3; 9 µM $MnSO_4$; 1 µM $ZnSO_4$; 0.2 µM $CuSO_4$; 0.1 µM Na_2MoO_4; and 60 µM Fe(III)-EDTA. Mill-Q water was used for the preparation and dilution of Hoagland's solution. During this experiment, nutrient solution was renewed every week and the plants and bottles were washed every week to protect them from the harmful effect of algae. Initial height of root and frond was measured with measuring scale. Initial weight was also recorded by analytic balance. The increase of height and weight was measured and recorded very carefully every month. Photographs were also taken to record the change of appearance in each and every month. The experiments were set up with five plants of approximately equal size and after analysis, results were calculated from the average of best three plants.

4.2.3 Application of *P. cretica* and *P. vittata* in an arsenic contaminated leachate

P. cretica as well as *P. vittata* were applied to an arsenic contaminated leachate that came from a dumping site (600,000 m^3 excavated soil has dumped) located in Akaishi area, Sendai, Miyagi, Japan [(Fig 4.1 (a)]. Initial arsenic concentration of that leachate was around 20-30 µg/L. In order to reduce the concentration to 10 µg/L (Japanese environmental standard), *P. cretica* and *P. vittata* was applied to two separate tanks that were come from the same entrance. Schematic diagram of treatment plant is given in Fig 4.1 (b). The height, weight and depth of each tank was 1.8m×0.9m×0.73m where flow rate of water to the tank was 0.5L/min. Retention time of the tank for 1.125 day (Huang et al 2017). Each tank contained 12 floating plates that kept the ferns remain floating like hydroponic cultivation. The ferns were adjusted to the plates so that the rhizome and frond was kept above the water level and roots were drowning into the water. Each plate contained 16 *P. vittata* (in case of tank 1) or 16 *P. cretica* (in case of tank 2). Both of the tanks contain total 192 plants. At November 2016 simple type greenhouse was made by using greenhouse plastic above the still frame to protect plants from severe cold.

Water sample was collected two times per month from entrance, tank 1 (treated by *P. vittata*) and tank 2 (treated by *P. cretica*) for analysis. Plant samples were also taken for analysis one time per month.

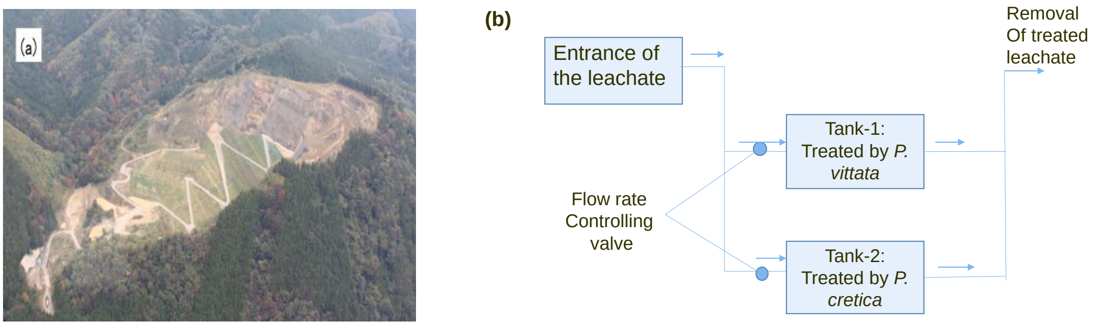

Fig 4.1 (a) Akaishi dumping area, Sendai, Miyagi, Japan; (b) Schematic diagram of treatment plant of leachate

4.2.5 Measurement of in-situ parameters in the leachate

In-situ parameters like outside and inside air temperature, water temperature and essential nutrient concentration were measured to ensure the suitable condition for effective arsenic removal by the ferns. Inside air temperature, outside air temperature and leachate water temperature was measured by the digital temperature recorder that have been fixed in the leachate area. Essential nutrient concentration like the concentration of phosphate (PO_4^{3-}) and nitrate (NO_3^-) was also measured by ICP-AES (Inductively coupled plasma - atomic emission spectrometry) and IC (Ion Chromatography) respectively to ensure that sufficient nutrients were available naturally for the healthy survival of plants.

4.2.6 Sample preparation and chemical treatment

After collecting water and plant samples from the leachate, plant roots and rhizomes were immersed in water for 5 min and then briefly rinsed in distilled water. Fronds were also washed three times with distilled water and then dried in an oven for 3 days. The dried plant samples (10 mg) were digested in 3 mL of concentrated HNO_3 on a heating block (ALB-121, Acinics, Japan) at 130 °C for 90 min. The digests were subsequently cooled and diluted 10-fold using Milli-Q water (Merck Millipore Corporation, Darmstadt, Germany), filtered through a PTFE 0.45 µm filter membrane (Merck Millipore Corporation) and then stored for analysis in 15 mL polypropylene tubes. Inductively coupled plasma mass spectrometry (ICP-MS; ELAN 9000, Perkin Elmer, SCIEX) was used for quantitative analysis of Arsenic in plant and water samples. The internal standard used during analysis was 10 µg/L indium (In). Standard reference materials were standard solution of Arsenic (Wako chemicals, Japan) and recovery rate was more than 100%. All glassware was washed five times with detergent solution, 3% HNO_3 and Milli-Q water. All reagents were of analytical grade.

4.2.7 Data analysis

The values reported in both text and figures are the mean ± SE (standard error of the mean). The statistical significance (at 95% confidence) was tested using analysis of variance, where appropriate, and the numbers of replicates are included in the figure legends.

4.3 Results and discussion

4.3.1 Limiting concentration of nutrient (Hoagland's solution) for natural growth of *P. vittata* and *P. cretica*

In order to ensure the proper limiting nutrient concentration for healthy survival of *P. vittata* and *P. cretica* in water, 5 months' hydroponic experiment with 6 different concentrations of nutrient (Hoagland's solution) and tap water has done in the growth chamber. After 5 months' assimilation, the highest growth of root was observed in case of 50 times diluted Hoagland's solution for both *P. vittata* and *P. cretica* [Fig 4.2 (a)]. For *P. vittata*, the increased height of root within 5 months was not significantly varied with the change of nutrient concentration ($P>0.05$) as it could survive even in the tap water. But in case of *P. cretica,* although after 5 months' growth of root in 5 to 1000 times diluted Hoagland's solution were not so varied, the growth of root in tap water was lower than others. So, tap water might not be sufficient for healthy survival of *P. cretica*. Lower concentration of nutrient could ensure the green survival of this temperate zone fern. The frond of *P. vittata* as well as *P. cretica* was not so significantly grown within 5 months' incubation ($P>0.05$).

After 5 months' assimilation, the weight gained by *P. vittata* and *P. cretica* was also measured to ensure the limiting concentration of nutrient for healthy survival. *P. vittata* has gained the highest weight at 100 times diluted Hoagland's solution where *P. cretica* has gained the highest at 500 times diluted Hoagland's solution [Fig 4.2 (b)]. Assimilation to tap water was decreased the weight of *P. cretica* but *P. vittata* has gained some weight (1.08g) after 5 months' assimilation without nutrient. So, this results of limiting concentration of nutrient could ensure that lowest concentration

(1000 times diluted) of nutrient (Hoagland's solution) is also enough for healthy survival of *P. cretica* where *P. vittata* can survive even in the tap water which will have helped us to maintain the proper nutrient concentration in the leachate for application of ferns.

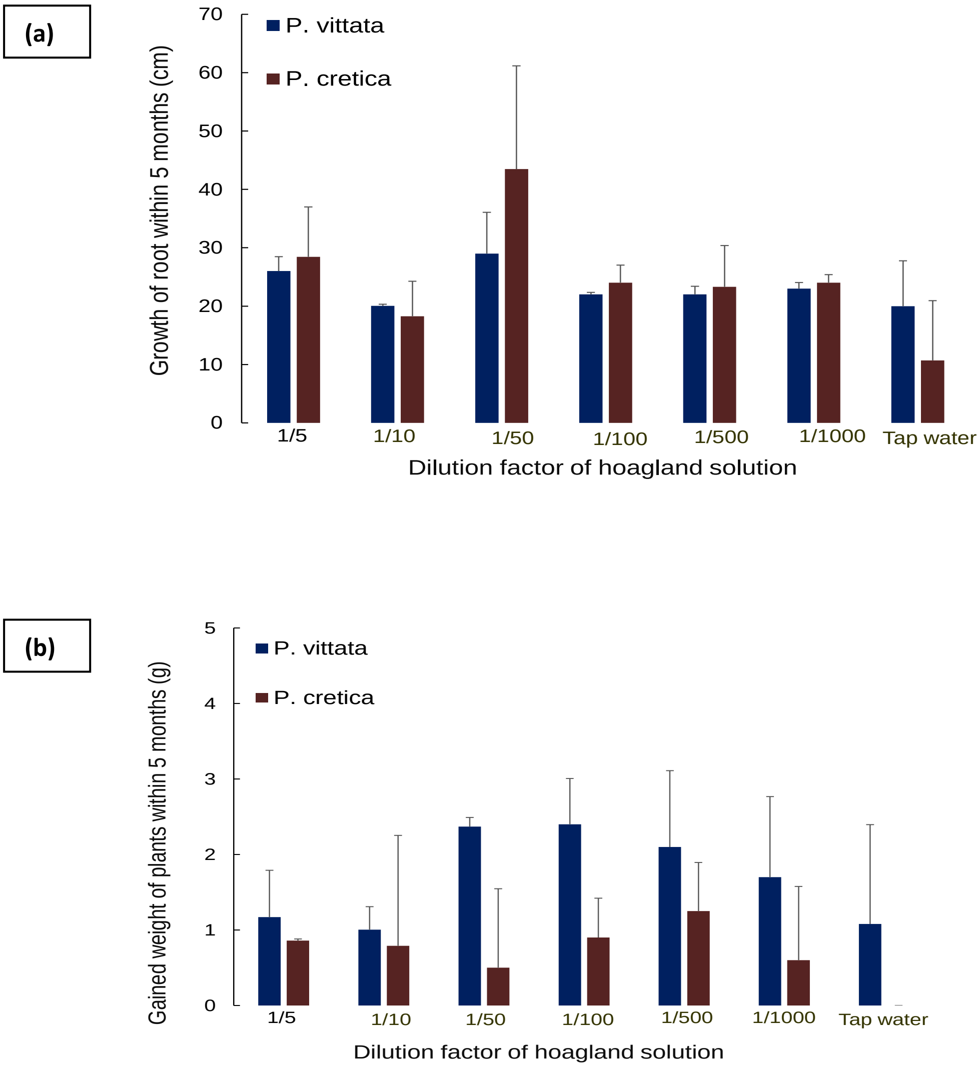

Fig 4.2: Increased height **(a)** and gained weight **(b)** of *P. vittata* and *P. cretica* during 5 months' incubation in different concentrations of hydroponic solution

Essential nutrient (PO_4^{3-} and NO_3^-) concentration in the leachate of Akaishi dumping site was also measured to ensure healthy survival of ferns in natural nutrient without any additional supply of nutrients. Phosphate and nitrate concentration in the leachate were around 3.4×10^{-3} mM and 3.2×10^{-2} mM respectively which are higher than 500 times diluted Hoagland's nutrient solution (Table: 4.1). Comparison with the result of limiting nutrient experiment proves that the remaining major nutrient concentrations in the leachate were enough for healthy survival and arsenic accumulation.

Table 4.1: Comparative concentrations of major nutrients at Akaishi leachate area And 500 times diluted Hougland's solution

	PO_4^{3-}	NO_3^-
Leachate nutrient concentration	3.4×10^{-3} mM	3.2×10^{-2} mM
500 times diluted Hougland's nutrient solution	2.0×10^{-3} mM	1.6×10^{-2} mM

Photographs of *P. vittata* and *P. cretica* after 5 months' assimilation in 7 different concentrations of nutrient solution are shown at Fig 4.3.

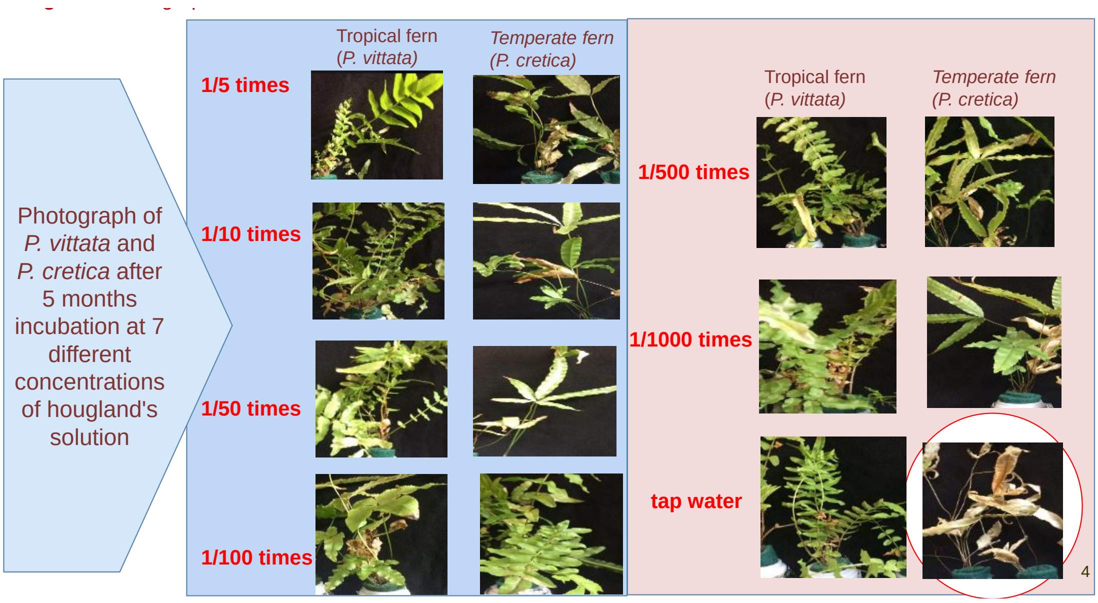

Fig 4.3: Photographs of *P. vittata* and *P. cretica* after 5 months' incubation in 7 different nutrient concentrations

4.3.2 Essential parameters for healthy survival of ferns

Essential parameter of leachate was monitored regularly for effective application of ferns to remove arsenic. Outside air temperature (outside of greenhouse), inside air temperature (inside of greenhouse) and leachate water temperature was measured regularly where outside temperature was lower than inside from November to March. During winter outside air temperature was almost same at 2015-2016 and 2016-2017 session, fluctuating from -5^{0}C to $+5^{0}$C [Fig 4.4 (a) (b)]. Lowest water temperature of leachate without greenhouse was around 5^{0}C which is critical for both *P. vittata* and *P. cretica*. By applying the simple type greenhouse lowest water temperature was controlled as 10^{0}C that ensured the plants health survival and accumulation [Fig 4.5 (a) (b)].

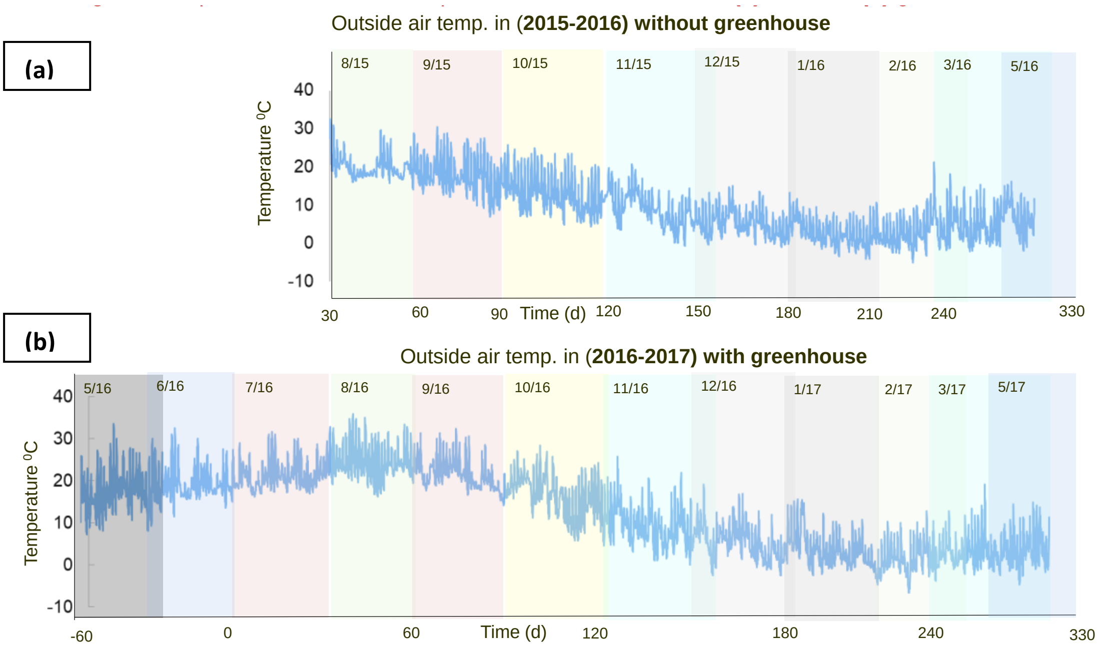

Fig 4.4 Comparative outside air temperature of *P. vittata* without **(a)** and with **(b)** greenhouse at low temperature

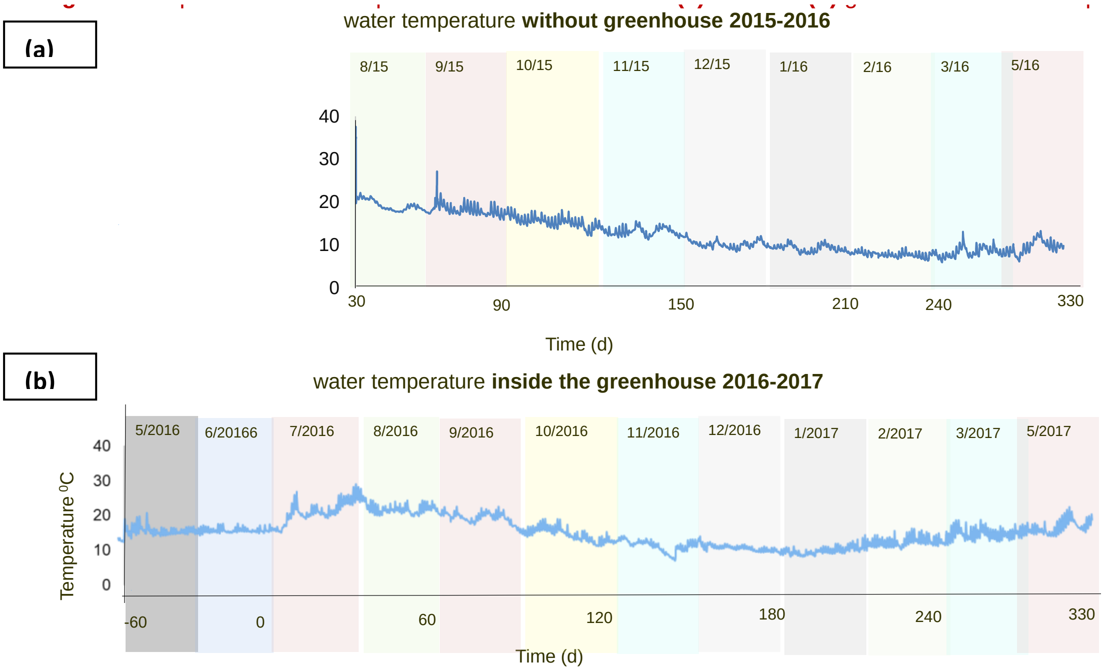

Fig 4.5: Comparative water temperature of *P. vittata* without **(a)** and with **(b)** greenhouse at low temperature

4.3.3 Determination of comparative potential of *P. vittata* without and with greenhouse at low temperature

At Akaishi dumping site, initially *P. vittata* was applied without greenhouse at July 2015. But that time *P. vttata* had started to damage by cold stress from November to March when temperature decreased to 0^0C/ below 0^0C. By the effect of low temperature, tropical fern *P. vittata* released arsenic to the leachate instead of accumulation. To protect plants from severe cold stress, at November 2016 simple type greenhouse was set-up which protected *P. vittata* from heat loss from the surface of fern by wind. Comparative change of biomass and arsenic accumulation capacity of *P. vittata* without and with greenhouse has shown in [Fig 4.6 (a) (b)] and [Fig 4.7 (a) (b)] respectively. Without greenhouse, *P. vittata* has started to damage from November when outside temperature decreased to 0^0C. Application of simple greenhouse helped the plant to recover biomass and accumulate arsenic again.

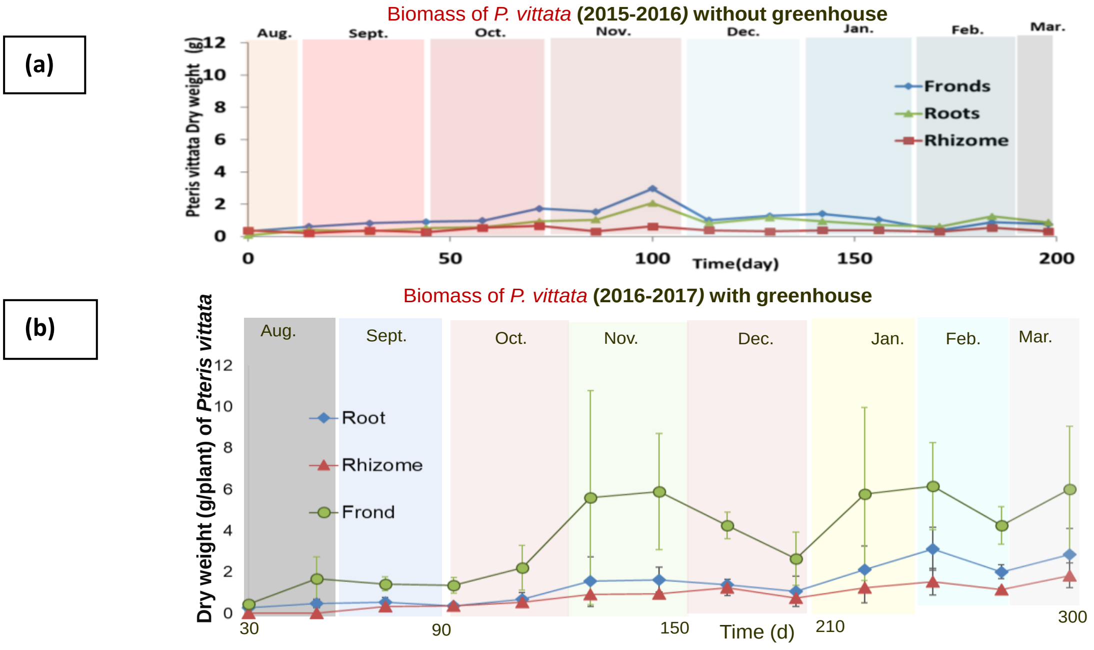

Fig 4.6: Comparative biomass of *P. vittata* without **(a)** and with **(b)** greenhouse at low temperature

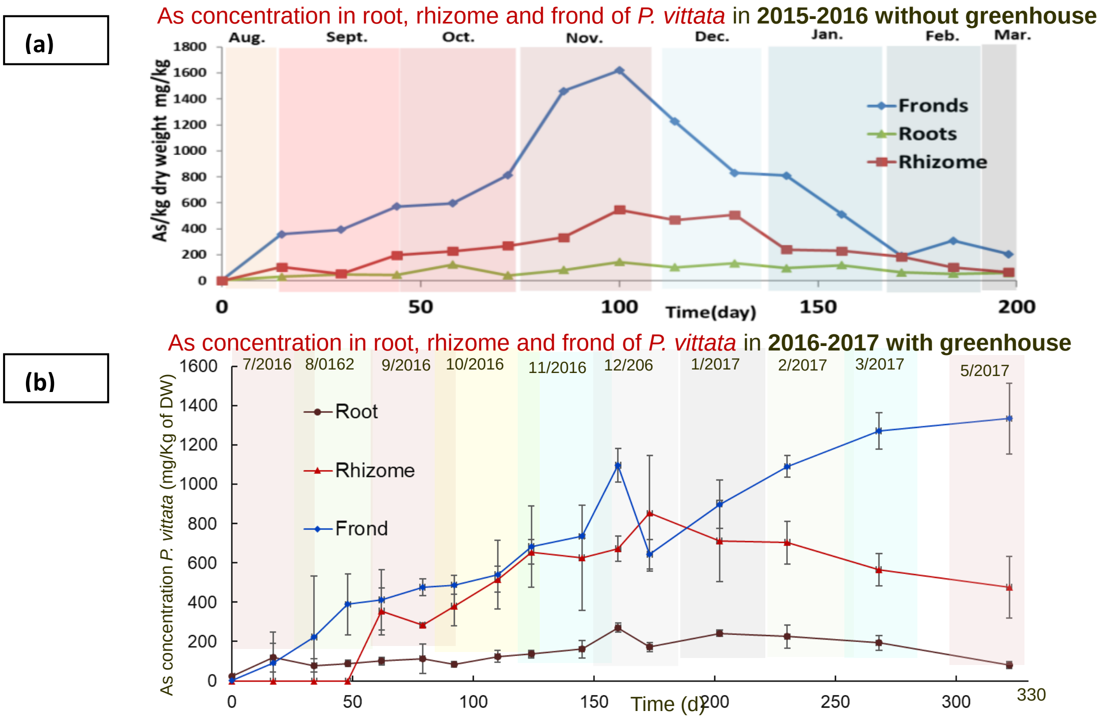

Fig 4.7: Comparative As concentration in root, rhizome and frond of *P. vittata* without (a) and with (b) greenhouse at low temperature

Arsenic concentration of the leachate was around 30 µg/L where during treatment with *P. vittata,* concentration of arsenic in tank 1 (treated by *P. vittata*) was fluctuating from 25 µg/L to 10 µg/L. Actually flow rate of leachate water to the tank was also fluctuating from 230L/day to 720L/day. However, it was really very difficult to control the fixed flow rate because sometimes, some solid particles or microorganisms came through the pipe to decrease the pre-set flow rate. Moreover, temperature or other parameters were also changing each and every moment naturally, even water chemistry and plant morphology were also affects arsenic removal potential of plants in leachate. So, it was difficult to control that kind of fluctuation of accumulation. Photograph of the applied ferns were taken two times per month to monitor the physical change of plants under cold stress. In summer, *P. vittata* appeared green and showed higher As accumulation efficiency from July to October which can be compared with *P. vittata* that grew healthily at sites highly contaminated by As (An et al., 2006; Wu et al., 2007). But during winter, it was slightly damaged even inside the simple type greenhouse. Because at every plant sampling time, greenhouse cover was taking off for 30-40 min and plants got stressed by cold. Fig 4.8 shows small damage by cold stress in *P. vittata* which has recovered very soon inside the greenhouse. Arsenic concentration in *P. vittata* has decreased at November-December by the effect of sudden low temperature (below 0^{0}C), but from January the plant started to accumulate again as greenhouse helped the plant to keep healthy. From November 2016 to May 2017 arsenic removal from tank-1 by *P. vittata* and accumulated amount of arsenic in root, rhizome and frond of *P. vittata* has shown in Fig 4.9 and Fig 4.10 respectively. Mass balance calculation between amount of arsenic removed from leachate by *P. vittata* and amount of arsenic gained by *P. vittata* at low temperature (from 11/2016 to 5/2017) showed that total amount of As accumulated in *P. vittata* was 997.3 mg which was almost the average value of maximum (1439.0 mg) and minimum (460.5 mg) amount of As removed from leachate by *P. vittata.* As the flow rate of leachate water was fluctuating each and every day, maximum and minimum amount of removed arsenic from leachate was calculated by using maximum (720L/day) and minimum (230L/day) flow rate respectively. So, the balance was good even at low temperature from November to May. Formula used for mass balance is given below-

Total As amount accumulated in *P. vittata*

= As amount per plant x 218 (total plant number in Tank1)

Result: 997.3 mg

Total As amount removed from leachate by *P. vittata*

= (influent As conc. - effluent As conc.) (mg/L) x 720 L/day (flow rate) x time interval (days)

Result: 460.5 mg (using minimum flowrate 230 L/day)
~ 1439.0 mg (using maximum flowrate 720 L/day)

- Water sample was collected two times per month. So It was difficult to keep the flow rate fixed for everyday

Fig 4.8: Showed small damage by cold stress in *P. vittata*

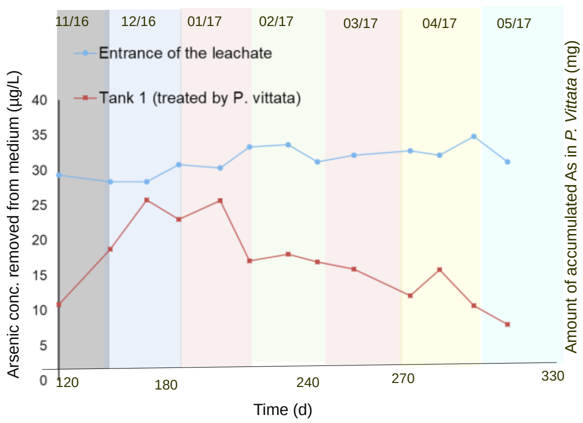

Fig 4.9: Arsenic concentration in the entrance of contaminated leachate and arsenic concentration removed from tank-1 (treated by *P. vittata*)

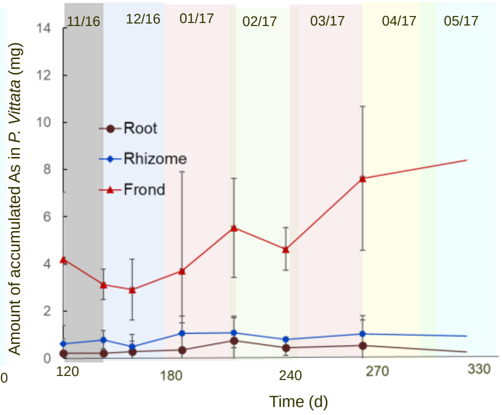

Fig 4.10: Amount of accumulated arsenic in root, rhizome and frond of *P. vittata*

4.3.4 First application of temperate zone fern *P. cretica* to Akaishi landfill leachate

To the best of our knowledge, temperate fern *P. cretica* has not yet been applied for the removal of arsenic from contaminated leachate at low temperature. Thus, this was the very first application of *P. cretica* to arsenic contaminated leachate. *P. cretica* with greenhouse has applied to the leachate at July 2016. This time, *P. cretica* has pre-cultivated for only one week at tank-2. Therefore, the initial biomass was low but total biomass of this fern was still increasing from November 2016 to May 2017 (Fig 4.11).

With the increase of dry biomass, total arsenic concentration in the plant was also increasing (Fig 4.12). Although *P. cretica* is a temperate zone fern, the result of laboratory experiment showed that low water temperature like 5^0C/ below 5^0C is still critical for *P. cretica* (chapter 3). So this time, simple type greenhouse made by greenhouse plastic above the still frame was used to protect the fern from the loss of heat and humidity. By using simple type greenhouse, the inside water temperature was controlled as 10^0C which was suitable for natural growth and arsenic accumulation. So, this simple type greenhouse was essential to protect *P. cretica* from heat loss by wind. Photograph of the plants has taken two times per month. Fig 4.13 shows the appearance of *P. cretica* looked green even at lowest temperature. *P. cretica* was applied to an arsenic contaminated leachate in temperate area that came from a dumping site having initial arsenic contamination around 30 µg/L. In order to reduce the concentration to 10 µg/L (Japanese environmental standard), *P. cretica* has applied to tank-2 where concentration of arsenic in tank 2 were fluctuating from 25 µg/L to 10 µg/L (Fig 4.14) as the flow rate of water to tank-2 was also fluctuating from 720L/day to 230L/day. The maximum accumulation capacity of *P. cretica* was lower (chapter 3), although each and every plant has different morphology. Further investigations are recommended to ensure the mechanism of arsenic accumulation by temperate zone ferns.

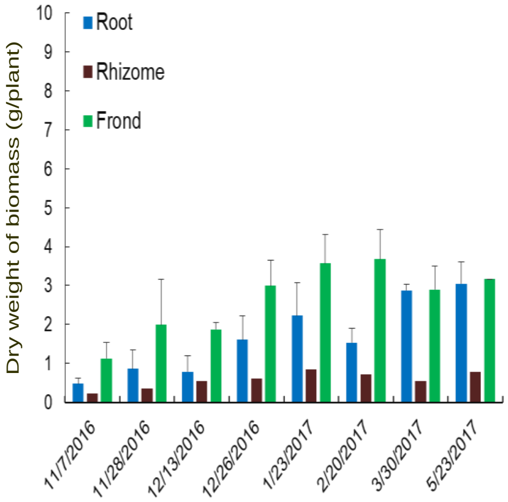

Fig. 4.11: Dry biomass of *P. cretica* (treated in tank-2)

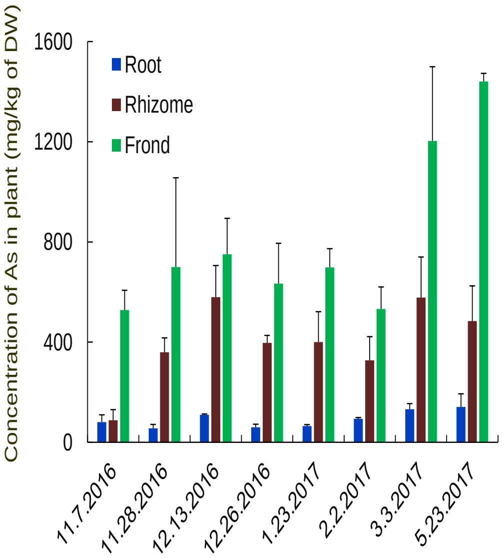

Fig. 4.12: concentration of arsenic in different parts (fronds, rhizomes and roots) of *P. cretica* (tank2)

Fig 4.13: Appearance of *P. cretica* at low temperature

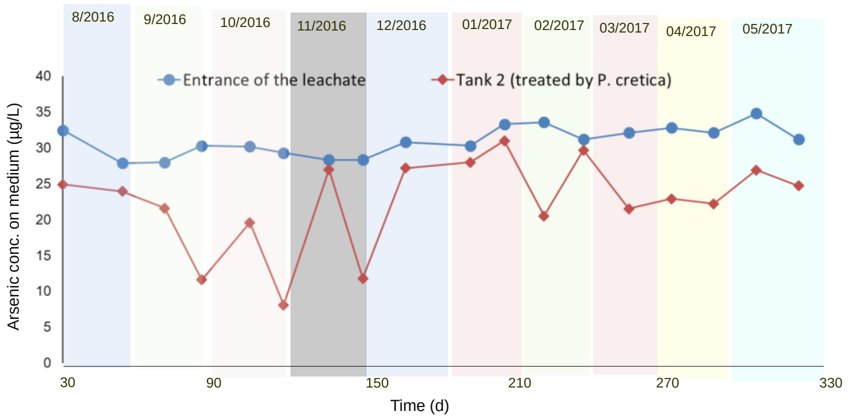

Fig 4.14: Arsenic concentration in the entrance of contaminated leachate and tank-2 (treated by *P. cretica*) solution

4.3.5 Arsenic transportation by *P. vittata* and *P. cretica* applied at the contaminated leachate

During application of ferns to the arsenic contaminated leachate, *P. vittata* as well as *P. cretica* has collected one time per month as sample plants to analyze the arsenic distribution in different parts of ferns. In most cases, maximum concentration of arsenic was storied in frond of *P. vittata* and *P. cretica* which supports the previous results of *P. vittata* (Su et al. 2008). Frond arsenic concentration of *P. cretica* and *P. vittata* was significantly higher than the concentration in root ($P<0.05$). Maximum accumulated arsenic concentration in *P. vittata* was (2038 ± 61) mg/Kg dry weight where *P. cretica* accumulated maximum (2066 ± 81) mg/Kg dry weight. Amount of arsenic in different parts of *P. vittata* and *P. cretica* has also shown significant transportation of arsenic to frond [Fig 4.15 (a) (b)].

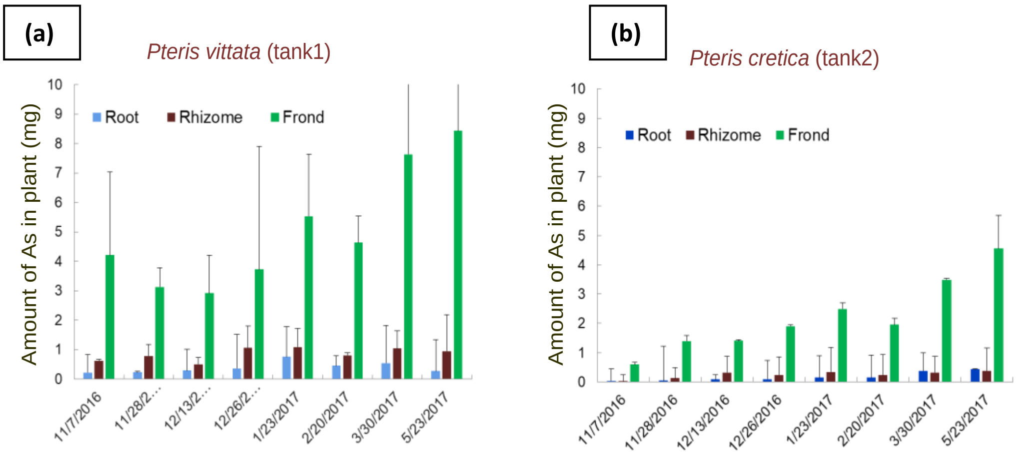

Fig 4.15: Transported amount of arsenic in different parts (fronds, rhizomes and roots) of *P. vittata* (tank1) **(a)** *and P. cretica* (tank2) **(b)**

4.3.6 Design for the treatment of total leachate that comes from the Akaishi dumping site

Final target is to treat the total leachate that comes from the Akaishi dumping site by using phytoremediation method. According to my laboratory results *P. multifida* will be the most effective fern for As removal from this temperate zone, northern part of Japan having high accumulation efficiency and cold tolerance.

The size of previously used trail tank was only 1.62m^2 where flow rate was 0.5L/min. Comparing with the total flow rate 30L/min, the area required for the total treatment was calculated as 97.2m^2. For getting the highest efficiency, this large sized tank should be applied for three times continuously. Three tanks should be connected to each other so that treated leachate from first tank will go to the second one and so on until the final removal to the environment. The design of future tank for the treatment of total leachate is given below-

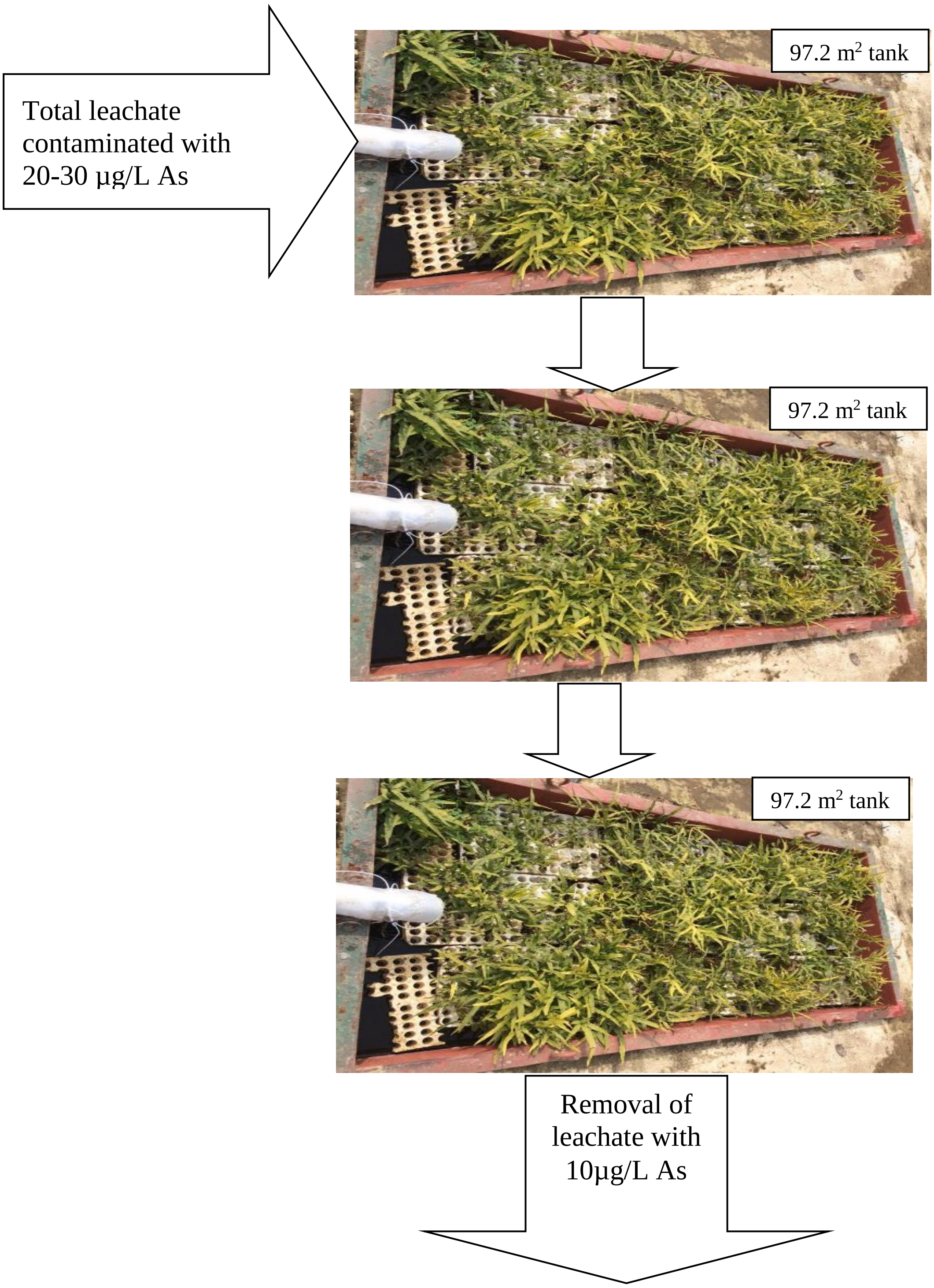

Fig 4.16: Design for the treatment of total leachate

4.4 Conclusion

This is the first time to apply temperate zone ferns as well as tropical zone ferns in As contaminated leachate at Akaishi, Miyagi, Sendai, the temperate zone of northern part of Japan. Leachate that produced from dumped treated soil such as construction soil generated by construction work etc. might be contaminated with harmful environmental pollutants. During the construction of Sendai City Subway Tozai Line which opened on December 6, 2015, about 600,000 m^3 excavated soil was dumped at Akaishi area from 2008 to 2014 that may dissolve arsenic and produce arsenic contaminated leachate that exceed the arsenic concentration of Japanese water quality standard (10 μg/L). This target dumping site generates about 30 L leachate water/ min which contains 20 to 30 μg As / L water. *P. vittata* as well as *P. cretica* removed arsenic from that contaminated leachate although removal efficiency was fluctuating each and every day with the fluctuation of flow rate of leachate water. At July 2015, *P. vittata* has applied firstly without greenhouse and damaged by cold stress. To protect plants from cold stress, simple type greenhouse has applied at July 2016 which kept *P. vittata* as well as *P. cretica* healthy. This greenhouse protected plants from heat loss by wind, kept proper humidity and also increased water temperature to ensure effective accumulation of arsenic from leachate. The very first application of *P. cretica* also showed that it has accumulated arsenic from leachate, gained maximum amount of arsenic in frond although the initial biomass was lower for this time. The growth rate and maximum accumulation capacity of temperate fern *P. cretica* was lower than tropical fern. Further investigations are recommended to ensure the mechanism of arsenic accumulation by temperate zone ferns.

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Chapter 5

Conclusion

Ferns are promising for phytoremediation of widely spread arsenic contaminated site. Although *P. vittata* is the most studied fern, it is limited to its cold tolerance. In this research, temperate zone fern *P. multifida* and *P. cretica* were introduced for highlighting their heavy metal accumulating potential at low temperature. Comparative potential of mostly studied tropical zone fern *P. vittata* was also determined to search the suitable fern for successful phytoremediation at low temperature. However, accumulated arsenic release tendency of those three ferns under cold stress has also been investigated. To date, there is no published data about the effect of low temperature on arsenic accumulation and release by ferns. In addition, temperate zone fern as well as tropical zone fern was applied for arsenic removal from leachate produced from Akaishi dumping site, Miyagi, Sendai for the first time. However, survival of the ferns under cold stress and continuous arsenic removal was a big challenge at this application.

In chapter-2, toxic heavy metal (arsenic, lead and cadmium) removal potential of temperate zone ferns (*P. multifida* and *P. cretica*) has determined. *P. multifida* and *P. cretica* were chosen because of their high cold tolerance in the field. The potential of *P. multifida* and *P. cretica* to remove As(III), Pb and Cd from solution and comparison with *P. vittata* was reported here for the first time. Our results showed that during the hydroponic experiment, heavy metal concentrations in the solution decreased continuously with time. *P. multifida* accumulated 90%, 36% and 50% of Pb, Cd and As, respectively, where *P. cretica* accumulated 90%, 71% and 43% of Pb, Cd and As, respectively from the mixed metal solution. The hydroponic experiment comparing temperate ferns and tropical fern revealed that 33% and 43% of As(III) was removed by *P. multifida* and *P. cretica* respectively, whereas *P. vittata* could not accumulate As(III) at all within 5 days'. As was transported to the frond and some Pb and Cd was also transported to the rhizome from the root during the hydroponic experiment. The results of the long-term (3 months) pot soil experiment provided confirmation of the As(V), Pb and Cd uptake and transportation potential of *P. multifida.* After 3 months, *P. multifida* grown in metal-spiked soil stored significantly higher concentrations of As, Pb and Cd compared with the control plants ($P < 0.01$). The heavy metal (As, Pb and Cd) accumulation capacity and transportation ability of temperate zone ferns were demonstrated in this study, providing evidence that it could be applied for the treatment of water and soil that are not only contaminated with As but also co-contaminated with Pb and/or Cd.

In chapter-3 effect of low temperature on As accumulating and releasing tendency of temperate zone ferns were investigated. Results from this study demonstrated that at low temperature, temperate ferns removed As from hydroponic solution faster and more effectively than tropical fern *P. vittata*. For being a temperate zone fern, at low temperature like 10^0C or 15^0C, *P. multifida* accumulated significantly higher concentration of As ($P<0.05$) compared with most studied tropical zone fern *P. vittata* . At 5°C or 25°C accumulation capacity of *P. multifida* was also higher than *P. vittata* but not at a level of significance ($P>0.05$). Another temperate zone fern *P. cretica also* accumulated significantly higher concentration of As ($P<0.05$) compared with *P. vittata* at low temperature (10^0C), although the maximum accumulated As by this fern was significantly lower ($P=0.005$) that *P. multifida* or *P. vittata*. Maximum amount of As was transported in the frond at all temperature for all three ferns. Analysis of comparative release properties showed that except 5^0C, initially released small concentration of As in Milli-Q water was accumulated again by *P. multifida* as well as *P. cretica* but tropical zone fern *P. vittata* could not accumulate total concentration of initially released As at 10^0C, 15^0C and 25^0C. Though temperate zone fern *P. multifida* and *P. cretica* released lower concentration than *P. vittata* at 5^0C but 5^0C temperature is still critical for all plants. So further investigations are recommended to make plants sustainable even at $\leq 5^0$C. Comparative potential of As accumulation, transportation and release of *P. multifida, Pcretica* and *P. vitata* in this study providing evidence that *P. multifida* could be the suitable fern for the treatment of As contaminated water at temperate zone area.

In chapter-4, temperate zone ferns as well as tropical zone ferns were applied for the first time to an As contaminated leachate at Akaishi, Miyagi, Sendai, northern part of Japan. During the construction of Sendai City Subway Tozai Line which opened on 6th December, 2015, about 600,000 m^3 excavated soil was dumped at Akaishi area from 2008 to 2014 that may dissolve arsenic and produce arsenic contaminated leachate. At July 2015, *P. vittata* has applied firstly without greenhouse and damaged by cold stress. To protect plants from cold stress, simple type greenhouse has applied at July 2016 which kept *P. vittata* as well as *P. cretica* healthy. This greenhouse protected plants from heat loss by wind, kept proper humidity and also increased water temperature to ensure effective accumulation of arsenic from leachate. Our target dumping site generated about 30 L leachate water/ min containing 20 to 30 μg As / L water that exceed the arsenic concentration of Japanese water quality standard (10 μg/L). Tropical fern *P. vittata* as well as temperate fern *P. cretica* were applied to removed arsenic from that contaminated leachate. At low temperature (from November 2016 to May 2017) *P. cretica* as well as *P. vittata* has removed almost 75% (maximum) As from the leachate although removal efficiency was fluctuating each and every day with the fluctuation of flow rate of leachate water. The very first application of *P. cretica* also showed that it has accumulated arsenic from leachate, transported maximum amount of arsenic in frond although the initial biomass was lower for this time. The growth rate and maximum accumulation capacity of temperate fern *P. cretica* was lower than tropical fern (chapter-3). Further investigations are recommended to ensure the mechanism of arsenic accumulation by temperate zone ferns.

In conclusion, final goal of this present study was to determine arsenic removal potential of temperate zone ferns at low temperature and apply to a contaminated leachate. Laboratory experiments of this study clarified the As accumulating potentials of temperate zone ferns (*P. multifida* and *P. cretica*) at low temperature (10^0C) and first application of *P. cretica* also removed As from the leachate of Akaishi dumping site . As comparative investigations between two temperate ferns showed that *P. multifida* has higher As removal potential than *P. cretica* at low temperature. Thus, P. *multifida* was recommended to apply in future as a more efficient cold tolerance hyperaccumulator.

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