Arsenic Biotransformation Potential of Marine and Freshwater Phytoplankton

Dissertation

Arsenic Biotransformation Potential of Marine and Freshwater Phytoplankton

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DOCTORAL DISSERTATION

ARSENIC BIOTRANSFORMATION POTENTIAL OF MARINE AND FRESHWATER PHYTOPLANKTON

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THIS DISSERTATION SUBMITTED TO THE GRADUATE SCHOOL OF NATURAL SCIENCE AND TECHNOLOGY OF KANAZAWA UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Dedicated to

our daughter
Sohana Afrin Mayumi

-who conveys light and smile from heaven in our life !

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ABSTRACT

The biotransformation of arsenic (As) species have been vibrant research topics due to significance to environmental and human health. Microalgae play an important role in As bioaccumulation and biogeochemical cycling in aquatic ecosystems. Microalgal growth and physiological activities in aquatic systems depend on various important environmental factors and data are available in the literature. However, effects of environmental factors on As biotransformation by aquatic microalgae has remain imprecise. This study aims to investigate the individual as well as integrated effects of such factors on As uptake, accumulation, and biotransformation potential of marine and freshwater microalgae. A series of laboratory-based experiments were designed using several marine and freshwater microalgae were cultured using f/2 and C medium, respectively. The following investigation was reported based on this study: (a) As biotransformation potential by marine phytoplankton under a salinity gradient, (b) effect of temperature and salinity on As biotransformation by marine diatom species, (c) integrated effects of important environmental factors on As biotransformation, (d) biotransformation of inorganic As to methylarsenic and organoarsenic, and (e) the effects of salinity stress on As biotransformation by freshwater phytoplankton. Conceptual model highlighting the microalgal species specifically associated with environmental factors-dependent As biotransformation mechanism was proposed.

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AAS	flame atomic absorption spectrophotometer
ANOVA	analysis of variance
ArsB	arsenobetaine
ArsC	arsenocholine
As	arsenic
As(III)	arsenite
As(-III)	arsine
AS(V)	arsenate
As3MT	arsenite methyltransferase
DMAA	dimethylarsinate
F_v/F_m	maximum quantum yield
GSH	glutathione
ICP-MS	Inductively coupled plasma mass spectrometer
LODs	limits of detection
methylAs	methylarsenic
MMAA	monomethylarsonate
orgAs	organoarsenic
PCs	phytochelatins
PE	photosynthetic efficiency
PO_4^{3-}	phosphate
RSD	relative standard deviation
SAM	S-adenosylmethionine
t-As	total As
UV-VIS	ultraviolet-visible

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2. Hiroshi Hasegawa*, **Rimana Islam Papry***, Eri Ikeda, Yoshiki Omori, Asami S. Mashio, Teruya Maki, M. Azizur Rahman*, "Freshwater phytoplankton: biotransformation of inorganic arsenic to methylarsenic and organoarsenic", *Scientific Reports*, **9** (2019) 12074 (Published: August 2019).
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EXECUTIVE SUMMARY

Arsenic (As), an environmental contaminant, is awfully toxic to environmental and human health at high concentrations. The occurrence of As in aquatic systems is of great concern due to its high bioavailability, bioaccumulation, and trophic transfer from the bases to the higher tropic level in aquatic food chain. Algal species occupy the lowest levels of aquatic food chains and significantly contribute to As speciation, distribution, and geochemical cycling in such ecosystems. Microalgal growth and other physiological activities in aquatic system highly depends on various important environmental factors including temperature, salinity, constituency of nutrient medium, light intensity, pH, concentrations of As species, length of exposure time and so on and data in this regards are available in the literature. In some cases, the combined effects of two or more factors on growth and development of microalgae are also found. However, the study on environmental factor(s) effects on As uptake, accumulation, and their biotransformation mechanisms by marine and freshwater microalgae are not done yet. Moreover, the knowledge on As biotransformation at various growth stages of aquatic microalgae are also imprecise. This study was aimed to fulfil the research gap by investigating As biotransformation potentials of various marine and freshwater microalgae under individual or combined stress of such factors. For this, a series of experiment was designed in the laboratory with the specific objectives. Three marine phytoplankton and six marine diatom species were collected and employed in the study to investigate their As biotransformation potentials. Six freshwater phytoplankton were used to know at which growth stages of microalgae the biotransformation of As species occur, and, finally, the effects of salinity stress on As biotransformation by these freshwater phytoplankton was also investigated. The marine and freshwater organisms were cultured in laboratory using f/2 and C medium, respectively. In addition, different concentrations of arsenate (As(V) and phosphate (PO_4^{3-}) were added to the culture medium in order to observe their effects on As uptake and biotransformation. As species was determined using the technique of cold trap hydride generation atomic absorption spectrophotometry (CT-HG-AAS). The combination of important environmental factors used in this study was temperature, salinity, concentrations of As(V) and PO_4^{3-}, and length of exposure period. Effects of individual as well as integrated effects of environmental factors on their growth, photosynthetic efficiency (PE), morphological changes, relationship between As biotransformation with other parameters were also studied. Each case, a conceptual model on growth and As biotransformation by these microalgae under the stress of various factors was proposed based on the findings of this study. The following research was conducted and reported giving emphasis on As biotransformation potentials by above marine and freshwater microalgae:

(a) Marine phytoplankton: Arsenic biotransformation under a salinity gradient

In this study, we investigated the As biotransformation potential of three marine phytoplankton species, namely *Prymnesium parvum*, *Oltmannsiellopsis viridis*, and *Eutreptiella gymnestica* under a

broad salinity gradient. Growth and As biotransformation potential were observed in As-poor and As-rich conditions in combination with phosphate (PO_4^{3-})-poor and PO_4^{3-}-rich conditions, and different salinities (0-50‰). For all species, the highest cell concentrations were observed on day 14 of culture. Among the three species, *O. viridis* showed maximum growth, photosynthetic efficiency, As biotransformation, and total As accumulation ability under a broad range of salinities (≥5‰ to ≤45‰). However, As biotransformation and other physiological activities decreased for all the phytoplankton species at ≤5‰ and ≥45‰. Phytoplankton growth and As biotransformation potential was significantly influenced by salinity and by the availability of As and PO_4^{3-} in the culture medium. A conceptual model of salinity effects on As uptake and biotransformation in the three marine phytoplankton species is proposed based on the findings of this study.

(b) Marine diatom species: Effect of temperature and salinity on arsenic biotransformation

This study reports the growth, and As biotransformation and speciation patterns at various temperatures and salinities of six marine diatom species: *Asteroplanus karianus*, *Thalassionema nitzschioides*, *Nitzschia longissima*, *Skeletonema* sp., *Ditylum brightwellii*, and *Chaetoceros didymus*. The growth rate and As biotransformation potentials of these species during three weeks of culture in f/2 based medium were significantly affected by wide temperature (0–35°C) and salinity (0.3–50‰) ranges. Growth and As biotransformation were higher at optimum temperatures of 10–25°C, and salinity of 10–35‰, whereas growth and arsenic biotransformation were lower at < 5°C and 5‰ and > 25°C and 35‰, respectively. The results showed that As(V) to As(III) biotransformation differed significantly ($p < 0.05$) between day 10 and 17. At optimum temperature and salinity levels, the cell size and As biotransformation were higher for all the species. A conceptual model on temperature and salinity effects on growth and As uptake and biotransformation mechanisms by these species has been proposed based on the findings of this study.

(c) Marine microalgae: Integrated effects of environmental factors on As intraformational

Herein, salinity and temperature are both considered in combination to investigate their influence on As uptake, biotransformation, and photosynthetic efficiency (PE). Two strains of marine diatom species, *A. karianus* and *Skeletonema* sp., were cultured under various temperatures (5.0, 20, and 35°C) and salinities (1.0‰, 10‰, 25‰, and 40‰) in association with As and phosphate-enriched (1.0 µmol L^{-1} of As(V) + 10 µmol L^{-1} of PO_4^{3-}) or deficient (20 nmol L^{-1} of As(V) + 1.0 µmol L^{-1} of PO_4^{3-}) conditions. For both species, maximum growth, As accumulation, biotransformation, and PE were recorded at 10 and 14 day of culture. Microalgal growth, As accumulation, biotransformation, and PE were maximum at 20°C with salinities of 10‰ and 20‰. Cell shape was also observed to be good at optimal at this temperature (20°C) and range of salinity (10‰ and 20‰). A conceptual model of integrated effects of environmental factors on growth and As accumulation and biotransformation

activities by these marine microalgae has been proposed. This study contributed to the elucidation of the relationship between environmental factors and As biotransformation mechanisms, which may further provide significant insight about As remediation processes.

(d) Freshwater phytoplankton: Biotransformation of inorganic arsenic to methyarsenic and organoarsenic

This study investigated the biotransformation of As species in freshwater phytoplankton at different growth phases to ascertain at which growth phase different types of biotransformation occur. At the logarithmic growth phase, arsenate (As^{V}) (> 90%) and arsenite (As^{III}) (> 80%) predominated in culture media when phytoplankton were exposed to 20 nmol L^{-1} and 1.0 µmol L^{-1} of As^{V}, respectively, and methylarsenic (methylAs) species were not detected in them at all. Intracellular As was mainly present in inorganic forms (iAs) at the logarithmic phase, while substantial amounts of organoarsenic (orgAs) species were detected at the stationary phase. At the stationary phase, As^{V} comprised the majority of the total As in culture media, followed by As^{III} and methylAs, although the methylation of As^{V} occurred slowly at the stationary phase. Biotransformation of As^{V} into As^{III} and As methylation inside phytoplankton cells occurred mainly at the logarithmic phase, while the biotransformation of As into complex orgAs compounds occurred at the stationary phase. Phytoplankton rapidly released iAs and methylAs species out of their cells at the logarithmic phase, while orgAs mostly remained inside their cells.

(e) Freshwater phytoplankton: The effects of salinity stress on As biotransformation

This study reports a distinctive pattern of As uptake, accumulation, and biotransformation by four axenic freshwater phytoplankton species, i.e., *Scenedesmus acutus*, *Closterium aciculare*, *Staurastrum paradoxum*, and *Pediastrum duplex*. Phytoplankton cells were incubated in sterilized C medium modified with varying salinity levels (0–5‰) in association with arsenate and phosphate concentrations. The biotransformation of arsenate (i.e., As(V)) to arsenite (As(III)) and to further methylated species decreased with increasing salinity in the culture medium. Among the four strains, only *S. acutus* and *S. paradoxum* converted As(V) to As(III), with no detected methylated species. In contrast, *C. aciculare* and *P. duplex* biotransformed As(V) to As(III) and further to methyl arsenic species, such as DMAA. *S. acutus* and *S. paradoxum* exhibited higher accumulation tendency than the other two species. *S. paradoxum* showed the lowest As reduction rate (i.e., As(V) to As(III)) compared to other species, although, without significant variations. The morphological changes were observed in phytoplankton cells in response to increased salinity stress. Moreover, As(V) concentrations in the culture medium significantly decreased by day 7 to 14. Thus, this study presents a conceptual model of the As biotransformation pattern by axenic freshwater phytoplankton.

Chapter 1:

Background of the study

1.1 General introduction

Arsenic (As), an environmental pollutant, is extremely toxic to living organisms at high concentrations. Naturally, As has four primary oxidation states (−3, 0, +3, and +5) with various physiological and chemical properties such as arsenate (As(V)), arsenite (As(III)), arsenic (As^0), and arsine (As(-III)) (Bahar et al., 2012). The occurrence and distribution of this toxic metalloid is recorded all over in soil, freshwater, and marine ecosystems. Inorganic arsenic (iAs), such as As(V) and As(III) found in aquatic ecosystems (Rodríguez-lado et al., 2013), which are more toxic than organarsenicals (Dopp et al., 2010; Sun et al., 2012; Alava et al., 2012). The toxicity of different As forms as determined by the 50% lethal dose (LD50) follow the order: As(III) (14) > As(V) (20) > monomethylarsonate (MMAA(V)) (700–1800) > dimethylarsinate (DMAA(V)) (700–2600) > arsenocholine (ArsC) (> 6500) > arsenobetaine (ArsB) (> 10,000) (Niegel and Matysik, 2010). In oxic waters, As(V) is thermodynamically stable, whereas As(III) found in a reduced-redox state (Hasegawa et al., 2010). Organic forms of As species, such as MMAA(V) and DMAA(V) are observed at low concentrations in the aquatic environment (Akter et al., 2005). The occurrence of As in aquatic systems is of great concern due to its high bioavailability, bioaccumulation, and trophic transfer from the bases of aquatic food chains through to higher trophic levels (Rahman et al., 2012). Arsenic in marine biota may not be a significant concern for human health because it is present among them in low concentrations. In freshwater systems As is likely to be a significant environmental and human health problem due to the high concentrations that can result from its direct input into these systems from natural and manmade sources (Rahman and Hassler, 2012).

Algal species occupy the lowest levels of aquatic food chains and significantly contribute to As speciation, distribution, and cycling in aquatic ecosystems (Hasegawa et al., 2001; Zhang et al., 2014). Microalgae as primary producers ensure As bioaccumulation and biogeochemical cycling in the aquatic ecosystem (Duncan et al., 2015; Zhang et al., 2014). They have eminent competence in the uptake of As from the environment, which makes these tiny species ecofriendly and cost effective in terms of the As remediation process (Bahar et al., 2013; Mahdavi et al., 2012; Wang et al., 2015). Biotransformation of As species by microalgae is a complex mechanism with different toxicity levels (Karadjova et al., 2008). The reduction of As(V) and subsequent methylation to methylated species (MMAA and DMAA) is considered to be a detoxification mechanism (Hasegawa et al., 2019; Wang et al., 2013).

The uptake, biotransformation, and accumulation of As(V) by phytoplankton is related to the accessibility of phosphate (PO_4^{3-}) (Hellweger et al., 2003). Because As(V) is considered an analog of PO_4^{3-} and microalgae uses the PO_4^{3-} transporter for uptake of As(V) inside the cell membrane (Guo et al., 2011). Moreover, PO_4^{3-} concentration has a significant influence on the uptake of As(V) by microalgae (Rodriguez Castro et al., 2015; Bahar et al., 2016; Duncan et al., 2013; Han et al., 2017). Biotransformation of As(V) to As(III) and subsequent methylation to MMAA and DMAA have also been reported to be affected by the concentration of As and PO_4^{3-} in the medium (Karadjova et al., 2008; Duncan et al., 2015). Methylated species in natural waters are believed to come not only from microalgae and bacteria but also from biodegradable components (Hasegawa et al., 2001), and several studies confirmed the presence of methylarsenic (methylAs) species in experiments with marine (Papry et al., 2020, 2019; Sanders et al., 1989) and freshwater microalgae (Maeda et al., 1992; Wang et al., 2017; Hasegawa et al., 2019).

However, microalgal, particularly phytoplankton and diatom species, growth and their physiological activities are influenced by various environmental factors (McLachlan, 1961; Ault et al., 2000). The data on environmental factor(s) effects on marine microalgal growth are available in the literature such as constituency of nutrient medium (Hasegawa et al., 2001), concentration of As species (Karadjova et al., 2008; Gong et al., 2008), pH (Maeda et al., 1992; Murray et al., 2003; Pawlik-Skowronska et al., 2004), light intensity (Karadjova et al., 2008; Bottino et al., 1978), temperature (Fujimoto et al., 1994; Raven and Geider, 1988), salinity (Abubakar, 2017; Bartley et al., 2013), and length of exposure period (Foster et al., 2008). In some cases, the combined effects of two or more factors on growth and development of marine microalgae are also reported (Adenan et al., 2013; Salarzadeh and Ebrahimi, 2016). In the marine environment, salinity variation affects the biochemical and physiological activities of phytoplankton, including growth, photosynthesis, and As uptake, accumulation, and biotransformation. Adaptation capacity of freshwater microalgae in terms of growth and physiological metabolism likely different from marine microalgae. In such instance salinity act as barrier in between fresh and marine ones (Lobban and Harrison, 1994). Even there is a significant variation among freshwater microalgae in terms of salinity tolerance and some of them are actively response to frequent salinity fluctuations (Maier Brown et al., 2006). Moreover, the tolerance to a particular factors of phytoplankton depends on the species type and its characteristics (Rao et al., 2007; Saros and Fritz, 2000). For example. *Rhizoclonium riparium* (Roth) Harvey a filamentous green alga is generally found in freshwater but also able

to survive in brackish and marine water. Salinity stresses lower the cellular growth because of high energy required for osmoregulation.

Although the study on how environmental factors effects on microalgal growth and physiological activities are vastly conducted and data in this regards are available in the literature, the study on environmental factors effects on As uptake, accumulation, and their biotransformation mechanisms by marine and freshwater microalgae are not done yet. This study was aimed to fulfil the research gap. A series of experimental setup was designed to examine the As biotransformation potential of marine and freshwater microalgae under the stress of various environmental factors. For this, different marine (three phytoplankton and six diatom species) and freshwater (six phytoplankton species) microalgae were collected and employed in the study to investigate their As biotransformation potentials. The marine phytoplankton species were *Prymnesium parvum, Oltmannsiellopsis viridis,* and *Eutreptiella gymnastica*, and diatom species were *Asteroplanus karianus*, *Thalassionema nitzschioides*, *Nitzschia longissima*, *Skeletonema* sp., *Ditylum brightwellii*, and *Chaetoceros didymus*. Six clonal axenic freshwater phytoplankton strains were *Achnanthidium minutissimum*, *Botryococcus braunii*, *Scenedesmus actus*, *Staurastrum paradoxum*, *Pediastrum duplex*, and *Closterium aciculare*. The marine and freshwater organisms were cultured in laboratory using f/2 and C medium, respectively. In addition, different concentrations of As(V) and PO_4^{3-} were added to the culture medium in order to observe their effects on As uptake and biotransformation. The combination of important environmental factors used in this study was temperature, salinity, concentrations of As(V) and PO_4^{3-}, and length of exposure period. Along with As biotransformation, effects of individual as well as integrated effects of environmental factors on their growth, photosynthetic efficiency (PE), changes in cell shapes, relationship between As biotransformation and other parameters were also studied. In case of freshwater microalgae, As biotransformation at different growth stages to understand at which growth phases biotransformation occur and at various salinity stress were studied. The conceptual model on growth and As biotransformation by these microalgae under the stress of various factors was proposed based on the findings of this study. This research contributes to a deeper understanding of the relationship among environmental factors, As uptake, and the biotransformation mechanism of marine and freshwater microalgae from the viewpoint of As remediation. In the following literature, an overview of the As speciation pattern by aquatic microalgae, their measurement procedures, effects of environmental factors on physiological activities are discussed in details.

1.2 Objective of the research

The **overall objective** of the research was to investigate the uptake, accumulation and biotransformation potentials of As species by various marine and freshwater microalgae under individual as well as integrated stress of important environmental factors such as temperature, salinity, nutrient concentrations and length exposure period. The effect of environmental factors on microalgal growth, PE, changes in cell shapes, and relationship between As biotransformation and other activities were also observed under this study. Conceptual model proposed highlighting the microalgal species specifically associated with environmental factors-dependent As biotransformation mechanisms, which may further provide significant insight regarding As remediation processes. This study contributed to the elucidation of the relationship between environmental factors and As biotransformation mechanisms, which may further provide significant insight about As remediation processes.

The **specific objectives** of the research are:

- **(a)** to observe the As biotransformation potentials by marine phytoplankton under a salinity gradient,
- **(b)** to scrutinize the effect of temperature and salinity on As biotransformation by marine diatom species,
- **(c)** to perceive the integrated effects of important environmental factors on As biotransformation by marine microalgae,
- **(d)** to investigate the biotransformation of As species in freshwater phytoplankton at different growth phases to ascertain at which growth phase different types of biotransformation occur,
- **(e)** to explore the salinity stress on As biotransformation by freshwater phytoplankton, and
- **(f)** to observe the physiological changes of microalgal cell under stress of temperature and salinity and the relationship with As biotransformation.

1.3 Organization of dissertation

This dissertation structured into 7 chapters. In the first chapter, a general introduction followed by the overall and specific objectives of the study, and organization of the dissertation is given. This chapter also includes overall background of the study including literature review

on the As toxicity, role of aquatic microalgae on As biogeochemical cycle, environmental factor affecting As biotransformation by microalgae are discuss.

In chapter 2, As biotransformation potentials of marine phytoplankton under a salinity gradient has been discussed. This chapter mainly focused on salinity effects on growth, photosynthetic efficiency, As uptake, accumulation, and their biotransformation by marine phytoplankton.

In chapter 3, As biotransformation potentials of six marine diatom species under a wide range of temperature and salinity are discussed. Marine diatom growth, PE, As biotransformation and changes in cell shapes are elaborated based on the findings of this study.

In chapter 4, Integrated effects of important environmental factors on marine diatom growth, PE, As uptake, accumulation and biotransformation, and physiological changes of cells are discourses. This chapter focuses on the combined application of temperature, salinity, nutrient concentrations and exposure time on the biotransformation of As species by two diatom species.

In chapter 5, the biotransformation of inorganic As species to methyl arsenic and organoarsenic by freshwater phytoplankton are discussed. This chapter emphases on the biotransformation of As species at different growth phases of freshwater phytoplankton to understand at which growth phase biotransformation occur.

In chapter six, salinity stress on As biotransformation by freshwater phytoplankton has been deliberated. A distinctive patter of diatom growth, PE, As uptake, accumulation and biotransformation, and physiological changes of cells are discourses.

In chapter seven, a general conclusion based on the present study and further scope and recommendation has been mentioned.

1.4 Review of the literature

1.4.1 Occurrence and distribution of As in the environment

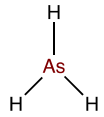
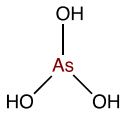
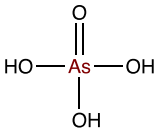
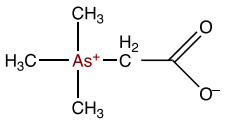
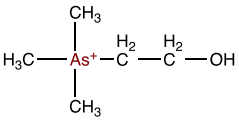
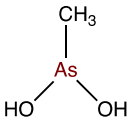
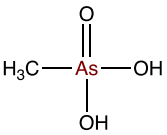
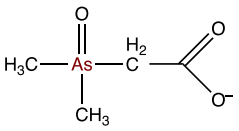
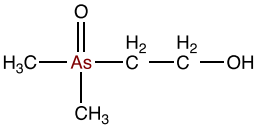
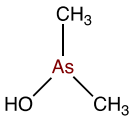
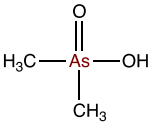
Arsenic was first discovered by German alchemist Albertus Magnus in 1250 A.D. (Singh et al., 2015) Since then it was one of the most contentious elements due to its insidious nature in the environment. As is a toxic metalloid which has both metal and non-metal property. Naturally occurred in ashes of volcanoes, fossil fuels, weathering of rocks and bioleaching processes (Nordstrom, 2002). Existence of As with higher quantity subjected to the topographical characteristics such as category of rock, land use pattern and morphological

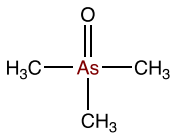
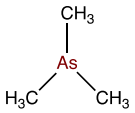
nature (Bhattacharya et al., 2007). Among different trace metals, it has been ranked as 20th, 14th and 12th according to its occurrence in earth crust, marine ecosystem and human body respectively (Mandal and Suzuki, 2002). As naturally occurs in groundwater of different countries all over the world such as India, China, Bangladesh, Chili, Hungary, Vietnam, Mexico, Taiwan, Romania, Argentina, and some parts of USA (Bissen and Frimmel, 2003). Approximately 240 minerals contain As, among all of them it causes strong interactions with Iron, Silicon, Phosphorus and sulphur (Bhattacharya et al., 2010). On earth, concentration of As may varies from 0.1 to 55 mg/kg with the total quantity of As in Oceans, earth`s crust, sediments, and atmosphere of about 3.7×10^6 kt (kiloton), 9.7×10^5 kt, 25×10^9 kt, and 8.12 kt respectively (Bissen and Frimmel, 2003).

1.4.2 Various forms of As species in the environment

In the environment, As found in four oxidation state (-3, 0, +3 and +5) (Sharma and Sohn, 2009). According to their chemical properties, As can be categorised into inorganic and organic form, where trivalent As species exhibits more toxic effect than pentavalent As species (Patel et al., 2005). As(V) is thermodynamically stable in oxic water but As(III) is predominant in anoxic condition (Zhao et al., 2013). The main iAs forms include Arsenate (As(V)), Arsenite (As(III)), As acids (H_3AsO_4, $H_2AsO_4^-$, $HAsO_4^{2-}$)) and arsenious acids (H_3AsO_3, $H_2AsO_3^-$, $HAsO_3^{2-}$) etc. Various forms of inorganic and organoarsenic species are listed in **Table 1.1**. However, forms of OAs create when combined with other molecules that contains carbon- or sulfur- such as arsenobetaine (AB), arsenocholine (AC) arsenosugars, arsenolipids, DMA, and MMA. The phenomenon where As can transform to organic or inorganic form actually depend on their physiological or biological state known as As speciation. However, toxic iAs converted to less toxic, volatile, and easily oxidizable methylarsine under the anaerobic environmental condition. In aquatic environment, the solubility of As species influenced by the pH and other ionic species available in the medium. Several studies mentioned the toxicity level of different As species as follows: MMA(III) > As(III) > As(V) > DMA(V) > MMA(V) (Wen et al., 2011) (Kile et al., 2011).

Table 1.1 Various arsenic species with symbol and structural formula.

Species	Symbol	Structural formula
Inorganic arsenic species		
Arsine	AsH_3	
Arsenite	As(III)	
Arsenate	As(V)	
Organoarsenic species		
Arsenobetaine	AB	
Arsenocholine	AC	
Monomethylearsenite	MMA(III)	
Monomethylarsenate	MMA(V)	
Dimethylarsinoylacetate	DMAE	
Dimethylarsinoylethanol	DMAE	
Dimethylarsenite	DMA(III)	
Dimethylarsenate	DMA(V)	

Trimethylarsineoxide	TMAO	
Trimethylarsine	TMA	

1.4.3 Biogeochemical cycle of arsenic

As considered as highly noxious metalloid found in environment that possesses severe risk to aquatic and terrestrial flora and fauna, as it is results of both natural and anthropogenic activities. As is found in rocks of earth crust and groundwater in different places all over the world (Smedley and Kinniburgh, 2002). Fate of As in the environment largely depends on the biotic (microflora) and abiotic (pH) component. Biogeochemical cycling of As in the environment actually involves the biotransformation reaction that oscillate between arsenate and arsenite (**Figure 1.1**). The overall activities of microbial processes, in combination with inorganic and physical processes represent the As cycle in the earth. The accessibility and mobility of As rely on redox reaction of As(V) and As(III) (Yamamura and Amachi, 2013). The biotransformation mechanism includes three main steps: firstly, redox reaction between As(III) and As(V), secondly, reduction and methylation processes of As and thirdly, biosynthesis of OAs species (arsenosugars and arsenolipids). Several thermal processes (e.g. volcanic eruption, coal fired power generation) and bioprocesses (e.g. methylation and microbial reduction) excreted As in the environment either aerobic state or anaerobic state (Bundschuh et al., 2011). Microorganisms plays an essential role in terms of As biotransformation and remediation processes. Biotransformation of As by microorganisms includes redox reaction of As (V) (Zouboulis and Katsoyiannis, 2005), series of methylation and demethylation by microorganism (Stolz et al., 2006) and occurrence of thiol based polypeptides such as glutathione and phytochelatins that make bondage with As to form organoarsenic compound (Meyer et al., 2007). In regards of As cycling, marine ecosystem play significant role as it is accumulates 3-4 times higher As than freshwater microalgae (Edmonds et al., 1997).

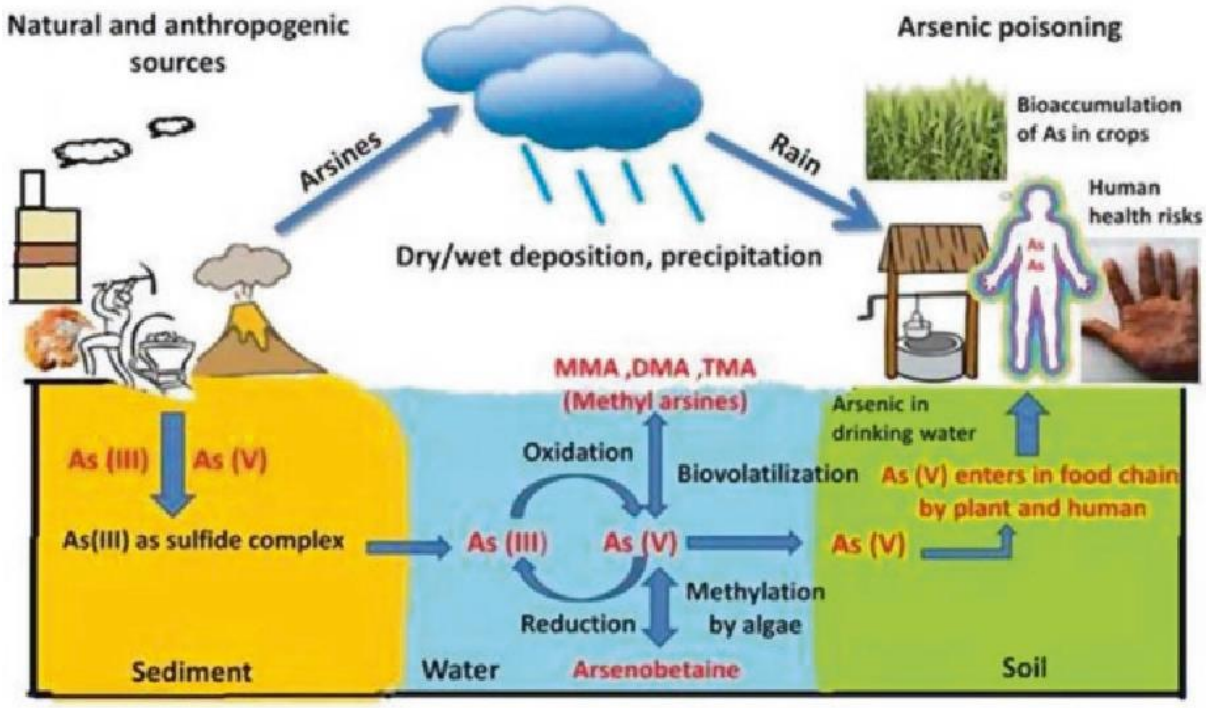


Figure 1.1 Biogeochemical cycle of Arsenic (adapted from (Hasanuzzaman et al., 2018)).

1.4.4 Microalgae for arsenic remediation

Microalgae are found in vast areas of aquatic habitats including ponds, lakes, rivers, oceans and even in wastewater too. Microalgae can live alone or in group with symbiotic way (Song et al., 2008) and showed variation in tolerance capacity in terms of light intensity, temperature, pH, and nutrient status in the environment. This suggests that microalgae could be an alternative source of resources for limited resources world as it has potentiality to modify themselves according to the extreme situation They are crucial element for coral reef and approximately 100000 microalgae or more are used for biodiesel production purposes (Satyanarayana et al., 2011). In recent years, considerable attention have been paid to biodiesel production from microalgae (Wijffels and Barbosa, 2010). Massive interest growing on microalgal study due to its rapid growth, high adaptation capacity and ability to accumulate useful nutrients at a cost-effective way. In aquatic ecosystem, they possess the most fundamental position as they are considered as the primary producer of food chain. Approximately 800,000 species exist worldwide but only 50,000 species identified so far (Mata et al., 2010).

1.4.4.1 Marine phytoplankton used in the study

P. parvum is a single-celled organism, often referred to as golden algae. It is a microscopic (~10 µm), flagellated alga, capable of producing the toxin prymnesin, which can cause extensive fish die-offs.

O. viridis is a marine colonial flagellate green alga in the phylum Chlorophyta. Of the three species in this phylum, *O. viridis* can form four-celled colonies.

E. gymnestica belongs to phylum Euglenozoa. This phytoplankton is ~10 µm in size and has two flagella, one reticulate chloroplast with a single pyrenoid, and a large stigma.

1.4.4.2 Marine diatom species used in the study

A. karianus is a pennate diatom distributed in coastal waters globally, but there is limited information available on its growth, physiology, and life cycle. This strain was isolated in June 2014 from Tsugaru Strait, Hokkaido, Japan.

T. nitzschioides is a yellow-brown pennate diatom with a wide range of salinity tolerance (12–38‰).

N. longissima is a free-living single-celled organism, which is motile and attached to the soft substratum of marine macrophytes, especially on seagrass leaves.

Skeletonema sp. is a centric, cylindrical diatom that can survive in water temperatures up to 30°C and causes water discolouration.

D. brightwellii is a marine centric unicellular photosynthetic autotroph. This strain was isolated in March 1989 from Hiroshima Bay, Japan (Yamaguchi, 1994).

C. didymus is a photosynthetic centric diatom that is connected in straight chains. This strain was isolated in June 2015 from Nanaehama, Hokkaido, Japan. The cell size and its effect on the biotransformation potentials were taken into consideration during the selection of these six species.

1.4.4.3 Freshwater phytoplankton used in the study

A. minutissimum is distinguished from other monoraphid diatoms by its small size, linear-lanceolate shape, and radiate striae. Cells are solitary or form very short chains and are often attached to the substrate by a stalk. This species' distribution is biased towards alkaline

waters, but it also appears in acidic waters (Watanabe, et al., 2005). It is widely adaptable to organic pollution and dominates in rivers polluted by heavy metals (Watanabe, et al., 2005).

B. braunii is a pyramid-shaped planktonic green microalga that belongs to the family Botryococcaceae. This microalga inhabits freshwater, with cells with a diameter of 10-20 µm that form aggregated colonies. This species is able to produce hydrocarbons (particularly triterpenes), which comprise around 30–40% of its dry weight (Metzger and Largeau, 2004). This organism synthesizes oil in its cells that is secreted extracellularly. The oil produced by *B. braunii* is expected to someday be used as an alternative fuel to gasoline.

S. actus is a green microalga belonging to the family Scenedesmaceae that has lanceolate cells. It always forms colonies, with colonies of 4 (or 2, 8, or 16) cells often connected in a line. It is widely distributed in freshwater environments, such as paddy fields, ponds, and swamps, as well as in soil. Its cells adhere to one another via the cell wall, and their positions do not change. In addition, the whole body may be wrapped in agar with extracellular polysaccharide secretions. The cells constituting the colonies have no flagella and are not motile.

S. paradoxum is a green microalga in the family Desmidiaceae with solitary floating cells. There is a constriction at the center of the cell, which divides each cell into two half-cells (desmids). The shape of the cell as seen from above (top-view) is a regular polygon. Four long arms extend from the central point. It is extremely common in various freshwater areas, such as lakes, ponds, paddy fields, rivers, and so on.

P. duplex is a species of freshwater green microalgae in the family Hydrodictyaceae. It forms colonies with specific numbers of cells (8 to 32 cells). The cell bodies are polygonal, granulated, and have horn-like projections. One cell has two protrusions, and there is a wide gap between the cells. The colonies are as large as single-celled algae, with a diameter that reaches tens to hundreds of microns, and the colonies have limited motility. This microalga is widely distributed in freshwater environments, such as paddy fields, ponds, and swamps. Most of these microalgae are free-floating, but there are also benthic forms.

C. aciculare is a crescent-shaped, unicellular, freshwater microalga. It can be found in almost all freshwater environments, from still-water ponds to running waters. There are 2 (rarely 4) chloroplasts in each cell, and they are divided in the center.

1.4.5 Arsenic biotransformation mechanism by microalgae

Inorganic arsenic possesses severe toxic effect which causes serious harmful impact on the growth and metabolic processes of aquatic microalgae. Therefore, microalgae adopted several processes to diminish the toxicity of As including adsorption and absorption process, As(V) reduction, As(III) oxidation, biomethylation, formation to arsenosugar or arsenolipid and excreted out of the algal cell. The biotransformation process especially oxidation-reduction reaction carried out by microalgae known as detoxification mechanism. This process takes place in microalgae likely to avoid As toxic effect (Levy et al., 2005) or to produce energy to assist cell growth (Mateos et al., 2006).

1.4.5.1 As adsorption and absorption by microalgae

Adsorption of arsenic takes place by the help of functional group remain on microalgae cell wall. Several functional groups such as carboxyl, hydroxyl, and amide groups effectively make bonds with As(V) and As(III) (Jasrotia et al., 2014) (Sarı et al., 2011). After adsorption, arsenic species enter the cell by absorption process. Absorption of As(III) requires little or no energy to enter the cell while As(V) needed high energy consumption (Bottino et al., 1978). As(III) is uptake by microalgae via aquaglyceroporine (AQP) and As(V) competitively uptake via phosphate transporter system (Wang et al., 2015).

1.4.5.2 Arsenite oxidation

The process regarding oxidation of As(III) to As(V) act as electron donor and recognized as an energy producing reaction (Páez-Espino et al., 2009). Chemical oxidation is much slower process than microbial oxidation of As(III) to As(V) (Stolz et al., 2006). It is a detoxification mechanism for prokaryotes and many microorganisms as As(V) possesses less toxic effect than As(III). In most of the microalgal species, As(III) oxidation takes place outside of cytoplasmic membrane by extracellular enzymes such as carbonic anhydrase and extracellular phosphatase (Qin et al., 2009). In case of anaerobic photosynthetic purple bacteria, oxidation reaction occurred on outer membrane of the cell (Kulp et al., 2008). In prokaryotes extensive research done on chemolithoautotrophic arsenite oxidizers (CAOs) and heterotrophic arsenite oxidizers (HAOs). Under anoxic and aerobic state, CAOs achieve energy

from oxidation reaction of As(III) but for HAOs only occurred at aerobic condition (Oremland and Stolz, 2005).

1.4.5.3 As(V) reduction

In the environment, promotion of As toxicity and mobility takes place due to reduction of As(V) to As(III) that commonly observed in prokaryotes and microalgae (Oremland and Stolz, 2003). Arsenate resistance microorganisms (ARMs) are mainly belongs to prokaryotes that reduce As(V) to As(III) through intracellular activities under aerobic conditions. Due to the similar chemical properties of As(V) and PO_4^{3-}, these microorganisms actively uptake the As(V) from the environment via phosphate transporter system and then As(V) reduce to As(III) and /or excreted out of the cell (Rahman and Hassler, 2014). ARMs contain `ars` genes connected to arsenate reductase is the best studied arsenate resistance mechanism (Rosen, 2002). This mechanism explained that reduction of As(V) to As(III) takes place by ArsC, a cytoplasmic arsenate reductase enzyme and excretion of As(III) out of the cell through arsenite efflux pump ArsB. If As(III) straight enters the microalgal cell, simply excreted out of the cell through sequestration into the vacuole (Levy et al., 2005). Inside vacuole, it forms stable complexion with glutathione (GSH) or phytochelatins (PCs) (Bleeker et al., 2006).

1.4.5.4 Arsenic methylation

Microorganisms such as prokaryotes and microalgae are known for its excretion of methylarsenicals (MethAs) into the aquatic environment. After uptake of Arsenate from the medium, As(V) reduced to As(III) and then subsequent methylated to MethAs by catalytic enzyme arsenite methyltransferase (AS3MT) (Páez-Espino et al., 2009). As(V) likely to be methylated to monomethylarsinate (MMA(V)) and dimethylarsinate (DMA(V)) which are known as less toxic As species than iAs species (Amend et al., 2014) (Qin et al., 2009). But not all the methylated As species are part of detoxification mechanism as they showed more toxicity than usual such as monomethylarsinite (MMA(III)) and dimethylarsinite (DMA(III)). During methylation reaction, trimethylarsine oxide (TMAO) and volatile trimethylarsine also generated. Due to the nontoxic and volatile nature, production of TMA is considered as important part of detoxification mechanism (Zhao et al., 2013). According to the literature, series of As toxicity may written as below: MMA(III) = DMA(III) > As(III) >As(V) >MMA(V) = DMA(V) > TMAO(V) = TMAO(III) > TMA (Wang et al., 2015) (Rahman and Hassler,

2014). Several studies suggested that high As concentration or longer exposer time may lead to produce methAs and their excretion by microalgae (Yin et al., 2012). Several studies suggested that phosphorus depletion condition promotes production of complex organic As compounds from methylation reaction at stationary growth phase (Hasegawa et al., 2001) (Hellweger et al., 2003).

1.4.5.5 Formation of arsenosugars and arsenolipids

Arsenic containing sugar as arsenosugars and arsenic containing lipid as arsenolipids are synthesize by many microalgae. Detailed study related to biosynthesize are conducted for marine ecosystem. Butseveral studies also observed arsenosugars in freshwater microalgae just after the reduction of As(V) to As(III) (Miyashita et al., 2011). Freshwater microalgae such as *Chlorella*, *Mono raphidium*, *Synechocystis*, *Nostoc*, *Chlamydomonas* able to produce complex forms of As species or arsenosugers (Murray et al., 2003) (Miyashita et al., 2012). In microalgae, the amount of arsenoribosides likely to be influenced by the several environmental factors including cell growth, analytical methods, As concentration in treatment, chromatographic status, nutrient medium and duration of exposure.

Arsenolipids and their possible effects on As biotransformation and detoxification mechanism are growing concern among researchers. Several microalgae succeeded to synthesize lipid soluble As such as *C. vulgaris*, *C. ovalis*, *C. pyrenoidosa*, *D. teriolecta*, *O. rubescence*, *S. costatum*, *P. tricornutum* and *T. pseudonana* (Duncan et al., 2013a) (Duncan et al., 2013b) (Foster et al., 2008) (Lunde, 1973) (Murray et al., 2003) (Xue et al., 2014). According to biosynthesis mechanism of arsenolipids (Irgolic et al., 1977), As could take part in biosynthesis of phospholipids as a result of replacement of phosphate or nitrogen in phosphatidylethanolamine (PE) and phosphatidycholine (PC). Several toxic As compounds likely to be produced from arsenolipids but needed to conduct more research on this issue and their impacts on environment.

1.4.5. Environmental factors affecting microalgal growth and physiological activities

1.4.5.1 Temperature

Microalgal growth and development are vastly influenced by temperature variation in the aquatic environment. The effect of temperature on both the marine and freshwater

microalgal as well as cyanobacterial growth, development and their chemical composition has been studied in detail. A review on microalgae and cyanobacteria covering marine, freshwater, arctic, and polar region, their maximum growth, and optimum temperature are compiled in **Table 1.2**. The optimum temperature and the temperature for maximum growth are varied based on species type and characteristics. For example, range of optimum temperature for the growth of marine diatom species *Chaetoceros calcitrans*, *Thalassiosira weissflogiito*, and *Thalassiosira allenii* were 25-30, 9-12 and 11-20 °C, respectively (Adenan et al., 2013; Fujimoto et al., 1994; Aydin et al., 2009). For freshwater algae, *Chlorella vulgaris*, the optimum temperature ranges from 25-30 °C (Attilio Converti et al., 2009). On contrarily, the performance of marine and freshwater cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa*, respectively, were found maximum at 25 °C (Gonçalves et al., 2016; Gonçalves et al., 2016). The tropical Australian microalgae *Chaetoceros* sp. was found grow well at 33-35°C, whereas *Isochrysis* sp. was reported found good condition at 27-30°C (Renaud et al., 2002). However, the optimum temperature for As uptake, accumulation and biotrasformation by marien and freswater microalgae was not found in the literature.

1.4.5.2 Salinity

Salinity is one of the most influential environmental factors which regulate microalgal growth and other physiological activities. The salinity stress on microalgal growth, photosynthetic activity, lipid content and so on were studied in detail. Experimental salinity, maximum growth, and optimum salinity for marine and freshwater microalgae found in the literature are listed in **Table 1.3**. The tolerance to salinity varies species to species and their characteristics. Optimum salinity for marine diatom species *Skeletonema ardens* and *Skeletonema grevillei* has been reported almost similar 8-35‰ (Balzano et al., 2011). Salinity tolerance and adaptation strategies by microalgal species largely depend on the group and environmental characteristics (Rao et al., 2007). The growth and salinity tolerance < 5‰ and > 35‰ of the marine diatom species of the reported by several previous studies (Huang et al., 2011; Takagi et al., 2006; Saros and Fritz, 2000). Whereas, the growth of marine phytoplankton was reported highest at 33‰ salinity and decreased at 45‰ (Brand, 1984). The optimum ranges of salinity for *Tetraselmis suecica* was 20-60‰ (Pugkaew et al., 2019). The salinity stress responses for freshwater microalgae is very strong, the physiological changes can be observed even at every 1‰ salinity variation. However, the effect of salinity changes on As biotransformation has not been reported before this study.

1.4.5.3 Nutrient concentrations

Another important environmental factor to regulate growth and development of aquatic microalgae is nutrient concentrations in the culture or environmental medium. The deficiency of N promote lipid formation in many microalgae (Adams et al., 2013). A significant reduction in growth and biomass was recorded with the reduction of N and P concentrations (Kalla and Khan, 2016). Changes in the macronutrient (carbon, nitrogen, phosphorus) at different concentrations enhance growth rate and biomolecular compositions such as chlorophyll, protein, carbohydrate, and lipid content in the BG-11 media of freshwater microalgae *Podohedriella* sp. (Ghosh et al., 2019). **Table 1.4** listed the species name, characteristics, nutrient medium and the concentrations of N and P that are available in the literature.

1.4.5.4 Other environmental factors (pH and light intensity)

The pH and light intensity are two important environmental factors which are vital for growth and development of aquatic microalgae. **Table 1.4** and **Table 1.5** are the list of the study conducted on pH and light intensity, respectively. For most of the cases, optimum pH for microalgal growth found between 6 to 8 (**Table 1.4**). The organism like *Chlorella ellipsoidea* and *Dunaliella bardawil* found grow well at higher pH value and ranges like pH 9-11 and pH 6-9, respectively. The effect of light intensity on microalgal development and physiological activities are listed in **Table 1.5**.

Table 1.2 Temperature as environmental factors affecting growth and physiological activities; species name, characteristics, experimental temperature, maximum growth, and optimum temperature for marine and freshwater microalgae found in the literature.

Species	Characteristics	Experimental temp.	Max. growth at temp.	Optimum temp.	References
		(°C)			
Alexandrium catenella	Marine planktonic dinoflagellates	10, 15, 20, 25, 30	25	20-30	(Jin et al., 2012)
Alexandrium tamarense	Single celled marine dinoflagellates	10,15, 20, 25, 30	15	10-20	(Jin et al., 2012)
Chaetoceros calcitrans	Marine diatom	20, 25, 30	30	25-30	Adenan et al., 2013)
Chaetoceros sp.	Colder adapted marine diatom	10,15, 20, 25, 30	16	12	(Liang et al., 2019)
Chaetoceros sp.	Warmer adapted marine diatom	10,15, 20, 25, 30	25	19	(Liang et al., 2019)
Chaetoceros sp.	Antarctic diatom	-1, 1, 4	-1, 1, 4	-1, 1, 4	(Bozzato et al., 2019)
Chlorella capsulata	Marine microalgae	20, 25, 30	25	25	(Ebrahimi and Salarzadeh, 2016)
Chlorella sp.	Marine phytoplankton	20, 25, 30	25	25	Adenan et al., 2013)
Chlorella vulgaris	Eukaryotic unicellular freshwater algae	15, 25, 35	25	25.3±1.1	(Gonçalves et al., 2016)
Chlorella vulgaris	Freshwater microalgae	25, 30, 35, 38	25-30	25-30	(Attilio Converti et al., 2009)
Euglena gracilis	Eukaryotic single-cell algae	25-33	27-31	27-31	(Kitaya et al., 2005)
Heterochlorella uteoviridis	Marine microalgae	22, 27, 32	27	27	(Menegol et al., 2017)
Isochrysis sp.	Australian marine microalgae	25, 27, 30, 33, 35	27	25-30	(Renaud et al., 2002)
Microcystis aeruginosa	Freshwater cyanobacterium	15, 25, 35	25	25.3±1.1	(Gonçalves et al., 2016)
Microcystis viridis	Freshwater cyanobacterium	14, 22, 25, 30	30	22-30	(Fujimoto et al., 1994)
Nannochloropsis oculata	Marine single- cell microalgae	15, 20, 30	20	20	(Attilio Converti et al., 2009)
Phaeocystis antarctica	Polar marine phytoplankton	-1, 1, 4	4	4	(Bozzato et al., 2019)
Pseudokirchneriella subcapitata	Used as bioindicator in freshwater	15, 25, 35	25	25.3±1.1	(Gonçalves et al., 2016)
Rhodomonas sp.	Australian marine microalgae	25, 27, 33, 35	27	25-27	(Renaud et al., 2002)
Skeletonema costatum	Marine diatom	20, 25, 30	30	27-30	(Ebrahimi and Salarzadeh, 2016)
Synechocystis salina	Marine cyanobacterium	15, 25, 35	25	25.3±1.1	(Gonçalves et al., 2016)
Thalassiosira allenii	Harmful marine diatom	4, 11, 16, 20	11	11-20	(Aydin et al., 2009)
Thalassiosira eissflogii	Marine unicellular diatom	8, 12, 16, 20, 24	12	9-12	(Montagnes and Franklin, 2001)
Thalassiosira pseudonana	Marine diatom	8, 17, 25	17	8-17	(Berges et al., 2002)
Thalassiosira pseudonana	Marine diatom	14, 18, 23	18	14-18	(Javaheri et al., 2015)

Table 1.3 Salinity as environmental factor affecting growth of microalgae; species name, characteristics, experimental salinity, maximum growth, and optimum salinity for marine and freshwater microalgae found in the literature.

Species	Characteristics of microalgae	Experimental salinity	Max. growth at salinity	Optimum salinity	References
		‰ (unless otherwise stated)			
Anabaena fertilissma	Marine cyanobacterium	2.5, 5, 10, 15, 20	2.5	2.5	(El Din, 2015)
Botryococcus braunii	Freshwater phytoplankton	17, 34, 51, 68 and 85 mM of NaCl	17 and 34 mM NaCl	17-85 mM of NaCl	(Rao et al., 2007)
Chaetoceros didymum	Marine diatom	0-45	33	15-45	(Brand, 1984)
Chlorella capsulata	Marine chlorophyte	20, 25, 30	25	25	(Ebrahimi and Salarzadeh, 2016)
Chlorella sp.	Freshwater green algae	0.0, 0.2, 0.5, 0.8 and 1.1M of NaCl	0.2 M	0.0, 0.2 and 0.5 M	(Rai et al., 2015)
Corethron hystrix	Marine diatom	12, 16, 20, 24, 28 and 32	32	24-32	(Aizdaicher and Markina, 2010)
Mesotaenium sp.	Freshwater microalgae	2, 8, 11, 18	2 and 8	2 and 8	(Alvensleben et al., 2016)
Nannochloropsis salina	Marine microalgae	10, 22, 34, 46, 58	22 and 34	33.3	(Bartley et al., 2013)
Pseudo-nitzschia delicatissima	Marine diatom	5-45	>45	15-40	(Thessen et al., 2005)
Rhodomonas salina	Marine microalgae	10, 20, 30	29.1±2.3	29	(Jepsen et al., 2019)
Scenedesmus obliquus	Freshwater green algae	0, 0.05, 0.3, 0.6, 1.0, 2.0 and 3.0 M of NaCl	0.05M NaCl	0-0.05 M NaCl	(Kaewkannetra et al., 2012)
Skeletonema ardens	Marine diatom	0-35	Between 15 and 20	8-35	(Balzano et al., 2011)
Skeletonema costatum	Marine diatom	20, 25, 30	30	30	(Ebrahimi and Salarzadeh, 2016)
Skeletonema grevillei	Marine diatom	0-35	10 and 30	10-35	(Balzano et al., 2011)
Skeletonema subsalsum	Brackish water centric diatom	0-35	3.5 - 5	3.5-25	(Balzano et al., 2011)
Skeletonema tropicum	Coastal diatom	0-35	25	7.5-3.5	(Balzano et al., 2011)
Tetraselmis suecica	Marine microalgae	10-60	60	20-60	(Pugkaew et al., 2019)
Thalassiosira weissflogii	Estuarine diatom	25, 30, 35, 40, 45, 50	25	25-30	(Garcia et al., 2012)
Thoracasphaera heimii	Marine microalgae	0-45	25 and 33	33	(Brand, 1984)
Chlamydomonas mexicana	Freshwater microalgae	0.43, 10, 25, 50 or 100 mM of NaCl	25 mM	0.43-50 mM	(Kim, 2013)

Table 1.4 Nutrient concentrations as environmental factors affecting microalgal growth and physiological activities; with list of the name of microalgae, nutrient medium and concentration (N and P).

Species	Characteristics	Nutrient medium	N concentration/ source	P concentration / source	References
Mixed microalgae	Heterotrophic cultivation		250 mg L^{-1} / $NaNO_3$	250 mg L^{-1} / Na_2PO_4	(Devi et al., 2012)
Scenedesmus sp.	Freshwater microalgae	BG-11	2.5, 5.0, 10.0, 15.0, and 25.0 mg L^{-1} / N-NO_3	0.1, 0.2, 0.5, 1.0, 2.0 mg L^{-1} / PO_4–P	(Xin et al., 2010)
Oleaginous green microalgae	Freshwater microalgae	BG-11	4 mM N (high stress) and 11 mM N (low stress) / $NaNO_3$ and KNO_3	0.34 mM / KH_2PO_4	(Adams et al., 2013)
Chlorella vulgaris	Unicellular freshwater algae	Synthetic wastewater	13.2–410 mg L^{-1} / NH_4Cl	7.7–99 mg L^{-1} / KH_2PO_4	(Aslan and Kapdan, 2006)
Scenedesmus sp	Freshwater microalgae	BG-11	15 g m^{-3} (Low TN) and 150 g m^{-3} (High TN)	5, 3, 1.5, 0.5 g m^{-3} (Low nutrient level) and 15, 10, 5, 1.5 g m^{-3}	(Zhuang et al., 2018)
Chlorella vulgaris	Unicellular freshwater algae	BG-11	1.5, 0.75 and 0.375 and 0.0 g L^{-1} / $NaNO_3$	0.04, 0.02, 0.01 and 0.0 g L^{-1} / KH_2PO_4	(Kalla and Khan, 2016)
Podohedriella sp.	Freshwater microalgae	BG-11	0.375, 0.75, 1.5, 3, and 6 g L^{-1} / $NaNO_3$	0.01, 0.02, 0.04, 0.08, and 0.16 g L^{-1} / K_2HPO_4	(Ghosh et al., 2019)
Chlorella sp.	Microalgae harvested from phycoremediation of swine wastewater	Waste-water	750 mg L^{-1} / NH_3-N	160 mg L^{-1} / PO_4–P	(Michelon et al., 2016)
Nannochloropsis salina	Marine microalgae	Artificial seawater	2.0 mM / NH_4SO_4	0.13 mM / H_3PO_4	(Davis et al., 2015)
Chlorella vulgaris	Freshwater algae	BG-11	1.5 g L^{-1} /$NaNO_3$	40 mg L^{-1} / K_2HPO_4	(Kwon et al., 2020)
Chlorella vulgaris	Freshwater algae	Standard medium at 10% cons.	0-56 mg L^{-1} / $NaNO_3$	0-19 mg L^{-1} / K_2HPO_4	(Alketife et al., 2017)

Table 1.5 The pH as environmental factors affecting microalgal growth and physiological activities; with list of the name of microalgae, experimental and optimum pH range.

Species	Character-istics	Experimental pH	Max. growth at pH	Opti-mum pH	Findings	References
Chlorella vulgaris	Unicellular freshwater algae	3.0, 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 9.5, 11.0	7.5	7.5	pH 3.0, 4.0 and 11.0 are extreme for this strain and pH 9.5 causes cell aggregation Fatty acid composition not related to pH.	(Sakarika and Kornaros, 2016)
Nannochloropsis salina	Marine microalgae	5, 6, 7, 8, 9, 10	8 and 9	7.7	pH values of 8 to 9 might be most conducive to increasing algae production and minimizing invading organisms	(Bartley et al., 2014)
Chlorella sorokiniana		6, 7, 8, 9	6	5.8, 6, 7	Biomass productivity increased with decreasing pH	(Qiu et al., 2017)
Chlorella vulgaris	Unicellular freshwater algae	2, 3, 4, 5, 6, 7, 8, 9	5 and 6	6	Alkaline condition is more suitable for microalgae biomass attachment than acidic condition	(Rosli et al., 2016)
Skeletonema costatum	Marine diatom	6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5	7.5	6.5-8.5	At >9.0 growth rate declined due to lower rate of some essential chemical reactions	(Taraldsvik and Myklestad, 2000)
Auxenochlorella pyrenoidosa,	Freshwater green algae	5, 6, 7, 8, 9	7	7	Alkaline pH increases the cell wall flexibility that prevent it from break down	(Mello and Chemburkar, 2018)
Dunaliella bardawil	Halotolerant green algae	4, 6, 7.5, 8, 9, 10, 11	7.5	6-9	Shifting the pH value towards the acidic or alkaline side significantly decreased the content of both protein and carbohydrate	(Khalil and Asker, 2010)
Chlorella ellipsoidea	Single cell marine algae	4, 6, 7.5, 8, 9, 10, 11	10	9-11	Alkaline pH is preferable for the growth of this species	(Khalil and Asker, 2010)
Dunaliella salina	Halotolerant microalgae	6, 6.5, 7, 8, 8.5, 9	7	7	*D. salina* can tolerate wide range of pH, and pH between 6 and 9 was found not to completely inhibit growth	(Ying et al., 2014)

Tetraselmis suecica	Marine green algae	Regulated (6,0, 6.5, 7,0, 7.5, 8.0) and unregulated pH	7.5	6.5-8.0	At pH 6.0, cells sticking to the wall of photobioreactor and form clump. At high pH, no clump formation observed	(Moheimani, 2013)
Scenedesmus obliquus	Freshwater green algae	6, 7, 8	6	6	The effect of pH on cell biomass is temperature dependent. Biomass growth is favoured by low pH and high temperature	(Guedes et al., 2011)

Table 1.6 Light intensity as environmental factors affecting microalgal growth and physiological activities; with list of the name of microalgae, experimental and optimum light intensity found in the literature.

Species	Characteristics	Experimental light intensity	Optimum	Photoperiod	Research outcomes	References
Chlorella vulgaris	Unicellular freshwater algae	70, 120, 180, 230 and 270 µmol m^{-2} s^{-1}	120 µmol m^{-2} s^{-1}		Mathematic models that considered light intensity, DIC concentration and time were proposed to predict the temporal dynamics of growth and CO_2 bio fixation rates in batch cultures.	(Chang et al., 2016)
Chlorella vulgaris	Unicellular freshwater algae	37.5, 62.5, and 100 µmol m^{-2} s^{-1}	62.5 µmol m^{-2} s^{-1}	16h light: 8h dark	Low light intensity decreases the growth and biomass	(Khoeyi et al., 2012)
Chlorella vulgaris	Unicellular freshwater algae	796 and 129 µmol $m^{-2}s^{-1}$	129 µmol $m^{-2}s^{-1}$	14h light:10h dark	Variation in light intensity changes the optimal growth rate of *C. vulgaris*	(Novriadi and Proano, 2018)
Desmodesmus sp	Unicellular marine microalgae	50, 150 and 300 µE m^{-2} s^{-1}	300 µE m^{-2} s^{-1}	16h light: 8h dark	Biomass production positively corelated with light intensity	(Nzayisenga et al., 2020)
Dunaliella viridis	Unicellular marine green algae	50 and 75 µmol m^{-2} s^{-1}	50 µmol m^{-2} s^{-1}	NM	growth at higher light intensity was faster, so pigment accumulation could not be promoted	(Ak et al., 2008)
Isochrysis galbana	Marine microalgae	50, 125 and 325 µmol m^{-2} s^{-1}	325 µmol m^{-2} s^{-1}	18h light: 6h dark	stress of high light for short period accelerates the cell growth more efficiently than low light intensity	(Mishra et al., 2019)
Nitzschia alexandrina	Benthic marine diatom strain	30, 100 and 400 µmol m^{-2} s^{-1}	30 µmol m^{-2} s^{-1}	14h light: 10h dark	High light intensity hampered the growth that inducing the lower nutrient consumption	(Eva Cointet et al., 2019)
Scenedesmus obliquus	Freshwater microalgae	60-540 µmol m^{-2} s^{-1}	420 µmol m^{-2} s^{-1}	NM	Excessive illumination reduces the biomass production and CO_2 fixation ability	(Ho et al., 2012)
Scenedesmus sp	Freshwater microalgae	27, 40.5, 54, 67.5, 81 and 94.5 µmol $m^{-2}s^{-1}$	81 µmol $m^{-2}s^{-1}$	16 light:8 dark	Light intensity plays significant role for biomass production during exponential growth phase	(Difusa et al., 2015)

Selenastrum minutum	Freshwater green algae	30-456 µmol m^2 s^{-1}	365 µmol m^2 s^{-1}	15h light: 9h dark	Growth of microalgae restricted by photoinhibition beyond the optimal light intensity	(Bouterfas et al., 2006)
Tetranephris brasiliensis	Freshwater green algae	50, 100 µmol m^{-2} s^{-1} and daylight	100 µmol m-2 s-1	18h light: 6h dark	Growth and biochemical composition significantly influenced by the light intensity and photoperiod	(Asfouri et al., 2019)

*NM=Not Mentioned

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Chapter 2:

Marine phytoplankton: Arsenic biotransformation potential under a salinity gradient

Rimana Islam Papry*, Yoshiki Omori, Shogo Fujisawa, M. Abdullah Al Mamun, Sohag Miah, Asami S. Mashio, Teruya Maki, Hiroshi Hasegawa*, "Arsenic biotransformation potential of marine phytoplankton under a salinity gradient", *Algal Research*, **47** (2020) 101842 (Published: February 2020).

2.1 Introduction

Arsenic (As) from both natural and anthropogenic sources is a potentially toxic contaminant in the aquatic environment (Sharma and Sohn, 2009). Of the various chemical elements found in marine waters, As is the 22nd most abundant, and its concentration is generally less variable in marine water than in freshwater systems (Neff, 1997). Arsenic naturally has four primary oxidation states (−3, 0, +3, and +5) with various physiological and chemical properties. In oxic waters, inorganic As (iAs) species such as arsenate (As(V)) are thermodynamically stable, whereas arsenite (As(III)) is found in a reduced-redox state (Hasegawa et al., 2010). However, organic forms of As species, such as monomethylarsonate (MMAA) and dimethylarsinate (DMAA) are observed at low concentrations in the aquatic environment (Akter et al., 2005). The reduction of As(V) and subsequent methylation to methylated species (MMAA and DMAA) is considered to be a detoxification mechanism (Hasegawa et al., 2019; Wang et al., 2013).

Algal and bacterial species occupy the lowest levels of aquatic food chains and significantly contribute to As speciation, distribution, and cycling in aquatic ecosystems (Hasegawa et al., 2001; Zhang et al., 2014). Moreover, algal species play an important role in As remediation due to their high capacity to take up As in contaminated water (Bahar et al., 2013; Wang et al., 2015). Firstly, marine phytoplankton takes up As(V) and biotransforms it to thermodynamically unstable As(III) (Sanders et al., 1989). The As(III) is then readily oxidized and converted to the more stable As(V) form (Francesconi and Edmonds, 1996). This process highlights the function of As(III)/As(V) ratios in the marine aquatic environment. The uptake, biotransformation, and accumulation of As(V) by marine phytoplankton is related to the accessibility of phosphate (PO_4^{3-}) (Hellweger et al., 2003) because As(V) and PO_4^{3-} possess similar chemical structures (Meharg and Macnair, 1992; Oremland and Stolz, 2003). As(V) uptake takes place competitively via the PO_4^{3-} transporter pathway (Zhao et al., 2009) and bioaccumulation can be reduced due to the presence of phosphate, particularly in eutrophic ecosystems (Guo et al., 2011).

Several factors influence the growth of marine microalgae, including phytoplankton, such as temperature, salinity, light intensity, pH, and nutrient availability (McLachlan, 1961; Ault et al., 2000). However, several marine phytoplankton species have a broad salinity tolerance (Brand, 1984). In the marine environment, salinity variation affects the biochemical and physiological activities of phytoplankton, including growth, photosynthesis, and As

uptake, accumulation, and biotransformation. The salinity tolerance of phytoplankton depends on the species type and its characteristics (Rao et al., 2007; Saros and Fritz, 2000). Several studies carried out in coastal and estuarine environments have shown the existence of multiple species in the genera *Cyclotella* (Burić et al., 2007), *Pyrodinium* (Phillips, 1990), and *Skeletonema* (Bergesch et al., 2009) along salinity gradients. Brown *et al.* (Brown et al., 2006) observed the growth and distribution patterns of *Karenia* species under different salinities in laboratory culture experiments. In spite of a large overlap in the growth curves and distribution patterns of the species, strains of *Karenia* isolated from the most saline areas exhibited lower salinity tolerance (Brown et al., 2006).

Various studies have examined the effects of salinity on marine phytoplankton growth. A recent study showed that As biotransformation by marine diatom species, along with other physiological activities, was significantly affected by temperature (0–35 °C) and salinity (0.3–50‰) (Papry et al., 2019). They found that at optimum salinity (10–35‰) and temperature (10–25 °C), As biotransformation of *Asteroplanus karianus*, *Thalassionema nitzschioides*, *Nitzschia longissima*, *Skeletonema* sp., *Ditylum brightwellii*, and *Chaetoceros didymus* were maximum whereas As biotransformation found lower at 5‰ and <5 °C, and 35‰ and >25 °C, respectively. However, the effects of salinity on As uptake and biotransformation by marine phytoplankton are not well understood and this is only the second study of this type. This study was designed to examine the growth and As biotransformation potential of three marine phytoplankton species, *viz. Prymnesium parvum, Oltmannsiellopsis viridis,* and *Eutreptiella gymnastica*, at various salinity levels. A broad range of salinities were used, and we observed the effects of salinity on growth and the As biotransformation process of the marine phytoplankton. In addition, different concentrations of phosphate and arsenate were added to the culture medium in order to observe their effects on As uptake and biotransformation.

2.2 Materials and methods

2.2.1 Reagents

Deionized water with resistivity of 18.2 MΩ (arium pro UV, Sartorius Stedim Biotech, Gottingen, Germany) was used throughout the experiment. Reagents were commercially available and used without further purification. Sodium hydroxide (NaOH; special grade reagent, Nacalai Tesque, Kyoto, Japan) and hydrochloric acid (HCl; Kanto Chemical, Tokyo, Japan) were used for pH adjustment of reagents and medium. Reagent 4-(2-hydroxyethyl)-1-

piperazinyl ethane sulfonate (HEPES; Nacalai Tesque) was used in the culture medium as a buffer. Phosphate and As(V) were supplied from sodium dihydrogen phosphate NaH_2PO_4 and disodium hydrogen arsenate heptahydrate ($Na_2HAsO_4.7H_2O$), respectively (both from Wako Pure Chemical, Osaka, Japan).

2.2.2 Phytoplankton

Phytoplankton strains, *P. parvum*, *O. viridis*, and *E. gymnestica* were used in the study (**Figure 2.1**), provided by Prof. Ichiro Imai of the Division of Marine Bioresource and Environmental Science, Hokkaido University, Japan. *P. parvum* is a single-celled organism, often referred to as golden algae. It is a microscopic (~10 µm), flagellated alga, capable of producing the toxin prymnesin, which can cause extensive fish die-offs. *O. viridis* is a marine colonial flagellate green alga in the phylum Chlorophyta. Of the three species in this phylum, *O. viridis* can form four-celled colonies. *E. gymnestica* belongs to phylum Euglenozoa. This phytoplankton is ~10 µm in size and has two flagella, one reticulate chloroplast with a single pyrenoid, and a large stigma.

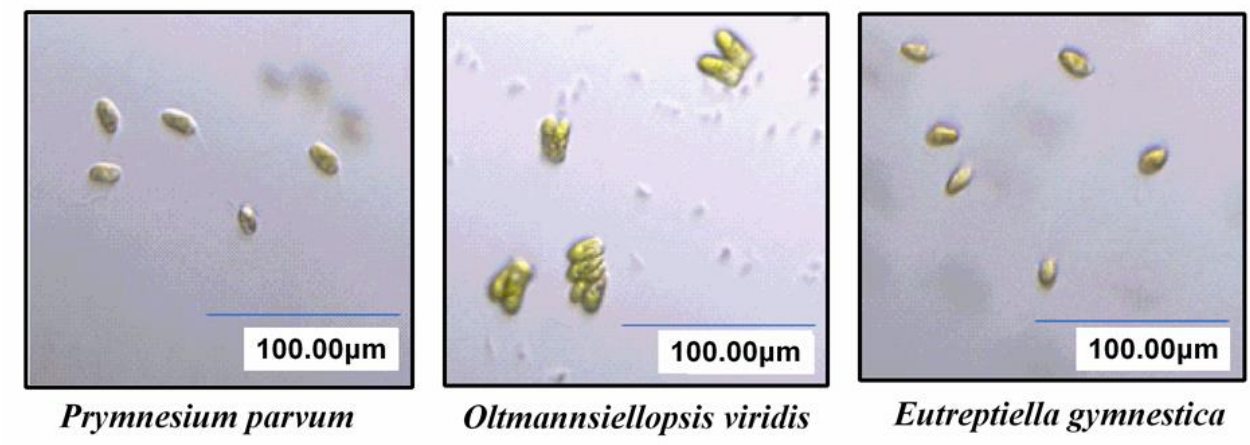

Figure 2.1 Images of three marine phytoplankton species used in this study, captured using a digital microscope (VHX-1000; Keyence, Osaka, Japan).

2.2.3 Preculture and maintenance

An f/2-based nutrient medium (see **Appendix 2.1**) with natural seawater was used for the maintenance of marine phytoplankton. The culture medium and the necessary apparatus (tips, bottles, vessels, micropipettes) were sterilized separately at 121ºC for 30 min in an autoclave (MLS 3780, Sanyo Electric, Japan). They were then placed in a clean bench (NK Clean Bench, VSF-1300A, Nippon, Japan) under UV irradiation for 20 min. Before using the phytoplankton in the experiments, cultures were maintained in the same medium for 1–2 weeks in polycarbonate bottles (Nalgene; Nalge Nunc International, Rochester, NY, USA) until they reach an exponential growth phase in a temperature- and light-controlled incubator (Koitotron3HN-35MLA, Koito Industries, Ltd., Yokohama, Japan). Experimental cultures were grown at 25°C under a 12:12 h light: dark photoperiod at a light intensity of 50 µE m^{-2} s^{-1} provided by cool white fluorescent lights.

2.2.4 Growth under various salinity conditions

Phytoplankton cultures were placed into 30 mL polycarbonate vessels with 30 mL of sterilized culture medium containing artificial seawater. Culture media with a range of salinity levels (0.3, 1.0, 3.5, 5.0, 10, 15, 20, 25, 30, 35, 40, 45, and 50‰) were used to investigate the effects of salinity on the uptake and biotransformation of As species (Papry et al., 2019). After incubating the phytoplankton, 0.1 µmol L^{-1} of As(V) and 1 µmol L^{-1} of PO_4^{3-} were added to the culture medium and grown for three weeks. Liquid samples were collected at several days' interval (days 4, 7, 14, and 21) and filtered with 0.45 µm pore size cellulose membrane filter paper (Toyo Roshi Kaisha, Tokyo, Japan). Phytoplankton growth was measured spectrophotometrically using a UV-VIS spectrophotometer at 540 nm and was calculated with an established cell density-to-absorbance ratio to estimate cell number. A digital microscope (VHX-1000; Keyence, Osaka, Japan) was used to count cell numbers. Growth rate per day was calculated as (Abu-Rezq et al., 1999)

$$\mu(\text{day}^{-1}) = \frac{(\ln N^1 - \ln N_0)}{t} \qquad (1)$$

where, N_1 = final cell density, N_2 = initial cell density, and t = time (day).

2.2.5 Chlorophyll fluorescence

The chlorophyll fluorescence of marine phytoplankton was determined from the maximum photochemical efficiency (quantum yield) using an AquaFlash^{TM} handheld active fluorometer (Turner Designs, San Jose, CA, USA). Before measurements were taken, samples were dark adapted for about 15 min. Maximum quantum efficiency of photosystem II was measured using the following equation (Dijkman and Kromkamp, 2006):

$F_v/F_m = (F_m–F_o)/F_m$

where F_m = maximum fluorescence yield, F_o = minimum fluorescence yield, F_v/F_m = maximum quantum yield, and F_v = maximum variable florescence yield ($F_m – F_o$).

2.2.6 Arsenic speciation analysis

Arsenic species in the culture media was determined using a hydride generation technique (Hasegawa et al., 1994). The technique was a combination of a flame atomic absorption spectrophotometer (AAS, 170-50A; Hitachi, Tokyo, Japan) and hydride generation device, followed by cold trapping (Mamun et al., 2019). Inorganic As [As(V) + As(III)], MMAA, and DMAA concentrations were analyzed by adding 5.0 mL of 0.20 mol L^{-1} EDTA·4Na and 5.0 mol L^{-1} HCl to 40 mL of the sample solution. For As(III), 5.0 mL of 0.20 mol L^{-1} EDTA·4Na and 0.5 mol L^{-1} KH_2PO_4 was added to 40 mL of the sample solution. Chromatograms representing each As species were recorded in a data processing device (Chromato-PRO, Runtime Instruments, Tokyo, Japan), and the concentrations of each species were determined from the height of the obtained peaks. The limits of detection (LODs) and the precision were calculated when the experiment was conducted. The LODs were 0.3, 0.8, and 0.7 nmol L^{-1} for the iAs [As(V) + As(III)], MMAA, and DMAA with a precisions (RSD, n=5) for 20 nmol L^{-1} mix sample (iAs [As(V) + As(III)], MMAA, and DMAA) were 2.2, 1.2, and 1.4%, respectively (Hasegawa et al., 2019). Certified standard reference material 1573a (tomato leaf from the National Institute of Standards and Technology (NIST), USA) was used to examine the accuracy of the analysis: recovery of As concentrations was 95.0% of the certified value (Hasegawa et al., 2019).

2.2.7 Determination of total arsenic concentration

Inductively coupled plasma mass spectrometer (ICP-MS, SPQ 9000; Seiko Instruments Inc., Chiba, Japan) was used to determine the total concentration of arsenic in the marine phytoplankton. Filter paper containing microalgal cells was digested using a microwave

digestion system (Multiwave 3000, Anton Paar GmbH, Graz, Austria) with concentrated HNO_3 (65%). The manufacturer's recommendations were taken into consideration regarding the optimal operating conditions for digestion. Digested liquors were co-washed with 15 mL ultrapure water and then transferred to heat resistant plastic containers (DigiTUBEs; SCP Science, Quebec, Canada). Tubes were then placed into a heat-block type thermal decomposition system (DigiPREP Jr.; SCP Science) at 100 ºC for 6–7 h. After evaporation, 10 mL of purified water was added and filtered with 0.45 µm pore size cellulose membrane filters. Finally, ICP-MS was used to quantify the total arsenic in the cells. The operational conditions for ICP-MS were high-frequency output of 1.2 kw, plasma gas flow rate of 16 L min^{-1}, auxiliary gas flow rate of 1.0 L min^{-1}, nebulizer gas flow rate of 1.0 L min^{-1}, and sample replacement time of 10 s. In addition, the accuracy of the digestion, extraction, and measurement procedures were checked according to certified standard reference materials 1571a (tomato leaf, NIST).

2.2.8 Growth and As biotransformation under As(V) and PO_4^{3-} exposure

Three marine phytoplankton strains were cultured in the sterilized f/2 medium, from low to high (0-50‰) salinity levels. The purpose of the experiment was to investigate the influence of arsenic and phosphate concentration on the growth and As biotransformation of marine phytoplankton species. Marine phytoplankton were cultured under the As(V) and PO_4^{3-} concentrations listed in **Table 2.1**. For clarity, As+P+ represents 1.0 µmol L^{-1} As(V) and 50 µmol L^{-1} PO_4^{3-}, As+P- represents 1.0 µmol L^{-1} As(V) and 1.0 µmol L^{-1} PO_4^{3-}, As-P+ represents 20 nmol L^{-1} As(V) and 50 µmol L^{-1} PO_4^{3-}, and As-P- represents 20 nmol L^{-1} As(V) and 1.0 µmol L^{-1} PO_4^{3-}.

Table 2.1 As(V) and PO_4^{3-} concentration used in the experiment.

As(V)	PO_4^{3-}	Treatment name	Symbol
1.0 µmol L^{-1}	50 µmol L^{-1}	As(V) rich: PO_4^{3-} rich	As+ P+
1.0 µmol L^{-1}	1.0 µmol L^{-1}	As(V) rich: PO_4^{3-} poor	As+P-
20 nmol L^{-1}	50 µmol L^{-1}	As(V) poor: PO_4^{3-} rich	As-P+
20 nmol L^{-1}	1.0 µmol L^{-1}	As(V) poor: PO_4^{3-} poor	As-P-

2.3 Results and discussion

2.3.1 Effect of salinity on the growth of marine phytoplankton

The growth rate of *O. viridis* was higher than that of *P. parvum* and *E. gymnestica* (**Figure 2.2a, b**). The response of each species to salinity differed, as reported in several previous studies (Huang et al., 2011; Hu and Gao, 2006); different species show variation in their adaptation to saline conditions in line with their grouping as halophilic or halotolerant species (Rao et al., 2007). For all species, the highest cell concentration was observed on culture day 14 (**Figure 2.2a**). The maximum cell concentrations for *P. parvum*, *O. viridis* and *E. gymnestica* were observed at 20‰ (cell density 2.1×10^5 cell mL^{-1} and growth rate 0.16 ± 0.005), 30‰ (cell density of 3.3×10^5 cell mL^{-1} and growth rate 0.20 ± 0.004), and 25‰ salinity (cell density of 1.9×10^5 cell mL^{-1} and growth rate 0.15 ± 0.003), respectively (**Figure 2.2a, b**). Moreover, the salinity levels had a significant impact on the growth of these three marine phytoplankton species. At lower (0–5‰) and higher (40–50‰) salinities, the conditions were unsuitable for cellular growth, whereas phytoplankton cell density increased between 10 and 35‰ (**Figure 2.2a, b**). Brand (Brand, 1984) also reported that the growth of marine phytoplankton was maximum at approximately 33‰ salinity and decreased at 45‰.

2.3.2 Effects of salinity on the photosynthetic efficiency of marine phytoplankton

Photosynthetic efficiency (F_v/F_m) of the three marine phytoplankton species was evaluated in regards of minimum (F_o), maximum (F_m), and variable fluorescence ($F_v = F_m - F_o$) under low to high salinity levels (**Figure 2.3**). The maximum quantum efficiency or photosynthetic efficiency of algal species denotes the non-cyclic electron flow capability via photosystem II (Cullen et al., 1997; Magnusson, 1997). F_m is the quanta transfer to the reaction centers of photosystem II and the F_v/F_m ratio determines the photochemical quantum efficiency of photosystem II (Cullen et al., 1997).

The maximum quantum efficiency (F_v/F_m) is useful for determining nutritional stress (Sakshaug et al., 2007; Kolber et al., 1990), functional state, and physiological changes in phytoplankton species (Garrido et al., 2013; Erga et al., 2014). The parameter (F_v/F_m) is also important in toxicological studies related to photoadaptation and photoinhibition (Alderkamp et al., 2011) of different algal species. In this study, significant changes were observed in the value of F_v/F_m when the culture medium was modified at the 0.3–50‰ salinity level. At 0.3–5‰ and 40–50‰, the quantum efficiency or photosynthetic efficiency decreased for all the

phytoplankton species. *P. parvum* showed the highest photosynthetic efficiency (F_v/F_m of 0.52 ± 0.10) at 30‰ salinity. Compared with the other two species, *O. viridis* had the highest photosynthetic efficiency. F_v/F_m values increased gradually from low to medium salinity and the highest F_v/F_m value (with average 0.7 ± 0.03) was measured at 25‰. A similar trend was observed for *E. gymnestica* in the values of F_v/F_m (average 0.47 ± 0.04) at 30‰. All three species showed broad adaptive capacity in response to salinity variation, but maximum photosynthetic efficiency and range of survival ability differed between species. The results of this study are in agreement with those of Qasim *et al.* (1972), who found that the photosynthetic rate decreased at very low salinity levels, such as 5‰. When salinity increased from 10–15‰, photosynthetic efficiency increased before declining again as salinity increased.

2.3.3 Effects of salinity on As biotransformation by marine phytoplankton

Speciation of As was analyzed to investigate the effect of salinity changes on As biotransformation by marine phytoplankton species (**Figure 2.4**). Biotransformation of As(V) to As(III) and methylated forms (MMAA and DMAA) was observed after day 4 of the incubation period. Among the three phytoplankton species, *O. viridis* showed significant biotransformation ability within a wide salinity range (5–45‰) (**Figure 2.4**). Changes in salinity level (low, medium, or high) may have affected the concentrations of iAs and methylarsenic species in the marine phytoplankton. For *P. parvum*, no biotransformation was observed at <10‰ or >40‰. At 10–20‰ and 30‰, As(V) was biotransformed to As(III), but no methylated species were detected. At 25‰ and 35–40‰, As(V) biotransformed to As(III) and then methylated to DMAA. At these salinities, As(V) concentrations were 23.8 ± 8, 24.1 ± 8, and 28.2 ± 5 nmol L^{-1}; As(III) concentrations were 22.2 ± 9, 35.6 ± 5, and 31 ± 11 nmol L^{-1}, and DMAA concentrations were 16.7 ± 7, 15.9 ± 6, and 2.41 ± 0.71 nmol L^{-1}, respectively. *O. viridis* showed no speciation at 0.3–3.5‰ or 50‰. Methylated As species were only detected in *O. viridis* samples at 30‰ and 35‰. At 30‰, the As(V) concentration was 44.1 ± 11 nmol L^{-1}, As(III) was 9.8 ± 1 nmol L^{-1}, MMAA was 8.8 ± 2 nmol L^{-1}, and DMAA was 9.3 ± 1 nmol L^{-1}; at 35‰, the concentrations of As(V), MMAA, and DMAA were 18 ± 4, 28.9 ± 3, and 18.5 ± 3 nmol L^{-1}, respectively. *E. gymnestica* showed a lower tolerance for high salinity levels and As biotransformation only took place at 10–35‰. Methylation of As(V) was observed at 15, 20, and 35‰ in this species, and the concentrations of As(V) were 21.4 ± 5, 31.3 ± 4, and 32 ± 6 nmol L^{-1}, As(III) were 38.8 ± 4, 21 ± 7, and 25 ± 3 nmol L^{-1}, and DMAA were 19 ± 4, 12.3 ± 4, and 11.6 ± 4 nmol L^{-1}, respectively. The variation in the occurrence of

inorganic and methylated species may also be dependent on phytoplankton species, due to differences in their biotransformation efficiencies and survival ability. It has been suggested that the biochemical and physiological properties of marine microalgae are largely influenced by salinity variation (Fava and Martini, 1988). In the present study, MMAA, as an intermediate product of As biomethylation (Challenger, 1945), was not detected with *P. parvum* and *E. gymnestica*. In a previous study with marine microalgae, similar results were observed and MMAA was not released as a free intermediate into the culture medium; rather, the cell metabolized the MMAA to DMAA for excretion into the medium (Papry et al., 2019; Cullen et al., 1994).

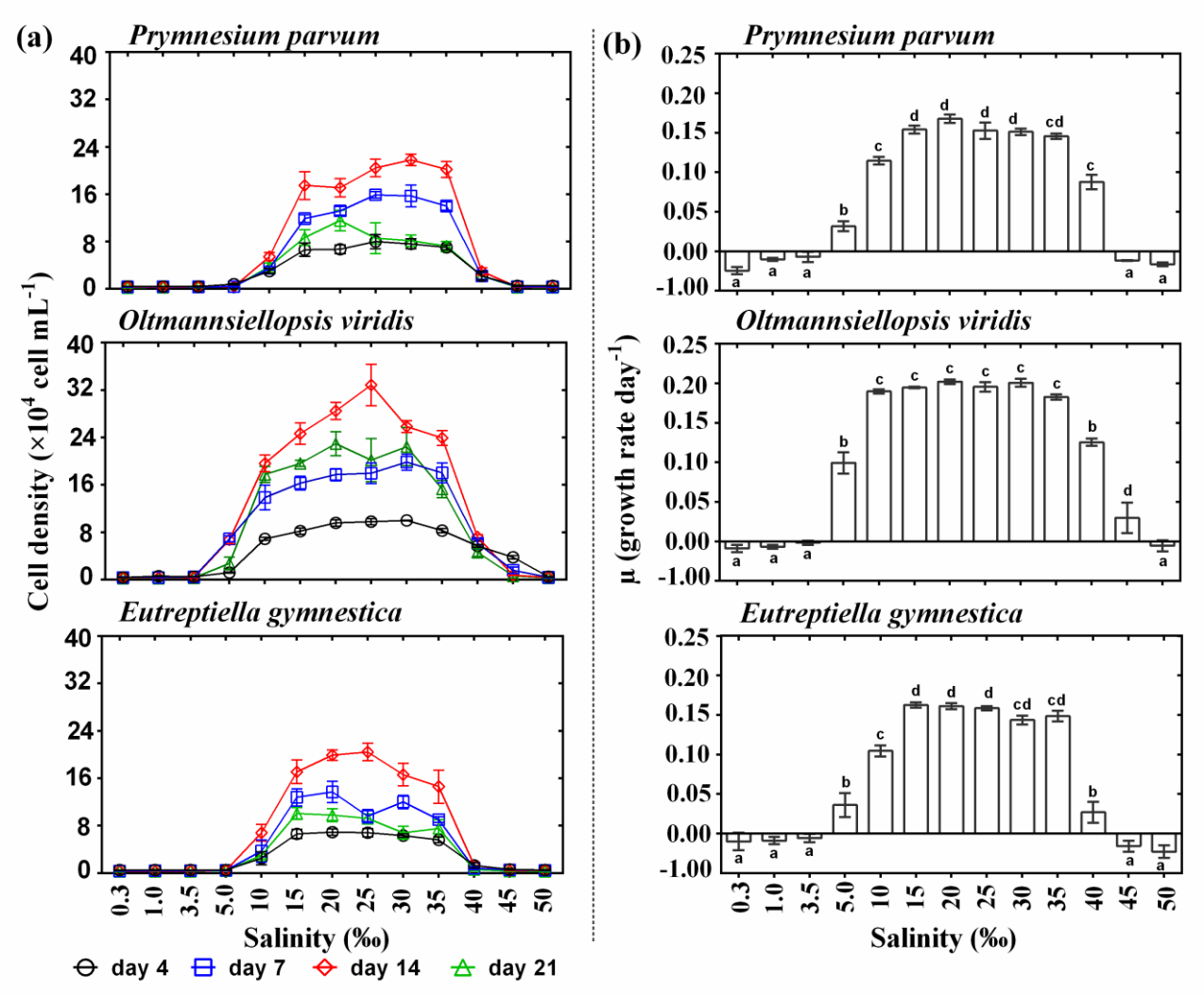

Figure 2.2 Effect of salinity (‰) on the growth of marine phytoplankton species: (a) cell density (cell mL^{-1}) and (b) growth rate day^{-1}. Data are means ± SD (*n*=3). Different lowercase letter in Figure 2(b) indicate significant differences between salinity levels (*p* <0.05).

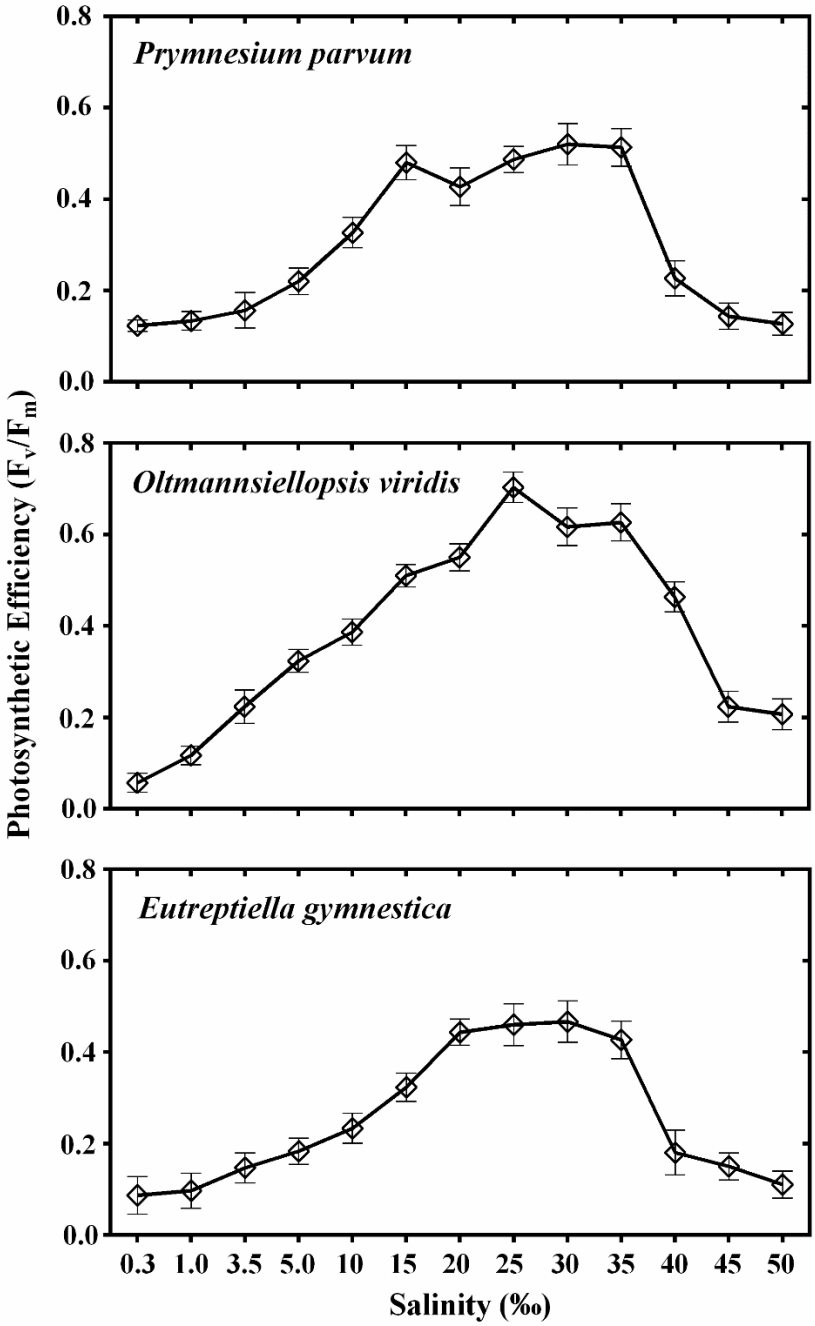

Figure 2.3 Photosynthetic efficiency or maximum quantum yield (F_v/F_m) of three marine phytoplankton species under different culture salinities. Data are means ± SD (*n*=3).

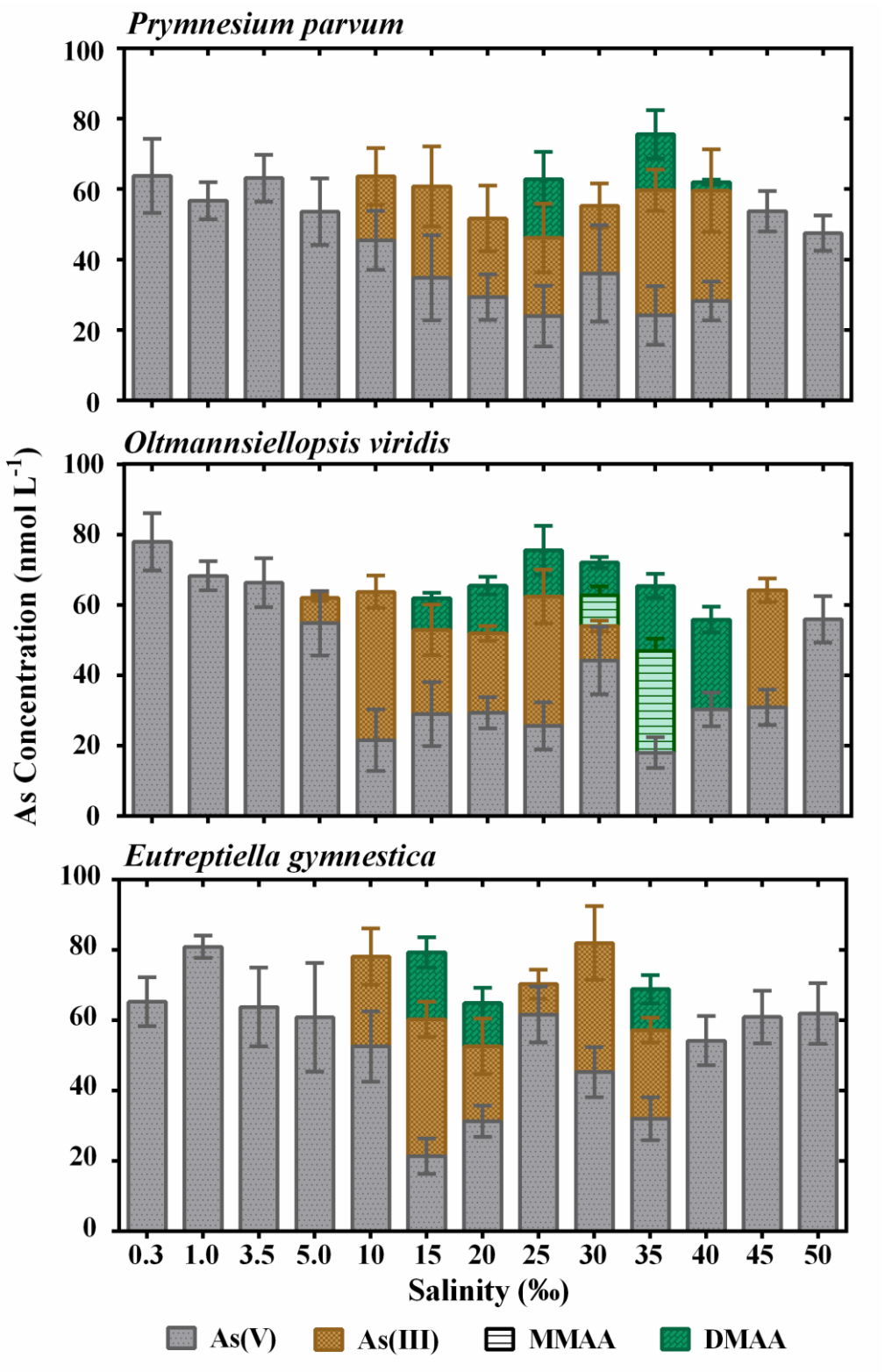

Figure 2.4 Effect of salinity (‰) on arsenic biotransformation [reduction of As(V) to As(III) and subsequent methylation to MMAA and DMAA] by three marine phytoplankton species. Data are means ± SD (*n*=3).

2.3.4 Effect of salinity on the accumulation of total arsenic

Arsenic accumulation by the three marine phytoplankton species is presented in **Figure 2.5**. The results showed significant variation as the salinity level increased. One-way ANOVA analysis showed that salinity had a significant effect on the uptake of arsenic by phytoplankton (p<0.05). Among the three species, *O. viridis* displayed higher As accumulation than the other two species. The maximum As concentrations for *P. parvum* (6.68 ± 1.8 nmol g^{-1} dry weight) and *O. viridis* (10.8 ± 0.5 nmol g^{-1} dry weight) were recorded at 25‰ salinity, whereas maximum As concentration for *E. gymnestica* (4.8 ± 0.2 nmol g^{-1} dry weight) at 30‰ (**Figure 2.5**). These results suggest that the uptake of As by phytoplankton varies with salinity level, and also depends on species-specific physiochemical metabolism. In addition, uptake of As is not only influenced by the As bioaccumulation and biotransformation processes of different algal species (Wang et al., 2013) but also by the environmental conditions where those species are generally found.

2.3.5 Effects of As(V) and PO_4^{3-} exposure on marine phytoplankton

2.3.5.1 Effects of As(V) and PO_4^{3-} exposure on phytoplankton growth

As(V), an analog of PO_4^{3-}, is thought to be taken up via PO_4^{3-} transporters in algal cells through competitive uptake (Zhao et al., 2009; Reed et al., 2015; Xie et al., 2018). As(V) and/or As(III) uptake by microalgae is notably influenced by PO_4^{3-} in the culture medium (Hellweger et al., 2003; Markley and Herbert, 2010; Slaughter et al., 2012). In this study, marine phytoplankton were cultured with different As(V) [20 nmol L^{-1} as poor (As-) and 1.0 µmol L^{-1} as rich (As+)] and phosphate [1.0 µmol L^{-1} as poor (P-) and 50 µmol L^{-1} as rich (P+)] concentrations under different salinity conditions (**Figures 2.6 & 2.7**). Phytoplankton growth was significantly influenced by the availability of As and PO_4^{3-} in the culture medium. In addition, salinity had its own effects under such conditions. When As(V) and PO_4^{3-} were insufficient in the culture medium, growth was lower. Moreover, high and low salinity conditions suppressed the growth of the phytoplankton under all culture conditions. For *P. parvum*, the maximum cell density of approximately 1.8×10^5 cell mL^{-1} with a growth rate of 0.16 ± 0.008 day^{-1} was recorded at 25‰ in both As(V)- and PO_4^{3-}-rich conditions (**Figures 2.6 & 2.7**). For all combinations of As(V) and PO_4^{3-} concentrations, a negative growth rate was observed at salinities of 0.3, 1.0, 45, and 50‰. Whereas at 3.5‰, negative growth rates of -0.013 ± 0.01 day^{-1} and -0.007 ± 0.004 day^{-1} were observed under As-P+ and As-P- culture

conditions, respectively (**Figure 2.7**). At 40‰, only As-P- conditions resulted in negative growth, while other culture conditions showed positive growth.

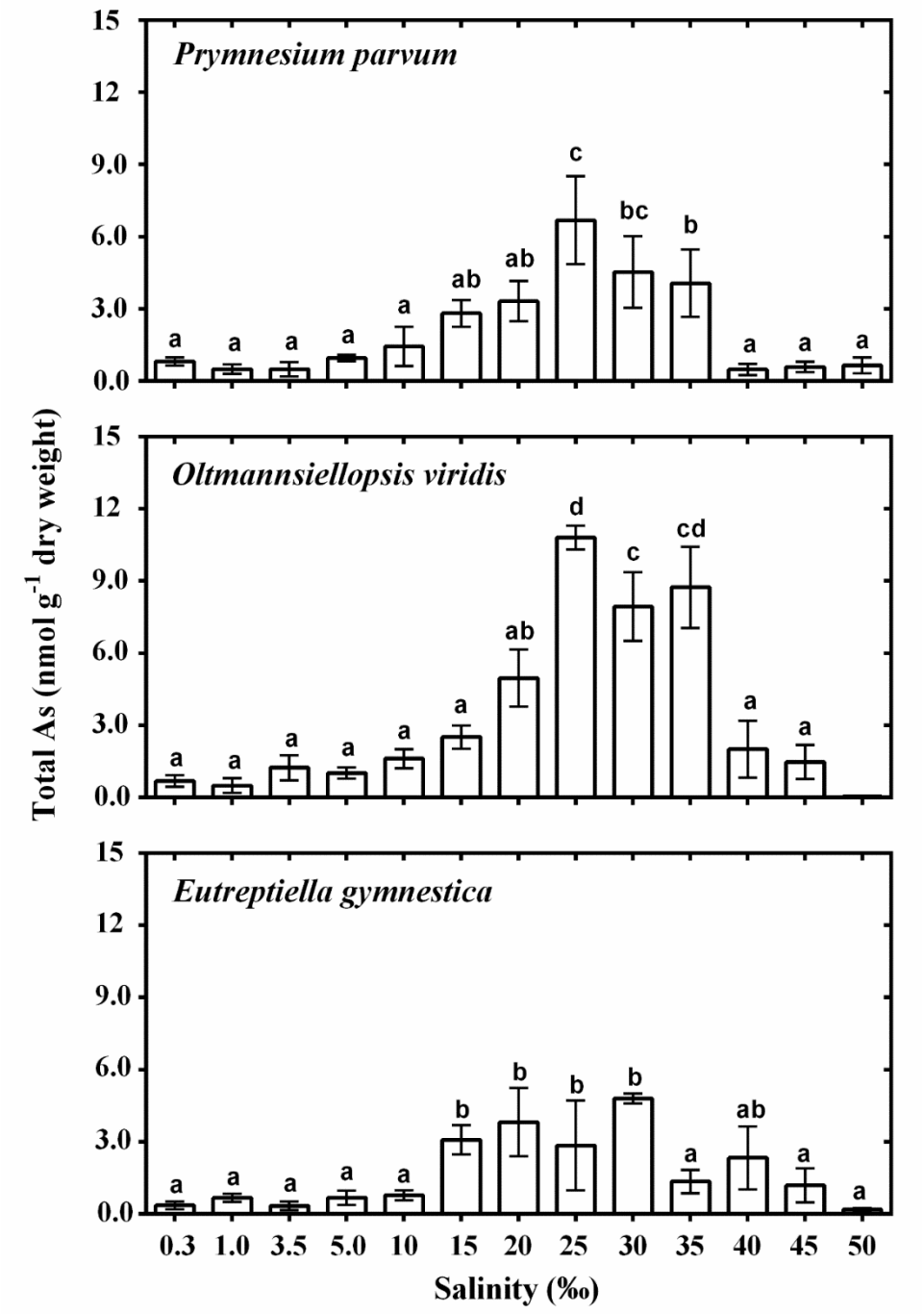

Figure 2.5 Effect of salinity (‰) on accumulation of total arsenic concentration (nmol g^{-1} dry weight) by three phytoplankton species. Data are means ± SD (*n*=3). Different lowercase letters indicate significant differences between salinity levels (p <0.05).

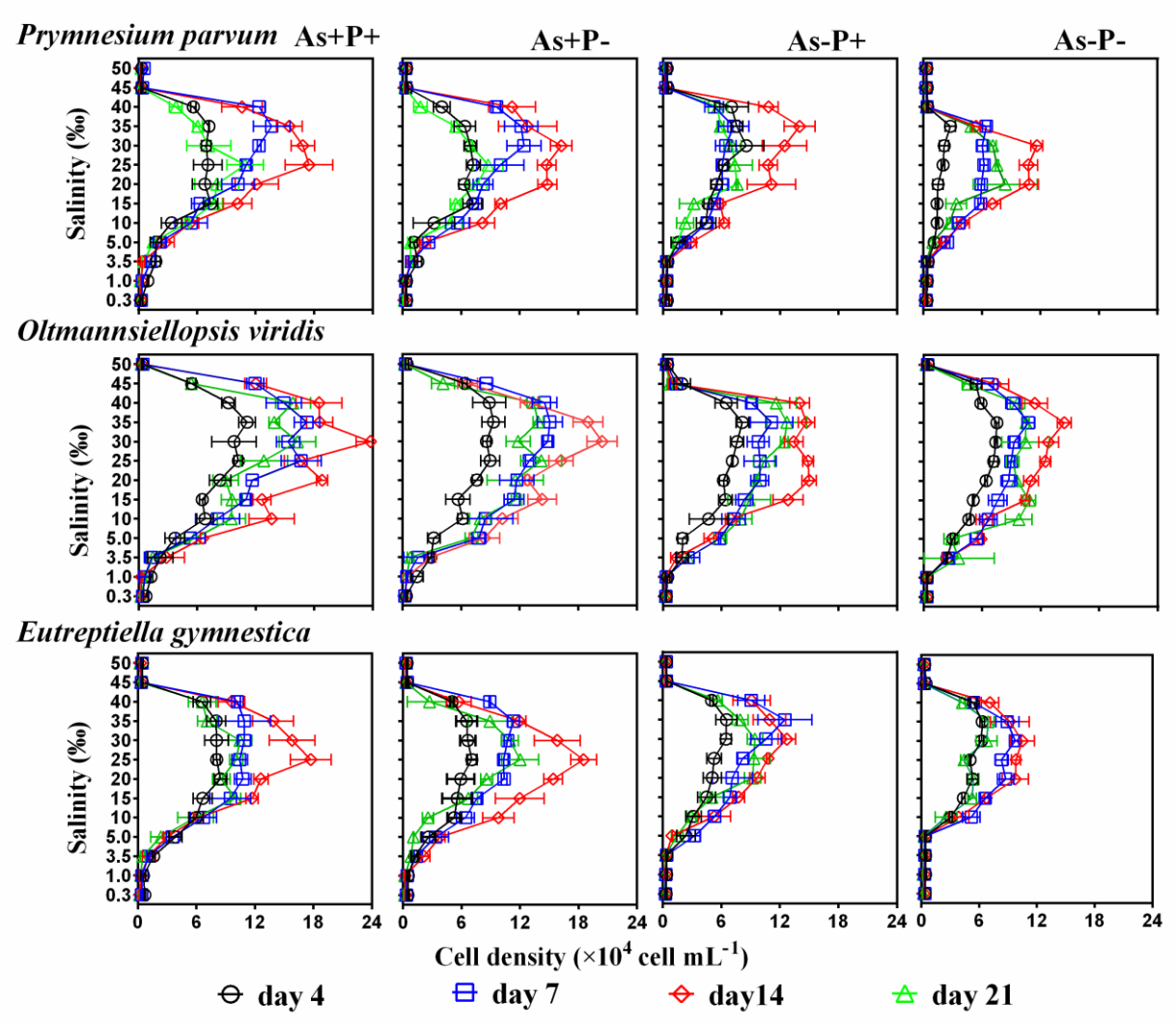

Figure 2.6 Effects of As(V) and PO_4^{3-} concentration with various salinities on the growth [as cell density (cell mL^{-1})] of marine phytoplankton species.

O. viridis had higher cell density in all combinations of As(V) and PO_4^{3-} concentrations. The maximum cell density (2.4×10^5 cell mL^{-1} / growth rate 0.19 ± 0.003 day^{-1}) for *O. viridis* was observed on day 14 at 30‰ under As(V)- and PO_4^{3-}-rich conditions (**Figures 2.6 & 2.7**). Negative growth rates of -0.02 ± 0.013 and -0.01 ± 0.008 day^{-1} were observed at 0.3 and 50‰ under all culture conditions, i.e., As+P+, As+P-, As-P+, and As-P-. At 1‰, the cell density was lower when cultured with As(V)-poor and PO_4^{3-}-rich (50 µmol L^{-1}) or PO_4^{3-}-poor (1µmol L^{-1}) conditions (**Figure 2.7**). *E. gymnestica* showed a high cell concentration (1.9×10^5 cell mL^{-1} / growth rate 0.17 ± 0.007 day^{-1}) at 25‰ under As+P- conditions (**Figures 2.6 & 2.7**). As with *P. parvum*, a negative growth rate was observed for *E. gymnestica* under all combinations of As(V) and PO_4^{3-} at salinities of 0.3, 1.0, 45, and 50‰. At the 3.5‰ level, negative growth was observed under As- conditions but at the 5‰ salinity level, the negative trend was only observed under As-P- conditions. These results imply that as salinity increased, high concentrations of As(V) and PO_4^{3-} tended to increase physiological metabolism and the growth of phytoplankton was higher in phytoplankton exposed to high As(V) levels in the culture medium. This response of phytoplankton species represents an adaptation to environmental conditions such as As(V) and PO_4^{3-} concentrations and salinity. In addition, the growth performance of phytoplankton under different culture conditions depends on the species type and characteristics, which is consistent with the results of previous studies (Levy et al., 2005; Rahman and Hassler, 2014). Planas and Healey (1978) tested five phytoplankton species and found that their responses to arsenate and phosphate ratios varied greatly. Among the five strains, the growth of two decreased when cultured with 1.0 µmol L^{-1} of As(V) but the other two strains remained unchanged since phosphate was abundant in the culture medium; they suggest that the As(V) and PO_4^{3-} ratios affect phytoplankton growth when insufficient phosphate is present (Planas and Healey, 1978).

2.3.5.2 Effects of As(V) and PO_4^{3-} exposure on As biotransformation by phytoplankton

In this study, *P. parvum* showed significant changes in As(V) biotransformation when exposed to the four different culture conditions, i.e., As+P+, As+P-, As-P+, and As-P-. *P. parvum* did not show As(V) biotransformation at salinity levels of 0.3–5‰ or 45–50‰ under As+P+ and As+P- conditions (**Figure 2.8**). However, in the same culture medium, As(V) was reduced to As(III) and then methylated to MMAA and DMAA at salinity levels of 10–40‰. MMAA was only detected at salinities of 25 and 35‰ under As+P+ culture conditions. With As-P+ conditions, As reduction was not observed at salinities of 0.3–10‰ or 45–50‰.

Moreover, at 15–40‰, the reduction of As(V) was not observed. In addition, no biotransformation occurred at salinities of 0.3–5‰ or 45–50‰ and no methylation was witnessed with As-P- conditions and salinities of 10–40‰. Among the three species, *O. viridis* showed higher biotransformation efficiency, with a broad salinity tolerance range. Under all combinations of As(V) and PO_4^{3-} concentrations, *O. viridis* was able to transform As(III) or methylated forms, i.e., MMAA and/or DMAA. At lower salinities such as 0.3–3.5‰ and higher salinities such as 50‰, *O. viridis* was unable to participate in As(V) reduction or methylation processes. Finally, for *E. gymnestica*, both As(V) reduction and methylation were observed under As+P+ and As+P- conditions at salinities between 10 and 40‰. However, only the reduction of As(V) to As(III) was observed at the same salinities under As-P+ and As-P- conditions.

Biotransformation of As species by marine phytoplankton are associated with the organism's growth rate and the surrounding nutrients status (Hellweger et al., 2003). The variations in the occurrence and the concentrations of iAs and methylarsenic species in the marine phytoplankton largely depends on the phytoplankton species and the biotransformation of the arsenic species inside the phytoplankton cells (Rahman et al., 2012). In this study, the ratio of As(V) and PO_4^{3-} played an important role in terms of phytoplankton growth and arsenic biotransformation mechanism. Previous studies suggest that phosphate has some impacts on As(V) reductase and greatly differs from species to species (Gladysheva et al., 1994; Mukhopadhyay et al., 2000). However, As(V) toxicity and its bioaccumulation by microalgae are not specifically determined by the total amount of phosphate, as speciation plays a significant role (Karadjova et al., 2008; Duncan et al., 2010).

2.3.6 Conceptual model of As uptake and biotransformation by phytoplankton

Phytoplankton species exhibited several adaptations to reduce or moderate the toxic effects of arsenic, especially iAs. These mechanisms included reduction of As(V) to As(III), formation of methylated species (methylation), conversion into either arseno-sugar or arseno-lipid, complex formation with As(III) and glutathione or phytochelatins, and excretion from the cell. A conceptual model of the arsenic uptake and biotransformation processes is described briefly in **Figure 2.9**.

a) The uptake of iAs by plants depends mainly on the balance of arsenic and phosphate as both are actively taken up by the cell through phosphate transporters (Asher and Reay, 1979). In marine aquatic systems, As(V) is the principal form and is taken up competitively by

phytoplankton via phosphate transporters as arsenate and phosphate possess similar chemical properties (Reed et al., 2015; Dixon, 1997) (**Figure 2.9a**).

b) This phenomenon antagonizes the As(V) and PO_4^{3-} uptake into phytoplankton cells (**Figure 2.9b**). Furthermore, difficulties in uptake can arise if the concentration of PO_4^{3-} is higher than As(V) in the medium; the opposite issue occurs in the presence of higher As(V) concentrations, which stimulate the active uptake of As(V) (Rahman and Hasegawa, 2012).

c) After uptake of As(V), phytoplankton can convert it to As(III) (Hasegawa et al., 2001; Hellweger et al., 2003; Karadjova et al., 2008) (**Figure 2.9c**). Hasegawa *et al.* (2001) noted that *Closterium aciculare* readily reduced As(V) to As(III) and at the exponential growth phase, the concentration of As(III) was highest. Two things may happen after the reduction of As(III) by phytoplankton: As(III) can either take part in methylation to produce methylated species (i.e., MMAA and DMAA) or is excreted out of the cell. Arsenate reductase as a catalyst and non-enzymatic reactions with glutathione (GSH) as an electron donor help in As(V) reduction to As(III) inside the cell. Low molecular thiol-based polypeptides that have metal binding capacities, such as GSH and phytochelatins (PCs), develop stable complexes with As(III) (Pawlik-Skowrońska et al., 2004). These complexes help to decrease toxicity through As(V) reduction and mitigate the competition between As(V) and PO_4^{3-}. Consequently, phytoplankton can uptake PO_4^{3-} as usual from arsenic-containing media.

Phytoplankton produce methylated species as a consequence of As(V) reduction to As(III) and subsequent methylation. As an oxidative procedure, a methyl group (CH_3^+) is actively used in the methylation reaction (**Figure 2.9c**). Arsenite methyltransferase (As3MT) is one of the most important catalysts, and its presence actually stimulates the methylation process inside phytoplankton cells (Zhang et al., 2013). In the methylation pathway, it was believed that the reaction of arsenic with GSH and glutathione S-transferase omega (GSTO 1) promoted the production of trivalent GSH conjugates. Subsequently, S-adenosylmethionine (SAM) acts as a methyl group donor through AS3MT and after the oxidization of the hydrolysis product, the methylation process proceeded sequentially (Hayakawa et al., 2005).

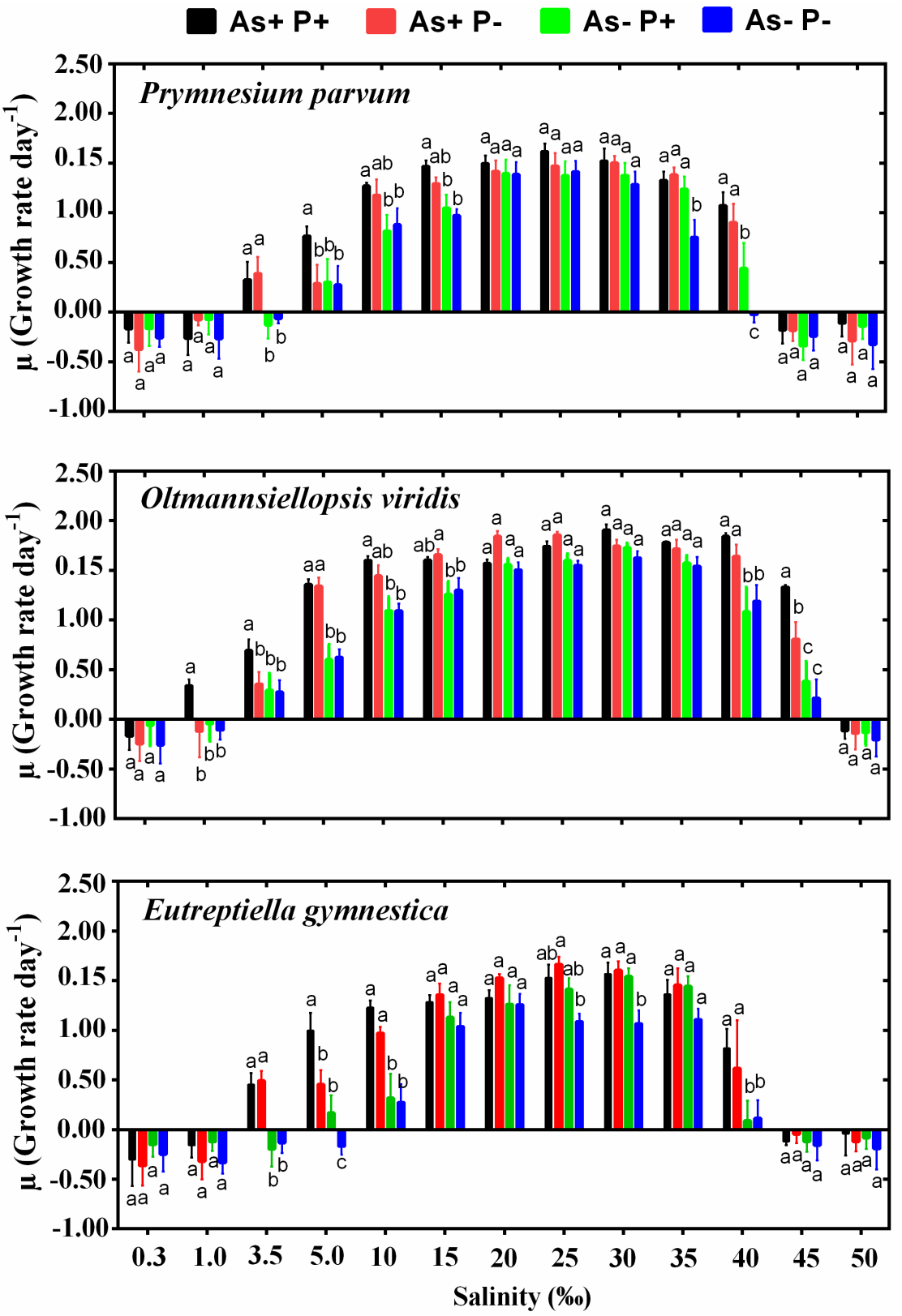

Figure 2.7 Effects of As(V) and PO_4^{3-} concentrations at various salinities on the growth (growth rate day^{-1}) of marine phytoplankton. Data are means ± SD (n=3). Different lowercase letters indicate significant differences between salinity levels (p <0.05).

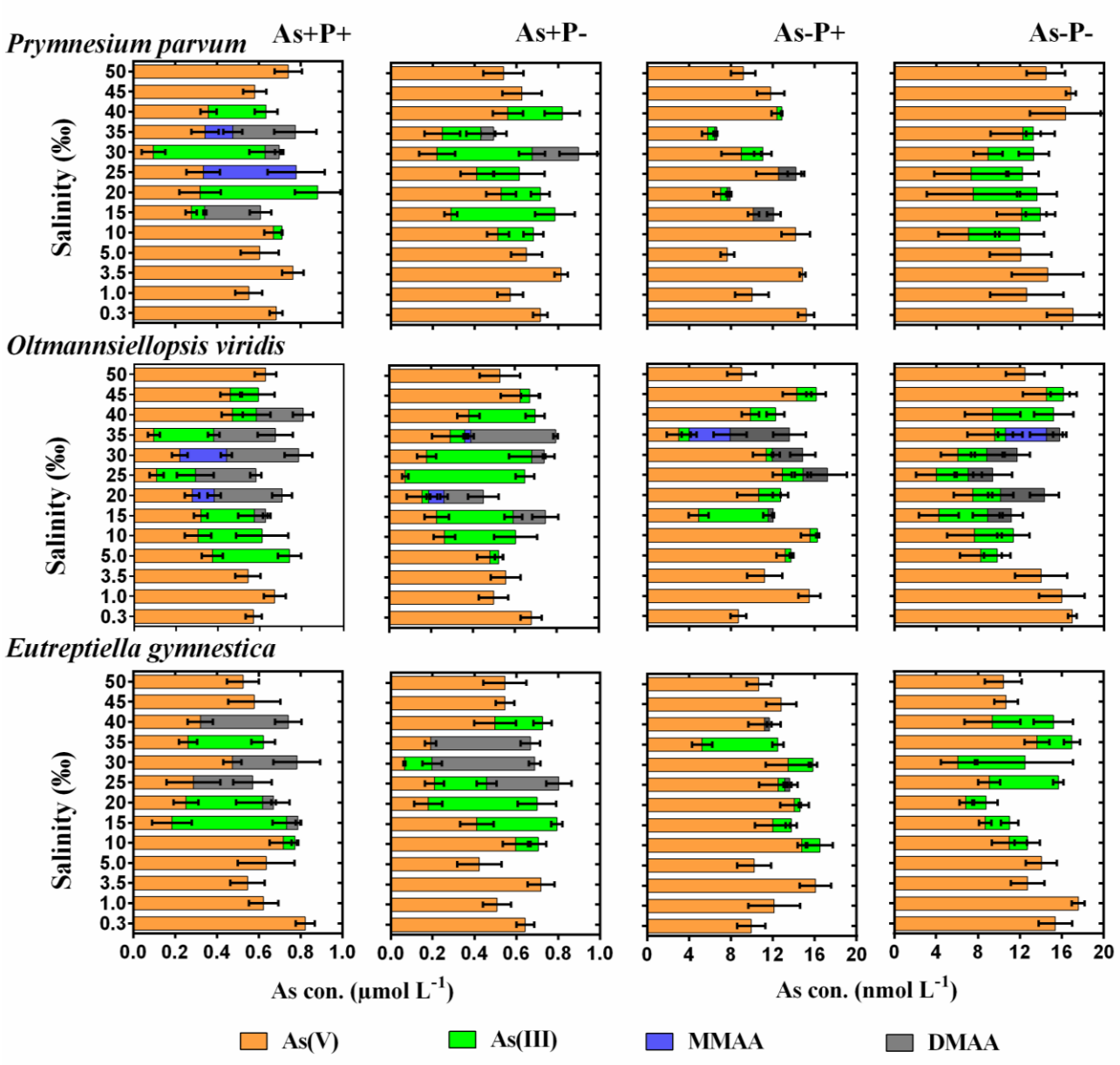

Figure 2.8 Effects of As(V) and PO_4^{3-} concentrations at various salinities on As biotransformation of marine phytoplankton species. Data are means ± SD (*n*=3).

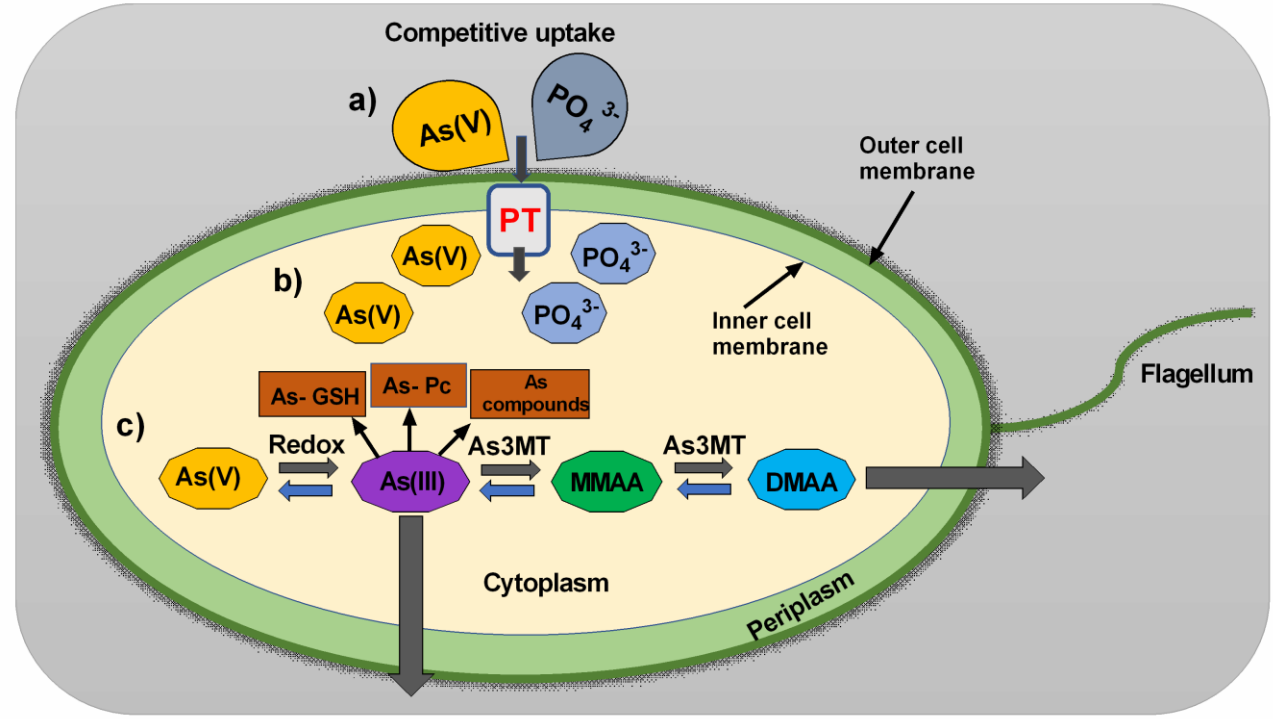

Figure 2.9 Arsenic uptake and biotransformation processes by marine phytoplankton. PT indicates phosphate transporter; As-red, arsenate reductase; As-GSH, arsenotriglutathione As(III) $(GS)_3$; As-Pc, arsenite-phytochelatins complex; Redox, reduction and oxidation reaction; As3MT, arsenic-methyltransferase. a) phytoplankton uptake As(V) competitively via phosphate transporters as As(V) and PO_4^{3-} possess similar chemical properties; b) As(V) and PO_4^{3-} uptake takes place inside the phytoplankton cell depending on the concentration of As(V) and PO_4^{3-} present in the medium; c) after uptake of As(V), phytoplankton are capable of converting it to As(III) and phytoplankton produce methylated species as a consequence of As(V) reduction to As(III) and their subsequent methylation.

2.4 Conclusions

This study revealed the As biotransformation potential of three marine phytoplankton species, *viz. P. parvum*, *O. viridis*, and *E. gymnestica* under a wide salinity gradient. Various concentrations of As(V) and PO_4^{3-} were added to f/2-based culture media with differing salinities in order to observe the effects of such conditions on the uptake and biotransformation of As species by marine phytoplankton. *O. viridis* showed maximum growth, As biotransformation, and total As accumulation ability under a broader range of salinity conditions (5–45‰) than the other phytoplankton species used in the experiment. The highest cell concentrations for all the species were observed on day 14 of culture. The photosynthetic efficiency was low for all the phytoplankton species at salinity levels of 0.3–5‰ and 40–50‰. The phytoplankton growth expectancy was significantly influenced by the availability of arsenic and phosphate in the medium, and salinity had its own effects under these conditions. A conceptual model of salinity effects on As uptake and biotransformation by the three marine phytoplankton species is proposed based on the findings of this study.

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Chapter 3:

Marine diatom species: Effects of temperature and salinity on arsenic biotransformation potential

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 Reports*, **9** (2019) 10226 (Published: July 2019).

3.1 Introduction

Arsenic (As), a toxic metalloid, is mostly found in soil, freshwater, and marine ecosystems. Anthropogenic activities, together with natural sources, contribute to increased contamination of surface and ground water. As has four oxidation states, such as arsenate (As(V)), arsenite (As(III)), arsenic (As0), and arsine (As(-III)), each of which comprises different physico chemical characteristics (Bahar et al., 2012). Biotransformation of As species by aquatic organisms is a complex mechanism with different toxicity levels (Karadjova et al., 2008). The toxicity of different As forms as determined by the 50% lethal dose (LD50) follow the order: As(III) (14) > As(V) (20) > monomethylarsonate (MMAA(V)) (700–1800) > dimethylarsinate (DMAA(V)) (700–2600) > arsenocholine (ArsC) (> 6500) > arsenobetaine (ArsB) (> 10,000) (Niegel and Matysik, 2010). Inorganic arsenic (iAs) is more toxic than organoarsenic (orgAs); however, the toxicity on aquatic organisms depends on the As concentration and its speciation (Mehrag and Hartley-Whitaker, 2002). Microalgae reduces the toxic effect of iAs through several processes, including As(V) reduction, As(III) oxidation, methylation, conversion to arsenosugars/arsenolipids, complex formation of As(III) with glutathione and phytochelatins, cell surface binding, and excretion from the cell (Wang et al., 2015). In marine environments, algal species uptake iAs (As(V) and As(III)) in the form of arsenic and biotransform it into methylated arsenic (methylAs) species and /or arsenosugars (AsS), such as orgAs species (Francesconi, K. A. & Edmonds, 1996). The concentration of iAs and methylAs species varies in marine microalgae cells, whereas As biomethylation produces methylAs species from iAs. This phenomenon is determined by the type of microalgae species and their characteristics because different species have various biotransformation abilities (Rahman et al., 2012). In the aquatic food chain, micro or macro algae, as members of lower trophic levels, take up As content more actively than higher trophic members (Maeda et al., 2006). According to Sanders et al. (1989), microalgae, as primary producer, accumulate As(V) from surrounding sea water and reduced it to As(III), and this biotransformation process elucidates the As(III) and As(V) ratio in marine aquatic environments. The chemical formation of As(III), MMAA) and dimethylarsinate (DMAA) are actively associated with primary productivity (Andreae, 1978) in marine water where microalgae play a pioneer role in the formation of such As species (Edmonds and Francesconi, 1998).

However, growth of microalga, particularly diatom species, and their As biotransformation mechanisms are influenced by several factors, including constituency of nutrient medium (Hasegawa et al., 20001), concentration of As species (Karadjova et al., 2008;

Gong et al., 2008), pH (Maeda et al., 1992; Murray et al., 2003; Pawlik-Skowronska et al., 2004), light intensity (Karadjova et al., 2008; Bottino et al., 1978), temperature (Fujimoto et al., 1994; Raven and Geider, 1988), salinity (Abubakar, 2017; Bartley et al., 2013), and length of exposure period (Foster et al., 2008). In this study, we focused on the two essential environmental factors, temperature and salinity, which regulate the growth and physicochemical metabolism in microalgae in marine ecosystems. The nutritional properties of microalgal species are stimulated by salinity and temperature variations in the environment (Hemaiswarya, et al., 2010). Moreover, temperature plays a significant role on the growth rate (Montagnes et al., 2001), chemical composition (Renaud et al., 2002), and metabolic processes of marine diatom species. Biochemical and physiological metabolism, such as growth, photosynthesis, and As accumulation and biotransformation are influenced by salinity in marine ecosystems. Although, adaptation and tolerance of microalgae varies between species, diatoms are directly or indirectly affected by salinity where the ion composition is one of the growth factors (Rao et al., 2007; Saros and Fritz, 2000). Many algal species can grow in a wide range of temperatures and salinities, exhibiting high tolerance to variation of such factors (Mclachlan, 1961).

The influence of temperature (Renaud et al., 2002; Aydin et al., 2009; Berges et al., 2002; Fiala and Oriol, 1990; Javaheri et al., 2015) and salinity (Garcia et al., 2012; Balzano et al., 2010; Aizdaicher and Markina, 2010) and their combined effect (Adenan et al., 2013; Salarzadeh and Ebrahimi, 2016) on the growth and development of marine diatoms have been studied in detail. However, limited information on the effect of temperature and salinity on As bioaccumulation, biotransformation, and speciation pattern, particularly by marine diatom species, is available. Moreover, the effects of salinity and temperature on cell sizes of marine diatom species and their interrelated influence on As biotransformation is also very limited in the literature. Therefore, this study aims to breech the research gap by investigating the influence of temperature and salinity on As biotransformation and speciation pattern by six marine diatom species. The interrelated influence of temperature, salinity, and cell size on As biotransformation was investigated. This study also highlighted the diatom species specifically associated with temperature- and salinity-dependent As biotransformation mechanisms, which may further provide important insight regarding As remediation processes.

3.2 Materials and methods

3.2.1 Marine diatom species

Six strains of marine diatom species, *A. karianus*, *T. nitzschioides*, *N. longissima*, *Skeletonema* sp., *D. brightwellii*, and *C. didymus*, were used in this study (**Figure 3.1**). Diatom species were supplied by Dr. Kanako Naito, Associate Professor of Hiroshima Prefecture University, Japan. *Asteroplanus karianus* is a pennate diatom distributed in coastal waters globally, but there is limited information available on its growth, physiology, and life cycle. This strain was isolated in June 2014 from Tsugaru Strait, Hokkaido, Japan. *Thalassionema nitzschioides* is a yellow-brown pennate diatom with a wide range of salinity tolerance (12–38‰). *Nitzschia longissima* is a free-living single-celled organism, which is motile and attached to the soft substratum of marine macrophytes, especially on seagrass leaves. *Skeletonema* sp. is a centric, cylindrical diatom that can survive in water temperatures up to 30°C and causes water discolouration. *Thalassionema nitzschioides*, *N. longissimi* and *Skeletonema* sp. strains were isolated in June 2015 from Nanaehama, Hokkaido, Japan. *Ditylum brightwellii* is a marine centric unicellular photosynthetic autotroph. This strain was isolated in March 1989 from Hiroshima Bay, Japan (Yamaguchi, 1994). *Chaetoceros didymus* is a photosynthetic centric diatom that is connected in straight chains. This strain was isolated in June 2015 from Nanaehama, Hokkaido, Japan. The cell size and its effect on the biotransformation potentials were taken into consideration during the selection of these six species.

3.2.2 Reagents

Deionized water (arium pro UV, Sartorius Stedim Biotech, Goettingen, Germany) with a resistivity of 18.2 MΩ was used for all experiments. Reagents were commercially available and used without further purification. Sodium hydroxide (NaOH; special grade, Nacalai Tesque, Kyoto, Japan) and hydrochloric acid (HCl; Kanto Chemical, Tokyo, Japan) were used for the pH adjustment of reagents and medium. 4-(2-hydroxyethyl)-1-piperazinyl ethane sulphonate (HEPES; Nacalai Tesque) was used as a buffer reagent in culture medium. Special grade sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen arsenate heptahydrate (As(V)), both from Wako Pure Chemical (Osaka, Japan), were used as the phosphate and arsenic sources, respectively, in the culture medium.

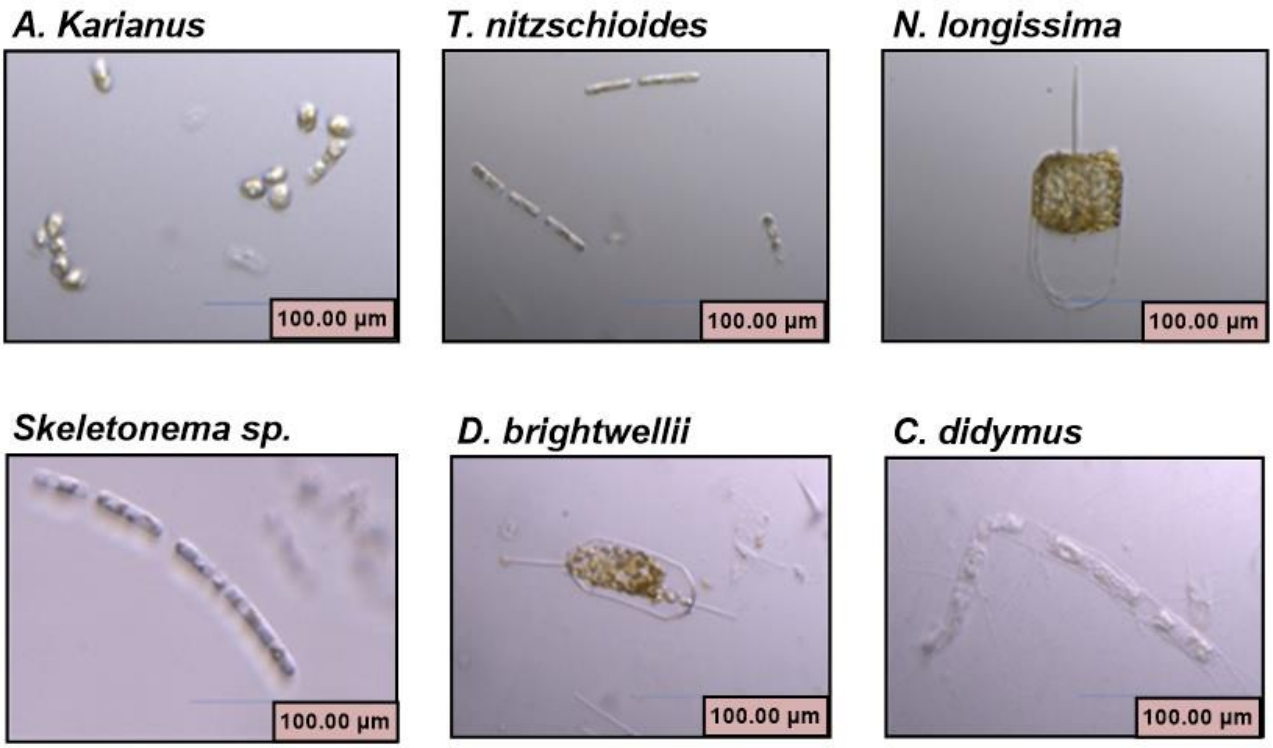

Figure 3.1 Images of six marine diatom species used in this study, captured using a digital microscope (KEYENCE, VHX-1000, Japan).

3.2.3 Preculture and maintenance of marine microalgae

Marine diatom species were maintained in f/2 based nutrient medium in natural sea water. Culture medium and the apparatus (tips, bottles, vessels, micropipettes) were sterilized separately at 121°C for 30 min in an autoclave (MLS 3780, Sanyo Electric, Japan), followed by UV irradiation for 20 min on a clean bench (NK Clean Bench, VSF-1300A, Nippon, Japan). Before using the diatom species in experiments, cultures were maintained in the same medium for 1–2 weeks in polycarbonate bottles (Nalgene, Nunc; Rochester, NY) until they reached an exponential growth phase in a temperature- and light-controlled incubator (Koitotron3HN-35MLA, Koito Industries, Japan).

3.2.4 Growth and As speciation under various temperature and salinity conditions

The growth and As speciation of diatom species were observed at different temperatures and salinities. For the temperature treatment, diatom cultures were placed into

polycarbonate vessels with 30 mL of sterilized culture medium containing natural sea water. The vessels for each batch of culture were kept in an incubator and set at a certain temperature (0, 5, 10, 15, 20, 25, 30, and 35°C). Artificial sea water (see **Appendix 3.1**) at various salinities (0.3, 1.0, 3.5, 5.0, 10, 15, 20, 25, 30, 35, 40, 45, and 50 ‰) was used to culture the diatom species for the salinity treatment. After incubating the diatom species, As(V) (0.1 µmol L^{-1}) and PO_4^{3-} (1 µmol L^{-1}) were added to the culture medium. The cultures were grown for three weeks. The liquid samples were collected at day 4, 7, 10, 14, 17, and 21, and filtered using 0.45 µm cellulose membrane filters (Toyo Roshi Kaisha, Tokyo, Japan). Growth of each species was measured spectrophotometrically using a UV-VIS spectrophotometer at 540 nm and was calculated with an established cell density-to-absorbance ratio to estimate cell number. Cell numbers were counted using a digital microscope (Keyence, VHX-1000, Japan). The initial cell concentration of the diatom species was measured as 2.4×10^3 cell mL^{-1}. Growth rate per day was calculated using equation (1) (Yang et al., 2012):

$$\mu(day^{-1}) = \frac{(\ln N_1 - \ln N_0)}{t} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where, N_1 = final cell density, N_2 = initial cell density, and t = time (day).

3.2.5 Arsenic speciation analysis

Arsenic species in culture media samples were determined using a hydride generation technique according to Hasegawa et al. (1994). A flame atomic absorption spectrophotometer (AAS) combined with hydride generation device followed by cold trapping (AAS, 170-50A, Hitachi, Japan) was used. Inorganic As (As(V)+As(III)), MMAA(V), DMAA(V) were analysed by adding 5.0 mL 0.20 mol L^{-1} EDTA·4Na (ethylenediaminetetraacetic acid; Kanto Chemicals) and 5.0 mol L^{-1} HCl to 40 mL of the sample solution. For As(III), 5.0 mL 0.20 mol L^{-1} EDTA·4Na and 0.5 mol L^{-1} potassium hydrogen phthalate (Kanto Chemicals) were added to 40 mL of sample solution. Arsenic species were recorded as a chromatogram on a data processing device (Chromato-PRO, Runtime Instruments, Tokyo, Japan) and the concentration was determined by the peak height. The lowest detectable concentrations of As(III), As(V), MMMA(V), and DMAA(V) were 0.02 , 0.11, 0.18, and 0.12 nmol L^{-1} with RSD values (n=3) of 1.3, 2.7, 2.5, and 2.3%, respectively (Mamun et al., 2019).

3.2.6 Cell size

A minimum of 35 individual cells was measured in each sample using a microscope (KEYENCE, VHX-1000, Japan) at 500X magnification. Cell diameter and height were measured as they appeared on the micro slide glass, considering the cells as cylinders. Cell surface and volume were then calculated in each temperature and salinity condition.

3.2.7 Statistics

Statistical analysis was carried out using SPSS 22.0 for Windows (IBM Co., USA) and Graph Pad Prism 7.0 (GraphPad Software Inc., USA). One-way and two-way analysis of variance (ANOVA) with a Tukey test was conducted to determine significant differences between the means for growth and As biotransformation of each diatom species at each temperature and salinity.

3.3 Results and discussion

3.3.1 Effects of temperature and salinity on the growth of marine diatom species

Temperature is a factor that can be controlled in microalgal cultivation and is a sensitive factor for algal growth and metabolic processes. Digital microscope images of six marine diatom species used in this study are shown in **Figure 3.1**. The maximum cell density (cells mL^{-1}) was recorded at temperatures between 10 and 25°C for all the species (**Figure 3.2**). Among the six species, *Thalassionema nitzschioides* and *Skeletonema* sp. showed exceptional cellular growth capacities with wide temperature tolerances. All the species exhibited higher cell densities on day 10 and 14 during the 3 weeks of culture. On day 14, the maximum cell concentration was recorded for *T. nitzschioides* (cell density 60.4×10^3 cell mL^{-1} / growth rate 0.11 ± 0.02 day^{-1} at 20°C) and *Skeletonema* sp. (cell density 64.6×10^3 cell mL^{-1} /growth rate 0.12 ± 0.01 day^{-1} at 15°C) (**Figure 3.2 and 3.3**). This result possibly suggests that these temperatures are optimum for the growth of these two species. Except for *T. nitzschioides* and *Skeletonema* sp., the remaining diatom species showed a similar growth pattern at various temperatures on different culture days. On day 10 at 15°C, *Asteroplanus karianus*, *Nitzschia longissima*, *Ditylum brightwellii*, and *Chaetoceros didymus* displayed higher cell growth with cell densities of 26.8×10^3 (growth rate 0.05 ± 0.01 day^{-1}), 18.8×10^3 (growth rate 0.04 ± 0.02

day^{-1}), 20.1 × 10^3 (growth rate 0.05 ± 0.03 day^{-1}), and 18 × 10^3 cell mL^{-1} (growth rate 0.05 ± 0.02 day^{-1}), respectively (**Figures 3.2 and 3.3**). The effects of temperature on algal species may vary depending on the species type, characteristics, and surrounding environment. Fujimoto et al. (1994) reported that the growth rate of a microalga (*Selenastrum capricornutum*) was higher when temperature was adjusted to 22°C, whereas that of *Microcystis viridis* was higher at 30°C. A positive correlation was observed between temperature and *Nannochloris oculata* growth in a temperature range of 20 to 25°C (Terlizzi and Karlander, 1980), below this temperature the growth rate decreased from 0.13 to 0.06 day^{-1} (Converti et al., 2009). The optimum temperature for *Scenedesmus* sp. growth was found to be between 20°C and 40°C (Sanchez et al., 2008; Martinez et al., 1999; Christov et al., 2001). Algal species, such as *C. vulgaris* grow well at 30°C; however, when the temperature was increased to 35°C, the growth rate decreased to 17% and the species died at 38°C (Converti et al., 2009). Therefore, the growth of our experimental species was dependent on the capability to survive in certain temperature ranges, which is optimum for their growth.

Salinity is an important factor that has significant effects on plant growth and their metabolic activities (Liska et al., 2004). A wide range of salinity levels (0.3–50‰) was employed to observe the cell concentrations and growth rate per day of the marine diatoms in the culture vessels. All diatom species showed maximum cell concentrations on day 10 of incubation at salinities between 10 and 35‰ (**Figure 3.4**). Similarly, to the temperature treatment, *T. nitzschioides* and *Skeletonema* sp. showed higher salinity tolerances than the other experimental species. On day 10 of culture, the highest cell concentration of 69.2 × 10^3 cell mL^{-1} (growth rate 0.13 ± 0.03 day^{-1}) at 20‰ and 94 × 10^3 cell mL^{-1} (growth rate 0.14 ± 0.12 day^{-1}) at 30‰ salinity level was recorded for *T. nitzschioides* and *Skeletonema* sp., respectively (**Figures 3.4 and 3.5**). A similar maximum growth rate (0.06 ± 0.02 day^{-1}) was observed for *N. longissima*, *D. brightwellii*, and *C. didymus*. A growth rate of 0.04 ± 0.01 day^{-1} with cell concentration of 28 × 10^3 cell mL^{-1} was observed for *A. karianus* at 25‰ salinity level (**Figures 3.4 and 3.5**). Different salinity tolerance levels and adaptation strategies by microalgal species largely depend on the group and environmental characteristics (Rao et al., 2007). In natural environments, multiplication of marine microalgae generally occurred between days 5 to 7 of their growth stage and they could survive for at least 21 days depending on the species. The growth and salinity tolerance (low growth expectancy between < 5‰ and > 35‰) of the marine diatom species of the present study were also supported by several previous studies (Huang et al., 2011; Takagi et al., 2006; Saros and Fritz, 2000).

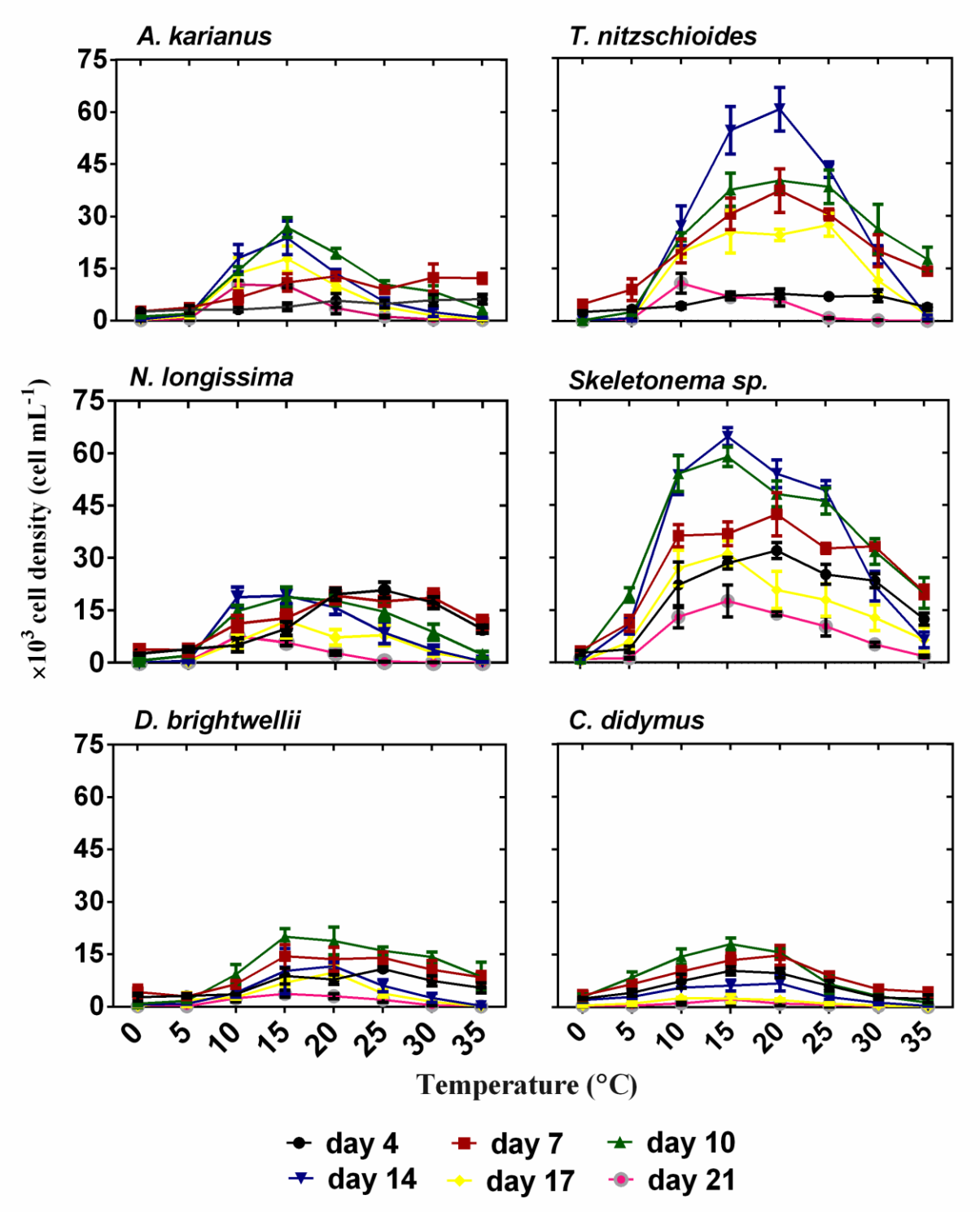

Figure 3.2 Effect of temperature (°C) on the growth (cell density (cell mL^{-1})) of marine diatom species. Data are means ± SD (n = 3).

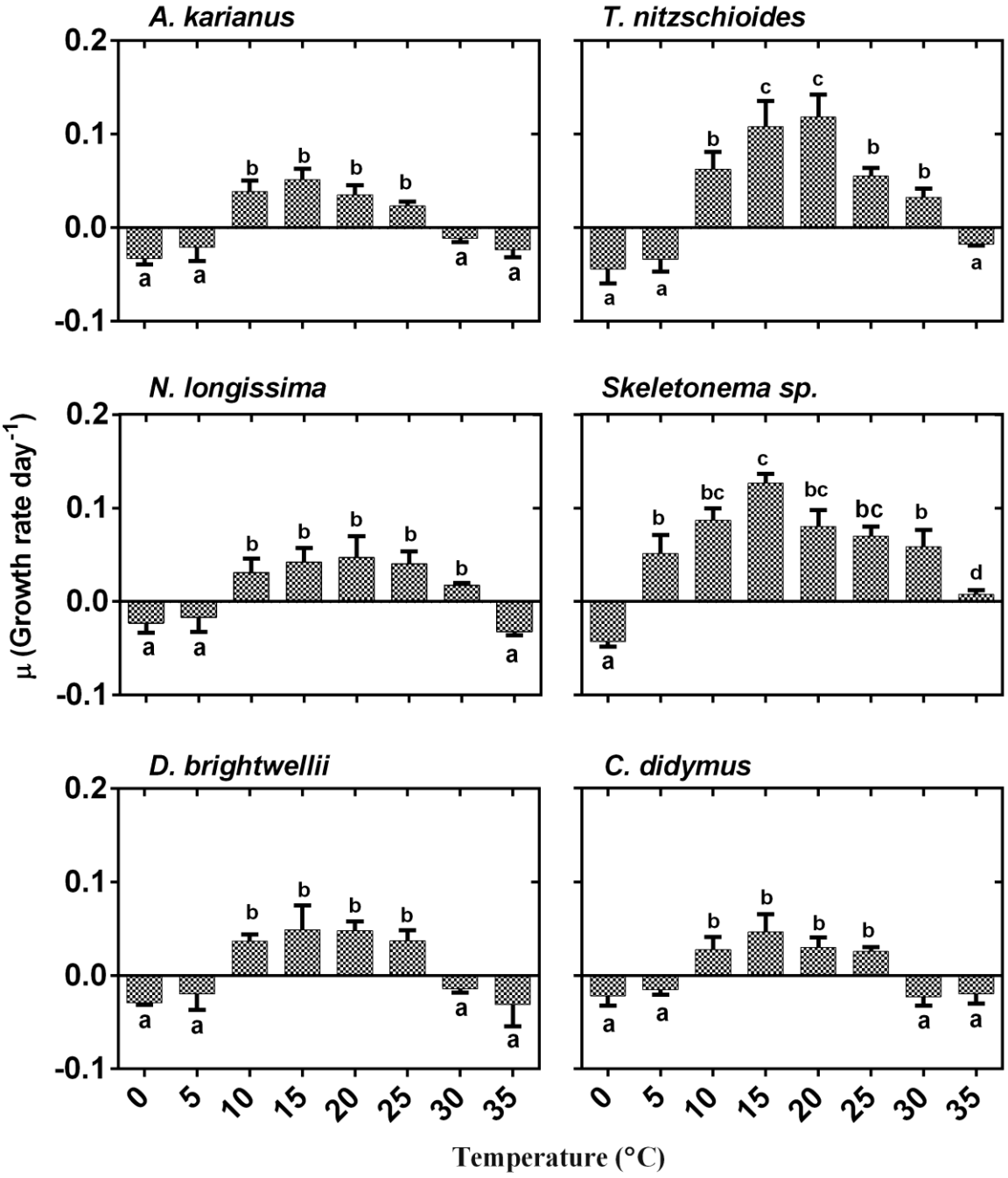

Figure 3.3 Effect of temperature (°C) on the growth (growth rate day^{-1}) of marine diatom species. Different lowercase letter indicates significant differences between temperature levels. Data are means ± SD (n = 3).

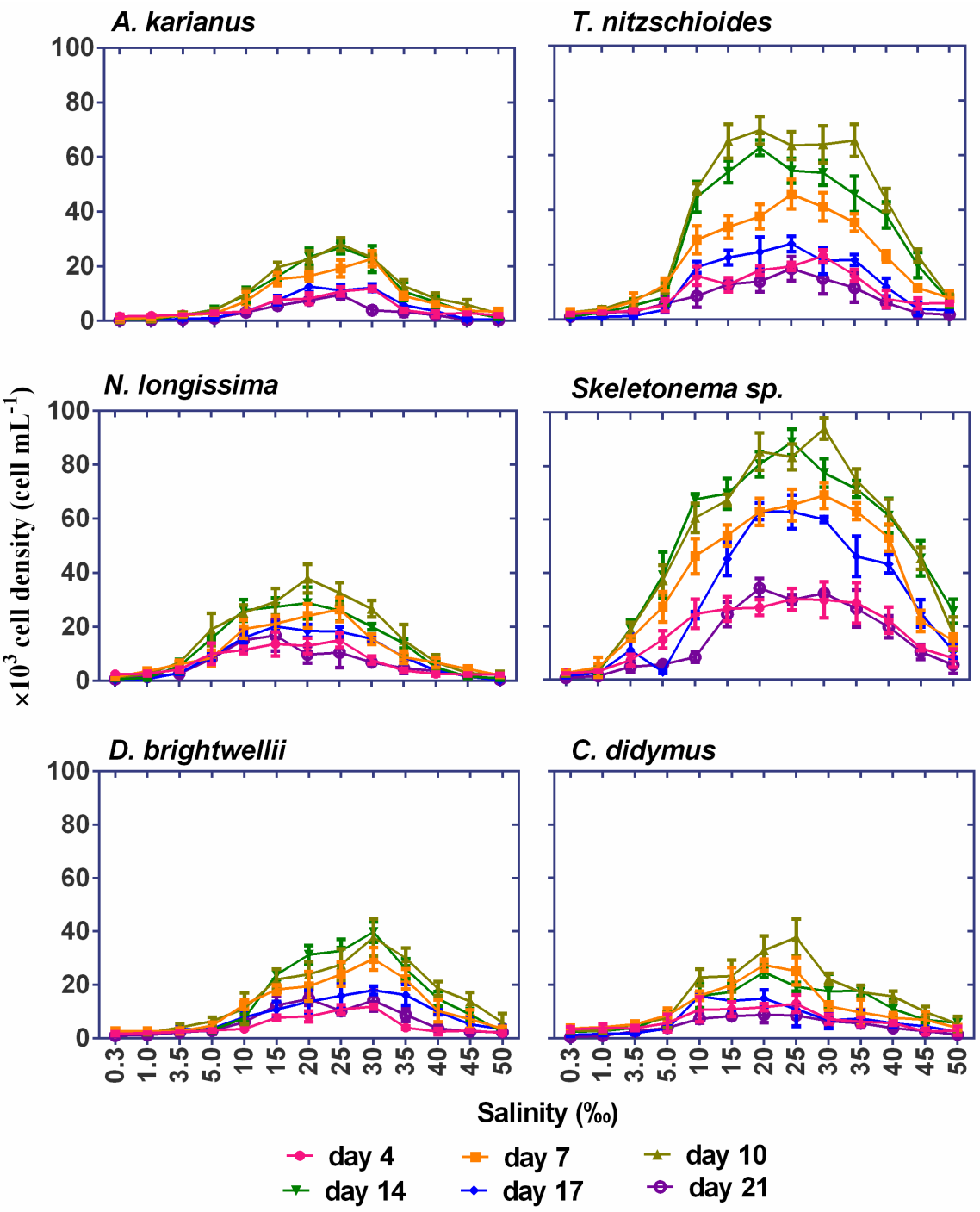

Figure 3.4 Effect of salinity (‰) on the growth (cell density (cell mL^{-1})) of six marine diatom species. Data are means ± SD (n = 3).

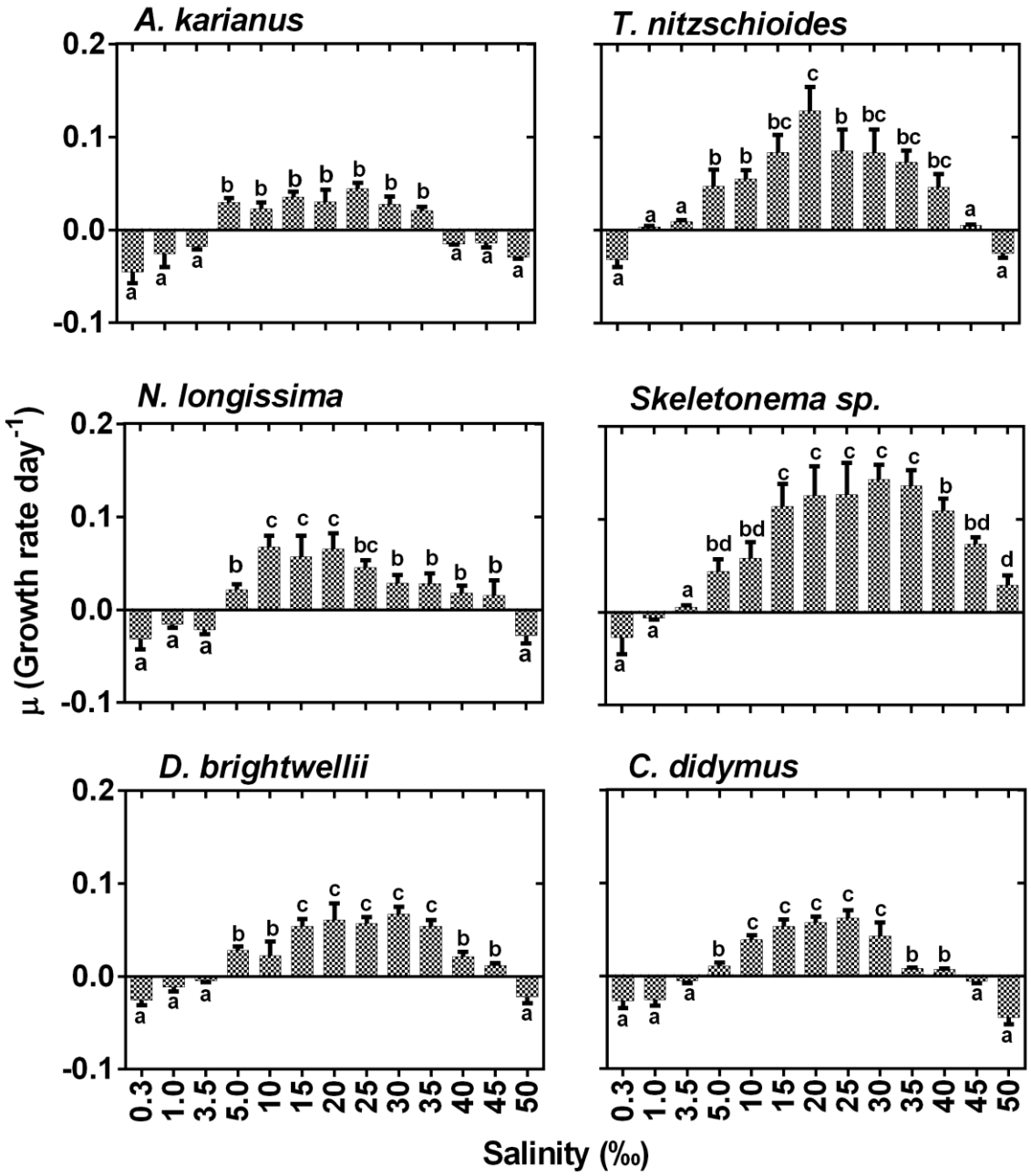

Figure 3.5 Effect of salinity (‰) on the growth (growth rate day^{-1}) of six marine diatom species, Different lowercase letter indicates significant differences between salinity levels. Data are means ± SD (n = 3).

3.3.2 Effects of temperature and salinity on As biotransformation

In this study, the cultures were exposed to 0.1 µmol L^{-1} Na_2HAsO_4 as As(V) and 1 µmol L^{-1} NaH_2PO_4 as PO_4^{3-} to influence the uptake mechanisms of the diatom species. Microalgae take up arsenic species in the form of As(V) using the phosphate pathway in the cell membrane owing to the physiochemical similarities between As(V) and PO_4 (Niegel and Matysik, 2010 Hasegawa et al., 2001; Suhendrayatna and Maeda, 2001). Marine microalgae reduce the toxic effect of As(V) by transforming As(V) to As(III) inside the cell (Baker and Wallschlager, 2016; Rahman and Hasegawa, 2012). This phenomenon occurs by a reduction of two electrons of pentavalent arsenate to trivalent arsenite facilitated by thiol, such as glutathione (Hughes, 2002). The conversion of As(V) to As(III) and the following biomethylation to methylated arsenicals, e.g. DMAA and MMAA, by microalgae largely depends on the species growth capability and concentration of phosphate and arsenic in the culture medium (Ye et al., 2012).

The potential of As biotransformation by six marine diatom species was investigated under various temperatures (0–35°C) at day 10 of culture (**Figure 3.6**). Except for *T. nitzschioides* and *Skeletonema* sp., none of the other microalgae showed biotransformation of As species at ≤ 5°C and ≥ 35°C. At 5°C, As biotransformation was detected only for *T. nitzschioides* (62.9 ± 13 nmol L^{-1} of As(V) and 8.4 ± 4 nmol L^{-1} of As(III)) and *Skeletonema* sp. (As(V) = 73.7 ± 10 nmol L^{-1} and As(III) = 6.3 ± 2.1 nmol L^{-1}), whereas *Skeletonema* sp. even biotransformed As at 35°C (As(V) = 68.4 ± 10.1 nmol L^{-1} and As(III) = 16.1 ± 4.3 nmol L^{-1}) (**Figure 3.6**). All the species transformed As(V) to As(III) and the methylated forms of As species i.e. DMAA and/or MMAA at 15 and 20°C. The reduction of As(V) to As(III) and/or DMAA was witnessed from 10–30°C for *A. karianus* and *N. longissima*, 5–30°C for *T. nitzschioides*, and 10–25°C for *D. brightwellii* and *C. didymus* (**Figure 3.6**). This phenomenon was observed for *Skeletonema* sp. even at a wide temperature range (5–35°C). The above results indicated that all the diatom species of this study could grow and potentially biotransform toxic As(V) to As(III) with further methylation to form methylAs species at certain temperature ranges at their logarithmic growth phase.

The present study elucidated the relationship between temperature on the growth of diatom species and As biotransformation. None of the species showed biotransformation at 0°C, suggesting that at such low temperatures these species are unable to reduce As(V) to As(III) and the subsequent methylation to MMAA and DMAA. However, the presence of small amounts of MMAA and MMAA not only assist in iAs methylation, but also help the fast release

of MMAA and DMAA when exposed to As(V) (Wang et al., 2013). Similarly, at 35°C, no As biotransformation occurred, except for *Skeletonema* sp. for growth and As biotransformation processes. However, As(V) was reduced to As(III) either for further methylation or efflux from the cell (Knauer and Hemond, 2000) by *D. brightwellii* and *C. didymus* at 10–25°C. The influence of temperature on As uptake mechanisms and speciation were very imprecise. However, several researchers exposed cultures to temperatures of 20–35°C to investigate As uptake and metabolism by phytoplankton (Duncan et al., 2010; Foster et al., 2008; Fujimoto et al., 1994), suggesting that high temperatures are important to reduce arsenic uptake. Cho et al. (2007) observed that maximum cellular growth of *Chlorella ellipsoidea* and *Nannochloris oculata* at 20°C and 30°C, respectively, but the study did not investigate the As uptake and biotransformation processes. These findings illustrated that As biotransformation depends on the specific species and their metabolism, in addition to adaptability to different temperature conditions.

The biotransformation of As species and the subsequent methylation by six diatom species occurred well between salinities of 10 to 35‰ (**Figure 3.7**). At low salinity levels (0.3–3.5‰), only As(V) was measured in the culture medium, indicating that at such low salinities all the species were unable to reduce As(V) to As(III) or methylated arsenicals. A 5‰ salinity was too low for cellular growth and As biotransformation of *N. longissima*, *D. brightwellii*, and *C. didymus*. At high salinity levels (45–50‰), no biotransformation was occurred, except for in *Skeletonema* sp. This species possesses a wide range of salinity tolerance (5–45‰) and could biotransform As(V) to As(III), even at a salinity of 45‰ (As(V) = 66.5 ± 9.8 nmol L^{-1} and As(III) = 8.8 ± 5.8 nmol L^{-1}) (**Figure 3.7**). This is might be because different species react differently in different salinities, which has been reported in several other studies (Huang et al., 2011; Takagi et al., 2006; Hu and Gao, 2006). This is an agreement a previous study indicating that *Skeletonema* sp. grow well under wide salinity conditions in the natural environment (Rijstenbil, 1988; Bergesch et al., 2009; Balzano et al., 2010). However, *C. didymus* showed As biotransformation under salinities between 10–30‰, whereas below 5‰ and above 35‰, the redox reaction, as well as methylation, was absent. This phenomenon may occur because (i) microalgal metabolism is affected by extreme high or low ion concentrations and (ii) certain metabolites required for cellular growth are depleted. In addition, salinity changes influenced changes in turgor pressure, which in turn modified the membrane thickness. The biochemical and biophysical processes of microalgal cells are possibly connected with the translocation rate of mobile charges located inside the membrane, which is regulated by the membrane thickness

(Kirst, 1990). The redox reaction by microalgae generally occurs as a protection mechanism to avoid metalloid toxicity (Maeda et al., 1992), or as a supportive system for cellular growth in the mode of energy production (Mateos et al., 2006). Biotransformation activity by marine species may be influenced by different salinity conditions, as it has an obstructive effect on the central metabolic activity (Liska et al., 2004).

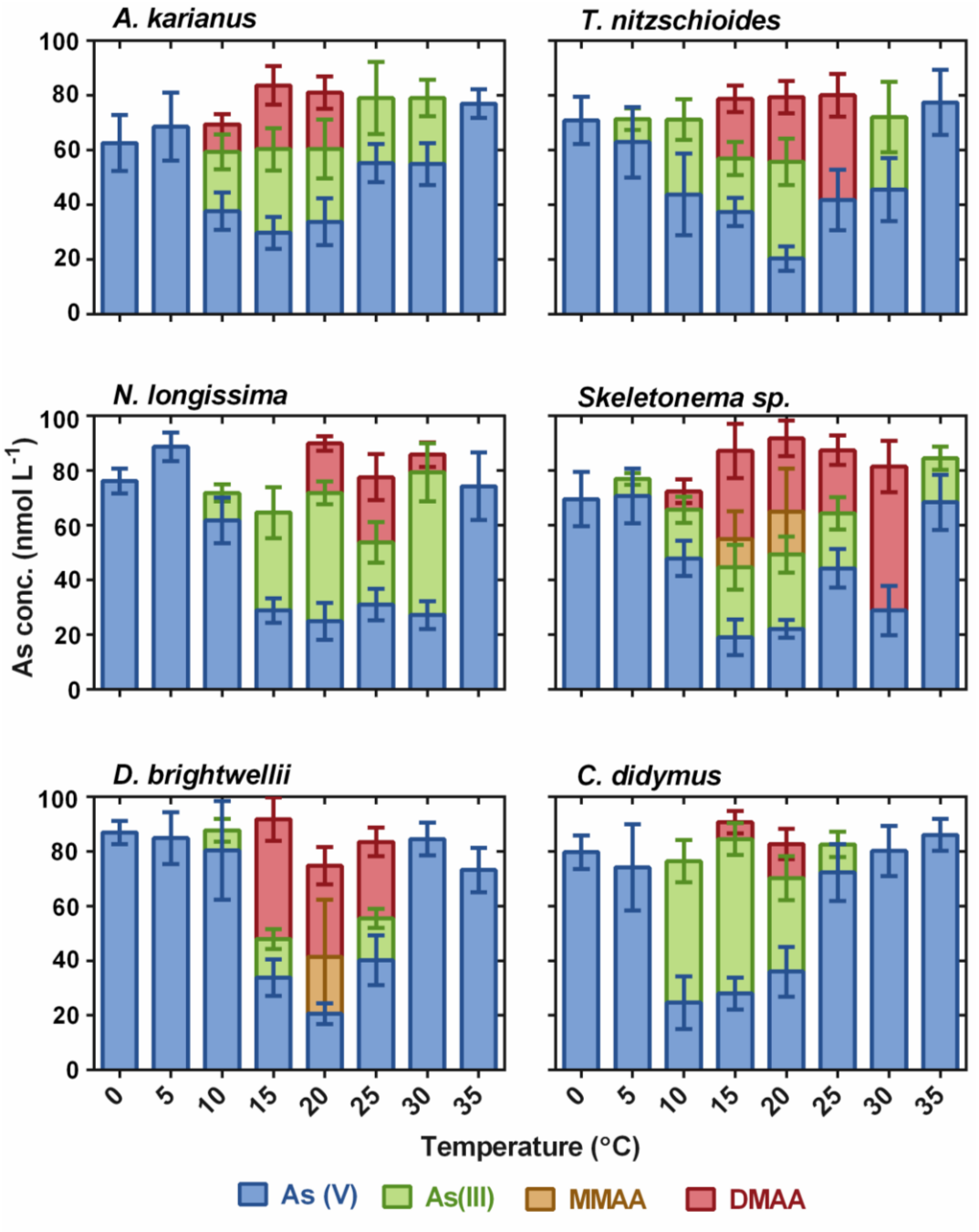

Figure 3.6 Effect of temperature (°C) on arsenic biotransformation processes (As(V) is reduced to As(III) and subsequent methylation to MMAA and DMAA) by six marine diatom species. Data are means ± SD (n = 3).

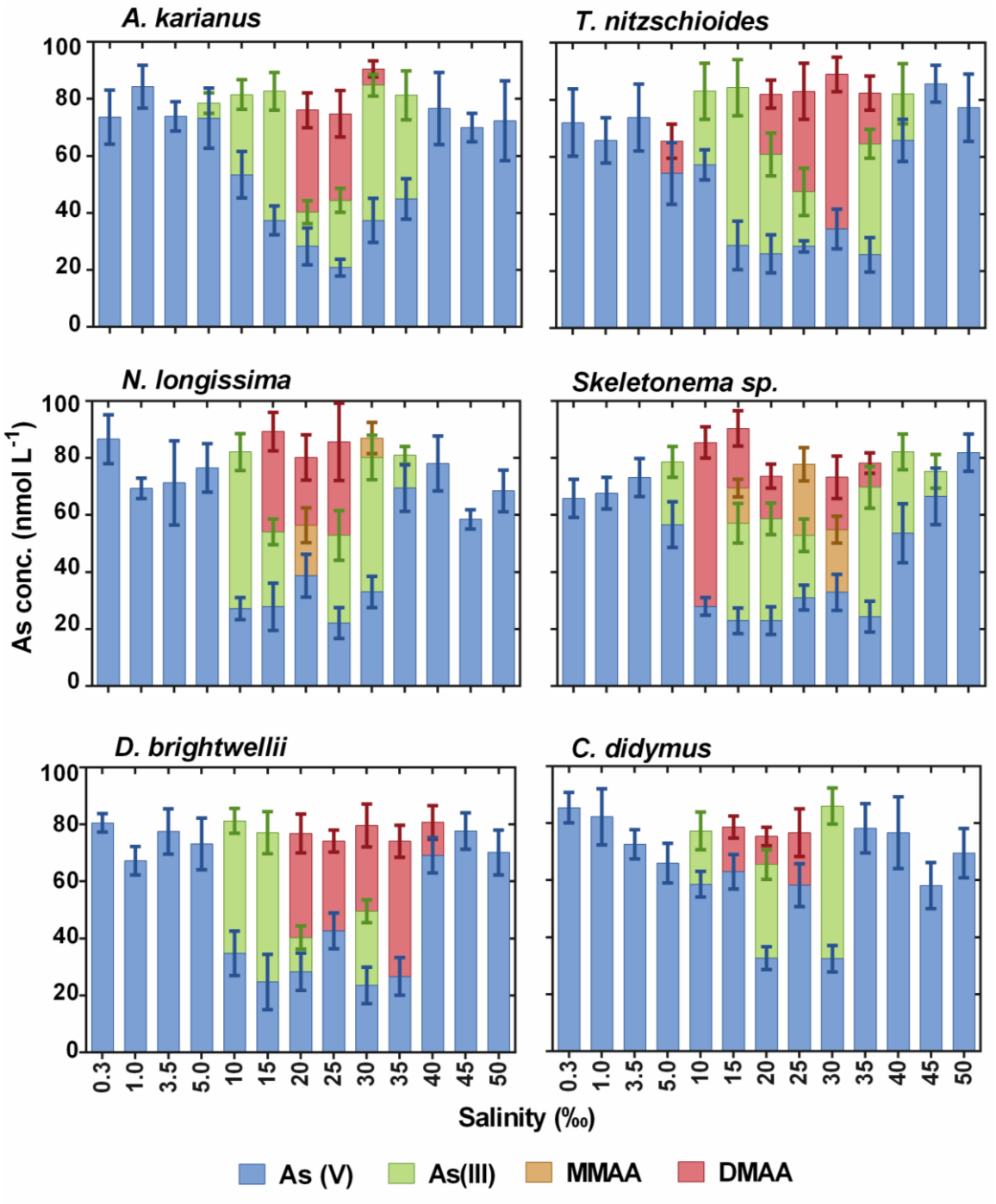

Figure 3.7 Effect of salinity (‰) on arsenic biotransformation processes (As(V) is reduced to As(III) and subsequent methylation to MMAA and DMAA) by six marine diatom species. Data are means ±SD (n = 3).

3.3.3 Effects of temperature and salinity on As speciation pattern: time dependency study

Arsenic speciation pattern by marine diatoms was observed at four different temperatures (10, 15, 20, and 25°C) in a week i.e. on day 10 and 17 of culture (**Figure 3.8**). These temperatures seemed optimum to all the species, as growth and As biotransformation were not properly observed for all the species below 10°C and above 25°C. The result indicated that in some cases, the biotransformation of As(V) to As(III) and subsequent methylated arsenicals was significantly different ($p < 0.05$) between day 10 and 17 of speciation. For example, on day 10 at 10°C, *A. karianus* contained 37.7 ± 5.6, 21.7 ± 5.2, and 10.1 ± 3.1 nmol L^{-1} of As (V), As(III), and methylated As component (MMAA+DMAA), respectively, whereas on day 17 at the same temperature, these amounts were 25.7 ± 4.9, 33.9 ±4 .0, and 23 ± 4.4 nmol L^{-1} (**Figure 3.8**). Regarding *T. nitzschioides*, the concentrations of As(V), As(III), and methylAs component were 43.8 ± 12.1, 27.4 ± 6.0, and 0 nmol L^{-1} on day 10 at 10°C, whereas these amounts were 33.6 ± 4.0, 35.9 ± 4.8, and 9.5 ± 5.2 nmol L^{-1} on day 17 at the same temperature, respectively (**Figure 3.8**). Other species, such as *N. longissima*, *Skeletonema* sp., *D. brightwellii*, and *C. didymus* showed significant As biotransformation from days 10 to 17. Lower As(V) concentrations on day 17 than on day 10 at each temperature indicated that As(V) decreased with time in the culture medium. The occurrence of As(III) and methylAs in the medium was mainly a biological reduction of As(V) to As(III). In addition, methylated arsenicals (MMAA and DMAA) were also found at higher concentrations on day 17 than that on day 10. The conversion efficiency of pentavalent arsenate (As(V)) to the reduced form of trivalent arsenite (As(III)) was considered to be the pioneer step for the methylation process (Hellweger and Lall, 2004). As biotransformation by marine diatom species in this study revealed that they are capable of redox reactions, methylation, and excretion of As in the medium.

Similarly, to temperature, a time dependency study was conducted at five salinity levels (15, 20, 25, 30, and 35‰), and As speciation was recorded on day 10 and 17. At all salinities, the biological reduction of As(V) by marine diatom species was recorded between day 10 and 17 (**Figure 3.9**). For example, at day 10 and 25‰ salinity condition in *T. nitzschioides*, the concentrations of As(V), As(III), and methylAs were 28.6 ± 1.6, 19.1 ± 6.8, and 35.2 ± 8.0 nmol L^{-1}, whereas on day 17 at the same temperature these concentrations were 11.9 ± 5.2, 22.2 ± 6.1, and 44.2 ± 3.9 nmol L^{-1}, respectively (**Figure 3.9**). In some cases, at certain salinity conditions on day 10, more than 60% As(V) remained in the medium. For example, on day 10 at 35‰ salinity, *N. longissima* and *C. didymus* species contained 69.4 ± 6.7 and 76.6 ± 7.0

nmol L^{-1} of As(V), respectively (**Figure 3.9**). However, *C. didymus* contained an As(V) concentration of 59.5 ± 4.8 nmol L^{-1} at day 17 at 35‰, which was the only exception found during the analysis of the data related to salinity-based time dependency, but is difficult to explain. As the regulation of As uptake is different for different species, it may be controlled by the intracellular As(V) concentration, and then its accumulation could be activated by its release from the cell (Wang et al., 2013; Wang e al., 2015). As(V) uptake or sequestration is enhanced by the physio-biochemical structure in association with individual species type and characteristics (Duncan et al., 2015).

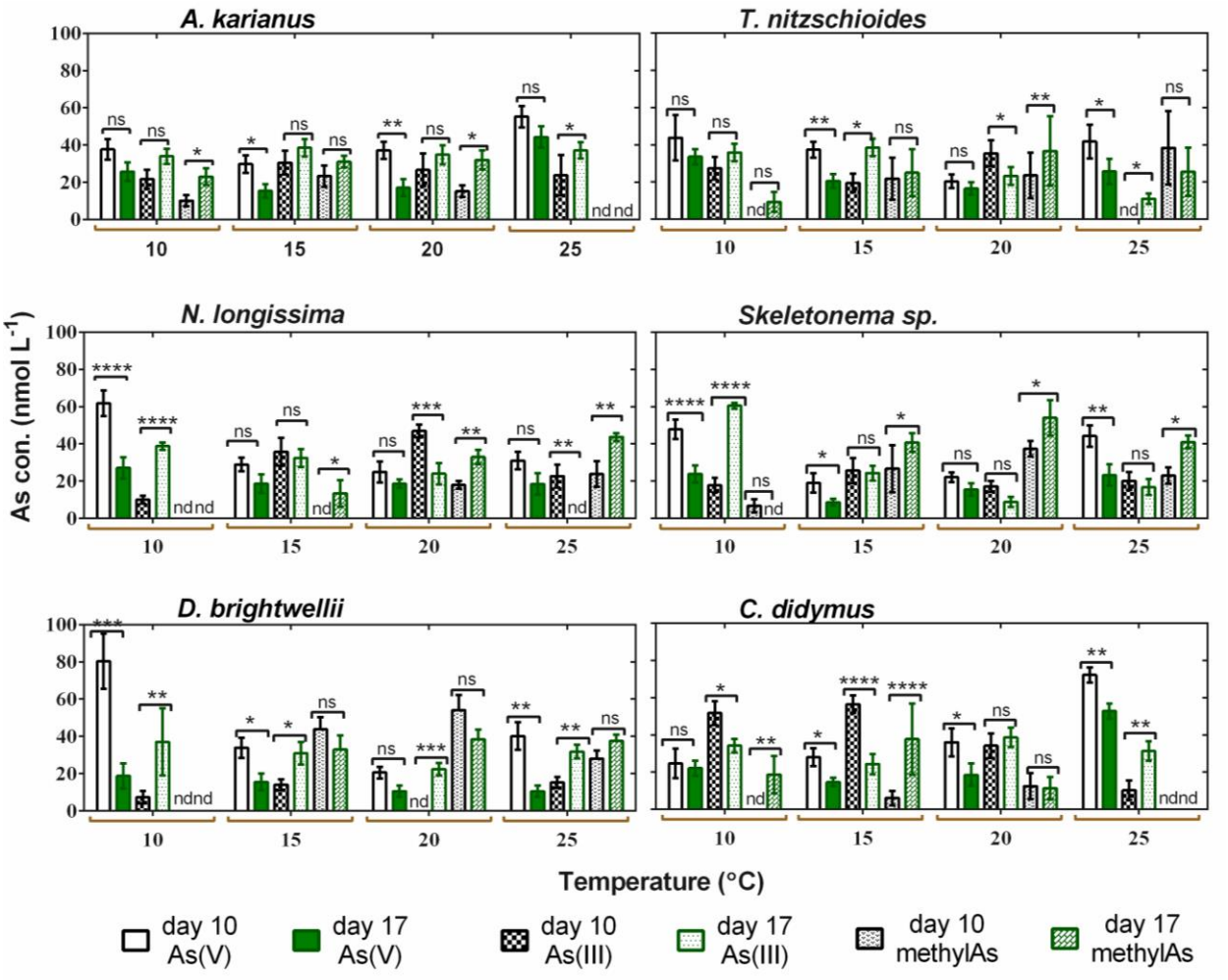

Figure 3.8 Time-dependent As speciation patterns by six marine diatom species. As speciation was observed on day 10 and 17 of culture at various temperatures (°C). The star marks above the bars showed significant differences at * $p \leq 0.01$, ** $p \leq 0.001$, and *** $p \leq 0.0001$ levels between day 10 and 17 within the same As species. 'ns' indicates non-significant, 'nd' indicates not detected. Data are means ±SD ($n = 3$).

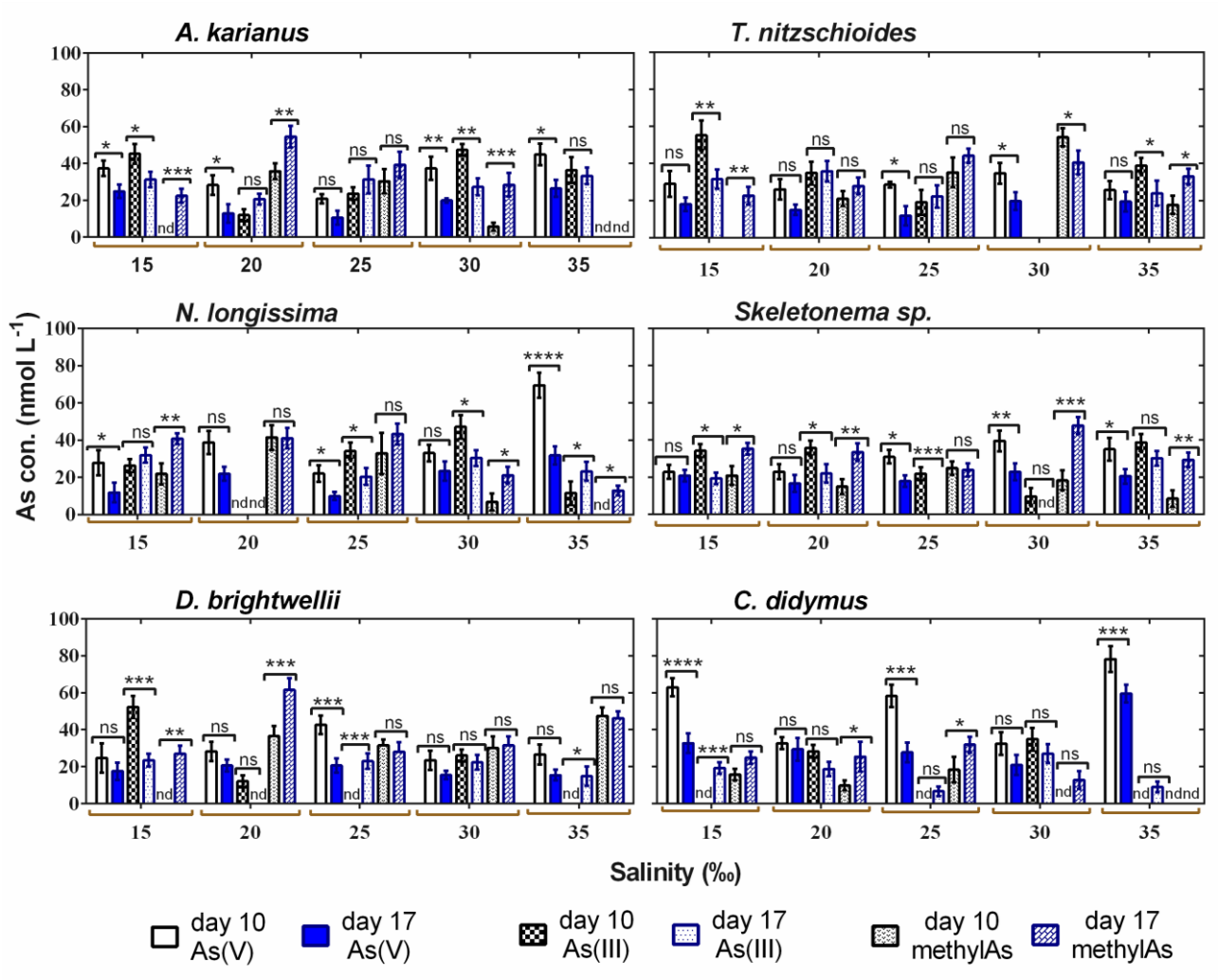

Figure 3.9 Time-dependent As speciation pattern by six marine diatom species. As speciation was observed on day 10 and 17 of culture at various salinity (‰) levels. The star marks above the bars showed significant differences at * $p \leq 0.01$, ** $p \leq 0.001$, and *** $p \leq 0.0001$ levels between day 10 and 17 samples within the same As species. ns indicates non-significant, nd indicates not detected. Data are means ± SD ($n = 3$).

3.3.4 Interrelated influence of temperature, salinity, and cell size on As biotransformation

Cell surface area (µm^2) of six diatom species was calculated at different temperatures (0– 35°C) and salinities (0.3–50‰) (**Table 3.1**). The maximum surface area of the cell was in the ranges of optimum temperature (10–25°C) and salinity (10–35‰) for all the species. Below and above these temperature and salinity levels, individual diatom cells had a reduced surface area for adaptation to the adverse environment. The interrelation between cell volume (µm^3) of each diatom species and As biotransformation potentials were evaluated (**Figures 3.10 & 3.11**). The cell volume and As biotransformation were positively correlated in almost all of the cases. Cell size of the six diatom species in the descending order: *N. longissima* > *D. brightwellii* > *C. didymus* > *Skeletonema* sp. > *T. nitzschioides* > *A. karianus*. At the optimum temperature and salinity levels, all the species could biotransform As(V) to As(III) or MMAA or DMAA at a maximum cell volume. The diatom species were unable to biotransform As species at temperatures < 5°C and > 30°C, and salinity < 5‰ and > 35‰ with some variations (**Figures 3.10 & 3.11**). The As biotransformation potentials of the six diatom species during temperature treatment was: *Skeletonema* sp. > *T. nitzschioides* > *N. longissima* > *A. karianus* > *D. brightwellii* > *C. didymus*, whereas during the salinity treatment the order was: *Skeletonema* sp. > *T. nitzschioides* > *A. karianus* > *D. brightwellii* > *N. longissima* > *C. didymus*.

3.3.5 Conceptual model of growth and arsenic uptake mechanisms

A conceptual model on the effect of temperature and salinity on the growth and As uptake and biotransformation mechanisms by six marine diatom species was developed based on the findings of this study (**Figures 3.12 & 3.13**). Microalgal growth is influenced by environmental conditions, such as temperature and salinity (Mclachlan, 1961; Latala et al., 1991; Abu-Rezq et al., 1999), which are considered as crucial environmental factors during their culture. The effect of both temperature and salinity on the growth of six marine diatom species showed similar trends based on the culture day. The maximum cell density (cell m L^{-1}) was recorded for all the diatom species on day 10 and 14 of culture for both temperature and salinity treatments. All the species showed optimum growth as cell density at temperatures between 10 and 25°C and salinity levels between 10 and 35‰ (**Figures 3.2, 3.4 & 3.12**). The temperature tolerance of algal species depends on rapid changes of temperature and the

physicochemical state of algae exposed to extreme temperature before, during, and after a period of time (Hirata et al., 1981). Moreover, salinity influences the growth of the species regulated by osmoregulatory mechanisms, which further affects the physiological and biochemical process of algal species in marine environments.

Table 3.1 Cell surface area (μm^2) of six diatom species under different temperature and salinity condition.

Temper-ature (°C)	***A. karianus***	***T. nitzschioides***	***N. longissima***	***Skeletonema sp.***	***D. brightwellii***	***C. didymus***
0	353±37^{a}	683±74^{a}	3236±398^{a}	768±140^{a}	3432±363^{a}	1298±66^{ac}
5.0	409±54^{a}	902±84 ^{a}	3737±378^{a}	1221±119^{b}	4090±384^{a}	1592±71^{ab}
10	524±33^{ab}	1018±64 ^{ab}	4187±415^{a}	1196±162^{b}	4114±363^{ab}	1702±103^{ab}
15	592±33^{b}	1020±63 ^{ab}	4312±462^{a}	1316±88^{b}	3544±277^{a}	1715±60^{ab}
20	594±57^{b}	920±98^{ab}	4950±463^{ab}	1271±125^{b}	3177±349^{a}	1703±60^{ab}
25	526±29^{ab}	856±61^{ab}	3873±293^{a}	1260±102^{b}	3137±371^{a}	1633±190^{ab}
30	360±41^{a}	642±91^{a}	3944±303^{a}	1236±79^{b}	3032±252^{a}	811±119^{c}
35	300±46^{a}	536±52^{a}	3923±355^{a}	919±58^{ab}	2696±340^{a}	617±102^{c}
Salinity (‰)	***A. karianus***	***T. nitzschioides***	***N. longissima***	***Skeletonema sp.***	***D. brightwellii***	***C. didymus***
0.3	252±55^{a}	1005±44^{a}	2255±113^{a}	753±113^{a}	3184±165^{ab}	1754±74^{ab}
1.0	292±22^{a}	1057±31 ^{a}	3677±471^{b}	794±126^{a}	3580±204^{ab}	1722±88^{ab}
3.5	399±57^{a}	1033±63 ^{a}	3908±157^{b}	961±80^{ab}	3841±292^{ab}	1905±99^{ab}
5.0	367±62^{a}	968±107 ^{a}	3374±85^{b}	1007±88^{ab}	4071±265^{a}	2104±218^{ab}
10	406±50^{a}	1221±117^{a}	3484±151^{b}	1170±40^{ab}	4112±90^{a}	2234±244^{a}
15	432±48^{ab}	1127±195^{a}	4133±287^{bc}	1274±51^{b}	4343±282^{a}	2240±267^{a}
20	499±41^{ab}	955±42^{a}	3945±463^{bc}	1158±127^{ab}	4032±221^{a}	2154±224^{a}
25	542±58^{ab}	1071±199^{a}	4242±156^{bc}	1147±132^{ab}	4086±251^{a}	2260±160^{a}
30	508±35^{ab}	945±53 ^{a}	4442±254^{bc}	1111±195^{ab}	4118±158^{a}	2064±258^{ab}
35	369±68^{a}	755±91^{ab}	4373±282^{bc}	901±114^{ab}	3788±299^{ab}	1846±223^{ab}
40	307±43^{a}	670±34^{b}	3762±283^{b}	844±65^{a}	3920±332^{ab}	1604±143^{ac}
45	326±35^{a}	6403±63^{b}	3924±298^{b}	865±152^{a}	3017±237^{ab}	1273±128^{c}
50	264±40^{a}	548±42^{b}	3278±406^{b}	797±145^{a}	2827±387^{ab}	1172±194^{c}

Different lowercase letter indicates significant differences between temperature and salinity levels (p <0.05). Data are means ±SD (n=3).

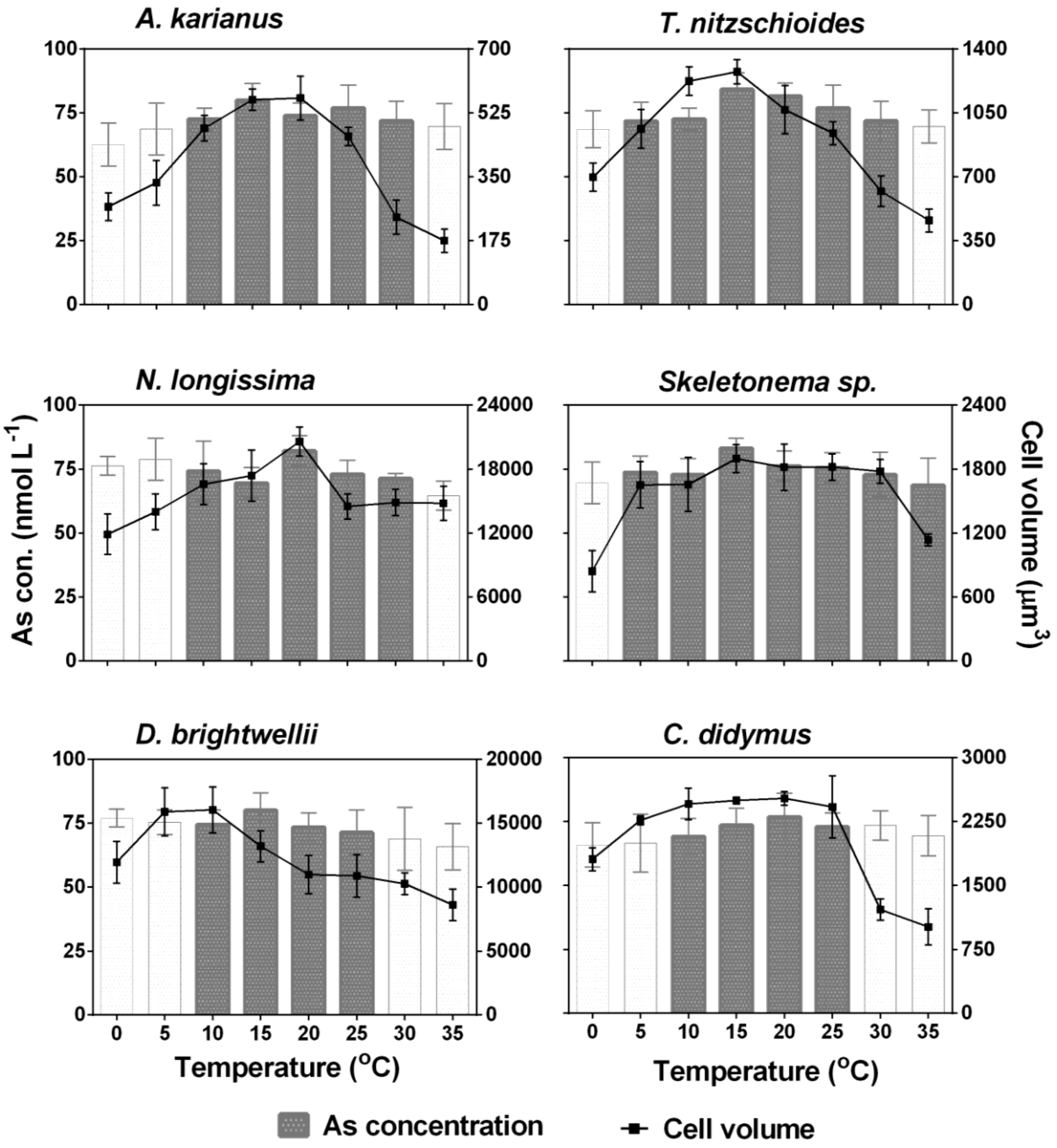

Figure 3.10 Relationship between cell size and As biotransformation of six marine diatom species at various temperature levels. Dark bars in the graph indicate As species biotransformation occurred, i.e. As(V) was reduced to As(III) and the subsequent methylation to MMAA and DMAA. White bars indicate no biotransformation occurred, i.e. only As(V) was detected in the culture medium. Data are means ± SD (n = 3).

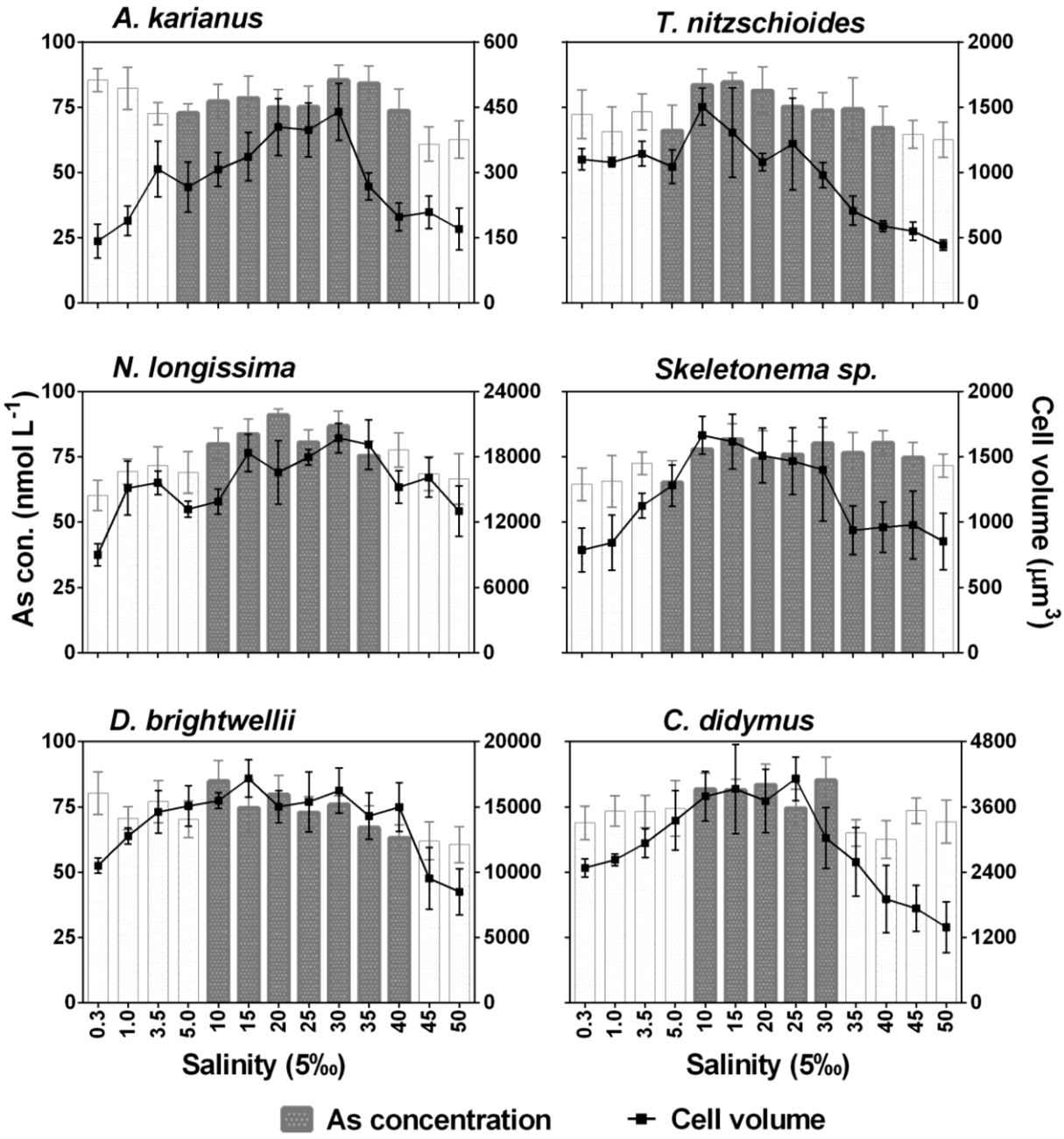

Figure 3.11 Relationship between cell size and As biotransformation of six marine diatom species at various salinity levels. Dark bars in the graph indicate As species biotransformation occurred, i.e. As(V) was reduced to As(III) and the subsequent methylation to MMAA and DMAA. White bars indicate no biotransformation occurred, i.e. only As(V) was detected in the culture medium. Data are means ± SD (n = 3).

In aquatic environments, As(V) accumulation and biotransformation to As(III) and subsequent methylation to MMAA and DMAA by photosynthetic microbial species e.g. algae and cyanobacteria (Ye et al., 2012), is affected by environmental factors, such as temperature, salinity, pH, and nutrients. These factors control the algal biochemical composition and behaviour in natural aquatic ecosystems. These microbial species play a significant role in regarding biogeochemical cycling of As content by biotransformation in natural ecosystems (Rahman and Hasegawa, 2012). As(V) and phosphate have similar physicochemical characteristics, and therefore algal species take up As(V) present in media via a phosphate transporter system (Reed et al. 2015) and As(V) biotransformation occurs inside the cell (Guo et al., 2011). The competitive uptake between As(V) and PO_4^{3-} suggested a possible active toxic mode of As. Inside the cellular portion of ATP, As(V) replaced the phosphate groups and established an unstable ADP-As complex that in turn interfered with several physiological process, e.g. energy flow (Ullrich-Eberius et al., 1989). Furthermore, after As(V) uptake, it is either transformed to trivalent As and excreted into the medium or subsequently methylated to methyl arsenicals. The reduced metabolites As(III) readily released from the cell leading to reduced the toxicity of As(III) inside of the cell (Hellweger et al., 2003; Rahman and Hassler, 2014). This process of biomethylation from iAs is regarded as the detoxification mechanism of aquatic algal species (Levy et al., 2005). In addition, excretion of As(V), As(III), or the organic component could be regarded as a significant arsenic detoxification mechanism (Zhao et al., 2009).

As(V) toxicity is based on the competitive inhibition of protein, along with other enzymes that use phosphate, as well as oxidative phosphorylation. The reduction reaction of As(V) to As(III) occurs in the presence of thiols and/or dithiols because of its tendency to combine with biochemical components, such as protein and non-protein thiols (Cullen et al., 1994). However, As(III) is more toxic than As(V) because of its actions, similarly to soft metal with thiols. In addition, the As(III) bond with monothiol is comparatively weak and its high concentration depletes glutathione within the cell. It develops strong bonds with dithiols, which inactivates different important enzymes and receptors (Yang et al., 2012). After As(V) uptake from surrounding marine water, As(V) is organized to form carbohydrate groups, which are further biosynthesized to orgAs species (Francesconi and Edmonds, 1996). However, orgAs contained by marine microalgae are mostly AsS that are regarded as pioneers in the metabolic channel to ArsB and ArsC (Hansen and Raab, 2003). ArsB acts as an antiporter that excludes As(III) from cells during the exchange by $H^+/As(OH)_3$ attached to the electrochemically

mediated proton gradient (Meng et al., 2004). ArsC is the substrate of ArsB that acts as an As(V) reductase. ArsC transforms As(V) to As(III) and increases the array of resistance to include As(V) (Gladysheva et al., 1994).

The biotransformation process of As(V) to As(III) takes place via aquaporin nodulin 26-like intrinsic protein (NIPs) (Zhao et al., 2009), which is a water channel that transports water molecules from extracellular to intracellular portions. This reduction reaction occurs in the presence of several reductases that act as electron donors, such as glutaredoxin, glutathione, or thioredoxin (Yin et al., 2011). As an electron donor, glutathione reduces As(V) to As(III) in aqueous solution and forms a complex of arsenotriglutathione, As(III) $(GS)_3$ that immediately gives As(III) to target species that contain dithiol groups (Scott et al., 1993). Methylation mechanisms by the As(III) $(GS)_3$ complex has been discussed in the study of Hayakawa et al. (2005).

Phytochelatins (PCs), with metal binding capacities, are intracellular thiol-based polypeptides of microalgae. These metalloproteins play a significant role in microalgal detoxification mechanisms. PCs encourage As to bind with thiol groups, i.e. glutathione has a significant function in As complexation and detoxification (Pawlik-Skowronska et al., 2004). It has been suggested that even at lower As concentrations, the rate of PC synthesis is sufficient to bond with As and cells of marine microalgae species (Morelli et al., 2005). The reduction mechanism of As(V) to As(III) is a rapid and important process for further sequestration into the vacuoles of algal cells (Zhang et al., 2013) where the majority of As(V) content is reduced within 1–2 days prior to sequestration into the vacuole (Levy et al., 2005; Cullen et al., 1994).

In the present study, the As(III) concentration gradually decreased owing to continuous abiotic oxidation indicating that As(V) constrained the intracellular As(III) accumulation in algal cells (Hellweger et al., 2003) (**Figure 11**). Further, methylation of As(III) to methylated species (MMAA and DMAA) occurred within the cells and their overall concentration gradually increased in the growth medium by their excretion from the cell. However, there are several steps associated with the biochemical metabolism of inorganic As to methylated arsenicals, i.e. mono-di and tri-methyl arsenic species. Some steps are activated by chemical reactions, whereas others are incorporated into enzymatic catalysis. Previously it was found that one catalyst, arsenic-methyltransferase (AS3MT), actively promoted the biotransformation of iAs to methylated arsenic species (Zhang et al., 2013). However, the methylation mechanism is an oxidative process where a methyl group (CH_3^+) is available for the reaction to progress.

But, oxidative stress occurred whenever active oxygen come in contact during the catalytic reaction with methyltransferase (Hu et al., 2002). During deviations in the redox state of arsenic, S-adenosylmethionine (SAM) donated methyl groups to As using AS3MT. For methylation mechanisms, SAM is required because the extent and pattern of methylation mechanisms largely depends on its availability (Thomas et al., 2001).

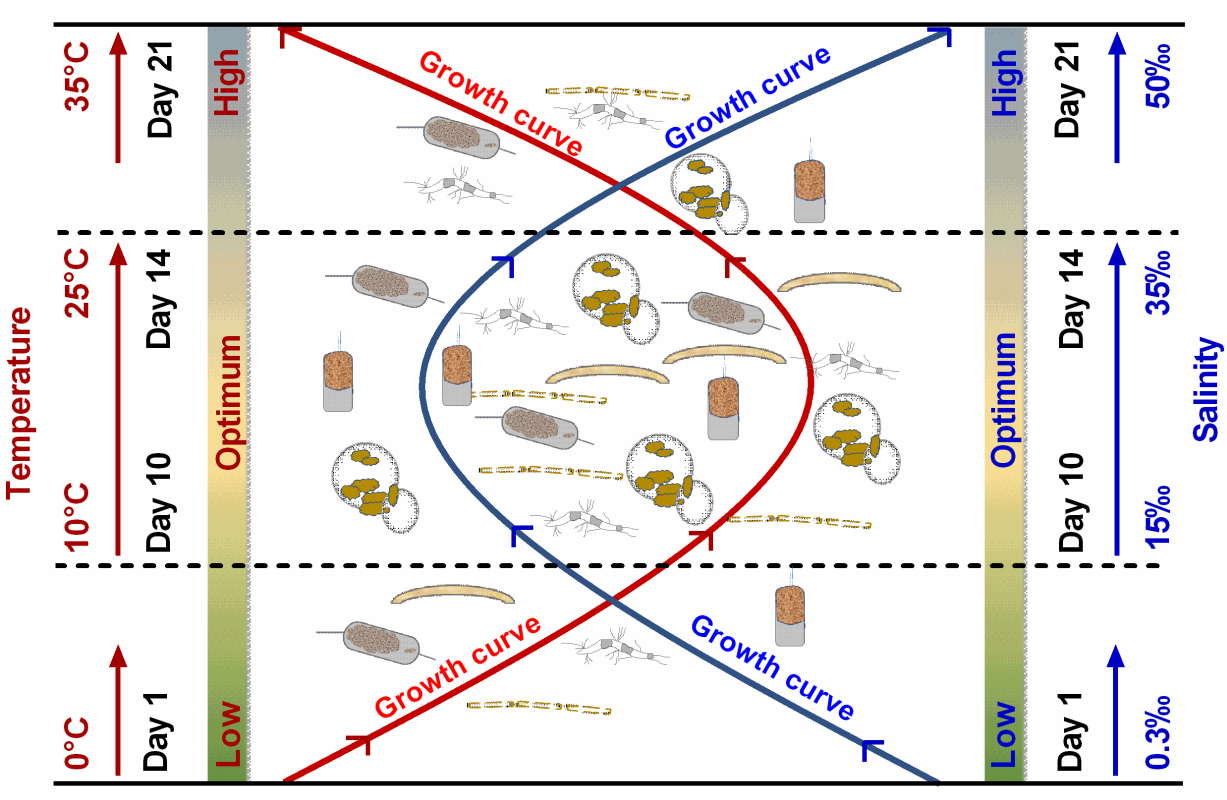

Figure 3.12 Conceptual model on effect of temperature and salinity on the growth of six marine diatom species.

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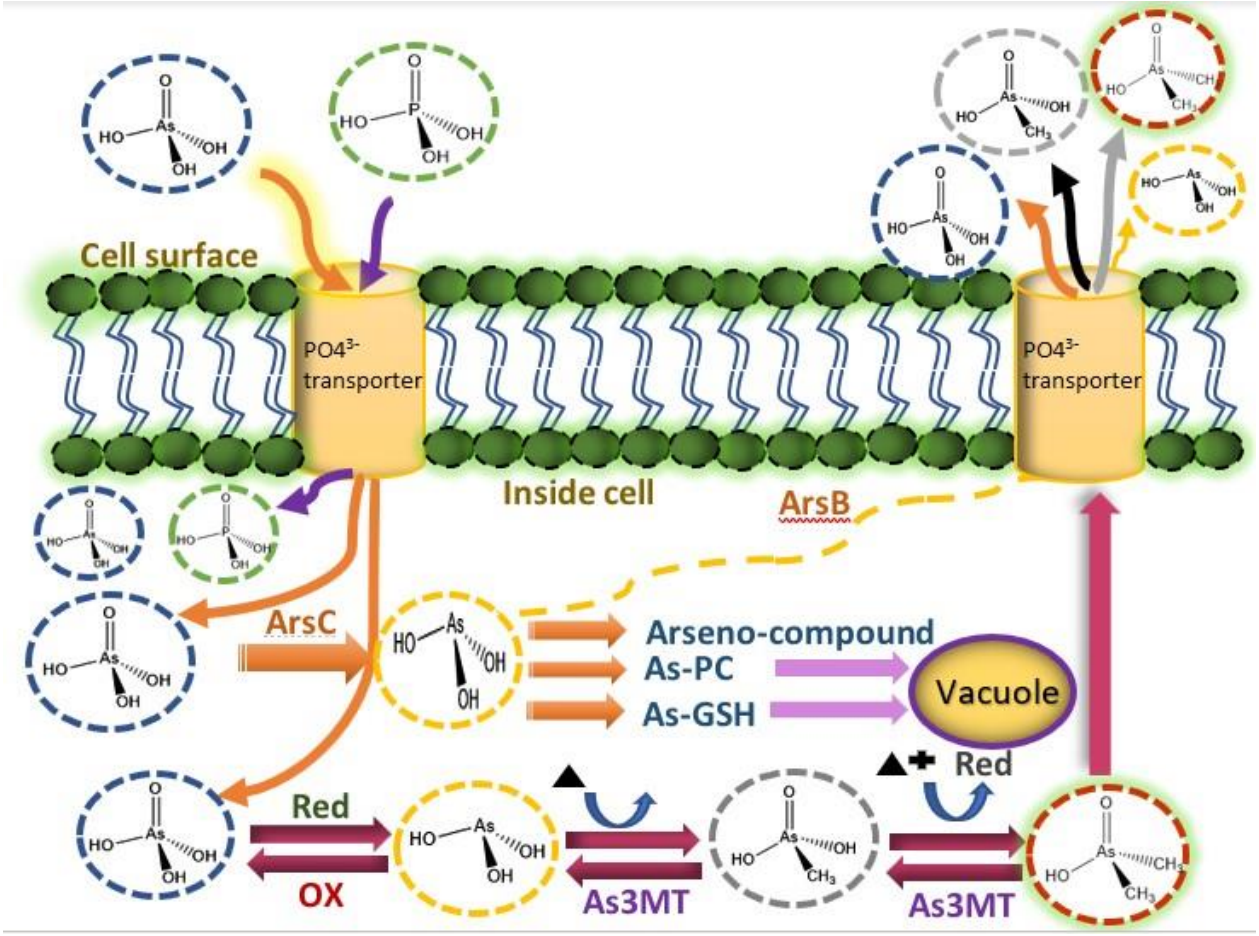

Figure 3.13 Conceptual model on effect of temperature and salinity on arsenic uptake and biotransformation mechanisms by six marine diatom species based on this study.

3.4 Conclusion

The potential growth and As biotransformation by six marine diatom species was investigated under various temperature (0–35°C) and salinity (0.3–50‰) conditions during three weeks of culture. Except for *T. nitzschioides* and *Skeletonema* sp., none of the species biotransformed As species at ≤ 5°C and ≥ 35°C. However, growth and As biotransformation and subsequent methylation were optimum between temperatures of 10 to 25°C and salinities of 10 to 35‰. At low salinity levels (0.3–3.5‰), only As(V) was measured in the culture medium, indicating that at such low salinity conditions all the species were unable to reduce As(V) to As(III) or methylated arsenicals. The biological reduction, i.e. biotransformation of As(V) to As(III) and subsequent methylated arsenicals, was significantly different between day 10 and 17 speciation at different temperature and salinity conditions. The interrelated influence of temperature, salinity, and cell size on As biotransformation was also reported for the first time. These results suggest that each species has an optimum temperature and salinity tolerance range suitable for their adaptation metabolism, such as growth and the biotransformation of toxic As(V) to As(III) and further methylation to form methylated As species.

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Chapter 4:

Marine microalgae: Integrated effects of important environmental factors on arsenic biotransformation and photosynthetic efficiency

Rimana Islam Papry*, Shogo Fujisawa, Zai Yinghan, Okviyoandra Akhyar, M. Abdullah Al Mamun, Asami S. Mashio, Hiroshi Hasegawa*, "Integrated effects of important environmental factors on arsenic biotransformation and photosynthetic efficiency by marine microalgae", *Ecotoxicology and Environmental Safety*, **201** (2020) 110797 (Published: June 2020).

4.1 Introduction

Arsenic (As) is a noxious trace metalloid that is allocated throughout the aquatic environment and has carcinogenic effects on human health (Smedley and Kinniburgh, 2002). As species are found in inorganic arsenic (iAs), such as arsenate (As(V)) and arsenite (As(III)) in aquatic ecosystems (Rodríguez-lado et al., 2013), which are more toxic than organarsenicals (Dopp et al., 2010; Sun et al., 2012; Alava et al., 2012). Moreover, several studies have implied that arsenic biotransformation is a very complicated process and the toxicity of each As species varies (Hirano et al., 2004; Karadjova et al., 2008; Petrick et al., 2000).

Microalgae as primary producers ensure As bioaccumulation and biogeochemical cycling in the marine ecosystem (Duncan et al., 2015; Zhang et al., 2014). They have eminent competence in the uptake of As from the environment, which makes these tiny species ecofriendly and cost effective in terms of the As remediation process (Bahar et al., 2013; Mahdavi et al., 2012; Wang et al., 2015). Diatoms are unique microalgae, which are abundant photosynthetic eukaryotes in the marine aquatic ecosystem that may account for almost 20% of global primary production. They play a noteworthy role in the biogeochemical cycling of essential nutrient elements (Rosenwasser et al., 2014).

As(V) is considered an analog of phosphate (PO_4^{3-}) and microalgae uses the PO_4^{3-} transporter for uptake of As(V) inside the cell membrane (Guo et al., 2011). The similarity in physiochemical characteristics of As(V) and PO_4^{3-} influences not only the growth of individual microalgal species but also their metabolic mechanisms, including uptake, accumulation, biotransformation, and excretion of arsenic species (Wang et al., 2014). Moreover, PO_4^{3-} concentration has a significant influence on the uptake of As(V) by microalgae (Rodriguez Castro et al., 2015; Bahar et al., 2016; Duncan et al., 2013; Han et al., 2017). Biotransformation of As(V) to As(III) and subsequent methylation to monomethylarsonate (MMAA) and dimethylarsinate (DMAA) have also been reported to be affected by the concentration of As and phosphate in the medium (Karadjova et al., 2008; Duncan et al., 2015). Methylated species in natural waters are believed to come not only from microalgae and bacteria but also from biodegradable components (Hasegawa et al., 2001), and several studies confirmed the presence of methAs species in experiments with marine (Papry et al., 2020, 2019; Sanders et al., 1989) and freshwater microalgae (Maeda et al., 1992; Wang et al., 2017; Hasegawa et al., 2019).

Environmental factors that act as important parameters for the growth and metabolic processes of microalgae vary from species to species (Huang et al., 2011). Most studies have focused on environmental factors, including salinity (Barron et al., 2007; Garcia et al., 2012), pH (Qiu et al., 2017; Cortés et al., 2018), temperature (Christov et al., 2001; Fujimoto et al., 1994; Raven and Geider, 1988), light intensity (Gunawan et al., 2018; Bernardi et al., 2013), and nutrient availability (Juneja et al., 2013; Markou et al., 2014). In this study, salinity and temperature were both considered in combination to investigate their influence on As uptake, biotransformation, and photosynthetic efficiency. Salinity is an essential environmental parameter whose presence affects growth rate and physiological activities, such as photosynthesis (Adam et al., 2004; Sudhir, 2004). Nevertheless, adaptation capacity in saline water depends on the species type and characteristics because they are categorized as halotolerant or halophilic species (Rao et al., 2007). It has been documented that variation in salinity differs for different microalgae (Huang et al., 2011; Takagi et al., 2006; Balzano et al., 2011). In addition, temperature is another environmental factor that controls growth, chemical composition, and metabolic metabolism of microalgal species (Aydin et al., 2009; Javaheri et al., 2015; Adenan et al., 2013).

Arsenic biotransformation potential and photosynthetic efficiency of marine microalgae in relation to arsenic and phosphate concentration (Papry et al., 2020; Y. Wang et al., 2017; Wang et al., 2017; Guo et al., 2011), salinity (Papry et al., 2019; Adam et al., 2004), and temperature (Papry et al., 2019; Renaud et al., 2002) have been reported in the literature. However, the integrated effect of these environmental factors on marine diatom species remains scarce and unclear. This study addresses the research gap by investigating the integrated effect of temperature and salinity in combination with As (V) and PO_4^{3-} deficient and sufficient conditions on As biotransformation potential and photosynthetic efficiency by two marine diatom species. This study contributes to a deeper understanding of the relationship among environmental factors, As uptake, and the biotransformation mechanism of marine microalgae from the viewpoint of As remediation.

4.2 Materials and methods

4.2.1 Marine diatom species

Two strains of marine diatom species, namely, *Asteroplanus karianus* and *Skeletonema* sp., were used (see **Figure 4.1**). Dr. Kanako Naito, Associate Professor of

Hiroshima Prefecture University, Japan, provided marine diatom strains. *A. karianus* is a pennate diatom, known as a bloom-forming pennate diatom, mainly found in the coastal aquatic environment. In the last decade, this bloom-forming species has had harmful effects on seaweed cultivation in Japan. *Skeletonema* sp. is a centric diatom species capable of surviving under high temperature and salinity conditions. It causes water discoloration because of algal blooms.

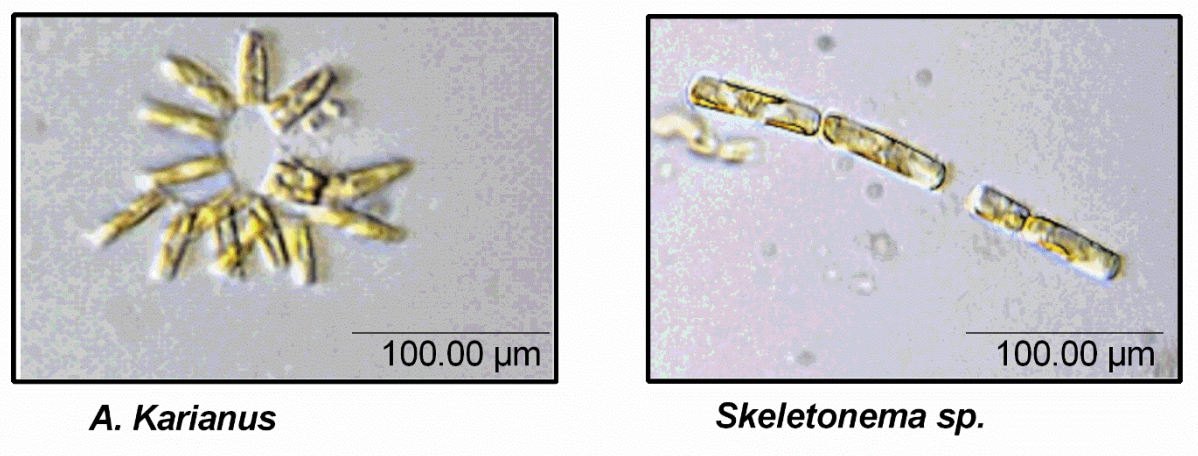

Figure 4.1 Images of marine diatom species captured using a digital microscope (KEYENCE, VHX-1000, Japan).

4.2.2 Reagents

Deionized water (arium pro UV, Sartorius Stedim Biotech, Goettingen, Germany) with a resistivity of 18.2 MΩ and a commercially available reagent without further purification were used for the experiment. Sodium hydroxide (NaOH; special grade, Nacalai Tesque, Kyoto, Japan) and hydrochloric acid (HCl; Kanto Chemical, Tokyo, Japan) were used for pH adjustment of the reagents and medium. The compound 4-(2-hydroxyethyl)-1-piperazinyl ethane sulfonate (HEPES; Nacalai Tesque) was used as a buffer reagent in the culture medium. Special grade sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen arsenate heptahydrate (As(V)), both from Wako Pure Chemical (Osaka, Japan), were used as PO_4^{3-} and As sources, respectively, in the culture medium.

4.2.3 Preculture and maintenance

The f/2-based nutrient medium along with natural seawater was used for the maintenance and culture of marine diatom species. An autoclave device (MLS 3780, Sanyo Electric, Japan) was used for the sterilization of the culture medium at 121°C for 30 min. The culture medium was kept inside a clean bench (NK Clean Bench, VSF-1300A, Nippon, Japan) under UV radiation flow for 20 min. Diatom species were cultured for 1–2 weeks in polycarbonate vessels (Nalgene, Nunc; Rochester, NY) inside an incubator (Koitotron3HN-35MLA, Koito Industries, Japan), where light and temperature were controlled. When cellular growth reached the exponential phase, samples were prepared for further experimental procedures.

4.2.4 Determination of the integrated effect of temperature and salinity

Integrated effects of temperature and salinity were observed with regard to cellular growth and arsenic biotransformation. Laboratory-prepared artificial seawater was used for changes to salinity levels in the culture medium. Certain temperatures (5.0, 20, and 35°C) and salinity levels (1.0, 10, 25, and 40‰) were pre-specified for further experimental procedures. Then, 30 mL of polycarbonate vessels containing diatom cultures were adjusted to predetermined temperature and salinity conditions in the incubator. Sequentially, various As and phosphate concentrations (As-phosphate enriched or As-phosphate deficient) conditions were applied to the culture medium (Papry et al., 2020; Y. Wang et al., 2017; Duncan et al., 2013), The 1.0 µmol L^{-1} of As(V) and 10 µmol L^{-1} of PO_4^{3-} was defined as enriched and 20 nmol L^{-1} of As(V) and 1.0 µmol L^{-1} of PO_4^{3-} as deficient concentrations in the experiment. During the 3 weeks of culture, culture samples were collected for cell density measurement using the cell counting method with a digital microscope (Keyence, VHX-1000, Japan). Initial cell concentration was adjusted to 2.0×10^3 cells mL^{-1}. The following equation was used for the calculation of growth rate (GR) per day during the experiment (Garcia et al., 2012):

$$\mu(\text{day}^{-1}) = \frac{(\ln N_1 - \ln N_0)}{t} \qquad (1)$$

where N_1 = final cell density, N_2 = initial cell density, and t = time (day).

4.2.5 Determination of arsenic species

The hydride generation technique was considered while determining the As species in culture media according to Hasegawa et al., (1994). The technique was comprising of a flame atomic absorption spectrophotometer and hydride generation device, followed by a cold trapping (AAS, 170-50A, Hitachi, Japan) (Papry et al., 2019; Hasegawa et al., 2019; Mamun et al., 2019). Inorganic As species, such as As(V) and As(III), and methylated arsenic species, such as MMAA(V) and DMAA(V), were determined during the quantitative analysis of liquid samples. While preparing samples, 5.0 mL 0.20 mol L^{-1} EDTA·4Na (ethylenediaminetetraacetic acid; Kanto Chemicals) and 5.0 mol L^{-1} HCl to 40 mL of the sample solution were added to the liquid samples. For As(III), 5.0 mL 0.20 mol L^{-1} EDTA·4Na and 0.5 mol L^{-1} potassium hydrogen phthalate (Kanto Chemicals) were added to 40 mL of the sample solution. A data analysis device (Chromato-PRO, Runtime Instruments, Tokyo, Japan) exhibited As species as the mean of the chromatogram and the peak height facilitated the measurement of the concentration of the As species. The lower detectable values of As(V), As(III), MMAA(V), and DMAA(V) were 0.11, 0.02, 0.18, and 0.12 nmol L^{-1} when RSD values (n = 3) included 1.3%, 2.7%, 2.5%, and 2.3%, respectively (Papry et al., 2019).

4.2.6 Measurement of chlorophyll fluorescence

Maximum photochemical efficiency (quantum yield) was considered while measuring the chlorophyll fluorescence of marine diatom species. Quantum yield was measured using an AquaFlash™ handheld active fluorometer (TURNER DESIGNS, San Jose, CA, USA). Liquid samples of cultures were dark adapted for 15 min before determination. The following equation (Dijkman and Kromkamp, 2006) was used for maximum photochemical efficiency:

$$F_v/F_m = (F_m - F_o)/F_m, \quad (2)$$

where F_m = maximum fluorescence yield, F_o = minimum fluorescence yield, F_v/F_m = maximum quantum yield, and F_v = maximum variable florescence yield, ($F_m - F_o$).

4.2.7 Statistics

Statistical software, including SPSS 22.0 for Windows (IBM Co., USA) and Graph Pad Prism 7.0 (GraphPad Software Inc., USA), were used to analyze the experimental data. A

one-way and two-way analysis of variance (ANOVA) with a 95% significance value was used when plotting the experimental data.

4.3 Results and discussion

4.3.1 Integrated effect of environmental factors on microalgal growth

In this study, *A. karianus* and *Skeletonema* sp. were cultured at various temperatures (5.0, 20, and 35°C) and salinities (1.0‰, 10‰, 25‰, and 40‰) under As and phosphate enriched or deficient conditions (**Figures 4.2 & 4.3**). The purpose of our study was to investigate the influence of temperature and salinity on marine diatom species under As and phosphate concentrations. *A. karianus* showed lower growth expectancy at all salinities at 5.0°C compared to that at 20 and 35°C. Higher cellular growth was observed at 10 and 25‰ with a cell density of approximately 71.0 ± 3.0 × 10^3 cell mL^{-1} (GR 0.14 ± 0.008) and 69.2 ± 4.0 × 10^3 cell mL^{-1} (GR 0.13 ± 0.02), respectively, under 20°C in arsenic-enriched conditions (**Figures 4.2 & 4.3**). At the same temperature, when cultured in deficient As concentrations, the cellular growth was 61.4 ± 4.0 × 10^3 cell mL^{-1} (GR 0.10 ± 0.005) and 40.8 ± 2.0 × 10^3 cell mL^{-1} (GR 0.08 ± 0.02). Under both conditions, high cell density was observed on the 14^{th} d of the culture experiment. The cellular growth of diatom species was at a maximum at 10‰, followed by 25‰ and was lower at 1.0‰ and 40‰ under all temperatures in both As-enriched and As-deficient concentrations (**Figure 4.2**). The effects of environmental factors on microalgal growth varies depending on the species categories, features, and neighboring environment. The optimum growth condition for *Chlorella ellipsoidea* and *Nannochloris oculata* were reported at salinity 10‰ and, temperature 15°C and 25°C, respectively (Cho et al., 2007). It was likely that the changes in environmental parameters, such as temperature, salinity, and nutrient concentration, had significant effects on the production of marine microalgal biomass (Cortés et al., 2018) in terms of species-specific adaptability and survival capacity under the environmental conditions.

Skeletonema sp. showed wide salinity adaptation capabilities, except for 1.0‰, under all temperature conditions and survived well between 10‰–40‰ (**Figures 4.4 & 4.5**). High cellular growth was determined at 25‰, followed by 10‰ and 40‰, indicating that physiological characteristics, such as growth and metabolic mechanisms of marine microalgal species were significantly induced by the culture environment where temperature and salinity were sensitive factors (Christov et al., 2001). Similar to *A. karianus*, *Skeletonema* sp. also

exhibited high cellular growth when culturation took place under As-enriched conditions, with a maximum cell density of 73.7 ± 6.0 × 10^3 cell mL^{-1} (GR 0.15 ± 0.008) observed at 25‰ under 20°C. A cell density of 46.8 ± 2.0 × 10^3 cells mL^{-1} (GR 0.08 ± 0.008) and 60.2 ± 1.5 × 10^3 cell mL^{-1} (GR 0.10 ± 0.03) occurred at 25‰ under 5 and 35°C, respectively, (**Figures 4.4 & 4.5**). However, under As-deficient concentrations, the cell density was 47.2 ± 2.0 × 10^3 cell mL^{-1} (GR 0.08 ± 0.008), 60.1 ± 4.2 × 10^3 cell mL^{-1} (GR 0.12 ± 0.01), and 52.2 ± 2.4 × 10^3 cell mL^{-1} (GR 0.20 ± 0.02) under similar conditions (**Figures 4.4 & 4.5**). Both species exhibited high growth capacity from the 7^{th} to 14^{th} d during the 3-week culture experiment. This result suggests that suitable temperature and salinity are important variables that control the growth rate of marine microalgae that encourage the uptake of chemical nutrients from the aquatic environments by marine algal species.

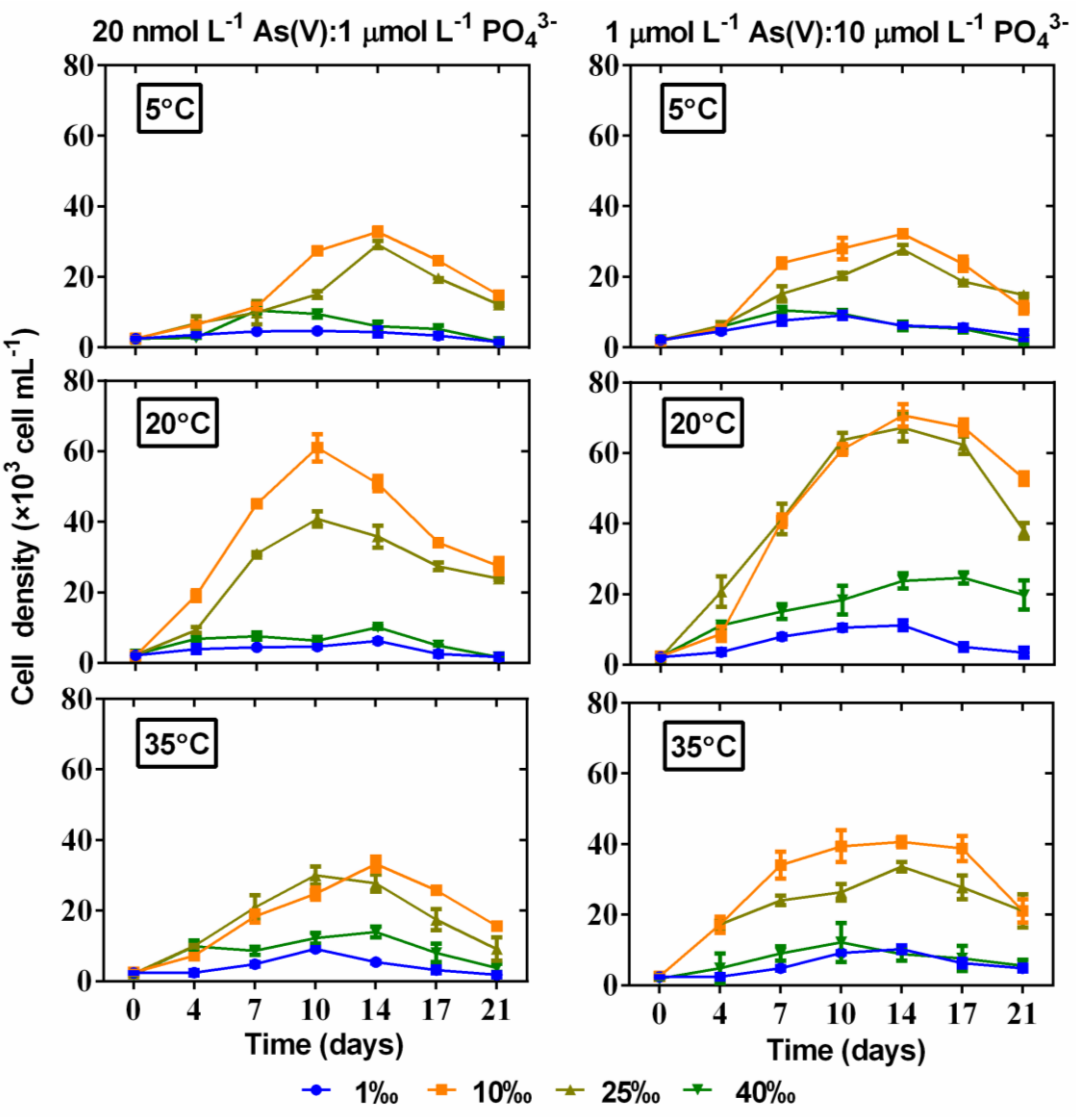

Figure 4.2 Integrated effects of environmental factors on the growth [cell density (cell mL^{-1})] of *A. karianus*. Data are presented as the mean ± SD (n = 3).

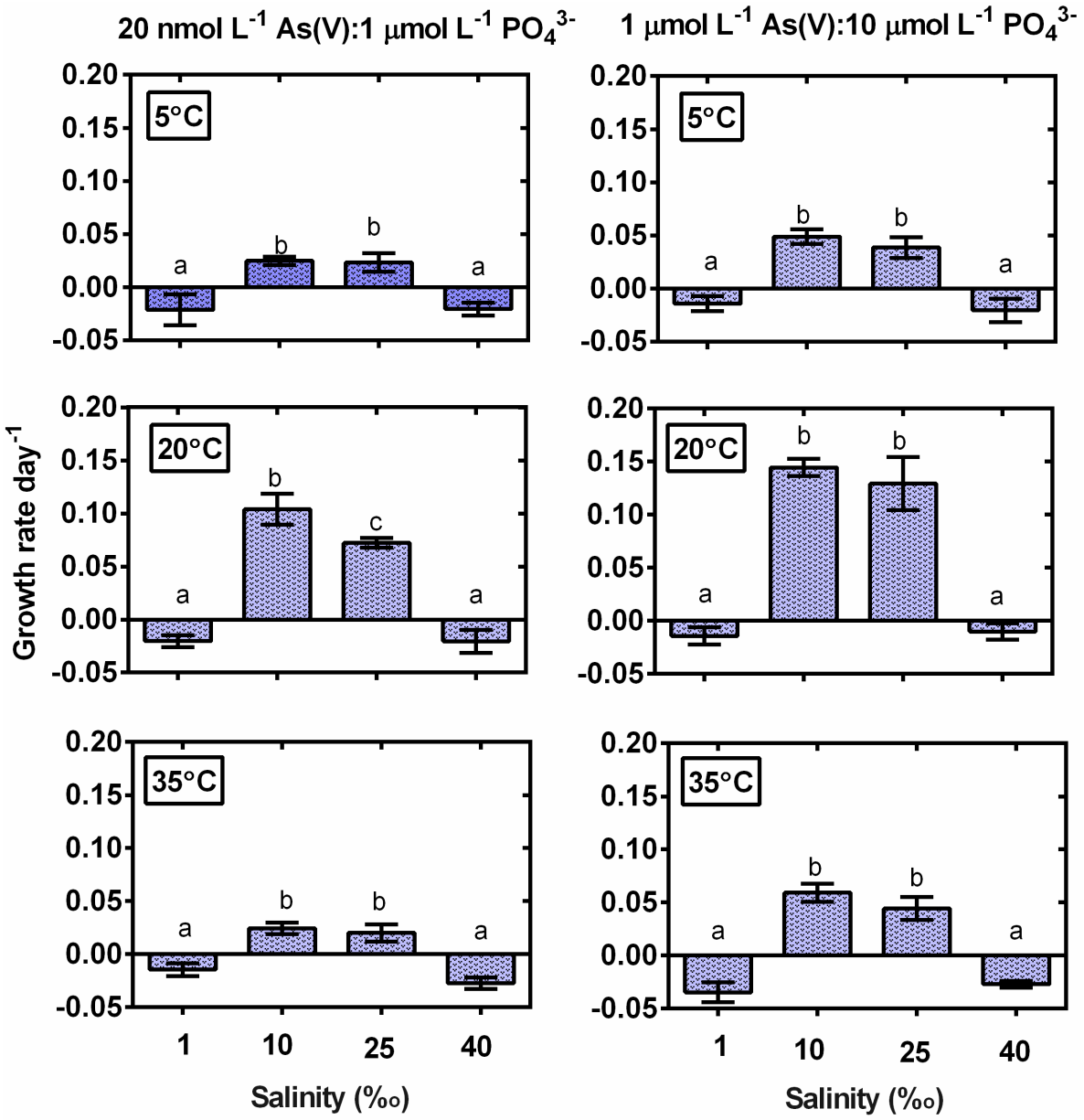

Figure 4.3 Integrated effects of environmental factors on the growth [growth rate d^{-1}] of *A. karianus*. Lowercase letters imply a 95% significance level between salinities under the same temperature. Data are presented as the mean ± SD (n = 3).

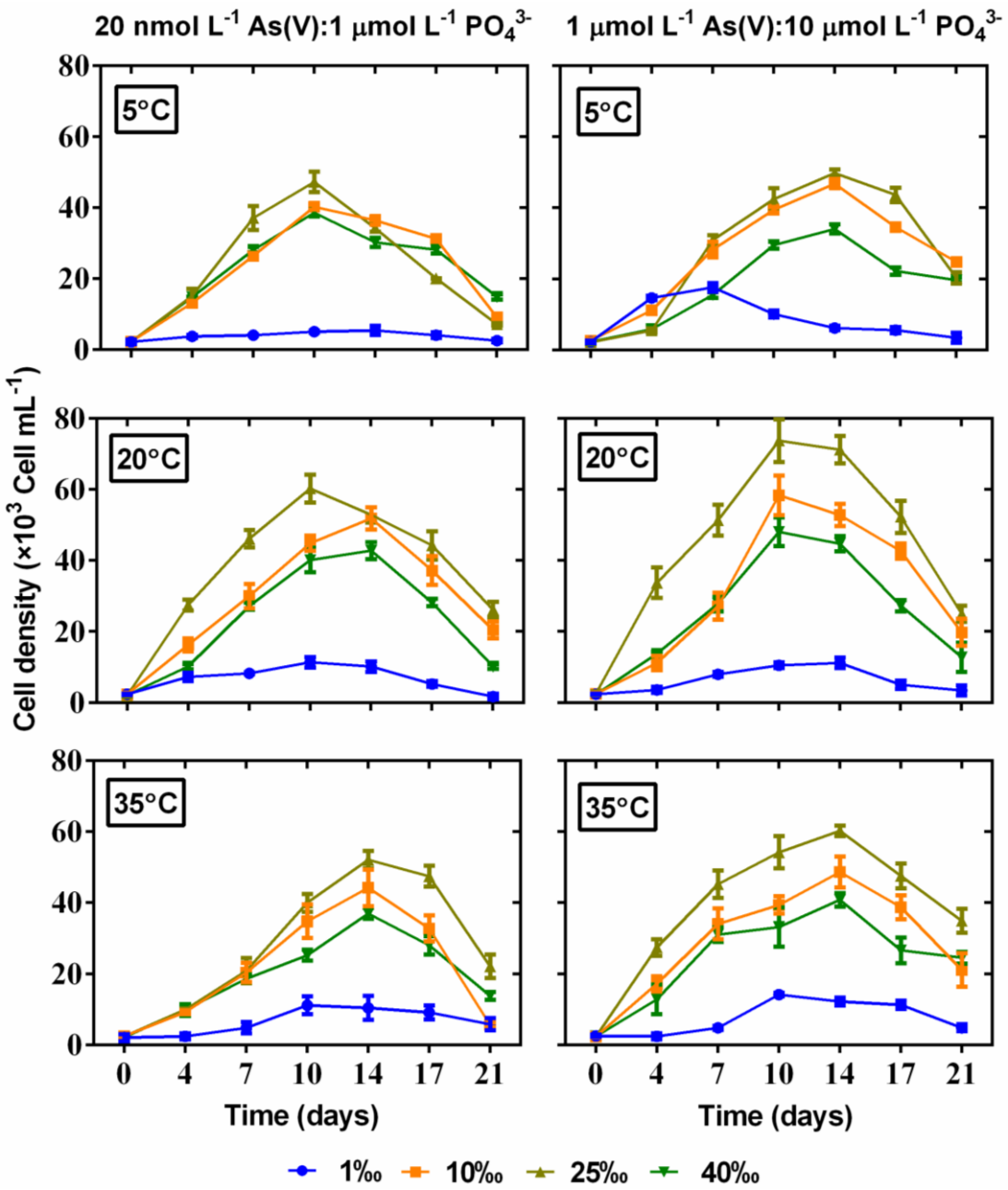

Figure 4.4 Integrated effects of environmental factors on the growth [cell density (cell mL^{-1})] of *Skeletonema* sp. Data are presented as the mean ± SD (n = 3).

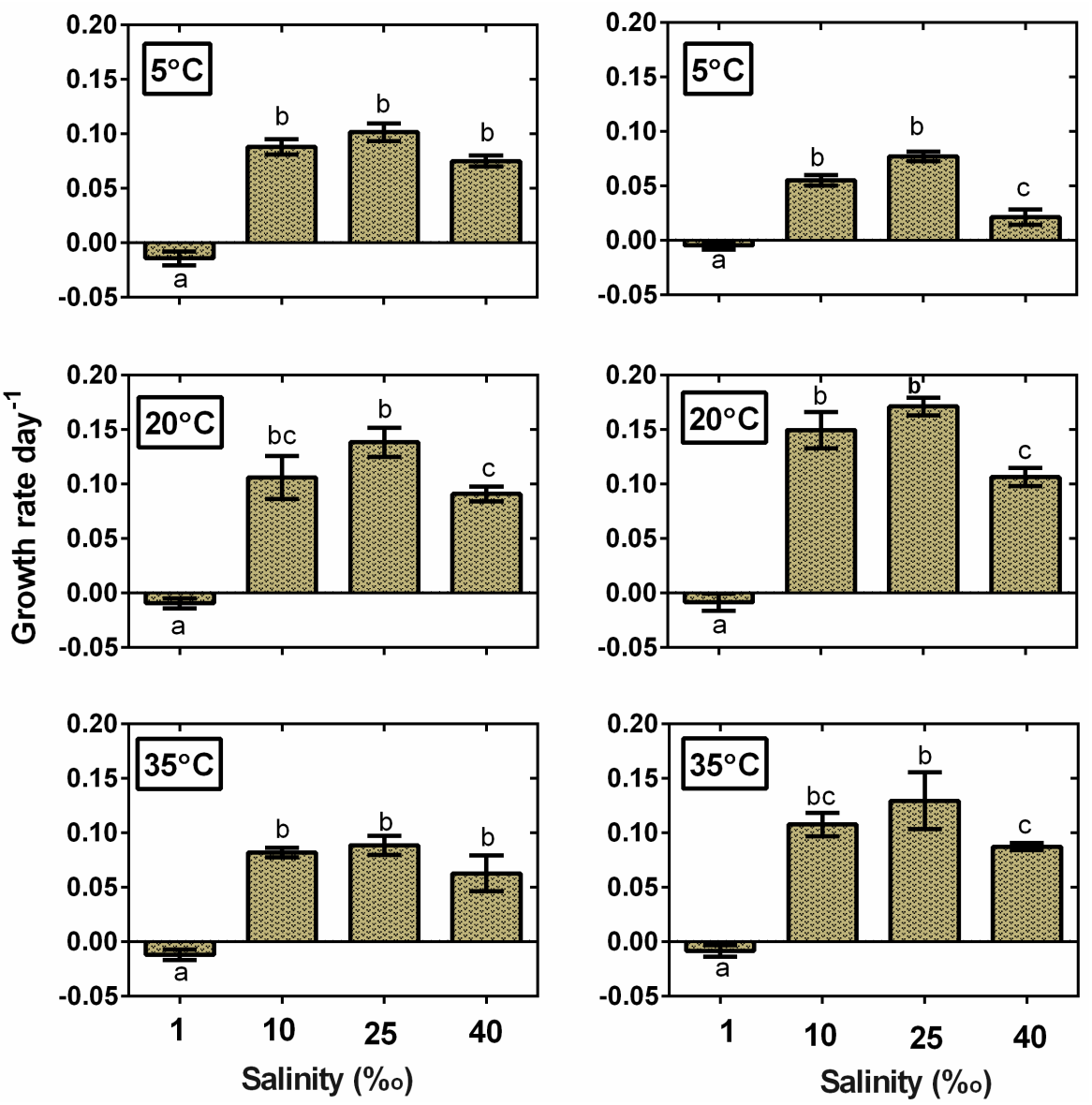

Figure 4.5 Integrated effects of environmental factors on the growth [growth rate d^{-1}] of *Skeletonema* sp. Lowercase letters imply a 95% significance level between salinities under the same temperature. Data are presented as the mean ± SD (n = 3).

4.3.2 As biotransformation by marine microalgae: Integrated effects of temperature, salinity and, As and PO_4^{3-} concentrations

The biotransformation mechanisms were considered in terms of the quantitative analysis of As reduction, such as As(V) to As(III), and/or methylation processes, such as the conversion of As(V) to MMAA and DMAA (**Figures 4.6 & 4.7**). In this experiment, at day 7, liquid cultures of *A. karianus* and *Skeletonema* sp. were assembled for further As species quantification because the species were cultured in As and PO_4^{3-} enriched and deficient concentrations. *A. karianus* showed no reduction of As(V) to As(III) or further methylation to produce methylated species at 1.0‰ and 40‰ at 5.0, 20, and 35°C in both the arsenic-enriched and -deficient treatments. Only As(V) reduced to As(III) at 2.7 ± 0.8 nmol L^{-1} under 25‰ and 3.6 ± 0.6 nmol L^{-1} under 10‰ occurred under 5.0 and 35°C in the As-deficient concentration treatment (**Figure 4.6**). Both As(V) reduction to As(III) at 4.5 ± 0.4 nmol L^{-1} and to a methylation product, such as DMAA at 3.0 ± 0.7 nmol L^{-1} occurred under 10‰, and As(III) at 9.0 ± 1.1 nmol L^{-1} and DMAA at 2.2 ± 0.7 nmol L^{-1} under 25‰ and DMAA at 3.2 ± 0.8 nmol L^{-1} were recorded under 5.0, 20, and 35°C, respectively, under As-deficient conditions. In liquid samples of the As-enriched treatment, As(III) at 0.28 ± 0.05 µmol L^{-1} and DMAA at 0.07 ± 0.01 µmol L^{-1} under 25‰, As(III) at 0.16 ± 0.03 µmol L^{-1} and DMAA at 0.12 ± 0.05 µmol L^{-1} under 10‰, As(III) at 0.10 ± 0.01 µmol L^{-1} and DMAA at 0.23 ± 0.04 µmol L^{-1} under 25‰, As(III) at 0.43 ± 0.06 µmol L^{-1} and DMAA at 0.09 ± 0.02 µmol L^{-1} under 10‰, and As(III) at 0.23 ± 0.04 µmol L^{-1} and DMAA at 0.12 ± 0.04 µmol L^{-1} under 25‰ were determined under 5.0, 20, and 35°C, respectively (**Figure 4.6**).

Skeletonema sp. showed higher As biotransformation ability across a wider range of salinity and temperatures than did *A. karianus*, indicating that different marine microalgal species have different competence with regard to iAs uptake and its biotransformation processes. However, no As reduction or methylation was observed at 1.0‰ under all temperatures in the As-enriched or -deficient treatments (**Figure 4.7**). Methylated As species, such as MMAA, were not detected in *A. karianus*, but occurred in *Skeletonema* sp. MMAA was determined only at 10‰ at 2.94 ± 0.7 nmol L^{-1} under 20°C in the As-deficient treatment. However, when *Skeletonema* sp. Was cultured with a rich arsenic concentration, MMAA at 0.13 ± 0.04 µmol L^{-1} at 10‰ and 0.16 ± 0.04 µmol L^{-1} at 40‰, 0.12 ± 0.02 µmol L^{-1} at 10‰ and 0.20 ± 0.06 µmol L^{-1} at 25‰ were detected under 5 and 20°C, respectively (**Figure 4.7**). A high tendency of As biotransformation to As(III) and methylated species (MMAA and/or DMAA) from As(V) were observed at 10‰ with a value for As(V) of 2.33 ± 0.60 nmol L^{-1},

As(III) of 1.58 ± 0.60 nmol L^{-1}, MMAA of 2.94 ± 0.7 nmol L^{-1}, and DMAA of 9.60 ± 0.90 nmol L^{-1} at 20°C in the As-deficient treatment. In terms of arsenic-rich conditions, similar tendencies were observed at 25‰ under the same temperature with As(V) of 0.10 ± 0.06 µmol L^{-1}, As(III) of 0.06 ± 0.01 µmol L^{-1}, MMAA of 0.29 ± 0.06 µmol L^{-1}, and DMAA of 0.38 ± 0.04 µmol L^{-1}, respectively (**Figure 4.7**).

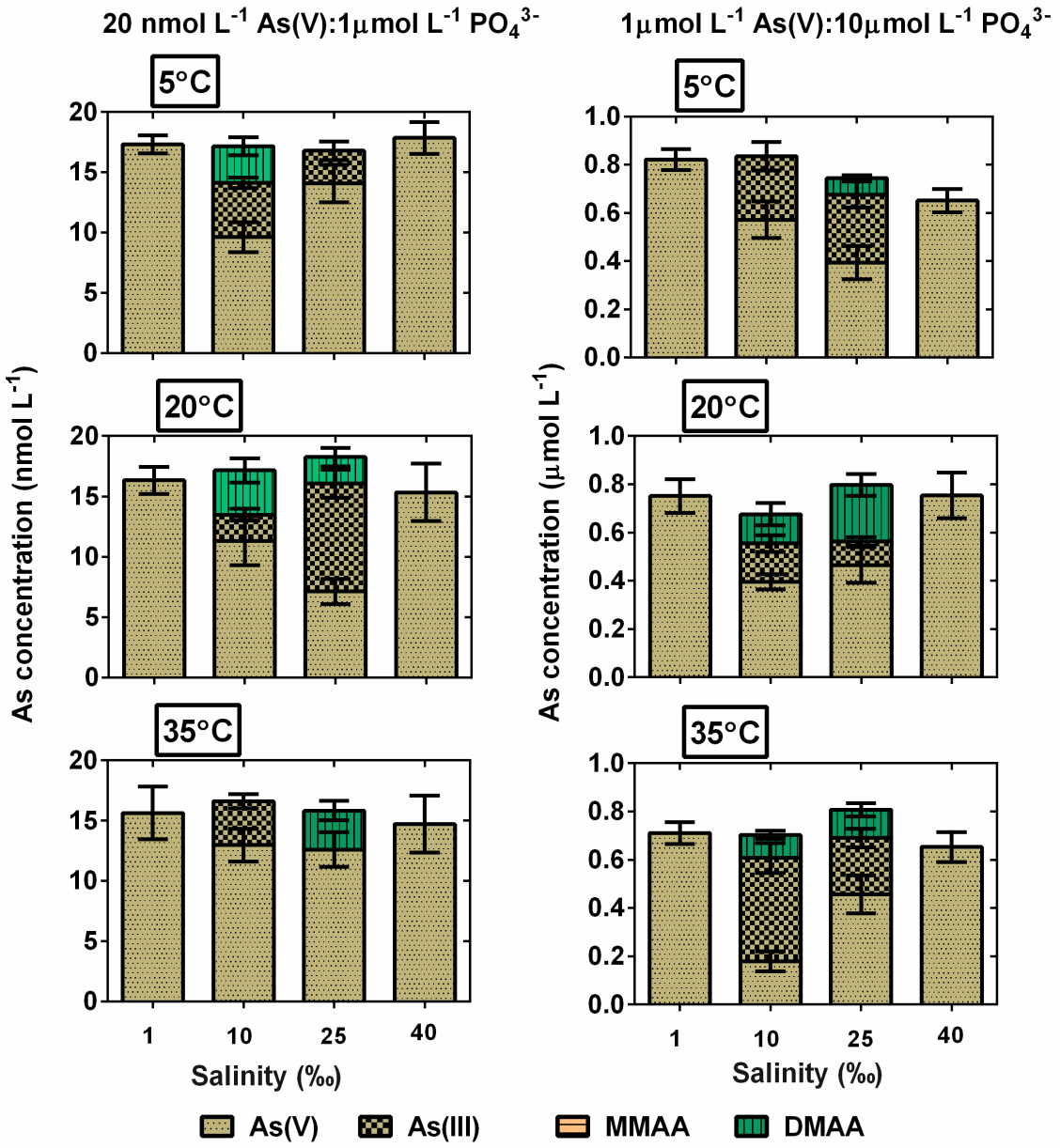

Figure 4.6 Integrated effects of temperature, salinity and, As and PO_4^{3-} concentrations on As biotransformation of *A. karianus*. Data are presented as the mean ± SD (n = 3).

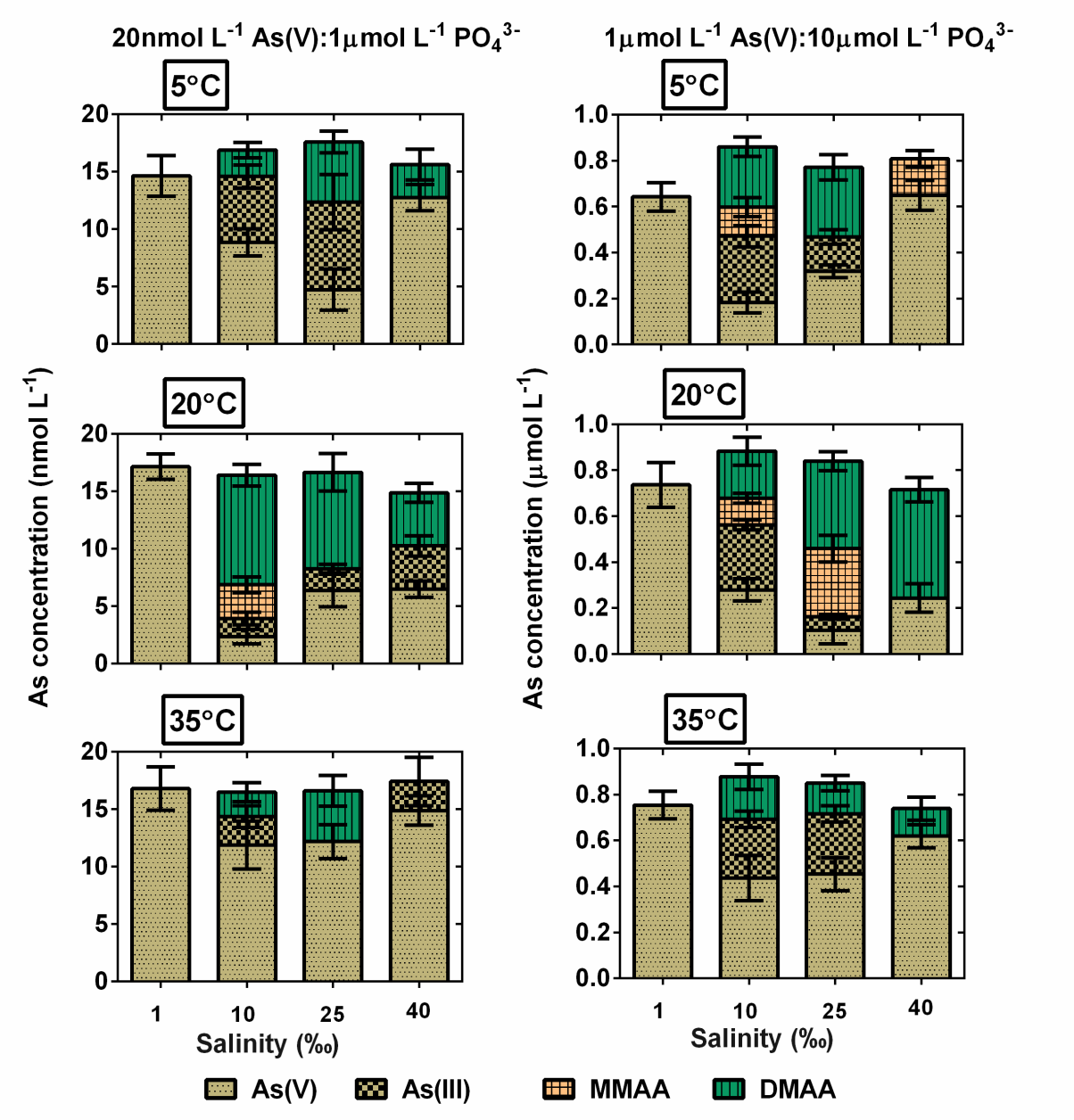

Figure 4.7 Integrated effects of temperature, salinity and, As and PO_4^{3-} concentrations on As biotransformation of *Skeletonema* sp. Data are presented as the mean ± SD (n = 3).

The integrated relationship between environmental factors and As biotransformation has been elucidated in this section. For both microalgae, no biotransformation of As species at low (1‰) salinity at all temperatures and As concentrations suggesting that this species incapable to biotrasform As(V) to As(III) and further methylation at such salinities (Papry et al., 2020, 2019). Whereas, for *A. karianus* and *Skeletonema* sp., the reduction of As(V) to As(III) and further methylation to DMAA(V) was recorded at optimum salinities (10 to 25‰

and 10 to 40‰, respectively) at all temperatures might be due to these species responded differently with various salinities (Cho et al., 2007; Hu and Gao, 2006; Takagi et al., 2006). The conversion of As(V) to other forms suggests that the algal cells took up As(V) and then excreted it in the form of As(III) or MMAA and DMAA (Fujimoto et al., 1994). Later, the uptake of As species was regulated by its excretion out of the cell. These excreted forms of As(V), As(III), or methylated species were regarded as important mechanisms of arsenic detoxification (Raven and Geider, 1988), where marine microalgae play a pioneering role in forming such As species.

4.3.3 Photosynthetic efficiency by marine microalgae: Integrated effects of temperature, salinity, and exposure time

In the present study, integrated with temperature and salinity, minimum (F_o), maximum (F_m), variable fluorescence ($F_V = F_m - F_m$), and maximum quantum photosynthetic efficiency (PE) (F_V/F_m) were investigated for diatom species under laboratory conditions (**Figure 4.8**). Furthermore, F_V/F_m acts as an indicator for the detection of nutritional stress (Gunawan et al., 2018), and functional and physiological variation in phytoplankton, photoadaptation, and photoinhibition mechanisms (Bernardi et al., 2013) related to toxicological studies. These studies are particularly important to the investigation of the effects of various substances on the photosynthetic efficiency (PE) of different kinds of microalgae (Juneja et al., 2013). PE of *A. karianus* and *Skeletonema* sp. were observed during 3 weeks of culture experiment. PE was recorded at a low temperature of 5°C when the cultures occurred at salinities of 1.0‰ and 40‰ for *A. karianus*. At this temperature, a maximum PE value of 0.31 ± 0.05 was determined at 10‰ on the 14th d of culture. The high PE was evaluated at 10‰ and 25‰ at all temperatures in this experiment. The PE values of 0.52 ± 0.03 and 0.49 ± 0.02 were determined at 10‰ and 25‰ under 20°C. *Skeletonema* sp. exhibited a decent PE at a wide range of salinities including 10‰, 25‰, and 40‰, but not 1.0‰. Considering the combined effect of temperature and salinity, this species displayed maximum efficiency at 25‰ of 0.68 ± 0.02 under 20°C followed that at 10‰ of 0.55 ± 0.04 and at 40‰ of 0.35 ± 0.01 (**Figure 4.8**). After investigating the PE value, it could be suggested that *A. karianus* and *Skeletonema* sp. followed the pattern of salinity 10‰ > 25‰ > 40‰ > 1.0‰ and 25‰ > 10‰ > 40‰ > 1.0‰, respectively. For temperature, both species followed a similar pattern of 20°C > 35°C > 5°C.

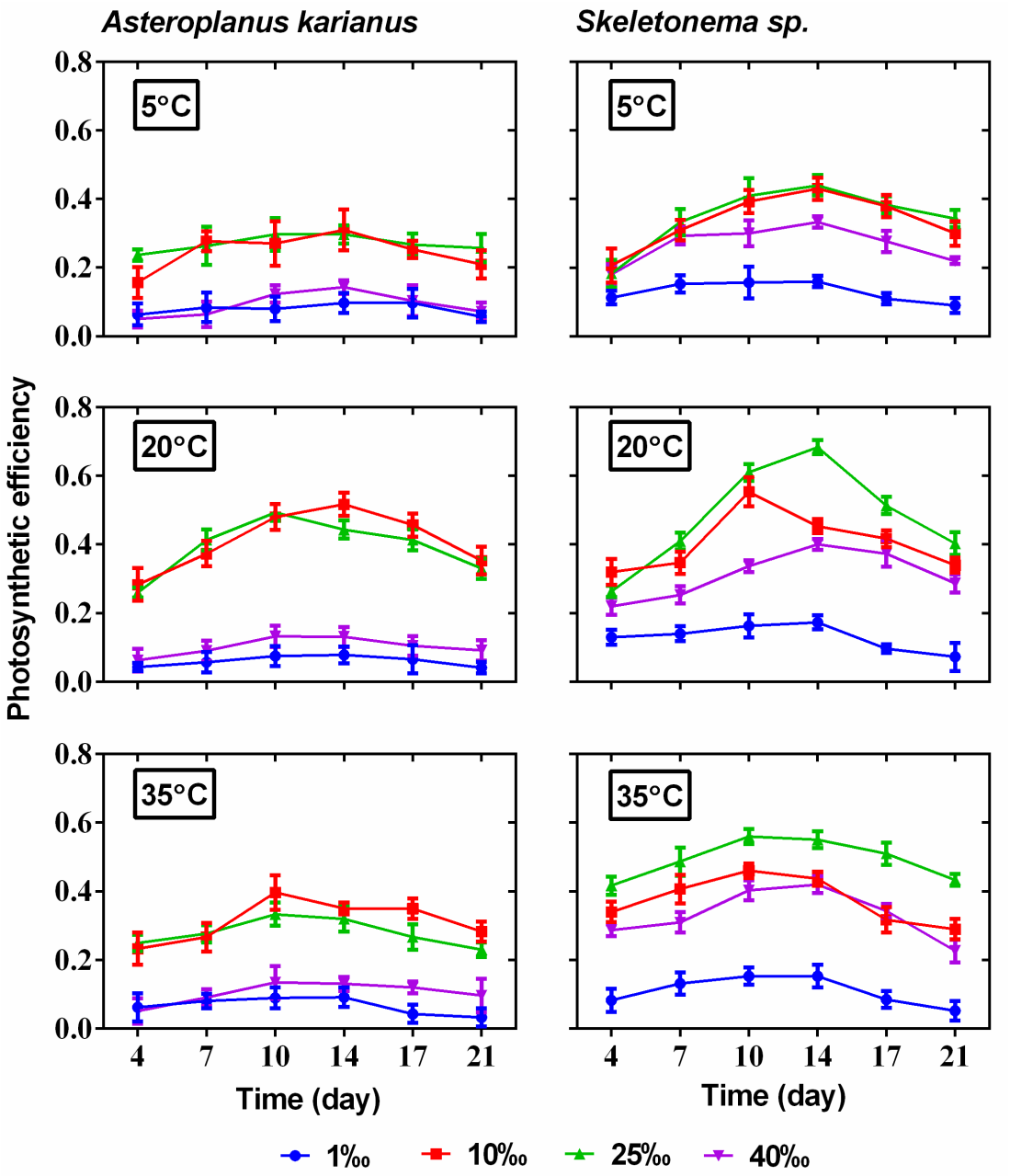

Figure 4.8 Integrated effects of temperature, salinity, and exposure time on photosynthetic efficiency by marine microalgae. Data are presented as the mean ± SD (n = 3).

4.3.4 Relationship between As biotransformation and photosynthetic efficiency: Integrated effect of environmental factors

In this section, experimental data were analyzed to detect whether any significant connection remained between As biotransformation and PE of marine microalgae cultured under certain temperature and salinity levels. Therefore, the 7th and 14th d liquid cultures of diatom species were analyzed with regards to As species of inorganic arsenic and methylated arsenic, and the maximum quantum efficiency of photosynthesis of F_V/F_m (**Figures 4.9 & 4.10**). *A. karianus* and *Skeletonema* sp. exhibited a significant interconnection in terms of As biotransformation and PE in this study. In addition, temperature and salinity also induced biochemical and physiological changes in marine diatom species. In this study, it was found that As biotransformation and photosynthesis were a phenomenon that was stimulated largely by salinity rather than temperature. However, the temperature was also a sensitive factor whose variation caused deterioration of the physiological activity of marine species.

In **Figure 4.9**, when a liquid sample of *A. karianus* was collected from both As-enriched and As-deficient conditions, only iAs was determined from the samples and no methylated species were detected at 1.0‰ and 40‰ under all temperatures. At 10‰ and 25‰, both iAs and methAs were determined, and interestingly, the F_V/F_m value was significantly increased in all samples. When the quantitative measurement of the 7th and 14th d samples was determined, the values for iAs were decreased and methAs were increased, and F_V/F_m also increased with time. For example, in the As-deficient treatment, the concentrations of iAs and methAs, and F_V/F_m were 12.3 ± 0.36 nmol L^{-1}, 5.1 ± 0.8 nmol L^{-1}, and 0.48 ± 0.03, respectively, at 10‰ under 20°C in the 7th d sample. Later, the values changed to 10.3 ± 0.4 nmol L^{-1}, 6.0 ± 0.8 nmol L^{-1}, and 0.54 ± 0.04, respectively, in the 14th d sample. Maximum As biotransformation and photosynthesis were observed at 10‰ under 20°C in the As-rich treatment. Under this condition, iAs, methAs, and F_V/F_m values were 0.42 ± 0.02 µmol L^{-1}, 0.45 ± 0.02 µmol L^{-1}, and 0.53 ± 0.04, respectively, in the 7th d sample and the concentrations were 0.32 ± 0.05 µmol L^{-1}, 0.55 ± 0.08 µmol L^{-1}, and 0.72 ± 0.04, respectively, in the 14th d sample. *Skeletonema* sp. was more efficient at 25‰ regarding arsenic biotransformation, methylation, and photosynthesis activities. This species also showed a similar tendency in the 7th and 14th d samples, regardless of As concentration (**Figure 4.10**). At 1.0‰, no methylation was observed under any temperature or arsenic treatment.

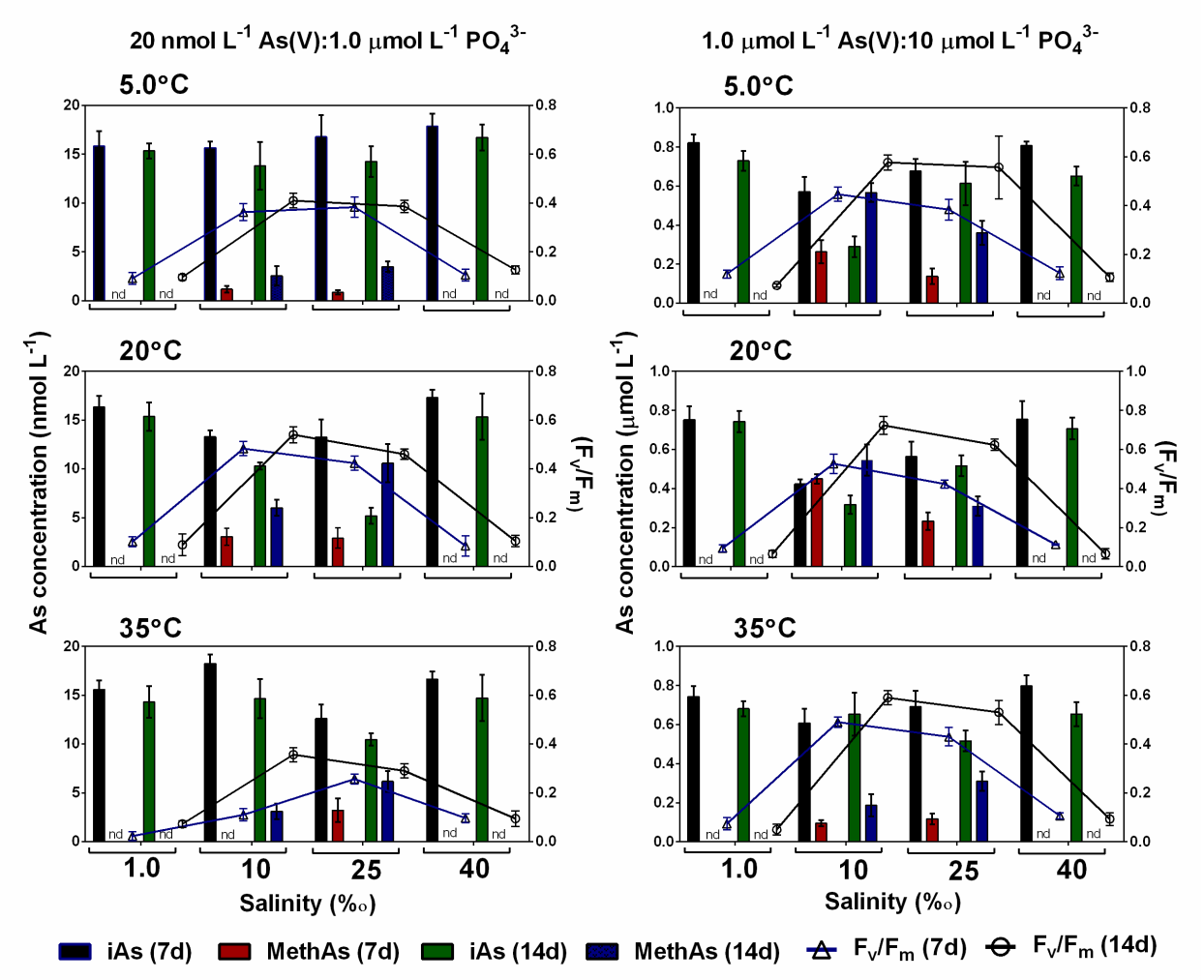

Figure 4.9 Integrated effects of environmental factors on the relationship between As biotransformation and photosynthetic efficiency by marine microalgae *A. karianus*. Data are presented as the mean ± SD (n = 3).

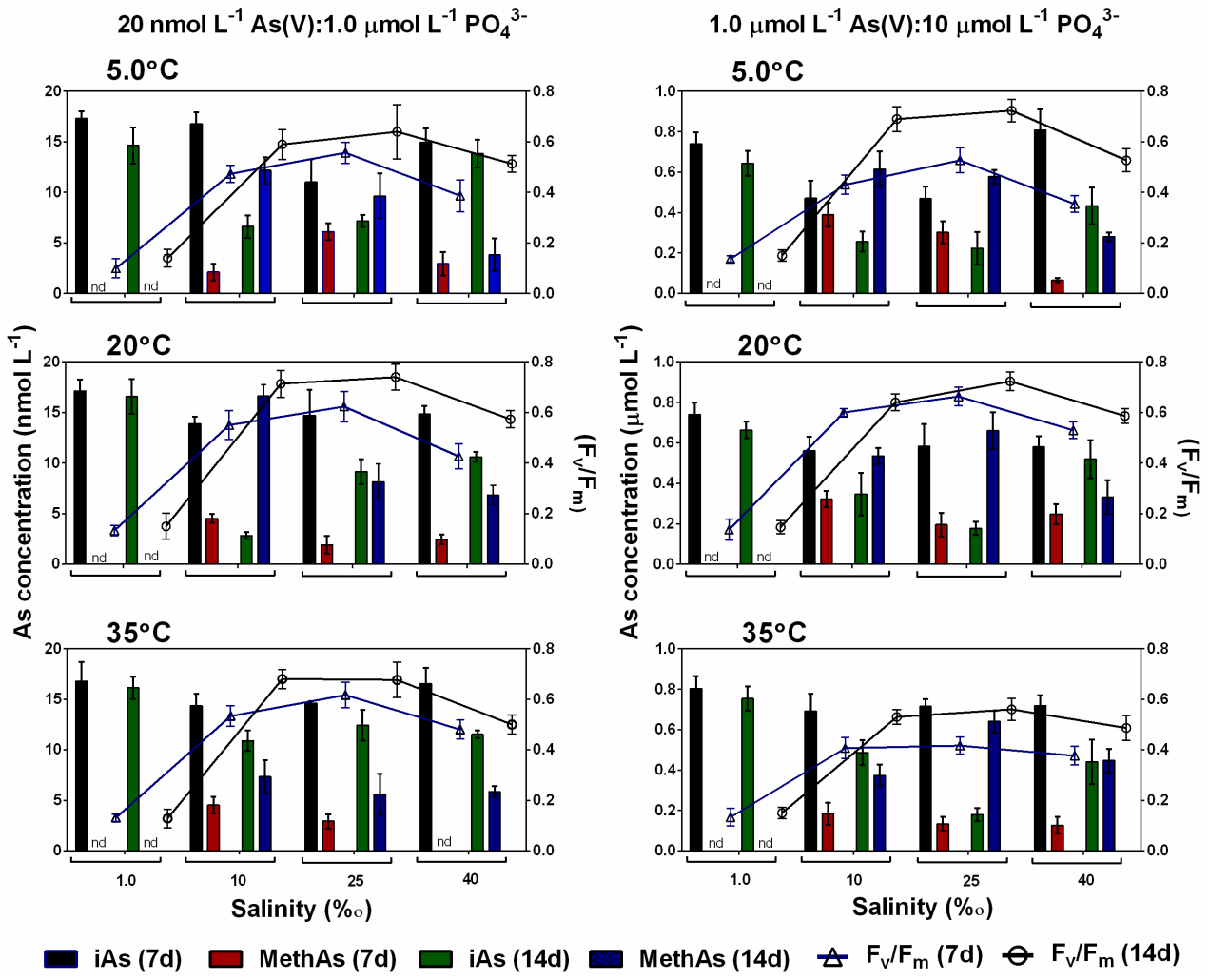

Figure 4.10. Integrated effects of environmental factors on the relationship between As biotransformation and photosynthetic efficiency by marine microalgae *Skeletonema* sp. Data are presented as the mean ± SD (n = 3).

4.3.5 Integrated effects of environmental factors on cell shape

The integrated effect of temperature and salinity on the changes in diatom cell shape was observed using a digital microscope (KEYENCE, VHX-1000, Japan). **Figures 4.11 and 4.12** show the changes in the two-diatom species *A. karianus* and *Skeletonema* sp., respectively. For both species, good shape was observed under salinity of 10‰ or 25‰ at 25 or 35 °C. *A. karianus* showed tremendous variation in their cellular pattern as salinity changed from low to high. When cultured at 10 and 25‰, their cellular shape remained the

same as in the normal environment. However, at 40‰ under all temperatures, the cellular shape changes were significant and formed clump-like structures. *Skeletonema* sp. is generally a rod-shaped diatom species. At 1‰ under 5°C and 20ºC, cell size was observed to be much smaller compared to cells cultured under 10‰ and 25‰ at all temperatures. At 40‰, the rod shape of *Skeletonema* sp. transformed into a U-shaped structure under all temperatures. This result indicated that the diatom cell shape mostly depended on the optimum salinity and temperature conditions in the marine environment. Under high salinity stress, they modified their cell structure to adapt themselves to the altered environment. The morphological anomalies by the marine organism to environmental changes as well as at various salinities denotes a common response to all aquatic diatom species. The diatom cell shape varies with the variations in surrounding environmental factors also reported by several researchers (Aizdaicher and Markina, 2010; Papry et al., 2019).

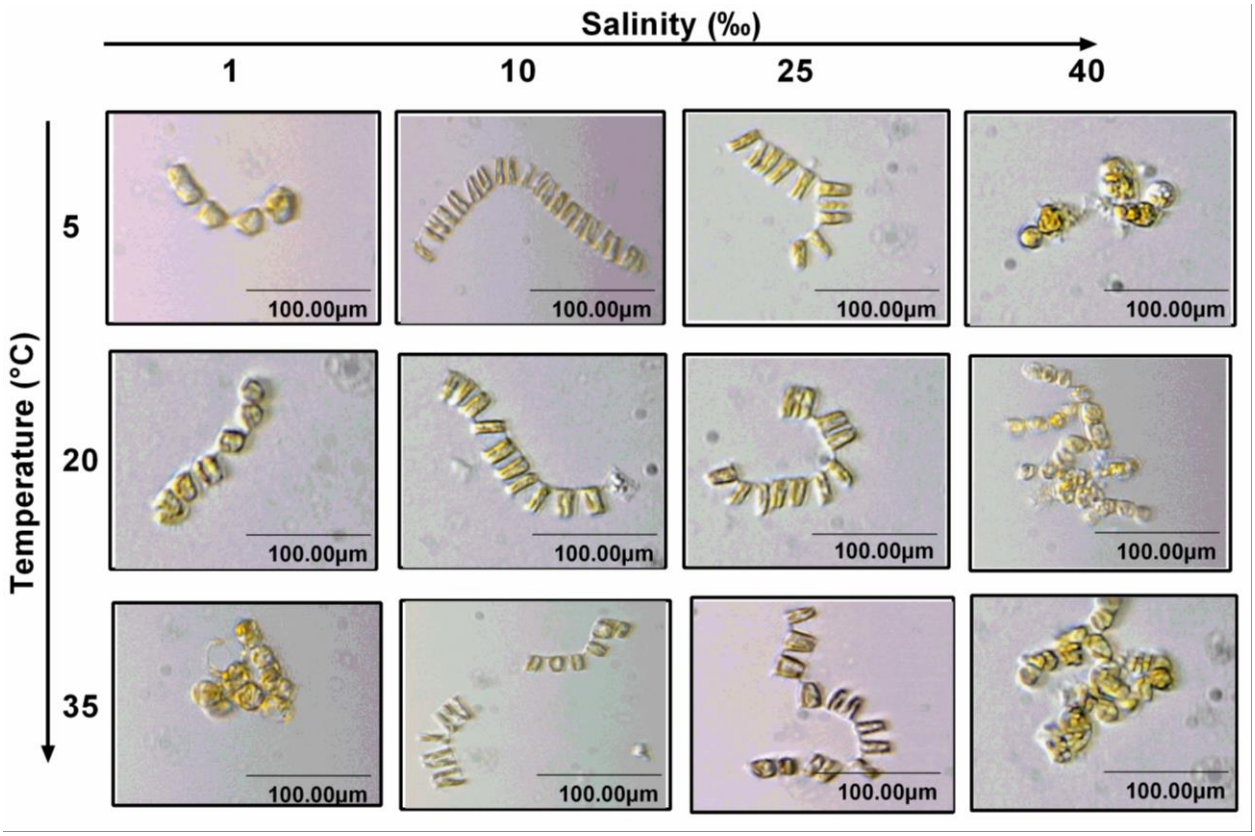

Figure 4.11 Integrated effects of environmental factors on the cell shape of *A. karianus*; the images were taken by a digital microscope (KEYENCE, VHX-1000, Japan).

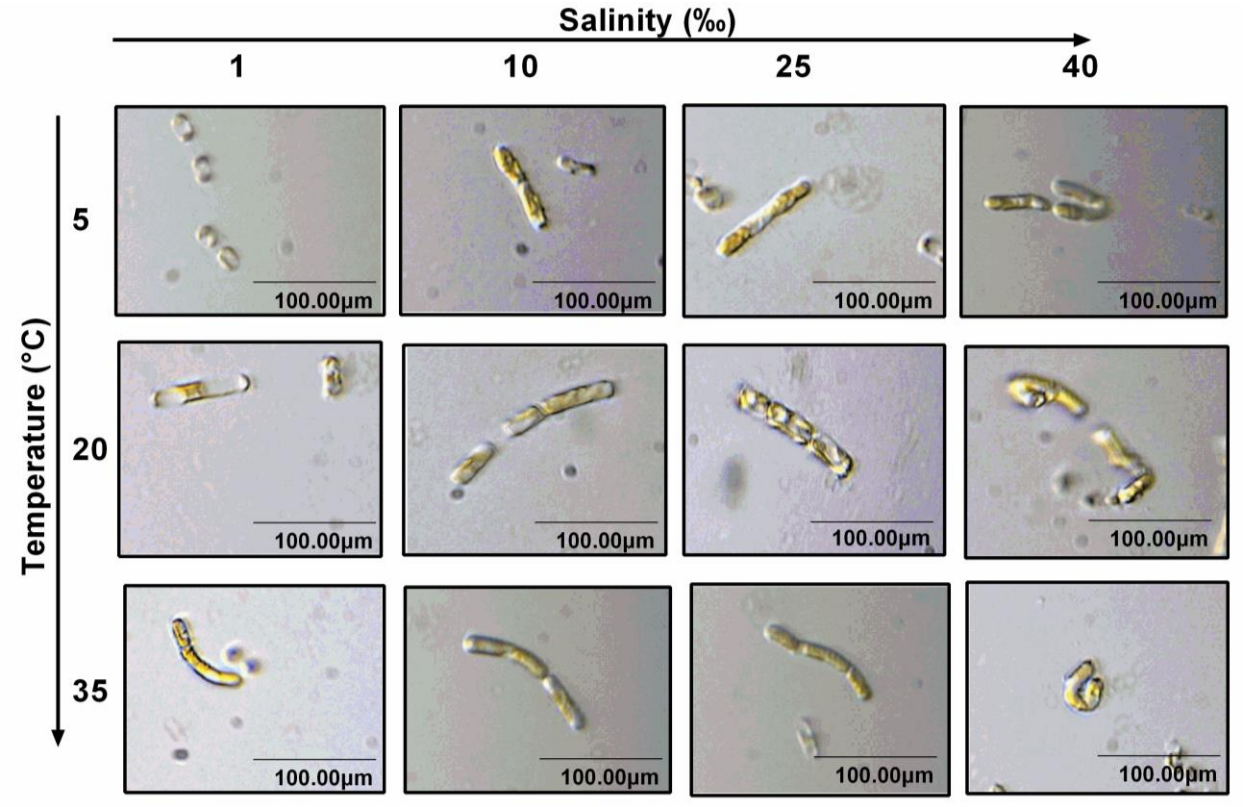

Figure 4.12 Integrated effects of environmental factors on the cell shape of *Skeletonema* sp.; the images were taken by a digital microscope (KEYENCE, VHX-1000, Japan).

4.3.6 A conceptual model of As biotransformation and photosynthetic efficiency

The model focused on As uptake, biotransformation, and excretion by marine diatoms, as well as their PE, which are regarded as essential mechanisms for their survivability in marine aquatic ecosystems (**Figure 4.13**). It also included various states of As species that are found predominantly in emerging water, such as As(V), As(III), MMAA, and DMAA. Conversely, other compounds, such as arsenosugars and complex organoarsenic species, covered a high fraction inside the algal cells (Francesconi and Edmonds, 1996). In the As biotransformation process, the first step involves the uptake of As(V) by diatoms, which is then transformation to As(III) or methylated into a methAs species (e.g., MMAA and/or DMAA). Metabolically stressed diatom species or microalgae are unable to absorb sufficient quantities of As(V) from the surrounding environment (Andreae and Klumpp, 1979). Because of its toxic nature, there is no specific metabolic mechanism for As(V); therefore, an active transport pathway is particularly designated for As(V). It has been suggested that

because of the similarities in chemical characteristics between As(V) and PO_4^{3-}, uptake of As(V) occurs via the phosphate transporter system and biotransformation takes place inside the cellular portion of microalgae (Hasegawa et al., 2001; Yin et al., 2011). In marine microalgae, oxidation and/or reduction reactions are the primary pathways for As biotransformation. The redox reaction acts as a detoxification process to reduce the toxic effect of As (Levy et al., 2005) or to produce momentum for cell evolution (Mateos et al., 2006). As(V) is reduced to As(III) by the ArsC pathway (Gladysheva et al., 1994), whereas As(III) is excreted from the cell through a specific pump, ArsB (Meng et al., 2004). Reduction of As(V) to As(III) facilitated by glutathione (Hughes, 2002) and oxidation of As(III) to As(V) is regarded as an energy production process (Páez-Espino et al., 2009). Reduction of As(V) to As(III) occurs by the interference of glutathione (GSH) and complexation of arsenotriglutathione, which readily releases As(III) to thiol-containing chemical groups (Scott et al., 1993). Furthermore, phytochelatins (PCs) have a metal-binding capacity to thiol groups, and their metalloprotein function encourages detoxification in algal cells (Pawlik-Skowrońska et al., 2004). Further sequestration of As(III) takes place as the major portion of As(V) is converted to As(III) before sequestration into the vacuoles of algal cells (Cullen et al., 1994). Intracellular ATP forms a complex As-ADP, which further takes part in various physiological processes, such as photosynthesis (Ullrich-Eberius et al., 1989). Inside the cell, methylation of As(III) to MMAA and DMAA occurs and then is excreted out of the cell. The quantity of methylated species increased with time in the growth medium of marine species. In the stage of methylation, one important catalyst, namely As-methyltransferase (AS3MT), helps in the biotransformation of iAs to methAs.

In this study, the combined stress of environmental factors on marine microalgae reduce or in some cases significantly lower the physiological processes like growth, As biotransformation and PE. However, at optimum condition, integrated application of various environmental factors remain influential for the microalgal physiological activities. According to Papry et al., (2019), at the optimum temperature (10–25°C) and salinity (15–35‰), marine microalgal growth and As biotransformation were maximum. On the contrary, at a temperature of <5°C and >25°C and salinity of <5‰ and >35‰ the microalgal physiological activities like growth and As biotransformation was minimum and in some cases, there was no biotransformation reported. The conceptual model was based on the findings of the present study where As accumulation and biotransformation by marine diatom species were illustrated. The model will help to understand the As biotransformation by

marine microalgae under integrated stress of temperature, salinity, nutrient concentrations and, culture time.

In the case of photosynthesis, chlorophyll pigments are necessary, which are seriously affected by As-stressed conditions. Several reasons behind this phenomenon may include the substitution of inorganic phosphate by As(V) during competitive uptake required for the biosynthesis of chlorophyll pigments and degradation of photosynthetic precursors (Mishra et al., 2016). Any modification in the biosynthesis process may directly affect the photosynthesis of microalgae. In addition, damage to PSII reaction centers causes a reduction of the potential to produce ATP and NADPH required for carbon fixation reactions. As(V), which replaces phosphate during photophosphorylation, causes the uncoupling of electron transport for ATP synthesis. As(V) binding with RuBisCo and decreases in the RuBisCo-mediated reactions cause obstacles to the Calvin cycle. The photosynthetic rate increases when NADP+ reductase catalyzes the production of NADPH essential to the assimilation of CO_2 and maintenance of energy flow (Dixit et al., 2015).

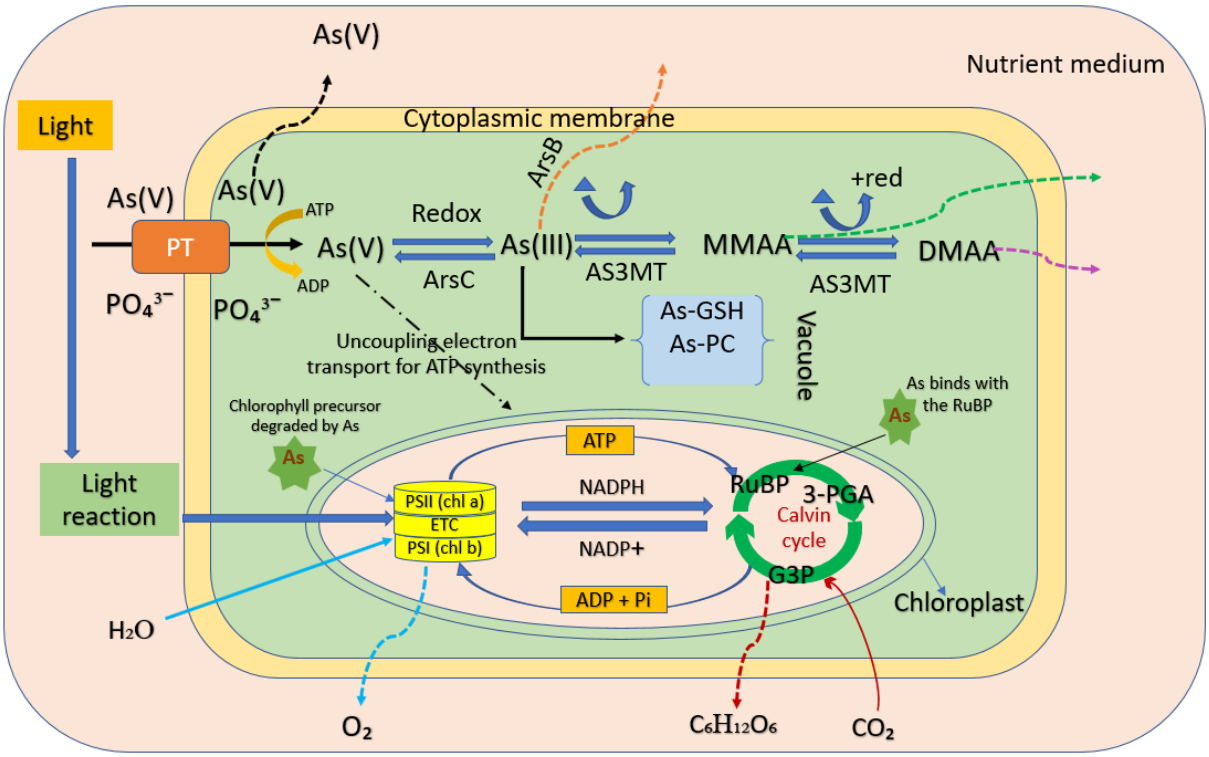

Figure 4.13 Conceptual model of the integrated effect of salinity, temperature, and nutrient concentrations on growth, As accumulation, biotransformation, and photosynthetic efficiency based on the findings of this study.

4.4 Conclusion

This study revealed the integrated effects of environmental factors, including salinity, temperature, As and phosphate concentrations, and culture time on growth, As accumulation, biotransformation, and PE by marine microalgae. The marine diatom species *A. karianus* and *Skeletonema* sp. were used in the experiments. Diatom species were exposed to the combination of temperatures and salinity in association with As and phosphate-enriched conditions. The maximum growth, As accumulation, biotransformation, and PE were recorded at 10 and 14 d of culture for both species. Microalgal growth, As accumulation, biotransformation, and PE were low at 5.0°C with salinities of 1.0‰ and 40‰. A conceptual model is given on the integrated effect of salinity, temperature, and nutrient concentrations on growth, As accumulation, biotransformation, and PE based on the findings of this study.

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Chapter 5:

Freshwater phytoplankton: biotransformation of inorganic arsenic to methylarsenic and organoarsenic

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5.1 Introduction

Arsenic (As), an environmental pollutant, is extremely toxic to living organisms at high concentrations. The occurrence of arsenic in aquatic systems is of great concern due to its high bioavailability, bioaccumulation, and trophic transfer from the bases of aquatic food chains through to higher trophic levels (Rahman et al., 2012). Arsenic in marine biota may not be a significant concern for human health because it is present among them in low concentrations. However, arsenic in freshwater systems is likely to be a significant environmental and human health problem due to the high concentrations that can result from its direct input into these systems from natural and manmade sources (Rahman and Hassler, 2012). Arsenic exists in different chemical forms in aquatic systems. Two major As species in aquatic systems are arsenate (As^{V}), which is the most thermodynamically stable form in oxic waters, and arsenite (As^{III}), which is predominant in reduced-oxygen environments (Hasegawa et al., 2010; Mamun et al., 2019). Through biotransformation processes, microorganisms like phytoplankton and bacteria can cause significant changes in the biogeochemistry of As in aquatic systems (Papry et al., 2019; Price et al., 2012; Rahman and Hasegawa, 2012; Navratilova et al., 2011). Photosynthetic microorganisms (*e.g.*, phytoplankton and cyanobacteria) are able to accumulate As^{V} and biotransform it into As^{III} and methylarsenic (methylAs) species, such as monomethylarsonate (MMAA) and dimethylarsinate (DMAA) (Ye et al., 2012). Although the toxicity of As^{III} is higher than that of As^{V}, As^{III} is predominantly excreted from cells, whereas As^{V} is excreted less. Therefore, a number of researchers have suspected that the reduction of As^{V} to As^{III} represents a detoxification mechanism of phytoplankton (Rahman et al., 2014).

As biotransformation by microorganisms plays a significant role in the occurrence, toxicity, and biogeochemistry of this toxic element in the aquatic environment, several pathways of As biotransformation have been proposed in different microorganisms that are mainly related to oxidation or reduction reactions (Oremland and Stolz, 2003). The microorganisms conduct these redox reactions either to protect themselves from the toxic effects of this metalloid (as a detoxification mechanism) (Levy et al., 2005; Maeda et al., 1992) or to produce energy to promote cellular growth (Mateos et al., 2010; Oremland and Stolz, 2003). Due to the physicochemical similarities between As^{V} (AsO_4^{3-}) and phosphate (PO_4^{3-}), phytoplankton actively take up As^{V} through the PO_4^{3-} uptake system, and then biotransform As^{V} inside their cells (Yin et al., 2011; Hasegawa et al., 2001; Suhendrayatna and Maeda, 2001). The high toxicity of As^{V} results because it binds to PO_4^{3-} receptors that have essential functions inside cells (Hellweger et al., 2003). To reduce its toxicity, phytoplankton

biotransform As^{V} inside their cells in a process that involves the two-electron reduction of As^{V} to As^{III}, which is mediated by glutathione (Hughes, 2002). The biotransformation of As^{V} into As^{III} and its subsequent methylation to form methylAs species in phytoplankton has been reported in many previous studies (Caumette et al., 2011; Miyashita et al., 2011; Llorente-Mirandes et al., 2010; Slejkovec et al., 2006; Hirata and Toshimitsu, 2005). Several studies also showed that the rates of As^{V} reduction and methylation in freshwater environments are generally dependent on the occurrence of phytoplankton blooms, which is related to nutrient enrichment and seasonal variables, such as light and temperature (Rahman and Hasegawa, 2012; Hasegawa et al., 2010; Hasegawa et al., 2009). Sohrin et al. (1997) reported an increase in levels of As^{III} in the water during the spring, which was correlated with the growth phases of two distinct phytoplankton blooms. They also observed that phosphate and As^{V} were also rapidly taken up by phytoplankton cells during these blooms. However, at the stationary bloom phase, when growth is limited by limited nutrient availability, the rates of As uptake and metabolism in phytoplankton were slow, which allowed for the further biotransformation of the pentavalent As^{V} into the trivalent As^{III} and its subsequent methylation to DMAA to occur (Hellweger et al., 2003). Although Sohrin et al. (1997) identified a relationship between the growth phases of phytoplankton blooms and the uptake and metabolism of As^{V}, little is known about the impacts of different phytoplankton growth phases on As biotransformation inside their cells, as well as the excretion of As metabolites out of their cells. The present study was carried out to address this knowledge gap and reveal the role of phytoplankton in As biogeochemistry in aquatic environments. The diversity in the biotransformation of and behavioral responses to As species by phytoplankton at different growth phases were also reported in this study.

5.2 Materials and methods

5.2.1 Reagents

Commercially available products were used as reagents without further purification in this study. Special-grade NaOH (NakaraiTesque, Kyoto, Japan) and HCl (Kanto Chemical, Japan) were used to adjust the pH of reagents and media. Special-grade disodium hydrogen formate heptahydrate (As^{V}) and arsenic trioxide (As^{III}) (Wako, Osaka, Japan), sodium cacodylate ($DMAA^{V}$) (NacaraiTesque, Kyoto, Japan), and arsenobetaine (AB) (Tri Chemical, Yamanashi, Japan) were used for the modification of culture media, and 4-(2-hydroxyethyl)-

1-piperazinyl ethanesulfonic acid (HEPES; NacalaiTesque, Kyoto, Japan) was used as a buffer reagent in culture media. KH_2PO_4 was purchased from Wako (Osaka, Japan) and ethylenediamine-N,N,N′,N′-tetraacetic acid (EDTA; > 98.0%) was obtained from Dojindo, Japan. These reagents were diluted to the desired concentration in ultrapure water. Water was purified using ultrapure water production equipment (arium pro UV, Sartorius StedimBiotech GmbH) with a resistivity of 18.2 MΩ.

5.2.2 Phytoplankton

Six strains of clonal axenic freshwater phytoplankton, namely *Achnanthidium minutissimum*, *Botryococcus braunii*, *Scenedesmus acutus*, *Staurastrum paradoxum*, *Pediastrum duplex*, and *Closterium aciculare*, were used in the present study. The strains of *A. minutissimum, B. braunii, S. actus*, *S. paradoxum*, and *P. duplex* used were obtained from the National Institute for Environmental Studies, Japan. The tested strain of *C. aciculare* (isolated from Lake Biwa) was provided by Dr. Naito of Hiroshima Prefecture University, Japan. The bioconcentration factors of the phytoplankton obtained under the same experimental conditions used in this study are provided in **Table 5.1**.

A. minutissimum is distinguished from other monoraphid diatoms by its small size, linear-lanceolate shape, and radiate striae. Cells are solitary or form very short chains and are often attached to the substrate by a stalk. This species' distribution is biased towards alkaline waters, but it also appears in acidic waters (Watanabe, et al., 2005). It is widely adaptable to organic pollution and dominates in rivers polluted by heavy metals (Watanabe, et al., 2005).

Table 5.1 Bioconcentration factor (BCF[a]) of freshwater phytoplankton used in this study.

Arsenic treatments (nmol L^{-1})	Culture period (days)	Chlorophyceae			Charophyceae		Bacilariophyceae
		B. braunii	*S. acutus*	*P. duplex*	*C. aciculare*	*S. paradoxum*	*A, minutissimum*
		Bioconcentration factors					
20	7	86.0	13.8	14.5	9.9	84.6	4.4
20	21	28.3	6.9	12.0	9.8	37.0	3.8
1000	7	12.8	1.5	1.0	0.3	8.9	0.3
1000	21	0.8	0.1	0.3	0.1	3.6	0.1

[a] $BCF = \frac{C_B}{C_W}$, where, C_B is the intracellular arsenic concentration per 1.0 g of cell dry weight and C_W is the arsenic concentration in culture media.

B. braunii is a pyramid-shaped planktonic green microalga that belongs to the family Botryococcaceae. This microalga inhabits freshwater, with cells with a diameter of 10-20 µm that form aggregated colonies. This species is able to produce hydrocarbons (particularly triterpenes), which comprise around 30–40% of its dry weight (Metzger and Largeau, 2004). This organism synthesizes oil in its cells that is secreted extracellularly. The oil produced by *B. braunii* is expected to someday be used as an alternative fuel to gasoline.

S. actus is a green microalga belonging to the family Scenedesmaceae that has lanceolate cells. It always forms colonies, with colonies of 4 (or 2, 8, or 16) cells often connected in a line. It is widely distributed in freshwater environments, such as paddy fields, ponds, and swamps, as well as in soil. Its cells adhere to one another via the cell wall, and their positions do not change. In addition, the whole body may be wrapped in agar with extracellular polysaccharide secretions. The cells constituting the colonies have no flagella and are not motile.

S. paradoxum is a green microalga in the family Desmidiaceae with solitary floating cells. There is a constriction at the center of the cell, which divides each cell into two half-cells (desmids). The shape of the cell as seen from above (top-view) is a regular polygon. Four long arms extend from the central point. It is extremely common in various freshwater areas, such as lakes, ponds, paddy fields, rivers, and so on.

P. duplex is a species of freshwater green microalgae in the family Hydrodictyaceae. It forms colonies with specific numbers of cells (8 to 32 cells). The cell bodies are polygonal, granulated, and have horn-like projections. One cell has two protrusions, and there is a wide gap between the cells. The colonies are as large as single-celled algae, with a diameter that reaches tens to hundreds of microns, and the colonies have limited motility. This microalga is widely distributed in freshwater environments, such as paddy fields, ponds, and swamps. Most of these microalgae are free-floating, but there are also benthic forms.

C. aciculare is a crescent-shaped, unicellular, freshwater microalga. It can be found in almost all freshwater environments, from still-water ponds to running waters. There are 2 (rarely 4) chloroplasts in each cell, and they are divided in the center.

5.2.3 Pre-culture and maintenance

CSi medium (**see Appendix 5.1**) was used for the maintenance of the six studied freshwater phytoplankton strains. For the growth experiment, the phosphate concentrations of the CSi medium were adjusted to 1.0 µmol L^{-1} or 50 µmol L^{-1}, and then media were modified

by supplementation with arsenic solutions. The culture medium and apparatus (tips, bottles, vessels, and micropipettes) were sterilized separately at 121ºC for 30 min using an autoclave (MLS 3780, Sanyo Electric, Japan). They were then placed in a clean bench (NK Clean Bench, VSF-1300A, Nippon Medical Equipment Co., Japan) under UV irradiation for 20 min. Before using the phytoplankton in the trials, the cultures were maintained in identical media for 1-2 weeks in polycarbonate bottles (Nalge, USA) until they reached at least the exponential growth phase in a temperature- and light-controlled incubator (Koitotron3HN-35MLA, Koito Industries, Ltd. Japan). Experimental cultures were grown at 25°C under a 12:12 h light/dark photoperiod, at a light intensity of 50 µE m^{-2} s^{-1} provided by cool white fluorescent lights. The axenic nature of the phytoplankton cultures was verified by performing the 4′,6-diamidino-2-phenylindole (DAPI) test and by the examination of cells under an epifluorescence microscope (Bohlool and Schmidt, 1980).

5.2.4 Growth experiments with various As concentrations

Phytoplankton cells acclimated to culture media at the exponential growth phase were incubated in 60-mL capacity polycarbonate vessels containing 60 mL of sterilized CSi culture medium (**see Appendix 5.1**). The culture medium was then modified by changing the concentration of phosphate (added as KH_2PO_4) to encourage the uptake of arsenic species. Two different arsenic concentration treatments, high- arsenic ($[As]_0$ = 1.0 µmol L^{-1}) and low-arsenic ($[As]_0$ = 20 nmol L^{-1}), were used in the experiment, in which As was provided in the form of NaH_2AsO_4. Initially, the cell density in the culture medium was less than 4.6×10^3 cells mL^{-1}. After incubating the phytoplankton for three days, four species of arsenic (As^V, As^{III}, $DMAA^V$, and AB) were added to the culture medium. The cultures were grown for 30 days. Phytoplankton growth was measured spectrophotometrically using a UV-VIS (ultraviolet-visible) spectrophotometer at 540 nm. The cell number was estimated with an established cell density-to-absorbance ratio. The number of cells was counted directly under a microscope. The growth rate (%) was defined by the following equation:

$$\text{Growth rate } [\%] = (\text{OD}_{Sample}/\text{OD}_{\text{Control}}) \times 100$$

where OD_{Sample} is the optical density of phytoplankton at 540 nm in arsenic-containing media and $OD_{Control}$ is the optical density of phytoplankton in arsenic-free media after seven days of cultivation.

5.2.5 Sample processing

On a pre-specified day, samples were collected and filtered through 0.45-µm membrane filters under low vacuum pressure (< 30 mm Hg). The filtrate sample was then stored in a cool and dark place after adding 0.50 mL of 1.0 mol L^{-1} HCl to them. After that, 20 mL of 3.0 mol L^{-1} HCl was added to the sample, and the mixture was heated on a hot plate at 100°C for 3 h to extract the arsenic both inside and outside of the freshwater phytoplankton cells. Thereafter, the mixture volume was adjusted to 40 mL with ultrapure water, and it was also adjusted to the same concentration as that of 1.3 mol L^{-1} HCl (pH = 0).

5.2.6 Analysis of arsenic speciation in microalgal samples

A hydride generation technique was used for the determination of arsenic species in culture media according to Hasegawa et al. (1994). The technique was a combination of a flame atomic absorption spectrophotometer (AAS, 170-50A, Hitachi, Japan) and hydride generation device followed by cold trapping (Papry et al., 2019; Mamun et al., 2019). Concentrations of inorganic As (As^{V} + As^{III}), MMAA, and DMAA were analyzed by adding 5.0 mL of 0.20 mol L^{-1} EDTA·4Na and 5.0 mol L^{-1} HCl to 40 mL of the sample solution[3]. For As^{III}, 5.0 mL of 0.20 mol L^{-1} EDTA·4Na and 0.5 mol L^{-1} KH_2PO_4 was added to 40 mL of the sample solution. The arsenic species detected were recorded in the form of a chromatogram using a data processing device (Chromato-PRO, Runtime Instruments, Tokyo, Japan), and their concentrations were determined based on the heights of the peaks obtained. A representative chromatogram of the occurrence and separation of peaks for each of the arsenic species is given in **Figure 5.1**. The limit of detections (LODs) for the iAs (As^{V} + As^{III}), MMAA, and DMAA were 0.3, 0.8 and 0.7 nmol L^{-1}, respectively. The precisions as relative standard deviation (RSD, n=5) for 20 nmol L^{-1} of iAs (As^{V} + As^{III}), MMAA, and DMAA were 2.2, 1.2 and 1.4%, respectively. The accuracy of the analysis was checked using the certified standard reference material 1573a (tomato leaf from NIST, USA) (Rahman et al., 2009). The recovery of As concentration was 95.0% of the certified value.

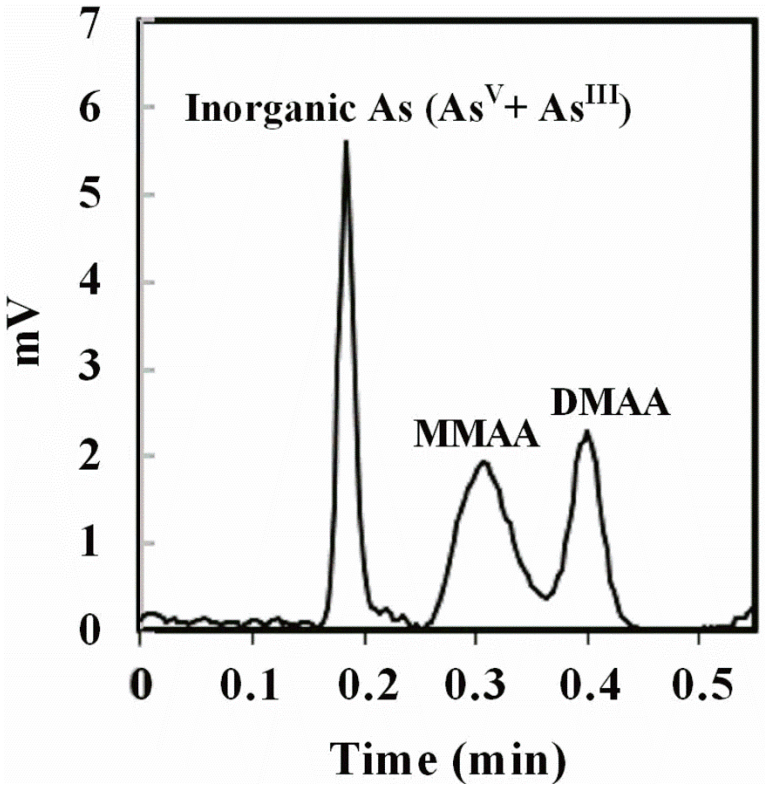

Figure 5.1 Chromatogram showing occurrence and separation of peaks representing each arsenic species.

5.2.7 Determination of the total arsenic concentrations in cell samples

The total concentrations of arsenic in the freshwater microalgae were determined by inductively coupled plasma mass spectrometry (ICP-MS, SPQ 9000, SEIKO, Japan). Filter paper containing microalgal cells was digested by a microwave digestion system (Multiwave 3000, Anton Paar) using the Sea 1-h method. After that, digested liquors were co-washed with 15 mL of ultrapure water and transferred to heat-resistant DigiTUBEs (DigiPREP Jr, SCP SCIENCE). Tubes were then heated on hot plates at 100 ºC for 6-7 h. After evaporation, 2 mL of deionized water was added to the samples, and they were then subjected to ICP-MS for the quantification of the total arsenic content in their cells. The operational conditions of the ICP-MS were the following: a high-frequency output of 1.2 kW; plasma gas flow rate of 16 L min^{-1}; auxiliary gas flow rate of 1.0 L min^{-1}; nebulizer gas flow rate of 1.0 L min^{-1}; and sample replacement time of 10 s.

The mean values in different treatments were compared using Duncan's multiple range test using the statistical program SPSS 22.0 for Windows (SPSS Inc., USA).

5.3 Results and discussion

5.3.1 Growth inhibition effects of arsenic species on freshwater phytoplankton

Inorganic As species had significant growth inhibition effects on freshwater phytoplankton (**Figure 5.2**). In general, the growth of phytoplankton was inhibited substantially by high As^{V} concentrations ($\geq 10^{3}$ nmol L^{-1}), except for *S. paradoxum*. The growth rates of *C. aciculare*, *S. actus*, and *P. duplex* were decreased by over 80% in the 10^{4} nmol L^{-1} arsenate treatment (**Figure 5.2a, c, e**). In contrast, the growth inhibition effects of As^{V} on *B. braunii*, *S. paradoxum*, and *A. minutissimum* at a high concentration (10^{5} nmol L^{-1}) were less than its effects on *C. aciculare*, *S. actus*, and *P. duplex* (**Figure 5.2b, d, f**). The growth inhibition effect of As^{III} on freshwater phytoplankton was lower than that of As^{V} (**Figure 5.2**). The toxicity of As^{III} reduced the growth rates of *C. aciculare*, *S. paradoxum*, and *A. minutissimum* by between 66 and 80% at an As^{III} concentration of 10^{5} nmol L^{-1} (**Figure 5.2a, d, f**). The freshwater phytoplankton *S. actus* and *P. duplex* were quite resistant to As^{III} toxicity, even at high concentrations (**Figure 5.2c, e**). These results indicate that As^{V} and As^{III} have different levels of toxicity to different types of freshwater phytoplankton.

Methyl- and organoarsenicals, such as $DMAA^{V}$ and arsenobetaine (AB), respectively, had little growth inhibition effects on the tested freshwater phytoplankton (**Figure 5.2**). These results suggest that the relative toxicity of different As species to freshwater phytoplankton decreased in the following order: $As^{V} \geq As^{III} \geq DMAA^{V} \geq AB$. This also suggests that the growth inhibition of phytoplankton by As species depends on the phytoplankton strain, which is consistent with the results of other studies (Levy et al., 2005; Rahman et al., 2014; Wang et al., 2013; Sharma and Sohn, 2009). Some strains of freshwater phytoplankton are more resistant to As^{V} and As^{III} toxicity, while others are more susceptible. This might be due to them having differential abilities to detoxify and/or biotransform As^{V} and As^{III} into less toxic organoarsenic compounds (Rahman and Hassler, 2014; Rahman et al., 2014).

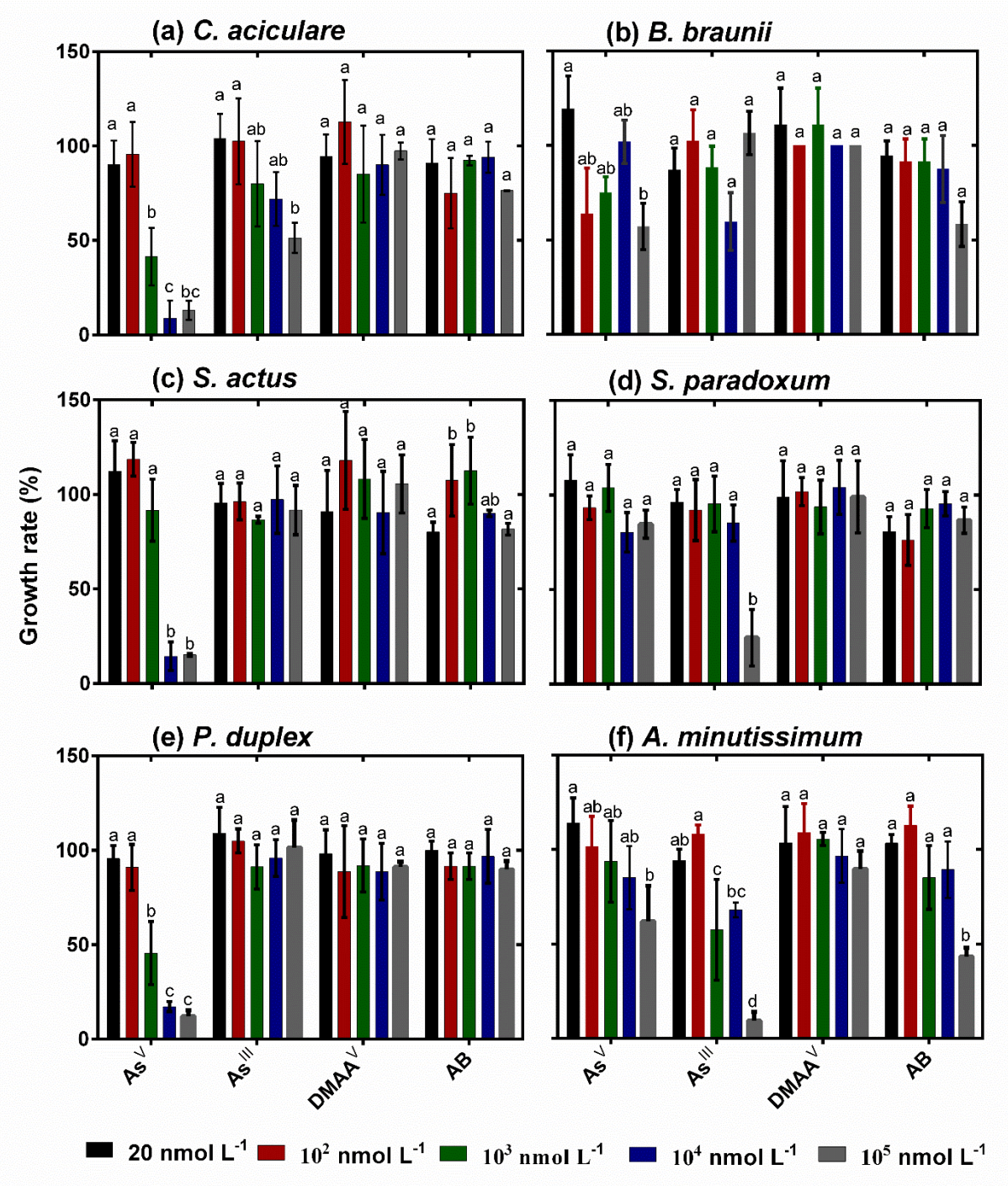

Figure 5.2 Growth of freshwater phytoplankton under various $[As]_0$ treatments. The different lowercase letters indicate significant differences between arsenic treatments ($p < 0.05$). The data presented are mean ± SD growth rates (n = 3).

Phytoplankton reduce As^{V} to As^{III}, which is then followed by the oxidative methylation of this As species to form intermediate trivalent methylAs species ($MMAA^{III}$ and $DMAA^{III}$) and pentavalent methylAs species ($MMAA^{V}$ and $DMAA^{V}$) in them (Hughes, 2002). A study by Hasegawa et al. (2001) reported that the freshwater phytoplankton *C. aciculare* converted approximately 80% of As^{V} into the less toxic $DMAA^{V}$, with trace concentrations of trivalent methylAs species ($MMAA^{III}$ and $DMAA^{III}$) also formed. The biotransformation of pentavalent As^{V} by freshwater phytoplankton has been discussed in detail elsewhere (Suhendrayatna and Maeda, 2001).

Based on the toxic effects (*i.e.* growth inhibition) of iAs species on them at high concentrations ($\geq 10^{4}$ nmol L^{-1}), the freshwater phytoplankton tested herein can be grouped into the following three distinct groups: (i) *B. braunii*, which is highly resistant to iAs and organoarsenic (orgAs) species; (ii) *S. actus* and *P. duplex*, which are highly resistant to As^{III} and orgAs species, as well as *S. paradoxum*, which is highly resistant to As^{V} and orgAs species; and (iii) *A. minutissium and C. aciculare*, which are highly susceptible to iAs species (As^{V} and As^{III}).

5.3.2 Biotransformation of iAs species at different growth phases

5.3.2.1 Biotransformation of As^{V} into As^{III}

The changes in As speciation in the phytoplankton culture media at the logarithmic and stationary growth phases at high and low As concentrations are shown in **Figure 5.3**. These results reflect the bioaccumulation and biotransformation of As species by the phytoplankton inside their cells and their subsequent excretion into the environment. In general, in the low-$[As]_0$ treatment (20 nmol L^{-1}), As^{V} was the predominant species in the growth medium, followed by As^{III}, at both the logarithmic and stationary growth phases (**Figure 5.3a, c**). However, in the high-$[As]_0$ treatment (1.0 µmol L^{-1}), As^{III} was the predominant species in the medium at the logarithmic growth phase (**Figure 5.3b**), while As^{V} was predominant at the stationary growth phase of all microalgae except *S. actus* (**Figure 5.3d**).

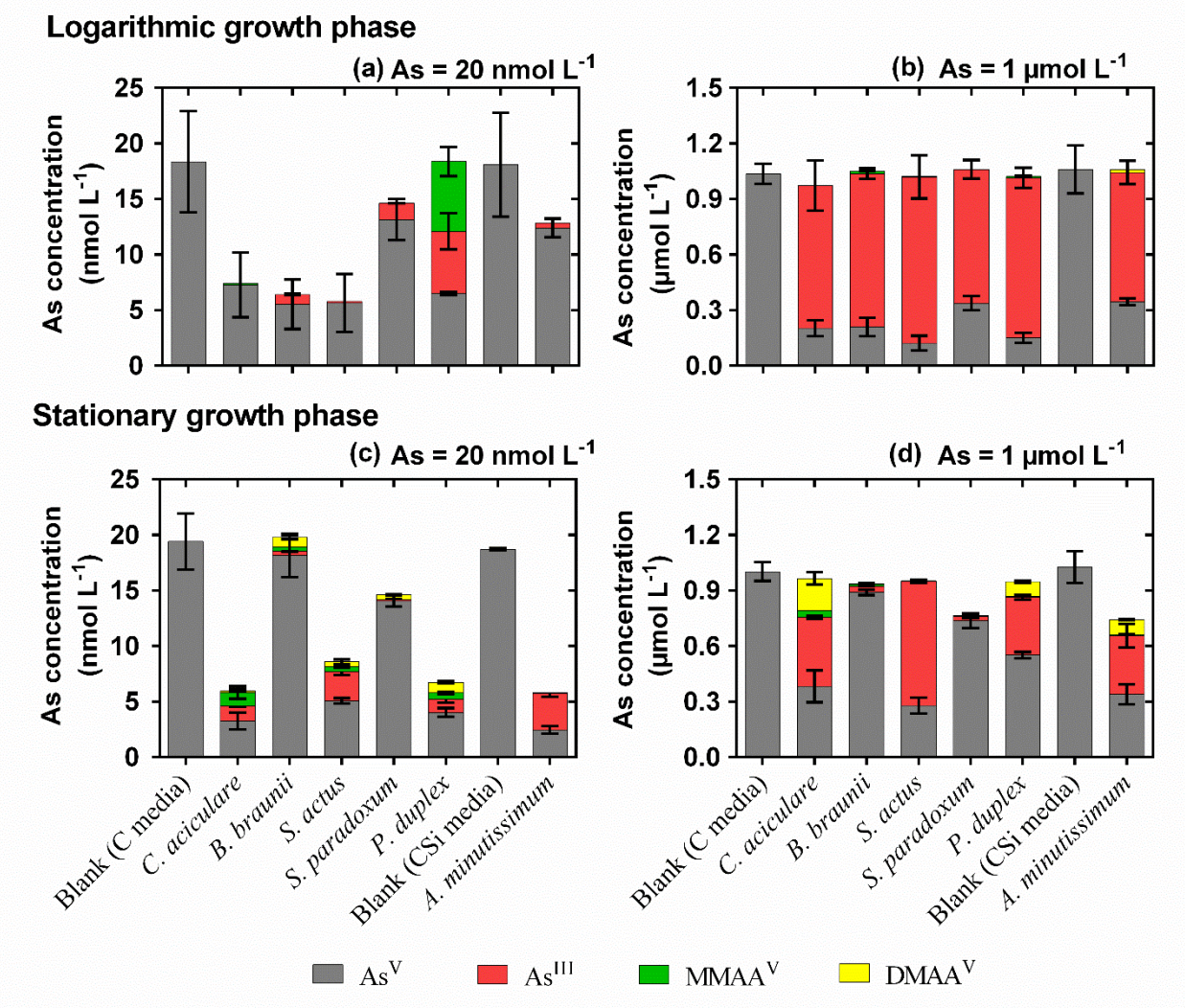

Figure 5.3 Arsenic speciation changes in the culture medium of freshwater phytoplankton at the logarithmic (a, b) and stationary (c, d) growth phases. Initially, the phytoplankton were grown in CSi culture medium with 20 nmol L^{-1} (a, c) and 1 µmol L^{-1} (b, d) of As^{V}. Mean ± SD As concentrations are shown (n = 3).

Phytoplankton plays significant roles in the biotransformation and biogeochemistry of As in aquatic systems, as illustrated in **Figure 5.4**. Because of the physicochemical similarities between arsenate and phosphate, phytoplankton actively uptake As^{V} through the PO_4^{3-} uptake system (Rahman and Hasslar, 2010; Hasegawa et al., 2001; Suhendrayatna and Maeda, 2001). In the present study, phytoplankton were exposed to $NaH_2AsO_4 \cdot 7H_2O$, and the concentration ratio of arsenate to phosphate in the growth medium was kept high to encourage arsenate uptake by phytoplankton. Arsenate has toxic effect on phytoplankton (as discussed in section 5.3.1.)

due to the binding of AsO_4^{3-} to places inside the cells where PO_4^{3-} binding is essential (Hellweger et al., 2003). The phytoplankton biotransform As^V into As^{III} inside their cells (Rahhman et al., 2014; Wang et al., 2013; Baker and Wallschläger, 2016), possibly to reduce the toxicity of As^V to them (Rahman and Hassler, 2014). The biotransformation process involves the two-electron reduction of the pentavalent As^V to the trivalent As^{III}, which is mediated by glutathione (Hughes, 2002).

The biotransformation of As^V into As^{III} and its subsequent methylation to form methylarsenic compounds (methylAs; *e.g.*, DMAA and MMAA) and more complex organoarsenic compounds (orgAs; *e.g.*, AB) by phytoplankton are correlated with the growth rate of the phytoplankton, and to their phosphorus nutrient status (Hellweger et al., 2003). The phytoplankton take up higher amounts of As^V than PO_4^{3-}, reduce As^V to As^{III}, and then excrete As^{III} out of their cells as their growth rates decline and under phosphate-depleted conditions. As^{III} is further biotransformed into methylAs and more complex orgAs species, which are then excreted out of the cells. The present study further showed that the biotransformation of As^V into As^{III} by freshwater phytoplankton was slow at the logarithmic growth phase in the low-$[As]_0$ treatment. However, most of the As^{III} was excreted out of the cells of the phytoplankton at this growth phase in the high-$[As]_0$ treatment.

In the high-$[As]_0$ treatment, the dominant species of As in the growth medium at the logarithmic phase was As^{III} (**Figure 5.3b**), whereas in the low-$[As]_0$ treatment As^{III} was the elast prevalent in the medium at this stage (**Figure 5.3a**). However, the relative abundances of As^V and As^{III} in the phytoplankton cells were similar in the high- and low-$[As]_0$ treatments (**Figure 5.5a, b**). These results demonstrated that the biotransformation of As^V into As^{III} inside the cells was not affected by the concentration of $[As]_0$ in the surrounding medium. However, the excretion of As^{III} out of the cells was affected by the $[As]_0$ in the surrounding medium. This might have been due to the fact that: (i) although As^{III} is more toxic than As^V is (Akter et al., 2005), As^{III} is more easily excreted than As^V from the cells (Rahman and Hassler, 2014) and/or (ii) the excretion of As^{III} out of the cells (efflux, which many researchers have agreed occurs to reduce the toxic effects of As^{III} (Rahman and Hassler, 2014; Papry et al., 2019; Hellweger et al., 2003) is negatively correlated to the As concentration in the surrounding medium due to equilibrium effects (Wang et al., 2013). The present study also showed that, unlike in the high-$[As]_0$ treatment, As^{III} excretion out of the phytoplankton cells in the low-$[As]_0$ treatment happened at the logarithmic and stationary growth phases. However, the excretion rate of As^{III} was higher at the logarithmic phase than that at the stationary phase.

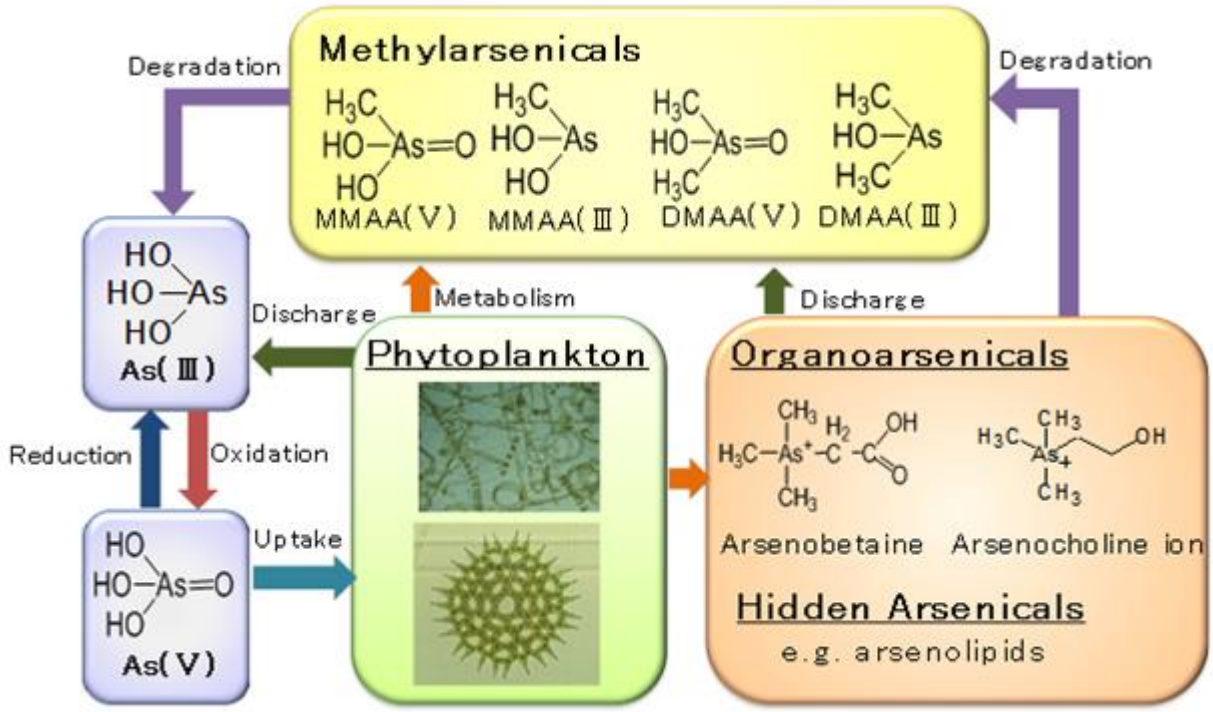

Figure 5.4 Biotransformation of As species by phytoplankton in aquatic systems.

5.3.2.2 Biotransformation of iAs into methylAs and orgAs

Irrespective of the $[As]_0$ treatment, iAs species ($As^V + As^{III}$) were the predominant As species in the growth medium at the logarithmic growth phase (**Figure 5.3a, b**). Intracellular As speciation results also showed that iAs species ($As^V + As^{III}$) were the main As species inside the cells at the logarithmic growth phase, although a small amount of methylAs (mainly MMAA) was also found inside the cells at this growth phase (**Figure 5.3a, b**). A significant proportion of the total As (t-As) inside the cells was represented by methylAs and orgAs species at the stationary growth phase (**Figure 5.3c, d**), while the methylAs and orgAs concentrations in the growth medium were insignificant compared to the iAs concentrations therein (**Figure 5.3c, d**). These results indicated that after the phytoplankton biotransformed iAs into methylAs and orgAs species inside their cells, these species were not excreted out of the cells by the organisms. This can be explained by the low toxicity and efflux rates of methylAs and orgAs species.

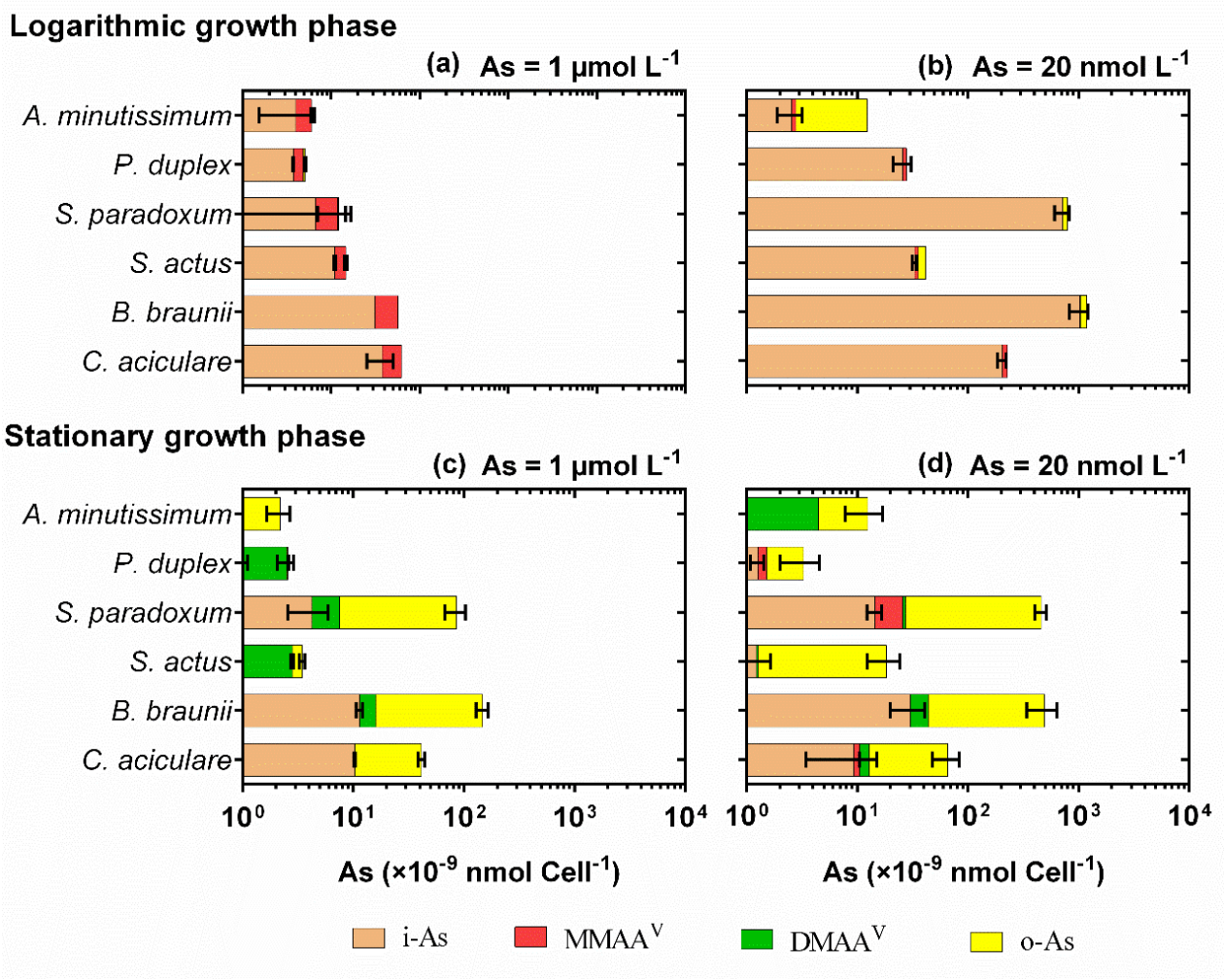

Figure 5.5 Arsenic speciation in freshwater phytoplankton cells at the logarithmic (a, b) and stationary (c, d) growth phases. Initially, the phytoplankton were grown in CSi culture medium with 1.0 µmol L^{-1} (a, c) or 20 nmol L^{-1} (b, d) of As^{V}. Mean ± SD As concentrations are shown (n = 3).

Inside the phytoplankton cells, As^{V} is reduced to As^{III}, which is then followed by the oxidative methylation of intermediate trivalent methylAs species ($MMAA^{III}$ and $DMAA^{III}$) to form pentavalent methylAs species ($MMAA^{V}$ and $DMAA^{V}$) (Hughes, 2002). A number of freshwater phytoplankton have been reported to biomethylate iAs (Caumette et al., 2011; Miyashita et al., 2011). It is known that methylAs and orgAs species are less toxic than iAs species (Akter et al., 2005). It is widely accepted that phytoplankton employ two main strategies to reduce the toxic effect of iAs: (i) the excretion of iAs (mainly As^{III}) out of their

cells; and (ii) the biotransformation of toxic iAs into less toxic methylAs and orgAs species (Rahman and Hassler, 2014). As methylAs and orgAs species are less toxic to them, phytoplankton do not need to excrete them out of their cells, and therefore a significant amount of methylAs and orgAs were found inside the phytoplankton cells. This result also suggests that the methylation of As^{V} occurs more slowly than its reduction, and also differs among strains of freshwater phytoplankton.

5.3.3 Diversity in As biotransformation by freshwater phytoplankton

An interesting pattern in the As biotransformation performed by the six freshwater phytoplankton strains studied in the present study was observed that potentially explains the diversity of As biotransformation by phytoplankton. Based on the biotransformation efficiency of different As species, the freshwater phytoplankton examined herein could be categorized into three groups: (i) phytoplankton that are efficient at biotransforming As^{III} into As^{V} (*e.g.*, *B. braunii* and *S. paradoxum*); (ii) phytoplankton that cannot efficiently biotransform As, and thus rather maintain As^{V} inside their cells (*e.g.*, *S. actus* and *P. duplex*); and (iii) phytoplankton that are efficient at biotransforming iAs into methylAs species (methylation) and complex orgAs species (*e.g.*, *A. minutissium,* and *C. aciculare*) (**Figure 5.6**; Groups A, B, and C, respectively).

Although there is no evidence to explain why different phytoplankton species have different As biotransformation efficiencies, this is likely related to the differential physiological activities of the phytoplankton, such as bioaccumulation, detoxification (transformation of toxic iAs species into less toxic methylAs and orgAs species), and excretion of As out of the cells. The fact that *B. braunii* and *S. paradoxum* (Group A phytoplankton; **Figure 5.6**) convert As^{III} to As^{V} indicates that, once they are released into the oxic medium, then As^{III}, MMAA, and DMAA are transformed back into the thermodynamically static As^{V}. In terms of As^{III} biotransformation, the bio-oxidation process plays a vital role in freshwater algal cells, and the transportation of As^{III} takes place via aquaporins (Zhao et al., 2009). In the case of *S. actus* and *P. duplex* (Group B phytoplankton; **Figure 5.6**), As^{V} was taken up simultaneously and accumulated inside the cell. The uptake of As^{V} by microalgae without transformation and their keeping it inside the cell likely occurs due to the detoxification strategies of these freshwater microalgae. The occurrence of this phenomenon in *S. actus* indicates that this species may be genetically incapable or energetically incompetent to take part in As biotransformation activities (Rose et al., 2007). The subcellular distribution and chemical forms of heavy metals may play important roles in the metal tolerance and detoxification responses in plants (Fu et

al., 2011). The ability of *A. minutissium* and *C. aciculare* (Group C phytoplankton; **Figure 5.6**) to transform As^{V} into As^{III} or methylated As forms confirm the occurrence of As biotransformation process in these microalgae. In general, bio-reduction occurs in association with the methylation process in response to As^{V} biotransformation. A study by Murray et al. (2003) on microalgae, including *D. salina* and *C. vulgaris*, provided evidence that this sort of biotransformation of arsenic species could be performed by these microalgae, which was in agreement with the findings for phytoplankton Group C in the present study. Phytoplankton converted As^{V} to As^{III}, and as the incubation period continued the As^{III} accumulated in the cells gradually began to undergo the methylation process, mainly to form DMAA, and was then likely excreted into the medium. The quantity of orgAs species present was related to the methylation/demethylation rate and release mechanisms of the microalgae. The presence (or absence) of MMAA in microalgal cell also confirmed the occurrence of the iAs methylation process in them. Therefore, biotransformation diversity among microalgae depends not only on the As speciation in the medium, but also on the As bioaccumulation and biotransformation methods used by each individual phytoplankton species.

The rates of arsenic uptake by the different microalgae used in this study varied, suggesting that the consumption or sequestration of arsenic species depends on the structural and biochemical properties of microalgae, and differs among microalgae belonging to different species or classes. Uptake of arsenic by cells depends on the valence of arsenic, as well as on several biotic and abiotic factors. Relevant biotic factors include the microalgal species and its uptake pathways, mode of detoxification, and whether it has had earlier exposure to As. On the other hand, abiotic factors include the arsenic species, phosphate concentration, pH, and time of exposure. Several studies suggested that living biota (both aquatic and terrestrial) have various methods they can use to detoxify arsenic-like metals and metalloids. These may involve: arsenic elimination from cells (Rosen, 1999), reduction of As^{V} to As^{III} (De Vos et al., 1992), secretion of polychelatins (*e.g.*, metal-binding proteins) (Cullen et al., 1994), and the subsequent methylation of As to form less toxic complexes (Reed et al., 2015). Nevertheless, the detoxification processes carried out by microalgae, due to their impacts on the excretion of As^{V}, As^{III}, or organic As species (Rose et al., 2007), have significant influences on their growth and As^{V} uptake and elimination.

This study showed that As biotransformation by and toxicity to freshwater phytoplankton was greatly influenced by the chemical species of As present, the type of phytoplankton species considered, and the phosphate concentration in the growth medium. Changes in experiment duration and conditions (such as exposure period and phosphate

concentration) altered the behavior and As biotransformation processes carried out by the phytoplankton species. The growth rate of each species varied even when they were treated with the same experimental procedures, suggesting that each species has its own specific As uptake mechanism(s).

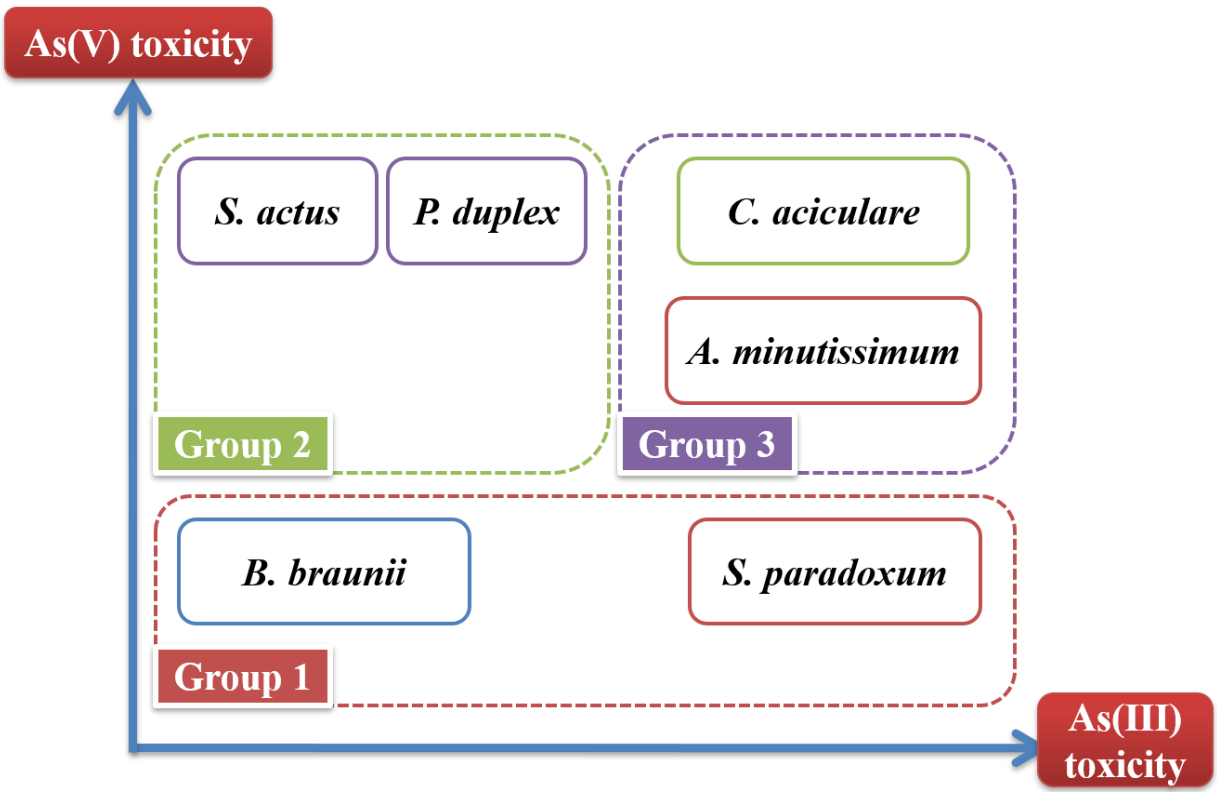

Figure 5.6 Diversity of arsenic biotransformation by freshwater phytoplankton. All of the six-freshwater phytoplankton tested converted As^{V} into As^{III} at the logarithmic growth phase. However, the phytoplankton could be grouped into the following three groups based on their As biotransformation ability at the stationary growth phase. **Group 1:** *B. braunii* and *S. paradoxum* converted As^{III} into As^{V}; **Group 2:** *S. actus* and *P. duplex* maintained As^{V} in their cells; and **Group 3:** *A. minutissium* and *C. aciculare* converted iAs into methylarsenic species.

5.3.4 A conceptual model of As metabolism in freshwater phytoplankton

A model of As biotransformation by freshwater phytoplankton based on the experimental results of this study is presented in **Figure 5.7**. In this model, the As^{V} present in the medium is adsorbed on the cell surface and is then taken into the cell via the phosphate transport system. This phenomenon happens due to the similar chemical properties of arsenate and phosphate. Arsenate can be actively taken up into the phytoplankton cells by the phosphate transporter pathway (Reed et al., 2015) and competing with phosphate uptake (Meharg and Hartley-Whitaker, 2002). This competitive uptake between As^{V} and phosphate in phytoplankton cells indicates the probable mode of action of the toxicity of As species. The As concentration in the cell proportionally increases with the As substrate concentration in the medium. This suggests that As uptake is influenced by metalloid availability in the environment (Smith et al., 2010; Maeda et al., 1992; Budd and Craig, 1981). As^{V} is reduced to As^{III} in the logarithmic growth phase and excreted into the growth medium via active transport. This reduction reaction may be carried out by thiols and/or dithiols, as As is likely to bind with the biochemical components of protein and non-protein thiols (Cullen et al., 1994). As^{III} is then methylated to form MMAA and DMAA, which then diffuse into the medium, suggesting that the chemical form of As species is changed depending on the As tolerance of the phytoplankton during the transition from the logarithmic growth phase to the stationary phase. Uptake of As^{V} causes the reduction of phosphate accumulation in the cell, likely due to As phytotoxicity or competitive uptake (Wang et al., 2002). Inside the cell, phosphate groups are replaced by As^{V} in ATP, which forms an unstable ADP-As complex that interferes with other physiological systems, such as energy flow (Ullrich-Eberius et al., 1989).

Uptake of As^{III} into the cell occurs via aquaporin nodulin26-like intrinsic proteins (NIPs). Aquaporins are a kind of water channel protein that can transport extracellular water molecules into the cell. Several studies have suggested that As^{III} likely interferes with enzymatic reactions (Planas and Healey, 1978) and the photosynthesis process (Blanck and Wängberg, 1988). Consequently, pigment, peptide, and lipid profiles can be altered in microalgal cells exposed to inorganic As species (Miazek eta l., 2015). There are several steps involved in the metabolic transformation of iAs into methylated species, such as mono-, di-, and tri-methylated arsenic compounds (Thomas et al., 2009). Among these steps, some are associated with chemical reactions, whereas others are enzymatically catalyzed. It was previously suggested that arsenic methyltransferase (AS3MT) acts as a catalyst that leads to the transformation of iAs into methylated compounds. However, the methylated compounds

yielded by this process are actually more reactive and toxic (Thomas et al., 2001).

The reduction of As^{V} to As^{III} occurs in the presence of several reductases that use glutaredoxin, glutathione, or thioredoxin as electron donors (Yin et al., 2011; Zhao et al., 2009). The methylation of As^{III} was reported to be slower than that of As^{V} to prevent As^{III} from building up inside the cell (Hellweger et al., 2003). As^{III} is excreted less and its concentration gradually declines as a result of ongoing abiotic oxidation. MMAA and DMAA occur inside the cell due to the methylation of As^{III}, and their excretion increases their concentrations in the culture medium. The production rates of DMAA and MMAA are comparatively slower than that of As^{III} at the logarithmic growth phase, likely due to the lower uptake of As^{V} at this phase (Hellweger et al., 2003). The presence of phytochelatins (PCs) in microalgae leads to the binding of As to thio groups, for example glutathione (GSH), and thus PCs play important roles in As complexation and detoxification (Pawlik-Showronska et al., 2004). PC synthesis takes place at a rate adequate to bind the As in the cell at lower As concentrations (Morelli et al., 2005). The presence of intracellular GSH along with PCs permits the effective accumulation of As inside the cell (Yamaoka et al., 1999). The reduction of As^{V} to As^{III} with GSH as the electron donor in an aquatic medium promotes the formation of arsenotriglutathione complexes. These complexes readily donate As^{III} to targets including dithiols (Delnomdedieu, et al., 1993). Hayakawa et al. (2005) reported that methylation mechanisms could occur via the formation of such complexes. The methyl group of S-adenosylmethionine (SAM) is transferred to As by AS3MT, and then the methylated compound undergoes hydrolysis and is oxidized, and the methylation process then proceeds sequentially. SAM is required for the As methylation reaction, in which it acts as a methyl donor, and if its availability changes this affects the patterns and extent of the As methylation process. The fact that the methylation mechanism of As involves AS3MT indicates that As methylation is an oxidative process. Chemically active oxygen was reported to initiate oxidative stress due to its involvement in the catalytic reaction of methyltransferases (Hu et al., 2002). Free radicals are generated due to the reaction of dimethylarsine with molecular oxygen during the metabolic reduction of $DMAA^{III}$. The mechanism of As uptake indicates that the pathway leading from iAs to methylated compounds includes several steps that generate several different intermediates and elements.

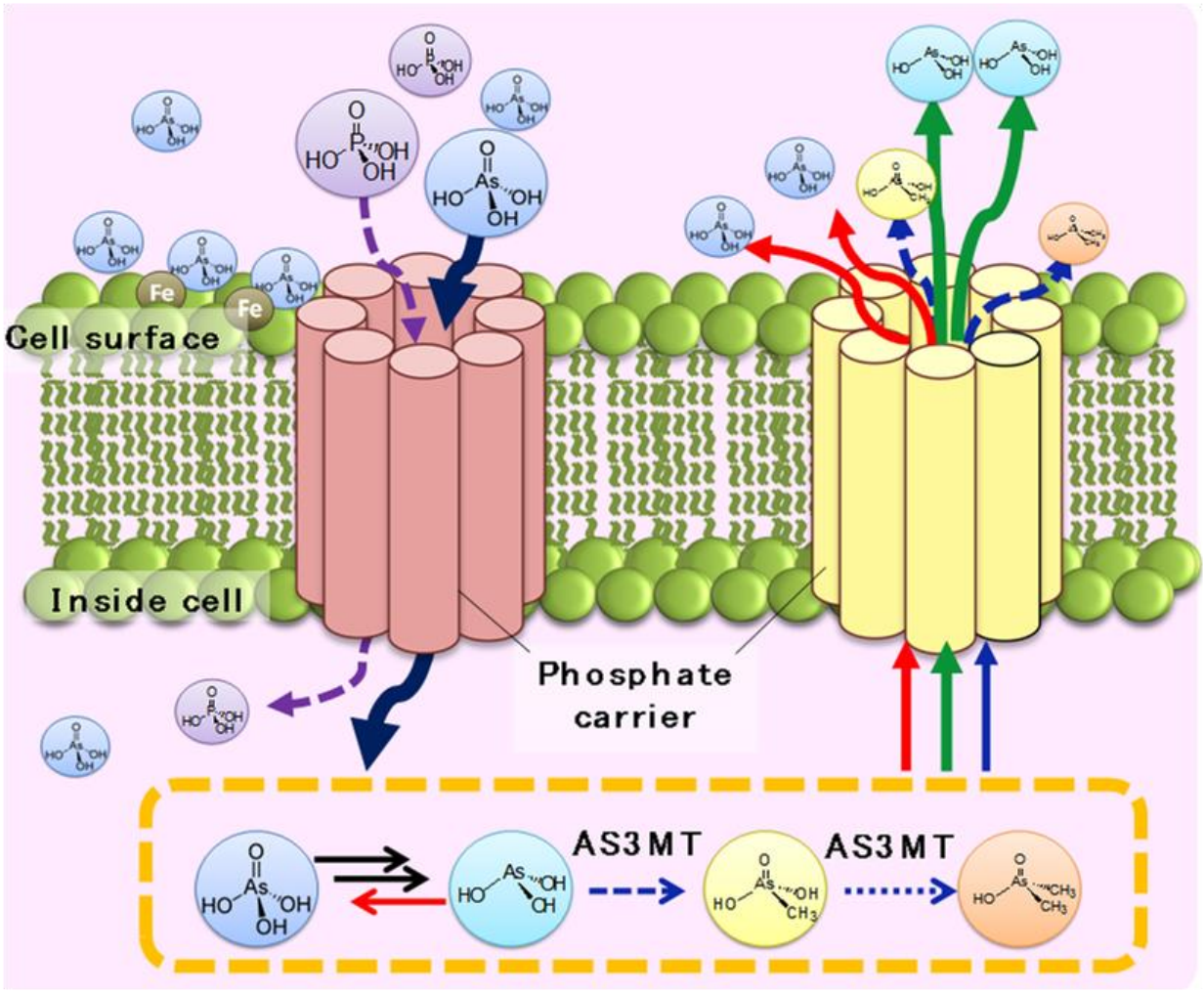

Figure 5.7 Conceptual model of arsenic metabolism in freshwater phytoplankton. Arsenic resistance in this study depended on the kinds of freshwater phytoplankton considered. In the logarithmic growth phase, freshwater phytoplankton took arsenate into their cells and reduced it to arsenite. In the stationary phase, freshwater phytoplankton transformed the chemical forms of the arsenic in the surrounding environment, and then released it from their cells rapidly. The arsenic resistance of freshwater phytoplankton has an interactive relationship with the changing availability of different arsenic species in the hydrosphere.

5.4 Conclusion

This study reported the toxicity, biotransformation, and release of different As species in freshwater phytoplankton, which may help us to better understand the biogeochemistry of As in freshwater systems. Inorganic As^{V} was taken up by the phytoplankton, biotransformed inside their cells, and then released into the culture medium as As^{III} during the exponential growth phase at a high As^{V} to phosphate ratio. The growth inhibition effects of As^{V} and As^{III} were significantly higher than those of orgAs, $DMAA^{V}$, and AB on the tested freshwater phytoplankton. The six freshwater phytoplankton strains examined could be categorized based on their As biotransformation patterns as follows: (i) those efficient in the biotransformation of As^{III} into As^{V}; (ii) those not efficient in As biotransformation, which rather maintained As^{V} inside their cells; and (iii) those efficient in the biotransformation of iAs into methylAs species (methylation) and complex orgAs species. These results reflect the differential bioaccumulation and biotransformation of As species by these phytoplankton inside their cells and their excretion of these As species into the environment. Further work is needed to assess whether the toxicity of As species differs for other aquatic microorganisms such as zooplankton, to better understand the influence of As on freshwater ecosystem dynamics.

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Chapter 6:

Freshwater phytoplankton: The effects of salinity stress on arsenic biotransformation

Rimana Islam Papry, Shogo Fujisawa, Yinghan Zai, Okviyoandra Akhyar, Asami S. Mashio, Hiroshi Hasegawa*, ‘‘Freshwater phytoplankton: the effects of salinity stress on arsenic biotransformation’’, *Environmental Pollution* (under review).

6.1 Introduction

Arsenic (As) is a toxic metalloid that occurs in the environment both naturally and anthropogenically, and exerts acute carcinogenic effects (Han et al., 2017). In freshwaters, the concentration of As varies from 0.5 to 5000 µg L^{-1} (Kunil et al., 2014; Pfeiffer et al., 2015). Inorganic forms of As, called arsenate (i.e., As(V)) and arsenite (i.e., As(III)), are the dominant species in the freshwater environment, particularly in oxic state (Hasegawa et al., 2010; Rodríguez-lado et al., 2013). To date, several studies have been performed on As accumulation and biotransformation metabolism in microalgae as well as bioremediation techniques to combat As pollution (Foster et al., 2008; Papry et al., 2020a; Wang et al., 2017, 2013).

Phytoplankton, as the primary producers, play a significant role in the circulation of As in the aquatic environment (Azizur Rahman and Hasegawa, 2012; Duncan et al., 2015). It contributes significantly to the As remediation process as it intakes As from contaminated water with a bioconcentration factor varying from 200 to 4000 (Levy et al., 2005). Therefore, microalgae are a better option for the As remediation process due to its cost-effective and eco-friendly approach (Sulaymon et al., 2013). Phytoplankton and cyanobacteria, as photosynthetic species, take up As(V) from the surroundings, convert it to As(III), and subsequently methylate it to methyl arsenic (i.e., methyl As) species (Ye et al., 2012). The uptake of As, its accumulation, and toxicity in microalgae, depend on its speciation in the surrounding atmosphere. Hence, it is critical to determine this process (Karadjova et al., 2008). Due to their similar chemical characteristics, As(V) and PO_4^{3-} compete for enzymatic reactions that constrain photophosphorylation and oxidative photophosphorylation. This results the interferences of cell related metabolic activities (Bhattacharjee et al., 2008). Microalgal reduction of As(V) to As(III) and methylation to organoarsenicals decreases As content from water, and this is an important step in As biogeocycling (Qin et al., 2006).

The chemical composition of aquatic microalgae is affected by several environmental factors(Papry et al., 2020a), including light intensity (Nzayisenga et al., 2020; Ota et al., 2015), temperature (Papry et al., 2019; Ras et al., 2013), salinity (Baek et al., 2011; Papry et al., 2020b), pH (Murray et al., 2003), nutrient concentration (Hasegawa et al., 2001) and species type (Duncan et al., 2015). Among these, salinity has the maximum influence on the survival of marine microalgae. The adaptive capacity of freshwater microalgae, in terms of growth and physiological metabolism, is likely to be different from that of marine microalgae. Therefore, salinity acts as a barrier between freshwater and marine microalgae (Lobban and Harrison, 1994). Although there is a significant variation in salinity tolerance among freshwater microalgae, some species actively respond to frequent salinity fluctuations (Maier Brown et al.,

2006). For example, *Rhizoclonium riparium* (Roth) Harvey, a filamentous green alga, is typically found in freshwater but also survives in brackish and marine waters. Salinity stress lowers cellular growth because of the high energy required for osmoregulation (Oren, 1999). Effects of salinity on the growth of freshwater phytoplankton and their metabolism have been discussed in the literature (Alvensleben et al., 2016; Chakraborty et al., 2011; Yun et al., 2019). However, to date, the As accumulation and biotransformation mechanism of freshwater phytoplankton under a salinity gradient has never been addressed. The aim of the present study was to determine the salinity stress effect on growth, As uptake, accumulation, and biotransformation potential of four axenic freshwater microalgae *viz.*, *Scenedesmus acutus*, *Closterium aciculare*, *Staurastrum paradoxum,* and *Pediastrum duplex*.

6.2 Materials and methods

6.2.1 Maintenance of phytoplankton strains

Four strains of freshwater phytoplankton, *viz.*, *Scenedesmus acutus*, *Closterium aciculare*, *Staurastrum paradoxum,* and *Pediastrum duplex*, were used in this study (**Figure 6.1**). Phytoplankton strains were collected from the National Institute for Environmental Studies, Japan. Phytoplankton cultures were maintained using C medium (**Appendix 6.1**). For growth, the culture medium strength was adjusted to 1.0 µmol L^{-1} phosphate (potassium dihydrogen phosphate (KH_2PO_4); Wako Pure Chemical, Osaka, Japan), and further modified by adding As(V) solution (special-grade disodium hydrogen formate heptahydrate ($Na_2HAsO_4.7H_2O$); Wako, Osaka, Japan). Sodium hydroxide (NaOH, special grade reagent; Nakarai Tesque) and hydrochloric acid (HCl; for measurement of harmful metals, Kanto Chemical, Tokyo, Japan) were used for pH adjustment of reagents and the medium. Ultrapure water, with a resistivity of 18.2 MΩ cm, was used in all experiments (arium pro UV, Sartorius Stedim Biotech GmbH).

Before the experiment, clonal axenic strains of *S. acutus*, *C. aciculare*, *S. paradoxum,* and *P. duplex* were incubated in the C medium for 1–2 weeks (Koitotron3HN-35MLA, Koito Industries, Ltd., Yokohama, Japan) to reach the exponential growth phase. Phytoplankton cultures were kept at 25 °C under a 12:12 h light/dark photoperiod and a light intensity of 50 µE m^{-2} s^{-1} provided by cool white fluorescent lights. The 4, 6-diamidino-2-phenylindole (DAPI) method was used to determine the axenic nature of phytoplankton under an epifluorescent microscope (Bohlool and Schmidt, 1980).

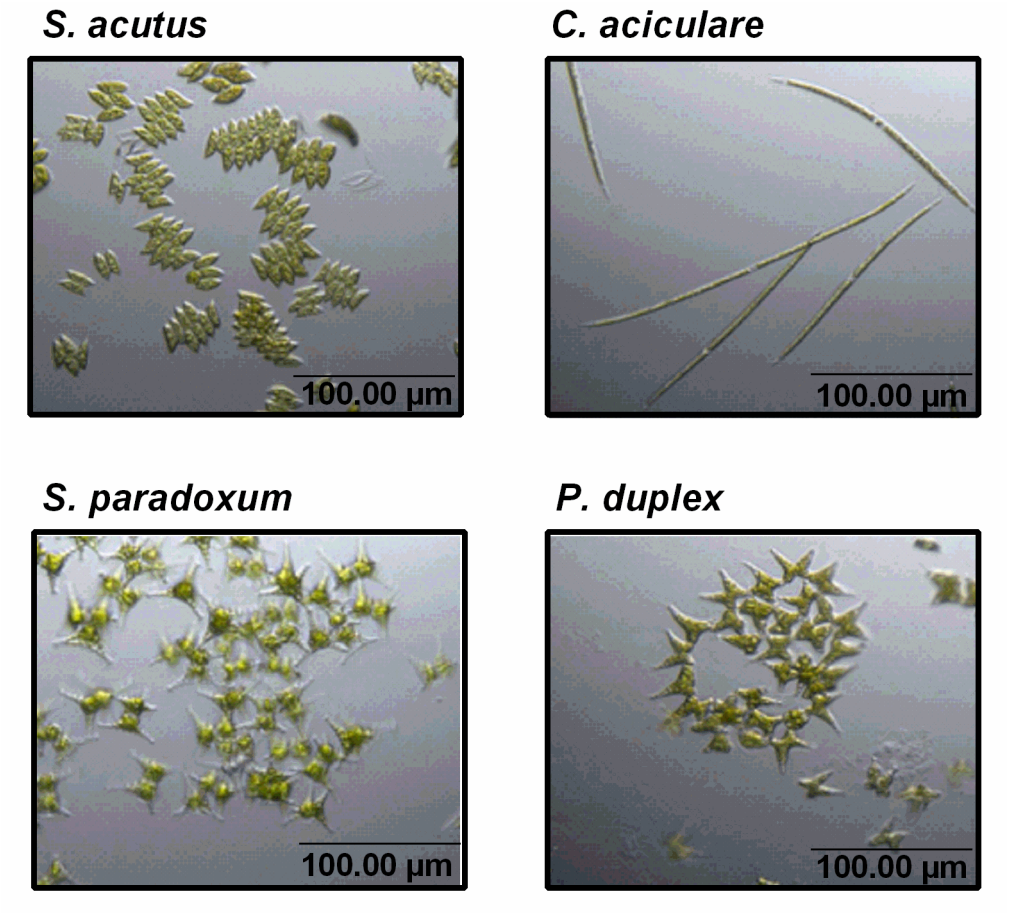

Figure 6.1 Images of four freshwater phytoplakton species used in this study, captured using a digital microscope (KEYENCE, VHX-1000, Japan).

6.2.2 Measurement of growth and As biotransformation under a salinity gradient

Phytoplankton cells (i.e., 30 mL) were incubated in sterilised C medium, modified with different salinities. The growth response and As biotransformation potential of phytoplankton were examined under 0, 1, 2, 3, 4, and 5‰ salinities. Artificial seawater (35‰), prepared in the laboratory (**Appendix 6.2**), was diluted to attain the salinities mentioned above. The initial cell density in the culture medium was $<4.5\times10^3$ cells mL^{-1}. Phytoplankton growth was measured using a UV-VIS spectrophotometer at 540 nm, and the cell number was estimated using an established cell density-to-absorbance ratio. Cell numbers were counted directly by using a digital microscope (VHX-1000; Keyence, Osaka, Japan). The growth rate (µ d^{-1}) was calculated using the following equation (Abu-Rezq et al., 1999).

Growth rate (µ/day)$= (\ln N1 - \ln No)/t$

where: *N*1, *N*o, and *t* denote the final cell density of phytoplankton (cell mL^{-1}), the initial cell density of phytoplankton (cell mL^{-1}), and time (d), respectively.

Further, two different concentrations of As(V), i.e., 20 nmol L^{-1} (low arsenic) and 1.0 µmol L^{-1} (high arsenic), under a salinity gradient of 0–5‰ were used to determine the As biotransformation potential of freshwater phytoplankton. During the experiment, 0.1 µmol L^{-1} or 100 nmol L^{-1} of As(V) was added as NaH_2AsO_4 and 1 µmol L^{-1} of PO_4^{3-} as KH_2PO_4; phosphate was added to the medium to enhance the uptake of As(V).

6.2.3 Arsenic speciation analysis in the culture medium

On the specified day, samples were collected and filtered through 0.45 µm membrane filters (Toyo Roshi Kaisha, Tokyo, Japan) under a low vacuum pressure (<30 mm Hg). The filtered sample was stored in a cool, dark place after adding 0.50 mL of 1.0 mol µmol L^{-1} HCl. A hydride generation technique (Hasegawa et al., 1994) was used to determine As species in the culture medium samples. The technique makes use of a flame atomic absorption spectrophotometer (AAS, 170-50 A, Hitachi, Japan) and hydride generation device (CT-HG-AAS), followed by cold trapping (Hasegawa et al., 2019; Papry et al., 2020b, 2019). Inorganic arsenic (V+III), monomethylarsonate (MMAA(V)) and dimethylarsinate (DMAA(V)) were analysed by adding 5.0 mL of 0.20 mol L^{-1} EDTA.4Na and 5.0 mol L^{-1} hydrochloric acid to 40 mL of the sample solution. In contrast, 5.0 mL of 0.20 mol L^{-1} EDTA.4Na and 0.5 mol L^{-1} of potassium hydrogen phthalate were added to 40 mL of the sample solution for As(III). Arsenic species were recorded as chromatograms, while concentrations were determined by the heights of their peaks.

6.2.4 Determination of total arsenic concentrations in the cell samples

Total As concentrations in the phytoplankton cell samples were determined by inductively coupled plasma mass spectrometry (ICP-MS, SPQ 9000; Seiko Instruments Inc., Chiba, Japan) (Papry et al., 2020b). The samples were collected after filtration of phytoplankton culture, on days 7, 14, and 21. A microwave digestion structure (Multiwave 3000, Anton Paar GmbH, Graz, Austria) was used for the digestion of phytoplankton cells comprising filter papers. Digested liquors were washed with 15 mL of ultrapure water and transferred to a heat-resistance DigiTube (DigiTUBEs; SCP Science, Quebec, Canada). Next, the tubes were heated on a hot plate at 100 °C for 6–7 h. After evaporation, 2 mL of deionised

water was added to the samples and samples were subjected to ICP-MS to quantify total As in the cells. The ICP-MS had a high-frequency output of 1.2 kW, plasma gas flow rate of 16 L min^{-1}, an auxiliary gas flow rate of 1.0 L min^{-1}, nebuliser gas flow rate of 1.0 L min^{-1}, and sample replacement time of 10 s. Moreover, the accuracy of the digestion, extraction, and measurement procedures were checked according to certified standard reference materials, 1571a (tomato leaf, NIST).

6.2.5 Statistical analysis

The data were analysed using statistical software SPSS 22.0 for Windows (IBM Co., USA) and Graph Pad Prism 7.0 (GraphPad Software Inc., USA). A one-way and two-way analysis of variance (ANOVA) was considered for experimental data.

6.3 Results and discussion

6.3.1 Salinity stress on freshwater phytoplankton growth

Phytoplankton showed variation in adaptation capacity towards salinity according to their halophilic or halotolerant characteristics (Rao et al., 2007). For marine microalgae, salinity is an essential environmental parameter that directly influences species growth and metabolic processes (Liska et al., 2004). In this study, phytoplankton species were cultured in 0–5‰ salinity for three weeks inside the culture incubator. Phytoplankton strains, i.e., *S. acutus*, *C. aciculare*, *S. paradoxum*, and *P. duplex*, exhibited tremendous variation in response to salinity. Among all the species, *S. acutus* had better growth response towards salinity, followed by *P. duplex, C. aciculare*, and *S. paradoxum*, respectively (**Figure 6.2a & b**). All the species displayed high growth performance at 0‰. This trend was expected as they belong to a freshwater ecosystem. The maximum cell density was 34.6±2×10^4 cell mL^{-1} with growth rate (GR) of 0.61±0.02 for *S. acutus*, 15.3±1×10^4 cell mL^{-1} with GR of 0.32±0.03 for *C. aciculare*, 12.8±2×10^4 cell mL^{-1} with GR of 0.29±0.02 for *S. paradoxum*, and 23.1±3×10^4 cell mL^{-1} with GR of 0.45±0.02 for *P. duplex* at 0‰ (**Figure 6.2a & b**). Besides, *S. acutus* exhibited a good growth rate not only at 0‰, but also at 1–5‰, indicating that this species has a higher salinity adaptation capacity than other species used in this study. The growth trend of freshwater phytoplankton was 0‰ >1‰ >2‰ >3‰ >4‰ >5‰. This also indicated that small changes in salinity cause significant effects on the growth viability of phytoplankton in freshwaters. In a

previous study (Alvensleben et al., 2016), four freshwater phytoplankton species, i.e., *Desmodesmus armatus, Scenedesmus quadricauda*, *Tetraedron* sp., and *Mesotaenium* sp., were cultured at 2, 8, 11, and 18‰. The study found no growth variation at 2, 8, and 11‰ except for 18‰. Moreover, among all the species, only *D. armatus* exhibited high salinity tolerance and could grow until 18‰. Another study cultured freshwater green alga, i.e., *Rhizoclonium riparium* (Roth) Harvey, with salinity variation from 0.1 to 34.0‰. The study found a remarkable growth at 13.6%; however, it was not able to survive at 0.1‰. Further, a reasonable photosynthesis rate was observed until 34.0‰ (Imai et al., 1997). Although the salinity range was vast when compared to this study, their results suggests that the response towards salinity tolerance varies by species types. Moreover, the physiological characteristics of individual microalga are directly or indirectly influenced by the interaction with ion composition in saline waters (Saros and Fritz, 2000).

6.3.2 Arsenic biotransformation by freshwater phytoplankton under salinity stress

Analysis of As biotransformation potential includes the reduction of As(V) to As(III), and methylation to MMAA and DMAA. Phytoplankton take up As(V) from the surrounding environment, and convert it to either As(III) which is excreted to the medium or methylated to methyl As species. This mechanism of methylation from inorganic arsenic (iAs) species by aquatic microalgae is referred to as a detoxification tool (Levy et al., 2005). The biotransformation potential of As by marine phytoplankton largely depends on the growth and nutrient availability in the aquatic environment (Hellweger et al., 2003). In contrast, freshwater phytoplankton may act differently under such circumstances. In this study, *S. acutus* and *S. paradoxum* absorbed As(V), converted it to As(III) and then excreted it to the medium. Contrarily, no methyl As species were identified during sample analysis, thus indicating that the two species could not perform methylation reaction (**Figure 6.3**). Furthermore, the conversion of As(V) to As(III) decreased with increasing salinity in the medium. For *S. acutus*, the values of As(III) were 37.0±6 nmol L^{-1}, 24.4±3 nmol L^{-1}, 21.7±5 nmol L^{-1}, 17.6±2 nmol L^{-1}, 9.2±2 nmol L^{-1}, and 6.5±1 nmol L^{-1} at 0, 1, 2, 3, 4, and 5‰, respectively. In the case of *S. paradoxum*, As(III) values were 38.8±4 nmol L^{-1}, 33.2±6 nmol L^{-1}, 24.2±5 nmol L^{-1}, 11.8±3 nmol L^{-1}, 2.6±0.8 nmol L^{-1}, and 1.8±0.7 nmol L^{-1} at 0, 1, 2, 3, 4, and 5‰, respectively. In contrast, *C. aciculare* and *P. duplex* were capable of biotransforming As(V) to methyl As species such as DMAA. MMAA, as an intermediate product, was not detected in any of the liquid samples of phytoplankton species during the methylation reaction. This was likely due

to the rapid cell mobilisation of MMAA to DMAA and subsequent excretion into the medium (Cullen et al., 1994). In the case of *C. aciculare*, DMAA were detected at 0, 1, and 2‰ with values of approximately 29.8±5 nmol L^{-1}, 32.7±4 nmol L^{-1}, and 10.3±2 nmol L^{-1}, respectively (**Figure 6.3**). In the liquid sample of *P. duplex*, DMAA values of approximately 42.2±4 nmol L^{-1}, 34.8±4 nmol L^{-1}, and 21.3±3 nmol L^{-1} at 0, 1, and 2‰, respectively, were recorded. During the quantitative analysis of both *C. aciculare* and *P. duplex* at 3–5‰, DMAA was not detected. This finding suggests that these salinity levels were not suitable for methylation reactions inside the phytoplankton cells. Previous studies reported that under optimum salinity for marine microalgae, As biotransformation is associated with cellular growth (Papry et al., 2019) (Papry et al., 2020b). Additionally, marine species that exhibit high cellular growth in response to salinity gradients such as diatoms, i.e., *Skeletonema* sp. and *Thalassionema nitzschioides* (Papry et al., 2019) and phytoplankton, i.e., *Oltmannsiellopsis viridis*, show high As biotransformation efficiency (Papry et al., 2020b). However, the cellular growth of freshwater phytoplankton, i.e., *C. aciculare* and *P. duplex*, was not as high as that of *S. acutus* but exhibited high As biotransformation efficiency, including methylation and reduction reactions. These findings suggest that the biotransformation potential of As differs among freshwater and marine phytoplankton.

6.3.3 Effects of salinity on total arsenic accumulation

Arsenic accumulation increased with salinity from 0–5‰. This trend was different from the As biotransformation process. Among all the species, *S. acutus* and *S. paradoxum* exhibited higher accumulation tendency (**Figure 6.4**). For *S. acutus*, the high As concentrations of 3.8±0.4, 4.0±0.6, 5.8±0.5, 7.4±0.3, 7.8±0.3, and 8.2±0.4 nmol g^{-1} dry weight, were observed on day 7 at salinity levels of 0, 1, 2, 3, 4, and 5‰, respectively. Further, As concentration gradually decreased in the sample collected on days 14 and 21. A similar trend in As accumulation was observed for *S. paradoxum*. In contrast, *C. aciculare* and *P. duplex* displayed opposite trends in As accumulation over the culture period of 3 weeks; As concentration was lower on day 7 and higher on day 21. However, As was not detected in *C. aciculare* and *P. duplex* on day 7 at 0‰, and only in *P. duplex*, at 1‰ and 2‰.

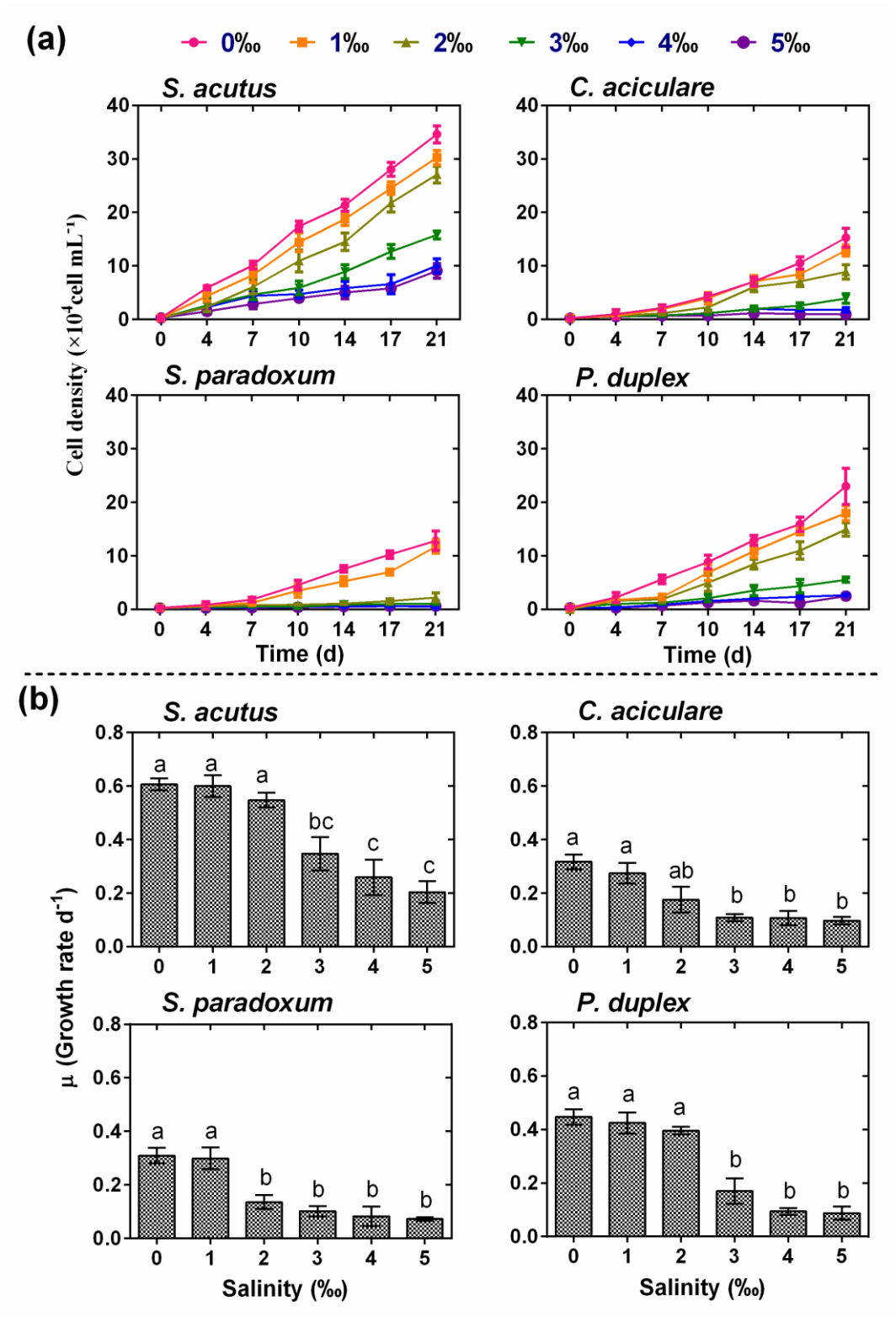

Figure 6.2 The growth of four freshwater phytoplankton species under various salinity stress conditions: (a) cell density ($\times 10^4$ cell mL^{-1}) and (b) μ (growth rate d^{-1}). The culture condition was As(V) = 0.1 μmol L^{-1} and PO_4^{3-} = 1.0 μmol L^{-1}. Lowercase letters in (b) indicate a 95% significance level between salinities under the same species. Data are presented as mean ± SD (n = 3).

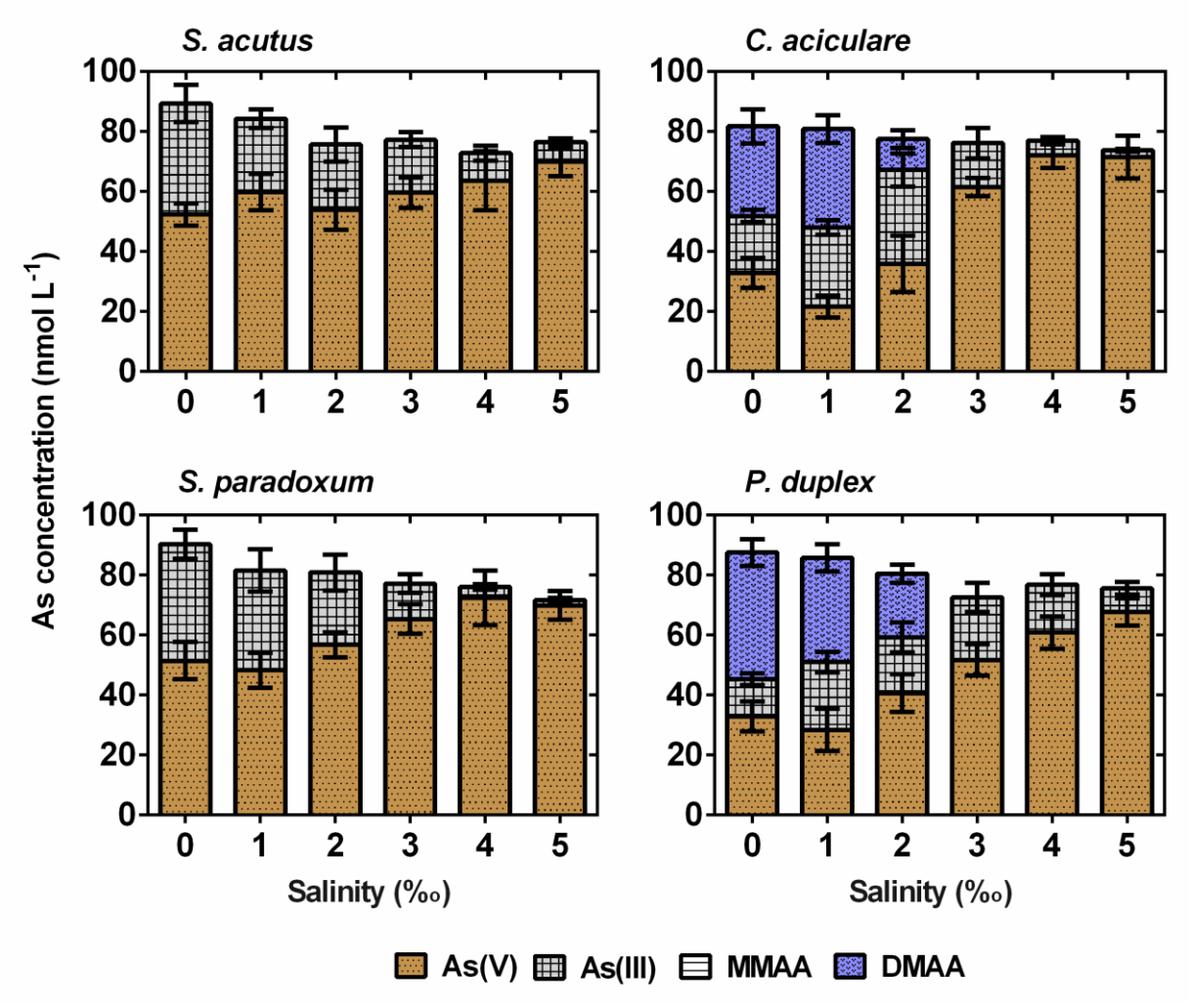

Figure 6.3 Arsenic biotransformation (reduction of As(V) to As(III) and further methylation to methylated species such as DMAA) by four freshwater phytoplankton under various salinity stress conditions. The culture condition was As(V) = 0.1 µmol L^{-1} and PO_4^{3-} = 1.0 µmol L^{-1}. Data are presented as mean ± SD (n = 3).

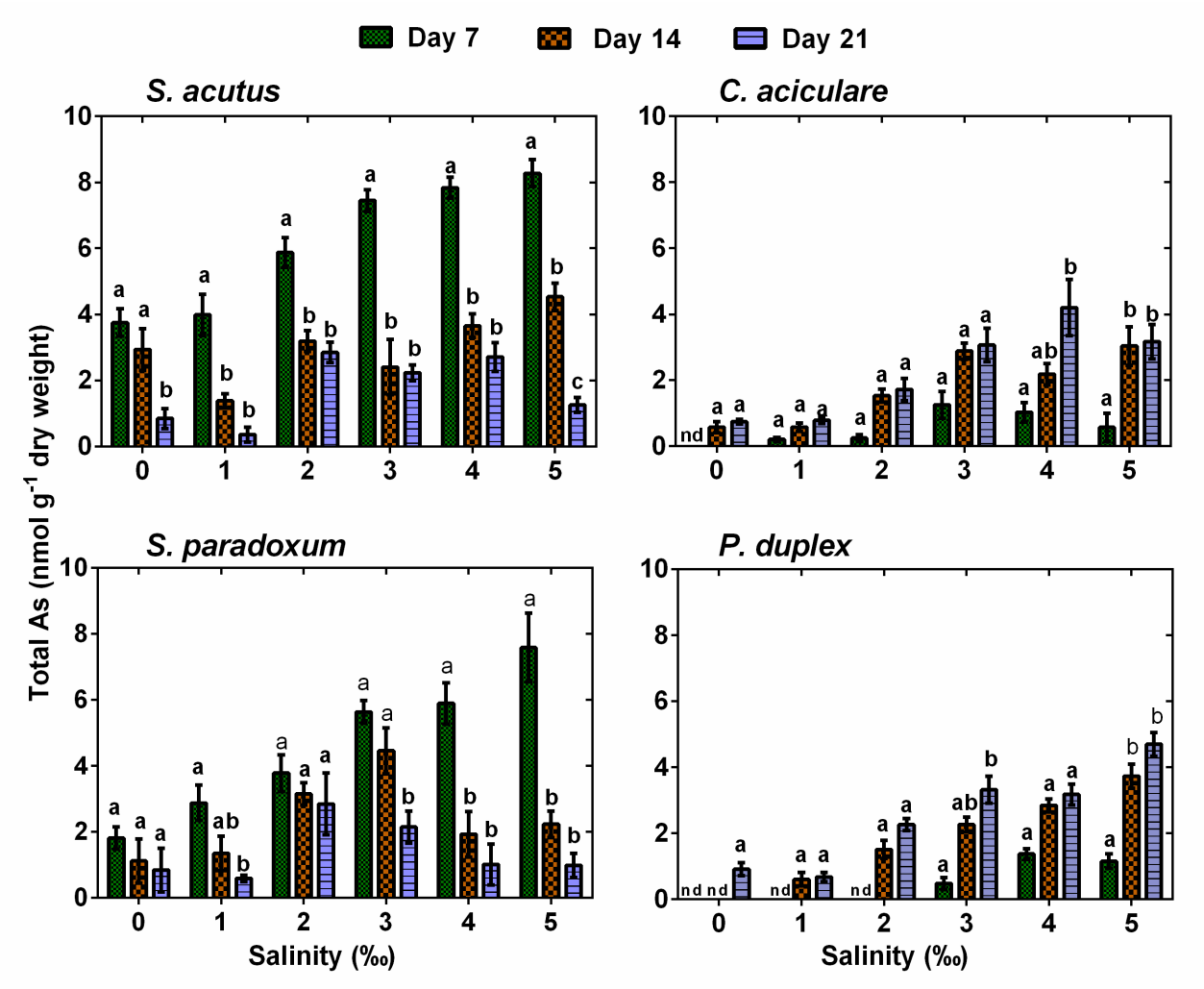

Figure 6.4 Accumulation of total As concentrations by four freshwater phytoplankton under salinity stress conditions at different culture days. The culture condition was As(V) = 0.1 µmol L^{-1} and PO_4^{3-} = 1.0 µmol L^{-1}. Lowercase letters indicate a 95% significance level between culture days under the same salinity of each species. Data are presented as mean ± SD (n = 3).

6.3.4 Arsenic reduction rate by phytoplankton cells in the medium

This study determined the reduction of As(V) to As(III) and methyl As species in the medium under 0–5‰ by phytoplankton cells (**Figure 6.5**). As(V) reduction rate by *S. acutus* gradually decreased from about 52.0±5 $\times 10^{-8}$ nmol $cell^{-1}$ at 0‰, to about 17.8±5 $\times 10^{-8}$ nmol $cell^{-1}$ at 5‰. *S. paradoxum* exhibited the lowest decrease compared to other species and no significant variation was observed. For *C. aciculare*, the higher trend of As reduction was observed at 0 and 1‰ with values of 89.2±6$\times 10^{-8}$ nmol $cell^{-1}$ and 92.4±4$\times 10^{-8}$ nmol $cell^{-1}$, respectively. *P. duplex* showed a higher reduction rate at 0‰ of 84.4±5$\times 10^{-8}$ nmol $cell^{-1}$. Collectively, these results showed that salinity has a significant impact on As biotransformation metabolism.

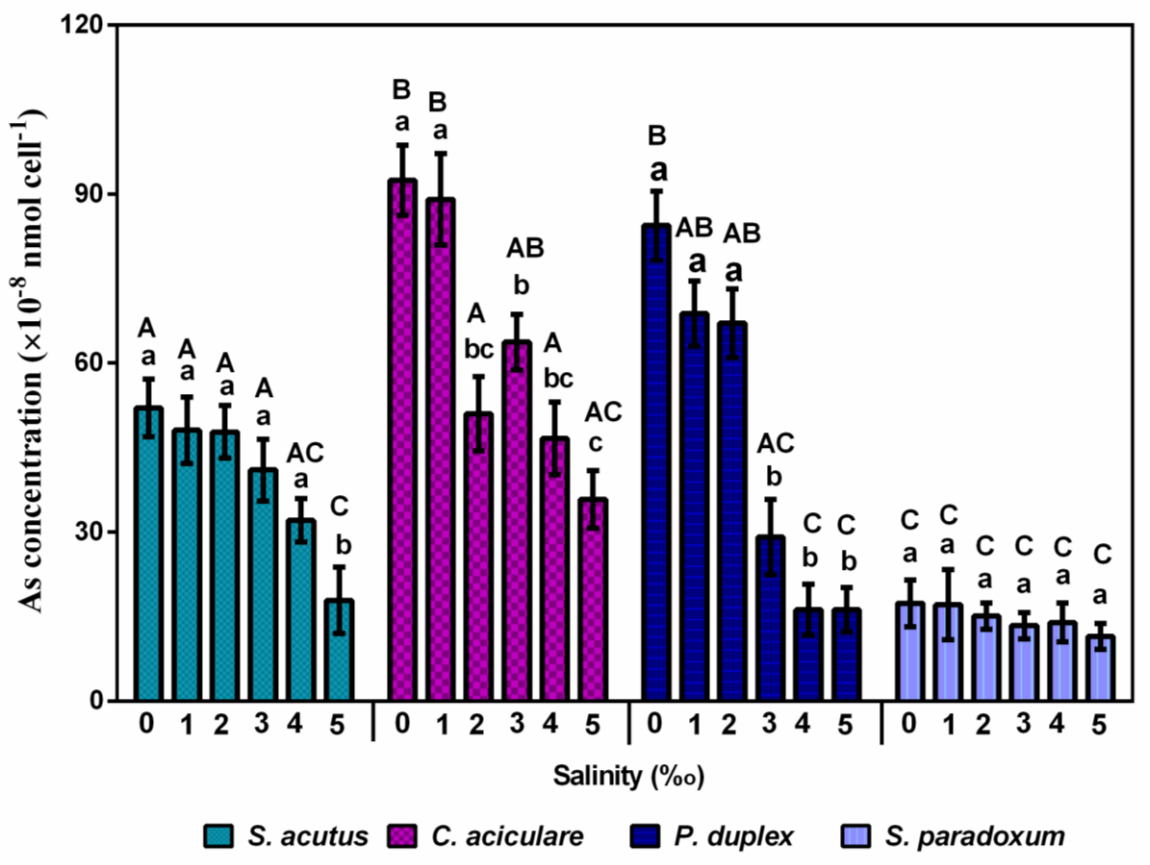

Figure 6.5 Reduction of As species (i.e., As(V) to As(III)) and further to the methylated species DMAA) by phytoplankton cells in the medium under salinity stress. Lowercase letters indicate a 95% significance level between salinities under the same species. Different uppercase letters indicate a 95% significance level between species under the same salinity. Data are presented as mean ± SD (n = 3).

6.3.5 Salinity stress on arsenic speciation pattern

The biotransformation of As(V) to As(III) and methylated As species (i.e., MMAA, DMAA, or both) was studied over time under varying salinity stress. We analysed liquid samples from two distinct time periods (days 7 and 14). Culture duration is one of the essential parameters for understanding the biotransformation mechanism of phytoplankton. In a time-dependent study, it was observed that the concentration of As(V) significantly decreased with increasing time, i.e., from day 7 to 14 (**Table 6.1**). *S. acutus* and *S. paradoxum* were unable to convert As(V) to methyl As, but only As(III) (**Figure 6.3**). The concentration of As(III) increased with decreased As(V) over time, while no significant reduction of As(V) was observed at 4 and 5‰. For *S. acutus*, As(V) concentrations were 72.5±7 nmol L^{-1} and 76.4±5 nmol L^{-1} on day 7, and 63.5±9 nmol L^{-1} and 69.9±4 nmol L^{-1} on day 14 when cultured with 4 and 5‰, respectively. In contrast, As(III) was not detected on day 7 at both salinity levels; on day 14, the concentrations were 7.2±2 nmol L^{-1} and 6.6±1 nmol L^{-1}, respectively. In the case of *S. paradoxum*, As(III) was only detected on day 14 with a value of 2.6±0.8 nmol L^{-1} when cultured with 4‰ and not detected at any other period at 5‰. The biotransformation of As(V) to methyl As was observed for *P. duplex* at 0‰. In the liquid samples, As(V) was 63.1±4 nmol L^{-1} and 22.8±5 nmol L^{-1}, while As(III) was 1.31±0.6 nmol L^{-1} and 12.4±2 nmol L^{-1}, and DMAA was 20.1±3 nmol L^{-1} and 42.2±4 nmol L^{-1} on days 7 and 14, respectively (**Table 6.1**). Significant outcomes of As biotransformation were observed at 1‰ in the *C. aciculare* sample. As(V) was 57.6±4 nmol L^{-1} and 21.4±3 nmol L^{-1}, while As(III) was 16.8±2 nmol L^{-1} and 30.3±2 nmol L^{-1}, and DMAA was 7.4±3 nmol L^{-1} and 41.8±5 nmol L^{-1} on days 7 and 14, respectively. This contributes to the knowledge of the detoxification mechanism of phytoplankton in the aquatic ecosystems.

6.3.6 Salinity stress on As biotransformation under As(V) treatment

Next, we used two different concentrations of As(V) under 0–5‰, i.e., 20 nmol L^{-1} (low As) and 1.0 µmol L^{-1} (high As) to determine the As biotransformation potential of freshwater phytoplankton. For all the species, the reduction rate of As(V) to As(III) and methyl As species decreased as the salinity increased, regardless of whether As concentration was high or low (**Figure 6.6 a and b**). In the case of *S. acutus*, As(V) reduction to As(III) occurred at low as well as high concentrations, but no methylation was observed at the high or low As levels. The tendency of biotransformation to As(III) was higher at 1.0 µmol L^{-1} with the values of 0.5±0.03 µmol L^{-1}, 0.4±0.02 µmol L^{-1}, 0.32±0.04 µmol L^{-1}, 0.21±0.03 µmol L^{-1},0.20±0.04

µmol L^{-1} and 0.03±0.006 µmol L^{-1} at 0, 1, 2, 3, 4, and 5‰, respectively, whereas *S. paradoxum* showed higher biotransformation ability at low As concentration. Under these conditions, the reduced As(III) concentrations were 7.5±0.8 nmol L^{-1}, 5.6 ±0.6 nmol L^{-1}, 3.4±0.6 nmol L^{-1}, 1.5±0.3 nmol L^{-1}, and 1.1±0.1 nmol L^{-1} at 0, 1, 2, 3, and 4‰, respectively. Similar to *S. acutus*, no methyl As species was observed in any of the experiments for *S. paradoxum.* Interestingly, *C. aciculare* and *P. duplex* showed methylation reactions at low As concentrations. DMAA with values of 2.9±0.4 nmol L^{-1} at 0‰, 6.5±0.62 nmol L^{-1} at 1‰, 2.8±0.4 nmol L^{-1} at 2‰ and 0.6±0.1 nmol L^{-1} at 3‰ was recorded for *C. aciculare*. In the case of *P. duplex,* methyl As species were also detected when cultured at low As concentrations. Moreover, As species was only detected at 0, 1, and 2‰ with the values of 4.1±0.5 nmol L^{-1}, 2.6±0.6 nmol L^{-1}, and 1.5±0.3 nmol L^{-1}, respectively; at high As concentrations, a minimal amount of As(V) was reduced to As(III) in the culture medium.

6.3.7 Morphological changes in phytoplankton

The morphological changes in phytoplankton cells were observed under a digital microscope (KEYENCE, VHX-1000, Japan). The phytoplankton cell shape changes with the increasing salinity. *S. acutus* and *S. paradoxum* cell shapes were in good condition until 2‰ salinity level. Beyond this level, the cells were segregated and reduced in numbers (**Figure 6.7**). These two species only played a role in the reduction of As(V) to As (III), and not methylation reaction. In contrast, the cell shape for *C. aciculare* and *P. duplex*, was good until 3‰ salinity, beyond which the cells were broken. Interestingly, these two species biotransformed As species from As(V) to As (III), and further methylated them to species such as DMAA. The latter two species were well-adapted to biotransform As species through physiological changes under salinity stress conditions.

Table 6.1 Salinity effects on arsenic speciation pattern by freshwater phytoplankton at different culture days. Different lowercase and uppercase letters indicate significance differences between culture days under the same salinities and species. Data are presented as mean ± SD (n = 3).

Species	Salinity	As(V)			As(III)			DMAA		
		7d	14d	SL	7d	14d	SL	7d	14d	SL
S. acutus	0	83.7±6A	52.5±4a	***	13.8±3B	36.4±6b	**	nd	nd	ns
	1	81.2±4A	59.8±6a	**	8.1±3B	24.4±3b	*	nd	nd	ns
	2	74.5±5A	53.9±7a	**	7.5±2B	21.7±6b	*	nd	nd	ns
	3	77.2±4A	59.6±5a	**	2.2±1B	17.6±3b	*	nd	nd	ns
	4	72.5±7A	63.5±9A	ns	nd	7.2±2B	ns	nd	nd	ns
	5	76.4±5A	69.9±4A	ns	nd	6.6±1B	ns	nd	nd	ns
P. duplex	0	63.1±4A	22.8±5a	****	1.31±0.6B	12.4±2b	*	20.1±3C	42.2±4c	***
	1	46.2±5A	28.4±7a	**	12.2±3B	32.6±3b	**	12.5±2C	33.0±5c	**
	2	48.4±6A	32.6±6a	**	7.1±2B	20.6±5b	*	0.7±0.1C	14.2±3c	*
	3	76.0±5A	54.7±5a	***	22.4±4B	20.7±4B	ns	nd	11.7±2c	*
	4	73.2±4A	60.9±5a	**	18.0±2B	16.0±3B	ns	nd	nd	ns
	5	82.3±4A	71.7±4a	ns	nd	7.9±1B	ns	nd	nd	ns
C. aciculare	0	72.1±4A	32.8±5a	****	3.2±1B	19.0±2b	**	8.1±3C	30.0±6c	***
	1	57.6±4A	21.4±3a	****	16.8±2B	30.3±2b	**	7.4±3C	41.8±5c	****
	2	53.7±5A	35.9±9a	**	12.1±2B	31.3±5b	**	0.7±0.2C	12.3±3c	*
	3	71.8±5A	65.5±5A	ns	3.0±0.8B	14.7±3b	*	nd	nd	ns
	4	80.0±6A	73.0±4A	ns	nd	nd	ns	nd	nd	ns
	5	82.6±4A	78.4±7A	ns	nd	nd	ns	nd	nd	ns
S. paradoxum	0	84.2±5A	63.4±6a	**	13.0±3B	28.8±5b	*	nd	nd	ns
	1	62.8±7A	48.3±6a	*	16.5±4B	33.3±7b	*	nd	nd	ns
	2	62.4±7A	46.7±4a	*	22.0±2B	37.1±6b	*	nd	nd	ns
	3	79.7±5A	63.3±5a	*	1.8±0.8B	16.8±3b	*	nd	nd	ns
	4	81.0±3A	72.4±9A	ns	nd	2.6±0.8B	ns	nd	nd	ns
	5	83.6±6A	76.1±5A	ns	nd	nd	ns	nd	nd	ns

SL = Significance level; ns = non-significance; nd= not detected; * indicates significance level at 0.05; ** = 0.01; *** = 0.001; and **** = 0.0001.

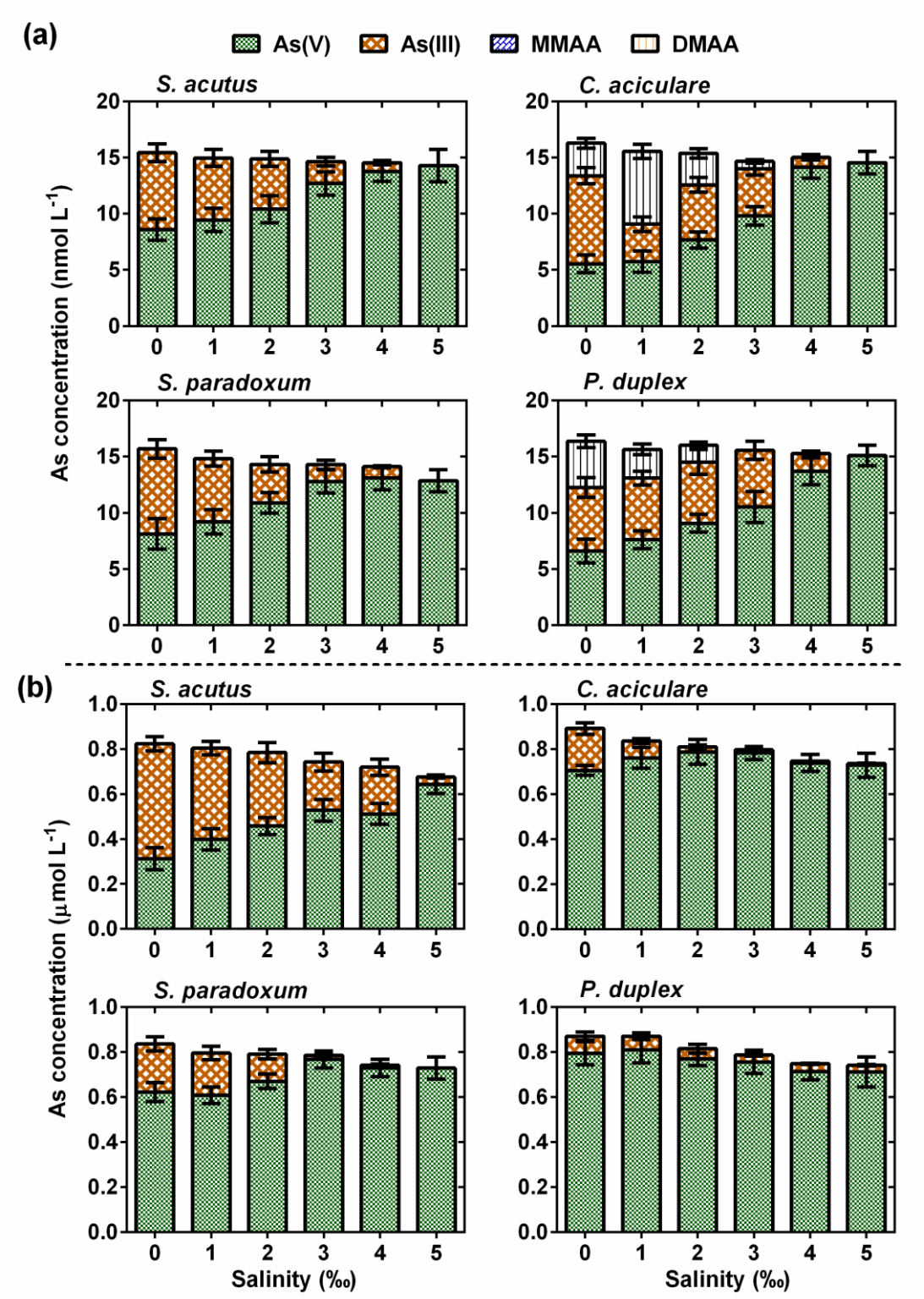

Figure 6.6 Salinity effects on As biotransformation by four freshwater phytoplankton species at low and high As(V) treatments; the conditions were as follows: (a) As(V) = 20 nmol L^{-1} and PO_4^{3-} = 1.0 µmol L^{-1}, and (b) As(V) = 1.0 µmol L^{-1} and PO_4^{3-} = 1.0 µmol L^{-1}. Data are presented as mean ± SD (n = 3).

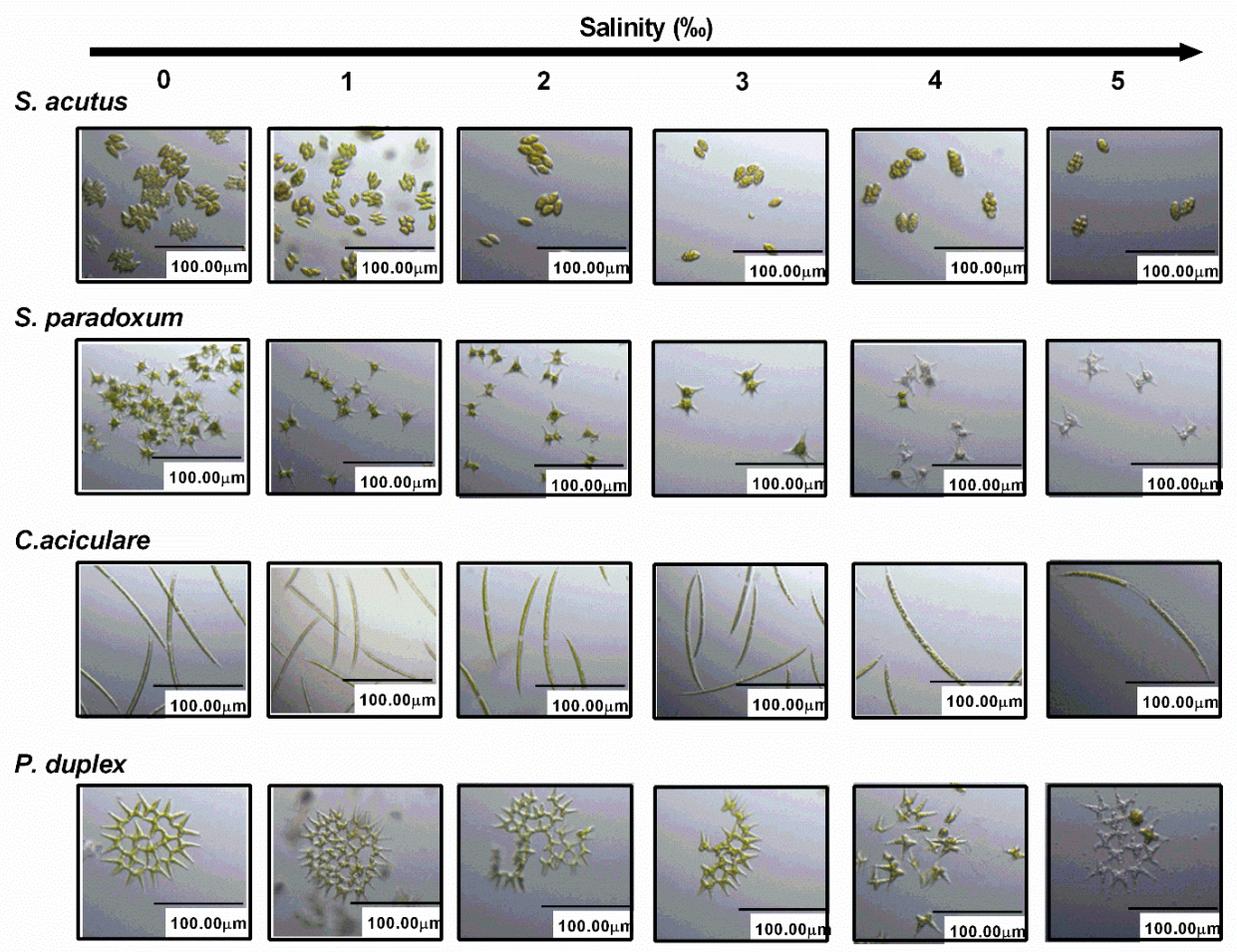

Figure 6.7 The changes in cell shape of four freshwater phytoplankton under salinity stress; the images were taken using a digital microscope (KEYENCE, VHX- 1000, Japan).

6.3.8 Arsenic biotransformation model

Based on the outcome of the present study (**Figure 6.8 a and b**), a conceptual model was developed to comprehend the As biotransformation mechanism of freshwater phytoplankton under salinity variation. It was observed that *S. acutus* and *S. paradoxum* biotransform As(V) to As(III) through a reduction reaction, while further methylation step was lacking. However, *C. aciculare* and *P. duplex* converted As(V) to As(III) by a reduction reaction and subsequently methylated to methyl As species. These results indicate that both reduction and methylation occurred simultaneously in the cells of *C. aciculare* and *P. duplex*. The methylation process is considered as a detoxification mechanism of photosynthetic microalgae, where iAs is biotransformed into less toxic methyl As species (Levy et al., 2005). For *S. acutus* and *S. paradoxum*, As(V) was reduced to As(III) under 0–5‰, but the reduction rate gradually decreased with increasing salinity. This was likely due to salinity stress that decreased the reduction rate of As(V) and its excretion from the cells (**Figure 6.8 a**). In the case of *C. aciculare* and *P. duplex*, As(V) was first reduced to As(III) and later methylated to methyl As species such as MMAA and DMAA (**Figure 6.8 b**). In this study, MMAA was not detected in any of the experiments. This is likely due to the prompt metabolic conversion of MMAA to DMAA rather than its excretion as an intermediate arsenic species (Cullen et al., 1994). After the uptake of As(V), it was reduced to As(III); and then, methylated to DMAA when cultured under 0–3‰. However, no methylation was observed at 4 and 5‰. Therefore, high salinity stress may inhibit the methylation process except for reduction to As(III) and its excretion to liquid medium (**Figure 6.8 b**).

As(V) and PO_4^{3-} have similar chemical characteristics that stimulate the competition for their uptake by the phytoplankton cells (Reed et al., 2015). An increased concentration of As(V) promotes its entry into the cell by a phosphate transporter (PT) through a competitive uptake process. Once inside the cells, As(V) is reduced to As(III) through As(V) reductase ArsC and later extruded from the cell via a specific pump ArsB (Meng et al., 2004). During cellular respiration, As(V), as the final electron acceptor, may be reduced to As(III). Later (i.e., at the time of cellular respiratory oxidation), As(III), as an electron donor, is oxidised to As(V). Glutathione (GSH) serves as an electron donor whose non-enzymatic reaction with arsenate reductase promotes As(V) reduction to As(III) (Yin et al., 2011). Phytochelatins (PCs) are thiol-based polypeptides that play an essential role in the detoxification process that promotes the formation of stable bonds with GSH (Morelli et al., 2005; Pawlik-Skowrońska et al., 2004) and further sequestering into the vacuoles of phytoplankton cells. During the methylation process,

arsenic methyltransferase proceeds as an active catalyst, which encourages the conversion of iAs to methyl As species (Zhang et al., 2013).

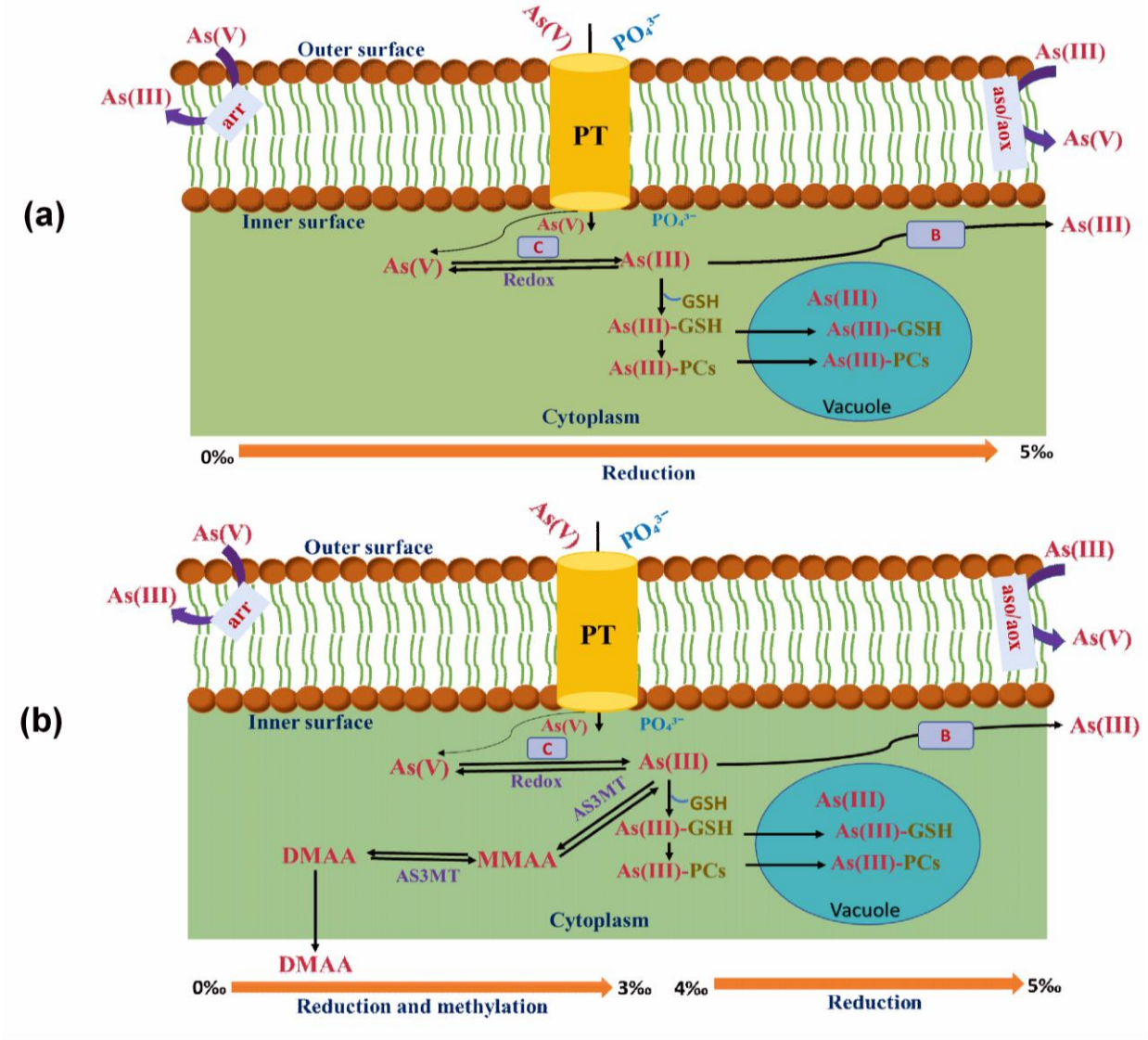

Figure 6.8 Conceptual model of As biotransformation by four freshwater phytoplankton under salinity stress; (a) *S. acutus* and *S. paradoxum* reduces As species (i.e., As(V) to As(III)) but does not participate in methylation reactions, and (b) *C. aciculare* and *P. duplex* participate in the reduction of As species as well as further methylation.

6.4 Conclusion

This study revealed a distinctive pattern of As accumulation, As biotransformation, and growth by four freshwater phytoplankton under various salinity stress conditions. Among the four phytoplankton, only two, i.e., *C. aciculare* and *P. duplex*, were able to reduce As(V) to As(III) and methylated species, i.e., DMAA. In contrast, *S. acutus* and *S. paradoxum*, did not exhibit methylation reaction. Moreover, these unique features were observed in the accumulation of total As in the cells of these organisms. The As reduction rate was lower in *S. paradoxum* than in other species. Further, under salinity stress, the studied phytoplankton cells adapted with corresponding physiological changes. Our results indicate that the pattern of As accumulation and speciation by freshwater phytoplankton, under low salinity stress, might be an insight for the As remediation point of view.

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Chapter 7:

Conclusion

7.1 Conclusion

To understand the As biotransformation potentials by aquatic microalgae, a series of experiments were conducted in the laboratory. Following conclusion was drown based on the findings of the study:

The study revealed the As biotransformation potential of three marine phytoplankton species, *viz. P. parvum*, *O. viridis*, and *E. gymnestica* under a wide salinity gradient. *O. viridis* showed maximum growth, As biotransformation, and total As accumulation ability under a broader range of salinity conditions (5–45‰) than the other phytoplankton species used in the experiment. The highest cell concentrations for all the species were observed on day 14 of culture. The photosynthetic efficiency was low for all the phytoplankton species at salinity levels of 0.3–5‰ and 40–50‰. The phytoplankton growth expectancy was significantly influenced by the availability of arsenic and phosphate in the medium, and salinity had its own effects under these conditions. A conceptual model of salinity effects on As uptake and biotransformation by the three marine phytoplankton species is proposed based on the findings of this study.

The potential growth and As biotransformation by six marine diatom species was investigated under various temperature (0–35°C) and salinity (0.3–50‰) conditions during three weeks of culture. Except for *T. nitzschioides* and *Skeletonema* sp., none of the species biotransformed As species at ≤ 5°C and ≥ 35°C. However, growth and As biotransformation and subsequent methylation were optimum between temperatures of 10 to 25°C and salinities of 10 to 35‰. The biological reduction, i.e. biotransformation of As(V) to As(III) and subsequent methylated arsenicals, was significantly different between day 10 and 17 speciation at different temperature and salinity conditions. The interrelated influence of temperature, salinity, and cell size on As biotransformation was also reported for the first time. These results suggest that each species has an optimum temperature and salinity tolerance range suitable for their adaptation metabolism, such as growth and the biotransformation of toxic As(V) to As(III) and further methylation to form methylated As species.

The integrated effects of environmental factors, including salinity, temperature, As and phosphate concentrations, and culture time on growth, As accumulation, biotransformation, and PE by marine microalgae was investigated. The marine diatom species *A. karianus* and *Skeletonema* sp. were used in the experiments. Diatom species were exposed to the

combination of temperatures and salinity in association with As and phosphate-enriched conditions. Microalgal growth, As accumulation, biotransformation, and PE were low at 5.0°C with salinities of 1.0‰ and 40‰. A conceptual model is given on the integrated effect of salinity, temperature, and nutrient concentrations on growth, As accumulation, biotransformation, and PE based on the findings of this study.

This study reported the toxicity, biotransformation, and release of different As species in freshwater phytoplankton, which may help us to better understand the biogeochemistry of As in freshwater systems. Inorganic As^{V} was taken up by the phytoplankton, biotransformed inside their cells, and then released into the culture medium as As^{III} during the exponential growth phase at a high As^{V} to phosphate ratio. The growth inhibition effects of As^{V} and As^{III} were significantly higher than those of orgAs, $DMAA^{V}$, and AB on the tested freshwater phytoplankton. The six freshwater phytoplankton strains examined could be categorized based on their As biotransformation patterns as follows: (i) those efficient in the biotransformation of As^{III} into As^{V}; (ii) those not efficient in As biotransformation, which rather maintained As^{V} inside their cells; and (iii) those efficient in the biotransformation of iAs into methylAs species (methylation) and complex orgAs species. These results reflect the differential bioaccumulation and biotransformation of As species by these phytoplankton inside their cells and their excretion of these As species into the environment. Further work is needed to assess whether the toxicity of As species differs for other aquatic microorganisms such as zooplankton, to better understand the influence of As on freshwater ecosystem dynamics.

This study reveals a distinctive pattern of growth, As accumulation, and their biotransformation by four freshwater phytoplankton under various salinity stress. Among four phytoplankton, only two such as *C. aciculare* and *P. duplex* were able to reduce As(V) to As(III) and further methylated species, DMAA, whereas *S. acutus* and *S. paradoxum* were unable to took part in methylation reaction. These unique features were also observed for accumulation of total As into the cell of these organisms. The As reduction rate was observed lower by *S. Paradoxum* than other species. Under salinity stress, studied phytoplankton cells were adapted with physiological changes. The pattern of As accumulation and speciation by freshwater phytoplankton under low salinity stress might be an insight for As remediation point of view.

Appendix 2.1 Chemical composition of modified f/2 culture medium in artificial seawater.

Chemicals	Concentration (mol L^{-1})
$NaNO_3$	8.82×10^{-4}
$NaH_2PO_4\cdot2H_2O$	3.84×10^{-5}
$Na_2SiO_3\cdot9H_2O$	3.46×10^{-5}
Na_2SeO_3	1.00×10^{-7}
$CoSO_4\cdot7H_2O$	4.27×10^{-8}
$ZnSO_4\cdot7H_2O$	7.31×10^{-8}
$MnCl_2\cdot4H_2O$	9.10×10^{-7}
$CuSO_4\cdot5H_2O$	2.80×10^{-8}
$Na_2MoO_4\cdot2H_2O$	2.89×10^{-8}
Vitamin B_{12}	3.69×10^{-10}
Thiamine HCl	2.96×10^{-7}
Biotin	2.05×10^{-9}

Appendix 3.1 Composition of artificial seawater (Lyman and Flemming, 1940).

Compounds	**Weight**
$MgCl_2\cdot6H_2O$	108 g
NaCl	240 g
$NaHCO_3$	1.96 g
KCl	6.8 g
Na_2SO_4	40.00
NaF	0.029 g
H_3BO_3	0.250 g
$SrCl\cdot6H_2O$	0.122 g
KBr	1.00 g
$CaCl_2\cdot6H_2O$	14.3 g
Purified water	
Total	**10 L**

Appendix 5.1 Chemical composition of CSi culture medium for freshwater phytoplankton used in this study.

Nutrients/Chemicals	**Concentrations**
$Ca(NO_3)_2 \cdot 4H_2O$	635 mmol L^{-1}
KNO_3	989 mmol L^{-1}
KH_2PO_4	213 mmol L^{-1}
$MgSO_4 \cdot 7H_2O$	162 mmol L^{-1}
$MnCl_2 \cdot 4H_2O$	0.546 mmol L^{-1}
$ZnSO_4 \cdot 7H_2O$	0.23 mmol L^{-1}
$CoCl_2 \cdot 6H_2O$	0.05 mmol L^{-1}
$Na_2MoO_4 \cdot 2H_2O$	2.73 mmol L^{-1}
$FeCl_3$	1.31 mmol L^{-1}
$Na_2EDTA \cdot 2H_2O$	8.06 mM
$Na_2SiO_3 \cdot 9H_2O$	10 mg mL^{-1}
Vitamin	1.0×10^{-6} mg mL^{-1}
4-(2-hydroxyethyl)-1-piperazine ethane sulphonate	50 mg mL^{-1}

Appendix 6.1 Composition of modified C medium used for the culture of four freshwater phytoplankton.

Reagent	**Concentration**
EPW + 35‰ artificial sea water	1 L
HEPES	500 mg L^{-1}
Tris(hydroxymethyl)aminomethane	150 mg L^{-1}
$Ca(NO_3)_2 \cdot 4H_2O$	100 mg L^{-1}
KNO_3	30.7 mg L^{-1} (225.6 µmol L^{-1})
KH_2PO_4	136.1 µg L^{-1} (1 µmol L^{-1})
$MgSO_4 \cdot 7H_2O$	40 mg L^{-1}
Vitamin B12 stock solution	1 µg L^{-1}
Thiamine-HCl stock solution	1 µg L^{-1}
Biotine stock solution	1 µg L^{-1}
PIV metal	3 mL L^{-1}
Chemical composition of PIV metal	
$Na_2EDTA \cdot 2H_2O$	1 g L^{-1}
$FeCl_3 \cdot 6H_2O$	196 mg L^{-1}
$MnCl_2 \cdot 4H_2O$	36 mg L^{-1}
$ZnSO_4 \cdot 7H_2O$	22 mg L^{-1}
$CoCl_2 \cdot 6H_2O$	4 mg L^{-1}
$Na_2Mo_4 \cdot 2H_2O$	2.5 mg L^{-1}

Appendix 6.2 Composition of artificial seawater used for culturing the freshwater phytoplankton.

Reagent	**Composition**
NaF	2.9 mg L^{-1}
H_3BO_3	25 mg L^{-1}
$SrCl_2 \cdot 6H_2O$	12.2 mg L^{-1}
KBr	100 mg L^{-1}
$NaHCO_3$	196 mg L^{-1}
KCl	683 mg L^{-1}
$CaCl_2 \cdot 2H_2O$	1.4346 g L^{-1}
NaCl	23.954 g L^{-1}
Na_2SO_4	4.004 g L^{-1}
$MgCl_2 \cdot 6H_2O$	10.787 g L^{-1}