Arsenic Speciation and Bioavailability to Macroalgae in Seawater

Dissertation

Arsenic Speciation and Bioavailability to Macroalgae in Seawater

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Dedicated to my beloved Students

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ABSTRACT

The metalloid arsenic (As) has no well-known biological function, but it is a widely distributed food chain contaminants and ranked a number one of the top priority hazardous substances in the environment. Arsenate (As(V)) and arsenite (As(III)) are the primary bioavailable inorganic forms in aquatic systems and are actively subjected to biotransform upon their exposure. Marine microalgae and macroalgae have shown enormous As accumulation and transformation capacity, and are the chief contributor of reduced, methylated, and/or other organic As species in seawater, hence play a critical link between cycling of As in the water column and other marine organisms. The recent discoveries of more than 50 arsenicals in marine organisms have extended the research field on As speciation. The information on the formation processes of As species, as well as their nature and distribution, is essential because of their complex chemistry and variable ecotoxicological effects on the marine ecosystems. As detoxification and/or biotransformation processes by the algae are habitat and species-specific, and it is necessary to investigate how marine macroalgae species interact, accumulate, detoxify, and produce As species in seawater and redistributed in marine food web with respect to their importance in As cycling. A series of laboratory culture experiments were designed with different macroalgal species namely *Undaria pinnatifida*, *Sargassum horneri*, *Sargassum patens*, and *Pyropia yezoensis* in seawater, and the following issues have been reported: (a) examination of the bioavailability of As species in terms of algal growth and photosynthetic activity; (b) observation of the accumulation, biotransformation, and extrusion behavior of As under different molar ratios of As and P; (c) investigation of the formation of Fe-plaque with or without coexisting Fe in the algal culture system; (d) demonstration of the modelling of As uptake rate; and (e) elucidation and comparison of the tolerance and metabolism diversity among macroalgae. The results of the proposed experiments are helpful in the understanding of the roles of macroalgae on As biogeochemical cycle in the marine environment.

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The author

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LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectrometry
AB	Arsenobetaine
AC	Arsenocholine
AFS	Atomic Fluorescence Spectrometry
ANOVA	Analysis of Variance
ArsC	Arsenate Reductase
As	Arsenic
As(III)	Arsenite (+3)
As(V)	Arsenate (+5)
As_2O_3	Arsenite
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
ATSDR	Agency for Toxic Substances and Disease Registry
BCF	Bioconcentration Factor
CRM	Certified Reference Material
CT	Cryogenic Trap
Cys	Cysteine
DIW	De-Ionized Water
DMAA(III)	Dimethylarsinous Acid
DMAA(V)	Dimethylarsinic Acid
DMAG	Dimethyl-Thio-Arsinite
DSA	Disodium Arsenate Heptahydrate
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic Acid
EDTA	Ethylenediaminetetraacetic Acid
Eh	Redox Potential
FAAS	Flame Atomic Absorption Spectrometry
FAFS	Flame Atomic Fluorescence Spectrometry
FW	Fresh Weight (Biological Samples)
GS	Glutathione (Complexed Form)
GSH	Glutathione
GSH	Reduced Glutathione (Non-Complexed Form)
GSSG	Oxidized Glutathione
GST	Glutathione S-Transferase
HGAAS	Hydride Generation Atomic Absorption Spectrometry
HPLC	High Pressure Liquid Chromatography
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectrometry
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
MIP	Major Intrinsic Protein
MMA	Monomethylarsonic Acid
MMAA(III)	Monomethylarsonous Acid
n	Number of Samples
ND/nd	Not Detected
PC	Phytochelatin
PC's	Phytochelatins
PCS	Phytochelatin Synthase
Pht1	Pi Transporter 1
pK_a	Acid Dissociation Constant

ppm	Parts Per Million
p-value	Probability Value
ROS	Reactive Oxygen Species
SAH	S-Adenosylhomocysteine
SAM	S-Adenosyl Methionine
SAM	S-Adenosylmethionine
SD	Standard Deviation
SE	Standard Error
SE	Standard Error of Mean
SEM	Scanning Electron Microscopy
SH	Free Thiol
TMA	Trimethylarsine
TMAO	Trimethylarsine Oxide
UV	Ultraviolet Radiation
XAFS	X-Ray Absorption Fine Structure Spectroscopy
XANES	X-Ray Absorption Near Edge Structure
XRF	X-Ray Fluorescence

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1. M. Abdullah Al Mamun, Ismail M. M. Rahman, Rakhi Rani Datta, Chika Kosugi, Asami S. Mashio, Teruya Maki, Hiroshi Hasegawa, "Arsenic speciation and biotransformation by the marine macroalga *Undaria pinnatifida* in seawater: A culture medium study", *Chemosphere*, **222** (2019) 705-713 (Published: May 2019).

2. M. Abdullah Al Mamun, Yoshiki Omori, Osamu Miki, Ismail M. M. Rahman, Asami S. Mashio, Teruya Maki, Hiroshi Hasegawa, "Comparative biotransformation and detoxification potential of arsenic by three macroalgae species in seawater: Evidence from laboratory culture studies", *Chemosphere*, **228** (2019) 117-127 (Published: August 2019).

3. M. Abdullah Al Mamun, Yoshiki Omori, Rimana Islam Papry, Chika Kosugi, Osamu Miki, Ismail M. M. Rahman, Asami S. Mashio, Teruya Maki, Hiroshi Hasegawa, "Bioaccumulation and biotransformation of arsenic by the brown macroalga *Sargassum patens* C. Agardh in seawater: effects of phosphate and iron ions", *Journal of Applied Phycology*, **31:(4)** (2019) 2669-2685 (Published: August 2019).

4. Most Sarmin Ashraf, M. Abdullah Al Mamun, Md. Mizanur Rahman, A. K. M. Mosharof Hossain, Hiroshi Hasegawa, "Integrated nitrogen management on nutrient contents, uptake and use efficiency of BRRI Dhan 29", *International Journal of Plant & Soil Science*, **23** (2018) 1-10 (Published: June 2018).

5. Rimana Islam Papry, Kento Ishii, M. Abdullah Al Mamun, Sohag Miah, Kanako Naito, Asami S. Mashio, Teruya Maki, Hiroshi Hasegawa, "Arsenic biotransformation potential of six marine diatom species: effect of temperature and salinity", *Scientific Report*, **9:10226** (2019) (Accepted: July 2019).

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INTRODUCTION

1.1 Arsenic in the Environment

Arsenic (As, atomic weight: 33 and atomic weight: 74.92) exhibits physical and chemical characteristics similar to metals and non-metals and sometimes called it as a metalloid. As is ubiquitous and found in all environmental compartments like soil, water, biota, and air. According to the abundance of As, it ranks 12th, 14th, and 20th in the human body, seawater and earth's crust, respectively (Woolson, 1975; Mandal and Suzuki, 2002). Chemically As is analogous to its neighboring phosphorus in the periodic table, this toxic element frequently changes its chemical forms in the environment, changing its solubility and volatility, by many physicochemical and biological processes. The different species and chemical forms are generally known as arsenicals. As forms different compounds of hydrides (arsine), halides, oxides, acids, and sulfides. A number of oxidation states of As (- 3, 0, +3, and + 5) exist in the nature as an inorganic As compounds (inorganic arsenicals) comprising water-soluble and volatile states, and as an organic As compounds (organoarsinicals) comprising water-soluble, lipid-soluble and volatile states (Miyashita et al., 2016). The lower oxidation states of As species (0 and -3) commonly occur under strongly reducing conditions and the species with their higher oxidation states (+5 and +3) are abundant under oxygenated and mildly reducing conditions.

1.2 Toxicity of Arsenic Species

As is a notorious and infamous metalloid in the general public's eye as a potent poison which was made famous by countless Agatha Christie novels. It has been proposed that nearly all As species are considered toxic and their toxicity is specific with particular As species which varies according to their composition and respective structure. As(III) and As(V) are considered as a group one carcinogens (Humans, 2012). In general, trivalent As species are more toxic than their pentavalent counterparts because of their binding affinity to thiol-rich proteins which are responsible for functional inhibition of essential enzymes present in cells. Methylated As of pentavalent species like methylarsonate, dimethylarsinate, and trimethylarsine oxide (MMAA(V), DMAA(V), and TMAO(V), respectively) are less toxic, and there is a general understating that the acute toxicity of As is decreased with increasing degree of methylation with the exception of TETRA. TETRA has lower acute LD_{50} value when compared with the other methylated compounds like MMAA(V), DMAA(V), TMAO(V) (Leermakers et al., 2006). As(III) showed to have 60 times more toxicity than As(V) while As(V) is 70 times more toxic than methylated species (MMAA(V) and DMAA(V)).

Arsenobetaine (AB) and arsenocholine (AC) are accepted as non-toxic forms of organic As (Hughes et al., 2011; Hughes, 2002) because of their no knew mutagenic, cytotoxic and immune toxicity. On the other hand, other forms of complex organoarsenic compounds including arsenosugars (As-sugars) (Feldmann and Krupp, 2011) and arsenolipids (Sele et al., 2012; Witt et al., 2017) which are predominantly found in marine algae and pose a theoretical risk of toxicity and still a subject of research in terms of their toxicity to humans (Borak and Hosgood, 2007). Though methylated pentavalent species (MMAA(V) and DMAA(V)) are less toxic, methylated trivalent forms (MMAA(III) and DMAA(III)) are exceptionally more toxic and genotoxic than their precursor compounds. It has been reported that the higher affinity of MMAA(III) for thiol ligands in biological binding sites than As(III)–thiolate complexes make the higher toxicity of MMAA(III) than inorganic As especially As(III) (Spuches et al., 2005). Besides, DMAA(III) also shown affinity to form complexes with sulfur-rich proteins (Naranmandura et al., 2006). The toxicity and mobility differences among As species and their roles in As metabolic studies have spurred the development of analytical methods for their determination. According to Niegel and Matysik (2010) and Cao et al. (2009), the toxicity of different As species as determined by the LD_{50} (mg kg^{-1}) follow the order: As(III) (14) > As(V) (20) > MMAA(V) (700 – 1800) > DMAA(V) (700 – 2600) > AC (> 6500) > AB (> 10,000). However, it is a well-known fact that As toxicity is considered based on the presence, nature (organic-inorganic) and concentration in the hydrosphere, lithosphere, and biosphere. A wide range of organisms, including aquatic plants, animals, and human showed less toxicity to the exposure of organic As species than exposure to inorganic As. The seafood contains a variety of organoarsenic like AB, AC, and AS and their dietary uptake considered to have a minimum or very low level of toxicity (Ng, 2005).

1.3 Need for Arsenic Speciation Analysis

The work presented in this thesis mostly focused on As speciation that deserves the importance and explanation of the word “Speciation.” The discovery of more than 50 As species in marine samples has reinforced the significance of As speciation analysis in recent years (Leufroy et al., 2011; Francesconi, 2010). The information on the elemental concentration of As alone may be insufficient and cannot be used solely to assess the toxicological risk and properties of As in natural systems for environmental studies. That is why, As toxicity and bioavailability investigation is entirely well correlated with the analysis of As species in a sample, i.e., As speciation and has been conserved as the norms of many years. In addition to the toxicity difference among the As species, speciation analysis is

tremendously critical but influential tools that provide far more information in terms of environmental fate, distribution, mobility, and transport characteristics, the prevention and mitigation approaches as well as efficiencies of remediation (Moe et al., 2016; Carlin et al., 2015; Gupta et al., 2012; Koch et al., 2013; Campbell and Nordstrom, 2014). Therefore, speciation of As is highly relevant and can reflect an appropriate level of hazard by providing meaningful risk assessment data on natural systems (Ng, 2005). A large number of research fields including food chemistry, environmental chemistry, health, and hygiene, as well as geology deliberately addressed the modern and reliable analytical methods on the speciation analysis of As (Chancho et al., 2017). The following terms generally used for the fractionation of elements and chemical speciation (Templeton et al., 2000):

IUPAC definitions:

a. *Chemical species*. Chemical elements: Specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure.
b. *Speciation analysis*. Analytical chemistry: Analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample.
c. *Speciation of an element*; speciation. Distribution of an element amongst defined chemical species in a system.
d. *Fractionation*. Process of classification of an analyte or a group of analytes from a certain sample according to physical (e.g., size, solubility) or chemical (e.g., bonding, reactivity) properties.

1.4 Phycoremediation and Arsenic Biotransformation

Phycoremediation is an emerging and advanced technology under complex environmental issues, which uses mainly various domains of algae (microalgae, macroalgae, and cyanobacteria) or lower plants and associated microflora for the elimination or biotransformation of contaminants, including heavy metals and nutrients etc. from contaminated water bodies and CO_2 from polluted air (Olguín, 2003; Sood et al., 2012; Ali et al., 2013; Franchino et al., 2013). Now a day, the utilization of living and non-living biomass of organisms including algae has gained significant interest to the researches over the conventional physicochemical and polymer-based As remediation technologies (oxidation, coagulation-flocculation, adsorption, ion exchange, and membrane-driven technique) and considered as a safe mitigation tool of As in a eco-friendly and cost-effective manner in an

aqueous system. Algae are abundantly found in freshwater and saltwater and can efficiently uptake and metabolize an adequate amount of all the species of As from the environment (Arora et al., 2018). Biosorption and bioaccumulation are two modes of As remediation by using algae and make them a potential budding tool in the field of As remediation. Abtahi et al. (2013) conducted biosorption study with dead marine biomass of *Colpomenia sinuosa* and showed that the maximum removal efficiency of As(III) and As(V) reached to 95.6 and 59.9 mg g^{-1} dry weight, respectively within 300 min from 100 mg L^{-1} As containing solution. Additionally, the living algae can detoxify As compounds and reduce the pollution in the environment. It is noteworthy that various macroalgae can tolerate and accumulate 5000 to 10000 times more As from the surrounding seawater followed by metabolic conversion and release of less toxic of As compounds. Jasrotia et al. (2014) showed that the living algae Cladophora able to biosorb nearly 100% As from 80 g L^{-1} enriched water with 9 to 10 days period.

The biotransformation of As by living organisms, including humans, plants, microorganisms produces both toxic and non-toxic forms of As. It has been well established that biotransformation is vital in the context of fate, bioavailability, mobility, and toxicity of environmental As levels. The predominant availability of inorganic As species in the environment (As(V) and As(III)) are continuously subjected to various biologically mediated transformations like oxidation, reduction, methylation, demethylation, and other reactions, and these reactions significantly emphasize the importance of biotransformation in living organisms in relation to biogeochemistry of As (Zhao et al., 2009; Zhu et al., 2014). The critical processes of transformation of As species acted by various biological organisms are summarized in *Figure 1.1*. The conversion of As(III) into As(V) through bio-oxidation is an energy-producing reaction and considered as a detoxification process by many bacteria and algae since As(III) is more toxic than As(V) (Páez-Espino et al., 2009; Biswas and Sarkar, 2019). The bioreduction (As(V) to As(III)) is a typical metabolic process in many organisms including algae and more easily release or excretion of As(III) from the cells is also believed to be a detoxification mechanism due to their toxicity difference inside the cell (Rahman and Hassler, 2014). Accordingly, formation and release of pentavalent methylated arsenicals upon intracellular As(III) methylation is clearly a detoxification strategy of many biological organisms. Bacteria, fungi, algae also employed to detoxify the inorganic As species into volatile arsenicals and contribute a significant part of biogeochemical cycling. The overall metabolic pathways in organisms greatly influence As cycling among environmental surroundings (terrestrial, aquatic, and atmospheric) (Qin et al., 2009; Yin et al., 2011; Zhu et

al., 2014). It could be questioned that whether As detoxification systems as well as species formation by the different organisms are unique or whether their potentiality to be cultured in the laboratory make them better understanding how they take part in the As cycle throughout the globe.

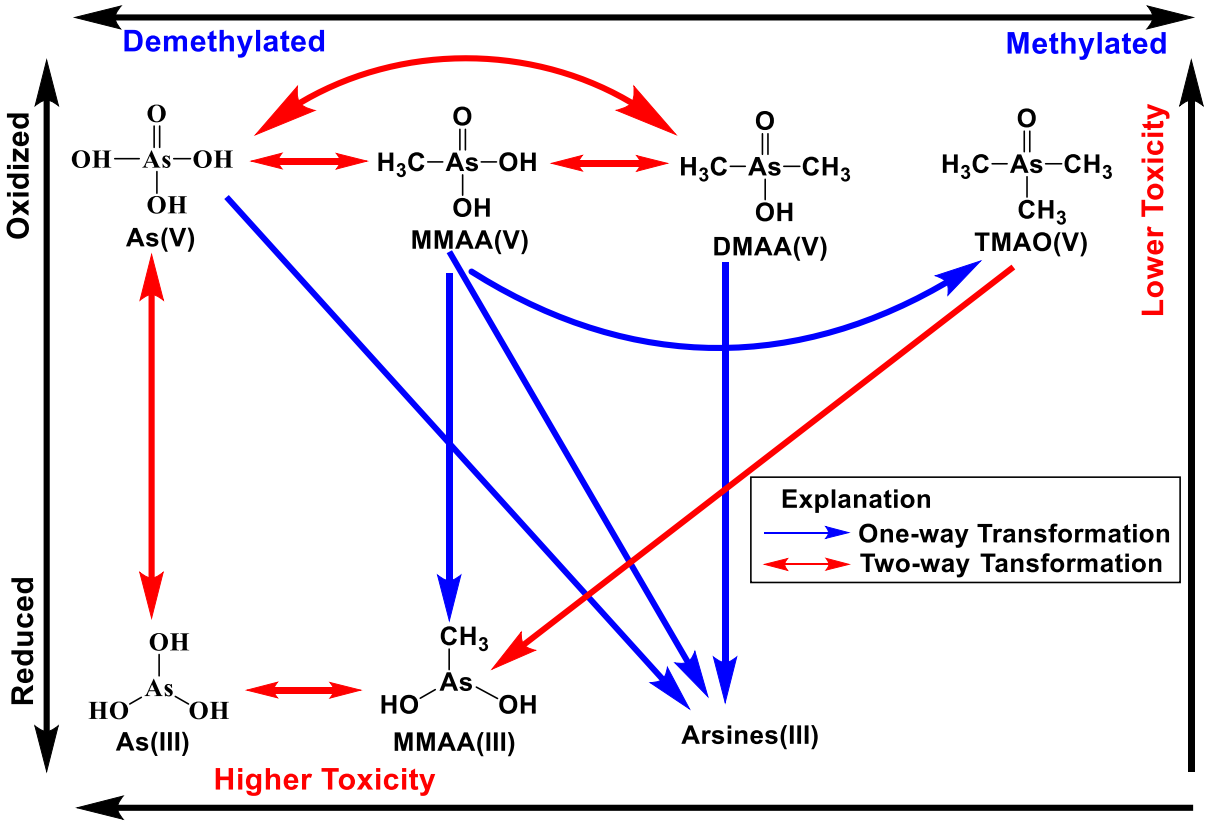

Figure 1.1: General overview of biological transformation processes of organic and inorganic As species.

Much research has been aimed at identifying the biological transformation pathways and focused mainly on the organisms belong to bacteria, archaea, phytoplankton. In contrast, there has been a little attention paid to the marine macroalgae, and their contribution to the biogeochemical cycles even though they are inhabiting in high As environment and have enormous accumulation and biotransformation potential. Surprisingly, despite the potential contribution of algae on As speciation as well as biogeocycle in the marine environment, there has yet been limited research on laboratory studies with marine macroalgae for assessing the transformation pathways (Kalia and Khambholja, 2015). It is also essential to better understand the link between enormous diversity of As transforming marine organisms especially macroalgae and their interactions with the As species in As cycling for managing and remediating As contaminated sites under various conditions.

1.5 Objectives of the Study

This study was mainly aimed to analyze the As speciation in the algal culture medium as a function of biotransformation potential of marine macroalgae species (*Undaria pinnatifida*, *Sargassum patens*, *Sargassum horneri*, and *Pyropia yezoensis*) in the aquatic system, especially in seawater. The interaction of As and P in algal uptake and modeling were investigated as well. Also, the influences of P, Fe, and As species on the accumulation as well as surface complexation by these algae species have been investigated. However, the specific objectives of this research are summarized as follows:

- To evaluate the toxicity of the macroalgae species upon exposure of As.
- To observe the bioavailability of As species to the macroalgae species in relation to their interaction on the growth and photosynthetic activity.
- To analyze the As speciation in contaminated seawater as a function of biotransformation under different As and P molar ratios.
- To compare and elucidate the mechanism of biotransformation potential among the macroalgae species.
- To investigate the diversity in metabolism and tolerance towards the exposure of As species.
- To investigate the formation of Fe-plaque in laboratory-based macroalgal culture with and/or with Fe.

1.6 Organization of the Study

The materials of this dissertation are divided and organized into seven chapters, and the chapter-specific objectives are as follows:

Chapter 1 provides a small introduction of the background information of As and its species with toxicity. The scope of utilizing macroalgae species in phycoremediation, and the associated metabolism As in contaminated water as well their practical application in As remediation is briefly explained.

Chapter 2 focuses a brief discussion on the abundance, sources, forms, speciation of As in marine environment. The toxicity, pathways of As metabolism in organisms, especially in algae, along with their interaction with different factors have been discussed briefly in this chapter. Besides, this chapter has reported the methodologies for As speciation.

Chapter 3 offers a brief outline of the macroalgae species. Also, different parameters and procedures that have been followed during the analysis and measurement are discussed here.

Chapter 4 describes the As biotransformation patterns of the laboratory cultured macroalga *Undaria pinnatifida*. The interaction of As(V) and P on the As speciation in the culture media as well as bioaccumulation in algal tissues have been explained. Algal surface complexation of As with the Fe-plaque has also been focused.

Chapter 5 discusses the comparison in metabolism and biotransformation of As(V) by *Sargassum horneri*, *Sargassum patens*, and *Pyropia yezoensis*. The surface complexation, bioaccumulation, and growth efficiency of algal species have been explained.

Chapter 6 deliberates the influence of Fe, P, and As (two IAs species) on the accumulation and transformation of As by *Sargassum patens*. The uptake kinetics, formation of Fe-plaque, and the interaction on the bioavailability of As species with the algae has been described.

Chapter 7 is based on the overall experimental results, concluding remarks, and suggestions on the future research scope. The limitation and difficulties of the laboratory macroalgae culture have also been included here.

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REVIEW OF LITERATURE

2.1 Chemistry of Arsenic in the Environment

2.1.1 Forms and Structure of Naturally Occurring Arsenic Species

As occurs mainly as +III and +V oxidation state in the biological samples. Both terrestrial and marine ecosystem, once As released into the environment, it cannot be degraded and destroyed, but form different arsenicals. Plants and micro and macroorganisms take part in the redistribution of As through bioaccumulation via biosorption, biomethylation, and volatilization and hence cycling of As in the environment. The diversified As species as well as their compounds are different in their chemical properties. The solubility of As compounds in water or oil are categorized as water-soluble and lipid-soluble As species, respectively. There are more than 50 naturally occurring water-soluble As species have been evidenced in the marine environment (Francesconi, 2010). The chemical structures and acronyms of important As species are represented in *Figure 2.1*.

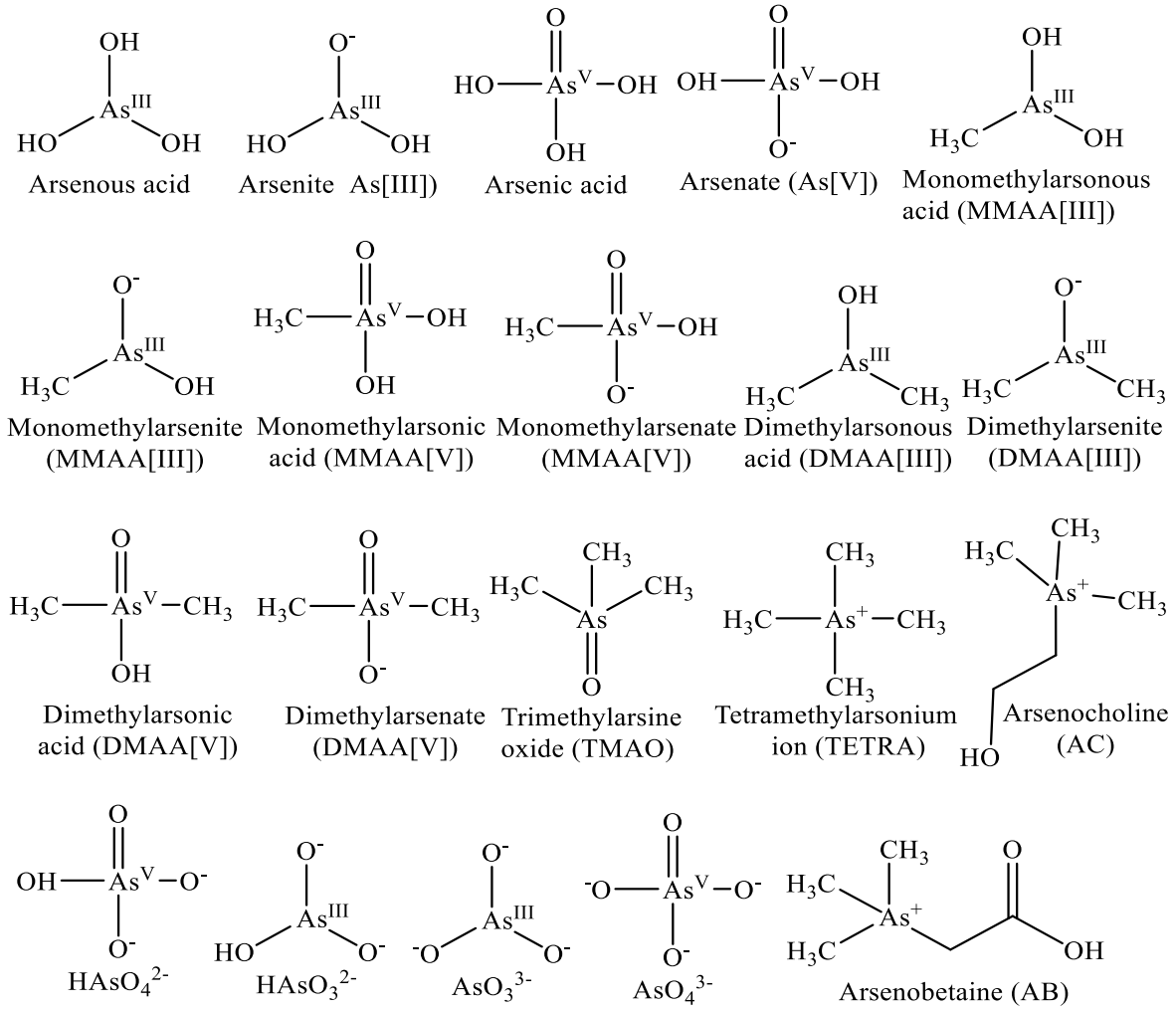

Figure 2.1: The presence of common As species and their anions in the environment

2.1.2 Sources of Arsenic in the Environment

As is ubiquitously distributed in earth crusts, sediments, soil, air, water, and living organisms long before human activities. Both man-made (anthropogenic), and natural sources are responsible for As contamination in the environment.

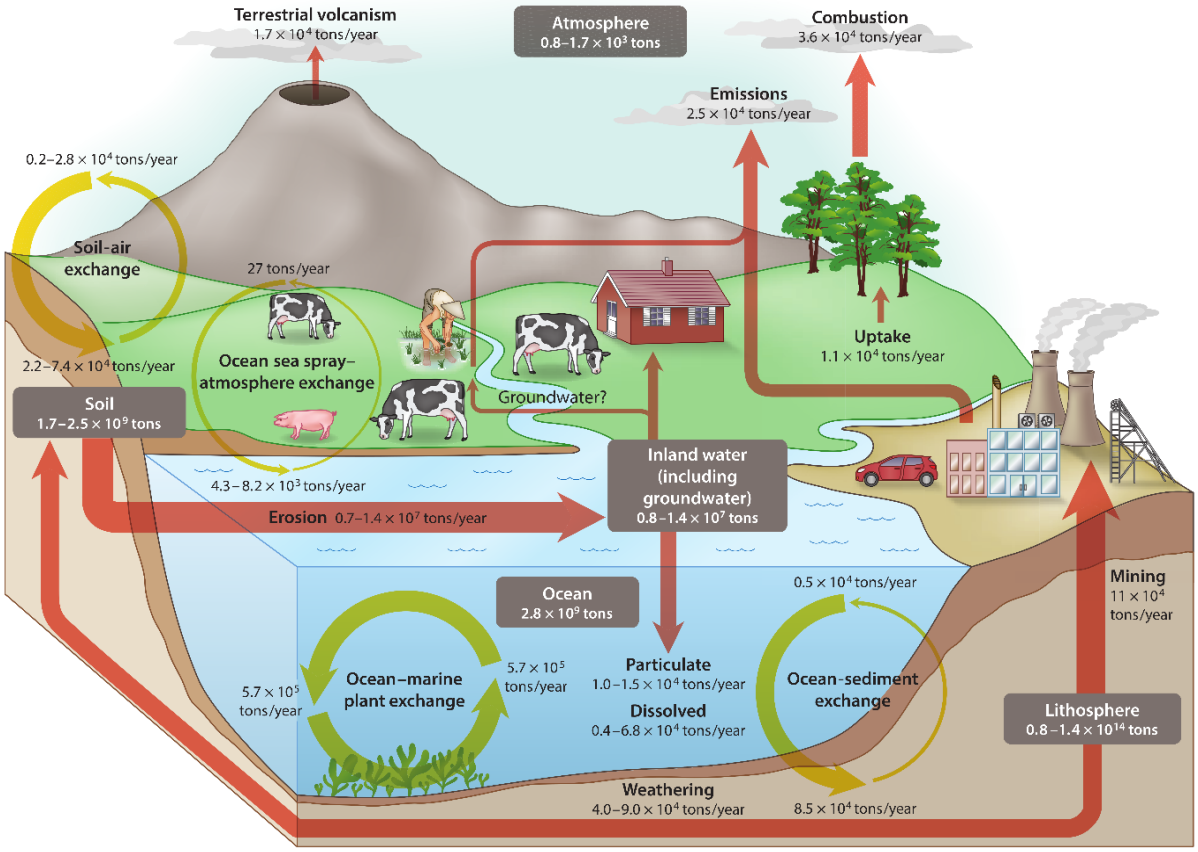

Figure 2.2: Schematic diagram showing stocks and fluxes of As in various earth components. Gray boxes indicate stocks of As in a given component, and arrows indicate the fluxes of arsenic toward a given component. (Reprinted from Zhu et al. (2014) with permission from NCBI).

The anthropogenic sources of As contamination is increasing day by day, and becoming an important issue, as they are transported via runoff and deposited in the aquatic ecosystem. The main anthropogenic activities through which As is introduced into the environment are mining and smelting of non-ferrous metals, combustion and production of energy from fossil fuels, agricultural inputs from manufacture and application of arsenical pesticides, herbicides, insecticides, feed additives, crop desiccants, and wood preservatives, medicinal application, industrial activities like chemicals, alloys, electronics, semiconductors, glass industries, leather preservatives, dyes, antifouling paints, cosmetics etc. On the other hand, natural contamination occurred through volcanic eruptions and hot springs, weathering reactions, and dissolution of

soils and sediments which are rich in As, biological activity, and geochemical reactions. Most of the environmental problems of As are associated with the natural mobilization process. Weathering of As-containing rocks and minerals form dissolved As in groundwater, and the sources of inorganic As in water are mostly derived from prevalent rocks such as As_2O_3, As_2S_3, AsFeS, As_4S_4 among 200 minerals of As. The concentration of As in water bodies, especially ocean water, are high in areas of geothermal activities (Smedley and Kinniburgh, 2002). The gaseous emission of As oxides such as As_2O_3 along with other arsines (AsH_3, CH_3AsH_2, $(CH_3)_2AsH$, $(CH_3)_3As$, and arsines from arsenoriboses) are released from the mining or microbial activities in soils, sediments, and water can oxidize in the air and settle back to the environment. The comprehensive transfer of As in the environment from different sources are presented in *Figure 2.2*.

2.1.3 Factors Affecting Arsenic Speciation in the Aqueous Environment

The speciation of As in natural condition is very complicated. There are several factors and processes which are directly or indirectly related to the speciation and behavior of As in water, air, and geologic materials such as sediments, soils, and rocks. Some of the factors are phosphate concentration, the interaction of As with the biological activities, chemical oxidation-reduction (redox conditions), changes in pH, other significant inorganic chemical reactions, methylation and demethylation, dissolution, adsorption, precipitation and/or coprecipitation, volatilization, condensation, and other unknown aqueous chemistry (Smedley and Kinniburgh, 2002; Pal, 2015).

The dissolved As in water generally exist as inorganic As(V) and As(III), and the speciation of As is mostly dependent on the redox potential (Eh). The prevalent As(V) anions like $H_2AsO_4^-$ and $HAs_2O4_2^-$ usually occur under an oxidizing condition having Eh value >100 mV and circumneutral pH level. On the other hand, $H_3AsO_3^o$ is the dominant inorganic As(III) form under reducing conditions (<100 mV) and circumneutral pH (*Figure 2.3*). Since the oxidation of As(III) in oxidizing water is often sluggish, As(III) may persist in that water (Boyle et al., 1998). It is generally pointed that the redox transformation of As is relatively slow (Masscheleyn et al., 1991), and both inorganic species (As(III) and As(V)) often exist in the same redox environment. Eh-pH diagrams are usually used to explain the dissolved As species and their precipitates which are exist at an equilibrium condition in respect to aqueous solutions (groundwaters, surface waters, laboratory solutions, and porewaters from soils, sediments, or rocks).

Table 2.1: The major anions of inorganic As species with their pK_a values

Arsenite [$H_nAs(III)O_3^{(3-n)-}$ while n = 1,2]	**pK_1**	**pK_2**	**pK_3**
$H_3AsO_3 = H_2AsO_3^-$	9.2	-	-
$H_2AsO_3^- = HAsO_3^{2-}$	-	12.1	-
$HAsO_3^{2-} = AsO_3^{3-}$	-	-	12.7
Arsenate [$H_nAs(V)O_4^{(3-n)-}$ while n = 1,2]			
$H_3AsO_4 = H_2AsO_4-$	2.2	-	-
$H_2AsO_4^- = HAsO_4^{2-}$	-	6.98	-
$HAsO_4^{2-} = AsO_4^{3-}$	-	-	11.53

Figure 2.3 indicates that As(III) is occurred as uncharged molecules throughout a wide pH from 0 to 9, while As(V) is predominant at high values of Eh and pH. In extremely acidic and alkaline condition, $H3AsO_4^0$ and AsO_4^{3-} are dominant forms, respectively. At a low pH level, $H_2AsO_4^-$ is dominant under oxidizing conditions. $HAsO4_2^-$ and $H_3AsO_3^0$ become dominant at pH more than ~9.9 and less than ~ 9.2, respectively. However, natural aqueous systems are not always at equilibrium condition, and such systems contain several metastable species that cannot be explained by Eh-pH diagrams. The metastable species like As(III) in oxygenated seawater is considered to result from biological activity. The redox boundary between As(III) and As(V) is approximately +300 mV at pH 4 and −200 mV at pH 9, respectively (O'Day, 2006). However, metastable As(III) and As(V) forms are widespread in natural waters. Smedley and Kinniburgh (2002) reported that the chemical oxidation and reduction of As species is generally a slower process when compared with biologically mediated As redox reactions, which may result in the presence of a variety of organoarsenicals. Though As(V) is dominant in surface water, its presence and distribution can be changed due to the stratification of seawater, anoxic bottom sediments, biological activities and their interaction with the As species as well as anthropogenic activities and/or inputs. The ratio of As(V) to As(III) is usually 0.1 to 250 which is driven by the biological transformation in oxygenated seawater, but theoretically, the order is 10^{-15} to 10^{-26} (Cullen and Reimer, 1989).

The distribution coefficient of As(V) is relatively higher than As(III) in an aqueous media (Smedley and Kinniburgh, 2002). Naturally, the (oxy)(hydr)oxides of Fe, Al, and Mn act as sorbents in waters and these oxides are essential in mobilizing As in natural water. It has been anticipated that anionic As(V) can sorb to the mineral surfaces more strongly at the pH levels of most natural waters. On the other hand, inorganic As(III) is generally remain as an

uncharged molecule (i.e., $H_3AsO_3^o$) under circumneutral pH and is less likely sorb to mineral surfaces (Korte and Fernando, 1991). In acidic and oxidizing conditions, the mobility of As(III) is five to six times faster than As(V). Though As(III) is more mobile than As(V), the mobility of As(V) is somewhat enhanced under near neutral to neutral pH condition when compared with the mobility under acidic conditions. However, both As species moved swiftly in alkaline and reduced condition (Gulens et al., 1979). In the presence of a high concentration of reduced sulphur or sulfide (HS^-, S^{2-}, and H_2S), the dissolved As-sulfide (As_2S_3), realgar (AsS) or other sulphide minerals are significantly formed at Eh values lower than −250 mV which is also favored by the acidic and reducing condition.

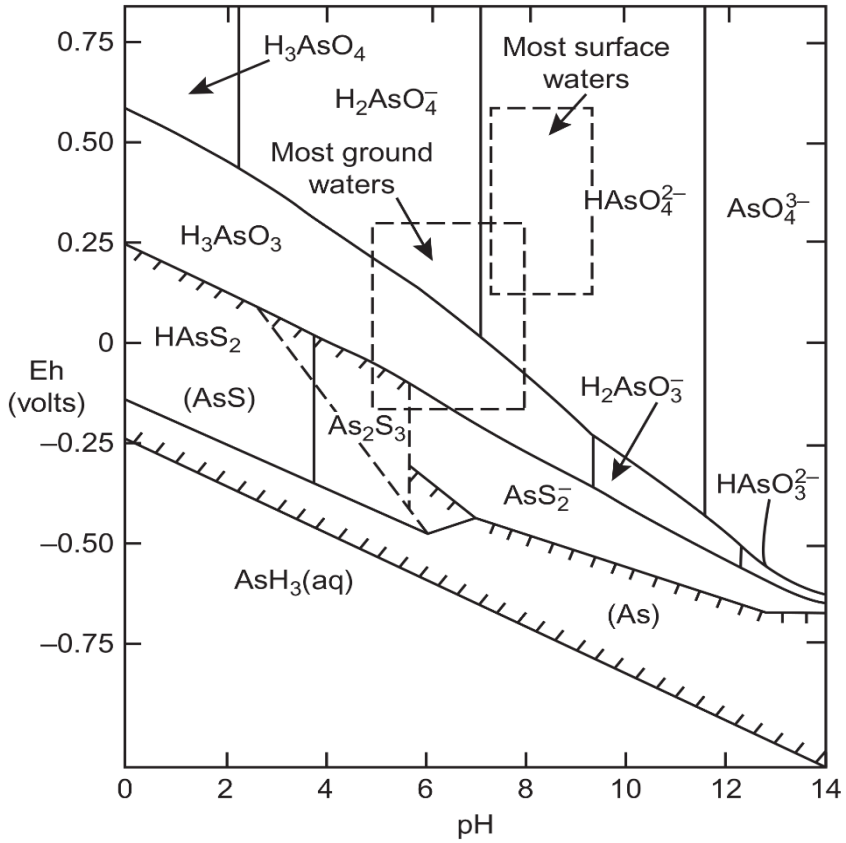

Figure 2.3: Eh-pH diagram for arsenic at 25 °C and 1 bar total pressure, with total As 10^{-5} M; symbols for solid species are enclosed in parentheses in crosshatched area, which indicates solubility less than 10^{-5} M [Reprinted from Pal (2015) with permission from Elsevier].

2.1.4 Arsenic Biogeochemical Cycling in the Marine Environment

In unpolluted marine environments, As is the most uniformly distributed elements, and in open waters, the concentrations are relatively constant. Anthropogenic processes as well as natural processes such as runoff from riverine, dry and wet atmospheric deposition, and

volcanic eruptions may contaminate the seawater in coastal areas, and estuaries result in an elevated concentration of As. *Figure 2.4* shows As cycling in the seawater. The dissolved inorganic As species are the primary forms that entered into the marine environment (Hasegawa, 1996). In the marine environment, As exist in various chemical forms either in water or in sediments or organisms. Recently more than 50 As species have been reported in the marine ecosystem. The knowledge of the nature and distribution of As species in marine ecosystems is necessary because As species show different ecotoxicological effects on marine organisms in terms of seafood-related dietary uptake (Francesconi, 2010).

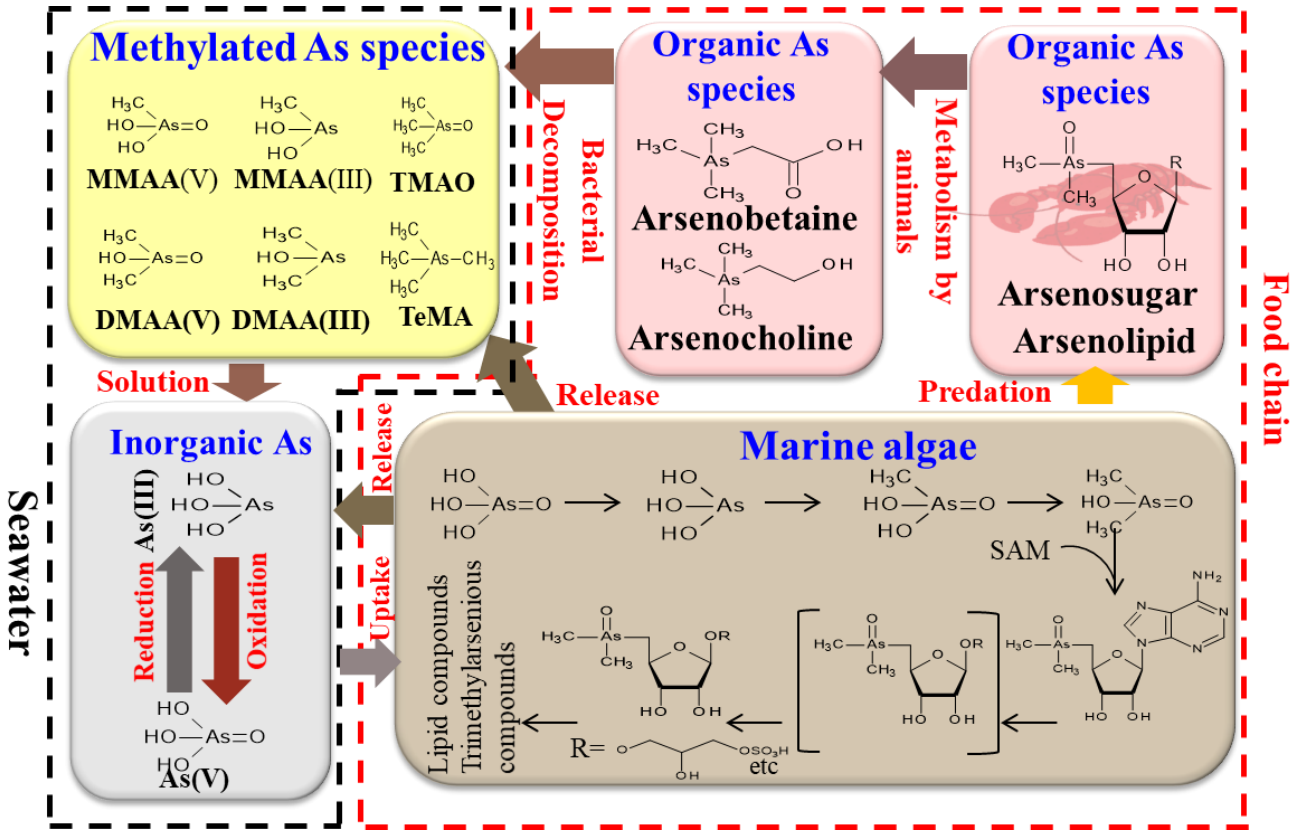

Figure 2.4: Schematic diagram showing the circulation of As species between seawater and food chain (Adopted and modified from Edmonds and Francesconi (2003)).

Elemental As is rarely occurred in natural seawater. Inorganic As species of the highest redox state (As(V) and As(III)) are most abundant species in seawater which are biologically active forms and continuously subjected to transform via marine organisms. The biotransformation processes such as reduction, methylation, demethylation, and other processes, etc. play a vital role for the availability of various ratios of As species in the marine ecosystem (Kalia and Khambholja, 2015). Algae are the most vulnerable groups of organisms subjected to the toxicity of inorganic As species. Therefore, they use different strategies to

detoxify As through which they can tolerate the toxicity (Rahman et al., 2012). The P concentration in seawater plays a critical role in regulating the As(V) absorption by the marine organisms, especially marine microalgae and macroalgae. The high accumulation of As(V) under P-limited and low P containing seawater enhance the uptake of As(V), and eventually increased the production of reduced and methylated As species as well as their release into the seawater. The occurrence of As(III) in seawater is believed to be resulted from As(V) bioreduction by the algae, but the concentration decreased rapidly below the thermocline because of its unstable nature. As(III) have shown low oxidation rate, hence its coexistence with As(V) might possible under deep seawater. By contrast, methylated arsenicals, which are formed after As(V) biotransformation and detoxification show chemically stable up to a few months (Kalia and Khambholja, 2015). Santosa et al. (1996) reported that increasing temperature and nutrient availability is the primary reasons for an elevated level of methylated As in Pacific surface seawater. Their results suggested the abundance of MMAA(V)/DMAA(V) are the consequence of biotransformation by the algal community in surface waters.

Marine algae accumulate As and stored arsenosugars, which are chiefly the largest group of naturally occurring arsenicals identified in 1981 from brown algae *Ecklonia radiata* (Francesconi, 2005; Kirby et al., 2002). Several studies have focused on how arsenobetaine is accumulated in marine animals. Marine organisms, mainly algae converted inorganic As(V) following uptake from seawater and forms organic species which then metabolized through the food chain and concentrated as arsenobetaine in marine animals (Santosa et al., 1996). Algae form arsenoribosides as a final product of As detoxification and the formation of arsenobetaine by marine animals are considered as a final metabolite of As in the marine food web (Edmonds and Francesconi, 2003). Although arsenosugar is abundant in marine macroalgae, there are also other kinds of organoarsinicals found in the tissues of marine animals, including arsenocholine, arsinylribosides, tetramethylarsonium ion, phosphatidylarsenocholine, other methylated products (Edmonds and Francesconi, 1997). The ingestion of organoarsenicals followed by successive food chains by higher trophic levels are immediately excreted out, and the small remainder parts are synthesized and/or incorporated within the organisms and/or released into seawater after their decomposition. The cycle may be completed either by exported to higher organisms or by deposited as dead material in marine sediments. In such way, the As species either as organoarsenicals or as freely anions in seawater

are degraded to complete the As cycle in the marine ecosystem (inorganic As to inorganic As via formation of organic arsenic) (Hanaoka et al., 1997).

Marine sediments are the harbor of As-containing dead tissues and undissolved biogenic particles The biodegradation of these compounds occurred either at the sediment levels or the euphotic seawater levels with inorganic As or lower forms of organoarsenicals, and then released back into the water column (Andreae and Klumpp, 1979; Andreae, 1978; Hasegawa, 1996). Hanaoka et al. (1991) identified the bacterial association for the decomposition of arsenobetaine to dimethylarsinic acid under aerobic conditions. The similar finding also found under laboratory studies by Hanaoka et al. (1988), who suggested that sedimentary microorganisms can transform complex forms of methylated As species into their simpler forms and even the conversion continued into the simpler and aerobically stable form of As(V). The hydrated metal (Fe, Al, and Mn) oxides can adsorb As in marine sediment. There is a common phenomenon that commonly found under the aquatic system is the co-precipitation of inorganic As species with metal oxides. Besides, surface active suspended particulate matters are involved in scavenging dissolved As from water column. Neff (2002) reported that much of the As in seawater are co-precipitated and/or adsorbed as $Fe_3(AsO_4)_2$ in the oxidized layer of sediments. The natural exchange of As between sediment and overlying water column has a significant influence on the biogeochemical cycle of As. Though sedimentary levels of As constitute a minor concentration, sedimentary diagenesis released As to pore water (Maher and Butler, 1988). It has been suggested that the adsorbed As released back into pore water due to the reductive desorption and/or dissolution of hydrous oxides (Neff, 1997). As in most marine sediments can react with available sulfides to form As containing insoluble minerals like realgar (AsS), arsenopyrite (FeAsS), or orpiment (As_2S_3) (Neff, 2002). Besides, marine sediments are the primary source of MMAA(V) and DMAA(V) to the overlying water column and some species of sediment inhabiting bacteria in aerobic and anaerobic nature have the potentiality to absorb As(V) and As(III) and convert them into methylated species.

2.1.5 Arsenic Contents and Speciation in Seawater

As in seawater shown to have a higher concentration than most of surface fresh water. The abundance of As in seawater ranked 14th among the other elements (Mandal and Suzuki, 2002), but in other studies, it ranks ranged between 24th and 28th (Haynes, 2011; Haraguchi, 2004). The concentration of As in open oceans showed a little variation with an average concentration of As in seawater is 2 µg L^{-1} (Ng, 2005), and the concentration generally ranged

from 1 to 2 µg L^{-1} in unpolluted seawater (Maher and Butler, 1988). Marine waters are contaminated by many sources like volcanic eruption, hot springs, atmospheric deposition through low-temperature volatilization, sea spray, riverine input, geysers, and marine hydrothermal fluids from active oceanic ridges. The higher concentration was found in contaminated sites of coastal areas suggesting the indispensable sources of As contamination in ocean waters caused primarily by the discharges and/or runoff of industrial mining effluents from land sources (Kalia and Khambholja, 2015).

Redox conditions, temperature, species composition and diversity, and biological activities, especially phytoplankton and macroalgae, are the critical factors in controlling the different proportions of inorganic and organic forms of As in marine and estuarine waters. The most abundant and thermodynamically stable forms of inorganic As in oxygenated fresh and brackish seawater under the normal pH conditions include As(V) and As(III). Though the As(V) as anionic forms ($H_2AsO_4^-$ and $HAsO_4^{2-}$) are predominant in seawater, the neutral As(III) as ($As(OH)_3$) accounted as much as 15 to 25% of total As content (Neff, 1997; Francesconi, 2005). As(III) is invariably present in seawater, but its presence is vital in the anoxic bottom waters. Ratios of As(V)/As(III) are typically in the range of 10–100 in open seawater (Andreae, 1978). Methylarsonic acid (MMAA(V)) and dimethylarsinic acid (DMAA(V)) is also frequently found in seawater, but their concentrations are relatively lower than inorganic As (Francesconi, 1994; Kalia and Khambholja, 2015). Though MMAA(V) and DMAA(V) are the dominant methyl As species in seawater, other organoarsenicals may also exist. However, the presence of all organoarsenicals in seawater and different biological samples are not always be detected by the available analytical procedures. IAs species (As(V) and As(III)) and organoarsinicals (methylated and other organic As species) comprise about 80% and 20% of the total As in the marine environment (Anninou and Cave, 2009). The methylated forms are believed to be formed because of the biological transformation of As(V) by the algae. Methyl As species such as MMAA(V)/DMAA(V) is relatively more stable As species than inorganic As(III) in seawater (Cutter and Cutter, 2006). Otherwise, these species degraded and decomposed through demethylation with the formation of inorganic As species (Anderson and Bruland, 1991). Thus, As(V), As(III), methylated As (MMAA(V) and DMAA(V)) species are generally considered as the important species in the context of biogeochemistry in seawater. *Table 2.2* shows the different As species in seawater of the different ocean waters in the world.

There is a strong connection exists between As(V) and phosphate concentration in ocean water, and it has been suggested that biological activity control the vertical distribution

of As in ocean. Cullen and Reimer (1989) also suggested that As(V) concentration minima in seawater often coincide with photosynthetic maxima evidenced by high concentrations of chlorophyll a. The low phosphate concentrations in seawater accelerate the uptake of As(V) as well as methylation by the marine algae. The increasing water temperature above 25 ^{o}C along the coast tends to increase the concentration of MMAA(V) and DMAA(V) which was found in the photic zone of oceans (Santosa et al., 1996; Francesconi, 1994). The variable concentration in MMAA(V)/DMAA(V) in seawater is resulted mostly from the excretion of marine organisms as well as from the chemical decomposition of more complex organoarsenicals. It has also been suggested the diffusion of methyl As-bearing surface waters and their circulation to greater depths leading to the abundance of these As species into deep ocean waters (Santosa et al., 1996). In oxygenated fresh, brackish and marine water, As(III) concentration is always higher than predicted when considering thermodynamic parameters alone. Biotic and abiotic reduction of As(V) along with atmospheric deposition, upwelling of anoxic waters, and input from hypoxic river basins are considered as the primary sources of thermodynamically unstable As(III) in seawater (Cutter, 1992; Neff, 2002).

Table 2.2: As speciation in different marine and estuaries waters in the world

Location	Seawater	As concentration (µg/L)	Reference
Antarctic Ocean	Filtered (0.45 µm)	As(V): 1.045, As(III): 0.003; MMAA(V): 0.007; DMAA(V): 0.023	Santosa et al. (1994)
Western Atlantic	Filtered (0.40 µm)	IAs: 8.0–23.0 (average: 16.3±2.1); [As(III): 0.1–2.0]	Cutter et al. (2001)
Atlantic Ocean (north, surface)	Unfiltered	1.0−1.3 As(V), 0.004−0.32 As(III)	Mlddelburg et al. (1988)
Australian coast: near Adelaide, South Australia	Filtered (0.45 µm)	DIAs: 1.10−1.61 [As(III): 1.2−4.3; rest: As(V)]	Maher (1985)
Indian Ocean: east	Filtered (0.45 µm)	As(V): 0.452; As(III): 0.232; MMAA(V):0.032; DMAA(V): 0.050	Santosa et al. (1994)
Indonesian Archipelago	Filtered (0.45 µm)	As(V): 0.418; As(III): 0.175; MMAA(V): 0.033; DMAA(V): 0.089	Santosa et al. (1994)
Oslofjord: Bunnefjord, Norway	Filtered (0.45 µm)	TAs: 0.64−2.02 [As(III): 0.0097−0.839)	Abdullah et al. (1995)
Puget Sound: Washington, USA: sediment porewaters: 65 analyses	Filtered (0.45 µm)	As(III) + As(V): 1.3−110	Peterson and Carpenter (1986)
Saanich Inlet: Washington, USA: sediment porewaters: 23 analyses	Filtered (0.45 µm)	As(III) + As(V): 0.6−19	Peterson and Carpenter (1986)
Thames Estuary: United Kingdom: February 1989	Filtered (0.45 µm)	As(III) + As(V): 7.33 ± 0.43	Millward et al. (1997)
Vestfjord: Oslofjord, Norway	Filtered (0.45 µm)	TAs: 0.70−1.57 [As(III) ≤ 0.40]	Abdullah et al. (1995)
Yellow Sea and East China Sea [surface (0.5–1 m beneath the surface) and bottom (2 m above the sediment-water interface)	Filtered (0.40 µm)	DIAs: 0.50–4.18 (surface) and 0.64–7.39 (bottom); As(III): 0.08 to 3.21 (surface) and 0.03 to 3.29 (bottom) As(V): 0.03 to 2.46 (surface) and 0.03 to 7.28 (bottom)	Wu et al. (2015)
Coastal of Johore, Malaysia (surface water)	Filtered (0.40 µm)	TAs: 0.38 ± 0.02 to 1.78 ± 0.04; As(III): 0.16 ± 0.0.02 to 0.48 ± 0.02; As(V): 0.23 ± 0.02 to 1.41 ± 0.03	Yusof et al. (1994)
Hon Do Island, Nha Trang Harbor, Vietnam	Filtered (0.45 µm)	TAs: 4.12; As(III): 2.65; As(V): 0.85	Le et al. (2011)

2.1.6 Availability and Speciation of Arsenic in Marine Macroalgae

Microalgae and macroalgae are considered as the primary producers at the base of food webs in the aquatic environment. These groups of organisms showed enormous accumulation and biotransformation capacity in seawater when compared with the other organism in higher trophic levels. The dietary uptake of algal-based food items may cause threats to human health. The speciation of As in macroalgae are of great concern and become essential issues now a days because of the toxicological difference of As species are dependent on their respective structure. As species in macroalgae not only provide the information concerning health risk but also give the information of As cycling in the marine environment because they play important link between the water column and other organisms in the food web.

Table 2.3: As speciation in the important and edible macroalgae species in the world

Algal Species	Location	As speciation (mg/kg)	Instrument	Remarks	Reference
Pyropia yezoensis	South China	TAs: 6.0 ± 0.1 to 8.2 ± 0.4; IAs: 0.054 ± 0.01 to 0.14 ± 0.01	TAs: AFS IAs: HPLC-HG-AFS	Environmental sample (site: 3)	Zhao et al. (2012)
Pyropia yezoensis	Japan	TAs: 14 ± 2; DMAA(V): 0.064 ± 0.005; As(V): nd; Gly-sug: 1.02 ± 0.07; PO_4^{3-}-sug: 13 ± 1	TAs: AFS IAs: HPLC-HG-AFS	Commercial dried sample	García-Salgado et al. (2012a)
Pyropia yezoensis	Spain	TAs: 25 ± 3; DMAA(V): nd; As(V): nd; Gly-sug: 1.6 ± 0.1; PO_4^{3-}-sug: 20.1 ± 0.3	TAs: AFS IAs: HPLC-HG-AFS	Commercial dried sample	García-Salgado et al. (2012a)
Porphyra umbilicalus	Kittery, New England	TAs: 20.73; IAs: 0.12; MMAA(V): 0.05; DMAA(V): 0.25; Gly-sug: 1.95; PO_4^{3-}-sug: 24.98; SO_3-sug: 0.09	TAs: ICP-MS; Others: LC coupled to collision cell ICP-MS	Environmental samples	Taylor and Jackson (2016)
Porphyra dentata	Taiwan	TAs: 31.6 ± 0.3; MMAA(V): 0.25 ± 0.003; DMAA(V): 0.24 ± 0.007; Gly-sug: 0.34 ± 0.02; PO_4^{3-}-sug: 27.3 ± 0.2	TAs: ICP-MS; Others: IC-ICP-MS	Commercial sample from local market	Hsieh and Jiang (2012)
Laminaria ochroleuca	Japan	TAs: 72 ± 2; DMAA(V): 0.36 ± 0.003; As(V): 32 ± 3; Gly-sug: 4.7 ± 0.3; PO_4^{3-}-sug: 22 ± 1	TAs: AFS IAs: HPLC-HG-AFS	Commercial dried sample	García-Salgado et al. (2012a)
Laminaria ochroleuca	France	TAs: 40 ± 2; DMAA(V): nd; As(V): 11 ± 2; Gly-sug: 11 ± 3; PO_4^{3-}-sug: 1.9 ± 0.3	TAs: AFS IAs: HPLC-HG-AFS	Commercial dried sample	García-Salgado et al. (2012a)
Undaria pinnatifida	Japan	TAs: 18 ± 3; DMAA(V): 0.025 ± 0.007; As(V): 4.5 ± 0.3; Gly-sug: 2.68 ± 0.03; PO_4^{3-}-sug: 10.10 ± 0.05	TAs: ICP-AES; Others: HPLC–(UV)–HG–AFS	Commercial dried sample	García-Salgado et al. (2012a)
Undaria pinnatifida	Japan	TAs: 26.2 ± 7.3; As(V): 1.7 ± 0.1; As(III) + MMAA(V): nd; DMAA(V): 0.56 ± 0.03; PO_4^{3-}-sug: 6.40 ± 0.04	TAs: ICP-MS; Other: HPLC-ICP-MS	Wet sample from local market	Hirata and Toshimitsu (2007)
Undaria pinnatifida	Japan	TAs: 49.07 ± 1.33; As(V): 0.24; As(III): 0.25; DMAA(V): 2.08	TAs: ICP-MS; Other: HPLC-ICP-MS	Environmental sample wet sample	Narukawa et al. (2012)
Undaria pinnatifida	Taiwan	TAs: 24.4 ± 0.8; MMAA(V): 0.22 ± 0.02; DMAA(V): 1.30 ± 0.10; Gly-sug: 22.0 ± 0.3	TAs: ICP-MS; Others: IC-ICP-MS	Commercial sample from local market	Hsieh and Jiang (2012)
Hizikia fusiformis	Japan	TAs: 72 ± 2; As(V): nd; DMAA(V): 0.44 ± 0.06; As(V): 50.3 ± 0.4; Gly-sug: 1.05 ± 0.03; PO_4^{3-}-sug: 0.4 ± 0.1; SO_3-sug: 0.7 ± 0.1; SO_4 sug: 2.7 ± 0.4	TAs: ICP-AES Others: HPLC–(UV)–HG–AFS	Commercial dried sample	García-Salgado et al. (2012a)
Palmaria palmata	Kittery, New England	TAs: 8.95 ± 4.80; IAs:0.06 ± 0.11; MMAA(V): 0.03 ± 0.01; DMAA(V): 0.96 ± 0.35; Gly-sug: 3.03 ± 1.50; PO_4^{3-}-sug: 4.35 ± 3.22; SO_3-sug: 0.16 ± 0.16; SO_4 sug: 0.01 ± 0.02	TAs: ICP-MS Others: LC coupled to collision cell ICP-MS	Environmental samples	Taylor and Jackson (2016)

Ascophyllum nodosum	Durham, New England	TAs: 23.14 ± 6.82; IAs:0.08 ± 0.03; MMAA(V): nd; DMAA(V): 0.21 ± 0.12; Gly-sug: 2.05 ± 0.77; PO_4^{3-}-sug: 0.59 ± 0.10; SO_3-sug: 1.10 ± 0.11; SO_4 sug: 5.18 ± 2.79	TAs: ICP-MS; Others: LC coupled to collision cell ICP-MS	Environmental samples	Taylor and Jackson (2016)
Fucus spiralis	Durham, New England	TAs: 16.27 ± 1.41; IAs:0.04 ± 0.05; MMAA(V): nd; DMAA(V): 0.19 ± 0.06; Gly-sug: 0.56 ± 0.45; PO_4^{3-}-sug: 0.31 ± 0.04; SO_3-sug: 3.63 ± 0.03; SO_4 sug: 1.51 ± 0.49	TAs: ICP-MS; Others: LC coupled to collision cell ICP-MS	Environmental samples	Tailor and Jackson (2016)
Fucus vesiculosus	Durham, New England	TAs: 28.89 ± 2.16; IAs: 0.06 ± 0.04; MMAA(V): nd; DMAA(V): 0.18 ± 0.08; Gly-sug: 0.50 ± 0.29; PO_4^{3-}-sug: 0.42 ± 0.04; SO_3-sug: 7.90 ± 3.94; SO_4 sug: 1.74 ± 0.65	TAs: ICP-MS; Others: LC coupled to collision cell ICP-MS	Environmental samples	Tailor and Jackson (2016)
Laminaria digitata	Kittery, New England	TAs: 50.38 ± 11.91; IAs: 8.32 ± 7.67; MMAA(V): 0.06 ± 0.13; DMAA(V): 0.97 ± 0.41; Gly-sug: 5.10 ± 0.91; PO_4^{3-}-sug: 10.63 ± 8.71; SO_3-sug: 10.23 ± 7.61	TAs: ICP-MS; Others: LC coupled to collision cell ICP-MS,	Environmental samples	Tailor and Jackson (2016)
Laminaria japonica	Taiwan	TAs: 32.6 ± 0.2; DMAA(V): 0.57 ± 0.008; Gly-sug: 23.7 ± 0.8; PO_4^{3-}-sug: 7.06 ± 0.15	TAs: ICP-MS; Others: IC-ICP-MS	Commercial sample from local market	Hsieh and Jiang (2012)
Saccharina latisama	Kittery, New England	TAs: 56.29; DMAA(V): 0.39; Gly-sug: 0.28; PO_4^{3-}-sug: 2.79; SO_3-sug: 15.45	TAs: ICP-MS; Others: LC coupled to collision cell ICP-MS	Environmental samples	Taylor and Jackson (2016)
Sargassum fusiforme	China	TAs: 65.3 ± 0.6 to 90.3 ± 1.6; DMAA(V): 0.6 to 1.8; As(V): 15 to 35	TAs: ICP-MS; Other: LC-ICP-MS	Wet samples from local market (n=12)	Han et al. (2009)
Sargassum cristaefolium	Taiwan	TAs: 4.73 ± 0.09; MMAA(V): 0.53 ± 0.03; As(III): 0.15 ± 0.01; DMAA(V): 0.43 ± 0.02; Gly-sug: 2.42 ± 0.08; PO_4^{3-}-sug: 0.83 ± 0.04	TAs: ICP-MS; Others: IC-ICP-MS	Commercial sample from local market	Hsieh and Jiang (2012)
Sargassum sp.	Isabel, Australia	TAs: 8.68 ± 0.25; As(III): 0.20; As(V): 4.63; MMAA(V): <0.2 BDL; DMAA(V): 0.16; AB: 1.24	TAs: ICP-MS Other: HPLC-ICP-MS	Environmental samples	Grinham et al. (2014)
Sargassum fulvellum	Japan	TAs: 206.9 ± 11.2; As(V): 112.4 ± 2.3; As(III) + MMAA(V): 1.7 ± 0.2; DMAA(V): 1.05 ± 0.40; PO_4^{3-}-sug: 9.62 ± 0.67; SO_3-sug: 2.71 ± 0.38; SO_3-sug: 11.9 ± 2.84	TAs: ICP-MS; Other: HPLC-ICP-MS	Wet sample from local market	Hirata and Toshimitsu (2007)
Hizikia fusiformis	Japan	TAs: 60.4 ± 0.4; As(V): 16.0 ± 1.6; As(III) + MMAA(V): 0.50 ± 0.10; PO_4^{3-}-sug: 1.09 ± 0.23; SO_3-sug: 0.17 ± 0.07; SO_3-sug: 1.17 ± 0.43	TAs: ICP-MS; Other: HPLC-ICP-MS	Wet sample from local market	Hirata and Toshimitsu (2007)
Sargassum piluliferum	Japan	TAs: 288.0 ± 16.8; As(V): 47.4 ± 3.3; As(III) + MMAA(V): 1.4 ± 0.2; DMAA(V): 2.7 ± 0.8; PO_4^{3-}-sug: 5.62 ± 0.39; SO_3-sug: 2.14 ± 0.30; SO_3-sug: 9.44 ± 0.86	TAs: ICP-MS; Other: HPLC-ICP-MS	Wet sample from local market	Hirata and Toshimitsu (2007)
Pelvetia wrightii	Japan	TAs: 14.5 ± 0.5; As(V): 0.6 ± 0.4; As(III) + MMAA(V): 0.13 ± 0.05; DMAA(V): 0.3 ± 0.2; AC: 0.12 ± 0.03	TAs: ICP-MS; Other: HPLC-ICP-MS	Wet sample from local market	Hirata and Toshimitsu (2007)

2.2 Arsenic Stress to Algae

The toxicity and sensitivity of As species to different algal species have been reported and showed a controversial scenario. The differences and converses might be due to the variation in toxicity response to the algal species and their complex chemistry in freshwater and marine water. It has been established that As(III) and As(V) cause sensitivity to marine and freshwater microalgae, respectively (Levy et al., 2005). Cullen et al. (1994a) and Takimura et al. (1996) showed that marine algal species *Polyphysa peniculus* and *Dunaliella* sp. was more sensitive to As(V) compared to As(III). The equal toxicity of As(V) and As(III) was also reported by freshwater alga *Stichococcus bacillaris* at pH 8.2 for a P range of 0.03 and 0.3 mg L^{-1} (Pawlik-Skowrońska et al., 2004). It has been noted that the toxic load exerted by the chemicals can be alleviated by using the high initial cell concentrations of microalgae (Franklin et al., 2002; Levy et al., 2005). Different species of algae showed unusual accumulation, biotransformation, and sensitivities toward As species. The quantity of internalized or synthesized As are entirely dependent on As-phytochelatins complexes, and the quality and quantity of phytochelatins are responsible for varying sensitivities towards different plant and/or algal species. Also, speciation of As might be changed due to biotic and abiotic transformation during the incubation period of algae. Moreover, the comparative and conclusive data on the phytotoxicity of methylated and inorganic species of As are scarce that lead to contradictory toxicological results in the literature. The toxicity and mode of action of As largely depends on several factors and is quite complicated. The complexity originated because As can form a very rich variety of species (Dopp et al., 2010). Though the mechanism of toxicity of As species is still not well understood, valence state (trivalent and/or pentavalent), charge at physiological pH, extent of methylation and electrostatic attraction and repulsion to active sites on important macromolecules, and several pharmacokinetic factors like absorption, distribution, metabolism, protein binding, and excretion are considered as major determinants associated with the toxicity of As (Flora, 2011).

It was generally observed that the growth rate of algae exposed to organic As species were found similar to those algae exposed under control treatment. It is still unknown why organic species lead to an increase in biomass of the algae or even no toxicity to the algae. It was reported that *Chlorella vulgaris* was able to uptake inorganic as well as methylated As species. Maeda et al. (1992b) suggested that methylated As species might be further biomethylated into the cells following their uptake that might cause less stress or toxicity to As. In a 72-h growth-inhibition test with the freshwater alga *Microcystis arcuatum*, Levy et al.

(2005) reported that As(V) is more toxic than As(III) to the algae with IC_{50} values of 14.5 and 0.25 mg L^{-1} for As(III) and As(V), respectively. Contrary to this, *Chlorella vulgaris*, which was isolated from As-contaminated freshwater (2000 mg L^{-1}) showed more sensitive to As(III) than As(V) (Maeda et al., 1985). The effect of As(III) and As(V) (concentration of both As species ranged between 3.75 to 375 mg L^{-1}) towards the growth of *Scenedesmus* sp. was investigated by (Bahar et al., 2013). The algae showed more toxicity toward As(V), and the growth of alga was inhibited even at a very low concentration under low phosphate containing BBM, whereas As(III) up to 75 mg L^{-1} did not inhibit the algal growth. The similar results and higher toxicity of As(V) compared to As(III) was reported in freshwater algae (*Monoraphidium arcuatum*) (Levy et al., 2005). On the other hand, *Chlorella salina*, marine water algae showed more tolerant towards As(III) than As(V) (Karadjova et al., 2008). Mkandawire et al. (2004) studied the concentration-dependent As toxicity to aquatic macrophyte *Lemna gibba* and reported that this macrophyte showed more toxicity toward As(III) concentration between 300 and 800 µg L^{-1}. However, when the As(V) concentration in the culture increased to above 800 µg L^{-1}, then As(V) was more toxicity than As(III).

Not unexpectedly, the higher exposure of external As concentration applied to the algae, the higher the total As accumulation in the alga and displaying more toxicity to the algae. The similarity of As(V) with the essential molecules P more often describe the mechanism of As(V) toxicity whereas high reactivity and affinity of As(III) to sulfur in proteins and peptides explain the toxicity potential of As(III). The inorganic As associated toxicity affect several mechanisms in the cells of algae include phosphate replacement, enzyme inhibition, oxidative stress, genotoxicity and alteration of DNA repair, signal transduction, gene transcription, DNA methylation and growth factors (Kevin, 2009). P is vital in controlling the metabolism of As and growth of macroalgae culture. It was seen that both increase in macroalgal biomass and daily growth rate is related to the increasing levels of P in the growth media (Mamun et al., 2019b). The reduced synthesis and regeneration of substrate in the Calvin-Benson cycle along with a decrease in the rate of necessary light utilization for carbon fixation are associated with the immediate effects of phosphorus limitation on algae (Chisti, 2013). The increases in PO_4^{3-} concentration (0.11 mg L^{-1} up to 1.3 mg L^{-1}) in culture media of marine algae *C. salina* showed a significant decrease the toxicity of As(III) and As(V). The effective concentration (EC_{50}) increased from 19.5 to 975 and 20.25 to 1200 µg L^{-1} for As(III) and As(V), respectively. However, the presence of PO43- did not affect the toxicity as well as intracellular MMAA(V) and DMAA(V) concentration (Karadjova et al., 2008).

It was reported that P deficiency could enhance the accumulation of astaxanthin and reduce chlorophyll a and protein content resulting in an overall reduced growth rate of microalgae (Juneja et al., 2013; Kobayashi et al., 1993). Algae utilize several ways to overcome the adverse effect of As toxicity, especially As(V). Studies showed that higher phosphate in algal culture inhibits the As(V) uptake, whereas the inhibitory effect of As(III) is independent of phosphate. The underlying fact might be the uptake regulation due to decline in the number of arsenate transporters or affinity on the cell membrane or competition for binding sites of cytoplasmic arsenate reductase which ultimately lowering the intracellular As content (Castro et al., 2015; Wang et al., 2013a; Karadjova et al., 2008), and reduce the burden of As stress inside the cell. It is also possible that As(V) may be released from the cells (Gupta et al., 2011). As(V) could be reduced to As(III) via various reductases which is either synthesized in the presence of glutathione and phytochelatins or methylated to less toxic As species (MMAA(V) and DMAA(V)) or excreted to the ambient environment. Another way of alleviating As(V) toxicity might be due to the distribution of As(V) at a subcellular level that might vary with its ambient concentration (Miot et al., 2009; Duncan et al., 2010).

2.3 Metabolic Mechanism of Arsenic by Algae

Algae are the prevalent inhabitant and primary producers in the aquatic systems which are responsible for bioaccumulation and biogeochemical cycling of As (Duncan et al., 2015). Both microalgae and macroalgae have developed different extracellular and intracellular mechanisms to tolerate and resist the exposure of toxic inorganic As compounds in the environment, a particularly complex matrix of natural water (freshwater and seawater). The extracellular mechanistic pathways through which algae reduce As stress include cell surface adsorption. By contrast, the intracellular metabolism with regard to As detoxification by algae include oxidation of trivalent As (As(III) to As(V)), reduction of pentavalent As(V) into As(III), complexation with thiolic compounds in the cell and cellular sequestration into vacuoles, methylation and other transformation process leading to less toxic organic As (arsenosugars or arsenolipids), and excretion from the cells.

2.4 Factors Affecting Arsenic Metabolic Activities by Algae

Different experimental conditions have been reported in the literature in terms of As uptake and metabolism by the various algal groups and species. Many factors have been evaluated on the metabolic pathways of As, but their comparison makes it difficult due to the difference in the experimental setup by different researchers. Besides, for reasons of experimental inconvenience, the laboratory macroalgal culture studies concerning As

metabolic studies under laboratory culture system have been evidenced in a limited number of publications. Most works of the literature with for As speciation in macroalgae has been conducted with commercially available and/or field collected samples because of their food safety and nutritional purposes. However, As metabolism in microalgae has been reported extensively. However, few researchers have been used macroalgae species in the As uptake and metabolism studies in the laboratory recently. Several factors that reinforced numerous studies for investigating the bioavailability and behavior of inorganic As species in water bodies as well their metabolism by organisms.

2.4.1 Environmental Factors

Light is essential for the photosynthetic reactions carried out by algae to produce energy and metabolites. The optimal conditions for this source of energy rely on intensity, quality, and periods of illumination. These conditions vary greatly among algae species as well as in the As metabolism studies in laboratory cultures. Hasegawa et al. (2001) reported that light/dark cycle had appreciably minimum and/or no influence on production rate of As(III) and DMAA(V) by freshwater algae *Closterium aciculare* when inoculated with a P-deficient As-high culture medium (12 µM As(V), 19 µM PO_4^{3-}, and 0.02 M NO_3^-) for 24 h. Their results also suggest that trivalent DMAA(III) production might be dependent on the light/dark cycle and production rate was comparably higher under the dark phase than a light phase. The low production rates of DMAA(III) during the light phase may be due to photochemical oxidation. The efflux or excretion of As by the algae species dependent on light intensity/illumination that require more photosynthetic energy than do As(V) absorption. For example, the marine microalgae (*Tetraselmis chui*) did not excrete ^{74}As during the stationary growth phase at an illumination of 7000 lux (100 µmol photons m^{-2} s^{-1}), but rapid As excretion was observed at 12500 (200 µmol photons m^{-2} s^{-1}) (Bottino et al., 1978). The short-term incubation of freshwater algae *Chlorella vulgaris* showed to decrease As(V) accumulation with the increase of light intensity from 0 to 7000 lux, and accumulation was higher under dark condition compared to the illuminated state (Maeda et al., 1985). Budd and Craig (1981) showed that the uptake of As(V) by the green algae was not influenced either by the light condition or external P.

Temperature also played a key in algal growth, and hence its effect on As metabolic differences cannot be ignored. The live alga *Chlorella vulgaris* was able to accumulate As(III) under 15 minute exposure period with the temperature maintained between 22 ºC and 37 ºC (Taboada-de la Calzada et al., 1999). The algal activity and growth are improved with rising water temperature during summer, and the situation played a crucial role in the fluctuation and

seasonal variation of As(III) as well as methylarsenicals in freshwater aquatic systems. It is believed that the significantly increasing trend of As(III) and/or methylarsenicals concentration in summer was related to temperature-mediated algal transformation like reduction and methylation, which could accelerated with lower levels of P during this time (Hasegawa et al., 2010; Yan et al., 2016; Hellweger et al., 2003; Zhang et al., 2014). The temperature change showed to influence the rate of release of methylated arsenicals by macroalga *Ascophyllum nodosum*. A two-fold and six-fold more release rate of MMAA(V) and DMAA(V), respectively, were reported when temperature increased between 5 and 15 °C (Millward et al., 1993). The environmental temperature in water bodies at elevated levels can help to increase the growth rate and metabolic activities of the macroalgae, and consequently enhance the rate of biotransformation (As(V) to organic As species) (Zhao et al., 2012).

2.4.2 Hydrogen Ion Concentration (pH) and Redox Potential (Eh)

Different culture compositions are designed to supply the proper nutrients and optimum environmental conditions, especially pH for the particular growth of algae species. Maeda et al. (1992a) suggested that pH could affect not only the accumulation of TAs but also affect the excretion of methylated As species in As free cultures when cells of *Chlorella vulgaris* were accumulated As in the presence of As. Their results indicated that increasing pH could increase the excretion of total As concentrations whereas the decreasing pH (from 9.0 to 5.3) levels could enhance the increasing amounts of methylated As. A reduced intracellular accumulation of As(V) in the freshwater algae *Stichococcus bacillaris* culture amended with HA was also observed when the pH of media changed from 6.8 to 8.2 (Pawlik-Skowrońska et al., 2004). Wang et al. (2017b) observed that As species transformation and distribution were slightly affected by pH levels in media whereas there had a strong effect of N, P, and As(V) on the transformation of As by the *Microcystis aeruginosa*. The high external As(V) levels correspond to high As(III) and DMAA(V) concentrations in media, but the reduction of As(V) to As(III) in media was achieved due to high N along with low P concentrations under slightly acidic environments. Their results suggested that methylated As in efflux media decreased with increasing levels of pH.

2.4.3 Duration of Incubation and Types of Culture

Biotransformation of As by the algae may be linked with the uptake of As taken up and time available for physiological activity of the algae. The influence of different incubation times after inoculation with As was evaluated in some studies. Maeda et al. (1992a) reported that the accumulation of As(V) (initial concentration 10 mg L^{-1}) by *Chlorella vulgaris* was

higher at the beginning of the lag phase which was maintained through the log phase, but at the stationary phase, accumulation was reduced. On the other hand, the maximum accumulation of As(III) (initial concentration 1 mg L^{-1}) reached at the beginning of the log phase and continued up to 48 h and then decreased dramatically. The algae were transformed As(III) into As(V), MMAA(V), DMAA(V), and trimethylarsine (TMA). Levy et al. (2005) observed that As(III) excretion in freshwater microalgae could not keep pace with As(V) reduction leading to As(III) accumulation in algae which then largely methylated to MMAA(V) and DMAA(V) and excreted into the medium at longer culture periods. Hasegawa et al. (2001) observed that freshwater green alga *C. aciculare* initially transformed most of the As(V) into As(III) which reached to peak concentration during the exponential phase of growth. Similarly, when marine microalgae *C. salina* was exposed to 75 µg L^{-1} As(V), the transformed 32 % of the total intracellular As(V) into As(III) for 72 h period (Karadjova et al., 2008). Some freshwater algae species can biotransform As(V) more rapidly, such as *C. reinhardtii* transformed As(V) into glycerol-arsenosugar (Gly-sug) and phosphate-arsenosugar (PO_4^--sug) within 24 h of the incubation period. *Synechocystis* and *Nostoc* also showed to transform As(V) into Gly-sug within 20 min (Miyashita et al., 2011; Miyashita et al., 2012).

2.4.4 Nutrients

The key nutrients N, P, and NP in culture composition play a key role in the metabolism of As(V) by the freshwater algae *Closterium aciculare* (Hasegawa et al., 2001). *D. tertiolecta* and *Thalassiosira pseudonana* produced PO_4^--riboside when the culture were enriched with nutrients, but this arsenosugar was not detected under nutrient-deficient cultures. This result was indicating the importance of culture composition on the uptake and metabolism of As by the algae (Duncan et al., 2013). Several studies have already investigated the relationship between P and As(V) in laboratory-controlled algal culture as well as in open environmental conditions. It has been well manifested that low ambient levels of P in algal culture have a complex interaction with the enhanced accumulation of As(V). The structural similarity between As(V) and P make competition for their cellular uptake via P transporter. These studies are also in line with the common assumption and mostly focused on the effect of P limitation on accelerated metabolism (reduction of As(V) to As(III)), and induced methylation (As(III) to MMAA(V) and/or DMAA(V)) as well as their induced excretion via active transport system in both microalgae and macroalgae species (Wang et al., 2013a; Mamun et al., 2019a; Granchinho et al., 2004; Levy et al., 2005).

Wang et al. (2013a) suggested that lower external P concertation in culture induce algal cells to synthesize more phosphate transporters to compensate for the phosphate deficiency in the medium, leading to enhanced As absorption in algal tissues. Hellweger et al. (2003) also demonstrated that P levels in culture media could regulate As(III) and DMAA(V) excretion. Since the ratio of As(V) to P influences intracellular As content as well as metabolism in marine algae but the response is not consistent across all algal species. It appeared that P had a minimum or no effect on the accumulation of As in phosphate replete environments and/or in concentration levels similar to natural seawater (2 µg L^{-1}) (Andreae and Klumpp, 1979; Sanders, 1979; Sanders and Windom, 1980). Many studies also reported P independent uptake and metabolism of As(V). Foster et al. (2008) showed that environmentally relevant concentration of As(V) and relative low concentration P had a little effect on As(V) uptake by the microalgae *D. tertiolecta* and the diatom *P. tricornutum.* Wang et al. (2016b) reported that lower intracellular P content in the cells of *Dunaliella salina* influence the As biotransformation as well as the efflux of metabolized As species. They showed that lower P added media (0.112 mg L^{-1}) had a reduced As content in *D. salina* compared to media containing high P (11.2 mg L^{-1}) upon the same concentration of As treatment (11.2 µg L^{-1}). Their result suggested that such difference is due to the enhanced As efflux resulted from lower intracellular P level which in turn leading to lower As in the cells of D. salina under the 0.112 mg L^{-1} P treatment.

Recently, some researchers not only focused on P nutrition but also focused on N and/or their interaction for the As biotransformation and metabolism by the algae (Che et al., 2018; Wang et al., 2014; Wang et al., 2017b). N is essential in influencing the bioaccumulation as well as regulating the intracellular As levels, particularly As(III) in *M. aeruginosa* and *C. reinhardtii* culture suggesting the importance of N on biogeochemical cycling of As (Wang et al., 2017b; Wang et al., 2014). The possible explanation is the algal growth acceleration under N-rich condition that could improve the As(III) accumulation and efflux, promoting the reduction of As(V) to As(III). Che et al. (2018) demonstrated a significant variation of an enhanced transformation of As(V) by *M. aeruginosa* under increasing levels of N (4 to 20 mg L^{-1}), though the metabolism was inhibited under increasing levels of P in the culture (0.5 to 1 mg L^{-1}). Their results also suggested that the addition of 10 to 20 mg L^{-1} N with 0.2 mg L^{-1} P to the culture facilitated the higher DMAA(V) production in algae. It has also been reported that N-limited *Chlamydomonas reinhardtii* were able to synthesize more As transporter leading to maximum As(III) accumulation into the cells (Wang et al., 2014). The accumulation of

As(V) by *Nostoc* sp. decreased with an accompanying increased NO_3^- concentration in the medium (Maeda et al., 1993). Wang et al. (2017b) found that the bioreduction of As (As[V] to As[III]) by the *Microcystis aeruginosa* was positively correlated with increasing N levels. These findings indicated the beneficial effect of N nutrition in the algal culture on the stimulation for glutathiones (GSH) synthesis (Downing et al., 2005), and might be the source of enhanced bioreduction potential of As(V) by the algae (Rosen, 2002). On the other hand, Yamaoka et al. (1996) found that N concentration above 18 mg L^{-1} could inhibit the As accumulation by *Chattonella antiqua*. These results implied that different algae species respond differently to As accumulation in association with changes in N concentrations.

2.4.5 Nature, Sources and Levels of Arsenic

As(V) is the most common As species used in the culture experimentation with algae which primarily focused on its uptake and transformation. Another important inorganic species As(III) has also been reported in many researches. The organic species like methylated As (MMAA(V) and DMAA(V)) are the subject of a limited number of studies. It has been well established that the increasing levels of As either as As(V) or As(III) promoted accelerated transformation process of reduction and methylation as well their resultant effect on producing increasing levels of metabolites in excretion (Mamun et al., 2019a; Wang et al., 2015). Zhang et al. (2013a) showed that living algae *Scenedesmus quadricauda* could absorb more monoanionic arsenite ($H_2AsO_3^-$) compared to neutral arsenite (H_3AsO_3). The brown macroalgae, *Fucus spiralis* and *Ascophyllum nodosum*, accumulate four times more As(V) than As(III) from equivalent concentrations in seawater (Klumpp, 1980). It has been suggested that low concentration of As along with high levels of P could facilitate the sequestration of As into the cells that might be released after the death of *M. aeruginosa* (Wang et al., 2017b). The source of As is also an important factor for their metabolism by different algal species. *Tetraselmis chui* and *Hymenomonas carterae* did not discriminate between radioactive and non-radioactive As(V) for their uptake (Bottino et al., 1978). The levels of As(V) have an influence on the speciation and transformation pattern of As(V) by *C. aciculare*. The low level of As(V) (10 nM) showed to have similar metabolic behavior for producing As(III) and DMAA(V) as like as high As(V) (12 µM), but MMAA(V), MMAA(III) and DMAA(III) were below the detection limit at low As(V) levels (Hasegawa et al., 2001). The concentration of inorganic As in algal species (*Fucus* sp.) under naturally contaminated sites had a relatively higher than control area, and thus suggesting the inefficient biotransformation of As when algae were inhabiting high level of As (Geiszinger et al., 2001).

2.4.6 Metabolic Inhibitors and Nanomaterials

The bioavailability and methylation of As are dependent on the association between As species and nano-TiO_2, which could substantially alter and influence the As speciation. It has been established that variable As metabolism occurred as a result of variable As forms and algae species as well as nanoparticles and environmental factors (Luo et al., 2018; Grill et al., 2001). However, it is still not clear as to what extent and how As bioavailability and methylation are influenced by this association. Luo et al. (2018) found an enhanced accumulation of As(V) as well as methylation of As in *S. obliquus* and *M. aeruginosa* in the presence of nano-TiO_2. Their results also speculated that nano-TiO_2 could markedly enhance the accumulation of As(V) in *M. aeruginosa* and As(III) accumulation in *S. obliquus*. The higher As methylation activity in the *M. aeruginosa* occurred when 2 mg L^{-1} nano-TiO_2 applied under As(III) treatment, and the situation was also similar for the *S. obliquus* under As(V) treatment. The introduction of nano-TiO_2 in microalgae culture contributes to an increased level of toxicity and stress to the algae, consequently leading to enhance greater As methylation in algae species. Maeda et al. (1985) reported that dinitrophenol, a respiratory inhibitor stopped the bioaccumulation of As in *Chlorella vulgaris*, whereas sodium azide, a photosynthesis inhibitor, did not affect bioaccumulation of As.

2.4.7 Algal Group and Species

The biotransformation and detoxification of As by the algal species are dependent on the genetic capability as well as cell composition and its interaction with the surrounding environment. It has been suggested that some algae species are not involved in As metabolism because the energy requirement for metabolic transformation is expensive to the algae (Ma et al., 2018). The significantly different uptake and metabolism of As(V) between *Dunaliella tertiolecta* and *Phaeodactylum tricornutum* was reported by Foster et al. (2008) and suggested the species-specific and/or classes structural/biochemical variation of algae are mostly associated with the different uptake pattern and transformation of As.

2.4.8 Algae and Associated Bacteria and Epiphytes

Microalgae and macroalgae are often associated with bacteria or other epiphytic organisms responsible for varying metabolic pathways of As (Borde et al., 2003; Wang et al., 2013b; Duncan et al., 2014). Wang et al. (2016a) observed that the symbiotic association of *Alteromonas macleodii* with *Dunaliella salina* could influence the transformation of extracellular and intracellular As, absorption and excretion. They observed a significantly less

As absorption in axenic cultures of *D. salina* when compared with non-axenic culture of *Alteromonas macleodii* + *D. salina* under 1.12 mg L^{-1} P level. However, the transformation of As(V) to As(III) along with its excretion by non-axenic *D. salina* was facilitated under 0.112 mg L^{-1} P levels that leads to less absorption between 7 and 9 days of the incubation period. On the other hand, Duncan et al. (2014) showed that the culture of *Dunaliella tertiolecta* alone or in combination with bacteria (*Vibrio* sp. or *Myxococcus* sp.) had a minimum comparable effect on the metabolism and cycling of As. Their results suggested the correctness of bacteria-contaminated phytoplanktonic laboratory cultures for investigating As biogeochemical cycling. The addition of bacteria in the algal culture showed little influence on the formation of water-solution As species by the marine algae *D. tertiolecta* (Foster et al., 2008; Duncan et al., 2013). The symbiotic association between *Alteromonas macleodii* and *D. salina* could be beneficial for As detoxification by *D. salina* as mentioned by Wang et al. (2016a).

2.5 Extracellular Uptake or Adsorption of Arsenic

Adsorption is technically distinct and different from the process of absorption. Absorption is considered as the accumulation of a substance in the interior of a non-aqueous phase. Adsorption is recognized as the accumulation of a substance at or near an interface relative to its concentration in the bulk solution, also called surface complexation. The two processes are often used together for defining the terms sorption, sorbate, and sorbent. In an aquatic matrix, metals and metalloids can be adsorbed onto the surface of photosynthetic organisms, including algae through a passive mechanism (Olguín and Sánchez-Galván, 2012). The interaction between trace-metals and marine biota are related to the chemistry of metals in seawater and their blending with cell-surface biomolecules (Sunda, 1989). The presence of negatively charged functional groups (carboxylates, sulfhydryls, phosphates, sulfates, and hydroxyls) in cell wall polymers are responsible for the surface binding of metallic cations while positively charged groups (amine and imidazole) can bind metallic anions (Crist et al., 1981).

The surface adsorption in an organism is a rapid process of accumulation and plays a promising role in the application of clean technology for As phytofiltration. The adsorption of As on the algal cell surface have generally overlooked and received little attention in previous laboratory studies. However, some algal species have been found to have an influence of As detoxification due to their surface adsorption phenomenon recently, and the species include *Dunaliella salina* (Wang et al., 2016b), *Sargassum patens*, *Sargassum horneri*, *Pyropia*

yezoensis, and *Undaria pinnatifida* (Mamun et al., 2019b; Mamun et al., 2019c; Mamun et al., 2019a). It was reported that *Dunaliella teritolecta*, *Undaria pinnatifida* and *Sargassum patens* could bind inorganic As species (As(V) and As(III)) extracellularly (Duncan et al., 2010), but there is no evidence of extracellularly surface adsorption of organic species like MMAA(V)/DMAA(V), and AB by *Dunaliella teritolecta* and other species of algae (Duncan et al., 2010). Wang et al. (2016b) showed that the metabolism of As(V) by *D. salina* was closely related with the adsorbed and absorbed As fractions in algae. The higher extracellular bound As in *D. salina* was associated with higher levels of As(V) exposure (112 and 1120 mg L^{-1}), while more As absorption was attributed to lower As(V) exposure (1.12 and 11.2 mg L^{-1}) within 24 hours of culture. Their results also indicated the inhibition of As(V) adsorption upon higher external P concentration. The similar finding was also in line with the other study (Levy et al., 2005). The electrostatic attraction, ion exchange, coordination, complexation, chelation, and micro-precipitation are the critical processes driven for passive surface adsorption (Dönmez et al., 1999). The mechanism involved with surface adsorption of As in algae species depends on the algae-specific surface properties of the cell wall.

2.6 Uptake and Transport Pathways of Arsenic

The metalloid As has no metabolic or nutritional function within the cells and there are no known dedicated transport and uptake systems for As. However, As enter into cells or crosses the cell membrane by using existing transporter systems that share similar molecular structure (Pantoja Munoz, 2014). It seems that the progress in the understanding of As transporters have been revealed their critical role in As metabolism in plants, including algae over the past decades (Zhao et al., 2010). Different biota, including algae, uses a variety of uptake routes of contaminants under diverse aqueous phases. The cellular absorption of toxic ions is generally occurring through selective ion channels or carriers. Algae uses different pathways to transport and uptake of inorganic and organic As species.

2.6.1 Transport of Inorganic Arsenic Species

It has been well established that phosphate transporting system (phosphate-specific transport, PST; phosphate inorganic transport, PIT) act as a primary channel/route for competitive absorption of pentavalent As(V) because of their analogous physicochemical characters along with similar internalization mechanisms (Guo et al., 2011; Murota et al., 2012). It was also reported that the activated PIT system in *Microcystis aeruginosa* could not differentiate between phosphate and As(V) under phosphate deprived condition. On the other hand, PST becomes activated and specific toward inorganic phosphate (Pi) under phosphate

excess conditions and allows the alga to selectively uptake phosphate from the growth medium (Guo et al., 2011). Phosphate independent uptake of As(V) has been reported by other researchers, indicating the insight of different transporting mechanism of As(V). In many marine algae, As(V) does not compete with phosphate for uptake, and there is more than one mechanism for As(V) uptake (Andreae and Klumpp, 1979).

The aquaporin membrane channels are considered as most relevant transport channels for assisting in the transport of water and neutral molecules and/or transport of metalloids, including As(III) by the plants (Li et al., 2014; Mukhopadhyay et al., 2014). The transportation of trivalent As(III) has been extensively studied under diverse plant species including rice roots, and other macrophytes and significant progress have been made for the identification and characterization of proteins involved with the As(III) uptake. As(III) exist as neutral anions in the environment; and it has been believed that As(III) could enter into the plant cells via aquaporin channels. These channels are recognized as a facilitator of passive transportation of uncharged soluble species into the root cells from surrounding media (Zhao et al., 2009). Based on the pore structure and substrate-specific selectivity and permeation function, aquaporin channels are dedicated to transporting (1) water, glycerol, and lactic acids, (2) urea and boric acid, and (3) silicic acids (Mitani et al., 2008).

As(III) uptake by the different microalgae species were studied by many researchers (Cullen et al., 1994a; Zhang et al., 2014; Levy et al., 2005); and they reported that the aquaglyceroporins channels could take part in the movement of As(III) into the algal cells from the surrounding media. As(III) is entered inside the cells via aquaglyceroporin channels and glucose permeases in *E. coli*, yeast and mammalian cells (Liu et al., 2004; Meng et al., 2004). However, there is evidence in some plants where As(III) is transported by aquaporin channels, which selectively carries water inside the cells particularly in rice and other plants growing under anaerobic flooded environments, where arsenite is predominant (Zhao et al., 2009). It has also been suggested that since phosphate channels are dedicated to As(V) transport, aquaporin channels are described for As(III) and passive diffusion is involved for DMAA(V) absorption, all efficient and rapid processes (Sun, 2010).

2.6.2 Transport of Organic Arsenic Species

As(III) and/or As methylated derivatives are considered to transport through aquaporins which belonging to PIPs, TIPs, and all three functional NIP groups (NIP-I, NIP-II, NIP-III). Li et al. (2009) showed that OsLsi1, the aquaporin NIP2;1, is essential for the uptake of

undissociated methylated As including MMAA(V) and DMAA(V) by rice roots. The rice mutant lacking OsLsi1 channels showed to reduce the uptake capacity of MMAA(V) (80%) and DMAA(V) (50%), suggesting the transport of these As species by aquaporin channels (Li et al., 2009). Besides, Rahman et al. (2011) reported that the entry of MMAA(V) and DMAA(V) into the rice roots occurred via aquaporins, which were a similar route of entry as glycerol. It has been suggested that methylated forms of As in their independent oxidation state have same physicochemical similarities to silicic acid, or glycerol or antimonite and can be transported and absorbed via silicon (Si)/glycerol influx-efflux systems in plants as well as in algae.

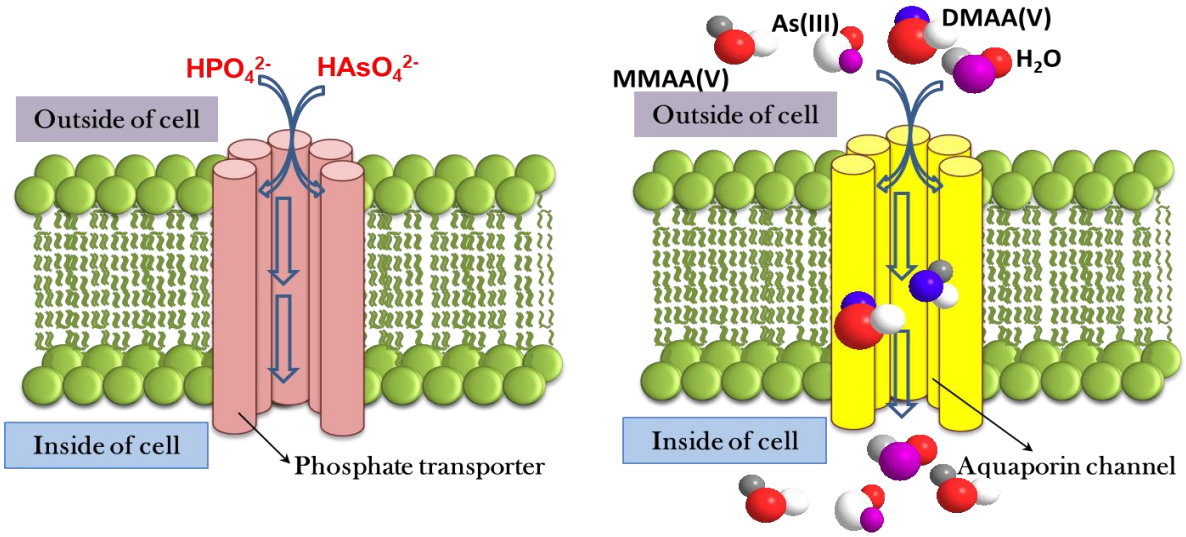

Figure 2.5: Schematic diagram showing the transport of different As species in algae via different transporters.

2.7 Metabolic Transformation Pathways of Arsenic

2.7.1 Arsenite Oxidation

Anderson et al. (1992) first purified and characterized the enzyme responsible for As(III) oxidation in *Alcaligenes faecalis* bacteria, namely arsenite oxidase, a molybdenum-containing hydroxylase. The rapid biological oxidation of As(III) in geothermal/hot spring waters are believed to be the activities of microbial activities, especially bacteria (Oremland and Stolz, 2003). For reasons of experimental inconvenience, the number of studies on As(III) exposure and its biotransformation in laboratory macroalgae culture system are relatively less, but a number of laboratory studies reported the transformation potential of As(III) to As(V) in microalgae which are reviewed by Chen and Zhang (2019). The species which have oxidation potential of As(III) in freshwater include *Chlorella* sp., *Chlorella vulgaris*, *Chlamydomonas*

reinhardtii, *Cyanidioschyzon* sp., *Leptolyngbya* sp., *Microcystis* sp., *Microcystis aeruginosa*, *Monoraphidium arcuatum*, *Nostoc* sp., *Scenedesmus* sp., *Spirulina platensis*, *Synechocystis* sp., *Westiellopsis* sp.) and in seawater include *Dunaliella salina*, *Ostreococcus tauri*. Though algae can accumulate As(III), it is still unclear if As(III) in laboratory cultures is taken up by algal cells as As(III) or if As(III) is oxidized to As(V) in cultures before uptake by cells (Sanders and Windom, 1980). Yin et al. (2012) observed that As is taken up by Synechocystis in the form of As(V) followed by surface oxidation of As(III) (approximately 83% of 2.67 µM As(III)), and then reduced intracellularly, and consequently release from the as a detoxification strategy (Zhang et al., 2014). The As biotransformation by three macroalgae species were investigated for the bioavailability and transformation of As in two marine fish (Zhang et al., 2016). Their results showed that the As(V) contents in *Gracilaria lemaneiformis*, *Gracilaria gigas*, and *Ulva lactuca* increased from 0.61 ± 0.04 to 28.3 ± 1.69, 1.29 ± 0.03 to 76.9 ± 1.97, and 1.20 ± 0.06 to 15.8 ± 1.48 µg g^{-1}, respectively from 1000 µg L^{-1} (III) exposure solution within 4 to 7 days. This results also indicated the oxidation potential of As(III) by the macroalgae.

It has been reported that the excretion of fatty acids from some microorganisms has shown to oxidize As(III) in the surrounding media containing As(III) (Frankenberger Jr, 2001). The association of fungus, bacteria, and epiphytes with microalgae and macroalgae are common in both in situ and in vivo conditions. Therefore, it has been believed that the oxidation of As(III) might be the presence of the bacteria or epiphytes associated with the algae (Ye et al., 2012). Traditionally, it has been speculated that As(III) oxidation is an intracellular metabolic process of algal following uptake of As(III). However, some researchers considered that oxidation of As(III) occurs outside of the cytoplasm compartment and the process might be due to the presence of extracellular enzymes like carbonic anhydrase and extracellular phosphatase (Qin et al., 2009). The surface oxidation of As(III) either in the periplasm or near the outer membrane of cyanobacteria *Synechocystis* sp. was also reported by Zhang et al. (2014). Their results suggested that the presence of 2,4-dinitrophenol, a respiration inhibitor and an uncoupling agent for oxidative phosphorylation, did not show any significant influence As(III) oxidation of *Synechocystis* sp. either under P-limited or P-added conditions within 24 hr of incubation. The presence of arsenite oxidase in algae remain unclear and not identified, and in the case of *Synechocystis* sp. has yet to be identified or does not exist (Zhang et al., 2014). Nevertheless, it should not be considered As(III) oxidation as a common process in photosynthetic organisms (Rahman and Hassler, 2014). Therefore, it has been recommended

to perform genetic and molecular studies for the clarification and understanding the As(III) oxidation mechanism in microalgae and macroalgae (Chen and Zhang, 2019). The enzyme arsenite oxidase is a periplasmic protein containing two heterologous subunits found outside the inner cell membrane which mediates the As(III) oxidation as a survival mechanism upon long term evolution of cyanobacteria (Zargar et al., 2010; Zhang et al., 2014). The structure of arsenite oxidase has been identified till date in different microorganisms include AioA, AioB, AioS, and AioR and the various isolated arsenite-oxidizing genes in bacteria might be helpful in genetic engineering in microalgae as well as remediation of As-containing water (Kumari and Jagadevan, 2016; Bahar et al., 2013).

2.7.2 Arsenate Reduction

The reduction of As(V) to As(III) is considered as a detoxification potential for many algae species even though the toxicity of As(III) is generally higher than As(V). The As(V) reducing microalgae species are reviewed by (Wang et al., 2015) and Chen and Zhang (2019) including *Chlorella vulgaris*, *Chlorella* sp., *Chlamydomonas reinhardtii*, *Chlorella aciculare*, *Cyanidioschyzon* sp., *Microcystis aeruginosa*, *M. arcuatum*, *Nostoc* sp., *Phormidium* sp., *S. obliquus*, *Synechocystis* sp. (freshwater inhabiting), and *Chlorella salina*, *D. salina*, *Dunaliella* sp., and *O. tauri* (seawater inhabiting). The macroalgae species also showed to reduce As(V) in similar ways as in microalgae and the reported species include *Fucus serratus*, *G. lemaneiformis*, *G. gigas*, *U. pinnatifida*, *S. patens*, *S. horneri*, *P. yezoensis* and *U. lactuca* (Mamun et al., 2019a; Granchinho et al., 2004; Zhang et al., 2016; Granchinho, 2000). There are some marine algae species which have relatively lower reduction as well as methylation capacity including *Hizika fusiforme*, *Laminaria* sp. and *Dunaliella tertiolecta* (Duncan et al., 2010; Ma et al., 2018; Kohlmeyer et al., 2002). The reduction is either mediated by arsenate reductase or by non-enzymatic reaction with glutathione. It has been conceived that once As(III) is formed inside the cells of algae, it can undergoes different ways to reduce the toxicity which include: rapid release from cells using As(III)-$(GSH)_3$ compounds, binding with thiolic compounds as As(III)-PCs, sequestration into vacuole, formation and excretion of MMAA(V)/DMAA(V), and even the volatile TMAs following methylation (Yin et al., 2011a; Qin et al., 2009; Xue et al., 2017). Therefore, the reduction of As(V) to As(III) is recognized as an essential and desired to the algae for two reasons: (i) decreasing the free As(III) ion in cells, and (ii) reducing concentration and competition of As(V) that could substitute P in photophosphorylation and oxidative phosphorylation (Knauer and Hemond, 2000; Ye et al., 2012; Zhu et al., 2014).

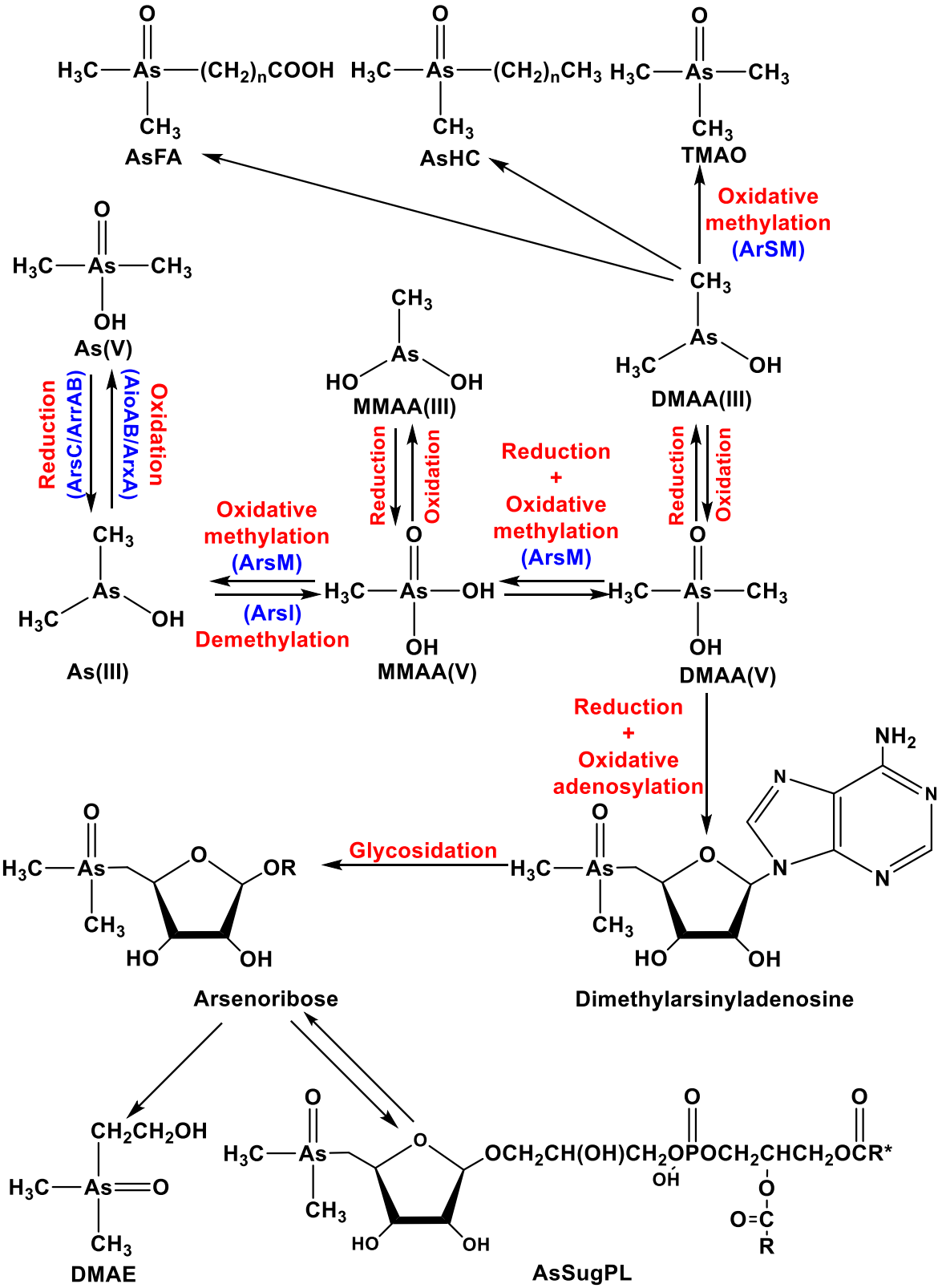

Figure 2.6: Schematic diagram showing the overall pathways of As biotransformation in algae [Redrawn and adapted from Chen and Zhang (2019)].

The "ars" gene is related to arsenate reductase which has been identified from extensive studies with microorganisms, and it plays a key role in As resistance. The bacterial resistance to As(V) has been confirmed by identifying cytoplasmic arsenate reductase (ArsC) genes (arsR, arsB, and arsC) in an "ars" operon, As(III) efflux pump (ArsB), transcriptional regulator (ArsR) and chaperone protein (ArsD) (Rosen, 2002; Kumari and Jagadevan, 2016). The microalgae *Synechocystis* related arsC genes have identified in the form of arsBHC operon (López Maury et al., 2003). This research group also identified two gene coding (arsI1 and arsI2) for As(V) reductase in *Synechocystis* sp. strain PCC 6803 whose products were biochemically characterized and known as ArsC and ArsI (Lopez-Maury et al., 2009). As(V) reductase genes (CrACR2s) in *C. reinhardtii* were cloned and expressed in disrupted arsC of *Escherichia coli*. The gene products were showed to reduce As(V) to As(III) in vivo (Yin et al., 2011b). It has been reported that the presence of As resistance genes (ars operon), arsenite efflux genes asr1102 (homologue of arsB) and alr1097 (transcriptional regulator arsR) in *Anabaena* sp. PCC7120 are necessary for their survival under As stress condition (Pandey et al., 2012). The unregulated gene expression of alr1097 and alr1105 encoded in As(III) efflux protein; arsB and As(V) reductase; arsC, respectively were also observed in *Nostoc* sp. under As stress (Pandey et al., 2013).

2.7.3 Methylation and Demethylation of Arsenic

Methylation is the addition of one or more methyl group (-CH_3) onto a chemical species. Inorganic As may be methylated into a number of methylated derivatives like MMAA(III), DMAA(III), trimethylarsine, trimethylarsine oxide, and other methyl species. There is limited evidence showing abiotic, i.e., chemical methylation of As; hence it has been suggested that the methylation of As is entirely or almost entirely dependent on biotic involvement (Frankenberger Jr, 2001; Frankenberger Jr and Arshad, 2002). This important As metabolism exists under a diverse group of organisms, including bacteria, archaea, fungi, algae, plants, animals, and humans (Ye et al., 2012).

The produced pentavalent arsenicals (MMAA(V) and DMAA(V)) in the methylation process are less toxic than IAs. However, the intermediate arsenicals (MMAA(III) and DMAA(III)) are more toxic compared to inorganic As and this step encompasses doubt over whether As biomethylation is a detoxification mechanism or not (Styblo et al., 2000; Thomas et al., 2007). However, MMAA(III) and DMAA(III) are unstable As species and usually under the detection limits in organisms and/or media suggesting As methylation is indeed a detoxification process. It was also evident that TMAA(III) is the end product of methylation

having little or almost no toxicity (Cullen, 2005). Methylation of As plays a critical role in algae that recognized as a detoxification mechanism (Hughes, 2009; Rahman and Hassler, 2014; Wang et al., 2015). It was evidenced that the presence of ArsM in *Escherichia coli* enhanced As(III) resistance (Qin et al., 2006; Kuramata et al., 2015; Ye et al., 2014; Zhang et al., 2015), while the absence/deletion of ArsM in *Pseudomonas alcaligenes* reduce the resistance to As(III) (Zhang et al., 2015). These studies suggested the detoxification mechanism of microorganisms through methylation. Different species of microalgae and macroalgae contained methylated As compounds in their cells as well as their excretion in culture media has been evidenced upon As(V)/As(III) exposure in the laboratory condition, or even in natural seawater suggesting the methylation is the key process involved with the algae. However, different organisms showed different efficiency of methylation and volatilization, which largely depend on the variation in the ArsM enzyme (Tang et al., 2016).

Though the exact pathway of As methylation is controversial, the Challenger mechanism is the first and most accepted pathways of As methylation in organisms (Challenger, 1945). According to this scheme, inorganic As transformed into methylated As via an alternating reductive and/or oxidative additions of methyl groups that lead to the formation of methylated organoarsenicals. Though the reductive methylation step is enzymatically catalyzed, GSH and lipoic acid have been found to act as reducing agents. On the other hand, S-adenosylmethionine (SAM) donate methyl in oxidative methylation step and converted to S-adenosylhomocysteine (Bentley and Chasteen, 2002). Other schemes of methylation have been proposed by Hayakawa et al. (2005) and (Naranmandura et al., 2006), in which As-glutathione complexes act as a substrate for the sequential methyl group transfer to the arsenicals during simultaneous reductive methylation rather than stepwise oxidative methylation. These models showed the direct methylation of As(III) to MMAA(III)/DMAA(III) and then oxidized to DMAA(V) as an end product of metabolism rather than their intermediate metabolites (Zhang et al., 2013b).

Recently, it has been suggested that As(III)–GSH complexes are the substrate for arsenite methyltransferase enzyme, and MMAA(III) or DMAA(III) conjugates along with GSH are the intermediates, which can either be dissociated from the enzyme or be hydrolyzed followed by oxidation with the formation of MMAA(V) or DMAA(V). The structure of ArsM binding domain as well as the relation between As with SAM binding sites have been described by Ajees et al. (2012) and supported such methylation pathways.

The overall methylation process involves the following step:

- Reduction: As(V) + thiol → As(III)
- Oxidative methylation: As(III) + SAM → monomethylarsonate (MMAA(V))
- Reduction: MMAA(V) + thiol → MMAA(III)
- Oxidative methylation: MMAA(III) + SAM → dimethylarsinate (DMAA(V))
- Reduction: DMAA(V) + thiol → DMAA(III)

The molecular mechanism of As methylation has been studied intensively over the past few years. It has been accepted that SAM in an organism act as a methyl donor and the presence of enzymes like ArsM, AS3MT has been identified in the pathways of methylation in different mammalian studies (Lin et al., 2002; Thomas et al., 2007). The genes account for the As methylation are lacking in higher plants (Ye et al., 2012), and this feature makes the higher plants incapable of methylating As (Lomax et al., 2012; Zhao et al., 2013). The microbes associated ArsM genes encoded arsenite S-adenosylmethionine methyltransferase (ArsM) that leads to catalyze the methylation process (Qin et al., 2006). ArsM genes CmarsM7 and CmarsM8 in thermophilic microalgae are responsible for promoting the sequential addition of methyl group to As(III) forming mono-, di-, and trimethyl arsenicals, also with the volatile trimethylarsine as the end product (Qin et al., 2006; Qin et al., 2009). The comparison of As biotransformation between wild type *Synechocystis* sp. and ΔarsM mutant strain were examined after exposure to As(V). The results revealed that ΔarsM mutant strain was unable to methylate As, indicating the necessary requirement of ArsM enzyme for methylation (Xue et al., 2014). ArsM (SpArsM) was also identified in *S. platensis*, which could show methylation activity in an in vitro assay (Guo et al., 2016).

Microorganisms played a significant role in demethylation process in which one or more methyl groups from organoarsenicals compound is removed, and ultimately form the inorganic As species. Though MMAA(V) and DMAA(V) are very stable and dissolved As species in water, some bacteria can demethylate them and other methyl As species into inorganic As (Santosa et al., 1996; Cullen and Reimer, 1989; Frankenberger Jr and Arshad, 2002). It was reported that arsenobetaine could rapidly demethylate to form dimethylarsenoylacetate and DMAA(V) in seawater (Khokiattiwong et al., 2001). The laboratory incubated bacteria *Mycobacterium neoaurum* showed to demethylate MMAA(III) and MMAA(V) into inorganic As. Results of their study indicated that at least some MMAA(V)

could reductively demethylate to inorganic As(III) (Lehr et al., 2003). The As-induced enzyme, namely ArsI in Nostoc sp. (NsArsI), has been identified by Yan et al. (2015), which can catalyze MMAA(III) demethylation. The similar demethylation of MMAA(III) was also reported in *Nostoc* sp. (Xue et al., 2017).

2.7.4 Formation of Arsenosugars

The biotransformation of As by marine organisms, including algae results in a variety of organoarsinicals. The most common intracellular As compounds found in marine animals and algae are arsenosugars. Samples originated from marine habitat naturally contain a relatively higher concentration of arsenosugar than their freshwater counterparts and terrestrial organisms (Niegel and Matysik, 2010). However, their relative amounts varied according to the class and order among algal taxonomy. Different side chains can attach to the main structure of arsenosugars and recently more 20 different types of arsenosugars have been identified in algae. Arsenosugar-Gly, arsenosugar-PO_4, arsenosugar-SO_3, and arsenosugar-SO_4 are the most common types arsenosugar found in marine organisms, but not much variation in algae (Cao et al., 2019; Francesconi and Edmonds, 1996). Marine algae are the primary producer of arsenosugars, which contribute up to above 85% of the soluble As species in most macroalgae species (Rose et al., 2007; Kalia and Khambholja, 2015). It was also speculated that absorbed inorganic As remains as much 1% of the total As in the macroalgae (Raber et al., 2000).

Several schemes for the biotransformation pathways of As are available in the literature, but the underlying pathways leading to arsenosugar biosynthesis in algae involve complex mechanisms and most of them are still not fully elucidated or poorly understood. It has been assumed that arsenosugars are the end metabolic products of absorbed As(V) from seawater by the algae species and considered as As detoxification metabolism. The possible pathways of arsenosugar synthesis include (i) algae uptake As(V) from seawater which was then sequentially reduced and methylated to form DMAA(V) in presence of methyl donor S-adenosylmethionine, and (ii) DMAA(V) was further transformed to DMAA(III) and the addition of the adenosyl group from SAM produced a glycosylation leading to the formation of dimethylarsinoylribosides (Murray et al., 2003; Edmonds and Francesconi, 2003). The glycophospholipids have been suggested to play an intermediary role in the pathway for the conversion of As(V) to arsenosugars in macroalgae (Geiszinger et al., 2001). It has also been suggested that lipid-soluble As species like arsenosugar-phospholipids synthesized by macroalgae, and arsenosugars are considered as the degradation products of the excess of arsenosugar-phospholipids. It has now well established that marine algae synthesize

arsenosugars by direct absorption of inorganic As from seawater (Tukai et al., 2002; Feldmann and Krupp, 2011). The formation of arsenosugars is connected with the environmental relevant levels of P in seawater, whereas arsenosugar-phospholipids are related with the phosphate-rich environments (García-Salgado et al., 2012b).

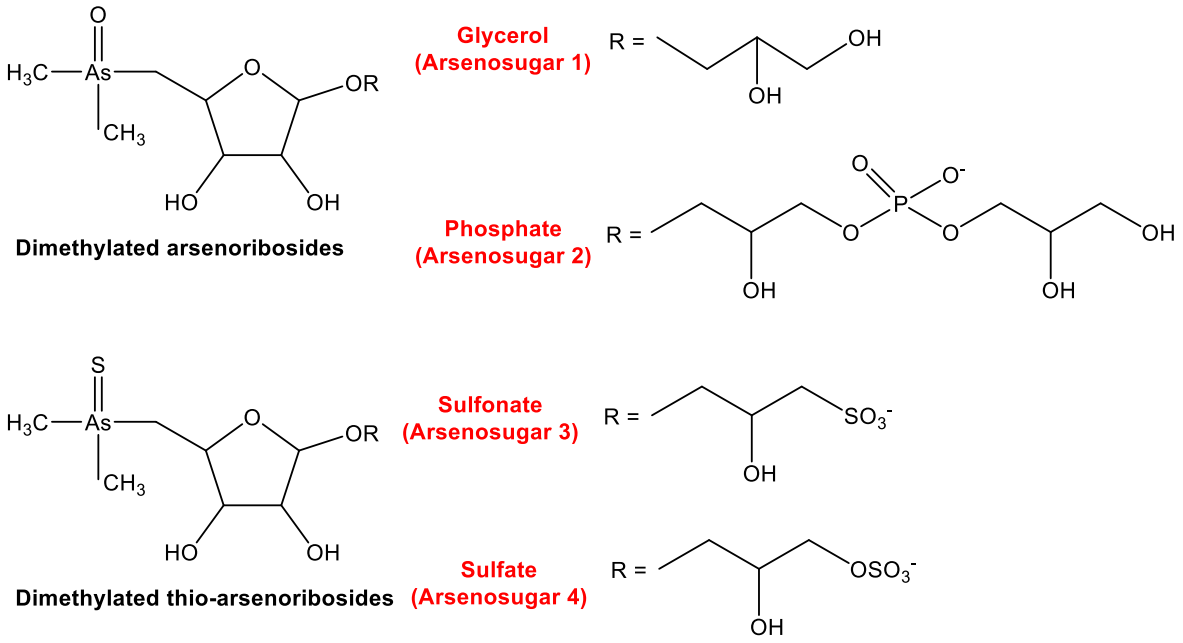

Figure 2.7: Chemical structures of common arsenosugars in the marine macroalgae.

2.7.5 Formation of Arsenolipids

Arsenolipids are lipophilic and considered as lipid-soluble organoarsenic compounds occurring mainly in marine organisms, especially microalgae, and macroalgae (Witt et al., 2017). According to chemical structure, As-containing hydrocarbons (AsHCs), As-containing fatty acids (AsFAs), As-containing phospholipids (AsPLs), and As-containing phosphatidylcholines (AsPCs) are the major groups of arsenolipids identified in marine organisms (Cao et al., 2019).

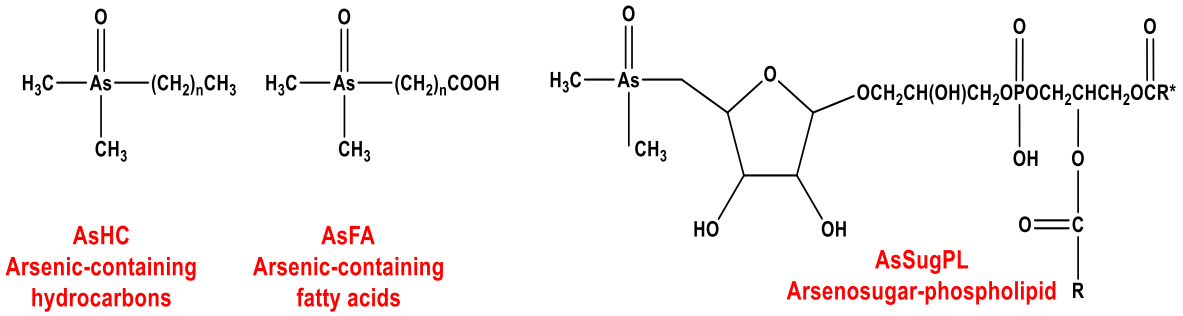

Figure 2.8: Chemical structures of common arsenolipids in the marine animals.

It appears that the distribution, concentration, and patterns of arsenolipids in various algae are species specific. AsHCs, AsFAs, and AsPLs have been identified in the marine macroalgae samples (García-Salgado et al., 2012b; Morita and Shibata, 1990). Kombu also contained AsHCs and AsPLs (Yu et al., 2018). The difference in arsenolipid patterns in algae is considered as their difference in habitat and genetic morphology. The biotransformation pathways of arsenolipid formation in algae or fish have not been proven clearly. The arsenosugar phospholipids in *Synechocystis* sp. (AsSugPL983 and AsSugPL985) and *Nostoc* sp. (AsSugPL982 and AsSugPL984) were identified. It was proposed that PO_4^--ribosides is the precursor of arsenosugar phospholipids formation in a cyanobacterium (Xue et al., 2014; Xue et al., 2017).

2.7.6 Formation of Other Organic Arsenic Species

The formation of more complex organoarsenicals through methylation and other transformation processes are recognized as a detoxification process of As in the marine ecosystem (Francesconi, 2010). Arsenobetaine, a water-soluble As species, probably the end metabolite of As metabolism in marine food web, unfortunately absent in microalgae, but has been identified in marine animals and also recently in a number of macroalgae species (*H. fusiforme*, *L. japonica*, *S. fulvellum*, *U. pinnatifida*, *P. haitanensis*, and *P. tenera*) as well as in algal extracts as a minor As species (Chen and Zhang, 2019). However, the predominant form of As in marine macroalgae is organic arsenosugars (Cullen and Reimer, 1989; Edmonds and Francesconi, 2003). Marine animals contain arsenobetaine (AB) and its precursor arsenocholine (AC) in water-soluble fractions of cells accounting above 80 % of the major As species. The other organoarsenicals which are occurred at relatively low concentration and proportion in decomposing macroalgae and marine animals include dimethylarsinoylethanol (DMAE), dimethylarsinoylacetate (DMAA), thioarsenoribosides, thio-dimethylarsinoylethanol (thio-DMAE), dimethylarsenopropionate (DMAP), and dimethylarsenopropionate (TMAP).

The formation of AB in macroalgae is uncertain as to whether the algae itself or its associated organisms like epiphytes, animals, and fungi are taken part in the source and/or synthesize of AB (Maher et al., 2009). Though macroalgae contain minor amounts of AB/AC, there is no report about the presence of AB and AC in microalgae (Duncan et al., 2015). It would hypothesize that both groups of algae, microalgae and macroalgae would produce AB because of their cellular similarities. Unfortunately, the axenic culture of microalgae (no epiphytic organisms) has shown no AB in their cells. The epiphytic organism are symbiotically

living with algae can produce AB and AC, and it is hardly or not yet be possible to eliminate the epiphytic association with macroalgae indicating the presence of macroalgal AB or AC might be related with epiphytes (Chen and Zhang, 2019).

2.8 Complexation and Accumulation Mechanism of Arsenic

Glutathione (GSH) and its derivatives such as phytochelatins (PC's) containing non-protein thiolic groups are the most important classes of metal-chelating polypeptides synthesized in microalgae, related eukaryotic photosynthetic organisms, fungi, and some nematodes to resist the As toxicity (Vatamaniuk et al., 2001; Perales-Vela et al., 2006). These compounds play a major role in sequestering the trivalent As species (As(III)) as organometallic compounds and can control the suitable cytoplasmic concentration of metalloid ions (Schmidt et al., 2007; Cobbett and Goldsbrough, 2002). Another important function of GSH and its derivatives is to alleviate the oxidative stress in cells as an antioxidative mechanism through the enzymatic or non-enzymatic way. The following equation shows the sequential reduction of inorganic As(V) and complexation with GSH:

$$\text{Inorganic As(V)} + 2\ \text{GSH} \rightarrow H_3AsO_3 + 3\ \text{GSH} \rightarrow \text{As(III)}(GS)_3$$

Once As(III) gets inside the cells, GSH can binds/chelates with As(III) and generates the formation of PC's. The multiple cysteine residues in PC's might chelate As(III) ions more strongly compared to GSH which contain single cysteine residue. The oxidized PC'n and reduced PC'n may act as redox buffer systems in a similar way of GSH/GSSG. The NADP/NADPH redox pair and glutaredoxin may also act as cofactors and reducing enzymes, respectively which might prevent the formation of reactive oxygen species (ROS) and cell damage due to oxidative stress (Hirata et al., 2005). Following uptake of As(V) in the cells, it can bind with several molecules and replace phosphate molecules and induce stress by reflecting elevated levels of free thiols and oxidative stress. Since the affinity of GSH to As(V) is much lower than that of As(III), no chelation or binding is reported with As(V).

2.8.1 Role of Glutathione

Frederick Gowland Hopkins discovered the compound glutathione (GSH) in 1922 (Masella and Mazza, 2009). In most eukaryotic cells, GSH and its homologues hGSH (where the C-terminal Gly is substituted by Ala, Ser, Gln or Glu or is absent) are a group of tripeptide thiols and present in high concentrations (W. Hedley and Chow, 1994). A functional N-terminal (γ-Glu), a central cysteine residue (Cys), and a variable C-terminal amino acid make up the GSH (Grill et al., 2001; Karp, 2009). Cysteine can form a disulfide linkage with another

cysteine residue in the GSH and facilitate a readily reversible covalent bond in vivo (GSSG: Glutathione [oxidized]; GSH glutathione [reduced]) (Masella and Mazza, 2009; Karp, 2009). GSH and GSSG acts as an intracellular reducing agents and responsible for different cellular functions including storage and transport of reduced sulphur, regulation of sulphur nutrition, compensation of oxidative stress, chemical anti-oxidant and co-substrate in enzymatic reactions, redox regulation and buffering, regulation of enzyme activity, mRNA translation and gene transcription, modification and transport of hormones and detoxification and transport of xenobiotics or heavy metals (Deneke, 2001).

Overall, GSH showed to play threefold roles in organisms which involves: (i) reduction of As(V) to As(III) and keeping the number of ROS at bay, (ii) decreasing the cellular bioavailability of As(III) due to the formation $As(GS)_3$ complexes with the help of free –SH group of the cysteine residue in the molecule of GSH, and (iii) act as a substrate in the PC synthase-mediated phytochelatin synthesis (Zenk, 1996). In an experiment with *C. vulgaris*, Jiang et al. (2011) detected the presence of GSH in all algal cells under 0.8 to 6.5 mg L^{-1} As(V) containing media, whereas PC's and their complexes with As were absent. Their results suggest the prominent role of GSH than PCs in the detoxification of As(V). Yamaoka et al. (1999) found that when microalgae *D. salina* cultured with an inhibitor of c-glutamylcysteine synthetase (buthionine sulfoxisamine, BSO) and 100 mg L^{-1} As(V), the algae accumulated eight times less As compared to BSO free medium which indicates the role of GSH on the As accumulation.

2.8.2 Role of Phytochelatins

PC's are found in microalgae, eukaryotic photosynthetic organisms, some nematodes, and fungi as organo-metallic complexes (Perales-Vela et al., 2006). PC's are formed in plants in the presence of enzyme phytochelatin synthase (PCS) and able to reduce metal toxicity by chelation and/or complexation with heavy metal(loids) including As (Wood et al., 2011). A number of metal ions ($Cd^{2+} > Hg^{2+} > As^{3+} > AsO_2^- > Cu^{2+} > Zn^{2+} > Pb^+ > AsO_4^{3-} > Mg^{2+} > Ni^{2+} > SeO_4^{2-}$) can induce the synthesis of PC (Sooksa-Nguan et al., 2009; Simmons et al., 2009). PCs form more stable complexes with As(III) compared to GSH because PC contain numerous cysteine residues in their molecule (Raab et al., 2004; Kitchin and Wallace, 2006). It was evidenced that As(III) and As(V) stress showed an influence on increasing the synthesis of PCs in the marine phytoplankton *Dunaliella salina* (Wang et al., 2017a).

2.8.3 Storage and Vacuole Compartmentalization of Arsenic

PC's are synthesized in the cytosol, and their complexes with metal(loids) are believed to be compartmentalized into vacuoles. The organo-metalloids compounds can also be partitioned inside vacuole and responsible for controlling the cytoplasmic concentration balance, and hence defending its toxicity to the cells (Cobbett and Goldsbrough, 2002). Since the As(III)-PC's complexes are more stable in the vacuole under suitable acidic condition, the chelation between As and PC's are the necessary step for complete As detoxification (Park et al., 2012). It has long been suggested that As(III)-PC complexes transport As(III) from the cytosol into the vacuole for storage and the hypothesis was eventually supported by the identification of the two vacuolar transporters AtABCC1 and AtABCC2 in *Arabidopsis thaliana* (Song et al., 2010).

2.9 Efflux or Excretion of Arsenic Species

The efflux systems are present in nearly every organisms, and it naturally evolved to get rid cells from toxic metalloids, especially As (Zhao et al., 2009). The biological transformation and excretion of As considered as an adaptive response of the organisms to accumulated As and intracellular content might being balanced with its discharge following biotransformation. Algae have shown extrusion of As in the aquatic habitats under an elevated level of As in field and laboratory condition indicating the As biotransformation and detoxification of algae (Wang et al., 2014; Granchinho et al., 2004; Mamun et al., 2019a).

Algae can regulate the intracellular As concentration by excretion of metabolites As shortly after inoculation of As(V) in the laboratory algal culture (Cullen et al., 1994a). The amount and proportion of As excretion is related to different factors such as algal species, the concentration of As, duration of As exposure, physiological and growth condition of the algae etc. Foster et al. (2008) showed that DMAA(V) in the water-soluble extract in *D. tertiolecta* was lower than *P. tricornutum*. Their result indicated algae-specific ability to metabolize As and a difference in excretion leading to less DMAA(V) content in *D. tertiolecta*. The uptake-metabolism-excretion model of As(V) in freshwater algae proposed by Hellweger et al. (2003), and suggested that the reduction and methylation occurred at a faster and slower rate inside the cell, respectively leading to either As(III) build up in the cell or rapid release from cells under non-P-limited (luxury uptake) medium during the exponential growth phase. Their results also depicted that the algae uptake As(V), reduced it to As(III), and As(III) was further methylated to DMAA(V) inside the algae, consequently leading to DMAA(V) excretion.

The undesirable growth of algae such as low nutrient concentration, high inorganic As exposure concentration and dark condition facilitate the readily excretion of both As(V) and As(III) and their (MMAA(V), DMAA(V) and TMAO(V)) (Suhendrayatna et al., 1999; Maeda et al., 1992b; Ohki et al., 1999). In many studies, DMAA(V) is the most common excreted organic metabolites from the algal cells, while MMAA(V) and other organic As species are hardly found in the culture medium. There was an instance of the non-detectable or minimum amount of excreted MMMA(V) in the macroalgal culture when treated with As(V) (Mamun et al., 2019c; Granchinho et al., 2004). MMAA(V) is the least transformed metabolized of As species and found to release less frequently from the cells as a free intermediate in unicellular marine algae (Cullen et al., 1994a). It has been suggested that MMAA(V) would not be lost from the cells due to its lower diffusion coefficient than DMAA(V), and therefore the cells would inevitability metabolize the MMAA(V) to DMAA(V) for excretion into the growth medium which leads to a lower or no excretion of MMAA(V) (Cullen et al., 1994a; Cullen et al., 1994b; Granchinho et al., 2001; Hellweger et al., 2003). However, the excretion of trivalent methylated species MMAA(III) was reported by Hasegawa et al. (2001) in *C. aciculare* culture and suggested that MMAA(III) is more susceptible to oxidation than As(III).

The liberation or release of As(III) from the cells is one of the most essential detoxification strategies adopted by a variety of organisms, including algae (Rahman and Hassler, 2014). It has been reported that carrier proteins like ArsB, ArsAB, and Acr3p involved in trivalent As (As(III)) efflux from the cells (Zhao et al., 2009). Also, genes asr 1102 and alr 1097 for As(III) efflux has been identified in *Anabaena* sp. PCC 7120 (Pandey et al., 2012). An ars operon encoding As(III) efflux protein was found to embed on the ars genes of microalgae *Synechocystis* sp. strain PCC 6803 (López Maury et al., 2003). However, methylated As species (MMAA(V)/DMAA(V)) efflux genes have not been discovered but warrant further investigation. Bienert et al. (2008) found that aquaporin channels like AtNIP5;1, AtNIP6;1, OsNIP2;1 and LjNIP5;1, and other transporters in the aquaporin family can efflux As(III). Zhao et al. (2010) observed only 15–20% of the total membrane efflux occurred via OsLsi1 channel and suggested that other As(III) exporters might also exist. There are little is known about the underlying mechanisms of pentavalent inorganic As (As[V]) efflux. It has been believed that some anion channels might be involved in As(V) efflux that was similar to phosphate excretion (Mimura, 1999; Zhao et al., 2009). Xu et al. (2007) found that the efflux of As(V) was significantly enhanced in the presence of carbonyl cyanide, an inhibitor of oxidative phosphorylation, whereas the presence of carbonyl cyanide

could inhibit the As(III) efflux. These results indicate the arsenite efflux is an energy-dependent and active process because ATP inhibitor did not produce any change in the efflux process. The efflux of radioactive As(V) in bacteria was correlated with the ATP content in the cell but not with the membrane potential (Bröer et al., 1993). Cullen et al. (1990) suggested that the methylated pentavalent forms can readily be passed in biological membranes and effluxed via a passive diffusion process.

2.10 Bioaccumulation of Arsenic

Bioaccumulation is defined as the uptake and removal of metals, which is metabolically controlled by living macroalgae. The contents of metals that are transferred onto and/or within the cellular membrane of algae depending on the age of the algae, physiological condition of the cells, and the availability of nutrients during growth as well as the environmental condition during absorption like pH, temperature, light intensity, salinity, and others. As bioaccumulation is distinct from the biosorption, which is defined as the uptake of As by dead biomass, which may be dried and/or chemically treated for this purpose (Volesky, 2007). The living biomass of algae employ the active and passive modes effectively for accumulating and metabolizing the toxic heavy metals, including As. That is why it has been suggested that the living biomasses are sometimes suitable and more efficient biosorbent choice than the dead biomass (Doshi et al., 2007).

2.11 Biosorption of Arsenic with Dead Algal Biomass

The primary focus of this work is the biotransformation of As by living biomass of macroalgae, and speciation of As in aqueous medium. However, some consideration must also be given to the mechanisms of heavy metal binding by non-living biomass of macroalgae in order to differentiate among the process of biotransformation, bioaccumulation, and biosorption.

Biosorption is a passive binding process by non-living biomass from an aqueous solution with the objective to remove heavy metals. The phenomenon of biosorption was observed in the early 1970s when the radioactive elements (also heavy metals) in the wastewater released from a nuclear power station were found to be concentrated by several algae (He and Chen, 2014). Now a day's algae-based biosorbent has much received much attention to the researchers. Biosorption includes ion-exchange, complexation, and coordination, and it involves the removal of heavy metals from an aqueous solution via a complicated passive binding to non-living biomass (Davis et al., 2003). Marine algal biomass

has developed as one of the most promising biosorbent types of heavy metals including As because of their rigid macro-structure, high uptake capacities, economically attractive, low cost, renewability as well as their ready abundance of the biomass in many parts of the world's oceans (Prasanna Kumar et al., 2007). The removal efficiency of biosorption process of As is entirely dependent on the biomass concentration, redox potential, pH of a solution, contact time, the temperature of As-contaminated solution. The macroalgae based sorption column is now available for the industrial application in heavy metal removal (Sivaprakash et al., 2010).

2.12 Methods for Speciation Analysis of Arsenic

Extraction, derivitization and/or separation, and detection are primarily three steps involved in most of all As speciation analysis for the environmental samples. As compounds and their species in water samples can be measured directly, whereas all solid samples are measured after proper extraction (Akter et al., 2005). However, micro XRD analysis like XANES and EXFAS provide an excellent opportunity to assess the As contents in solids samples directly. As extraction can affect both qualitative and quantitative results of As speciation, it is necessary to ensure two essential considerations during extraction: (i) no transformation of As species (ii) all As species should be extracted efficiently.

The ideal and universal chemical extraction is not always possible and currently unavailable (Yuan et al., 2005), and hence, a mixture of various extractants is essential to reach a complete extraction by using polar organic solvents or water (Francesconi and Kuehnelt, 2004; Goessler and Kuehnelt, 2001). The extraction techniques which are used in speciation analysis include solvent extraction, microwave-assisted extraction, and pressurized liquid extraction. Suitable and reliable separation of As compounds are not always possible because of variable nature and chemical properties of As species (Francesconi and Kuehnelt, 2004). That is why a combination of different separation steps commonly used to the speciation of As including hydride generation (HG), liquid chromatography (LC), gas chromatography (GC), and capillary electrophoresis (CE).

The analytical devices or most commonly available methods that determine As speciation require a separation step before detection. With the advancement of analytical instruments, a separation step is not necessary to investigate As species, e.g. XANES (X-ray absorption near-edge spectroscopy) and EXAFS (extended X-ray absorption fine structure (Bluemlein et al., 2009). Separation methods include HPLC, GC (Gas Chromatography), and CE (Capillary Electrophoresis) (Francesconi and Kuehnelt, 2004).

2.12.1 Hydride Generation

Hydride generation is most extensively used techniques for the As speciation analysis and can measure trace levels of As(III)/As(V), MMAA(V)/MMAA(III), DMAA(V)/DMAA(III) and TMAO(V) after conversion to their respective arsines (Goessler and Kuehnelt, 2001). Sample preparation and conversion of volatile arsine from all separated hydride generation active As species are the important steps in HG technique (Francesconi and Kuehnelt, 2004). At an acidic medium, the volatile arsine is quantified when As(V) treated with sodium borohydride ($NaBH_4$). On the other hand, most of the organoarsenic compounds do not produce volatile As containing products like AB or only with low efficiency (Urgast et al., 2010; Regmi et al., 2007). As the gaseous hydrides are only introduced into the detector, this technique can eliminate spectral and chemical interferences from the matrix that encountered in the detection system. The transportation of As in the gaseous phase to the detector is also more efficient compared to the liquid phase. As derivitization is most common in HG techniques and it can be used either as pre-column or post-column derivitization. In pre-column derivitization, volatile arsines are initially formed and cryogenically trapped and subsequently desorbed, and introduced to the detector (Gomez-Ariza et al., 2000). The first report on measuring trivalent methyl arsenicals (MMAA(III)/DMAA(III)) in natural waters by using CT-HG-AAS was reported by (Hasegawa et al., 1994). As species are also separated with the help of HPLC by using post-column derivitization method for the improvement of the limit of detection (Sloth et al., 2003). The following equations are adopted from Shraim (1999) which summarize the overall reduction and hydride (arsine) formation from different As species:

$R_nAs(O)(OH)_{3-n} + H^+ + BH_4^- \rightarrow R_nAs(OH)_{3-n} + H_2O + BH_3$ ……1

$R_nAs(OH)_{3-n} + (3-n)BH_4^- + (3-n)H^+ \rightarrow R_nAsH_{3-n} + (3-n)BH_3 + (3-n)H_2O$ ……..……..2

$BH_3 + 3H_2O \rightarrow H_3BO_3 + 3H_2$ ……3

Equation 1 and 2 expressed the reduction of As(V) to As(III) and arsines production, respectively.

It is possible to connect HG techniques to the various detection systems including AAS, AFS, ET-AAS, ICP-AES, ICP-MS and offers the improvement of sensitivity up to 100-fold over the commonly used liquid sample nebulization process (Goessler and Kuehnelt, 2001). Since it is possible to handle large volumes of sample, a reasonable detection limit can also be obtained by using HG. However, this method infers some drawbacks including (i) laborious, (ii) limited only for volatile arsine forming materials, (iii) the reaction conditions need to be

strictly controlled, and (iv) the presence of some interfering elements can reduce the efficiency of HG.

2.12.2 Liquid Chromatography

Liquid chromatography (LC) can determine both organic and inorganic As species and most frequently used and popular techniques in the field of As speciation of environmental matrices (Ammann, 2011; B'Hymer and Caruso, 2004). A mobile and stationary phase commonly employed in LC mass where samples and analytes are transported into the column via a mobile phase and As species are selectively retained on a stationary phase, and subsequently separated. High-Performance Liquid Chromatography (HPLC), Ion Exchange Chromatography (IEC), and Ion Interaction Chromatography (IIC) are the most common types of LC techniques. LC offers direct measurement of waters, extracts, and complex organic matrices, and there is no need to use the complicated derivitization step (Akter et al., 2005). Both HPLC and IEC based As speciation is based on the different dissociation constants of As species because As(III), As(V), MMAA(V), and DMAA(V) showed to form weak acid with significantly different pK_a values (Shraim, 1999; Akter et al., 2005).

In IEC, mobile phase column offers to transport the analyte where As species are competing for oppositely charged functional groups in the stationary phase and the separation of As species occurs by displacement of mobile phase ions. The detection of sub-nanogram levels of As species can be determined by using some of hyphenated IEC-ICP-MS. Kohlmeyer et al. (2002) reported 17 different types of organic and inorganic arsenicals in marine biota using IEC-ICP-MS. Reversed-phase liquid chromatography (RPLC) consists of a polar mobile phase and a non-polar stationary phase; and the separation of As species are achieved through an aqueous mobile phase containing different ratios of organic modifiers like methanol, ethanol, acetonitrile, or THF which increase the selectivity among the species (Hu et al., 2019).

In ion pair RPLC, ionic or ionizable compounds are separation by using an ion-pairing reagent having a polar head and a non-polar tail. When an ion-pair reagent is used, neutral compounds are formed between ion pair reagents and targeted arsenic species based on the electrostatic attraction, and hence the retention of arsenic species on RPLC columns would be improved. IP-HPLC offers high capacity over IEC technique because it can separate cationic, neutral, and anionic arsenic species at the same time. Ion-pair agents are noncompatible with ESI-MS, and there is a restriction in qualitative determination of As species in the algal extract by using ion-pair HPLC. However, co-elution of species with similar physicochemical

properties is a common problem in LC and some organic solvents that are used as the mobile phase have a limited UV transparent range, which limits their use with a UV detector. LC can be easily interfaced with many other detection systems such as ICP-MS, HG-AFS, and MS.

2.12.3 Gas Chromatography

In the GC separation technique, gas is used as both mobile phase and carrier and the separation of As species are performed by a gas phase using gas chromatograph. The GC-based method was developed in 1975 for the determination of As species in algae samples (Talmi and Bostick, 1975). On the basis of stationary phase, GC technique is two categories: (a) gas-solid adsorption chromatography where the separation is based on the difference in adsorption abilities between solute and solid, and (b) gas-liquid partition chromatography where the separation is based on the difference in partition coefficient caused by the difference of dissolution ability between the solutes in the stationary phase.

Gas chromatography (GC) is often used with mass spectrometry (MS) (GC-MS) and GC-MS/MS for the speciation of As. The use of GC is certainly employed for As speciation because most As compounds are non-volatile. This method can be used to examine landfill gases for the determination of volatile As compounds but is unsuitable for biological samples. In some studies, the samples were extracted in dichloromethane after different derivitization agents and injected in GC/MS in SIM mode to measure the As species (Namera et al., 2012; Takeuchi et al., 2012; Kang et al., 2016; Campillo et al., 2008). Pantsar-Kallio and Korpela (2000) also determined volatile As species like arsines, methylarsines, dimethylarsine and trimethylarsine in the air by GC-MS. High resolution, fast analysis speed, and low-cost are the main advantages of GC technique. Very high precision and accuracy with a low detection limit of 0.08 pg can be measured by using GC-MS/MS technique (Sankararamakrishnan and Mishra, 2018).

2.12.4 Capillary Electrophoresis

CE often called high-performance capillary electrophoresis (HPCE) that consists of a capillary separation channel driven by a high voltage DC electric field, which depends on the mobility between the components of the sample. The separation of As species in CE depends on their charge-to-size ratio, which is controlled by a suitable choice of pH and buffer constituents (Qu et al., 2015; Naidu et al., 2000). Separation processes like capillary electrochromatography (CEC), micellar electrokinetic capillary chromatography (MECC), isotachophoresis (ITP), isoelectric focusing (IEF), and capillary zone electrophoresis (CZE) can be combined with CE (Michalke, 2003). This technique offers a great advantage over GC

and HPLC which include: low cost and environmentally friendly, fast analysis with high separation efficiency, simple in sample pretreatment, low sample consumption, and many operation modes. However, the application of this technique in real sample analysis is limited due to the insufficient detection and poor sensitivity as well as low tolerance of the sample matrix.

2.12.5 Others

There are some other kinds of non-chromatographic separation techniques utilized for the As speciation analysis which include liquid-liquid extraction (LLE), liquid phase microextraction (LPME) solid phase extraction (SPE), and solid phase microextraction (SPME). LLE methods are based on differences in solubility of two liquids in a sample matrix usually achieved through water and a solvent of organic nature. LPME techniques are similar to LLE, where extraction and preconcentration are prepared in a microliter volume of the targeted compounds of interest. The advantage of this technique is the requirement of very low solvent for sample extraction and easy to operate. Single-drop microextraction (SDME), hollow fibre liquid phase microextraction (HF-LPME), dispersive liquid-liquid microextraction (DLLME), and solidified floating organic drop microextraction (SFODME) are the common types of LPME. In the SPE technique, target compounds of a sample matrix are extracted by using a solid absorbent and target As species can be eluted by heating or using an eluent. SPME techniques which are commonly used for the separation technique include stir bar sorptive extraction (SBSE), capillary extraction (CME), and fibre SPME (Hu et al., 2019). A field-deployable method (FDM) has been introduced recently for the on-site monitoring as well as in situ measurement of inorganic As species in a wide range of samples including macroalgae within a shorter time of 1 h. The results of this method showed functional recovery of 80–95% and had limited comparable uncertainties when analyzed with HPLC-ICP-MS (Bralatei et al., 2017).

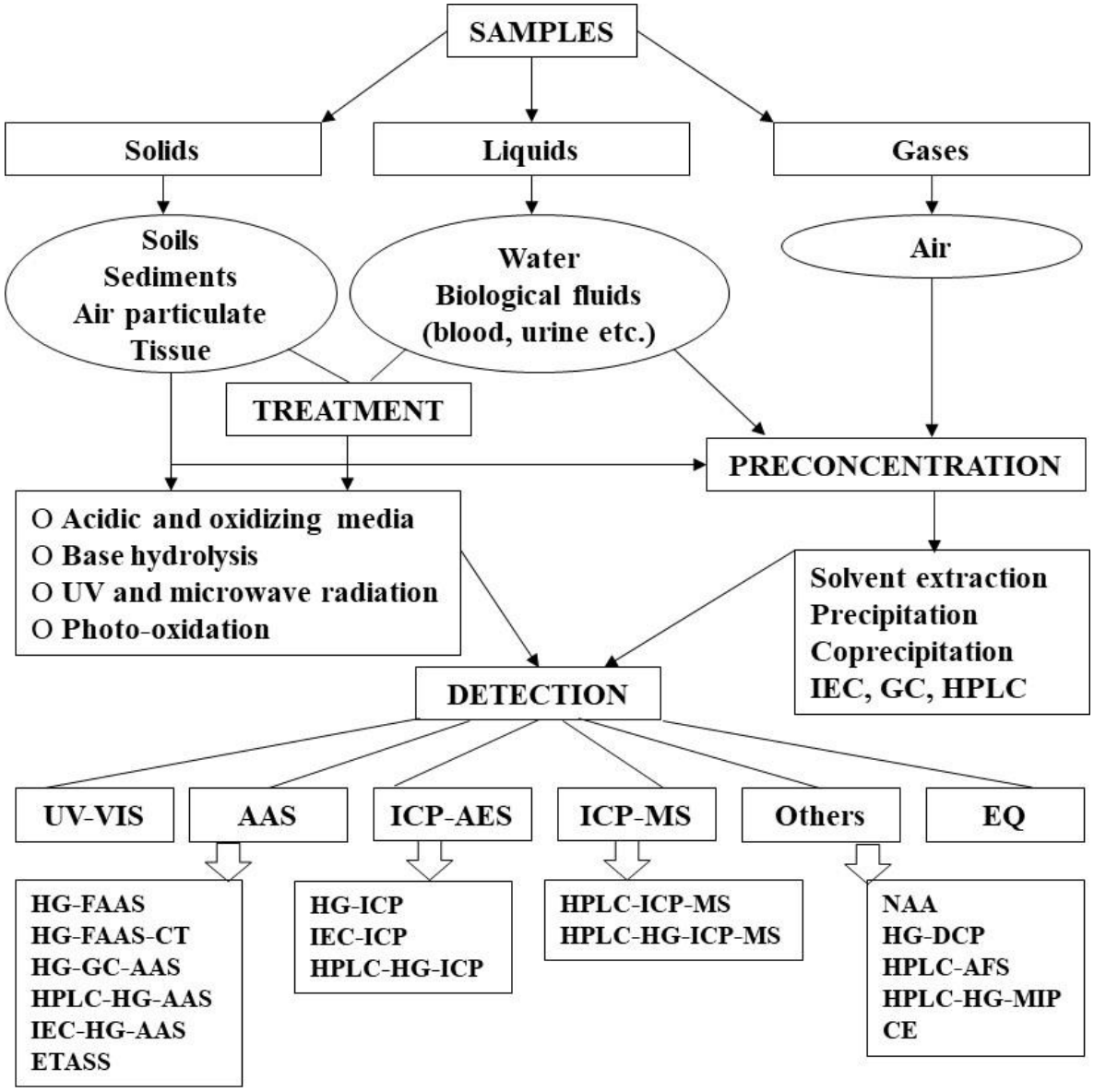

Figure 2.9: Methods for determining As speciation in different matrixes [Redrawn, modified and adapted from Burguera and Burguera (1997)].

2.12.6 Detection Techniques and Instruments

The detection techniques of As species, i.e., identification and quantification are usually achieved by using atomic spectrometry and mass spectrometry. The element-specific and selective detectors for As speciation include AAS, HG-AAS, AFS, HG-AFS, ET-AAS, ICP-AES, ICP-MS, etc. These detectors combined with suitable separation techniques like CEUV, HG-AAS, IC-ICP-MS, HPLC-ICP-MS, and HPLC-HG-AFS are commonly employed for As determination for a wide range of biological samples. The use of HPLC-ICP-MS is the most reliable detection system in the field of speciation analysis because of its high sensitivity (ppb level) (Hu et al., 2019). The cost of instrumentation, operation as well as maintenance in HPLC-ICP-MS make inconvenient for the user of developing countries which lead to alternative means of measurement. In this regard, HG-AAS or HG-AFS coupled with HPLC has gained much popularity because of their simple, fast, and inexpensive for As speciation analysis (Welz, 1998).

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MATERIALS AND METHODS

3.1 Overview of Macroalgae

Algae in a general term include a wide variety of photosynthetic organisms lacking a high degree of differentiation and hemicellulose and lignin, as opposed to plants that do differentiate in forming roots, leaves, stems and xylem/phloem vascular networks and withstand with their structural support. Plant and algae share some common characteristics including (i) both produce the same storage compounds, (ii) use similar defense strategies, and (iii) share similar morphological functions (Barsanti and Gualtieri, 2014). The marine macroalgae are multicellular, visible by the naked eyes and often called as seaweeds that fall under the general category of 'plants' (Guiry, 2019b). The characteristics pigments in their chloroplasts make them as brown, red, and green seaweeds group, but the actual color of the seaweeds tends to vary a great deal. Marine algae have shown to convert photon more efficiently, and they can consequently synthesize biomass more rapidly (Subhadra and Edwards, 2010).

3.2 Classification of Macroalgae

The extensive work on the classification of algae was carried out by Bold and Wynne (1985). There is four division as the members of macroalgae/seaweeds among the seven broad divisions of algae. The larger visible seaweeds are the division of Cyanophyta (blue-green algae), Chlorophyta (green algae), Rhodophyta (red algae) and Phaeophyta (brown algae) (Lee, 1986). The division Cyanophyta comprise eubacteria and have distinct evolutionarily characteristics when compared with macroalgae (Davis et al., 2003).

3.3 Structure and Morphology of Macroalgae

Macroalgae have a wide array of body parts and structures. The entire body of seaweed is recognized as the thallus. The holdfast and blades are the main body parts of most of the seaweed's thallus. The holdfast made up of many fingerlike projections called haptera usually responsible for secure attachment of the seaweeds to a surface, e.g., rock, sand, coral rubble, other plants, or animals). The blades/fronds are the leading site for photosynthesis and nutrient uptake from the surrounding seawater. Blades can support the reproductive structure in some species. Stipe, a stiff and cylindrical flexible shaft, rising from the holdfast, sometimes present in certain species of seaweed (e.g., *Undaria pinnatifida*, *Saccharina japonica*, etc.) that help to support the remainder body parts of algae. Some species of seaweed also contain air bladders which can assist for the flotation of the photosynthetic tissues of a thallus (e.g., *Sargassum patens*) (Bold and Wynne, 1985). *Figure 3.1* illustrates the morphology of large macroalgae.

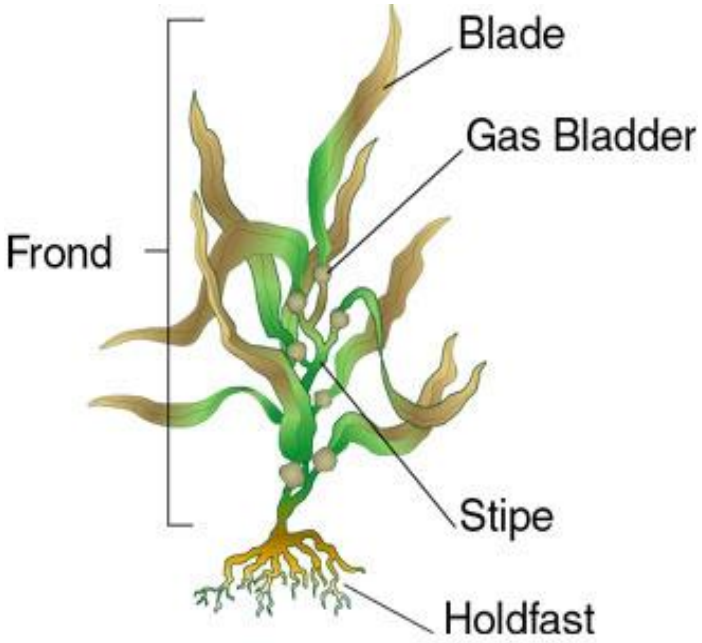

Figure 3.1: Schematic diagram showing different morphological structure of a giant kelp *Macrocystis pyrifera* (Reprinted from http://www.ncl.ac.uk).

3.4 Cell Wall Composition of Different Group of Macroalgae

A fibrillar skeleton and an amorphous embedding matrix are the most common and principal layers of typical algal cell walls of Phaeophyta and Rhodophyta (Davis et al., 2003). Cellulose is the main product of the fibrillar skeleton of most groups of algae that ensures the rigidity to the cell wall (Park et al., 2005). Cellulose can be replaced by xylan in the Chlorophyta and Rhodophyta in addition to mannan in the Chlorophyta (Davis et al., 2003). Though alginic acid, a long chain heteropolysaccharide, is the dominant embedding matrix in the cell wall of brown algae, some sulphated polysaccharides like fucoidan are also present. The Rhodophyta algal matrix is usually sulphated galactans including agar, carrageenan, and porphyrin etc. The compounds associated with the cell wall of different algae contain a number of functional groups and make them potential material of heavy metals biosorption. The typical cellular morphology of brown macroalgae is given in *Figure 3.2*.

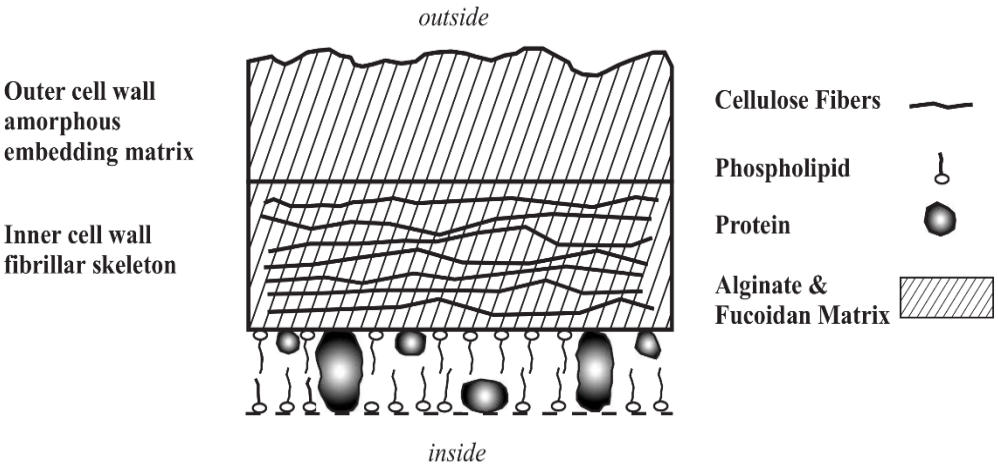

Figure 3.2: Schematic diagram showing typical cell wall structure of the brown macroalgae [Reprinted from Davis et al. (2003) with permission from Elsevier].

3.5 Macroalgae Under the Study

There are four species of macroalgae which are used for studying the different growth characteristics and other analysis. The simple description of the algae species has been discussed in this section. The pictorial view of different species of macroalgae is shown in *Figure 3.3*.

3.5.1 *Undaria pinnatifida*

Undaria pinnatifida (Harvey) Suringar, 1873 (Class: Phaeophyceae, Order: Laminariales) is usually known as "Wakame" by the Japanese people and is one of the most important species for seaweed mariculture in China, Japan, and Korea. This species is native to the Japan Sea, particularly on the coasts of Japan, west of Hokkaido, coasts of Korea, and parts of China (Guiry, 2019a). The thallus fixed by a ramified holdfast with numerous haptera, the origin of a flat stipe with denticulated margins (in young individuals); frond blade-like (lanceolate), extending from the tip of the plant for half to three-quarters the length of the plant, and reaching an overall length to about 0.6 m or even up to 1.0 m. The thallus consists of a midrib with undulating wing-like pinnate blades at the base. The sporophyte is golden-brown, with a lighter colored stipe (FAO, 2019).

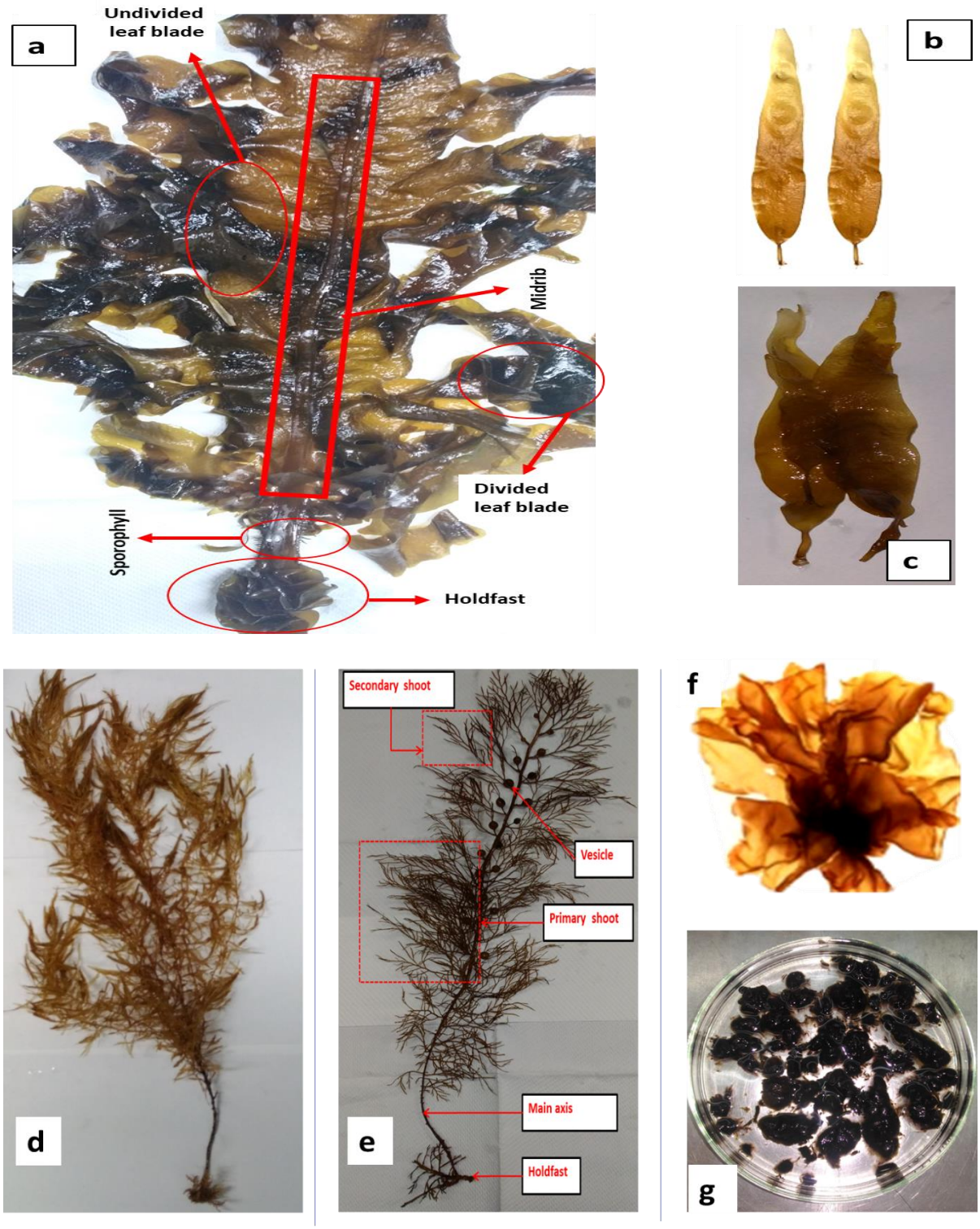

Figure 3.3: Photographs showing the mature thalli of *Undaria pinnatifida* (a), *Sargassum horneri* (d), *Sargassum patens* (e), *Pyropia yezoensis* (f), the young sporophyte of *U. pinnatifida* (b & c), and the spore stage of *P. yezoensis* (g) grown in the laboratory.

3.5.2 *Sargassum horneri*

Sargassum horneri (Turner) C. Agardh 1820 is an important edible brown seaweed species (Class: Phaeophyceae; Order: Fucales; Family: Sargassaceae) in eastern Asia (Guiry, 2019a). This species is found in Japan (except Taiwan and the Ryukyu Archipelago), the northeast coasts of China to Russia, the Korean Peninsula (Komatsu et al., 2014). *Sargassum horneri* is attached by a solid holdfast and grows erectly. Sometimes the thallus also produces free-floating mats. The young thallus looks like ferns with different opposite leafy blades which are spreading from a main central axis. The blades are broad, deeply incised with ragged tips. At the growing stage, the thallus becomes a single frond which is loosely branched with a zigzag pattern. When the plants reached to mature stage, the blades turn into thinner as well as narrower, and the branches develop many small size ellipsoidal air bladders and larger spindle-shaped reproductive receptacles on the stalks. The air bladders offer the seaweed to keep straight in the water column. The mature plants can reach up to 2.5 to 5 m in height at some locations of Japan (Fofonoff et al., 2019; Yoshida et al., 2004; Choi et al., 2007).

3.5.3 *Sargassum patens*

Sargassum patens C. Agardh 1820, Japanese name Yatsumatamoku, is a brown species of seaweed species (Class: Phaeophyceae; Order: Fucales; Family: Sargassaceae) found in the area of Honshu, Shikoku, Kyushu, the Nansei Islands in Japan; Korean Peninsula, and China (Guiry, 2019a). The thallus of *Sargassum patens* grows up to 0.5 to 1 m long. The main branch that expanded in winter becomes 3 to 4 m in spring, and the tip may reach to the sea level so that it can be seen from land. The main branch will disappear when the algae reach to the maturation stage. The stem is cylindrical in shape, and several main branches are originated from the top of the stem. The main branch is flat and thin on both sides, and alternately branches into wings. The distinction between leaves and branches is unclear, and it feels like flat twigs become leaves. The upper leaves of the main branch are linear, and the edges are smooth or rough with a shallow jaggedness. The vesicles are oval, oval or nearly spherical. It has a flat handle as long as the length of the vacuole. At the apical end of the vacuole, it has one crown leaf or a branched crown leaf.

3.5.4 *Pyropia yezoensis*

Pyropia yezoensis (Ueda) M.S. Hwang and H.G. Choi (Rhodophyta, Bangiaceae) known as ‘Nori’ in Japan which was previously recognized as *Porphyra yezoensis* (Sutherland et al., 2011). The aquaculture of ‘nori’ has been started in Asia since the seventeenth century. Susabi-nori (*Pyropia yezoensis*) is one of the most profitable mariculture crops in the world

and ranks highly valued marine crops in East Asia, as well as in Japan (Nakamura et al., 2013). This species grows on the upper and middle part of the intertidal zone, and the major areas of distribution include Hokkaido, Honshu Pacific north coast (Chiba prefecture), Honshu Japan. The size of the thallus is generally 5 to 20 cm in length with 2 to 8 cm width, but it reached up to the length of 50 cm and width of 20 cm. The body has a thin film and ovate in shape and looked like a bamboo leaf. The edges are wavy with a dense greenish base. The color is reddish brown, and the touch is smooth and soft. The natural distribution is only limited in northern Japan, but it is also cultivated in farms.

3.6 Laboratory macroalgae culture and maintenance

The culture is defined as the artificial medium, which is supplemented with various chemicals similar to the natural environment in order to the proper growth of any organisms, including macroalgae (Devi and Sahoo, 2015). As it is expected to grow unialgal culture for maintenance in the laboratory, isolation of bacteria free (axenic culture) of algae is necessary. Various culture media and environmental conditions have been employed to grow of various algal species because the habitat and nutrient requirements of algae vary from one species to another. Algal culture media are divided into two broad classes viz Freshwater culture media and marine culture media.

Aseptic or sterile culture environment is a necessary requirement for maintaining pure culture. That is why sterilization is the first and most essential step before the start of any freshwater or marine algae culture. Autoclaving is the most commonly used sterilization techniques and is usually performed for sterilizing glasswares, pipettes, pipette tips, plastic wares, utensils and medium (both solid and liquid) etc. Autoclaving is done in a closed chamber where steam pressure maintained at 15 PSI (pounds per square inch) with 121 °C temperature for 15 minutes (Yue and Chen, 2005). The following considerations should be followed before the start of culturing algae:

- All the sterile pipette, loop or any material which are going to be used in culture should be UV radiated or flamed and should be cooled before used.
- Laminar flow should be turned on before the start of the culture work.
- The working surface should be cleaned with 70% ethanol.
- Hands should be cleaned with 70% ethanol after organizing all requirements under the laminar flow.

Macronutrients, trace elements, and vitamins are the three key components which might be considered during media preparation. Each of the components often called as stock solutions are made separately as the concentrated solution and subsequently diluted to prepare the final media concentration with an expected volume of 100 to 1000 mL. The required amounts of chemicals weighed and dissolved in a flask by using distilled or deionized water. Proper care should be necessary during the complete dissolution of chemicals before making the desired volume and concentration of stock with distilled or deionized water. Tightly sealed glass bottle or plastic bottle containing stock solutions are kept in a refrigerator at 4 °C for storage (Sahoo and Seckbach, 2015).

The field collection of marine macroalgae are generally brought to the laboratory and washed immediately with natural seawater and cleaned manually to remove attached epiphytes and epifauna. As macroalgae are highly potential in regeneration, the unialgal culture of macroalgae is generally practiced by cutting off the vegetative cells. It is also possible to culture macroalgae, which can be easily developed from zoospores and planogametes, zygotes, carpospores, tetraspores, or aplanospores (Kawai et al., 2005). The thallus of macroalgae should be acclimatized for 4 to 5 days in sterilized natural seawater with desired salinity before initiation of any culture work. The algal samples are generally cultured in sterilized glass or petridishes containing an enriched medium. The enriched medium is necessary for providing nutrients and other micronutrients in the media for the growth of algae. F/2 medium and PES medium are widely used and most common for macroalgae culture. The composition of PES media used in this study are shown in Appendix A. The cultures of macroalgae are maintained in a chamber, especially in an incubator and/or growth chambers where the temperature, light, and humidity can be operated. Culture media are usually changed after a week or even after 2 to 4 weeks based on the algal species and environmental conditions, especially light and temperature.

3.7 Growth Rate of Algae

The increasing amounts of research with different macroalgal culture both in laboratory and field conditions is directed towards the growth rate determination. Different studies have investigated different attempts for the estimation of growth rates under stress conditions. Growth rate can be defined as the speed of growth of algae over time, for example, the growth rate of 5 g day^{-1} indicates growth of 5 g every day, or 10% day^{-1} represents 10% increase of weight (fresh weight or dry weight) or diameter every day. For the growth performance and response of a target culture of macroalgae species, growth rate calculation is a basic

measurement. It has been suggested that the time interval between growth rate data is recommended as short as weekly for more accurate measurement (Yong et al., 2013). Table 3.1 shows the equations used for the growth rate determination.

Table 3.1: Currently available formulae for the determination of growth rate of macroalgae and aquatic macrophytes.

Formula name	Formula	Unit	Reference
Daily growth rate	[ln(Wt/Wo)] X 100%	% day^{-1}	Loureiro et al. (2010)
Growth rate	$[(Wt/Wo)^{1/t} - 1]$ X 100%	% day^{-1}	Hayashi et al. (2011)
Growth rate	[(Wt/Wo) - 1] X (100/t) %	% day^{-1}	Schmidt et al. (2010)
Relative growth rate	[ln(Wt) – ln(Wo)] X 100/t	g g^{-1} day^{-1}	Huang et al. (2013)
Specific growth rate	[lnWt – lnWo]/t X100%	% day^{-1}	Luhan (2010)

Wt and Wo is the final and initial weight (g), respectively, and t is the culture period (day).

3.8 Photosynthetic Activity of Macroalgae

Algae are photosynthetic organisms and the measurement of photosynthetic activity upon different stress conditions like light, PAR, temperature, drought and nutrient and toxic metal stress like As. Autotrophic organisms including algae produce Chlorophyll a, b and other pigments as part photosynthetic machinery. Measurements of these pigments in vivo and in vitro under different stresses have been used over the years. The measurement of fluorescence value is informative regarding photosynthetic processes, plant health, and plant stress.

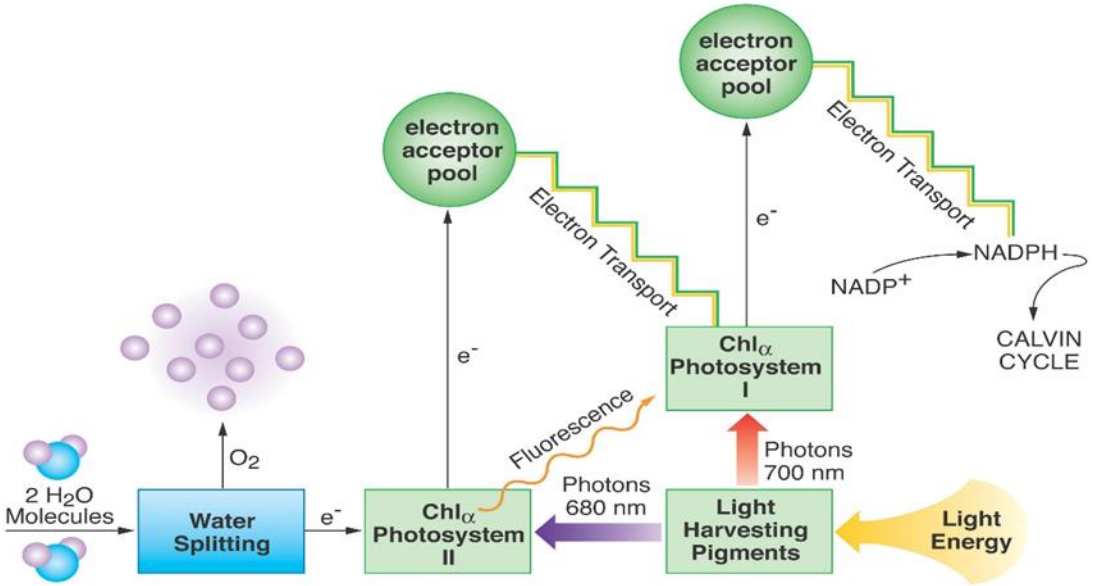

Figure 3.4: Simplified diagram of the "light reaction" of photosynthesis. Chlorophyll fluorescence emanates from chlorophyll a in Photosystem II [Reprinted from Ritchie (2006)].

3.8.1 Chlorophyll Fluorescence

"Chlorophyll (Chl) a fluorescence" is a sensitive indicator of the photosynthetic electron transport activity upon toxic metal exposure (Popovic et al., 2003), and provides more detailed insight into the physiological state of the photosynthetic apparatus (Mallick and Mohn, 2003). Different aspects of photosynthetic apparatus can be explained by one of the most important and widely applicable measurements of chlorophyll a fluorescence both in vitro and, non-invasively, in vivo (Schreiber et al., 1995; Kalaji et al., 2014; Kalaji et al., 2017). Chlorophyll absorbs light most effectively in the red and blue parts of the visible spectrum. Chlorophyll fluorescence can be defined as the re-emitted light at a longer wavelength after being absorbed by chlorophyll molecules at a shorter wavelength. The sole origin of variable chlorophyll fluorescence is occurred in chlorophyll "a" in photosystem II (PSIII). That is why, chlorophyll fluorescence measurement of photosystem II is of great interest in most of the studies because photosystem I emit invariably very lower level of chlorophyll fluorescence (Strasser et al., 2004).

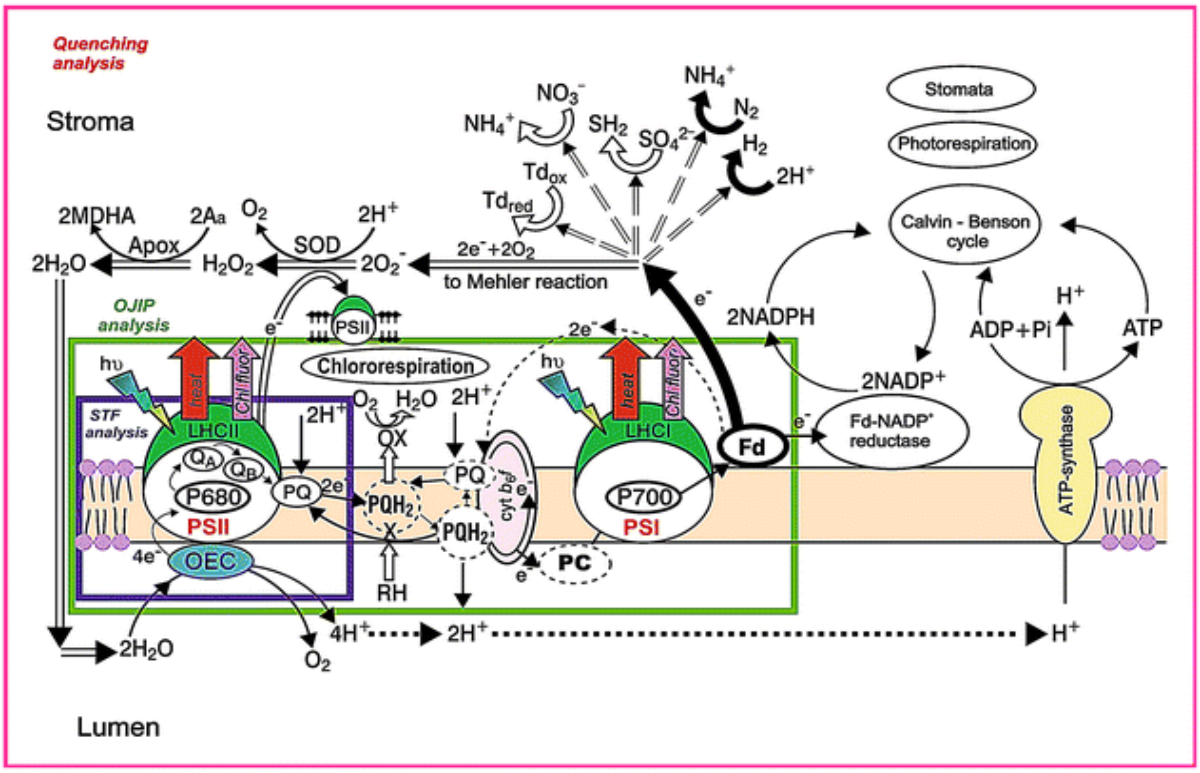

Figure 3.5: Diagram showing different processes analyzing during fluorescence decay following a single turnover flash. Purple line for the fluorescence decay kinetics; green line for ETR inside PSII and OJIP transients; and rose line for the redox state of the photosynthetic ETC, the stoichiometry of the components of the ETC and the relative PSII antenna size and the quenching analysis [Reprinted from Kalaji et al. (2014) with permission from Springer].

The maximum quantum yield of PSII photochemistry is most frequently used parameter in the photosynthetic status of organisms in environmental researches. The maximum quantum yield or maximum photochemical efficiency of a stable charge separation for the dark-adapted state is in the literature defined as F_V/F_M. On the other hand, the light phase determination of photochemical yield is Fv'/Fm'. Chlorophyll fluorescence measurements in dark adaptation technique used to fix a non-stressed reference point relative to various measurements (Maxwell and Johnson, 2000). Fv/Fm measures the performance of PSII of all samples under the same known dark-adapted state. The average and maximum Fv/Fm reading of most plant species, including algae under stress-free condition is ranged between 0.79 and 0.84 (Maxwell and Johnson, 2000).

3.8.2 Measurement of Chlorophyll Fluorescence

Different handy fluorimeters have been developed during the last few decades. The available pulse amplitude modulated (PAM) fluorometry devices are mainly employed for measuring the different aspects of photosynthesis and on various properties of Chl a fluorescence. These devices offer rapid and non-invasive measurement of the photosynthetic parameters of many terrestrial and aquatic plant species, including marine macroalgae.

The OS1p Modulated Fluorimeter (PAM, OS1p) is a battery powered, and light weight device and the graphical touch screen and operating system are effortless to use that making it an excellent choice in the field of photochemistry studies. This multipurpose portable instrument designed precisely for the measurement of chlorophyll fluorescence for both field and laboratory studies. The basic OS1p is equipped to make several different kinds of tests including (i) dark-adapted maximum quantum yield (F_v/F_m) and (ii) light adapted Yield (Y) or F'/Fm' or Y(II). The OS1p devices along with different operating parameters are shown in *Appendix B.*

3.8.2.1 Dark Adaptation or Dark-adapted Measurement

Dark-adapted measurement is recognized as the measurement of samples (an area of a plant, or the entire plant) under the dark for an extended period of time before measurement. The time required for dark adaption may vary for dark-adapted tests and depending on the test species. Twenty to sixty minutes are generally practiced for dark adaptation in order to get reliable test results depending on the test species and light history. However, longer dark adaptation is commonly performed for measuring non-photochemical quenching parameters.

The following formula is used for describing the maximum photochemical yield of dark-adapted tissues (Cosgrove and Borowitzka, 2010):

$$F_v / F_m = (F_m – F_o) / F_m$$

where, F_v/F_m = maximum photochemical efficiency (quantum yield) of open RCIIs; F_o = minimum fluorescence yield or dark fluorescence yield (dark adapted, all RCIIs open); F_m = maximum fluorescence yield (dark adapted, all RCIIs closed with no NPQ) or fluorescence yield at point P (FP) of the fluorescence induction curve is equivalent to F_m if irradiance is saturating; and $(F_m – F_o) = F_v$ = maximum variable fluorescence yield.

3.8.2.2 Light Adaptation or Light-adapted Measurement

Light-adapted measurement indicates the amount of energy used in photochemistry by PSII system taken under steady-state photosynthetic lighting conditions. About 15 to 20 minutes is necessary for a plant to reach steady state photosynthesis at a specific light level (Maxwell and Johnson, 2000). This measurement may vary with the closure of reaction centers and heat dissipation caused by non-photochemical quenching (Kalaji et al., 2014). Therefore, it is necessary to compare the samples with a similar light history before measurement. The measurement of maximum photochemical efficiency under light adaptation is a more sensitive test than measurement under dark adaptation. The following equation describes the light-adapted maximum photochemical yield (Cosgrove and Borowitzka, 2010):

$$Y(II) = F_v' / F_m' = (F_m' – F_o') / F_m'$$

where, $Y(II)$ or F_v'/F_m' = maximum PSII photochemical efficiency or quantum yield in actinic light; F_o' = minimum fluorescence yield in light-acclimated state usually measured with the application of far-red light; F_m' = maximum fluorescence yield in actinic light; and F_v' or $(F_m' – F_o')$ = Variable fluorescence yield in actinic light.

3.9 Measurement of Arsenic

3.9.1 Microwave Digestion

The heating and wet digestion of biological matrices by using microwaves has become increasingly popular and significantly accelerated the measurement of trace elements including As because of decreasing the contamination risk as well as the loss of volatile chemical elements. Microwave-assisted digestion systems are more efficient in speed up the overall digestion procedures by overcoming some of the difficulties while dissolving the complex biological matrices.

It has been suggested that the organic matrix of samples could easily be decomposed at an elevated temperature and pressure in a closed vessel of microwave (Kingston and Jassie, 1988). Microwave digestion under relatively high temperatures and pressures often allow the single use of nitric acid for complete digestion of sample matrix and consequently eliminate the requirement of more dangerous acid mixtures. The Teflon reactor along with nitric acid is widely used method in the microwave assisted digestion for the As analysis of macroalgae (Thodhal Yoganandham et al., 2019; Hwang et al., 2010; Dominguez-Gonzalez et al., 2010). However, Teflon vessels which are fitted with pressure-relief valves required extensive cleaning between use due in order to overcome vapor entrainment in the cap and relief valve.

Before starting the quantitative determination of As in solid samples, especially the algal tissues should first be converted to a solution. The conversion process is achieved by mineralization or digestion in a microwave reaction system (Multiwave 3000; Anton Paar GmbH, Graz, Austria). The system was equipped with a microwave power system along with an operator selectable output of 1400 W via two magnetrons. The system also consists of eight-position rotor and vessels attached with a hydraulic pressurized sensing system, a cavity exhaust fan, and tubing to vent fumes which can be programmed with different stages. The vessels allow controlled pressure from 6 MPa to a maximum of 12 MPa with the maximum temperature of 2600 ^{o}C.

3.9.2 Measurement of Total As by ICP-MS

The measurement of total As contents of the microwave-assisted samples has been carried out by ICP-MS in this study. The ICP-MS apparatus consisted of a SPQ-9000 system (Seiko Instrument, Tokyo, Japan). The ICP-MS was operated under the conditions of a high-frequency ICP quartz torch (RF-power output 1.2 kW), a plasma gas flow rate of 16 L min^{-1}, an auxiliary gas flow rate of 1.0 L min^{-1}, a nebulizer gas flow rate of 1.0 L min^{-1}, sample scan range of 30 to 220 amu, and a sample replacement time of 10 s. The diluted sample is introduced into the introduction system, which is allowed to nebulize and deliver into plasma torch section with a carrier argon gas. The generated ions are then introduced into the mass spectrometer. The characteristics isotopic pattern of an atomic element is evaluated automatically for its identification with sensitivity (1 amu). The counts of ions in the sample is quantified by subtracting the minuend of the ion counts of each blank from the actual count of ion. The ion lens, the width of the scheme cone, and the pressure of developed gas can affect the level of ion counts in each determination series. Therefore, the appearance of the ion between 40 and 41 amu was cut to exclude the developed argon gas. For total As analysis, the

m/z 75 for arsenic and m/z 115 for indium were monitored. A blank, standard, and certified reference material (CRM) were measured in every batch of samples.

3.9.3 Arsenic Speciation by CT-HG-AAS

3.9.3.1 Apparatus of CT-HG-AAS

The quantitative determinations of arsenicals in the growth medium samples have been accomplished by using an atomic absorption spectrophotometer (AAS) in association with hydride generation apparatus followed by cold trapping (AAS, 170-50A, Hitachi, Japan). An As hollow cathode lamp was used as the light source (PerkinElmer, Atomax, USA). Table 3.2 shows the conditions of the analyzer, while Figure in *Appendix C* shows the chromatogram of As species that have been recorded on a chromatogram data processing device (Chromato-PRO, runtime Instruments, Hitachi).

Table 3.2: Instrumental parameters for As speciation analysis in CT-HG-AAS

Parameter	**Condition**
Measurement mode	Absorbance
Wavelength	193.7 nm
Lamp current	10 mA
Slit width	1.3 nm
Atomizer	Air-C_2H_2
Oxidant (Air) flow rate	1.5 L min^{-1}
Fuel (C_2H_2)	1.0 L min^{-1}
Quartz tube temperature	900 ^oC
Time constant	2 min
ChromatoPRO parameter	
Minimum height	0 mV
maximum height	40 mV
Measurement time	1.0 min

The apparatus also includes a reaction vessel, a Teflon four-way valve, a U-trap, and a quartz cuvette (*Appendix C*). The reaction vessel along with tubings was connected on the straight with Teflon joints. The top port of the vessel contained a glass cap stopper through a ground glass joint. A helium gas inlet was connected with the glass cap and a ball filter. The side arm port allowed to introduce the samples inside the vessel, and when the port was fitted with a natural rubber septum, $NaBH_4$ solution was injected through a Teflon tube via a

peristaltic pump. The other side-arm port was connected to the U-trap for the gas outlet. A gas chromatographic packing was packed into three-fifths of the U-tube which was stopped with glass wool. Nichrome wire was wired the U-tube for the purpose of heating. The distance of tubing between U-tube and the quartz cell was about 27 cm. The internal surface of the glasswares other than the vessels was deactivated using silylation reagents to prevent the irreversible adsorption of arsines. Silanization process was repeated when the deterioration of column performance was detected. Before measurement, the column was heated in stream flow (0.3 L min^{-1}) of helium gas for 1 hr. The quartz cuvette was heated by a flame of the acetylene-air burner which was mounted on the atomic absorption spectrometer.

3.9.3.2 Procedure of Arsenic Speciation in CT-HG-AAS

The hydride generation from the liquid samples has been performed following the technique by (Hasegawa et al., 1994). The fractionation of As(V) and As(III) was carried out by adjusting the pH during the reduction of the sample matrix. Inorganic As (As(V) + As(III)), MMAA(V), and DMAA(V) were reduced as arsine at pH 0.5 or less strongly acidic range, whereas only As(III) was reduced to arsine at the weakly acidic range.

The measurement of As(V) was done by subtracting the quantified As(III) concentration at pH 4.0 from the total inorganic As concentration at a strongly acidic pH of 0.5. Prior to measurement, the U-tube was heated through an electrical heating wire at 5 V along with He gas flow of 0.3 L min^{-1} for 1 h. The sample solution was prepared by adding 40 mL of distilled water followed by 5 mL of 0.2 M EDTA·2Na and 5 M HCl for the measurement of IAs, MMAA(V), and DMAA(V). The He gas flow (0.3 L min^{-1}) was done for 3 min as bubbling into the sample solution in order to remove the oxygen. Next, 10 mL of 3 % $NaBH_4$ was injected through the peristaltic pump for 1.5 min. The reduced arsines were collected in the U-tube and cooled with liquid nitrogen for another 1.5 min. The He gas pathway was redirected to pass directly towards the U-tube at 1.2 L min^{-1} immediately after cold trapping. At this time, liquid nitrogen was promptly removed, and heating of U-tube was increased at 20 V for re-gasify the hydride of arsenic that was collected and concentrated during trap.

The arsines were then reached into the quartz cell of the atomic unit of flame atomic absorption spectrometer where they were atomized. The atomic absorption of the arsenic species was recorded as a chromatogram. The concentration of each arsenic species was determined from the peak height. Before batch measurement of the samples, calibrations were made by using standards solution mixture of different As species.

3.10 Measurement of P and Fe in ICP-AES

The Inductively coupled plasma atomic emission spectroscopy (ICP-AES) (iCAP 6300; Thermo Fisher Scientific, Waltham, MA) was used for the determination of Fe and P concentration in algal tissues, as well as phosphate concentration in culture media samples. This apparatus consists of ICP and optical spectrometer. The ICP torch includes an EMT duo quartz glass tubes. The peristaltic pump is designed to introduce and deliver the liquid sample into a concentric gas nebulizer and allowed to deliver the samples directly inside the plasma flame. The outer-shell electrons of the elements in a sample are thermally excited due to plasma flame. The excited electrons are then returned to the ground state by emitting photons of light or spectrum of light wavelength with an energy characteristic of the element. The separated emissions are then detected by optical spectrometer through a specific wavelength for each element of a sample mixture. Each line of wavelength is compared with the measured intensities of the standards with known concentrations. During measurement, ICP was operated by maintaining radio frequency power at torch: 1.15 KW, plasma gas flow rate: 12 L min^{-1}, auxiliary gas flow rate: 1 min^{-1}, the nebulizer gas flow rate: 0.5 min^{-1}, and an integration time of 30 s.

3.11 Washing Extraction of Surface Adsorption

This surface adsorption phenomenon has accelerated the development of researches and protocols with different washing chemicals linking to separate the pool of surface-bound iron and its role in iron acquisition studies (Tovar-Sanchez et al., 2003). The significance of biologically surface-bound iron has generally been overlooked in many cases since iron present in the media was in the form of very stable Fe-EDTA chelate. However, the possibility and existence of surface binding were persisted even in medium contained excess EDTA (Miller et al., 2013). Numbers of washing protocols with their variety of chemicals employed for partitioning the surface-bound/extracellular iron plaque and associated metals from internalized metals under a diverse group of organisms including marine phytoplankton and macroalgae.

In this research work, TiCE and CBE reagents were used for the extraction of surface-bound Fe and associated As and P. The preparation of Ti(III)-citrate-EDTA reagent was followed according to (Hudson and Morel, 1990). Simply, different salts such as citrate, EDTA, NaCl, and KCl were prepared at a concentration of 0.047, 0.047, 0.35, and 0.01 M, respectively by dissolving in deionized water. The pH of the solution was adjusted to ~8 by using NaOH, followed by adding 20% $TiCl_3$ (0.047 M). The pH of the final solution was again adjusted to 8

with NaOH. The recommended incubation time of TiCE (2 min) with algae followed prior and after rinsing with artificial seawater was carefully performed during the extraction. The CBE solution was prepared by using 0.03 M citrate salt, 0.125 M bicarbonate, 0.05M EDTA, 0.25M KCl and 0.25 M NaCl. There were only some modifications for preparing the CBE solution by using additional NaCl and KCl and pH adjustment to 8.0 from the original preparation (Rahman et al., 2008).

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Arsenic Speciation and Biotransformation by the Marine Macroalga *Undaria pinnatifida* in Seawater: A Culture Medium Study

4.1 Introduction

Arsenic (As) is a ubiquitous and well-known metalloid that exists in four oxidation states, namely, elemental arsenic (0), arsine (-III), arsenite (+III), and arsenate (+V), under different redox conditions, and the latter two are the most abundant inorganic species in the environment (Tseng, 2009). The most critical determinant of As toxicity depends upon the oxidation state in organic and methylated forms. For example, arsenite (As(III)) is typically more toxic than arsenate (As(V)) (Le et al., 2004), whereas methylated species of pentavalent states, such as monomethylarsonic, dimethylarsinic, and trimethylarsinic acids (MMAA(V), DMAA(V), and TMAA(V), respectively), are less toxic than As(V) and As(III) (Kucuksezgin et al., 2014). Some species of As such as arsenobetaine and arsenocholine are found in marine ecosystems and considered to be nontoxic (Francesconi, 2010). As(III) and As(V) are subjected to chemically and biologically mediated oxidation, reduction, methylation, demethylation, and other reactions, and these processes are the primary reasons for the availability of various species of As in the ecosystem. As contamination has received particular attention because of its possible human health risk concerns from dietary intake and resulted in research on As biogeochemistry, including its bioaccumulation and biotransformation in both freshwater and marine food chains (Rahman et al., 2012). Considering the wide range of toxicity of the different chemical forms of As, identification and examination of factors that affect speciation have become an important issue (Kalia and Joshi, 2009). Speciation analysis is often used as a starting point for understanding how As is transported, accumulated, transformed, and released from an organism.

As enters the marine environment from both natural and anthropogenic sources (Francesconi, 2010), and it is actively transformed by biological systems to create the As biogeocycle (Bhattacharjee and Rosen, 2007). Marine macroalgae are primary producers in marine ecosystems, and they bioaccumulate 1000 times more As from seawater and show sensitivity to environmental changes in As concentrations in seawater (Chaudhuri et al., 2007). Aquatic and terrestrial biota, including algae, have developed multiple accumulation and

biotransformation pathways to detoxify metals and metalloids such as As (Bhattacharya et al., 2015; Mitra et al., 2017; Zhu et al., 2014). The toxicological effects of As on different organisms are considered to be similar, whereas the actual mechanisms, for the most part, underlying toxicity and detoxification are still unclear (Andrewes et al., 2004). Biotransformation of As in algae is initiated with the absorption of As(V), which is reduced to As(III), and subsequent methylation leads to the formation of methylated forms and excretion. Inorganic phosphate (P) has physiochemical properties similar to As(V) and interferes with the cellular uptake of As(V) through phosphate transporters (Sanders and Windom, 1980; Wang et al., 2013; Wang et al., 2015a). However, many studies have shown indiscriminate and P-independent uptake of As(V), suggesting the existence of more than one As(V) uptake mechanism in algae (Duncan et al., 2013; Foster et al., 2008; Klumpp, 1980). Furthermore, biotransformation of As occurs outside and inside the cells of freshwater microalgae, and it is mostly dependent on P concentration in the culture media (Levy et al., 2005). Most of the laboratory studies used unicellular algae from both freshwater and marine habitats for As uptake experiments (Geiszinger et al., 2001; Wang et al., 2015a). However, there have been limited studies on the uptake and biotransformation of As by macroalgae under laboratory conditions with respect to different levels of As(V) and P.

Commercially available edible and environmental samples of macroalgae have been used more extensively for As speciation because of food safety and nutritional studies worldwide (Diaz et al., 2012; Foster and Maher, 2016; Khan et al., 2015b; Ronan et al., 2017; Taylor and Jackson, 2016). However, few studies have reported the biotransformation of arsenicals by marine macroalgae in growth/culture medium (Cullen et al., 1994a; Cullen et al., 1994b; Granchinho et al., 2001). Granchinho et al. (2004) investigated the metabolism of As(V) using marine macroalga *Fucus gardneri* from environmental samples and reported the presence of biotransformed As metabolites in the culture medium. Understanding of the nature and distribution of As species is essential because of their complex chemistry, behavior, and eco-toxicological effects on marine ecosystems. Moreover, different organisms use different processes to metabolize or detoxify As and hence contributes to varying As species in seawater.

It is also necessary to examine the influence of each aquatic species on changes in As speciation through biotransformation. We selected *Undaria pinnatifida* as our study organism because of its As(V) methylation capacity from our preliminary work (Mamun et al., 2017). Metabolic studies of As with this marine macroalga have not been performed previously, and it is unclear whether it can tolerate and biotransform As(V) as many marine organisms do.

Therefore, the laboratory culture experiment was conducted with different As(V) and P abundance ratios in seawater. The objectives of this study were to: (1) analyze As speciation in the culture medium and observe the uptake and biotransformation behavior; (2) examine the bioavailability of As(V) with respect to growth efficiency as well as how As is bioaccumulated in the presence of As(V) and P along with ions like iron (Fe) in the culture medium; and (3) elucidate the mechanism of As(V) tolerance and metabolism by marine macroalgae. To the best of our knowledge, this is the first time biotransformed As species has been measured on a daily basis in the culture medium of macroalgae under laboratory conditions.

4.2 Materials and Methods

4.2.1 Chemicals and Standards

In this study, analytical reagent grade chemicals were used without further purification, unless otherwise stated. Stock solutions and/or working standards were at nM and/or µM levels and prepared by dilution on a weight basis. Either HCl or NaOH (1 M) was used for adjusting the pH when necessary. Standard chemicals of As(V) from $Na_2HAsO_4 \cdot 7H_2O$, MMAA(V) from CH_3AsO_3, and P from KH_2PO_4 were purchased from Wako Pure Chemical Ind. Ltd., Tokyo, Japan; As(III) from As_2O_3, Merck, Tokyo, Japan; and DMAA(V) from $(CH_3)_2AsO_2Na.3H_2O$, Nacalai Tesque, Kyoto, Japan. The solutions were made up with deionized water. The standard metal solution ICP Multi-Element Standard solution IV 1000 ppm (Merck, Tokyo, Japan) was prepared by diluting it with 0.1 M nitric acid and deionized water.

Low-density polyethylene bottles and micropipettes (Nichiryo, Tokyo, Japan) and other laboratory wares used throughout the experiments were cleaned according to the procedure described by Hasegawa et al. (2017). All glassware used for storing the standard solutions or for the algae culture experiments were autoclaved.

4.2.2 Pre-culture and Maintenance of Algae

The juvenile sporophytes of *Undaria pinnatifida* (each ~2 to 3 cm in length) were purchased from Sakaiovex Co. Ltd. (Fukui, Japan) and cultivated at 17 °C in the Environmental Research Laboratory of Nippon Steel and Sumitomo Metal Corporation (Chiba, Japan). Fifty-day-old sporophytes (average length, 4.5 cm; n = 10) were then transported to our laboratory in insulated cool boxes. Individual sporophytes were rinsed in a sterile beaker approximately five times with sterile natural seawater (NSW). Before the main experiments, the sporophytes were allowed to acclimatize for 15 d in 1000 mL glass bottles containing fresh, sterilized NSW enriched with 1% Provasoli enriched seawater (PES) medium (pH: 8.1 ± 0.2). The PES medium

was prepared with deionized water and synthetic salts, according to the method described by Provasoli (1968), and autoclaved at 121 °C for 30 min (pH = 8). The composition of the PES medium is listed in a Table of *Appendix A*. The seawater was collected from the Toyama Bay, Toyama, Japan (37°86′ N, 137°13′ E) and supplied by Namerikawa Ocean Deep Sea Water Facility "Aqua Pocket." The seawater contained no detectable amount of any As species, and seawater quality assessment was performed as described previously (Miki et al., 2016). The seawater used throughout the experiments was filtered through 0.45 µm membrane filters to remove particulates, autoclaved at 121 °C for 30 min, and UV-irradiated for 20 min on a clean bench sprayed with 70% ethanol. The stock culture for the macroalga was maintained in an incubator at an irradiance of 90 µmol photons m^{-2} s^{-1}, 12 h/12 h light/dark cycle, and 10 °C.

4.2.3 Arsenate and Phosphate Treatments and Inoculation of Algae

Culture solutions used for the primary experiment were prepared with 500 mL sterilized NSW enriched with 1% PES medium (without P) in 1000 mL polycarbonate bottles. Two test concentrations of P (1 and 10 µM) and three test concentrations of As(V) (0, 0.1, and 1 µM) were added to each bottle. Different culture media containing As(V) and P in the test solutions are listed in *Table 4.1*. The pH and salinity were adjusted to 8 and 35%, respectively. Before inoculation, acclimated sporophyte of *U. pinnatifida* from the stock culture was removed, washed with deionized water, and rinsed three times with artificial seawater. The composition of artificial seawater was as reported by Lyman and Flemming (1940) and listed in a Table of *Appendix D*. The holdfast from each thallus of algae was removed to minimize excessive nutrient uptake from the medium. Four sporophytes weighing more than 1 g (each ~ 4 to 5 cm in length) were inoculated in each test bottle. The experiment was followed a randomized design with three replications. Cultures without algae were used as the chemical control for monitoring abiotic transformation. All steps performed during the inoculation of the algae were performed under sterile conditions on a clean bench, which ensured minimum biological contamination. The culture bottles were then incubated in an incubator at 45 µmol photons m^{-2} s^{-1}, 18 °C, and 12 h/12 h light/dark period. To ensure an adequate supply of dissolved oxygen to the medium, a silicon plug and tube were connected to the air pump (e-Air 4000WB, GEX) for continuous stirring. The media were not changed during the exposure of As(V) to the cultures. The exposure experiment was performed for 7 d, and 10 mL of the culture medium samples were collected on a daily basis after 24 h of inoculation. The samples were stored in a refrigerator by adding 1% of 1 M HCl, and speciation analysis of As was immediately performed.

Table 4.1: Initial concentrations of arsenate (As(V)) and phosphate (P) in the culture media at the start (day 0) of incubation of *Undaria pinnatifida*.

Type of culture medium	Initial nominal concentration (µM)		Initial measured concentration (µM)		Abbreviation[a]
	As(V)	P	As(V)[b]	P[b]	
No As(V) and low P	0	1	nd[c]	1.84 ± 0.11	$As_0 + P_1$
No As(V) and high P	0	10	nd[c]	10.70 ± 0.91	$As_0 + P_2$
Low As(V) and low P	0.1	1	0.09 ± 0.02	1.71 ± 0.16	$As_1 + P_1$
Low As(V) and high P	0.1	10	0.10 ± 0.01	10.78 ± 0.36	$As_1 + P_2$
High As(V) and low P	1	1	0.96 ± 0.12	1.68 ± 0.29	$As_2 + P_1$
High As(V) and high P	1	10	0.94 ± 0.10	10.72 ± 0.65	$As_2 + P_2$

[a] used in text including figures and tables, [b] values in this column indicate mean ± SD (n = 3), [c] not detected

4.2.4 Analytical Procedures

As species in the growth medium samples were measured using an improved hydride generation technique (Hasegawa et al., 1994). The technique was performed using a flame atomic absorption spectrophotometer (AAS, 170-50A, Hitachi, Japan) and hydride generation apparatus, followed by cold trapping. An As hollow cathode lamp (PerkinElmer, Atomax, USA) was used as the light source. Chromatograms for As were recorded on a chromatogram data processing device (Chromato-PRO, Runtime Instruments) connected to the atomic absorption spectrophotometer. The minimum detectable concentrations were 0.02, 0.11, 0.18, and 0.12 nM for As(III), As(V), MMMA(V), and DMAA(V), respectively.

The total As (TAs) contents in the algal cells were determined by an inductively coupled plasma mass spectrometer (ICP-MS; SPQ 9000, Seiko, Japan). A microwave heat decomposition device (Multiwave 3000; Anton Paar GmbH, Graz, Austria) was used for the sample digestion. Concentrated HNO_3 (65%) and optimized operation conditions for the digestion were selected according to the manufacturer's recommendations. The mixtures after the instrumental reaction procedures were transferred to heat-resistant plastic containers (DigiTUBEs; SCP Science, Japan) with 5 mL of purified water and placed in a heat-block type thermal decomposition system (DigiPREP Jr; SCP Science) for about 5 h at 100 °C until dryness. Then, the contents were redissolved with 2 mL of purified water and filtered through cellulose membrane filters of 0.45 µm pore size (Advantec, Tokyo, Japan).

For analytical quality control and digestion, certified reference material (National Metrology Institute of Japan [NMIJ] CRM 7405-a, No. 265: Trace Elements and Arsenic Compounds in Seaweed [Hijiki]) was purchased from NMIJ, Ibaraki, Japan; it was processed as the samples. The uncertainties of recoveries for As concentrations were within 5% of the certified values.

P and Fe contents of the algal cells were quantified in an inductively coupled plasma atomic emission spectrometer (ICP-AES, iCAP 6300; Thermo Fisher Scientific, Waltham, MA) by using the microwave-assisted digested samples. The P content of the medium samples was also analyzed using ICP-AES.

For chlorophyll fluorescence intensity measurement, pulse amplitude modulation fluorometry (PAM, OS1p; Opti-Sciences, USA) was used. Arium Pro water purification system from Sartorius Stedium Biotech GmbH (Gottingen, Germany) was used to produce the purified water (resistivity > 18.2 MΩ cm).

4.2.5 Data Analysis

4.2.5.1 Growth Rate

Fresh weight of the sporophytes before the experiments (before incubation) and at the end (after incubation) was measured after blotting them dry. The average fresh weight of each replicate was calculated, and daily growth rates (GR [% d^{-1}]) were calculated using the following equation (Loureiro et al., 2012):

$$GR = \left[\frac{ln\left(\frac{W_t}{W_i}\right)}{t}\right] \times 100 \qquad (1)$$

where W_i = initial algal fresh weight (g), W_t = algal fresh weight after 7 d (g), and t = experimental time (d).

4.2.5.2 Chlorophyll Fluorescence

Chlorophyll fluorescence was measured by quantifying the maximum photosystem II (PSII) photochemical efficiency (quantum yield) in actinic light. Maximum quantum yield (F_v'/F_m') was measured using the following equation:

$$\frac{F_v'}{F_m'} = \frac{F_m' - F_o'}{F_m'} \qquad (2)$$

where $F_v´/F_m´$ is the maximum quantum yield (the fraction of energy photochemically converted in PSII), $F_o´$ is the minimum fluorescence yield in light-acclimated state, F_m' is the maximum fluorescence yield in actinic light, and $F_v´$ is the variable fluorescence yield in actinic light ($F_m' - F_o'$).

4.2.5.3 Extracellular and Intracellular As, P, and Fe Determination

After 7 d of culture, the algal biomass was harvested and divided into two parts and weighed. One part of the algal sample was used for the intracellular uptake measurement and washed immediately with 0.05 M Ti(III)-citrate-EDTA reagent for 2 min to remove surface-bound Fe oxides and associated As and/or P. Artificial seawater was used before and after washing with the washing reagent. This reagent is widely used for differentiating extracellular and intracellular trace metals from phytoplankton and macroalgae surfaces (Hudson and Morel, 1989; Miller et al., 2016). The other parts of the algae were washed only with deionized water and used to measure total uptake. The extracellular uptake was calculated by subtracting the intracellular uptake from the total uptake.

4.2.5.4 Release Rate Determination

The release rate of As metabolites (As[III] and DMAA[V]) from the algal biomass into the medium was calculated using by the following equation (Che et al., 2017):

$$R_{As} = \frac{C_{ex} \times V}{DW_a} \times \frac{1}{t} \qquad (3)$$

where R_{As} (μM g^{-1} DW d^{-1}) is the release rate; C_{ex} (μM) represents the concentration of As in the medium; V (L) is the volume of the medium; DW_a (g) represents the algal dry-weight; and t (d) represents the incubation period.

4.2.5.5 Statistical Analysis

Concentrations in the algal tissues were calculated on a dry weight basis. The data of the different parameters were analyzed using one-way analysis of variance (ANOVA) and the statistical package SPSS 22.0 for Windows (IBM Co., NY, USA). Pearson's correlation coefficient (r) was determined using the same statistical package. A significance level of $p < 0.05$ was accepted for all the statistical analysis. The figures were prepared using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

4.3 Results and Discussion

4.3.1 Effects of Arsenate and Phosphate on the Growth of *U. pinnatifida*

After incubation for 7 d with different levels of As(V) and P, the macroalga grew considerably and showed an increase in weight when compared with the initial weight (*Figure 4.1a*). No signs of toxicity were observed during the incubation period, and the thallus looked healthy and remained brown (*Appendix E*). The color of the culture medium samples was also unchanged over the course of the experiment. The growth rate of the alga did not differ significantly under the culture conditions ($p > 0.05$). The highest growth rate (2.4% d^{-1}) was noted in the control culture ($As_0 + P_2$), whereas the lowest (0.5% d^{-1}) was found in the high As(V) and low P-enriched culture ($As_2 + P_1$). It is possible that the higher increase in algal biomass was due to higher uptake of P from As(V)-free cultures (*Figure 4.1a*). Increasing the P supply may have enhanced the tolerance of the alga to As(V). The slightly reduced growth rate may be related to both As stress and intracellular transformation of As(V) to As(III). According to the study by Klumpp (1980), two brown macroalgae, *Fucus spiralis* and *Ascophyllum nodosum*, exposed to 1000 µg L^{-1} As(V) for 14 d showed no adverse effects, although these algae were collected from As-contaminated water. Brown macroalgae accumulate higher amounts of As from the environment and possibly have greater tolerance to As(V) than that by other algal groups. The specific growth rate of *Microcystis aeruginosa* was significantly increased when P was added, which suggests that P reduces As toxicity (Yan et al., 2014). The cell growth of various marine phytoplankton species under 0.5–1 mg L^{-1} P treatments showed comparatively reduced As stress (Karadjova et al., 2008; Wang et al., 2014a), although freshwater algal cells synthesize and use more P transporters to compensate for the P deficiency in the medium (Wang et al., 2013). The brown alga *Fucus serratus* remained healthy in appearance for up to 16 weeks when exposed to 20 µg L^{-1} As(V) (Geiszinger et al., 2001).

4.3.2 Effects of Arsenate and Phosphate on Photosynthetic Activity

Chlorophyll fluorescence was measured to monitor the photosynthetic activity of the macroalga. Different PAM devices offer suitable options for estimating the photosynthetic function of PSII in diverse plant species, including algae and seagrasses. Chlorophyll fluorescence parameters (maximum photochemical yield) are frequently used in field and laboratory experiments, and they provide a lot of evidence on photosynthetic efficiency under particular stresses (Enríquez and Borowitzka, 2010; Mamun et al., 2019a). In this study,

chlorophyll fluorescence values of the alga were recorded before and after incubation with As. The $F_v´/F_m´$ value of algae incubated for 7 d showed no significant differences among different culture conditions ($p > 0.05$). Before As exposure, the average $F_v´/F_m´$ value of the different treatments was 0.72 ± 0.01 (n = 6). However, after 7 d of As exposure, the value of $F_v´/F_m´$ decreased slightly (0.10 to 1.83%), irrespective of As(V) to P ratios in the medium (*Figure 4.1b*). The decreasing trend was more pronounced in the medium with low levels of P. The $F_v´/F_m´$ value in brown algae ranged from 0.7 to 0.8 (Büchel and Wilhelm, 1993), which was consistent with our results (initial $F_v´/F_m´$). The observable and optimum $F_v´/F_m´$ is close to 0.832 ± 0.004 in a wide range of healthy and unstressed vascular plants and macroalgae, whereas lower values indicate stress or photoinhibition and sometimes downregulation of photosynthesis (Chaloub et al., 2010; Maxwell and Johnson, 2000).

4.3.3 Biotransformation and Arsenic Speciation in the Culture Medium

Uptake of As(V) by the macroalga was confirmed by a relative decrease in its concentration in As(V) cultures from the first day of inoculation, followed by a gradual decrease during the rest of the experiment. After the uptake of As(V), different proportions of reduced and methylated As species (As(III) and/or DMAA(V)) were observed throughout the incubation period, indicating biological transformation of As(V) because these species were not detected in the abiotic controls (no algae). The decrease in As(V) was accompanied by an initial increase in As(III) in the early incubation period, along with a gradual increase in DMAA(V) later, presumed by the excretion of biotransformed metabolites from the algal cells. MMAA(V) was under the detectable limit under all the studied culture conditions. However, minimum or no amount of MMAA(V) was detected when the algae were treated with As(V). MMAA(V) is the least transformed metabolite of As and less frequently released from the cells as a free intermediate of unicellular marine algae (Cullen et al., 1994a). The degree of uptake and subsequent transformations of As(V) in a time-dependent manner is related to the P levels in the culture medium. The one-way ANOVA showed that the detected As species under individual culture conditions were significantly different with the increasing exposure time ($p < 0.05$).

4.3.3.1 Low Arsenate and Low Phosphate Medium

The As(V) concentration decreased by 19% on the 1st day of incubation, and the concentration decreased to 72% on the 5th day of incubation. At the end of incubation, As(V) declined by 78% (0.022 µM) from its initial concentration. During the incubation period,

As(III) was found on 2 d, 5 d, and 7 d (0.016, 0.01, and 0.004 µM, respectively). DMAA(V) was observed from the 2nd day (0.008 µM), and the concentration increased and peaked (0.04 µM) on the last day of incubation (*Figure 4.2a*). The rate of decrease in P concentration was comparatively higher than that of As(V), reaching below the detection limit at the end of incubation (*Figure 4.3a*). As a result, the As(V) to P ratio increased in the medium, indicating enhanced uptake of As(V) by the alga. Relatively higher amounts of DMAA(V) were detected in this culture when compared with the cultures of high P and the same amount of As(V) (As_1 + P_2). As shown in *Table 4.2*, DMAA(V) concentration in the medium had a significant positive correlation with simultaneous As(V) depletion. The average release rate of As(III) and DMAA(V) in the medium was 0.0036 and 0.027 µM g^{-1} dry weight d^{-1}, respectively (*Figure 4.4*). Granchinho et al. (2004) found that the addition of a low concentration of P in *Fucus gardneri* culture exposed to As(V) resulted in higher amounts of biotransformed As(III) and DMAA(V) after 28 d. The results suggest the enhanced uptake of As(V) under lower levels of P in the medium for macroalgae.

Table 4.2: Correlation between arsenate (As(V)) depletion and dimethylarsinic acid (DMAA(V)) production during the incubation period of *Undaria pinnatifida*.

Type of culture medium	Pearson correlation (*r*)	Significance (*p*)
Low As(V) and low P (As_1 + P_1)	0.98	0.0002**
Low As(V) and high P (As_1 + P_2)	0.93	0.0025**
High As(V) and low P (As_2 + P_1)	0.92	0.0039**
High As(V) and high P (As_2 + P_2)	0.89	0.0068**

** Correlation is significant at the 0.01 level (2-tailed).

4.3.3.2 Low Arsenate and High Phosphate Medium

Gradual depletion of As(V) concentrations was observed, and the concentration was 0.04 µM on the 7th day. The concentration of As(III) was the highest on the 2nd day (0.018 µM), and it then decreased rapidly on the 3rd day (0.007 µM). The minimum concentration of As(III) was found on the last day of incubation. DMAA(V) was detected from the 3rd day (0.01 µM). The maximum amount of DMAA(V) was observed on the 7th day (0.023 µM) (*Figure 4.2b*). The P in the medium was depleted by 26.7% on the 3rd day of incubation; then, the rate of depletion slowed, ranging from 4.6 to 15.1% (*Figure 4.3a*). In this culture, the growth rate of algae was found to be higher than that in the other cultures exposed to As(V). This might be possible because of the elevated uptake of P. Macroalgae can accumulate large amounts of P

even when the external P levels are high (Schramm, 1991), which is consistent with our results. When the P level increased from 1 to 10 µM at a constant As(V) concentration, the As(V) uptake decreased and P consumption increased. Consequently, lower transformation of As(V) inside the cell along with limited amounts of excretion were observed. The daily As(III) and DMAA(V) release per gram dry biomass of algae was lower when compared with the same concentration of As(V) (*Figure 4.4*). The Pearson correlation analysis showed that the release of DMAA(V) was positively correlated with the relative decrease of As(V) (*Table 4.2*). The results of the lower detectable As(III) and DMAA(V) in this culture were similar to that of *Fucus gardneri* culture with higher P levels (Granchinho et al., 2004).

4.3.3.3 High Arsenate and Low Phosphate Medium

In this culture, As(V) depletion ranged from 0.02 to 0.55 µM during the exposure period, with the production of 0.01 to 0.14 µM As(III) and 0.015 to 0.323 µM DMAA(V) (*Figure 4.2c*). As(III) was detected on 1 d, and it then increased 1.5-fold on 2 d, but decreased by 14.3 to 19.5% from 3 d to 7 d of incubation (*Figure 4.2c*). Higher As(III) levels in the culture medium containing high As(V) (1 µM) at an early stage of incubation may be due to the tolerance mechanism of the algae to overcome intracellular toxicity. Result shows that the As(III) release rate was 0.008 µM g^{-1} dry weight d^{-1} (*Figure 4.4*). Rapid intracellular reduction of As (As(V) to As(III)) at the initial stage of freshwater microalgal culture was also found by Hasegawa et al. (2001); however, in the later stage of incubation, abiotic oxidation or surface oxidation of the algal cells enhanced subsequent decrease of As(III) in the media (Guo et al., 2011; Zhang et al., 2014a). It is also possible to increase the cellular uptake of As(III) through the aquaglyceroporin channel, which is independent of the P transporter. As(III) in the cells needs to be methylated to form DMAA(V) and subsequently released into the medium through passive diffusion. The level of DMAA(V) was maximum on 7 d (0.32 µM), with an average release rate of 0.23 µM g^{-1} dry weight d^{-1} (*Figure 4.4*). Levy et al. (2005) reported that reduction of As(V) to As(III) inside the cells of microalgae and its excretion decreased with the increase in culture period because of additional methylation and release of DMAA(V) into the medium. The depleted concentration of As(V) in the medium was positively correlated with the abundance of DMAA(V) (*Table 2*). P consumption was lower when compared with the culture containing the same concentration of P with 0.1 µM As(V) (*Figure 4.3b*). This may have been due to the competitive behavior of As(V) and P for similar uptake pathways. In fact, the initial As(V) to P ratio in this culture was 1:1.

4.3.3.4 High Arsenate and High Phosphate Medium

The significantly variable contents of As(V) ranged from 0.47 to 0.82 µM during the incubation period. Minimum (0.08 µM) As(III) level was detected on the 1st day, and the maximum (0.12 µM), on the 2nd day of incubation. A constantly decreasing trend of As(III) was observed on four days (2 d to 5 d of incubation). This culture medium showed the highest release of As(III) (0.014 µM g^{-1} dry weight d^{-1}). An increasing trend of DMAA(V) production was observed, except on the 5th day (*Figure 4.2d*). The average release rate was 0.18 µM g^{-1} dry weight d^{-1} (*Figure 4.4*), which was lower and comparable with the low P-containing culture treated with the same concentration of As(V). P was consumed more rapidly (<90%) within a period of five days and remained about 7% on the last day. In contrast, the As(V) uptake rate was relatively lower in the initial period and indicated active phosphate transporters, which helps take up more P. The increased P supply in this culture may explain the inhibition of As(V) uptake, which contributed to reduced methylated metabolites in the culture medium. Similar results have also been reported in microalgal cultures (Guo et al., 2011). Besides, DMAA(V) production and excretion has been observed to decrease with increasing molar ratios of As(V) to P, particularly in freshwater microalgal culture (Hasegawa et al., 2001; Levy et al., 2005).

4.3.4 Bioaccumulation of Total As, Phosphate, and Iron in *U. pinnatifida*

The uptake of total As, P, and Fe in the algal cells was measured (*Figure 4.5*). The extracellular and intracellular fractions as well as the total uptake of As and P by the macroalga were significantly different among the culture conditions ($p < 0.05$). However, Fe content was not significantly affected in the different cultures ($p > 0.05$) (*Appendix F*). As was not detected in the algal tissues of control culture (no As(V)). The uptake of As either intracellularly or extracellularly was lower than that of P and Fe. As shown in *Figure 4.5a*, the extracellular and intracellular concentrations of As increased with increased initial As(V) exposure in the cultures. In addition, relatively more As was bound extracellularly (adsorption) than intracellularly in the high As(V)-containing cultures, irrespective of P addition. When the addition of P was increased to 10 µM from 1 µM, 2.4- and 1.4-fold less total accumulation of As were observed in the media containing 0.1 and 1 µM As(V), respectively. The P uptake (extracellular and intracellular) in the control cultures was comparatively higher than that in the As(V) cultures. Significantly higher contents of P were recorded in the high P-containing cultures, irrespective of As(V) addition. Increasing molar ratio of As(V) to P (1:1) enhanced both intracellular and extracellular P uptake, which was confirmed by increasing ratios of

As(V) to P at the later stage of incubation. The intracellular or absorbed As levels were significantly higher in media with initially low P levels. The competitive behavior of As(V) and P for the same cellular uptake pathways increased the absorption of As(V). The effect of P on the inhibition of As(V) uptake is a common phenomenon in the laboratory culture of macrophytes and macroalgae, and our results are in accordance with those of Rahman et al. (2007) and Mamun et al. (2019a). A considerable amount of P and Fe was adsorbed extracellularly in the control as well as As(V)-treated cultures (*Figure 4.5b & c*). This may be because the stock culture of the alga was maintained in P- and Fe-rich PES medium. Besides, algae grown in the laboratory or occurring naturally have shown evidence of an external pool of Fe (Fe-plaque) on their surfaces (David et al., 1999), and they can sequester a number of nutrients and trace elements, including As (Khan et al., 2016). The Fe-plaque has a strong binding affinity for inorganic anions like $H_2AsO_4^-$ and/or $HAsO_4^{2-}$ and PO_4^{3-}. Although insignificant variations in Fe content were observed in the different cultures, intracellular Fe content was higher than the extracellular content (*Figure 4.5c*). A significant positive correlation was observed between the extracellular Fe and As contents (r = 0.83, p < 0.05), whereas the correlation was insignificant between the intracellular Fe and As contents (r = 0.80, p > 0.05) (*Appendix G*). P not only competes with As(V) during cellular uptake but also adsorbs on Fe oxides/hydroxides. Therefore, reduced As accumulation due to P addition has not always been successful (Zhao et al., 2010). Intracellular As accumulation was negatively correlated with the intracellular P content (*Table S4*). These results indicate that increasing concentrations of As(V) in the culture solution enhanced the competition between As(V) and P for absorption.

4.4 Conclusion

Few studies have focused on the growth of macroalgae with respect to As uptake and biotransformation within the growth media. Moreover, uptake and biotransformation of As(V) by *Undaria pinnatifida* have not yet been elucidated under laboratory conditions. This study shows that the young sporophytes were less susceptible to abiotic stress due to As with respect to growth and photosynthetic activity. P in the medium had a significant inhibitory effect on As(V) uptake, biotransformation, and excretion. The reduction (As(V) to As(III)) and methylation (As(III) to DMAA(V)) of As might be coupled with the biotransformation of As(V) by this alga, but the possible intermediate MMAA(V) was not observed. Biomethylation was found to be the dominant metabolism, and up to 40% and 32% methylated DMAA(V) was excreted from the initially low (0.1 µM) and high (1 µM) As(V)-containing cultures with a constantly low P level (1 µM), respectively. The results also demonstrated that the presence of

Fe in the medium can lead to the formation of Fe-plaque, which sequesters As and P. These findings contribute to a better understanding of the role of As(V) and P molar ratios in As(V) biotransformation, and our insights with respect to the magnitude of the bioavailability and bioaccumulation patterns of As, P, and Fe under laboratory macroalgal culture conditions will contribute to the understanding of As biogeochemical cycling.

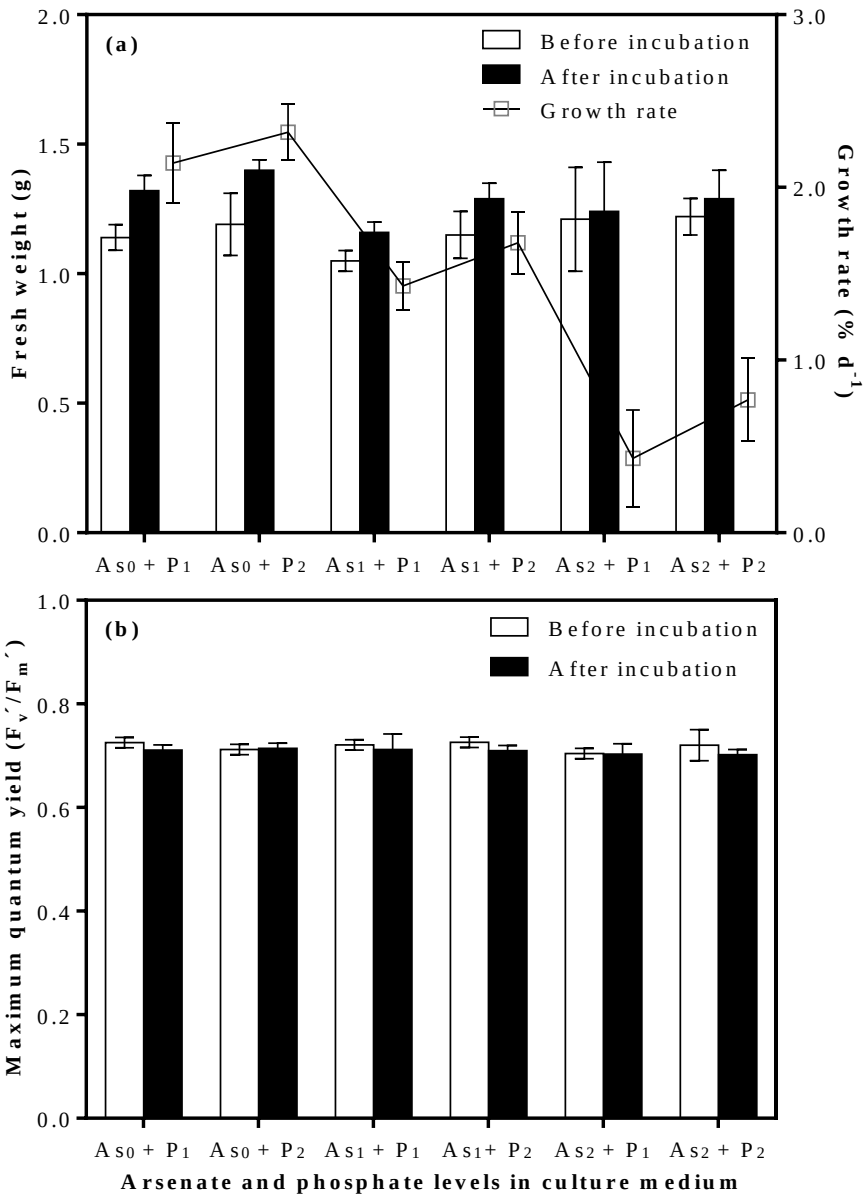

Figure 4.1: Effects of different levels of arsenate and phosphate on the changes in fresh weight and growth rate (a) and maximum quantum yield (b) of *U. pinnatifida*. As_0, As_1, and As_2 denote 0, 0.1, and 1 µM As(V), respectively. P_1 and P_2 denote 1 and 10 µM P, respectively. Data are means ± standard deviation (n = 3).

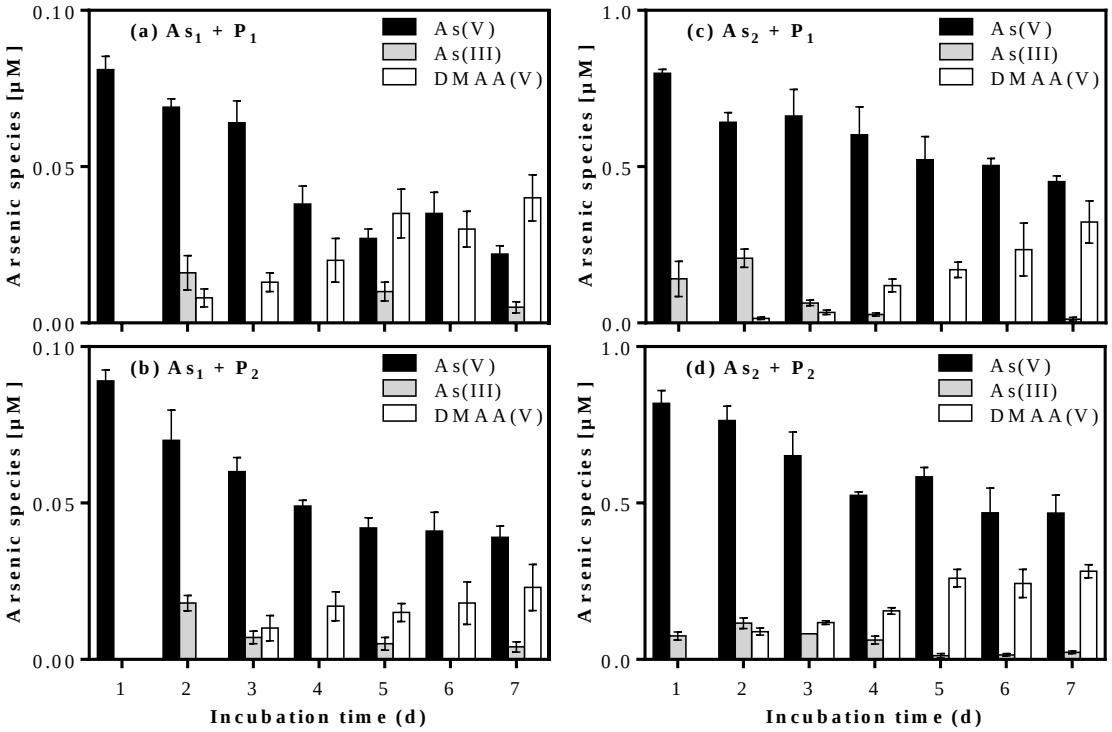

Figure 4.2: Arsenic speciation in the culture medium of *U. pinnatifida* for seven days under $As_1 + P_1$ (a), $As_1 + P_2$ (b), $As_2 + P_1$ (c), and $As_2 + P_2$ (d) growth conditions. As_1 and As_2 denote 0.1 and 1 µM As(V), respectively. P_1 and P_2 denote 1 and 10 µM P, respectively. Data are means ± standard deviation (n = 3).

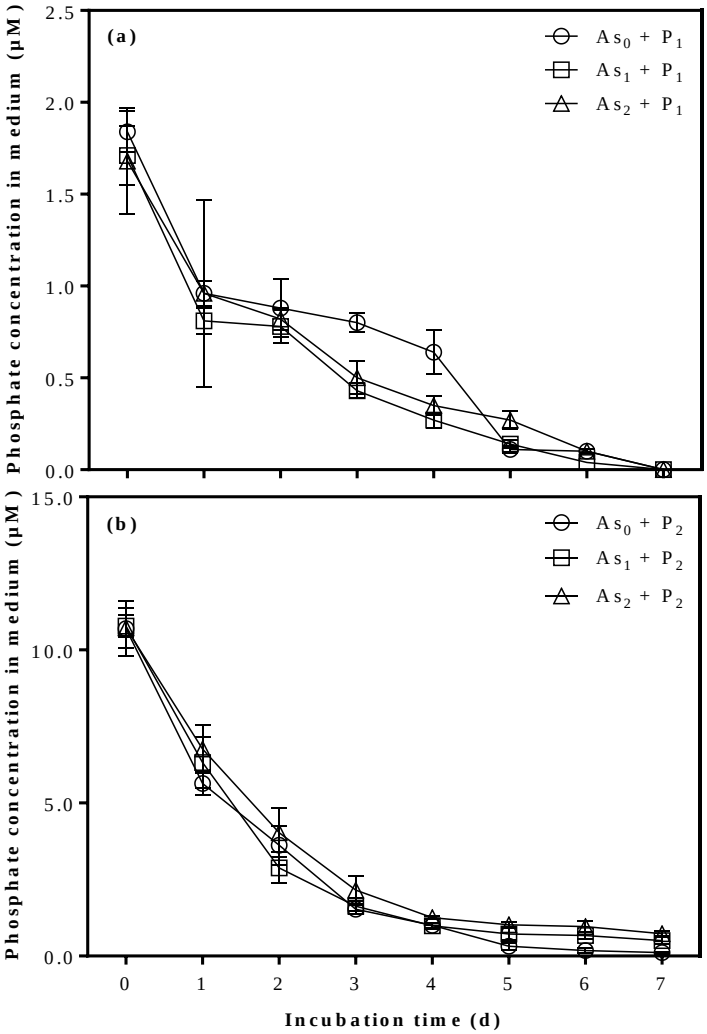

Figure 4.3: Phosphate concentration in the culture medium during the incubation of *U. pinnatifida* exposed to As_0, As_1, and As_2 under P_1 (a) and P_2 (b) growth conditions. As_0, As_1, and As_2 denote 0, 0.1, and 1 µM of As(V), respectively. P_1 and P_2 denote 1 and 10 µM P, respectively. Data are means ± standard deviation (n = 3).

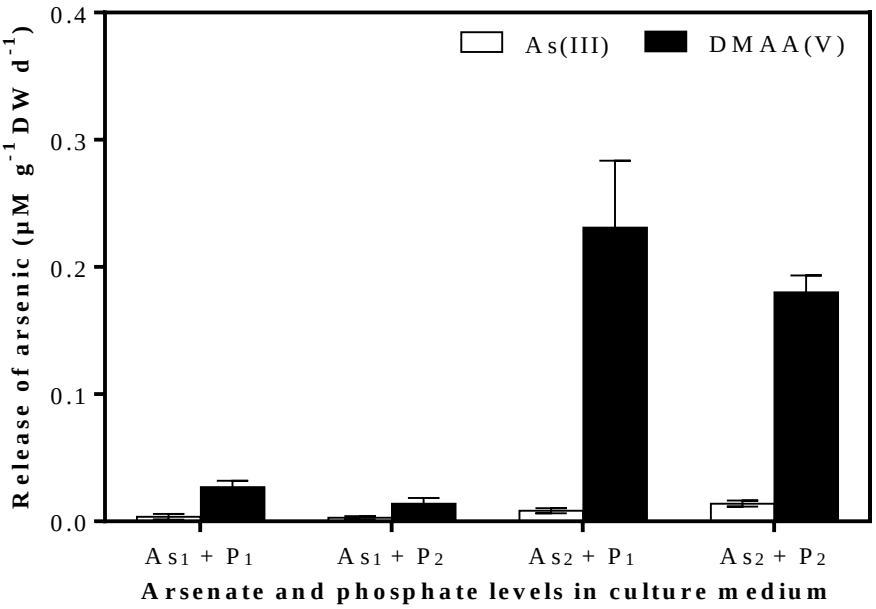

Figure 4.4. The average release rates of different arsenic species after seven days into the culture medium of *U. pinnatifida* exposed to indicated concentrations of arsenate and phosphate. As_1 and As_2 denote 0.1 and 1 µM As(V), respectively. P_1 and P_2 denote 1 and 10 µM P, respectively. Data are means ± standard deviation (n = 3).

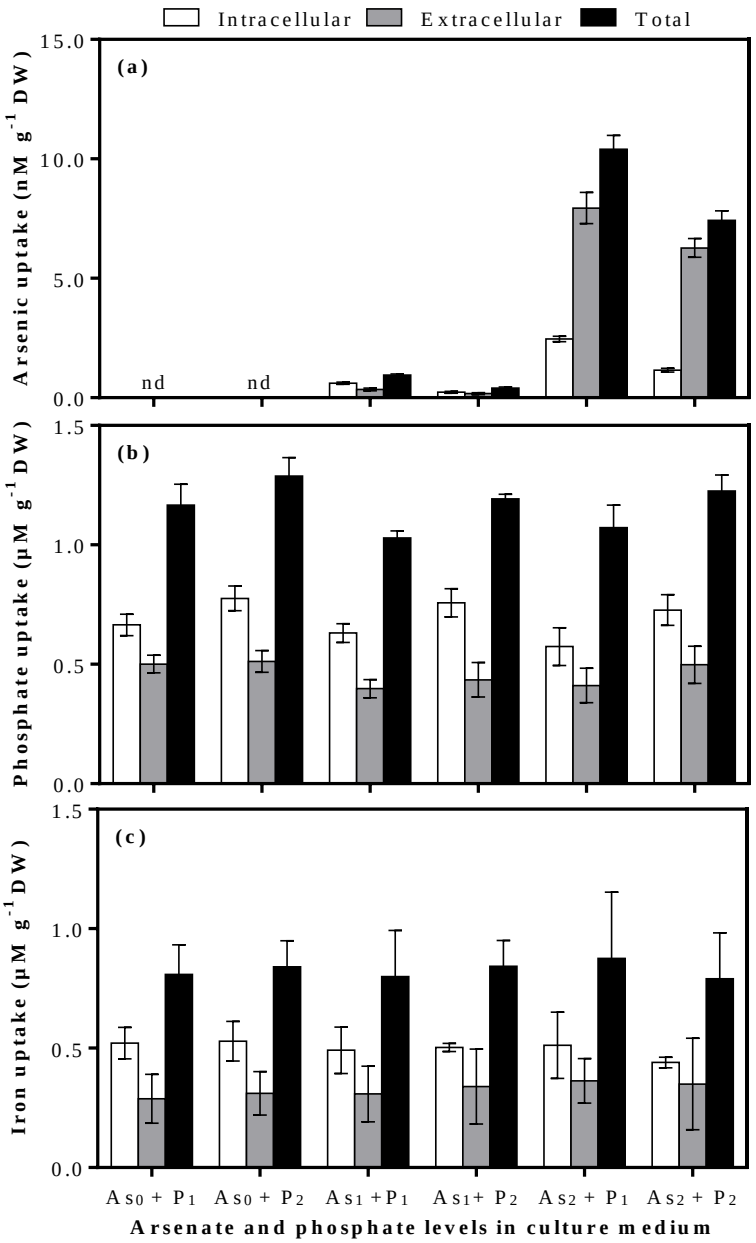

Figure 4.5: Accumulation of total arsenic (a), phosphate (b), and iron (c) by *U. pinnatifida* after seven days of exposure to various indicated concentrations of arsenate and phosphate. As_0, As_1, and As_2 denote 0, 0.1, and 1 µM As(V), respectively. P_1 and P_2 denote 1 and 10 µM P, respectively. Data are means ± standard deviation (n = 3). "nd" indicates not detected.

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Comparative Biotransformation and Detoxification Potential of Arsenic by Three Macroalgae Species in Seawater: Evidence from Laboratory Culture Studies

5.1 Introduction

The metalloid arsenic (As) is found under diverse contamination pools in the environment and considered as a king of poisons (Hughes et al., 2011). As exists in nature as a variety of chemical forms commonly known as arsenicals which are originated by organically and inorganically (Mitra et al., 2017). Different arsenicals in the marine ecosystem have been investigated, and the presence of more than 50 arsenicals in marine organisms have been evidenced recently (Francesconi, 2010). Inorganic As (IAs) like arsenite (+III) and arsenate (+V) are the most abundant forms in aquatic systems and exhibit their potential toxicity to all lives (Bissen and Frimmel, 2003; Tseng, 2009). The methylated As species like mono, di, and trimethylarsinic acids (MMAA(V), DMAA(V) and TMAA(V), respectively) as well as trimethylarsine oxide (TMAO), tetra-methylarsonium ion (TMA) and dimethylarsinoylriboside derivatives (arsenosugars) are comparatively less toxic compared to IAs (Kucuksezgin et al., 2014). It does also appear that arsenobetaine and arsenocholine, the end products of As metabolism in marine plants and animals, are non-toxic and living organisms can tolerate upon their exposure (Francesconi, 2010; Mato-Fernández et al., 2007). As(V), the highest oxidation states of IAs, is the primary bioavailable form in seawater and subjected to chemically- and biologically-mediated transformation with the existence of various arsenicals. Different organisms including algae use different ways to resist and detoxify adverse effect of As including cell-surface adsorption, absorption or storage in the cells (binding with glutathione and/or sequester to vacuoles or vesicles), oxidation, reduction, methylation, demethylation, and excretion (Ma et al., 2018; Mitra et al., 2017; Zhu et al., 2014). The detoxification of As has gained interest to researchers to identify the processes involved with an organism during biotransformation and to investigate how the different biotransformed arsenicals are essential in the context of As biogeochemistry.

Macroalgae efficiently take part in As cycling because of their high affinity for trace metals and showed sensitivity to changes in the concentration of As in seawater (Chaudhuri et al., 2007). The uptake pathways and mode of toxicity of As are entirely different among the organisms, depending on the family, genus, and species (Zhao et al., 2009). It is suggested that

P plays a vital role in cellular uptake and speciation of As in the environment (Sanders and Windom, 1980; Wang et al., 2015a). Algae absorb As in the form of As(V) through phosphate transporters, because of its physicochemical similarities with P ($H_2PO_4^-$ versus $H_2AsO_4^-$) (Taylor and Jackson, 2016). The P-independent uptake of As(V) has also been reported by many researchers indicating the existence of more than one and/or different uptake mechanism in algae (Duncan et al., 2013; Foster et al., 2008; Klumpp, 1980). The biotransformation of As(V) occurs via reduction (As(V) to As(III)) and then via methylation (As(III) to DMAA(V)) inside the cell along with subsequent excretion outside the cells. Freshwater microalgae also exhibit As biotransformation outside and inside of cells depending on P concentration in growth medium (Levy et al., 2005; Wang et al., 2017b). Besides, algae grown in the laboratory or occurring naturally showed evidence of an external pool of Fe (Fe-plaque) on their surface and can sequester As and P (David et al., 1999; Mamun et al., 2019b). However, few studies have dealt with the macroalgal interspecies or intragroup differences on As metabolism, and their biotransformation potential under laboratory culture conditions. Also, there is little information presently available on tolerance to As toxicity and As efflux mechanisms in macroalgae.

Freshwater and marine microalgae have been used more extensively for evaluating their tolerance to As species as well as their uptake and biotransformation potential (Wang et al., 2015a). A number of studies have also investigated the As species of commercially and naturally occurring macroalgae because of food safety and nutritional quality (Diaz et al., 2012; Ma et al., 2018; Rose et al., 2007b; Taylor and Jackson, 2016). However, there have been limited studies on the uptake of As by living macroalgae, and their multiplicity on biotransformation in growth medium (Cullen et al., 1994a; Geiszinger et al., 2001; Sanders and Windom, 1980). Different species of algae use a different mechanism to metabolize As and hence contribute to varying As species in the surrounding environment. Some species of algae such as *Cystoseira* spp. and *Sargassum* spp., have a higher accumulation capacity of IAs from seawater but have limited capacities to biotransform IAs into organoarsinicals (Malea and Kevrekidis, 2014). Other species like *Fucus gardneri* and *Undaria pinnatifida* have the potential to biotransform As(V), and excrete a significant amount of biotransformed As metabolites into surrounding medium (Granchinho et al., 2004; Mamun et al., 2019b). Therefore, considering species-specific As metabolism and detoxification, the responses of specific macroalgae should be investigated to fill in the knowledge gaps. Also, it is important to identify and examine the factors involved in the biotransformation of As. Therefore, a series

of culture experiments were carried out with three different species of macroalgae (Phaeophyceae: *Sargassum horneri*, *Sargassum patens*; and Rhodophyceae: *Pyropia yezoensis*). The objectives of this study include (a) demonstrating how As(V) is biotransformed and extruded from algal species under different molar ratios of As(V)/P; (b) examining the bioavailability of As(V) with respect to growth efficiency as well as how As is accumulated in the presence of As(V) and P along with coexisting ions like iron (Fe) in the culture medium; and (c) elucidating the tolerance and diversity in metabolism of As by the laboratory-grown macroalgae species.

5.2 Materials and Methods

5.2.1 Pre-culture and Maintenance of Macroalgae

The mature and large *S. horneri* and *S. patens*, average length of 50 cm (n = 5), were collected from the coast of Noto Peninsula (facing the Sea of Japan), Ishikawa, Japan, and washed thoroughly on site with seawater. The algae were brought to the laboratory in a cooler box and instantly rewashed with filtered natural seawater (NSW) to remove debris and epiphytic organisms are adhering to the algae. Each alga was then kept in large bottles containing 20 L NSW (30% salinity) enriched with 1% Provasoli enriched seawater (PES) medium (pH: 8.1 ± 0.2). The composition of the PES medium is presented in *Appendix A*. A constant temperature (20 °C) and light (12/12 h light and dark period) with aeration of the bottles were maintained in a constant chamber (RZ-2S, Oriental Giken Industry Co., Ltd., Tokyo, Japan). The seawater medium was refreshed twice a month and 1% PES was added once a week and kept up to three months for maintaining the healthy growth conditions. When new buds appeared and dead tissues from the algae were shredded out, several short shoots (length of 3–4 cm) were excised from each of the large algae, leaving 10 cm between the lower and upper parts of the whole thallus. To reduce the negative effects of excision, these shoots were also kept in 5 L glass flasks containing NSW seawater enriched with 25% PES medium for 24 h (Endo et al., 2013). Asexual monospores of *P. yezoensis* were regenerated in the laboratory and kept in glass bottle containing NSW with 1% PES medium up to two months. The shoots (*S. horneri* and *S. patens*) and spores (*P. yezoensis*) of algae were also maintained in an incubator for another 48 h with 1% PES medium (P-free) until the start of the main experiments. Light was provided by white fluorescent tubes in incubators with a photon flux density of 45 μmol photons m^{-2} s^{-1}, temperature of 20 °C, and photoperiod of 12/12 h light and dark period for *S. horneri* and *S. patens*. In case of *P. yezoensis*, the temperature and light were 15 °C and 10/14 h of light and dark, respectively.

5.2.2 Arsenate and Phosphate Addition to the Culture Medium

Culture solutions used for the main experiment were prepared by 500 mL sterilized NSW enriched with 1% PES medium (P-free) in 1000 mL polycarbonate bottle. Each bottle was then modified by adding three test concentrations of As(V) (0, 0.1 and 1.0 µM) and another two test concentrations of P (1 and 10 µM). A total of six culture combinations were replicated three times following a randomized design for each algal species and presented in *Table 5.1*.

Table 5.1: Initial added concentrations of arsenate (As(V)) and phosphate (P) in the culture media for incubation of macroalgae species

Type of culture medium	Initial nominal concentration (µM)		As(V)/P ratio	Abbreviation[a]
	As(V)	P		
No As(V) and low P	0	1	0:1	$As_0 + P_1$
No As(V) and high P	0	10	0:10	$As_0 + P_2$
Low As(V) and low P	0.1	1	1:10	$As_1 + P_1$
Low As(V) and high P	0.1	10	1:100	$As_1 + P_2$
High As(V) and low P	1	1	1:1	$As_2 + P_1$
High As(V) and high P	1	10	1:10	$As_2 + P_2$

[a] as used in text including figures and tables. The detectable concentrations of PO_4^{3-}–P, total As and Fe were 0.77 ± 0.10, 0.008 ± 0.002, and 0.005 ± 0.001 µM in natural seawater, respectively.

Cultures without algae were set as a chemical control for monitoring abiotic transformation. Before inoculation of algae, acclimated shoots and spore bodies from the stock culture was removed, washed with deionized water (DW) and rinsed three times with artificial seawater (ASW). The composition of ASW is presented in *Appendix D*. Randomly selected shoots of *S. patens* and *S. horneri* (~1.5 to 2 g), and spores of *P. yezoensis* (~0.8 to 1 g) were inoculated in each of the test vessels. All steps performed during the inoculation of algae were performed under sterile conditions on a clean bench (MCV-711ATS, Sanyo Electric Co. Ltd., Osaka, Japan), which ensured minimum biological contamination. The culture flasks with algae were then maintained in an incubator with light/dark cycle (12/12 h for *S. horneri* and *S. patens* and 10/14 h for *P. yezoensis*), irradiance of 45 µmol photons m^{-2} S^{-1}. The temperature was maintained at 20 °C for *S. patens* and *S. horneri*; and 15 °C for *P. yezoensis*. The algae-specific light/dark cycle, temperature, and irradiance were set according to their optimum

growth condition as observed in the pre-culture and other studies with the particular algae (Baba, 2014; Choi et al., 2007; Fukui et al., 2014). Silicon plug and tube was connected to the air pump (e-Air 4000WB, GEX Co. Ltd., China) for continuous stirring to ensure adequate dissolved oxygen in the medium. The media were not changed during the exposure of As(V)/P to the culture. 10 mL of culture medium samples were taken just after inoculation and continued to collect daily up to 7 d. Samples were stored in a refrigerator after adding 1% of 1 M HCl and analyzed for As speciation.

5.2.3 Analytical Procedures

As speciation in the growth medium samples were done by an improved hydride generation technique (Hasegawa et al., 1994). The technique involved using a flame atomic absorption spectrophotometer (AAS) in association with hydride generation apparatus followed by cold trapping (AAS, 170-50A, Hitachi, Japan). An As hollow cathode lamp (PerkinElmer, Atomax, USA) was used as the light source. Chromatograms of As were recorded on a chromatogram data processing device connected to an AAS (Chromato-PRO, Runtime Instruments, Tokyo, Japan). The minimum detectable concentrations were 0.02, 0.11, 0.18, and 0.12 nM for As(III), As(V), MMAA(V), and DMAA(V), respectively. The relative standard deviation (RSD) of As(III), As(V), MMAA(V), and DMAA(V) were 1.3, 2.7, 2.5, and 2.3%, respectively.

The total As (TAs) in the algal cells were determined by an inductively coupled plasma mass spectrometer (ICP-MS; SPQ 9000, Seiko, Japan). The algal biomass after harvesting was divided into two parts and weighed. The surface adsorbed As, P, and Fe from one part of algae were extracted using 0.05 M Ti(III)-citrate-EDTA reagent for 2 min. The concentration of As, P and Fe in the tissues of TiCE washed samples were regarded as an intracellular content. This reagent is widely used for extracting Fe-plaque and associated nutrients and trace metals adsorbed on macroalgae surfaces (Mamun et al., 2019b; Miller et al., 2013). ASW was used before and after washing with the washing reagent followed by thoroughly rinsing with 0.05 M NaCl and DW. The other parts of algae were washed only with DW and used to measure total uptake. The extracellular content was calculated by subtracting the intracellular content from the total content. A microwave heat decomposition device (Multiwave 3000; Anton Paar GmbH, Graz, Austria) was used for the sample digestion. Concentrated HNO_3 (65%) and pre-optimized operating conditions were selected for the digestion following the manufacturer's recommendations. The mixtures obtained after the reaction procedures were transferred to

heat-resistant plastic containers (DigiTUBEs; SCP Science, Japan) with 5 mL DW and placed in a heat-block type thermal decomposition system (DigiPREP Jr; SCP Science) for about 5 h at 100 °C until dryness. Then, the contents were redissolved with 10 mL DW and filtered through cellulose membrane filters (0.45 μm; Advantec, Tokyo, Japan). For analytical quality control and digestion, certified reference material (National Metrology Institute of Japan [NMIJ] CRM 7405-a, No. 265: Trace Elements and Arsenic Compounds in Seaweed [Hijiki]) was purchased from NMIJ (Ibaraki, Japan) and processed as the samples. The uncertainties of recoveries for As concentration was within 5% of the certified values.

P and Fe contents of the algal cells were quantified in an inductively coupled plasma atomic emission spectrometer (ICP-AES, iCAP 6300; Thermo Fisher Scientific, Waltham, MA) by using the microwave-assisted digested samples. The P content of the medium samples was also analyzed using ICP-AES.

For chlorophyll fluorescence intensity measurement, pulse amplitude modulation fluorometry (PAM, OS1p; Opti-Sciences, USA) was used. Arium Pro water purification system from Sartorius Stedium Biotech GmbH (Gottingen, Germany) was used to produce DW (resistivity > 18.2 MΩ cm).

5.2.4 Chemicals and Standards

Analytical reagent grade chemicals were used throughout the study without further purification unless otherwise stated. Stock solutions and/or working standards were nM and/or μM levels and prepared on the day of analysis. Either HCl or NaOH (1 M) was used for adjusting the pH when necessary. Standard solutions of As(V) from $Na_2HAsO_4.7H_2O$, MMAA(V) from CH_3AsO_3, and P from KH_2PO_4 were purchased from Wako Pure Chemical Ind. Ltd. (Tokyo, Japan); As(III) from $NaAs_2O_3$ was purchased from Merck (Tokyo, Japan); and DMAA(V) from $(CH_3)_2AsO_2Na.3H_2O$ was purchased from Nacalai Tesque (Kyoto, Japan). The standard solution of Fe was prepared by dissolving $FeCl_3.6H_2O$ (Wako Pure Chemical Ind. Ltd.) in 1 M HCl and deionized water.

Low-density polyethylene bottles and micropipettes (Nichiryo, Tokyo, Japan) and other laboratory wares used throughout the experiments were cleaned according to the procedure described by Hasegawa et al. (2017). Glassware used for storing the standard solutions or utilized for the algal culture were autoclaved.

5.2.5 Data Analysis

5.2.5.1 Measurement of Chlorophyll Fluorescence

Chlorophyll fluorescence was measured by quantifying the maximum photosystem II (PSII) photochemical efficiency (quantum yield) of open RCIIs. Replicated samples were adapted to the dark for about 15 min before getting the measurements. Maximum quantum yield (F_v/F_m) was measured using the following equation:

$$\frac{F_v}{F_m} = \frac{F_m - F_o}{F_m} \quad (1)$$

In Eq (1), F_v/F_m is the maximum quantum yield; F_o is the minimum fluorescence yield (dark adapted, all RCIIs open); F_m is the maximum fluorescence yield (dark adapted, all RCIIs closed with no NPQ); and F_v is the maximum variable fluorescence yield, ($F_m - F_0$).

5.2.5.2 Measurement of Growth Rate

Fresh weight of the sporophytes before the experiments (before incubation) and at the end (after incubation) was measured after blotting them dry. The average fresh weight of each replicate was calculated, and daily growth rates (GR [% d^{-1}]) were calculated using the following equation:

$$GR = \left[\frac{ln\left(\frac{W_t}{W_i}\right)}{t}\right] \times 100 \quad (2)$$

In Eq (2), W_i is the initial algal fresh weight (g), W_t is the algal fresh weight after 7 d (g), and t is experimental time (d).

5.2.5.3 Release Rate Determination

The release rate of As metabolites (As[III] and DMAA[V]) from the algal biomass into the medium was calculated using by the following equation (Che et al., 2017):

$$R_{As} = \frac{C_{ex} \times V}{DW_a} \times \frac{1}{t} \quad (3)$$

In Eq (3), R_{As} (μM g^{-1} DW d^{-1}) is the release rate; C_{ex} (μM) represents the concentration of As in the medium; V (L) is the volume of the medium; DW_a (g) represents the algal dry-weight, and t (d) represents the incubation period.

2.5.4 Statistical Analysis

Elemental concentrations of As, P, and Fe in tissues were calculated on a dry-weight basis and presented as the mean value ± SD (n = 3). The IBM SPSS 22.0 for Windows (IBM Co., NY, USA) was used to perform one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Also, the *Pearson* correlation coefficient (r) was also determined using the same statistical package. The values of $p < 0.05$ were considered as significant differences among the treatments. The figures were prepared using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

5.3 Results and Discussion

5.3.1 Effects of Arsenate and Phosphate on Chlorophyll Fluorescence

The detection of chlorophyll fluorescence by different PAM devices offer a suitable option for estimating the photosynthetic function of the PSII of diverse plant species including algae and seagrasses. The activity of PSII largely depends on a wide range of stresses like temperature, light, radiation, salinity, drought, metals, pesticides, salts, etc. Chlorophyll fluorescence parameters (maximum photochemical yield, F_v/F_m) are frequently used in field and laboratory experiments and provides a wealth of evidence on the photosynthetic efficiency for particular stresses on plants (Suggett et al., 2010). The F_v/F_m value of the studied macroalgae species were not significantly affected by different cultures after 7 d incubation with As(V) and P ($p > 0.05$). Before As(V) exposure, F_v/F_m of *S. horneri* ranged from 0.70 ± 0.01 to 0.75 ± 0.02, with an average of 0.73 ± 0.02. Similarly, F_v/F_m of *S. patens* and *P. yezoensis* ranged from 0.67 ± 0.02 to 0.75 ± 0.02 (average 0.71 ± 0.02) and 0.52 ± 0.02 to 0.60 ± 0.02 (average 0.56 ± 0.02), respectively. After 7 d of incubation of algal species, results indicated a slightly but non-significantly decreasing trend of F_v/F_m when compared with their respective initial F_v/F_m values (0.7 to 3.1%, 2.1 to 3.0% and 4.8 to 6.1% in *S. horneri*, *S. patens*, and *P. yezoensis*, respectively). These results suggested that As can partly inhibit chlorophyll fluorescence of the tested concentrations of As(V). However, the situation was not consistent with the changes in F_v/F_m of control cultures (no As(V)). It was observed that initial high As(V) concentration (1 µM) produced reasonably lower F_v/F_m of the algae, but cultures enriched with 10 µM P showed relatively higher F_v/F_m than cultures containing 1 µM P (*Figure 5.1*). It was argued that healthy and unstressed vascular plants including macroalgae showed optimum F_v/F_m of 0.832 ± 0.004, whereas the value would be below 0.1 in case of dead materials (Chaloub et al., 2010; Maxwell and Johnson, 2000).

The lower F_v/F_m in algae may reveal stress or photoinhibition and sometimes downregulation of photosynthesis (Velez-Ramirez et al., 2017). The F_v/F_m in brown algae ranged from 0.7 to 0.8, which was in accordance with our results (initial F_v/F_m of *S. horneri* and *S. patens*) (Büchel and Wilhelm, 1993). It was noted that the red algae *P. yezoensis* had comparatively lower F_v/F_m when compared with the brown algae. Different red algae species showed F_v/F_m of around 0.6 (Figueroa et al., 1997; Lüder et al., 2001). The average F_v/F_m value of 0.65 was also reported for four strains of *P. yezoensis* (Zhang et al., 2014b). It was suggested that the pigment composition and cell structures are responsible for variable F_v/F_m value among different taxa and species (Suggett et al., 2009). As toxicity in plants can damage to chloroplast membrane, and disturb to membrane structure resulting in reduced biosynthesis of chlorophyll content (Miteva and Merakchiyska, 2002; Stoeva et al., 2005).

5.3.2 Effects of Arsenate and Phosphate on Growth Rate of the Macroalgae

The exposure of As(V) for up to 7 d did not cause significant visual damages (no observable signs of toxicity) of the studied algal species, looked healthy in appearance and remained intact during the incubation period. The color of culture media also remained unchanged throughout the experiment. Two brown macroalgae (*Fucus spiralis* and *Ascophyllum nodosum*) showed no ill effects when exposed to 1000 µg L^{-1} As(V) for 14 d, although these algae were collected from As contaminated sites (Klumpp, 1980). The brown algae *Fucus serratus* remained healthy in appearance up to 16 weeks when exposed to 20 µg L^{-1} As(V) (Geiszinger et al., 2001). There had a significant difference in the growth rate of algae upon the addition of different concentrations of As(V)/P ($p < 0.05$).

The fresh weight of all algal species after incubation indicated a substantial increase over their initial weight at each culture conditions (*Figure 5.2*). It suggested that the algae were able to adapt and continue their photosynthetic activity resulting in an increase in biomass. The growth rate of different cultures ranged from 0.21 ± 0.07 to 1.04 ± 0.25, 0.43 ± 0.04 to 0.99 ± 0.21 and 0.85 ± 0.35 to 2.05 ± 0.24% d^{-1} in *S. horneri*, *S. patens* and *P. yezoensis*, respectively (*Figure 4.2*). The relatively lower growth rate of all algal species was linked to cultures treated with high As(V) (1 µM). It was also evidenced that control culture with 10 µM P brought about the maximum growth rate for all algal species. Significantly higher growth rate was also noted from As added treatments at 10 µM P, which reflected the positive advantage of P nutrition on As stress and indicate that increase in biomass of algae might be due to higher uptake of P. The increasing P supply in the medium might have the effect on reduced uptake of As(V) that

enhanced the tolerance and stress response to As. Consequently, the increased As(V) accumulation as well as intracellular transformations occurred in cultures with 1:1 ratio of As(V)/P that might be connected with As stress showing reduced growth of the algal species. As(V) is thought to inhibit the synthesis of ATP, causing harmful interference on cellular metabolism (Bhattacharya et al., 2015), and inhibiting cellular division under extreme stress (Levy et al., 2005). P-enriched cultures with As(V) and As(III) up to 13 d had a significantly higher specific growth rate of *Microcystis aeruginosa* than P-limited cultures, suggesting the positive effect of P on reducing As toxicity (Yan et al., 2014). As is found to be accumulated in the plant tissues, thereby reducing the biomass due to the dysfunction of metabolic activities (Drličková et al., 2013). The toxicity of As(V) and As(III) was investigated by Thursby and Steele (1984) using marine macroalga Champia parvula. The result of their study suggested that increasing P concentration (0–9.1 μM) had an inhibitory effect on As(V) toxicity upon vegetative and reproductive growth of the algae.

5.3.3 Biotransformation and Arsenic Speciation in the Culture Medium of Macroalgae

The As(V) uptake by the three species of macroalgae was confirmed by a relative decrease of its concentration from coexisting cultures just after inoculation followed by a simultaneous depletion during rest days of the incubation. Irrespective of algal species, uptake of As(V) and the presence of different proportions of reduced (As(III)) and methylated (DMAA(V)) As species were detected in culture samples throughout the incubation period. It indicated biological transformations because these species (As(III) and/or DMAA(V)) were not detected in the abiotic controls (no algae). The decline of As(V) was accompanied with a concurrent increase in the concentration of As(III) and DMAA(V) in the later days of incubation confirmed the As metabolic process (excretion). MMAA(V) was under the detectable limit by any of the cultures. However, there was an instance of the non-detectable amount of MMAA(V) from the macroalgal culture when treated with As(V) (Mamun et al., 2019b). MMAA(V) is the least transformed metabolized of As species and found to release less frequently from the cells as a free intermediate in unicellular marine algae (Cullen et al., 1994a). The time-dependent magnitude of uptake and excretion of biotransformed As species in the cultures were related to the initial concentration of P. As speciation of individual culture condition was significantly different according to increasing exposure period and algal species ($p < 0.05$).

5.3.3.1 Biotransformation and Arsenic Speciation in the Culture Medium of *S. horneri*

The As(V) concentration in different exposure solutions of *S. horneri* decreased just after inoculation resulting statistically significant differences in concentrations of As(V) in media samples between different days of incubation ($p < 0.05$). The extent of transformation and excretion of As species were varied according to As(V)/P ratios and days of incubation. The minimum As(V) concentration was reached at the end of incubation which was 20, 34, 29 and 40% of initial concentration from $As_1 + P_1$, $As_1 + P_2$, $As_2 + P_1$ and $As_2 + P_2$ cultures, respectively. It was observed that As(III) concentrations constantly increased with increasing time of exposure under all the cultures. It also appeared that relatively higher As(III) were detected in $As_1 + P_1$ and $As_2 + P_1$ cultures, amounting 37 and 17% of initial concentration, respectively (*Figure 5.3*). Though As(V) was the major species detected at each sampling times except 6th and 7th day of $As_2 + P_1$ culture, DMAA(V) was found from cultures with high As(V) levels (1 µM) at the later stage of incubation (5–7 d). After 7 d, the decreasing concentration of As species in $As_2 + P_1$ and $As_2 + P_2$ cultures follow the order: As(V) > As(III) > DMAA(V). When the concentration of P in cultures reached to 10 µM from 1 µM, a 36.6% less As(III) content was recorded with a constant 0.1 µM As(V) at the last day of incubation. Similarly, a 16.3% lees of As(III) and 32.9% less of DMAA(V) was recorded from 1 µM As(V). The release rate of As(III) and DMAA(V) (12.7 ± 1.9 and 1.8 ± 0.5 nM g^{-1} fresh weight d^{-1}, respectively) were maximum in $As_2 + P_1$ culture in *Appendix H*. It was suggested that the low P levels (0 to 0.5 mg L^{-1}) in *Fucus gardneri* culture for 28 d exposure with As(V) had relatively higher amounts of biotransformed metabolites of As(III) and DMAA(V) Granchinho et al. (2004), which was in accordance with our results from low P (1 µM) containing cultures.

The enhanced uptake of As(V) under low P feeding microalgae as well as macroalgae culture might be due to competitive behavior of As(V) and P for cellular uptake through the same transporter. Algal taxonomy, incubation period, and the external As(V) and nutrient concentrations (N and/or P) are the critical factors relating to the pathway of As(V) uptake-transformation (reduction and/or methylation)-excretion process (Wang et al., 2017b). For example, the reduction of As(V) to As(III) and its excretion was accelerated by a low level of external P (Wang et al., 2014b). The possible explanation of methylation under high levels of As(V) (1 µM) in *S. horneri* might be the production of intracellular As(III) at levels where it became saturated leading to As(III) methylation and subsequent excretion. The increasing concentration of As(V) (11.2 to 1120 µg L^{-1}) might induce the production of a minimum amount of DMAA(V) in the *Dunaliella salina* (Wang et al., 2016). The detectable methylated

As species (DMAA(V)) was only found at above 7.5 mg L^{-1} of As(V) exposure concentration in photosynthetic cyanobacteria (Yin et al., 2011).

5.3.3.2 Biotransformation and Arsenic Speciation in the Culture Medium of *S. patens*

As(V) is the dominant As species during the seven days of the exposure period. The As(V) concentration decreased by 70 and 63% from 1 and 10 µM P with a constant 0.1 µM As(V) containing cultures, respectively. Similarly, the decrease was 61 and 57% in high (1 µM) As(V) enriched cultures. The reduced metabolites As(III) was recorded from the 4th and 2nd day under low (0.1 µM) and high (1 µM) As(V) treated cultures, respectively. The rapid uptake of As(V) at the early days of incubation result in a gradual increase of As(III) concentration along the incubation at later days. As(III) concentration found higher at the last of incubation (0.03 ± 0.01, 0.02 ± 0.01, 0.15 ± 0.03, and 0.12 ± 0.03 µM in $As_1 + P_1$, $As_1 + P_2$, $As_2 + P_1$, and $As_2 + P_2$ cultures, respectively) (*Figure 5.4*). This alga showed comparatively lower growth rate under As(V) containing cultures when compared with *S. horneri* and *P. yezoensis*. It is possible that As(III) in combination with a higher ambient As(V) level aggravating As toxicity to this alga. Daily As(III) release (nM) per gram fresh biomass was 2.5 ± 1.2, 2.0 ± 1.1, 12.8 ± 2.1, and 10.5 ± 1.1 in $As_1 + P_1$, $As_1 + P_2$, $As_2 + P_1$, and $As_2 + P_2$ cultures, respectively in *Appendix H*. It was also observed that high levels of P in cultures had a comparatively lower abundance of As(III). Similar results were also reported by Granchinho et al. (2004).

In our previous study, this algae can reduce As(V) to As(III) when exposed to As(V) (Mamun et al., 2019a). The methylation process related to the presence of methyltransferase enzyme in organisms require S-adenosyl-L-methionine (SAM) as the methyl donor and glutathione as the reducing agent. However, it is unknown whether *S. patens* has methyltransferase enzymes in our results. Wang et al. (2016) found that intracellular P could mediate efflux of biotransformed metabolites in the *Dunaliella salina*, and the process was enhanced under low level of intracellular P. *S. patens* contained significantly less amount of cellular P compared to other algal species that could favor the excretion of reduced As(III) into the media probably by an active transport system before being methylated. The discrepancy of metabolism among the macroalgae species could be attributed to the difference in species, and cellular morphology and the transformation process of *S. patens* might support with other studies of microalgae (Cullen et al., 1994a; Levy et al., 2005).

5.3.3.3 Biotransformation and Arsenic Speciation in the Culture Medium of *P. yezoensis*

The biotransformation metabolites in *P. yezoensis* culture showed that As(III) and DMAA(V) were present in each of the cultures during the incubation period (*Figure 5.5*). As(V) was the dominant As species along the incubation period (0–7 d), ranged from 33 to 94%, 52 to 97%, 35 to 101%, and 49 to 103% of initial concentration in As_1 +P_1, As_1 + P_2, As_2 + P_1, and As_2 + P_2 cultures, respectively (*Figure 5.5*). As(III) and DMAA(V) in As_1 + P_1 culture was observed on the 2nd and 6th day of incubation, whereas in As_1 + P_2 culture these species were recorded on the 4th and 6th day. In As_2 + P_1 and As_2 + P_2 cultures, As(III) and DMAA(V) was detected on the 2nd day of incubation with the exception of DMAA(V) from As_2 + P_1 on the 3rd day. It was noted that the detected DMAA(V) concentration was higher than As(III) in both P containing cultures with constantly high levels of As(V) (1 µM), but the more noticeable result was inclined to low P levels (1 µM). It suggests that rapid reduction of As(V) (As(V) to As(III)) and methylation (As(III) to DMAA(V)) inside the cells of this algae lead to higher efflux amounts of DMAA(V) in the medium.

Phytoplankton's usually uptake As(V), subsequently reduce and methylate; and excrete a major portion into the surrounding media. Biotransformation of IAs species plays a key role in As biogeochemistry (Sanders and Windom, 1980). It was evidenced that low levels of external P concentrations could enable As(V) reduction and excretion in microalgae culture (Wang et al., 2014b). The release rate of As(III) and DMAA(V) (7.0 ± 1.0 and 18.8 ± 2.1 nM g^{-1} fresh weight d^{-1}, respectively) in As_2 +P_1 culture was found maximum, whereas in As_1 + P_2 culture rate was minimum (*Appendix H*). The effect of P on the regulation of excretion of As (As(III) and DMAA(V)) in freshwater microalgae was also reported by Hellweger et al. (2003).

The minimum efflux of As(III) and DMAA(V) was found at low and high As(V) containing cultures with a constant 10 µM P. This result highlights the fact that As(V) metabolism and efflux ratio of As is essentially affected by the availability of P in the medium. It was noteworthy that the growth rate of this red algae was relatively higher than the other two species of brown algae. It might be due to the efflux of less toxic organic As species over As(III) in *P. yezoensis* and keep alleviating the inorganic As toxicity. The conversion of intracellular As(V) to As(III), and the subsequent methylation of As(III) to simple methylated and arsenosugars are closely related to the algae-specific growth rate. These biotransformation processes in organisms prevent As(V) from substituting inorganic P in ATP, and As(III) from reacting with the sulfhydryl group of enzyme (Miyashita et al., 2016). This suggested the

intracellular high P in *P. yezoensis* that could prevent the disruption of cellular metabolism. The simultaneous reduction and methylation along with their excretion associated with this alga was similar to *Fucus gardneri* (Granchinho et al., 2004) and such detoxification metabolism might favor the relatively higher growth rate.

5.3.4 Depletion of Phosphate from the Culture Media

The depletion of P occurred so rapidly than As(V) from all the algal culture depending on As(V)/P ratios and significantly varied with the days of incubation. The decreasing trend of P followed the expected pattern, a reckless decline in low of P (1 µM) and reaching below the detection at the end days of incubation. In all species of algae, P depletion from control treatments (no As(V)) occurred rapidly compared to As(V) added treatments. Irrespective of algal species, the P was depleted within the first four days of incubation under a control culture with a low P level. However, the depletion time extended to 1 or 2 days more in As(V) added culture depending on algal species. The P concentration in As(V) added treatments dropped half of their initial concentration within the first few days based on initial addition. When 1 µM P was supplied to *S. horneri*, the least remaining concentration (µM) was 0.07 ± 0.01 (4 d), 0.06 ± 0.02 (5 d), and 0.08 ± 0.03 (6 d) under $As_0 + P_1$, $As_1 + P_1$, and $As_2 + P_1$ cultures, respectively. Accordingly, the concentration (µM) was 0.05 ± 0.02 (4 d), 0.16 ± 0.02 (4 d), and 0.40 ± 0.14 (5 d) in *S. patens* and 0.03 ± 0.01 (4 d), 0.2 ± 0.1 (4 d), and 0.02 ± 0.01 (5 d) in *P. yezoensis*. When the initial concertation of P was 10 µM in *S. horneri*, P levels reached to 0.6, 10.7 and 15.6% ($As_0 + P_2$, $As_1 + P_2$, and $As_2 + P_2$ cultures, respectively) of their initial concentration at the last day of incubation. Similarly, the P levels were 0.7, 5.0, and 13.9% in *S. patens* and 0.8, 1.3, and 4.9% in *P. yezoensis* (*Figure 6.6*). It was observed that P was consumed more quickly by *P. yezoensis* from both low and high P containing cultures than other algal species. P is recognized as a major nutrient to macroalgae required for their growth. It is suggested that macroalgae can accumulate large amounts of P from the high levels of external P (Schramm, 1991).

5.3.5 Bioaccumulation of Total As, P, and Fe in the Macroalgae

The concentrations of As, P, and Fe in the algal cell (extracellular and intracellular fractions as well as the total content) were measured. As and P content in each algal species were significantly different among the cultures ($p < 0.05$), whereas the variation was insignificant for Fe content ($p > 0.05$) in all algal species (*Appendix I*). The extracellular or intracellular accumulation of As was lower than that of P and Fe, and the accumulation was

increased with increasing concentration of initial As(V) exposure to all cultures and algal species (*Appendix J*).

The intracellular As in tissues of all algal species were inhibited due to the addition of high levels P (10 µM) in cultures. When the concentration of P increased from 1 to 10 µM, 21.3, 8.5 and 27.7% reduced uptake of intracellular As were recorded from *S. horneri*, *S. patens*, and *P. yezoensis* culture with the exposure of 1 µM As(V), respectively (*Appendix K*). The intracellular P contents in all species of algal tissues under As(V) free culture were comparatively higher than that in the tissues receiving As(V), whereas the opposite was found in the extracellular content. The intracellular P content was significantly higher in cultures containing 10 µM P, but the concentration decreased with increasing As(V) addition. A negative correlation was observed between intracellular As and P uptake (r = – 0.47, – 0.49, and – 0.69 in *S. horneri*, *S. patens*, and *P. yezoensis*, respectively; $p > 0.05$). It suggested the competitive behavior of As(V) and P for the same cellular uptake pathways, and consistent with increased absorption of As(V) under low levels of P. The effect of P on the inhibition of As(V) uptake was reported by other researchers in the laboratory culture of macroalgae (Mamun et al., 2019a; Mamun et al., 2019b).

A considerable amount of P and Fe was adsorbed extracellularly in control as well as As(V)-treated cultures (*Appendix J*). This happening might be due to the pre-culture and maintenance of algae under P- and Fe-rich PES medium and/or naturally inhabit during collection. It was evidenced that algae grown in the laboratory or occurring naturally showed an external pool of Fe (Fe-plaque) on their surfaces (David et al., 1999). The extracellular and intracellular Fe concentration (mg kg^{-1} dry weight) in different cultures ranged from 10.12 ± 0.6 to 12.05 ± 1.2 and 50.81 ± 6.7 to 55.40 ± 4.4 (*S. horneri*), 8.09 ± 0.8 to 9.30 ± 0.7 and 70.77 ± 7.5 to 75.22 ± 4.9 (*S. patens*), and 2.51 ± 0.8 to 3.71 ± 0.7 and 62.40 ± 3.7 to 68.35 ± 3.5 (*P. yezoensis*), respectively (*Appendix J*). It was observed that the extracellular As and P contents were positively correlated with the extracellular Fe contents for all algal species (*Appendix K*). The Fe-plaque has binding affinity for certain nutrients and trace elements including inorganic anions like $H_2AsO_4^-$ and/or $HAsO_4^{2-}$ and PO_4^{3-} and function as a metal ion buffer for regulating their uptake during deficiency period depending on external concentrations (Jiang et al., 2009; Khan et al., 2016).

5.4 Conclusion

Algae are well known for cycling of As in seawater. However, the role of individual species remains mostly unknown in this context. Moreover, there have been limited studies that have focused on the growth of macroalgae concerning uptake and metabolism of As within the growth media algae system. In this study, accumulation and biotransformation behavior of As by three selected marine macroalgae species were investigated and compared. The results demonstrated that P had a positive influence on the mitigation of As toxicity and algal growth rate, though the different culture conditions showed an insignificant decrease in chlorophyll fluorescence. The cellular uptake of As(V), biotransformation and excretion process is different among the algal species. The overall biotransformation suggests that reduction (As(V) to As(III)) and methylation (As(III) to DMAA(V)) is existed as a detoxification mechanism of As(V) by *Pyropia yezoensis* and *Sargassum horneri*, and only reduction by *Sargassum patens*. Increasing As(V) concentration and/or increasing As(V) to P molar ratios in the exposure solution lead to increasing the excretion of metabolized As. Results also indicated the evidence of Fe plaque on the algal surface that sequestered a certain proportion of As and P. The findings contribute to a better understanding of the species-specific ability to metabolize As(V), and the magnitude of the bioavailability and bioaccumulation patterns of As and P under laboratory macroalgal culture conditions will contribute to the understanding of contamination risks and As biogeochemical cycling.

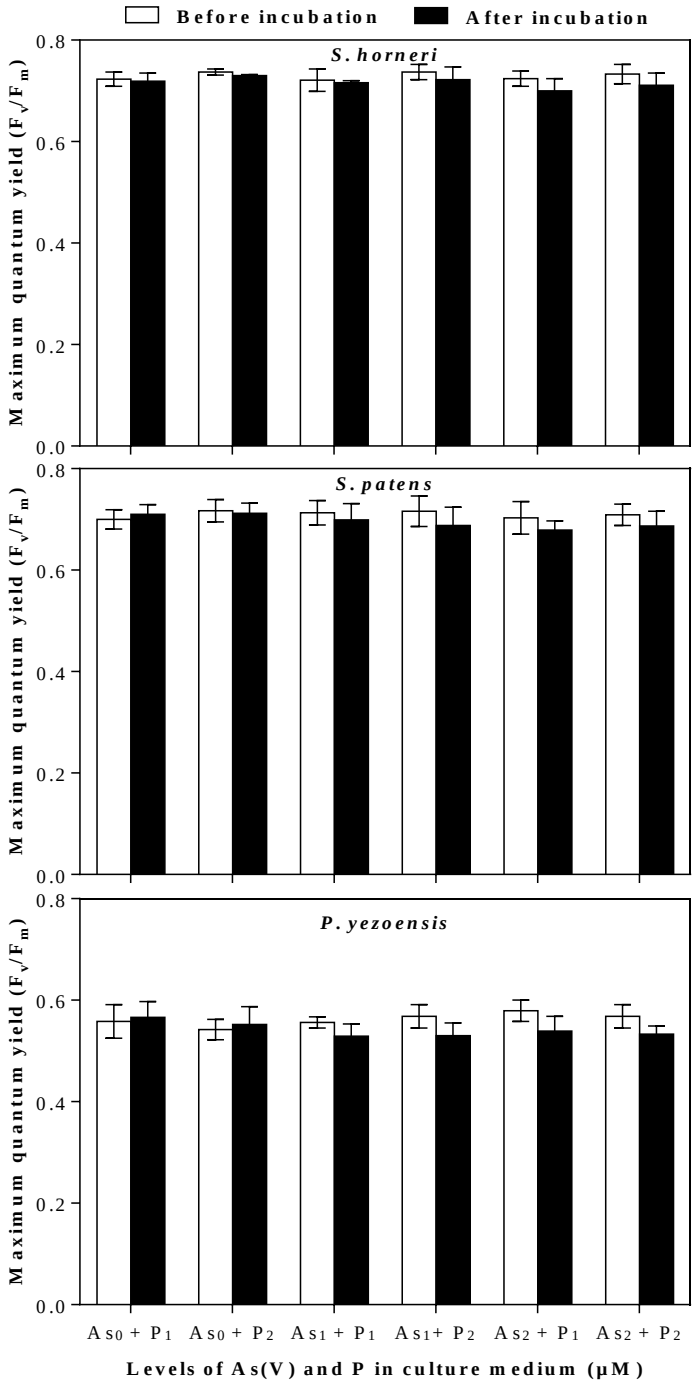

Figure 5.1: Effects of different levels of As(V) and P on the changes in photosynthetic activity measured as maximum quantum yield (F_v/F_m) of three species of macroalgae. As_0, As_1, and As_2 denote 0, 0.1, and 1 µM As(V), respectively. P_1 and P_2 denote 1 and 10 µM P, respectively. Data are means ± standard deviation (n = 3).

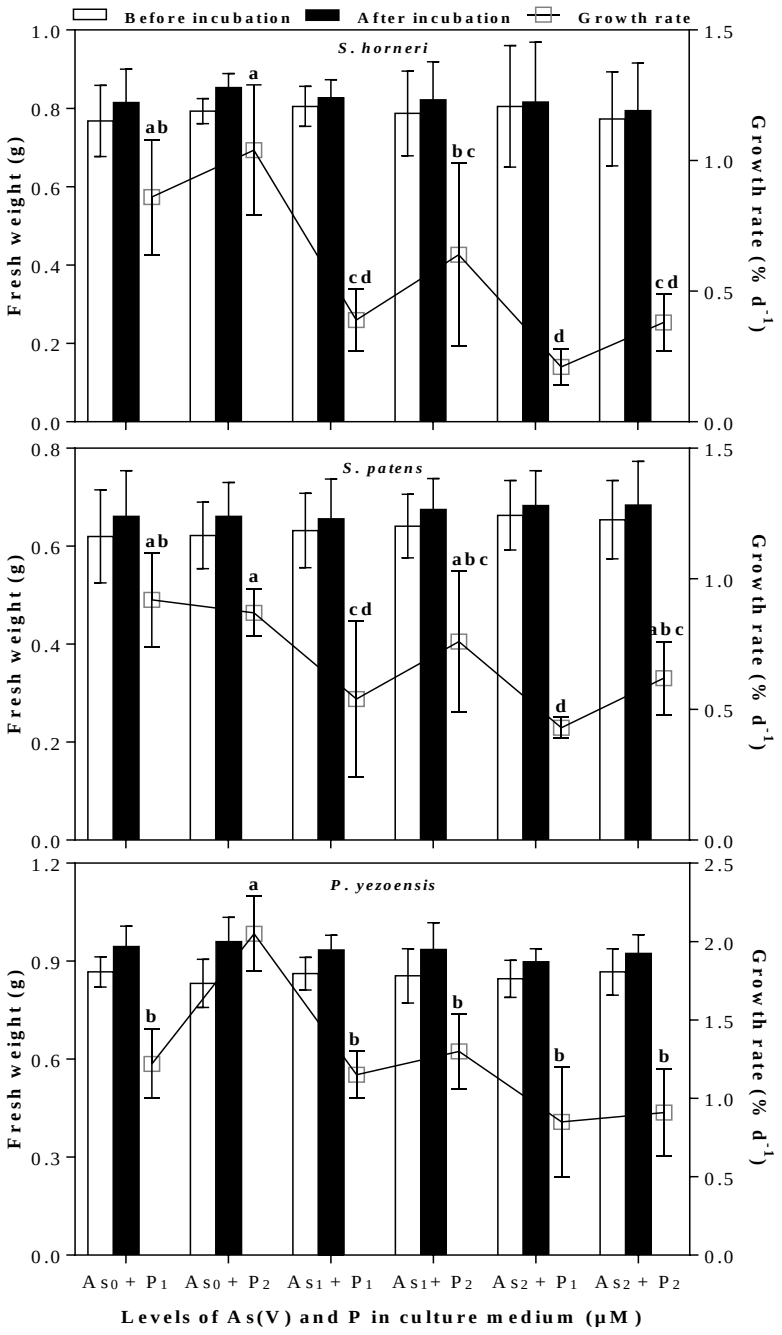

Figure 5.2: Effects of different levels of As(V) and P on the changes in fresh weight and growth rate of three species of macroalgae. As_0, As_1, and As_2 denote 0, 0.1, and 1 µM As(V), respectively. P_1 and P_2 denote 1 and 10 µM P, respectively. Different small letter(s) indicate significant differences among the culture conditions ($p < 0.05$). Data are means ± standard deviation ($n = 3$).

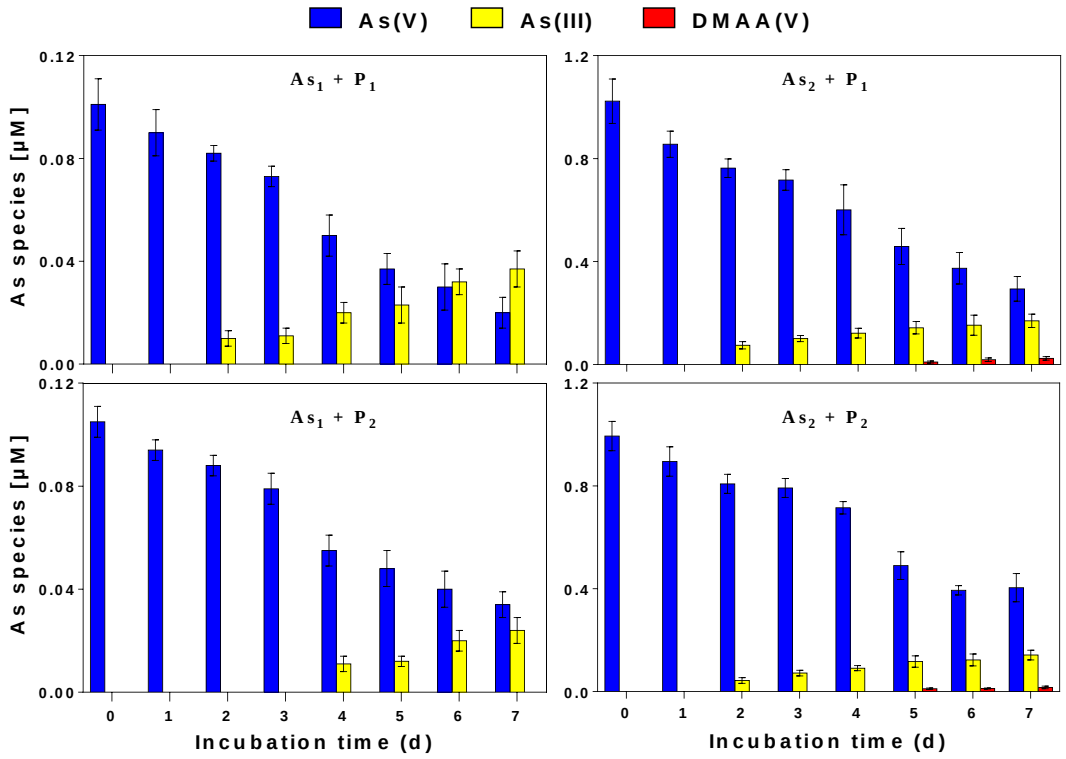

Figure 5.3: Arsenic speciation in the culture medium of *S. horneri* after seven days incubation under $As_1 + P_1$, $As_1 + P_2$, $As_2 + P_1$ and $As_2 + P_2$ culture conditions. As_1 and As_2 denote 0.1 and 1 µM As(V), respectively. P_1 and P_2 denote 1 and 10 µM P, respectively. Data are means ± standard deviation (n = 3).

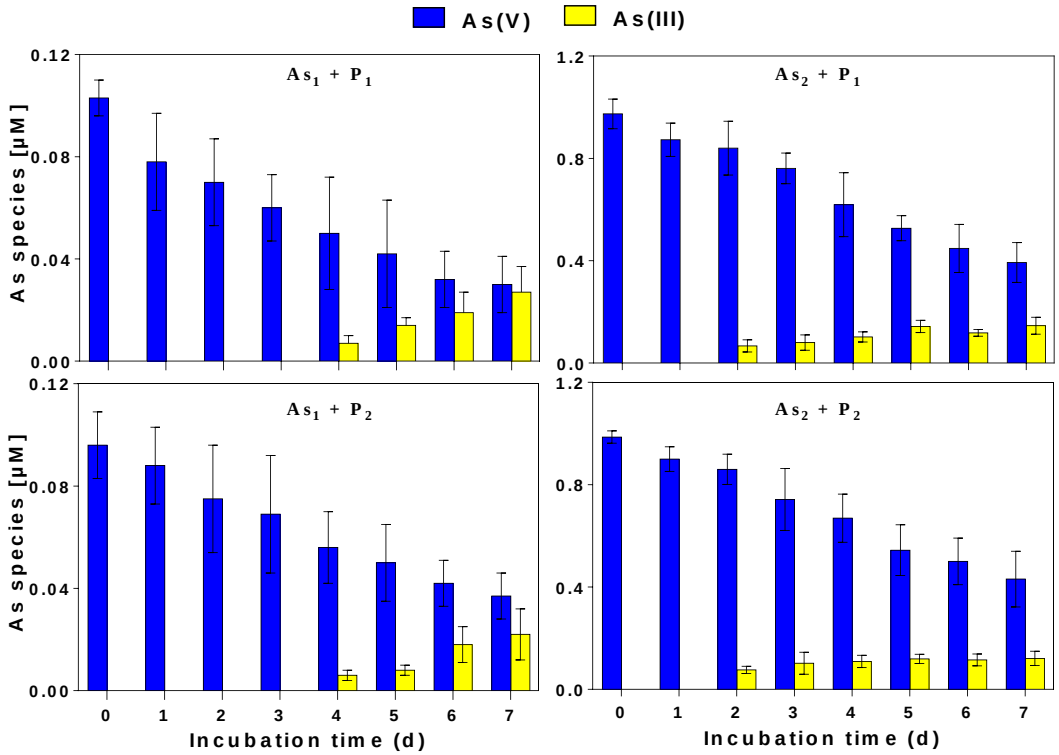

Figure 5.4: Arsenic speciation in the culture medium of *S. patens* after seven days incubation under $As_1 + P_1$, $As_1 + P_2$, $As_2 + P_1$ and $As_2 + P_2$ culture conditions. As_1 and As_2 denote 0.1 and 1 µM As(V), respectively. P_1 and P_2 denote 1 and 10 µM P, respectively. Data are means ± standard deviation (n = 3).

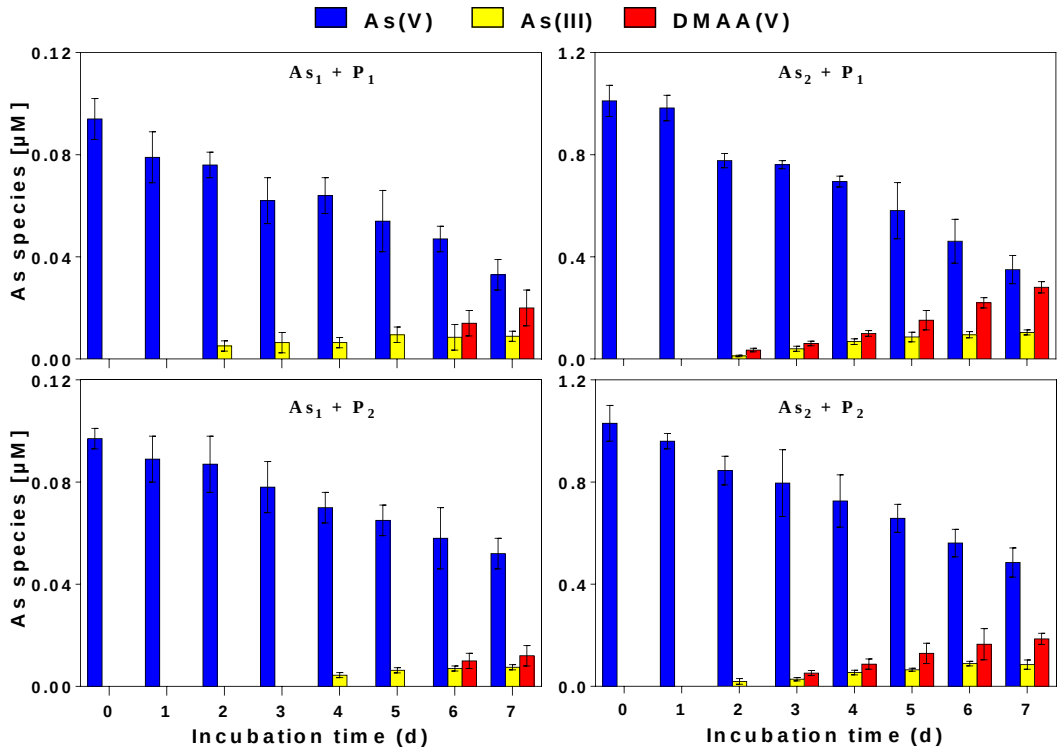

Figure 5.5: Arsenic speciation in the culture medium of *P. yezoensis* after seven days incubation under $As_1 + P_1$, $As_1 + P_2$, $As_2 + P_1$ and $As_2 + P_2$ culture conditions. As_1 and As_2 denote 0.1 and 1 µM As(V), respectively. P_1 and P_2 denote 1 and 10 µM P, respectively. Data are means ± standard deviation (n = 3).

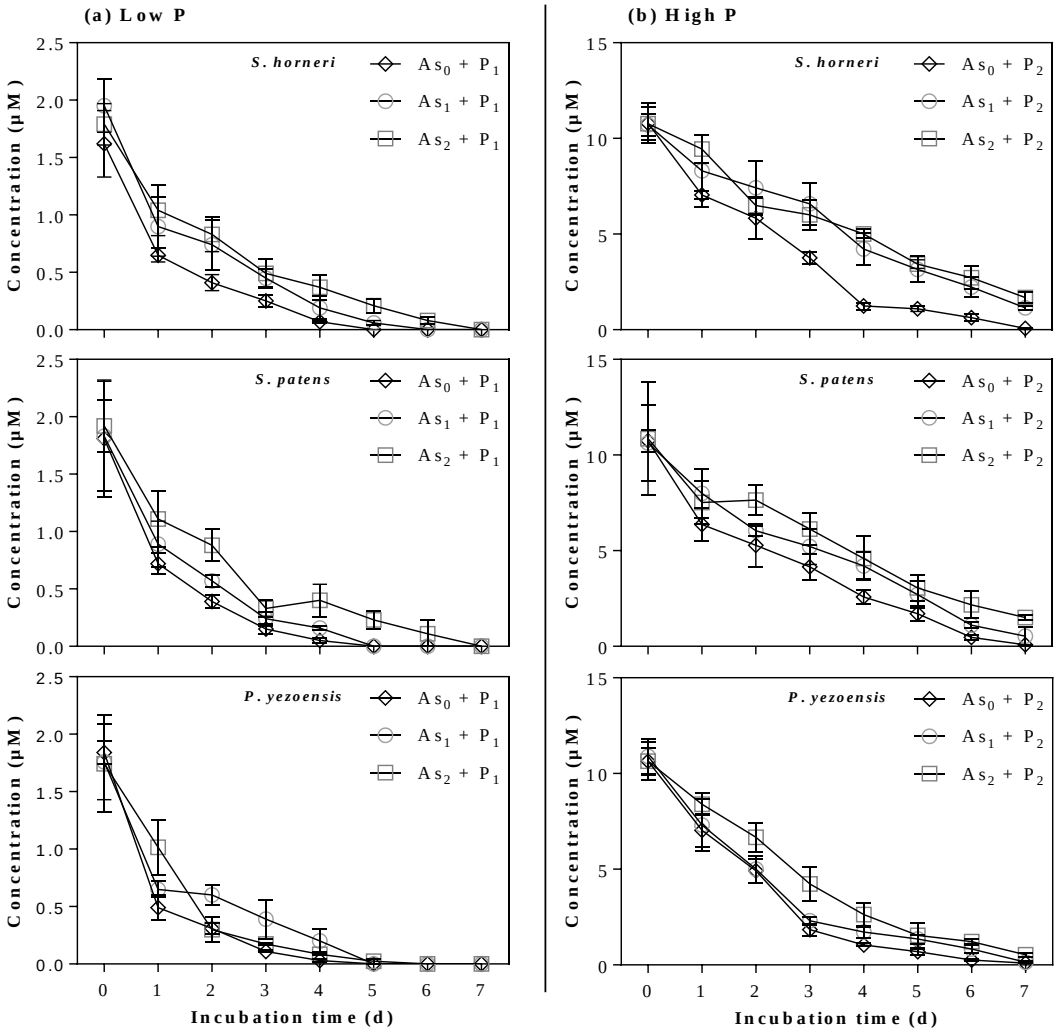

Figure 5.6: Phosphate concentration in the culture medium during the incubation period of macroalgae species exposed to low (a) and high (b) P containing cultures with As(V). As_0, As_1, and As_2 denote 0, 0.1, and 1 µM of As(V), respectively. P_1 and P_2 denote 1 and 10 µM P, respectively. Data are means ± standard deviation (n = 3).

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Bioaccumulation and Biotransformation of Arsenic by the Brown Macroalga *Sargassum patens* C. Agardh in Seawater: Effects of Phosphate and Iron Ions

6.1 Introduction

Arsenic (As) is ubiquitous and the most diverse food chain contaminant in the environment that is mainly released from natural and anthropogenic sources (Mohan and Pittman Jr, 2007). This notorious and toxic metalloid exists in four oxidation states—As(V), As(III), As(0), and As(-III), with a variety of chemical forms that are formed organically and inorganically (Mitra et al., 2017). Arsenate (As(V) as $H_2AsO_4^-$ and $HAsO_4^{2-}$) is the predominant inorganic As (IAs) species in aqueous and aerobic environments, and is strongly adsorbed onto the surface of several aquatic organisms and oxidized minerals of Fe, Mn, and Al. Arsenite (As(III) as $H_3AsO_3^0$ and $H_2AsO_3^-$), another IAs species, is highly abundant under anoxic environments, but is adsorbed weakly onto fewer minerals, making it a more mobile oxyanion (Smedley and Kinniburgh, 2002). In aqueous environments, As has a very diverse and complex chemistry, and IAs species are the major bioavailable forms in seawater. IAs species are subjected to chemically- and biologically-mediated oxidation, reduction, methylation, demethylation, and other reactions through which different organisms resist the adverse effect of As.

The uptake pathways and mode of toxicity of IAs are completely different among the organisms (Zhao et al., 2009). As is absorbed by algae in the As(V) oxidation state through phosphate transporters, owing to its physicochemical similarities with P (H_2PO^{4-} versus H_2AsO^{4-}) (Taylor and Jackson, 2016), whereas As(III) is thought to be transported through an aquaglyceroporin channel as neutral $As(OH)_3$ (Meharg, 2004). Freshwater and marine algae are the group of organisms most vulnerable to IAs toxicity (Rahman et al., 2012). A number of studies have been performed on aquatic organisms, mostly on microalgae, to evaluate their tolerance to IAs, as well as IAs bioaccumulation and biotransformation. Numerous studies have also investigated As speciation and biosorption in the nonliving biomass of both field-observable and commercially available edible macroalgae around the world (Rose et al., 2007a; Pennesi et al., 2012; Khan et al., 2015a; Taylor and Jackson, 2016). However, there have been limited studies on IAs toxicity and bioaccumulation in living macroalgae under laboratory culture conditions.

Marine macroalgae have well-recognized mechanisms for retention of potential toxic metallic ions through biochemical, chemical, and physical processes. Bioaccumulation is one of the most important intracellular mechanisms, whereby pollutants are incorporated in cystine rich proteins, as well as immobilized and/or sequestered in vacuoles and vesicles through enzymatic action (Pinto et al., 2003; Li et al., 2015). Bioavailability and bioaccumulation of As by the marine organisms largely depend upon the As species (Casado-Martinez et al., 2010; Maher et al., 2011), and therefore, the abundance of inorganic and/or organic species of As are potentially important to explain the difference in bioaccumulation among marine algae. The accumulation of As is affected by the family, genus, and species of macroalgae, and the total As concentrations in algal tissues were found to be 1000 to 50000-fold higher than that in seawater (Ma et al., 2018). Macroalgae, particularly the members of Phaeophyceae (brown algae), can resist and tolerate environments highly polluted with metal (Davis et al., 2003; Luna et al., 2010), and have been demonstrated to have enormous accumulation capacities for As, when compared with other groups such as Chlorophyceae (green algae) and Rhodophyceae (red algae) (Tukai et al., 2002; Farias et al., 2007; Brito et al., 2012; Malea and Kevrekidis, 2014; Sartal et al., 2014; Ma et al., 2018; Squadrone et al., 2018). The cell wall compounds, especially alginate and fucoidan of brown algae, are largely responsible for the higher metal chelation capability of these algae (Davis et al., 2003; Ghimire et al., 2008). Besides, algae grown in the laboratory or occurring naturally showed evidence of an external pool of Fe (Fe-plaque), which are non-specifically adsorbed and tightly complexed or scavenged on their surface in the presence of strong natural chelates that can sequester a number of nutrients and trace elements including As (Rue and Bruland, 1995; David et al., 1999; Khan et al., 2016). However, little is known about the accumulation potential of As species in terms of Fe-plaque and macroalgal interspecies or intragroup differences.

A number of *ex* and *in situ* studies have been carried out concerning the accumulation of trace elements in macroalgae, but less frequently reported elements include As. A literature survey demonstrated that *Sargassum* spp. is associated with relatively higher biomass production and metal binding capacity than the other genera of algae (Hashim and Chu, 2004). Also, there are some species, such as *Cystoseira* spp., that have a higher retention capacity of IAs from seawater but have limited capacities to biotransform IAs into organoarsinicals (Malea and Kevrekidis, 2014). Therefore, considering the species-specific accumulation capacity of As, the responses of specific macroalgae to particular metal tolerance and sensitivity, especially that of As, should be investigated, to fill in the gaps in knowledge in this respect. We selected

Sargassum patens as our study organism because of its higher As uptake capacity (Mamun et al., 2017); until now, the studies with this alga in laboratory conditions have been lacking. This study examined the difference between the bioavailability of two important and dominant IAs species—As(V) and As(III)—to *Sargassum patens*, in relation to growth efficiency, as well as investigated how As bioaccumulation and biotransformation is controlled in the presence of Fe and P ions in the growth medium. The effects of the differences in As species on the interactions of As-Fe and/or P ions due to surface adsorption of Fe-plaque was also investigated.

6.2 Materials and Methods

6.2.1 Pre-culture and Maintenance of Macroalgae

The mature and large *S. patens* specimens, average length of 45 cm (n = 10), were collected from the coast of Noto Peninsula (facing the Sea of Japan), Ishikawa, Japan, and washed thoroughly on site with seawater. The algae were brought to the laboratory in a cooled box and instantly rewashed with filtered natural seawater (NSW) to remove debris and epiphytic organisms adhering to the algae. Individual specimens were then kept in large buckets containing 20 L of NSW (30% salinity) enriched with 1% Provasoli enriched seawater (PES) medium (pH: 8.1 ± 0.2). The composition of PES medium is presented in (*Appendix A*). The buckets were placed in a constant temperature (20 °C) and light (12:12 h light and dark period) chamber with aeration (RZ-2S, Oriental Giken Industry Co., Ltd., Tokyo, Japan). The medium was refreshed every week and kept up to six weeks for maintaining the healthy growth conditions of algae. When new buds appeared and dead tissues from the algae were shredded out, several short shoots (length of 4–5 cm) without vesicles were excised from each of the large algae, leaving 10 cm between the lower and upper parts of the thallus. To reduce the negative effects of excision, these shoots were also kept in 5 L glass flasks containing NSW seawater enriched with 25% PES medium for 24 h (Endo et al., 2013). The shoots were also maintained in an incubator for another 48 h with 1% PES medium (P and Fe free), under aeration and optimal growth temperature (20 °C) until the start of the main experiments. Light was provided by white fluorescent tubes in incubators with a photon flux density of 45 µmol photons m^{-2} s^{-1} and photoperiod of 12:12 h light and dark period.

6.2.2 Iron, phosphate, and arsenic treatments of the culture

Culture solutions were prepared in 500 mL polycarbonate bottle containing 250 mL sterilized NSW enriched with 1% PES medium (without P and Fe). The test concentrations of As (0.25, 0.5, 1.0, 2.0 and 4.0 µM) either as As(V) or As(III) along with a control (0 µM) were added to four different P and Fe containing culture groups shown in *Table 6.1*. A total of 24

treatment combinations were replicated three times following a randomized design. Fe and P was used as Fe^{3+}-EDTA and KH_2PO_4, respectively. The sources of As(III) and As(V) were $NaAs_2O_3$ and $Na_2HAsO_4.7H_2O$, respectively. Before inoculation and growth experiments, shoots from the stock culture were removed and rinsed thrice with deionized water. Three to four randomly selected shoots (fresh weight between 1.5 to 2.0 g) were inoculated in each of the test vessels. All steps of the inoculation were performed under sterile conditions in a clean bench (MCV-711ATS, Sanyo Electric Co. Ltd., Osaka, Japan). The culture flasks were then incubated for seven days in an incubator with a 12/12 h light/dark cycle, irradiance of 45 µmol photons m^{-2} S^{-1}, and 20°C. Culture samples (10 mL) were collected at the last day of incubation and stored in a refrigerator by adding 1% of 1 M HCl.

Table 6.1: Initial added concentration of P and Fe into the culture medium of *Sargassum patens*

P and Fe treatments	Concentration (µM)		Abbreviation*
	P	Fe	
P-limited + Fe-limited	0	0	–P–Fe
P-limited + Fe-rich	0	10	–P+Fe
P-rich + Fe-limited	10	0	+P–Fe
P-rich + Fe-rich	10	10	+P+Fe

'*' used for next parts of the manuscript including Tables and Figures. The detected PO_4^{3-}–P, total As and Fe concentration in NSW was 0.34, 0.01 and 0.006 µM, respectively.

6.2.3 Analytical procedures

As species in the growth medium samples were measured by an improved hydride generation technique (Hasegawa et al., 1994). The technique involved using a flame atomic absorption spectrophotometer (AAS) in association with hydride generation apparatus followed by cold trapping (AAS, 170-50A, Hitachi, Japan). An As hollow cathode lamp (PerkinElmer, Atomax, USA) was used as the light source. Chromatograms of As were recorded on a chromatogram data processing device connected to an AAS (Chromato-PRO, Runtime Instruments, Tokyo, Japan). The minimum detectable concentrations were 0.10, 0.11, 0.18, and 0.12 nM for As(III), As(V), monomethylarsinic acid (MMAA(V)), and dimethylarsinic acid (DMAA(V)), respectively.

The surface adsorbed As, P, and Fe from the algae were extracted using the citrate-bicarbonate ethylenediaminetetraacetate (CBE) technique (Rahman et al., 2008b). The CBE solution was prepared from 0.03, 0.125, and 0.05 M of sodium citrate, sodium bicarbonate, and

EDTA, respectively. Additionally, 0.025 M NaCl and 0.05 M KCl were added to the CBE solution (pH was adjusted to 8.0 using $NaHCO_3$). The shoots after incubation were treated with 30 mL of CBE solution for 30 min at room temperature (25 °C). The samples were then washed and rinsed with deionized water for three times, and rinsed water was added to the CBE extracts to a volume of 50 mL. The concentrations in CBE extracts and tissues were measured as extracellular and intracellular fractions, respectively.

The total As content in the tissues and CBE extracts were determined by an inductively coupled plasma mass spectrometer (ICP-MS, SPQ 9000, Seiko, Japan) following microwave digestion with concentrated HNO_3 (65%). A microwave heat decomposition device (Multiwave 3000, Anton Paar GmbH, Graz, Austria) and optimized operation conditions for the digestion were chosen according to the manufacturer's recommendations. The mixtures after the instrumental reactions were transferred to heat resistant plastic containers (DigiTUBEs, SCP Science, Japan) with 5.0 mL of purified water and placed into a heat-block type thermal decomposition system (DigiPREP Jr, SCP Science, Japan) for about 5 h at 100 °C, until the samples dried. Then, the contents were redissolved with 10 mL of purified water and filtered through cellulose membrane filters with a 0.45 µm pore size (Advantec, Tokyo, Japan). The certified standard reference materials 1571a (Tomato leaf, NIST, USA) was used for checking the accuracy of digestion, extraction, and measurement procedures. The uncertainties of recoveries for As concentrations were within 5% of the certified values. The Fe and P content of the digested samples were quantified in an inductively coupled plasma atomic emission spectrometer (ICP-AES, iCAP 6300, Thermo Scientific, Waltham, MA). Arium Pro water purification system from Sartorius Stedium Biotech GmbH (Gottingen, Germany) was used to produce the purified water (resistivity > 18.2 MΩ cm).

6.2.4 Chemicals and Standards

Analytical reagent grade chemicals were used throughout the study without further purification unless otherwise stated. Stock solutions and/or working standards were nmol and/or µmol levels and prepared on the day of analysis. Either HCl or NaOH (1 M) was used for adjusting the pH when necessary. Standard solutions of As(V) from $Na_2HAsO_4.7H_2O$, MMAA(V) from CH_3AsO_3, and P from KH_2PO_4 were purchased from Wako Pure Chemical Ind. Ltd. (Tokyo, Japan); As(III) from $NaAs_2O_3$ was purchased from Merck (Tokyo, Japan); and DMAA(V) from $(CH_3)_2AsO_2Na.3H_2O$ was purchased from Nacalai Tesque (Kyoto, Japan). The standard solution of Fe was prepared by dissolving $FeCl_3.6H_2O$ (Wako Pure Chemical Ind. Ltd.) in 1 M HCl and deionized water.

Low-density polyethylene bottles and micropipettes (Nichiryo, Tokyo, Japan) and other laboratory wares used throughout the experiments were cleaned according to the procedure described by Hasegawa et al. (2017). Glasswares used for storing the standard solutions or used for the algal culture were autoclaved.

6.2.5 Measurement of Chlorophyll Fluorescence

The measurement of chlorophyll fluorescence was carried out by quantifying the maximum photochemical efficiency (quantum yield) of open RCIIs by using pulse amplitude modulation fluorometry (PAM, OS1p, Opti-Sciences, USA). Replicated samples were adapted to the dark for about 15 min prior to getting the measurements. The following equation was used for expressing maximum quantum yield that described elsewhere (Cosgrove and Borowitzka, 2010):

$$F_v/F_m = [(F_m - F_0)/(F_m)]$$

Where, F_v/F_m is the maximum quantum yield; F_0 is the minimum fluorescence yield (dark adapted, all RCIIs open); F_m is the maximum fluorescence yield (dark adapted, all RCIIs colsed with no NPQ); and F_v is the maximum variable fluorescence yield, ($F_m - F_0$).

6.2.6 Measurement of Growth Rate

The fresh weights of the shoots prior to the experiment (before incubation) and at the end (after final incubation) were measured using an electrical balance (0.10 mg accuracy) after drying. The average fresh weights for each replicate were calculated, and daily growth rates (GRs) were calculated using the following equation (Loureiro et al., 2012):

$$GR\ [\%\ day^{-1}] = \left[\frac{\ln\left(\frac{Wt}{Wi}\right)}{t}\right] \times\ 100$$

Where, W_i = initial weight, W_t = weight after 7 days, and t = experimental time in days.

2.7 Modeling of Uptake Kinetics

The Michaelis-Menten equation was used to describe the uptake kinetics of As over time in a concentration-dependent manner:

$$V = \frac{Vmax\ [As]}{Km + [As]}$$

Where [As] is the concentration of As in μM. The maximum uptake rate expressed as *V*max (nM) indicates the maximum uptake rate of As. *K*m (μM) is the Michaelis-Menten constant. The uptake rates of As were estimated by $K = (C_{t1} - C_{t2}) / (t_1 - t_2)$, where C_{t1} and C_{t2} are the concentrations of As at time t_1 (initial) and t_2 (final), respectively.

6.2.8 Statistical Analysis

Elemental concentrations were calculated on dry weight basis and presented as the mean value ± SD ($n = 3$). The IBM SPSS 22.0 for Windows (IBM Co., NY, USA) was used to perform statistical analyses. One-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) was performed to determine significant differences between different concentrations of As under individual growth conditions, or between conditions at each concentration of As. In addition, Pearson correlation coefficient (r) was also determined using the same statistical package. The values of $p < 0.05$ were considered as significant. Graphical works as well as Michaelis-Menten Curve fitting was carried out using Graph Pad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

6.3 Results and Discussion

6.3.1 Effect of Arsenic on Chlorophyll Fluorescence

The detectable signal of chlorophyll fluorescence can be measured in a non-invasive way by different PAM devices (Schreiber et al., 1995). This technique offers a suitable option for estimating the photosynthetic function of the photosystem II (*PSII*) of algae and seagrasses under diverse stresses (metals, pesticides, salts, temperature, and radiation etc) in field and laboratory experiments (Gong et al., 2008; Enríquez and Borowitzka, 2010; Kittle and McDermid, 2016; Farias et al., 2017; Terada et al., 2018). In this study, F_v/F_m values were taken before and after incubation, revealing that F_v/F_m of the shoots of *S. patens* were not significantly affected by different conditions of P and Fe in both As(V)- and As(III)-containing medium ($p > 0.05$). The significant changes in F_v/F_m were due to the application of different concentrations of As(III) ($p < 0.05$), but the As(V) treatments showed an insignificant effect ($p > 0.05$) (*Figure 6.1a*).

Before As exposure, the F_v/F_m values ranged from 0.68 ± 0.05 to 0.71 ± 0.002, with an average of 0.70 ± 0.01 ($n = 144$ of mean values). It was noted from the results that F_v/F_m values were decreased slightly after exposure for seven days to both species of As, when compared with the F_v/F_m of initial conditions (1.0 to 7.1%, and 0.2 to 14.3% in As(V) and As(III), respectively). Irrespective of the P and Fe effects, it was observed that the average decline in F_v/F_m was 0.68 ± 0.01 ($n = 72$) and 0.66 ± 0.03 ($n = 72$) in the As(V)- and As(III)-treated samples, respectively. However, a significantly decreasing trend of F_v/F_m was more pronounced with high initial levels of As(III), especially at 2.0 and 4.0 µM (*Figure 6.1b*). However, algae under +P+Fe culture with As species had little comparable effect on F_v/F_m. The observable and optimum F_v/F_m value comes close to 0.832 ± 0.004 in a wide range of

healthy and unstressed vascular plants and macroalgae, whereas the value would be below 0.10 in case of dead materials (Maxwell and Johnson, 2000; Chaloub et al., 2010). Büchel and Wilhelm (1993) reported that F_v/F_m in brown algae ranged from 0.7 to 0.8, which was in accordance with our results (initial F_v/F_m). However, it was also suggested that the pigment composition and cell structures are responsible for variable F_v/F_m value among different taxa and species (Suggett et al., 2009). Miteva and Merakchiyska (2002) and Stoeva et al. (2005) observed that damage to chloroplast membrane and disturbance of membrane structure were due to the effect of As toxicity in plants, resulting in reduced biosynthesis of chlorophyll content. Photosynthesis is strongly coupled with chlorophyll fluorescence, and for this reason, measurements of F_v/F_m can be used to detect damage to photosynthetic efficiency.

6.3.2 Effect of arsenic on growth rate

The exposure of As either as As(V) or As(III) for up to seven days did not cause significant visual damages or color changes in the shoots of *S. patens*. *Figure 6.2* shows that there was a significant difference in growth rate due to the addition of different concentrations of As(V) and As(III), whereas growth rate was unaffected by the different conditions upon the addition of P and Fe. The control conditions (no As) had the maximum growth rate of shoots and ranged from 0.72 to 1.02% per day, irrespective of P and Fe addition. The growth rate under As(V) added conditions ranged from 0.21 to 0.73, 0.22 to 0.95, 0.24 to 0.84, and 0.31 to 1.02% per day in –P–Fe, –P+Fe, +P–Fe, and +P+Fe cultures, respectively. It was evidenced that the growth rate under As(III) treated conditions was comparatively lower than that in the As(V) culture medium. The negative growth rate was also noted under elevated levels of As(III), particularly at 2.0 and 4.0 µM **(***Figure 6.2b*).

As has been found to be accumulated in the plant tissues, thereby reducing the biomass due to dysfunction of metabolic activities (Drličková et al., 2013). The growth rate of algae decreased with the increase of As(V) and As(III) concentrations in nutrient solutions, which seems to be connected with the decrease in photosynthesis and respiration. The toxicity of As(V) and As(III) were studied by Thursby and Steele (1984) using marine macroalga *Champia parvula*. The study suggested that P concentration up to 9.1 µM had a minimum, or no effect on As(III) toxicity upon vegetative and reproductive growth of the algae, and which were in agreement with our results. Their study also indicated about the inhibitory action of increasing P concentration (0 to 9.1 µM) in the growth medium upon As(V) toxicity. A slightly increased growth rate was noted with As treatments under +P+Fe culture, when compared with –P–Fe culture. It is possible that P and Fe nutrients prevented the algae from having a stress

response to As. Significantly positive response of growth rate in shoots exposed to control treatments or low concentrations of As probably resulted from an increased uptake of P. In addition, Fe-rich medium might be helpful in maintaining photosynthetic activity, because Fe plays a great role in the synthesis of chlorophyll. As(V) is thought to inhibit the synthesis of ATP, causing harmful interference of cellular metabolism (Bhattacharya et al., 2015), and inhibiting cellular division under extreme stress (Levy et al., 2005). Contrarily, toxicity of As(III) is mainly associated with disruption in activities of enzymes containing -SH groups that results in membrane degradation and cell death via reactive oxygen species formation (Wang et al., 2015b).

6.3.3 Accumulation of Arsenic

6.3.3.1 Effect of Arsenic Species on Accumulation of Arsenic

The accumulation of extracellular, intracellular, and total of As accumulation by *S. patens* exposed to As(V) and As(III) solutions with different P and Fe combinations are presented in *Figure 6.3* and *6.4*. One-way ANOVA analysis revealed that accumulation of As varied significantly by different levels of As, as well as by the influence of P and Fe in the culture medium ($p < 0.05$). The algae exposed to As(V) treatments had comparatively higher concentrations of As, when compared with the algae exposed to As(III). In both species of As, increasing the initial concentrations in the incubating solutions led to an elevated concentration and/or uptake rate in the tissues. On exposure to 4.0 µmol L^{-1} As(V), the total accumulation was: 617.1 ± 22.7, 628.4 ± 21.9, 582.5 ± 14.0, and 558.1 ± 29.4 nM g^{-1} dry weight from –P–Fe, –P+Fe, +P–Fe and +P+Fe cultures, respectively. In contrast, when the alga was exposed to the same concentration of As(III), the total accumulation was: 288.6 ± 15.7, 292.0 ± 21.0, 331.7 ± 23.8 and 324.2 ± 12.9 nM g^{-1} dry weight from –P–Fe, –P+Fe, +P–Fe and +P+Fe cultures, respectively. This species of algae accumulated 1.7- to 2.12-fold less As from As(III) solutions than from As(V) solutions. Irrespective of P and Fe effects, the total accumulation of As showed a strong positive correlation with its initial concentrations in culture solutions ($r = 0.99$ and 0.97 for As(V) and As(III), respectively; $p < 0.01$).

The results from the present study are consistent with those of previous studies conducted with laboratory-cultured microalgae, showing steady-state accumulation of As in relation with the external As concentrations (Duncan et al., 2013; Baker and Wallschlager, 2016; Wang et al., 2017a). However, the concentration factor (µM dry mass of As/µM exposure of As) decreased with increasing exposure concentrations of As. For example, the concentration factor at 0.25 µM (–P+Fe culture) was 231.5 and 113.8 for As(V) and As(III),

respectively, which was decreased by 1.5- and 1.6-fold upon exposure to 4.0 µM of As(V) and As(III), respectively. We observed that –P+Fe culture ranked top for uptake of As(V), which was closely followed by –P–Fe culture. The higher accumulation of As from As(III) was attributed to +P–Fe culture. The toxicity of As depends on its chemical speciation and bioavailability. It has been reported that As(V) is generally 10 times less toxic than As(III) (Srivastava et al., 2011), and the relatively lower accumulation of As in As(III) treatments might be due to differences in toxicity and growth rate. Selective uptake of As species by the macroalgae from seawater (the predominant form of As in seawater is As(V)) may be another possibility. The uptake kinetics from the disappearance or depletion of As(V) and As(III) over the incubation period were calculated and adequately described by the Michaelis-Menten function presented in *Figure 6.5 & 6.6*. The Michaelis constant (*K*m) under –P–Fe, –P+Fe, +P–Fe and +P+Fe cultures with As(V) were 2.67 ± 0.30, 2.71 ± 0.23, 2.84 ± 0.37 and 3.11 ± 0.32 µM, respectively, while in As(III) solutions *K*m values were: 2.01 ± 0.81, 1.96 ± 0.63, 1.85 ± 0.38, and 1.89 ± 0.49 µM L^{-1}, respectively. The lower *K*m in As(V) treatments was recorded under the growth condition of –P–Fe, whereas the higher values were recorded in +P+Fe conditions. This result suggested that As(V) accumulation in algae were inhibited by the presence of P and Fe in the medium. On the contrary, lower *K*m was associated with +P–Fe culture condition in As(III), indicating a lack of inhibition of As(III) uptake due to P.

6.3.3.2 Effect of Phosphate on Extracellular and Intracellular Accumulation of Arsenic

Algae can resist and tolerate stress of heavy metals either through limited intracellular accumulation for metabolism or through a non-active adsorption–biosorption process (Chekroun and Baghour, 2013). The adsorption phenomena is commonly found in aquatic organisms where suspended oxides of Fe adsorb on the surface of organisms that can accumulate As (Robinson et al., 2006). In this study, when the algae were treated with As(V) and As(III), significant amounts of As were surface-adsorbed or extracellularly bound and varied according to the presence and/or absence of P and Fe ($p < 0.05$). *Figures 6.3a and 6.4a* show that the extracellular content of As was significantly higher in As(V) treatments than in As(III). The extracellular As concentrations (nM g^{-1} dry weight) in culture medium containing 4.0 µM As(V) increased in the following order: +P–Fe (63.4 ± 4.3) < –P–Fe (72.5 ± 7.6) < +P+Fe (85.9 ± 7.2) < –P+Fe (104.1 ± 5.0). It was observed that, under the same concentration of As(V) (at 4.0 µM) in the Fe-limited medium, adsorption of As increased by 14.4% under –P–Fe culture, when compared with +P–Fe culture. Similarly, a 21.2% increase in As adsorption was found under Fe-rich medium. It was noticed that the presence of P in both +P–Fe and

+P+Fe cultures with As(V) treatments had a reducing effect on As surface adsorption. On the contrary, P had a positive influence on surface adsorption of As under As(III) treatments, though the amount of adsorption was lower compared to As(V). The algae grown under +P–Fe and +P+Fe cultures with 4.0 µM of As(III) contained 31.5 ± 7.1 and 43.1 ± 5.4 nM g^{-1} dry weight of extracellular As, which was 19.7 and 17.7% more when compared with –P–Fe and –P+Fe cultures, respectively. Adsorption studies found that Fe-oxides/hydroxides had a strong binding affinity for inorganic anions like $H_2AsO_4^-$ and/or $HAsO_4^{2-}$ and PO_4^{3-} compared with that for $H_2AsO_3^-$ (Meng et al., 2002). In other words, As(III) associated with Fe-oxides/hydroxides may be much more easily desorbed than As(V). Similar findings were also observed in other studies (Chen et al., 2005). It was suggested that P not only competes with As(V) in cellular uptake, but also for adsorption sites of Fe-oxides/hydroxides.

The descending order of intracellular As concentrations (nM g^{-1} dry weight) in the tissues under As(V) containing medium at 4.0 µM was: 544.6 ± 7.6 (–P–Fe) > 524.3 ± 21.7 (–P+Fe) > 519.1 ± 15.3 (+P–Fe) > 472.5 ± 33.29 (+P+Fe). When the concentration of P reached 10 µM from 0 µM under +P–Fe culture, a 4.7% reduction in intracellular As content was recorded with a constant 4.0 µM of As(V). Similarly, under +P+Fe culture, the decrease was 9.9%. The intracellular uptake of As (nM g^{-1} dry weight) in algae exposed to 4.0 µM As(III) were 262.3 ± 11.2, 255.5 ± 22.9, 300.2 ± 16.7, and 281.1 ± 13.5 under –P–Fe, –P+Fe, +P–Fe, and +P+Fe culture, respectively (*Figure 6.3b and 6.4b*). Irrespective of Fe in the medium, the P-rich and As(V) cultured algae showed substantially lower absorption of As, whereas the opposite was observed for As(III). This may have been due to the competitive behavior of As(V) and P for similar uptake pathways. In fact, interference of P on reduced As uptake is a common phenomenon, and our results were in accordance with those of Rahman et al. (2007). It was also noted that the presence of P can give rise to an increase in the uptake of As from As(III) solutions. The absorption of As(III), which occurs primarily through the plasma membrane aquaporin channel, was not affected by the presence of P in the P-rich medium, but the accumulation of As(V) was disrupted by the presence of P. (Meharg and Jardine, 2003; Ma et al., 2008). Such channel for the As(III) uptake pathways might be independent of P absorption, and the overall intracellular As(III) accumulation had no effect due to the presence of soluble P in the medium.

6.3.3.3 Effect of Iron on Extracellular and Intracellular Accumulation of Arsenic

In many studies, Fe-plaque have been demonstrated to sequester As and can act as reservoir, facilitator, and barrier (Khan et al., 2016). A significant variation in extracellular As

in both As(V) and As(III) treatments were obtained by the addition of Fe in culture medium ($p < 0.05$). With the same concentration of 10.0 μM P (P-rich condition) and 4.0 μM As(V), extracellular As increased by 35.4% under +P+Fe culture when compared with +P–Fe culture, whereas in the P-limited conditions, the concentration was increased by 43.5% in –P+Fe culture. On the contrary, when the algae was cultured with the same concentration of As(III), the extracellular As increased by 36.6% in +P+Fe culture. Likewise, under –P+Fe culture, the increase was 38.8%. The analogous properties of P with As(V) might be the reason for the reduced extracellular As under +P+Fe culture when compared with –P+Fe culture. It was observed that the Fe-rich conditions had a positive influence on the adsorption of As either supplied through As(V) or As(III). Irrespective of the growth conditions modified by P and Fe, adsorption of As onto the algae surface in both As(V) and As(III) containing media was positively correlated with their initial concentrations ($r = 0.91$ and 0.92 for As(V) and As(III), respectively; $p < 0.05$). However, it was noted that the amount of adsorption was consistently lower in As(III)-mediated cultures and was comparable with that of As(V). This may be due to the low adsorption affinities for As(III) when compared with that for As(V) on the adsorption site (Chen et al., 2005). Contrasting results were also found by Raven et al. (1998), who noted stronger binding affinities of both As(III) and As(V) in Fe-oxides.

The proportion of intracellular uptake of As out of total uptake under As(V) at 4.0 μM were 88.2, 83.4, 89.1, and 84.6% in –P–Fe, –P+Fe, +P–Fe, and +P+Fe cultures, respectively. Conversely, in the As(III) treatment solutions, the proportions were 90.9, 87.5, 90.5, and 86.7%. When the Fe concentration increased from 0 to 10 μM with an exposure of 4.0 μM of As(V), 3.7 and 9.0% reduced uptake of intracellular As were recorded from –P+Fe and +P+Fe cultures, respectively. The results indicated that P with Fe in the medium inhibited the absorption of As when algae were grown with As(V). Contrarily, the maximum intracellular As in tissues from 4.0 μM of As(III) was found under +P–Fe culture (300.2 ± 16.7 nM g^{-1} dry weight), while the least was in the –P+Fe culture (255.5 ± 22.9 nM g^{-1} dry weight). There may have been no competition between As(III) and P, and hence, the P-rich medium enhanced the mobility of As to the absorption site under +P–Fe culture condition. It was noted that As(III) treatments under Fe-limited conditions resulted in higher intracellular accumulation of As irrespective of the P effect. This suggested that the additional Fe in the medium can significantly decrease the As concentrations in hydroponically grown rice roots, but increase the Fe concentrations in both extracellular and intracellular portions (Shaibur et al., 2015). Similar findings were also observed in aquatic macrophytes by other researchers (Robinson et al., 2006; Rahman et al., 2008b).

6.3.4 Biotransformation of Arsenic in Culture Medium

The culture medium samples were analyzed for speciation of biotransformed As metabolites after seven days of incubation. Following uptake of As(V) and As(III), different proportions of reduced and oxidized As species were detected from the algal culture medium, indicating biological transformations because these transformed metabolites were not detected in the abiotic controls (no algae) of coexisting media. However, no culture media containing detectable organic species such as MMAA(V) and DMAA(V) were observed. This suggested that bioconversion of IAs to organic As and/or arsenosugars is the most common metabolic pathway of As in macroalgae, but some species, such as *Hizikia fusiforme* and *Sargassum piluliferum*, do not utilize metabolic conversion but can accumulate significant proportions of IAs as As(III) and As(V) (Ma et al., 2018). The uptake and subsequent transformations, as well as the simultaneous depletion of As(V) and As(III) in the media, were related to the levels of P and Fe and varied significantly depending on the levels of As treatments ($p < 0.01$). When the algae were incubated in As(V) solution, the predominant metabolites were As(V), along with a trace of As(III). On the contrary, when algae were incubated in As(III) solution, the major As species was As(III) with a small amount of As(V) accounting for 2.0 to 3.7% of total As (*Figure 6.7*). The proportions of As(III) were observed to increase with increasing external concentrations of As(V) in the culture medium. With 4.0 µM of As(V), the detectable As(III) were 4.9, 4.6, 4.2 and 3.5% of total As under –P–Fe, –P+Fe, +P–Fe, and +P+Fe cultures, respectively. In our previous study, we found that this species of macroalgae can transform As(V) to As(III) in As(V) containing culture medium (Mamun et al., 2017). As(III) is more easily excreted from cells than As(V) due to toxicity difference, and for this reason the reduction of As(V) to As(III) was considered a detoxification process (Rahman and Hassler, 2014). Our results of As accumulation and speciation were in agreement with those reported by Malea and Kevrekidis (2014), who suggested that higher retention of As by macroalgae was attributed to a lower rate of excretion into the surrounding environment.

6.3.5 Effect of Arsenic Species on Accumulation of Phosphate

Aquatic hydrophytes under solution culture significantly binds P ions in Fe-oxides/hydroxides and function as reservoirs during a deficiency period (Jiang et al., 2009). However, in our results, the P in CBE extracts, such as the extracellular P, was not detected in either P-limited or P-rich conditions. The intracellular P concentrations in algal tissues after As(V) treatments ranged from 47.3 ± 7.1 to 55.7 ± 3.9 and 46.5 ± 10.9 to 56.6 ± 11.42 µM g^{-1} dry weight under +P–Fe and +P+Fe cultures, respectively. Conversely, after the As(III)

treatments, the concentrations were 53.1 ± 4.5 to 56.5 ± 7.0 and 51.9 ± 10.0 to 56.5 ± 8.6 μM g^{-1} dry weight (*Appendix L*). The correlation results summarized in *Table 6.2* showed that the intracellular P uptake by *S. patens* under P-rich conditions decreased negatively with the intracellular As(V) uptake (r = - 0.92, $p < 0.01$ and r = - 0.91, $p < 0.05$ for +P–Fe and +P+Fe cultures, respectively). On the contrary, As(III) treatments had a significantly positive relationships on its uptake (r = 0.96, $p < 0.01$ and r = 0.89, $p < 0.05$ for +P–Fe and +P+Fe cultures, respectively). However, there was an insignificant and negative correlation observed between P and As accumulation under P-limited (–P–Fe and –P+Fe) conditions of As species. These results indicated that the increasing concentration of As(V) in solution also increases the competition between P and As(V) for absorption sites, and a subsequent decrease of P in the tissues of algae receiving increasing concentrations of As(V). On the contrary, the interference of As(III) over P uptake might not be inhibited due to variations in uptake pathways, and hence, P absorption was independent in the presence of As(III). Our results were in agreement with the results of a previous study on aquatic macrophytes (Rahman et al., 2008b).

3.6 Effect of Arsenic Species on Accumulation of Iron

The significance of biological surface-bound Fe has generally been overlooked in many cases due to the fact that Fe present in the media was in the form of a very stable Fe-EDTA chelate. However, the possibility and existence of surface binding has persisted even in algal medium containing excess EDTA (Miller et al., 2013). Both As(V) and As(III) treatments had significant influence on intracellular and extracellular Fe content under Fe-rich (–P+Fe and +P+Fe) conditions ($p < 0.05$). The results in *Table 6.3* showed that As(III)-feeding algae contained reasonably higher concentrations of intracellular Fe, when compared with As(V). The intracellular Fe in –P+Fe and –P+Fe culture ranged from 0.79 ± 0.11 to 1.59 ± 0.12 and 0.83 ± 0.14 to 1.84 ± 0.13 μM g^{-1} dry weight under As(V) and As(III) media, respectively. On the contrary, the amounts of extracellular Fe in As(V) treatments were higher than that in the As(III) treatment, irrespective of P in medium. It was also observed that intracellular Fe concentration increased with increasing As concentration in tissues of algae receiving As in the forms of As(V) (r = 0.95 and 0.97 for –P+Fe and +P+Fe conditions, respectively, $p < 0.01$) and As(III) (r = 0.87 and 0.90 for –P+Fe and +P+Fe conditions, respectively; $p < 0.05$) (*Appendix M*). The surface adsorbed extracellular Fe was detected under both –P+Fe and +P+Fe cultures. A significantly positive correlation was also found for extracellular Fe and As content under both As(V) (r = 0.97 and 0.95 for –P+Fe and +P+Fe cultures, respectively; $p < 0.05$) and As(III) (r = 0.93 and 0.94 for –P+Fe and +P+Fe cultures, respectively; $p < 0.01$). An insignificant but

positive correlation was noted for intracellular uptake of Fe and As from –P–Fe and +P–Fe cultures with As(V) and As(III) treatments (*Appendix N*). These results suggest that the presence of As(V) and As(III) had no inhibitory effect on intracellular Fe uptake by *S. patens*, but adsorption may be enhanced by the addition of Fe in the media. Hu et al. (2005) reported that As can be extracted from dithionate-citrate-bicarbonate (DCB) in the Fe-plaque of three rice cultivars, which had a strong positive correlation with the amounts of Fe in Fe-plaque. Similar findings were also reported by Rahman et al. (2008a), who noted about 56% and 44% of total As accumulation in the tissues of *Spirodela polyrhiza* and CBE extracts.

6.4 Conclusion

Algae has received much attention due to their potential in metal ion remediation and water quality improvement in aquatic systems. However, uptake and remediation studies on As using different IAs by living *Sargassum patens* has not yet been elucidated under laboratory conditions. Furthermore, there have been limited studies that have focused on the growth of macroalgae in relation to IAs uptake within the growth media algae system. The results in this study demonstrated that As(III) and As(V) were not equally toxic to this species of algae. Reduced photosynthetic activity and growth rates were mostly associated with toxicity of As(III). Trivalent As was considerably more toxic than pentavalent As due to its tendency to react as a soft metal with thiols. From the overall accumulation and biotransformation results, this algae can sufficiently remove As from an As(V) source, when compared with As(III). The P and Fe in the medium significantly inhibited the intracellular uptake of As(V). Besides, Fe in the medium leads to the deposition or formation of Fe-plaque on the surface of algae, and the amounts of Fe-plaque are greatly enhanced with an increase of external As. This study showed that As accumulation inside/outside of tissues may be used as a measure of bioavailability of As in the habitat in the context of contamination risks. This study also contributes to a better understanding of the adverse effects of IAs on macroalgal growth, and insights obtained herein on the diverse variations in bioaccumulation patterns under laboratory conditions will contribute to understanding As biogeochemical cycling.

Table 6.2: Pearson's correlation matrix between intracellular arsenic and phosphate accumulation from arsenate and arsenite treatments.

Pearson correlation coefficient			Intracellular uptake							
			–P–Fe		–P+Fe		+P–Fe		+P+Fe	
			As(V)	P	As(V)	P	As(V)	P	As(V)	P
Intracellular uptake	–P–Fe	As(V)	1							
		P	-0.38	1						
	–P+Fe	As(V)	0.99**	-0.39	1					
		P	-0.68	0.34	-0.66	1				
	+P–Fe	As(V)	0.99**	-0.40	0.99**	-0.66	1			
		P	-0.95**	0.52	-0.93**	0.75	-0.92**	1		
	+P+Fe	As(V)	0.99**	-0.39	1.00**	-0.66	1.00**	-0.92**	1	
		P	-0.93**	0.54	-0.91*	0.66	-0.91*	0.99**	-0.91*	1
Pearson correlation coefficient			Intracellular uptake							
			–P–Fe		–P+Fe		+P–Fe		+P+Fe	
			As(III)	P	As(III)	P	As(III)	P	As(III)	P
Intracellular uptake	–P–Fe	As(III)	1							
		P	-0.58	1						
	–P+Fe	As(III)	0.99**	-0.55	1					
		P	-0.50	0.14	-0.51	1				
	+P–Fe	As(III)	1.00**	-0.58	0.99**	-0.50	1			
		P	0.96**	-0.56	0.95**	-0.37	0.96**	1		
	+P+Fe	As(III)	1.00**	-0.58	0.99**	-0.51	1.00**	0.96**	1	
		P	0.89*	-0.59	0.89*	-0.70	0.89*	0.76	0.89*	1

'*' and '**' indicates correlation is significant at the 0.05 and 0.01 level (2-tailed), respectively.

Table 6.3: Intracellular and extracellular Fe concentrations (μM g^{-1} dry weight) from arsenate and arsenite treatments.

As(V) treatments (μM)	–P+Fe		+P+Fe	
	Content of Fe (μM g^{-1} DW)		Content of Fe (μM g^{-1} DW)	
	Intracellular	Extracellular	Intracellular	Extracellular
Control	0.79 ± 0.11 c	0.48 ± 0.11 d	0.87 ± 0.11 d	0.40 ± 0.11 c
0.25	1.00 ± 0.21 bc	0.73 ± 0.12 c	0.98 ± 0.11 cd	0.59 ± 0.11 bc
0.50	1.05 ± 0.14 bc	0.81 ± 0.13 bc	1.13 ± 0.15 bcd	0.72 ± 0.15 ab
1.0	1.23 ± 0.25 b	0.92 ± 0.11 bc	1.18 ± 0.09 bc	0.81 ± 0.16 ab
2.0	1.25 ± 0.14 b	1.01 ± 0.17 ab	1.28 ± 0.18 b	0.87 ± 0.14 a
4.0	1.59 ± 0.12 a	1.16 ± 0.12 a	1.68 ± 0.21 a	0.96 ± 0.15 a
As(III) treatments (μM)				
Control	0.83 ± 0.14 c	0.38 ± 0.05 c	0.79 ± 0.19 d	0.46 ± 0.16 c
0.25	1.21 ± 0.23 bc	0.62 ± 0.19 bc	1.10 ± 0.23 cd	0.58 ± 0.16 bc
0.50	1.48 ± 0.29 ab	0.69 ± 0.16 b	1.39 ± 0.20 bc	0.74 ± 0.16 ab
1.0	1.61 ± 0.27 a	0.70 ± 0.20 b	1.55 ± 0.14 ab	0.77 ± 0.12 ab
2.0	1.73 ± 0.16 a	0.76 ± 0.13 ab	1.65 ± 0.26 ab	0.81 ± 0.10 ab
4.0	1.84 ± 0.13 a	1.01 ± 0.15 a	1.79 ± 0.16 a	0.90 ± 0.13 a

The lowercase letters in each column indicate significant differences between arsenic treatments at each culture conditions modified with iron and phosphate ($p < 0.05$). All data are means ± SD ($n = 3$).

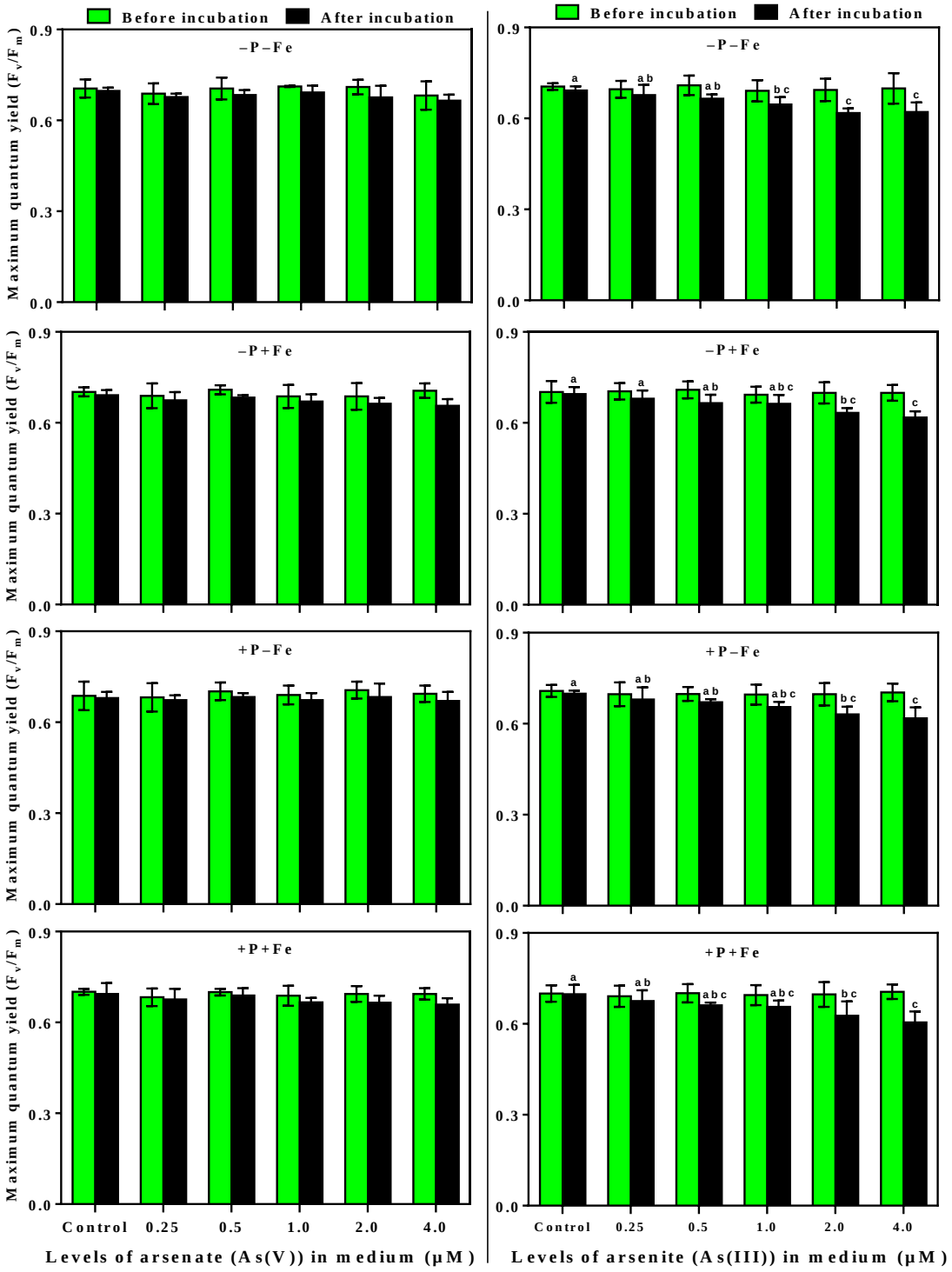

Figure 6.1: Changes in photosynthetic activity measured as maximum quantum yield (F_v/F_m) of *Sargassum patens* due to the effect of arsenate (a) and arsenite (b) treatments. The lowercase letters in Fig. 1b indicate significant differences between levels of arsenite treatments from culture medium modified with iron and phosphate ($p < 0.05$). Data are means ± SD ($n = 3$).

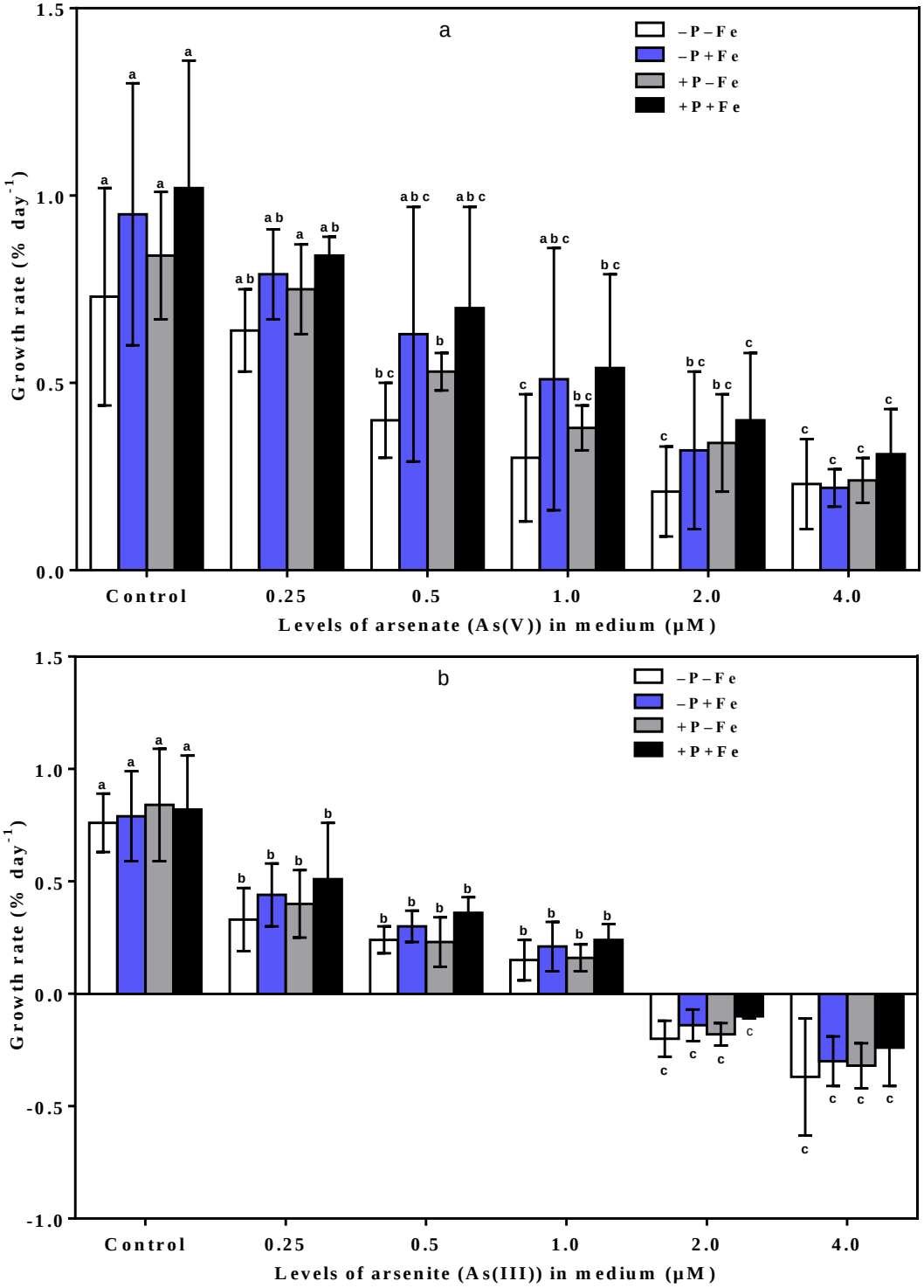

Figure 6.2: Changes in the relative growth rate of *Sargassum patens* due to the effect of arsenate (a) and arsenite (b) treatments. The different lowercase letters indicate significant differences between levels of arsenic treatments ($p < 0.05$) of individual culture medium modified with iron and phosphate. Data are means ± SD ($n = 3$).

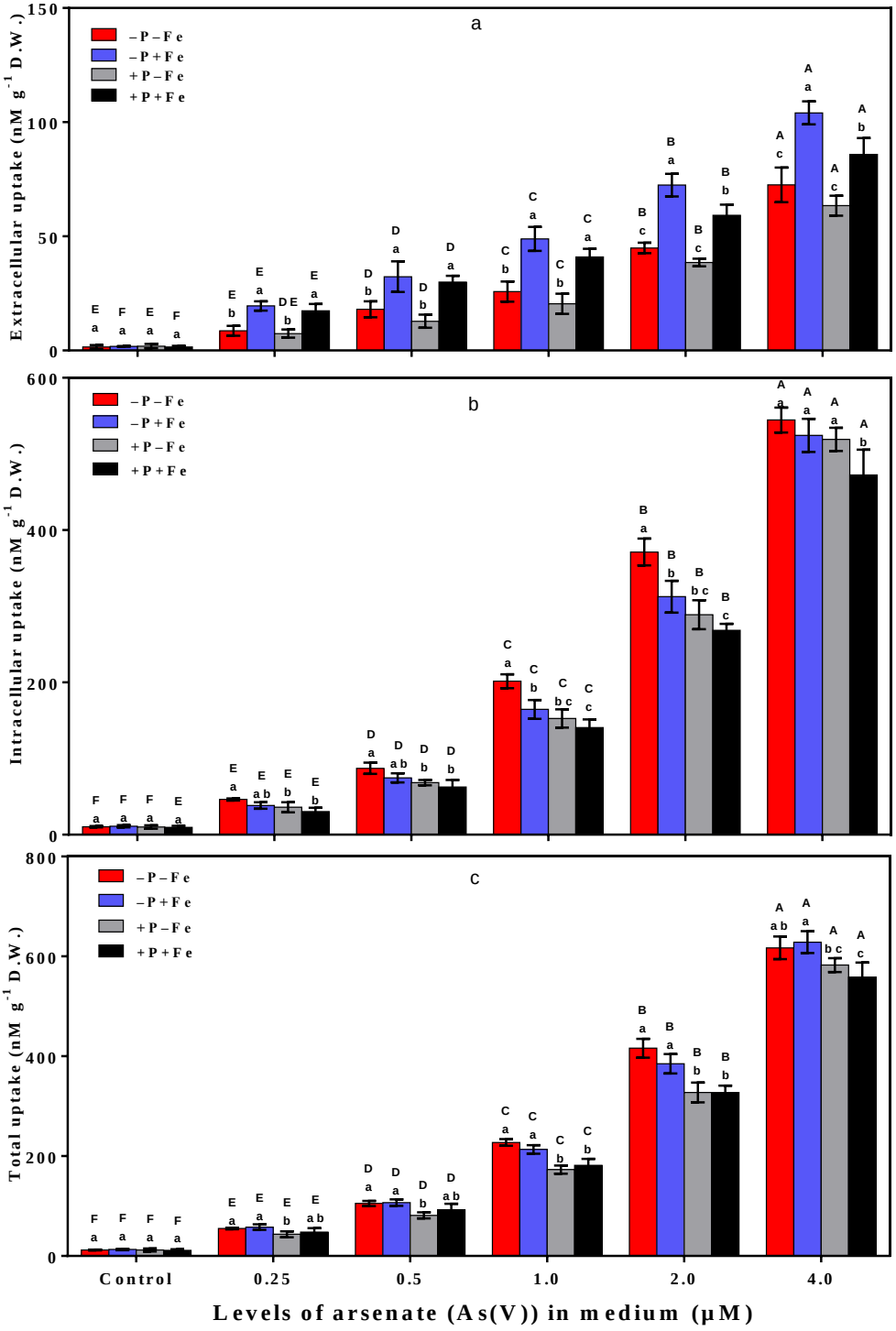

Figure 6.3: Effect of arsenate on extracellular (a), intracellular (b), and total (c) accumulation of arsenic by *Sargassum patens*. The different uppercase letters indicate significant differences between levels of arsenate treatments in individual culture medium, and different lowercase letters indicate significant differences between culture mediums modified with iron and phosphate at each level of arsenate ($p < 0.05$). Data are means ± SD ($n = 3$).

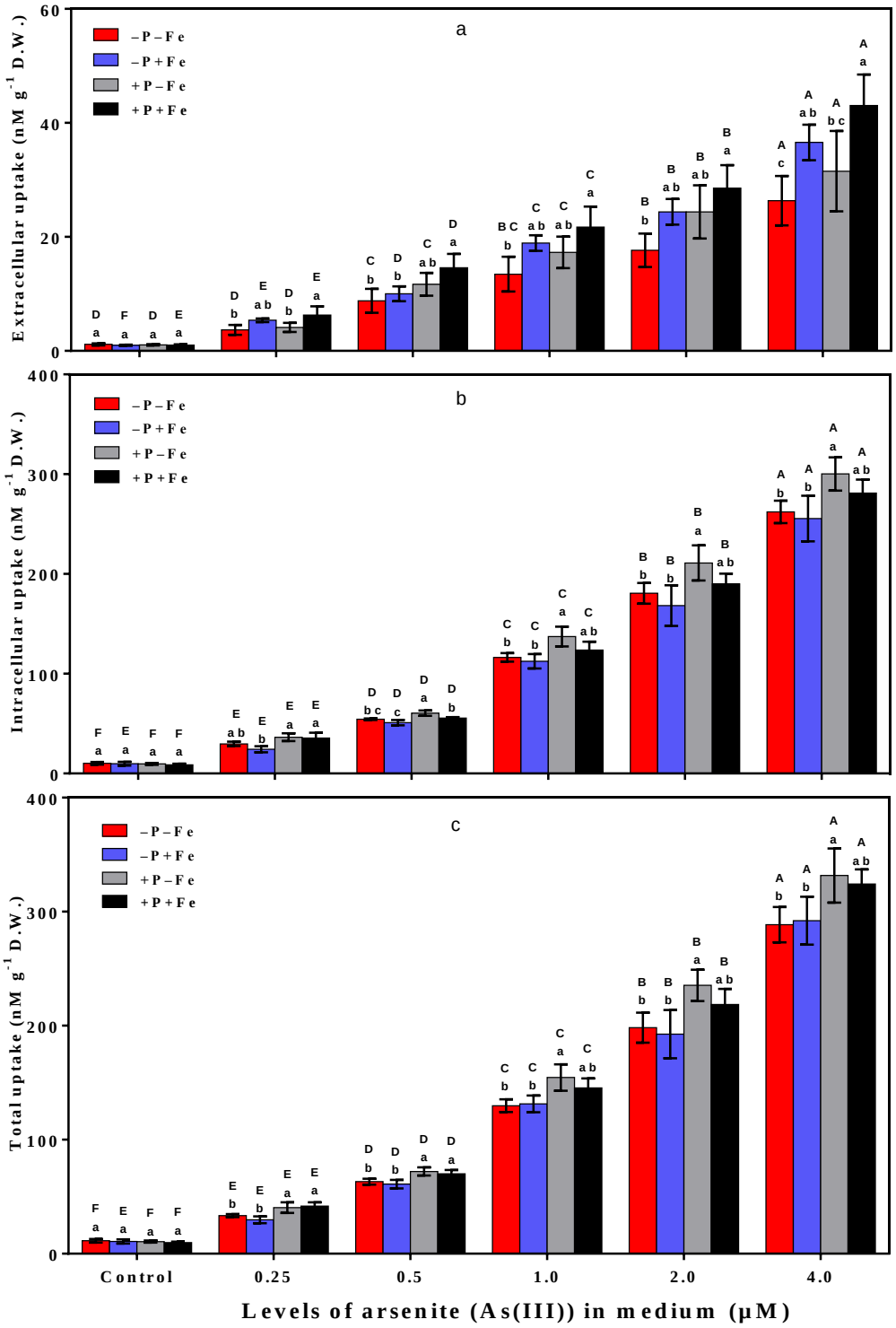

Figure 6.4: Effect of arsenite on extracellular (a), intracellular (b), and total (c) accumulation of arsenic by *Sargassum patens*. The different uppercase letters indicate significant differences between levels of arsenate treatments of individual culture medium, and the different lowercase letters indicate significant differences between culture mediums modified with iron and phosphate at each level of arsenite ($p < 0.05$). Data are means ± SD (n = 3).

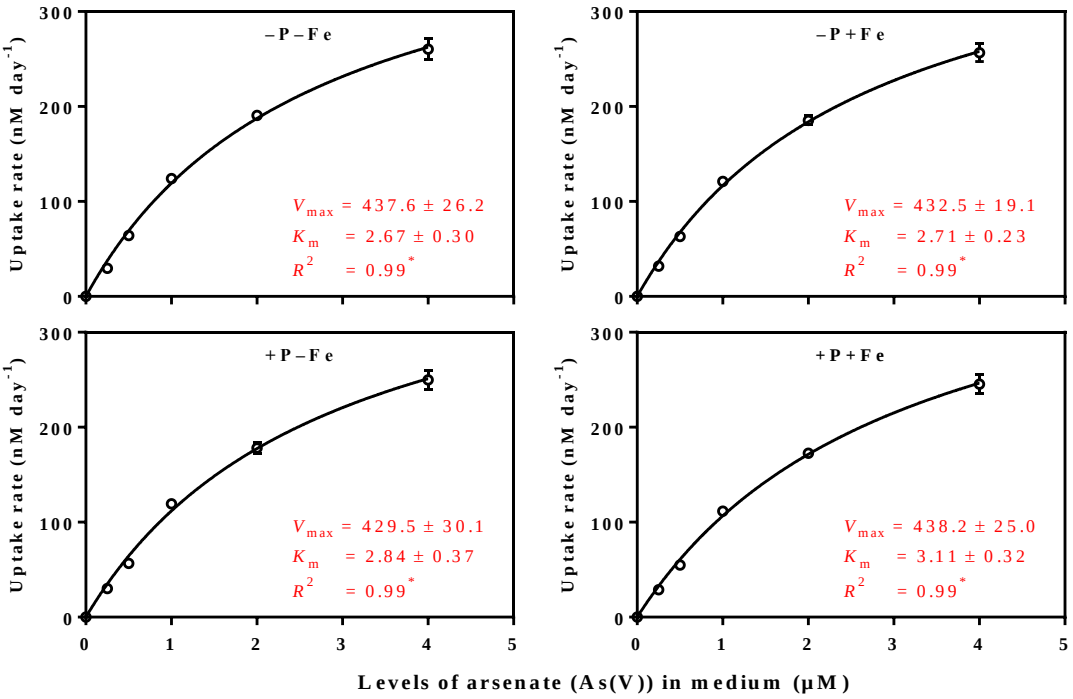

Figure 6.5: The concentration dependent arsenate uptake by *Sargassum patens* for the determination of *V*max and *K*m in Michaelis menten equation. The unit of *V*max and *K*m is nM day^{-1} and µM, respectively. The symbol '*' indicates significant differences ($p < 0.05$). Data are means ± SD (n = 3).

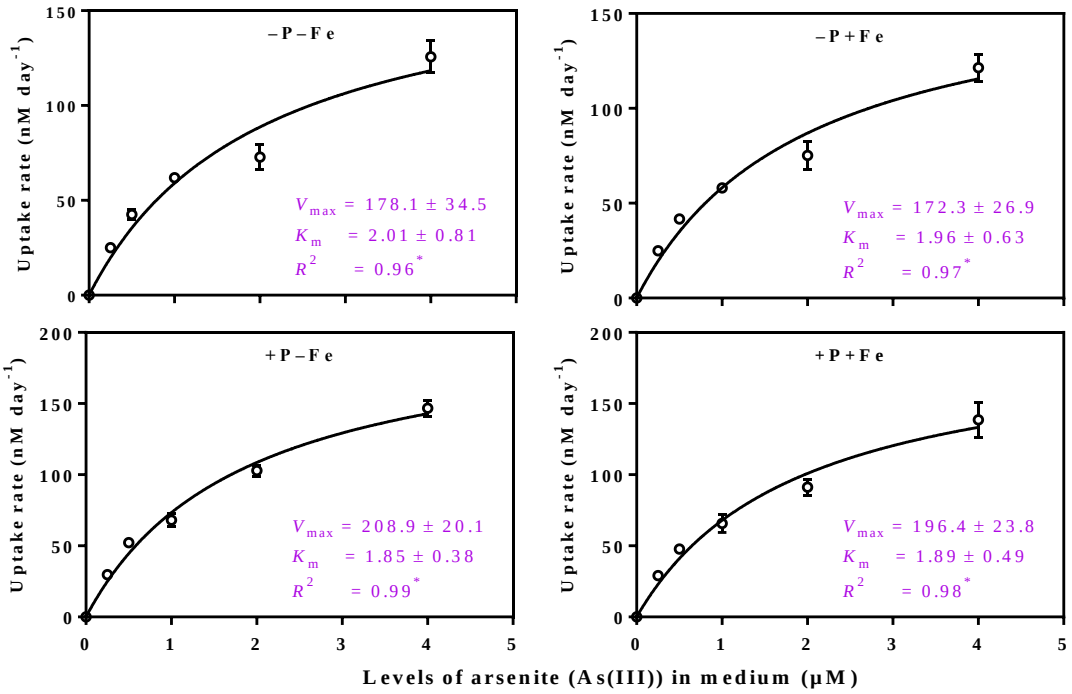

Figure 6.6: The concentration dependent arsenite uptake by *Sargassum patens* for the determination of *V*max and *K*m in Michaelis menten equation. The unit of *V*max and *K*m is nM day^{-1} and µM, respectively. The symbol '*' indicates significant differences ($p < 0.05$). Data are means ± SD ($n = 3$).

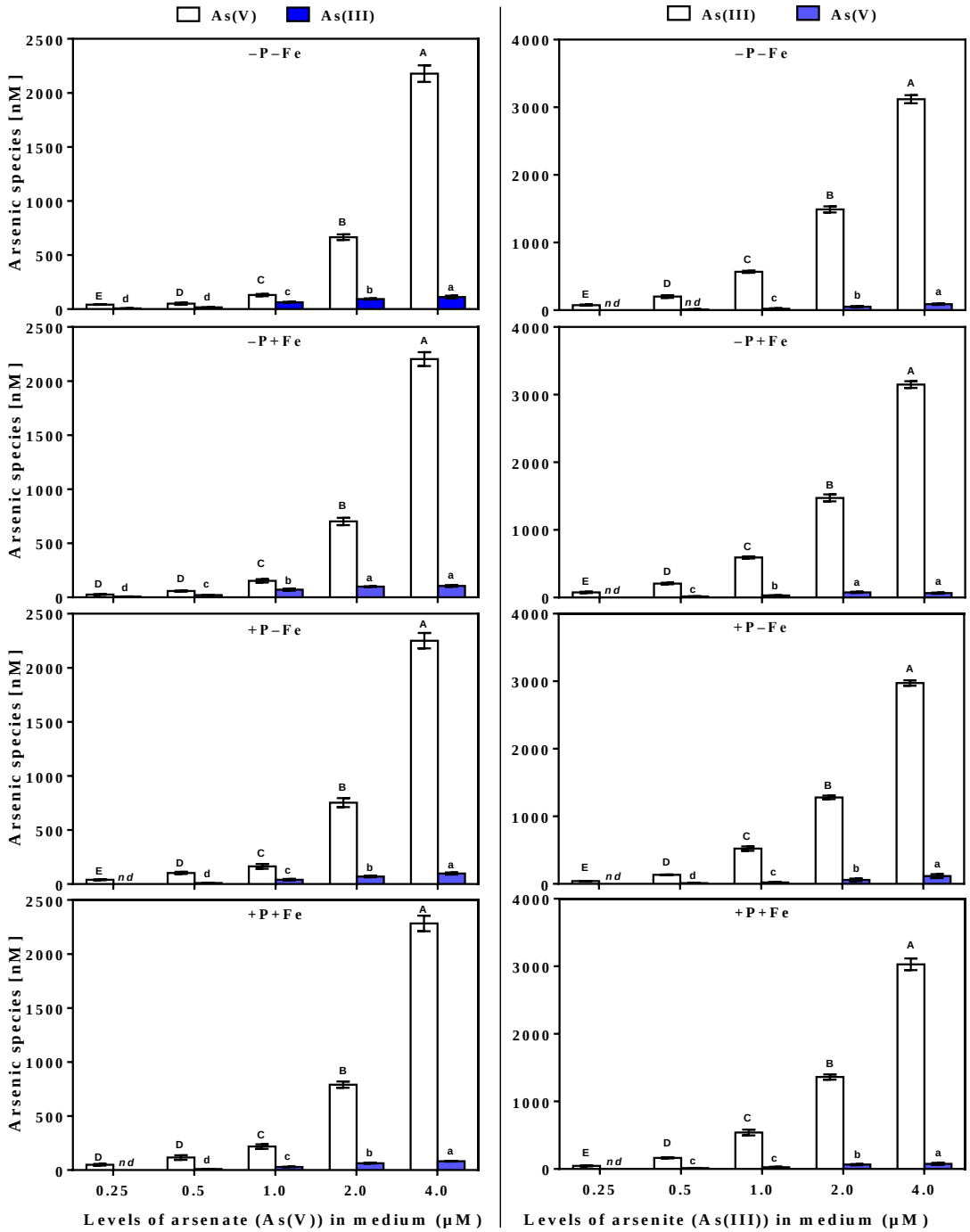

Figure 6.7: Arsenic speciation in culture medium of *Sargassum patens* under arsenate (a) and arsenite (b) treatments. The different uppercase and lowercase letters indicate significant differences between levels of arsenic treatments and between biotransformed arsenic metabolites from coexisting arsenic treatments modified with iron and phosphate ($p < 0.05$), respectively. '*nd*' indicates not detected. Data are means ± SD ($n = 3$).

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SUMMARY AND CONCLUSIONS

In this study, the laboratory culture of macroalgae was conducted with different proportions or molar ratios of added As(V) and P. Another study was also conducted with the interaction of inorganic As species (As(V) and As(III)) with or without P and Fe. The As(V) metabolism by the macroalgae species would provide information regarding what extent various algae could affect the uptake, transformation, and release of As species following metabolism (*Figure 7.1*). All the macroalgae species instantly interact with As-contaminated seawater differently at the cellular level, expressing different responses and tolerance strategies. Though As is toxic to the algae, the underlying mechanism of reducing As toxicity would be related to the P nutrition in the algal growth medium. The algal species were able to resist under high As stress by taking more P in the absorption site or by synthesizing more phosphate transporter to inhibit or minimize As uptake. All the experimental results were indicating the potentiality of the macroalgae species for metabolizing As following uptake of As(V) within seven days culture period. The speciation of As in algal culture system was attributed to the absorption, adsorption, and excretion capacities that were dependent on the levels of As and P as well as algae-specific metabolism. It was found that the metabolism would active under the culture conditions containing limited P nutrients, which would ultimately mediate the transformation processes, i.e., reduction and methylation. The cellular concentration of all the algal species was inhibited under P rich condition, which further pinpointing the competitive absorption of both the anions for the same transporter.

The transport of As species through intercellular and extracellular mechanism after As exposure involves a combination of biochemical, kinetic, and thermodynamic processes. The cell wall is the initial barrier against metal ion uptake especially As, but some channels and pumps in the wall provide pathways for inwardly or outwardly moving metal ions. The algal surfaces are composed of different functional groups that can chelate several ions from the media. The algae species were showed to bound a portion of As in their surfaces and the amount of surface-bound As was increased with an increasing amount of As in the media. The barrier effect of iron-plaques under Fe-enriched culture systems and/or coexisting media containing Fe have also shown to regulate the transport of both intracellular As species and P. It was found that Fe-plaque could significantly adsorb a certain portion of added As(III)/As(V) by the *sargassum patens* and reduce the intracellular As burden which implies the Fe-As co-precipitating bondage in the algal cell surface. However, As surface-binding effect was not

predominant under higher P added conditions as well as in As(III) exposed medium indicating the barrier of both high Fe and P for the absorption site of As by the algae or less binding affinity of As(III) to the Fe-plaque.

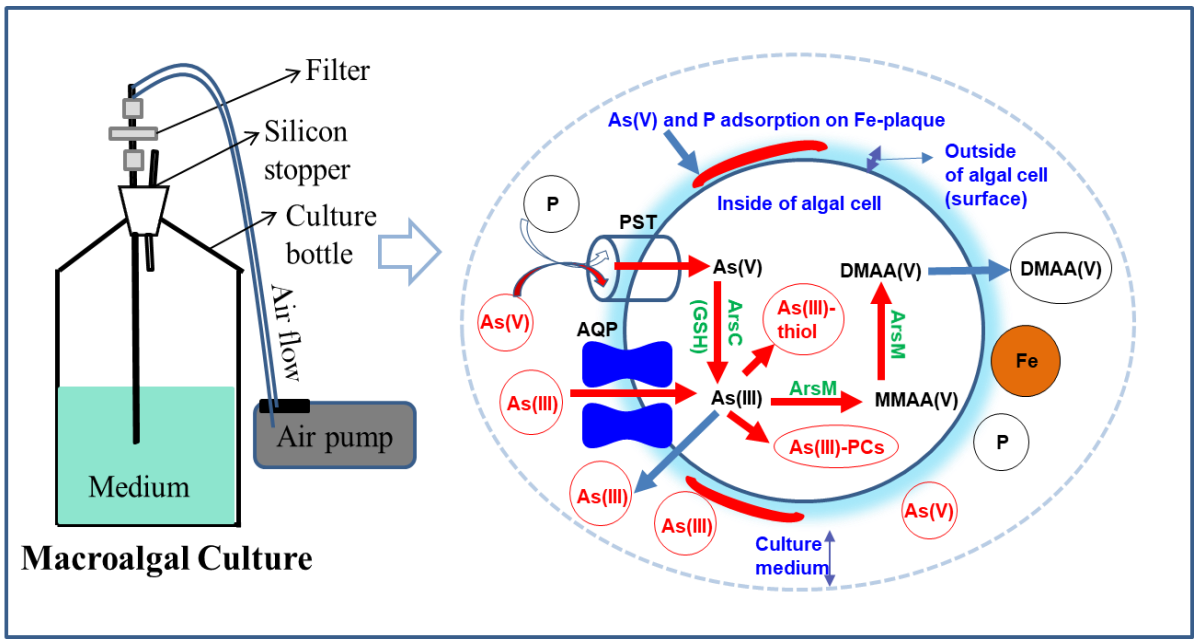


Figure 7.1: Schematic diagram showing the metabolism of As under macroalgal culture system

Though most of the pioneering works have focused on the treatment of As exposure by various organisms including microalgae, very few studies on a macroalgal route for detoxification of As has been reported so far. Also, there are fewer attempts to explore the macroalgal growth and its relation with the uptake-transformation-excretion processes. To sum up this study, the highest As methylating algae species (*Undaria pinnatifida* and *Pyropia yezoensis*) could be useful for the phytofiltration of As-contaminated water or soil. On the other hand, the higher accumulation behaviour of As in macroalgal tissues including the algae species *Sargassum patens* and *Sargassum horneri* may be used as a measure of the bioavailability of As in the habitat or utilizing for biomonitoring purposes. The sensitivity of macroalgae species toward different As species could be employed in assessing the toxicity of water contaminants as well. The possible changes of As upon growth-media-macroalgae system would also provide insights into the biogeochemistry of As in the real environment. Therefore, this study can be an excellent contribution to literature and will increase our understanding of the adverse effects of As in aquatic ecosystems.

Scope of Future Research

1. The details knowledge on the pathways of biotransformation could make a linkage between the diversity of macroalgal species and the predominant pathways on As

speciation in a particular environment. That is why the molecular mechanisms of biotransformation in both microalgae and macroalgae warrants fundamental importance because of their impact on the cycling of As in the environment. As cannot be destroyed and/or degraded and will always exist in nature, identifying and managing its biological transformation processes can redistribute its chemical speciation.

2. The macroalgae *Undaria pinnatifida* and *Pyropia yezoensis* exhibits higher methylation capacity, which implies that there is a scope for utilizing genetic engineering in improving the methylation.
3. The identification of enzymes and functional genes participating and/or regulating the uptake and transformation pathways as well as release processes in dominating algal species is necessary to construct models in mitigating the trophic transfer of As and associated health risks with the seafood consumptions. The understanding of the responses of these specific genes with the particular algae is also required for knowing their functions under variable nutrient concentration in the laboratory culture studies.
4. The investigation of optimum concentration of iron and/or P levels in the algal culture system is necessary to minimize the surface adsorption phenomena of As and/or P and their related influence on the uptake and transformation.
5. Though it is anticipated that laboratory experiments have provided useful information on the As species formation and excretion, there is still necessary to understand how such metabolism might perform under real marine environmental conditions.

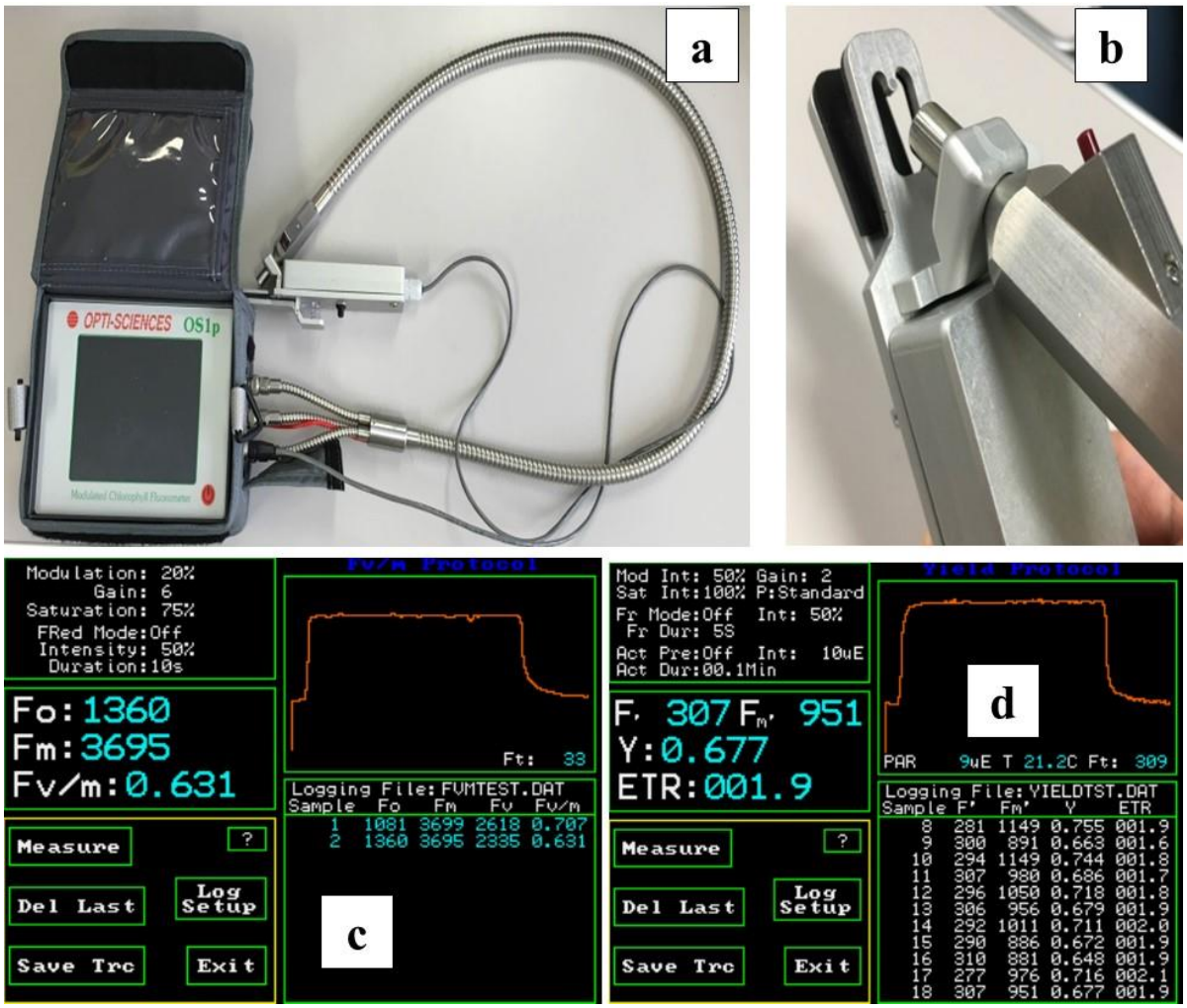
APPENDIX

Appendix A: Composition of PES media (Provasoli, 1968)

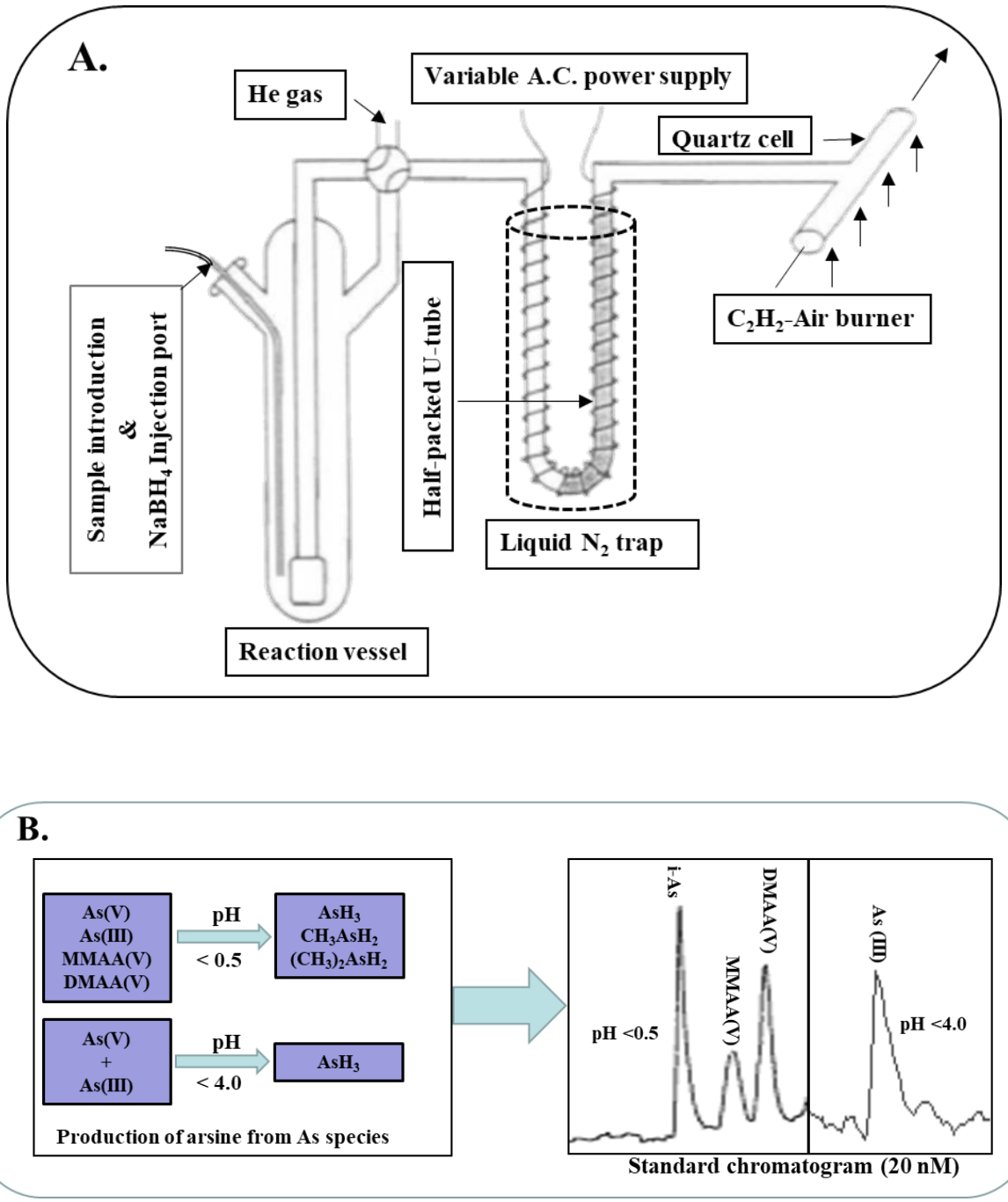
Compounds	**Concentrations**
TRIS (hydroxyl methyl)-aminomethane	5.0 g L^{-1}
$NaNO_3$	3.5g L^{-1}
KH_2PO_4*	2.0 mM
Fe stock solution[1]	250 mL L^{-1}
P-2 metal mix[2]	250 mL L^{-1}
Vitamin B_{12} stock solution (0.1 mg mL^{-1})	1.0 mL L^{-1}
Thiamine-HCl stock solution (0.1 mg mL^{-1})	5.0 mL L^{-1}
Biotine stock solution (0.1 mg mL^{-1})	0.5 mL L^{-1}
[1]**Fe Stock Solution*:**	
$Na_2EDTA \cdot 2H_2O$	660 mg L^{-1}
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	702 mg L^{-1}
[2]**P-2 Metal Mix:**	
$Na_2EDTA \cdot 2H_2O$	1.0 g L^{-1}
H_3BO_3	1.14 g L^{-1}
$FeCl_3 \cdot 6H_2O$*	49 mg L^{-1}
$MnSO_4 \cdot 4H_2O$	164 mg L^{-1}
$CoSO_4 \cdot 7H_2O$ stock solution (4.8 mg mL^{-1})	1.0 mL L^{-1}
$ZnSO_4 \cdot 7H_2O$	22 mg L^{-1}

*The concentrations were changed upon experimental setting.

APPENDIX



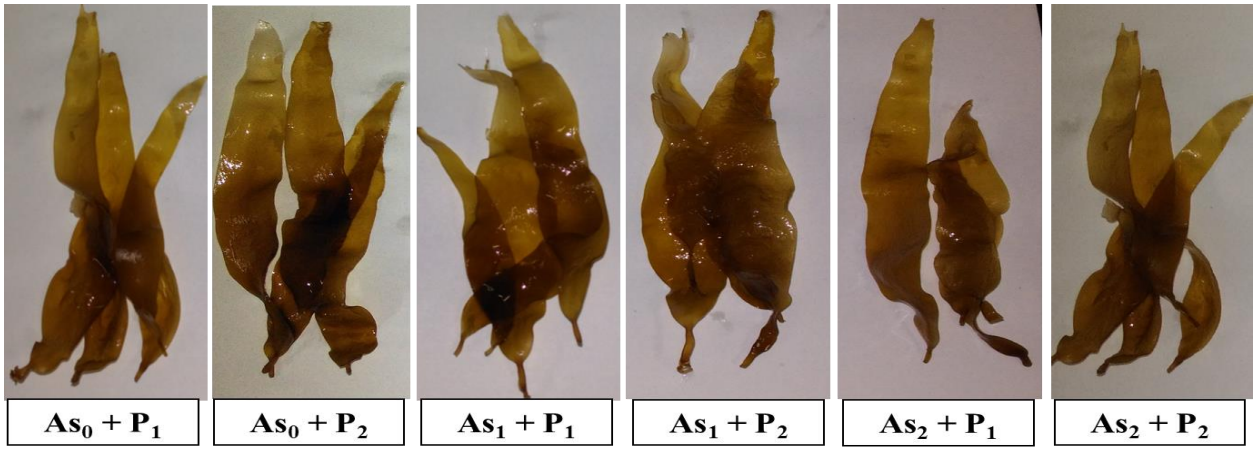
Appendix B: OS1p PAM device (a), PAR clip and sample holder (b), display of fast dark adapted (Fv/Fm) measurement (c), and fast light adapted Y(II) measurement (d).



Appendix C: The accessory apparatuses of CT-HG-AAS (A) (Reprinted from Hasegawa et. al., (1994), and chromatographic separation of As species in CT-HG-AAS (B).

Appendix D: Composition of artificial seawater (Lyman and Flemming, 1940).

Compounds	**Weight**
NaF	0.029 g
H_3BO_3	0.250 g
$SrCl \cdot 6H_2O$	0.122 g
KBr	1.00 g
$NaHCO_3$	1.96 g
KCl	6.8 g
$CaCl_2 \cdot 6H_2O$	14.3 g
$MgCl_2 \cdot 6H_2O$	108 g
Na_2SO_4	40.00
NaCl	240 g
Purified water	
Total	10 L



Appendix E: The appearance of sporophyte of *Undaria pinnatifida* after seven days incubation under various concentrations of As(V) and P containing growth media. As_0, As_1 and As_2 denote 0, 0.1 and 1 µM of As(V), respectively. P_1 and P_2 denote 1 and 10 µM of P, respectively.

APPENDIX

Appendix F: Unifactorial ANOVA for six culture conditions (six treatments) for measuring different parameters

Source of variation	Sum of Squares	df	Mean Square	F	Significance
Growth rate (GR)	8.49	5	1.70	1.97	0.16 ^{ns}
Maximum quantum yield ($F_v´/F_m´$)	0.00	5	0.00	0.27	0.92 ^{ns}
Intracellular Fe	0.02	5	0.00	0.94	0.49 ^{ns}
Extracellular Fe	0.01	5	0.00	2.06	0.14 ^{ns}
Total Fe	0.02	5	0.00	0.61	0.70^{ns}
Intracellular P	0.09	5	0.02	5.06	0.01**
Intracellular P	0.04	5	0.01	3.01	0.05*
Total P	0.21	5	0.04	19.56	0.00**
Intracellular As	13.51	5	2.70	687.36	0.00**
Intracellular As	199.09	5	39.82	410.08	0.00**
Total As	309.16	5	61.83	729.22	0.00**

^{ns} = nonsignificant

‘*’ and ‘**’ indicate significant at 5% and 1% confidence level, respectively.

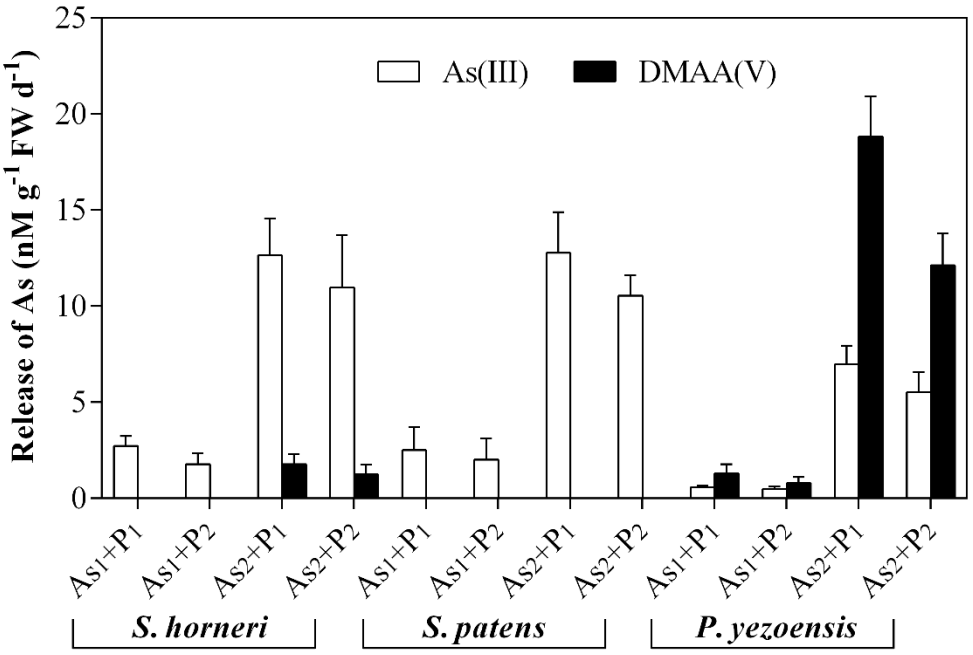
APPENDIX

Appendix G: Correlation matrix among the concentration of arsenic, phosphate and iron in different fractions of *Undaria pinnatifida*

Pearson correlation	Intracellular As	Extracellular As	Total As	Intracellular P	Extracellular P	Total P	Intracellular Fe	Extracellular Fe	Total Fe
Intracellular As	1.00								
Extracellular As	0.93**	1.00							
Total As	0.96**	1.00**	1.00						
Intracellular P	- 0.69	- 0.47	- 0.52	1.00					
Extracellular P	- 0.49	- 0.18	-0.24	0.63	1.00				
Total P	- 0.49	- 0.20	- 0.27	0.88*	0.86*	1.00			
Intracellular Fe	- 0.28	- 0.47	- 0.44	- 0.07	- 0.00	0.01	1.00		
Extracellular Fe	0.80	0.83*	0.83*	- 0.16	- 0.34	- 0.08	- 0.44	1.00	
Total Fe	0.442	0.27	0.31	- 0.21	- 0.31	- 0.06	0.60	0.46	1.00

**Correlation is significant at the 0.01 level (2-tailed)

*Correlation is significant at the 0.05 level (2-tailed).



Appendix H: The average release rates of different biotransformed As species after seven days incubation under indicated concentrations of As(V) and P. As_1 and As_2 denote 0.1 and 1 µM As(V), respectively. P_1 and P_2 denote 1 and 10 µM P, respectively. Data are means ± standard deviation (n = 3).

APPENDIX

Appendix I: ANOVA for six culture conditions (six treatments) for measuring different parameters of macroalgae species

Source of variation		**Type III Sum of Squares**	**df**	**Mean Square**	**F**	***p* value**	**Sig.**
Maximum quantum yield	*S. horneri*	0.00	5	0.00	1.87	2E-01	NS
	S. patens	0.00	5	0.00	1.03	4E-01	NS
	P. yezoensis	0.00	5	0.00	0.85	5E-01	NS
Growth rate	*S. horneri*	1.51	5	0.30	6.80	3E-03	**
	S. patens	0.73	5	0.15	3.33	4E-02	**
	P. yezoensis	2.78	5	0.56	8.60	1E-03	**
Extracellular As	*S. horneri*	14.06	5	2.81	61.33	4E-08	**
	S. patens	19.00	5	3.80	75.89	1E-08	**
	P. yezoensis	0.50	5	0.10	94.75	3E-09	**
Intracellular As	*S. horneri*	400.54	5	80.11	621.35	5E-14	**
	S. patens	295.58	5	59.12	289.08	5E-12	**
	P. yezoensis	29.00	5	5.80	329.38	2E-12	**
Total As	*S. horneri*	563.45	5	112.69	381.62	9E-13	**
	S. patens	458.68	5	91.74	445.12	3E-13	**
	P. yezoensis	37.09	5	7.42	696.19	2E-14	**
Extracellular P	*S. horneri*	54.58	5	10.92	9.10	9E-04	**
	S. patens	147.46	5	29.49	25.24	6E-06	**
	P. yezoensis	129.63	5	25.93	7.66	2E-03	**
Intracellular P	*S. horneri*	678.76	5	135.75	3.40	4E-02	**
	S. patens	1018.47	5	203.69	15.17	8E-05	**
	P. yezoensis	655.92	5	131.18	3.64	3E-02	**
Total P	*S. horneri*	995.27	5	199.05	4.76	1E-02	**
	S. patens	1707.36	5	341.47	18.26	3E-05	**
	P. yezoensis	1054.29	5	210.86	4.46	2E-02	**
Extracellular Fe	*S. horneri*	2.48	5	0.50	0.41	8E-01	NS
	S. patens	4.16	5	0.83	0.92	5E-01	NS
	P. yezoensis	4.16	5	0.83	0.92	5E-01	NS
Intracellular Fe	*S. horneri*	66.60	5	13.32	0.30	9E-01	NS
	S. patens	80.80	5	16.16	0.23	9E-01	NS
	P. yezoensis	126.57	5	25.31	0.56	7E-01	NS
Total Fe	*S. horneri*	89.84	5	17.97	0.48	8E-01	NS
	S. patens	63.16	5	12.63	0.18	1E+00	NS
	P. yezoensis	172.16	5	34.43	0.78	6E-01	NS

'NS' indicate nonsignificant, '**' indicates significant at 1% level of confidence

APPENDIX

Appendix J: Concentration of total As, P, and Fe (mg kg^{-1}) in different fractions of macroalgae

Element	Type of Culture	*Sargassum horneri*			*Sargassum patens*			*Pyropia yezoensis*		
		Extracellular	Intracellular	Total	Extracellular	Intracellular	Total	Extracellular	Intracellular	Total
Total As	As_0+P_1	0.00 ± 0.0	0.01 ± 0.0 d	0.01 ± 0.0 c	0.00 ± 0.0	0.04 ± 0.0 b	0.04 ± 0.0 c	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0 d
	As_0+P_2	0.00 ± 0.0	0.01 ± 0.0 d	0.01 ± 0.0 c	0.00 ± 0.0	0.03 ± 0.0 b	0.03 ± 0.0 c	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0 d
	As_1+P_1	0.10 ± 0.0 c	0.87 ± 0.2 c	0.97 ± 0.2 c	0.12 ± 0.0 c	0.78 ± 0.1 b	0.90 ± 0.1 c	0.05 ± 0.0 c	0.20 ± 0.0 c	0.25 ± 0.0 c
	As_1+P_2	0.07 ± 0.0 c	0.70 ± 0.1 c	0.78 ± 0.1 c	0.08 ± 0.0 c	0.50 ± 0.1 b	0.58 ± 0.1 c	0.04 ± 0.0 c	0.19 ± 0.0 c	0.23 ± 0.0 c
	As_2+P_1	2.26 ± 0.3 a	11.49 ± 0.6 a	13.76 ± 0.8 a	2.69 ± 0.5 a	9.30 ± 0.8 a	11.99 ± 0.4 a	0.43 ± 0.0 a	3.17 ± 0.2 a	3.60 ± 0.2 a
	As_2+P_2	1.43 ± 0.4 b	9.05 ± 0.6 b	10.47 ± 1.0 b	1.52 ± 0.3 b	8.51 ± 0.8 a	10.03 ± 1.0 b	0.30 ± 0.1 b	2.29 ± 0.2 b	2.59 ± 0.2 b
P	As_0+P_1	5.07 ± 1.1 b	101.49 ± 5.0 bc	106.56 ± 4.1 b	2.89 ± 0.5 b	77.08 ± 2.2 bc	79.97 ± 2.7 c	8.87 ± 1.1 b	109.01 ± 5.5 abc	117.88 ± 4.5 bc
	As_0+P_2	8.70 ± 1.0 a	114.70 ± 10.3 a	123.41 ± 10.8 a	8.43 ± 1.4 a	91.85 ± 4.1 a	100.29 ± 5.2 a	12.82 ± 1.9 a	119.26 ± 6.0 a	132.08 ± 7.8 a
	As_1+P_1	5.97 ± 0.7 b	99.72 ± 4.8 bc	105.69 ± 4.1 b	3.65 ± 0.9 b	71.97 ± 3.3 cd	75.62 ± 4.1 c	9.14 ± 1.8 b	104.85 ± 2.2 bc	113.98 ± 1.4 c
	As_1+P_2	8.90 ± 1.3 a	110.56 ± 5.8 ab	119.46 ± 5.5 a	9.08 ± 1.2 a	83.36 ± 4.4 b	92.44 ± 5.2 b	14.12 ± 1.8 a	113.57 ± 6.9 ab	127.68 ± 8.6 ab
	As_2+P_1	6.71 ± 0.8 b	97.21 ± 4.9 d	103.92 ± 5.2 b	3.86 ± 0.8 b	68.88 ± 4.1 d	72.74 ± 3.4 c	9.24 ± 1.8 b	100.63 ± 5.7 d	109.87 ± 7.2 c
	As_2+P_2	9.94 ± 1.5 a	105.59 ± 5.2 abc	115.53 ± 6.6 ab	9.81 ± 1.4 a	79.26 ± 3.3 b	89.06 ± 4.7 b	15.64 ± 2.4 a	106.73 ± 8.1 bc	122.37 ± 8.5 abc
Fe	As_0+P_1	11.05 ± 1.4	50.81 ± 6.7	74.54 ± 8.0	8.14 ± 0.8	75.22 ± 4.9	102.17 ± 6.6	2.56 ± 0.8	62.40 ± 3.7	80.55 ± 4.3
	As_0+P_2	10.12 ± 0.6	52.42 ± 4.0	76.57 ± 3.7	8.09 ± 0.8	72.38 ± 7.0	98.56 ± 8.9	2.51 ± 0.8	64.33 ± 3.1	82.92 ± 3.9
	As_1+P_1	11.53 ± 0.9	53.37 ± 6.1	78.25 ± 7.1	9.01 ± 0.9	74.16 ± 3.1	101.71 ± 4.0	3.42 ± 0.9	68.35 ± 3.5	88.87 ± 3.6
	As_1+P_2	11.62 ± 1.1	55.40 ± 4.4	80.86 ± 5.9	9.03 ±1.3	70.89 ± 6.9	97.64 ± 9.2	3.45 ± 1.3	67.61 ± 6.0	87.96 ± 7.6
	As_2+P_1	12.05 ± 1.2	55.00 ± 3.7	80.80 ± 3.4	9.13 ± 0.9	70.77 ± 7.5	97.58 ± 8.4	3.54 ± 0.9	66.87 ± 5.6	87.12 ± 6.5
	As_2+P_2	11.77 ± 1.1	53.31 ± 6.4	78.41 ± 7.1	9.30 ± 0.7	71.29 ± 9.1	98.41 ± 11.4	3.71 ± 0.7	67.61 ± 8.4	88.22 ± 10.8

Different small letter(s) in each column indicate significant differences among the cultures ($p > 0.05$).

APPENDIX

Appendix K: Correlation matrix among the concentration of As, P, and Fe in different fractions of macroalgae species

Sargassum horneri						
Pearson correlation (r)	Extracellular As	Intracellular As	Extracellular P	Intracellular P	Extracellular Fe	Intracellular Fe
Extracellular As	1	0.99**	0.16	- 0.50	0.66	0.45
Intracellular As	0.99**	1	0.23	- 0.47	0.68	0.44
Extracellular P	0.16	0.23	1	0.69	0.25	0.40
Intracellular P	- 0.50	- 0.47	0.68	1	- 0.67	-0.03
Extracellular Fe	0.66	0.68	0.25	- 0.67	1	0.63
Intracellular Fe	0.45	0.44	0.40	- 0.03	0.63	1
Sargassum patens						
Pearson correlation (r)	Extracellular As	Intracellular As	Extracellular P	Intracellular P	Extracellular Fe	Intracellular Fe
Extracellular As	1	0.96**	- 0.07	- 0.56	0.59	- 0.59
Intracellular As	0.96**	1	0.10	- 0.49	0.67	- 0.60
Extracellular P	- 0.07	0.10	1	0.70	0.22	- 0.63
Intracellular P	- 0.56	- 0.49	0.70	1	- 0.53	- 0.11
Extracellular Fe	0.59	0.67	0.22	- 0.53	1	- 0.59
Intracellular Fe	- 0.59	- 0.60	- 0.63	- 0.11	- 0.59	1
Pyropia yezoensis						
Pearson correlation (r)	Extracellular As	Intracellular As	Extracellular P	Intracellular P	Extracellular Fe	Intracellular Fe
Extracellular As	1	0.99**	0.05	- 0.71	0.67	0.41
Intracellular As	0.99**	1	0.08	- 0.69	0.64	0.38
Extracellular P	0.05	0.08	1	0.46	0.29	0.31
Intracellular P	- 0.71	- 0.69	0.46	1	- 0.63	- 0.40
Extracellular Fe	0.67	0.64	0.29	- 0.63	1	0.90*
Intracellular Fe	0.41	0.38	0.31	- 0.40	0.90*	1

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

APPENDIX

Appendix L: The intracellular arsenic and phosphate concentration (μM g^{-1} dry weight) under different concentrations of arsenate and arsenite treatments.

As(V) treatments (μM)	Intracellular P uptake (μM g^{-1} DW)			
	–P–Fe	–P+Fe	+P–Fe	+P+Fe
Control	47.57 ± 4.35	47.64 ± 6.51	55.71 ± 3.86	56.56 ± 11.42
0.25	47.01 ± 6.08	48.81 ± 8.03	53.91 ± 6.60	53.43 ± 9.65
0.50	47.20 ± 5.74	47.20 ± 5.95	52.26 ± 9.50	51.47 ± 8.04
1.0	46.85 ± 6.00	46.12 ± 5.60	50.30 ± 6.97	50.77 ± 6.88
2.0	47.39 ± 6.89	46.97 ± 5.60	48.94 ± 4.34	48.68 ± 7.54
4.0	46.88 ± 9.28	46.21 ± 9.02	47.29 ± 7.07	46.53 ± 10.86
As(III) treatments (μM)				
Control	49.60 ± 11.26	48.62 ± 3.34	53.05 ± 4.50	51.85 ± 9.96
0.25	47.35 ± 9.03	47.92 ± 9.07	53.27 ± 10.21	52.61 ± 7.59
0.50	47.70 ± 7.62	47.29 ± 7.65	53.24 ± 3.18	54.51 ± 7.40
1.0	48.62 ± 5.16	46.47 ± 4.80	55.01 ± 9.42	54.10 ± 8.41
2.0	46.69 ± 8 .27	48.24 ± 4.77	56.47 ± 8.69	54.41 ± 5.76
4.0	47.23 ± 4.47	46.69 ± 5.32	56.53 ± 6.69	56.47 ± 8.54

APPENDIX

Appendix M: Pearson's correlation matrix between intracellular arsenic and iron concentrations.

Pearson correlation coefficient			Intracellular uptake							
			–P–Fe		–P+Fe		+P–Fe		+P+Fe	
			As(V)	Fe	As(V)	Fe	As(V)	Fe	As(V)	Fe
Intracellular uptake	–P–Fe	As(V)	1							
		Fe	0.02	1						
	–P+Fe	As(V)	0.99**	0.01	1					
		Fe	0.95**	-0.05	0.95**	1				
	+P–Fe	As(V)	0.99**	0.01	0.99**	0.95**	1			
		Fe	0.48	0.28	0.46	0.60	0.45	1		
	+P+Fe	As(V)	0.99**	0.02	1.00**	0.95**	1.00**	0.45	1	
		Fe	0.96**	0.09	0.97**	0.98**	0.97**	0.58	0.97**	1
Pearson correlation coefficient			Intracellular uptake							
			–P–Fe		–P +Fe		+P–Fe		+P+Fe	
			As(III)	Fe	As(III)	Fe	As(III)	Fe	As(III)	Fe
Intracellular uptake	–P–Fe	As(III)	1							
		Fe	0.16	1						
	–P+Fe	As(III)	0.99**	0.14	1					
		Fe	0.88*	0.47	0.87*	1				
	+P–Fe	As(III)	1.00**	0.16	0.99**	0.88*	1			
		Fe	-0.05	0.80	-0.06	0.13	-0.06	1		
	+P+Fe	As(III)	1.00**	0.14	0.99**	0.87*	1.00**	-0.07	1	
		Fe	0.90*	0.44	0.89*	0.99**	0.90*	0.11	0.90*	1

‘*’ and ‘**’ indicates correlation is significant at 0.05 and 0.01 level (2-tailed), respectively.

APPENDIX

Appendix N: Pearson's correlation matrix between extracellular arsenic and iron concentrations.

Pearson correlation coefficient			Extracellular uptake							
			–P+Fe		+P+Fe		–P+Fe		+P+Fe	
			As(V)	Fe	As(V)	Fe	As(III)	Fe	As(III)	Fe
Extracellular uptake	–P+Fe	As(V)	1							
		Fe	0.97**	1						
	+P+Fe	As(V)	0.99**	0.97**	1					
		Fe	0.94**	0.99**	0.95**	1				
	–P+Fe	As(III)	0.99**	0.96**	0.99**	0.94**	1			
		Fe	0.95**	0.97**	0.96**	0.95**	0.93**	1		
	+P+Fe	As(III)	0.99**	0.97**	0.99**	0.95**	0.99**	0.94**	1	
		Fe	0.93**	0.98**	0.94**	0.99**	0.92**	0.94**	0.94**	1

'*' and '**' indicates correlation is significant at the 0.05 and 0.01 level (2-tailed), respectively.