Dissertation

Molecular responses of phytoplankton to iron limitation

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Doctoral Dissertation

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ABSTRACT

The present study focused on the proteomic study of phytoplankton to Fe limited conditions. It was found that marine phytoplankton *Prymnesium parvum* employ several strategies to compensate Fe stress. It was found that marine phytoplankton biosynthesize different proteins under low total Fe and ligand-induced Fe-limited conditions. To understand responses of phytoplankton to Fe deficiency, responses to nitrogen and phosphate deficiency were also studied and was found that several proteins were differentially expressed in marine phytoplankton *P. parvum* in response to different exposure levels of nitrate, phosphate and iron. The expression levels of an 83 kDa protein in *P. parvum* can be used as biomarker of N-status, while a 121 kDa protein can be used as a biomarker of P-deplete condition in aquatic systems. In addition, two protein can be used as biomarker of Fe-status (deplete or replete conditions) in aquatic systems. Under Fe-limited condition, *P. parvum* may increase Fe uptake efficiency by increasing ABC transporters. Under Fe-limited condition, the phytoplankton may also increase photorespiration which needs high metabolic energy. The phytoplankton may satisfy the demand of high metabolic energy (for photorespiration and ABC transporter) by increasing ATP synthase in chloroplast. Fe stress may cause oxidative stress in phytoplankton which is thought to be defended by up-regulating oxidative stress response proteins MnSOD and STK. Carbohydrate degradation and glycolytic activity was thought to be increased under Fe-limited conditions. Marine phytoplankton *P. parvum* also alters its cellular biochemical processes by up-regulating several proteins involved in photosynthesis. The phytoplankton also increased biosynthesis of some PSII component proteins under Fe-limited conditions.

Experiment 1:

In the first experiment, growth and proteomic responses of three marine phytoplankton strains (*Pleurochrysis roscoffensis, Prymnesium parvum* and *Skeletonema marinoi-dohrnii* complex) under Fe limitation by low total Fe and ligand induced Fe limitation condition were studied. Compared to Fe-rich conditions (Fe = 1 μM), the growth of phytoplankton decreased substantially under low total Fe-limited condition (Fe = 0.07 μM for *P. roscoffensis* and *S. marinoi-dohrnii*

complex, and Fe = 0.03 µM for *P. parvum*). The marine phytoplankton express different proteins under Fe-limited and Fe-rich conditions and the protein expression differ among the phytoplankton. In low total Fe condition, *P. parvum* expressed three proteins (19, 32 and 42 kDa), which were very similar to those expressed by *P. roscoffensis* in the same condition. In addition, *S. marinoi-dohrnii complex* expressed a 55 kDa protein, which was not expressed by the other phytoplankton in low total Fe condition. The phytoplankton expressed different proteins under ligand induced Fe-limited conditions. Both *P. parvum* and *S. marinoi-dohrnii complex* expressed a common protein (19 kDa), while *S. marinoi-dohrnii complex* produced an additional protein of 33 kDa under desferrioxamine B (DFO-B) induced Fe-limited conditions. A 19 kDa protein was expressed by *P. roscoffensis* under low total Fe and ligand-induced Fe-limited conditions; however, the *P. roscoffensis* expressed a new protein of 27 kDa under diethylenetriamine-N,N,N',N'',N''-pentaacetate (DTPA) induced Fe-limited conditions instead of the 33 kDa protein that was expressed under low total Fe and DFO-B-induced Fe-limited conditions. The results indicate that marine phytoplankton alters their Fe acquisition strategy under low total Fe and ligand-mediated Fe-limitations by expressing different proteins.

Experiment 2:

Nitrogen (N), phosphorus (P) and Iron (Fe) are important nutrients for phytoplankton, and are key limiting nutrients in marine systems. In the second study, growth and protein expression of marine phytoplankton *Prymnesium parvum* under different nitrate, phosphate and iron conditions were investigated in order to evaluate whether proteins and their expression level can be used as biomarker of N, P, and Fe conditions in aquatic systems. The growth of *P. parvum* increased with the increase of nitrate, phosphate and iron concentrations in the culture medium. Protein expression levels also differed significantly ($p < 0.001$) for different nitrate, phosphate and iron conditions in the culture medium. The expression level of an 83 kDa protein at 0 and 5 µM nitrate treatments differed significantly ($p < 0.001$) from those at 20, 30, 50 and 100 µM nitrate treatments, indicating the expression levels of this protein as a biomarker of N status in the culture medium. A 121 kDa protein

was expressed at phosphate stress conditions ([P] ≤ 1.0 μM), while this protein was not expressed at phosphate replete conditions ([P] ≥ 5 μM). Therefore, the expression of 121 kDa protein in *P. parvum* is indicative of phosphate deplete condition in aquatic systems. The expression level of a 42 kDa protein was significantly higher ($p < 0.01$) at Fe-stress condition ([Fe] = 0.01 μM) than Fe-replete conditions ([Fe] ≥ 0.1 μM). In addition, a new protein of 103 kDa was only expressed under Fe-deplete condition ([Fe] = 0.01 μM). Therefore, the 42 and 103 kDa proteins can be used as a biomarker of Fe-limitation condition of aquatic systems. However, further studies (two dimensional gel electrophoresis and mass spectrometry) are needed to identify and characterize these proteins in *P. parvum*.

Experiment 3:

Iron is a vital limiting factor for phytoplankton in vast regions of oceans, notably the high nutrient low chlorophyll (HNLC) regions. Therefore, it is needed to be acquainted with the adaptation mechanisms of marine phytoplankton under Fe-limited condition. In third experiment, *Prymnesium parvum* was grown under Fe-deplete (0.0025 μM) and Fe-rich (0.05 μM) conditions, and proteomic responses were compared. Compared to 0.05 μM Fe concentration (Fe-rich condition) *P. parvum* showed substantially reduced growth under 0.0025 μM Fe concentration (Fe-limit condition). In sodium dodecyl sulfate gel electrophoresis, 7 proteins (16, 18, 32, 34, 75, 82, and 116 kDa) were highly expressed under Fe-deplete condition, while one protein (23 kDa) was highly expressed under Fe-rich condition. The proteins were subjected to 2-dimensional gel electrophoresis to differentiate individual proteins, and were identified by MALDI-TOF-MS analysis. The results showed that under Fe-deplete condition *P. parvum* increases the biosynthesis of ABC transporters and a flagellar associated protein which may change their Fe acquisition strategy in order to facilitate Fe acquisition under Fe stress condition. Under Fe-deplete condition, *P. parvum* increases the synthesis of RuBisCO and/or pyruvate dehydrogenase, phosphoribosylaminoimidazole-succinocarboxamide synthase, malate dehydrogenase, glycosyl hydrolase, glyceraldehyde-3-phosphate dehydrogenase, and two Fe-independent oxidative stress response proteins, MnSOD and Serine threonine kinase. These proteins are assumed to be involved in a number

of cellular biochemical processes, such as photorespiration, glycolysis followed by degradation of stored polysaccharides, and managing of iron limitation induced oxidative stresses that facilitate marine phytoplankton to cope with Fe-limitation.

Experiment 4:

Iron (Fe) is essential for photosynthesis, a process used by autotrophic organisms to convert light energy into chemical energy, of autotrophic organisms. Fe limitation may influence the growth and productivity of microalgae by reducing photosynthetic efficiency. In the fourth experiment, the effect of Fe-limitation on growth and photosynthetic activities of marine microalga (*Prymnesium parvum*) were investigated. Marine microalga *P. parvum* was grown in f/2 medium in artificial seawater under Fe-limit (0.0025 µM) and Fe-rich (0.05 µM) conditions. Compared to Fe-rich condition (the highest of 156 cell mL^{-1} d^{-1} at 10^{th} day), *P. parvum* showed substantially reduced growth rate under Fe-limit condition (the highest of 97 cell mL^{-1} d^{-1} at 8^{th} day). Proteomic responses of *P. parvum* to Fe-limitation were also studied for understanding the strategies of marine microalgae that the organism employ in order to maintain photosynthetic activity and productivity. Under Fe-limit condition, *P. parvum* was found to up-regulate eleven proteins, which were identified by matrix-assisted laser desorption-ionization-time of flight-mass spectrometer (MALDI-TOF-MS) analysis. Results showed that *P. parvum* increases the biosynthesis of several proteins associated with photosystem II (PSII), which is assumed to be a strategy of the microalga in order to cope with the Fe-limitation. The up-regulation of chloroplast ATP synthase biosynthesis would be a strategy of the microalga to meet the cellular energy under Fe-limit condition. Thus, microalgae alter the biosynthesis of several photosynthetic proteins in order to sustain Fe-limit condition.

1

INTRODUCTION

The global atmospheric carbon dioxide (CO_2) level was around 280 ppm during the preindustrial and last interglacial periods and lowest during the glacials (around 200 ppm during 18,000 years B.P.) (Barnola *et al.*, 1987). This change in CO_2 concentration was predicted due to photosynthetic utilization of CO_2 by marine phytoplankton (Martin, 1990). Before the Industrial Era atmospheric CO_2 concentration was 280 ppm for several thousand years and it has risen continuously since then, reaching to 367 ppm in 1999 (Ipcc, 2001), 379 ppm in 2005 (Ipcc, 2005), and 384 ppm in 2007 (Bernstein *et al.*, 2007). Potential global warming induced by the accumulation of green house gases (GHGs) such as CO_2 has become an important environmental issue. CO_2 alone is responsible for 77% anthropogenic GHGs emission and about 80% of radiative forcing of all GHGs for global warming and climate change (Bernstein *et al.*, 2007). The rate of growth of CO_2 emissions was much higher during the 10-years period of 1995-2004 (0.92 $GtCO_2$ per year) than during the previous period of 1970-1994 (0.43 $GtCO_2$-eq per year) and this rate is thought to be increased in the recent years due to anthropogenic CO_2 emission by burning fossil fuels for industrialization, transportation, energy supply, forestry, etc. (Bernstein *et al.*, 2007). The increasing atmospheric CO_2 is the reason for

importance of investigations of methods for minimizing and removing anthropogenic CO_2 emission. Some hypotheses have proposed for the removal of the anthropogenic CO_2 which can be divided in two broad categories:

1. Chemical reaction-based removal of CO_2
2. Biological CO_2 mitigation

Chemical reaction-based CO_2 removal processes are relatively costly, energy-consuming, have question of environmental issue and the mitigation benefits are not so appreciating (Yang *et al.*, 2008). Therefore, biological CO_2 mitigation processes, in other word, biofixation of armospheric CO_2 by photosynthesis is the suitable alternative. About 71% of the earth surface is covered by ocean which provides approximately 300 times more space for life than that provided by land and freshwater combined (Lalli & Parsons, 1997). Phytoplankton grow only within the well-illuminated sunlit surface region and is responsible for the primary productivity of oceanic food web. Marine phytoplankton account for more than half of the global primary production, and play a major role in regulating global climate and sequestering carbon dioxide (CO_2) from the atmosphere (Benner, 2011, Field *et al.*, 1998).

Growth of phytoplankton and their photosynthesis activity mostly depends on the availability of a variety of inorganic elements called nutrients and light. Well illuminated light is available all over the ocean except ice cover and little seasonal effect (Lancelot et al., 2000). Among different nutrients, nitrogen (N), phosphorus (P) and iron (Fe) has been found to limit phytoplankton productivity in a variety of aquatic systems, in the open oceans, coastal upwelling areas, as well as lakes (Ammerman *et al.*, 2003, Moore *et al.*, 2008, North *et al.*, 2007, Vuorio *et al.*, 2005). Nitrogen has historically been considered to be the predominant limiting nutrient in many coastal and deep sea ecosystems (Elser *et al.*, 2007, Howarth & Marino, 2006), which is assumed to be responsible for the decrease of phytoplankton growth of those aquatic systems. Several studies have also reported inhibition of primary production due to P limitation in many areas of seas all over the world (Ammerman *et al.*, 2003, Moore *et al.*, 2008, Van Mooy *et al.*, 2006, Wu *et al.*, 2000). Several

investigation of low or limited phytoplankton growth in a vast area of high nutrients containing ocean have reported which open a new dimension of research (Abbott & Zion, 1985, Cullen, 1991, Eppley, 1972, Sand-Jensen & Søndergaard, 1981). In this high nutrient containing phytoplankton community, chlorophyll content was found to be low which was assumed to be due to low iron availability (Behrenfeld *et al.*, 1996, Martin & Fitzwater, 1988, Martin *et al.*, 1990, Morel *et al.*, 1991). Phytoplankton bloom was also achieved by artificial and natural iron fertilization in these high nutrient low chlorophyll (HNLC) areas which confirm iron as the limiting factor of phytoplankton growth (Banse, 1991, Blain *et al.*, 2007, Boyd *et al.*, 2007, Coale *et al.*, 1996, Martin *et al.*, 1994), and ultimately, speculation that these blooms could be utilized to capture and sequester carbon (CO_2) from atmosphere (Morrissey & Bowler, 2012, Tortell *et al.*, 2008).

Phytoplankton need Fe for photosynthetic and respiratory electron transport (Morrissey & Bowler, 2012), and is directly involved in nitrate, nitrite and sulfate reduction, nitrogen fixation, chlorophyll biosynthesis, and a number of other biosynthetic and degradative reactions, including those involved in detoxification of O_2 radicals (Geider & La Roche, 1994). The Southern Ocean is the largest HNLC region which plays a major role in the climate system, and is recognized as the oceanic body most sensitive to climate change (Blain *et al.*, 2007) where Fe deficiency is limiting phytoplankton growth in the. Still, limited iron availability impairs phytoplankton growth in as much as 40% of the ocean, notably in the Southern Ocean, Gulf of Alaska, equatorial Pacific Ocean, and north Pacific Ocean (Moore *et al.*, 2001, Morrissey & Bowler, 2012). It is also difficult to measure total and available proportion of Fe for phytoplankton in a marine system. Cellular macromolecules such as proteins, phytochelatins, glutathione, phospholipid, fatty acid, and RNA have been used as bioindicators of the status of nutrients, metals, and environmental pollutants (Bartell, 2006, Kawakami *et al.*, 2006). In addition, several studies have shown that the expression levels of certain protein or the ratio of certain proteins (Inda & Peleato, 2002, Kawakami *et al.*, 2006) can be used as bioindicator of a certain element's status in an ecosystem. Therefore, it is important to identify a suitable biomarker to know Fe status (also N, P) of a plankton community.

Sub-nanomolar concentration of dissolved Fe in open oceans can effectively confines phytoplankton growth (Marchetti *et al.*, 2006, Watson *et al.*, 2000). Some environmental events can also reduce Fe availability for phytoplankton. For example, dissolution of atmospheric CO_2 in the ocean leads to the changes in the chemistry of seawater (Tortell *et al.*, 2008), commonly described by the term 'ocean acidification' that may create Fe-limited conditions for phytoplankton by decreasing the bioavailability of dissolved Fe (Shi et al., 2010). In addition, more than 90% of total dissolved Fe is bound to organic ligands (Gledhill & Van Den Berg, 1994, Rue & Bruland, 1995), which can increase the Fe solubility in seawater, but also can dramatically reduce the inorganic Fe fraction (i.e. ionic Fe and Fe-hydroxides). Whether these organic ligands increase or decrease the availability of Fe for individual phytoplankton species is still a question of debate (Hutchins *et al.*, 1998, Hutchins *et al.*, 1999, Maldonado & Price, 1999). Therefore, it is also important to understand Fe uptake mechanism under low total Fe and ligand-induced Fe-limited conditions.

Prymnesium parvum is an ichthyotoxic phytoplankton grown in geographically widespread areas of the open oceans including temperate waters, both in the northern and southern hemispheres (Fistarol *et al.*, 2003), vast areas of temperate and tropical open seas (Edvardsen & Imai, 2006), Baltic Sea (Edler *et al.*, 1984) and also of vast coastal plankton community (Carvalho & Granéli, 2010, Edvardsen & Imai, 2006). Dimension of this phytoplankton is generally 8-19 µm × 4-10 µm (Manton & Leedale, 1963). Because of its geographical widespread and have a major effect on natural communities, it has been the focus of considerable research during the last decade (Carvalho & Granéli, 2010, Fistarol *et al.*, 2003, La Claire, 2006, Maki *et al.*, 2008, Susan *et al.*, 2010). Except some coastal areas, most of the above areas where *P. parvum* is naturally grown are low in Fe content (Allen *et al.*, 2008, Mills *et al.*, 2004, Vuorio *et al.*, 2005, Wells *et al.*, 1995). Therefore, we have selected *P. parvum* to study the molecular responses and adaptation mechanisms under Fe limited condition.

Objectives of the Study

The main objective of the present study is to know the molecular and proteomic responses of marine phytoplankton *Prymnesium parvum* to Fe-limited condition. Fe-limited condition can be occurs by low total Fe content or by chelating of Fe by ligands, therefore, we investigate responses under both these Fe-limitation conditions. The specific aims and objectives of the study are:

1. To investigate the protein expressions of marine phytoplankton under low total Fe and ligand-induced Fe-limited conditions

2. To investigation whether protein expression level of phytoplankton can be used as bioindicator of nitrogen, phosphorus and iron conditions

3. To know the proteomic responses and cellular adaptation of *Prymnesium parvum* to Fe limitation

4. Photosynthetic adaptation of *Prymnesium parvum* to Fe limitation

References

Abbott, M. R. and Zion, P. M. (1985) Satellite observations of phytoplankton variability during an upwelling event. *Contin. Shelf Res.,* **4,** 661-680.

Allen, A. E., Laroche, J., Maheswari, U., Lommer, M., Schauer, N., Lopez, P. J., Finazzi, G., Fernie, A. R. and Bowler, C. (2008) Whole-cell response of the pennate diatom *Phaeodactylum tricornutum* to iron starvation. *Proc. Natl. Acad. Sci.,* **105,** 10438.

Ammerman, J. W., Hood, R. R., Case, D. A. and Cotner, J. B. (2003) Phosphorus deficiency in the Atlantic: An emerging paradigm in oceanography. *Eos, Transactions American Geophysical Union,* **84,** 165.

Banse, K. (1991) Rates of phytoplankton cell division in the field and in iron enrichment experiments. *Limnol. Oceanogr.,* **36,** 1886-1898.

Barnola, J. M., Raynaud, D., Korotkevich, Y. S. and Lorius, C. (1987) Vostok ice core provides 160,000-year record of atmospheric CO_2. *Nature,* **329,** 408-414.

Bartell, S. M. (2006) Biomarkers, bioindicators, and ecological risk assessment- A brief review and evaluation. *Environmental Bioindicators,* **1,** 60-73.

Behrenfeld, M. J., Bale, A. J., Kolber, Z. S., Aiken, J. and Falkowski, P. G. (1996) Confirmation of iron limitation of phytoplankton photosynthesis in the equatorial Pacific Ocean. *Nature,* **383,** 508-511.

Benner, R. (2011) Loose ligands and available iron in the ocean. *Proceedings of the National Academy of Sciences,* **108,** 893-894.

Bernstein, L., Bosch, P., Canziani, O., Chen, Z., Christ, R., Davidson, O., Hare, W., Huq, S., Karoly, D., Kattsov, V., Kundzewicz, Z., Liu, J., Lohmann, U., Manning, M., Matsuno, T., Menne, B., Metz, B., Mirza, M., Nicholls, N., Nurse, L., Pachauri, R., Palutikof, J., Parry, M., Qin, D., Ravindranath, N., Reisinger, A., Ren, J., Riahi, K., Rosenzweig, C., Rusticucci, M., Schneider, S., Sokona, Y., Solomon, S., Stott, P., Stouffer, R., Sugiyama, T., Swart, R., Tirpak, D., Vogel, C. and Yohe, G. (2007) Climate Change 2007: Synthesis Report: An Assessment of the Intergovernmental Panel on Climate Change. Intergovernmental Panel on Climate Change (IPCC), Valencia, Spain., pp. 1-52.

Blain, S., Quéguiner, B., Armand, L., Belviso, S., Bombled, B., Bopp, L., Bowie, A., Brunet, C., Brussaard, C. and Carlotti, F. (2007) Effect of natural iron fertilization on carbon sequestration in the Southern Ocean. *Nature,* **446,** 1070-1074.

Boyd, P. W., Jickells, T., Law, C. S., Blain, S., Boyle, E. A., Buesseler, K. O., Coale, K. H., Cullen, J. J., De Baar, H. J. W. and Follows, M. (2007) Mesoscale iron enrichment experiments 1993-2005: Synthesis and future directions. *Science,* **315,** 612-617.

Carvalho, W. F. and Granéli, E. (2010) Contribution of phagotrophy versus autotrophy to *Prymnesium parvum* growth under nitrogen and phosphorus sufficiency and deficiency. *Harmful Algae,* **9,** 105-115.

Coale, K. H., Johnson, K. S., Fitzwater, S. E., Gordon, R. M., Tanner, S., Chavez, F. P., Ferioli, L., Sakamoto, C., Rogers, P., Fillero, F., Steinberg, P., Nightingale, P., Cooper, D., Cochlanl, W. P., Landry, M. R., Constantinou, J., Rollwagen, G., Trasvina, A. and Kudela, R. (1996) A massive phytoplankton bloom induced by an ecosystem-scale iron fertilization experiment in the equatorial Pacific Ocean. *Nature,* **383,** 10.

Cullen, J. J. (1991) Hypotheses to explain high-nutrient conditions in the open sea. *Limnol. Oceanogr.,* **36,** 1578-1599.

Edler, L., Hällfors, G., Niemi, Å. and Tiedeakatemia, S. (1984) A preliminary check-list of the phytoplankton of the Baltic Sea. *Acta Bot. Fenn.,* **128,** 1-26.

Edvardsen, B. and Imai, I. (2006) The ecology of harmful flagellates within Prymnesiophyceae and Raphidophyceae*Ecology of Harmful Algae.* Springer, pp. 67-79.

Elser, J. J., Bracken, M. E. S., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., Ngai, J. T., Seabloom, E. W., Shurin, J. B. and Smith, J. E. (2007) Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecology letters,* **10,** 1135-1142.

Eppley, R. W. (1972) Temperature and phytoplankton growth in the sea. *Fish. Bull,* **70,** 1063-1085.

Field, C. B., Behrenfeld, M. J., Randerson, J. T. and Falkowski, P. (1998) Primary production of the biosphere: Integrating terrestrial and oceanic components. *Science,* **281,** 237-240.

Fistarol, G. O., Legrand, C. and Granéli, E. (2003) Allelopathic effect of *Prymnesium parvum* on a natural plankton community. *Mar. Ecol. Prog. Ser.,* **255,** 115-125.

Geider, R. J. and La Roche, J. (1994) The role of iron in phytoplankton photosynthesis, and the potential for iron-limitation of primary productivity in the sea. *Photosynthesis research,* **39,** 275-301.

Gledhill, M. and Van Den Berg, C. M. G. (1994) Determination of complexation of iron (III) with natural organic complexing ligands in seawater using cathodic stripping voltammetry. *Mar. Chem.,* **47,** 41-54.

Howarth, R. W. and Marino, R. (2006) Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: Evolving views over three decades. *Limnology and Oceanography,* **51,** 364-376.

Hutchins, D. A., Ditullio, G. R., Zhang, Y. and Bruland, K. W. (1998) An iron limitation mosaic in the California upwelling regime. *Limnol. Oceanogr.,* **43,** 1037-1054.

Hutchins, D. A., Franck, V. M., Brzezinski, M. A. and Bruland, K. W. (1999) Inducing phytoplankton iron limitation in iron-replete coastal waters with a strong chelating ligand. *Limnol. Oceanogr.,* **44,** 1009-1018.

Inda, L. and Peleato, M. L. (2002) Immunoquantification of flavodoxin and ferredoxin from *Scenedesmus vacuolatus* (Chlorophyta) as iron-stress molecular markers. *Eur. J. Phycol.,* **37,** 579-586.

Ipcc (2001) *Climate change 2001: The scientific basis. Contribution of working group I to the third assessment report of the intergovernmental panel on climate change.* Vol., Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Ipcc (2005) *IPCC special report on carbon dioxide capture and storage. Prepared by working group III of the Intergovernmental Panel on Climate Change.* Vol., Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Kawakami, S. K., Gledhill, M. and Achterberg, E. P. (2006) Production of phytochelatins and glutathione by marine phytoplankton in response to metal stress. *J. Phycol.,* **42,** 975-989.

La Claire, J. (2006) Analysis of expressed sequence tags from the harmful alga, Prymnesium parvum (Prymnesiophyceae, Haptophyta). *Mar. Biotechnol.,* **8,** 534-546.

Lalli, C. and Parsons, T. (1997) *Biological oceanography: An introduction.* Vol., Butterworth-Heinemann Ltd, Linacre House, Jordan Hill, Oxford OX2 8DP.

Lancelot, C., Hannon, E., Becquevort, S., Veth, C. and De Baar, H. J. W. (2000) Modeling phytoplankton blooms and carbon export production in the Southern Ocean: Dominant controls by light and iron in the Atlantic sector in Austral spring 1992. *Deep Sea Research Part I: Oceanographic Research Papers,* **47,** 1621-1662.

Maki, T., Suzuki, T., Kido, K., Nakahara, A., Higashi, T., Hasegawa, H., Ueda, K. and Saijoh, K. (2008) Effect of Iron Stress on Gene Expression in Harmful Microalga *Prymnesium Parvum*. *J. Ecotechnol. Res.,* **14,** 13-16.

Maldonado, M. T. and Price, N. M. (1999) Utilization of iron bound to strong organic ligands by plankton communities in the subarctic Pacific Ocean. *Deep Sea Research Part II: Topical Studies in Oceanography,* **46,** 2447-2473.

Manton, I. and Leedale, G. F. (1963) Observations on the fine structure of prymnesium parvum carter. *Arch. Mikrobiol.*, **45,** 285-303.

Marchetti, A., Sherry, N. D., Kiyosawa, H., Tsuda, A. and Harrison, P. L. J. (2006) Phytoplankton processes during a mesoscale iron enrichment in the NE subarctic Pacific: Part I-biomass and assemblage. *Deep Sea Research Part II: Topical Studies in Oceanography,* **53,** 2095-2113.

Martin, J. H. (1990) Glacial-interglacial CO_2 change: The iron hypothesis. *Paleoceanography,* **5,** 1-13.

Martin, J. H., Coale, K. H., Johnson, K. S., Fitzwater, S. E., Gordon, R. M., Tanner, S. J., Hunter, C. N., Elrod, V. A., Nowicki, J. L., Coley, T. L., Barber, R. T., Lindley, S., Watson, A. J., Van Scoy, K., Law, C. S., Liddicoat, M. I., Ling, R., Stanton, T., Stockel, J., Collins, C., Anderson, A., Bidigare, R., Ondrusek, M., Latasa, M., Millero, F. J., Lee, K., Yao, W., Zhang, J. Z., Frederich, G., Sakamoto, C., Chavez, F., Buck, K., Kolber, Z., Yungel, J., Turner, S., Nightingale, P., Hatton, A., Liss, P. and Tindale, N. W. (1994) Testing the iron hypothesis in ecosystems of the equatorial Pacific Ocean. *Nature,* **371,** 123.

Martin, J. H. and Fitzwater, S. E. (1988) Iron deficiency limits phytoplankton growth in the north-east Pacific subarctic. *Nature,* **331,** 341-343.

Martin, J. H., Fitzwater, S. E. and Gordon, R. M. (1990) Iron deficiency limits phytoplankton growth in Antarctic waters. *Global Biogeochem. Cycles,* **4,** 5-12.

Mills, M. M., Ridame, C., Davey, M., La Roche, J. and Geider, R. J. (2004) Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. *Nature,* **429,** 292-294.

Moore, C. M., Mills, M. M., Langlois, R., Milne, A., Achterberg, E. P., La Roche, J. and Geider, R. J. (2008) Relative influence of nitrogen and phosphorous availability on phytoplankton physiology and productivity in the oligotrophic sub-tropical North Atlantic Ocean. *Limnology and Oceanography,* **53,** 291.

Moore, J. K., Doney, S. C., Glover, D. M. and Fung, I. Y. (2001) Iron cycling and nutrient-limitation patterns in surface waters of the World Ocean. *Deep Sea Research Part II: Topical Studies in Oceanography,* **49,** 463-507.

Morel, F. M. M., Rueter, J. G. and Price, N. M. (1991) Iron nutrition of phytoplankton and its possible importance in the ecology of ocean regions with high nutrient and low biomass. *Oceanography,* **4,** 56-61.

Morrissey, J. and Bowler, C. (2012) Iron utilization in marine cyanobacteria and eukaryotic algae. *Frontiers in microbiology,* **3**.

North, R. L., Guildford, S. J., Smith, R. E. H., Havens, S. M. and Twiss, M. R. (2007) Evidence for phosphorus, nitrogen, and iron colimitation of phytoplankton communities in Lake Erie. *Limnology and Oceanography,* **52,** 315-328.

Rue, E. L. and Bruland, K. W. (1995) Complexation of iron(III) by natural organic ligands in the Central North Pacific as determined by a new competitive ligand equilibration/adsorptive cathodic stripping voltammetric method. *Marine Chemistry,* **50,** 117-138.

Sand-Jensen, K. and Søndergaard, M. (1981) Phytoplankton and epiphyte development and their shading effect on submerged macrophytes in lakes of different nutrient status. *Int. Rev. Ges. Hydrobiol. Hydrograph.,* **66,** 529-552.

Shi, D., Xu, Y., Hopkinson, B. M. and Morel, F. M. M. (2010) Effect of ocean acidification on iron availability to marine phytoplankton. *Science,* **327,** 676-679.

Susan, V. J., Theodore, W. V., Daniel, L. R., James, P. G. and Bryan, W. B. (2010) Probabilistic ecological hazard assessment of microcystin-LR allelopathy to *Prymnesium parvum*. *J. Plankton Res.,* **00,** 1-14.

Tortell, P. D., Payne, C. D., Li, Y., Trimborn, S., Rost, B., Smith, W. O., Riesselman, C., Dunbar, R. B., Sedwick, P. and Ditullio, G. R. (2008) CO_2 sensitivity of Southern Ocean phytoplankton. *Geophys. Res. Lett.,* **35,** L04605.

Van Mooy, B. A. S., Rocap, G., Fredricks, H. F., Evans, C. T. and Devol, A. H. (2006) Sulfolipids dramatically decrease phosphorus demand by picocyanobacteria in oligotrophic marine environments. *Proceedings of the National Academy of Sciences,* **103,** 8607-8612.

Vuorio, K., Lagus, A., Lehtimäki, J. M., Suomela, J. and Helminen, H. (2005) Phytoplankton community responses to nutrient and iron enrichment under different nitrogen to phosphorus ratios in the northern Baltic Sea. *Journal of Experimental Marine Biology and Ecology,* **322,** 39-52.

Watson, A. J., Bakker, D. C. E., Ridgwell, A. J., Boyd, P. W. and Law, C. S. (2000) Effect of iron supply on southern ocean CO_2 uptake and implications for glacial atmospheric CO_2. *Nature,* **407,** 730-733.

Wells, M. L., Price, N. M. and Bruland, K. W. (1995) Iron chemistry in seawater and its relationship to phytoplankton: A workshop report. *Mar. Chem.,* **48,** 157-182.

Wu, J., Sunda, W., Boyle, E. A. and Karl, D. M. (2000) Phosphate depletion in the western North Atlantic Ocean. *Science,* **289,** 759-762.

Yang, H., Xu, Z., Fan, M., Gupta, R., Slimane, R. B., Bland, A. E. and Wright, I. (2008) Progress in carbon dioxide separation and capture: A review. *J. Environ. Sci.,* **20,** 14-27.

2

LITERATURE REVIEW

The present study is closely related to climatic change, specially, the consequences of mitigation of anthropogenic CO_2 from atmosphere using phytoplankton by Fe fertilization of the ocean known as “Iron Hypothesis”. Anthropogenic CO_2 emission is the primary cause of climate change. Therefore, a brief review is presented here on the rules of marine phytoplankton and primary production on climate change and the background of t study.

2.1. Climate Change:

According to Intergovernmental Panel on Climate Change (IPCC), climate change refers to any change in climate over time, whether due to natural variability or as a result of human activity (Bernstein et al., 2007).

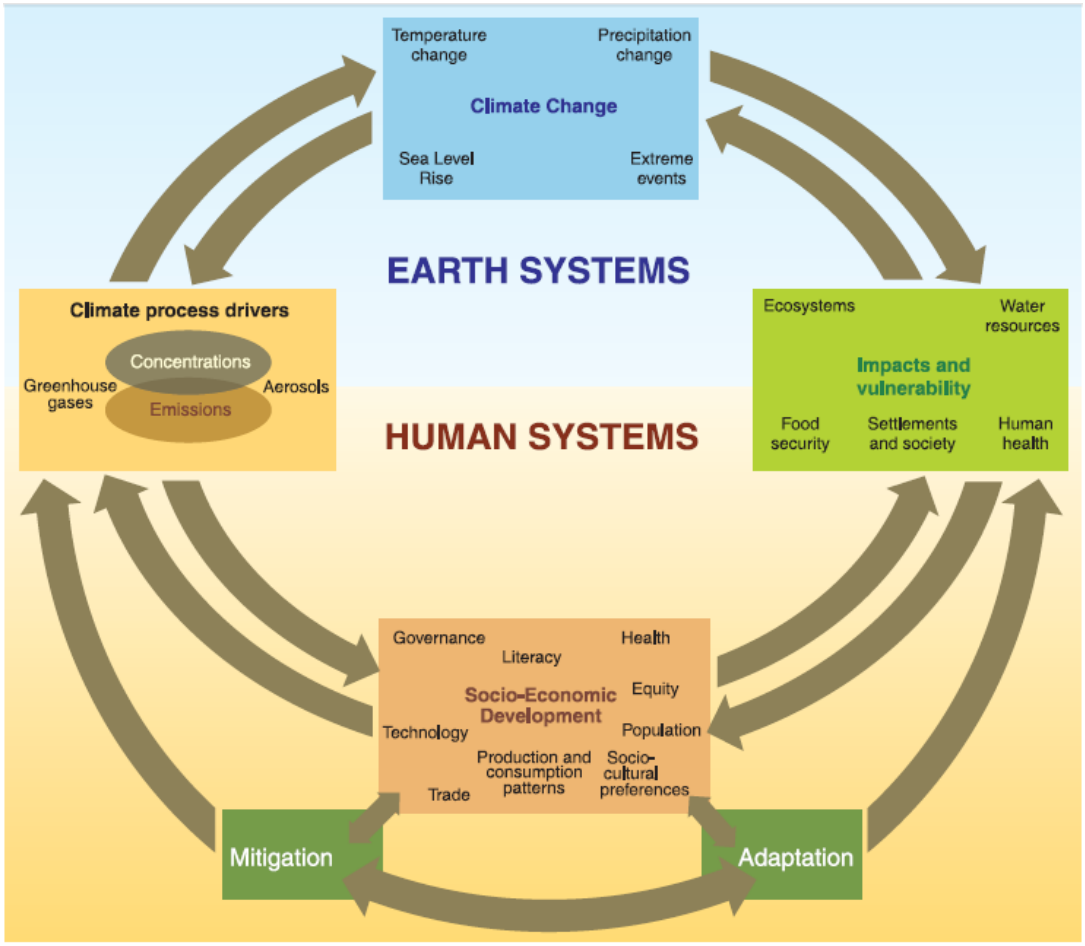

Fig. 2.1: A schematic framework representing anthropogenic drivers, impacts of and responses to climate change, and their linkages (clockwise). Possible development pathways and global emissions constraints that would reduce the risk of future impacts that society may wish to avoid are also presented (counterclockwise) (Bernstein *et al.*, 2007).

2.2. Effect of Climate Change:

2.2.1. Increase in temperature

Eleven of the last twelve years (1995-2006) rank among the twelve warmest years in the instrumental record of global surface temperature (Bernstein *et al.*, 2007). The temperature increase is widespread over the globe and is greater at higher northern latitudes. The warming trend over the 50 years from 1956 to 2005 (0.13 [0.10 to 0.16]°C per decade) is nearly twice that for the 100 years from 1906

to 2005 (Bernstein *et al.*, 2007). Recent regional changes in temperature have had discernible impacts on physical and biological systems.

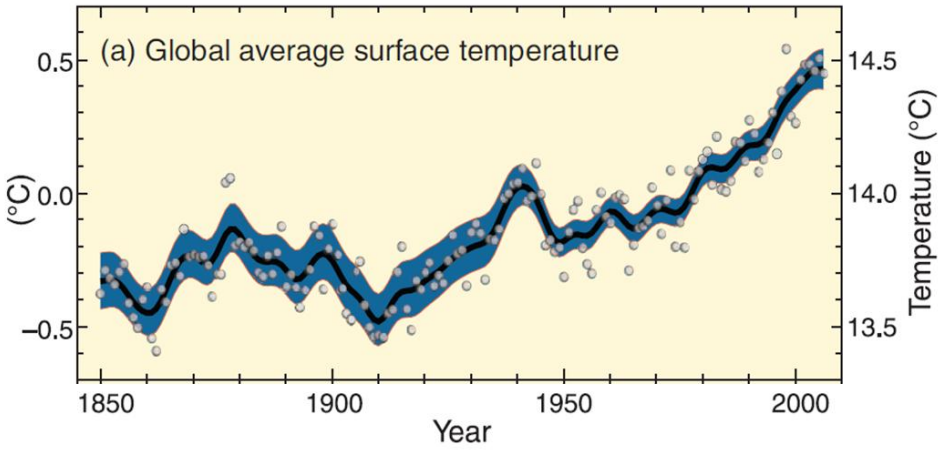

Fig. 2.2: A schematic diagram of global temperature over last 150 years.

2.2.2. Sea level rise

Sea level rise is consistent with warming. Global average sea level rose at an average rate of 1.8 [1.3 to 2.3] mm per year over 1961 to 2003 and at an average rate of about 3.1 [2.4 to 3.8] mm per year from 1993 to 2003 (Bernstein *et al.*, 2007). This faster rate for 1993 to 2003 than 1961 to 2003 reflects decadal variation or an increase in the longer term trend.

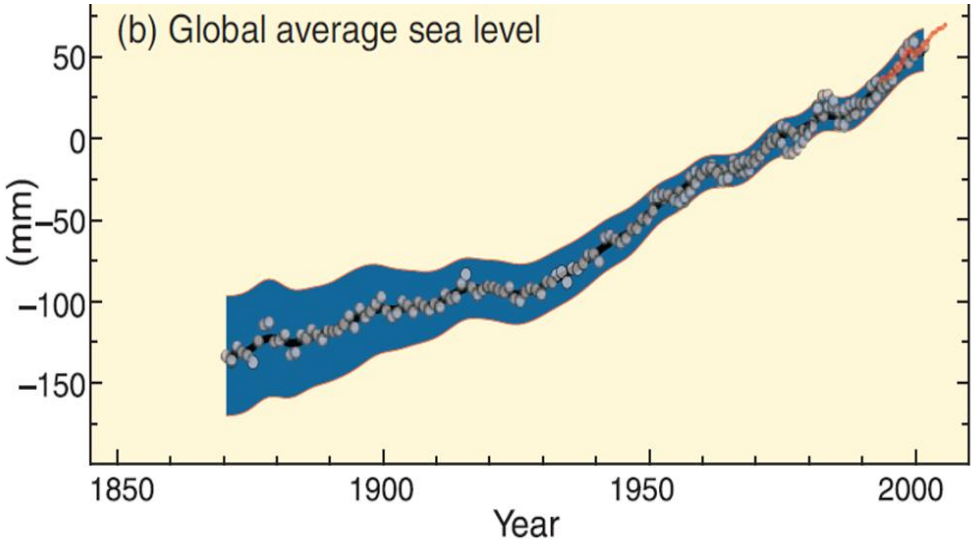

Fig. 2.3: A schematic diagram of sea level increase over last 130 years.

2.2.3. Decrease in snow and ice content

Observed decreases in snow and ice extent are also consistent with warming. Satellite data since 1978 show that annual average Arctic sea ice extent has shrunk by 2.7 [2.1 to 3.3]% per decade, with larger decreases in summer of 7.4 [5.0 to 9.8]% per decade (Bernstein *et al.*, 2007). The maximum areal extent of seasonally frozen ground has decreased by about 7% in the Northern Hemisphere since 1900, with decreases in spring of up to 15%.

2.2.4. Change in precipitation

According to IPCC report precipitation trend from 1900 to 2005 showed significantly increased precipitation in eastern parts of North and South America, northern Europe and northern and central Asia whereas precipitation declined in the Sahel, the Mediterranean, southern Africa and parts of southern Asia. Globally, the area affected by drought has *likely* increased since the 1970.

2.2.5. Some other events

- It is very likely that cold days, cold nights and frosts have become less frequent over most land areas, while hot days and hot nights have become more frequent.
- It is likely that heat waves have become more frequent over most land areas.
- It is likely that the frequency of heavy precipitation events (or proportion of total rainfall from heavy falls) has increased over most areas.
- There is observational evidence of an increase in intense tropical cyclone activity in the North Atlantic since about 1970, and in some other regions where concerns over data quality are greater.

2.3. Effect of Climate Change on Marine Phytoplankton Community

Observational evidence from all continents and most oceans shows that many natural systems are being affected by climate changes. For example, Changes in some Arctic and Antarctic ecosystems, including those in sea-ice biomes, and predators at high levels of the food web (Bernstein *et al.*, 2007, Tortell *et al.*, 2008). There is high confidence, based on substantial new evidence, that observed changes in marine and freshwater biological systems are associated with rising water temperatures, as well as related changes in ice cover, salinity, oxygen levels and circulation (Forster *et al.*, 2007). Shifts in ranges and changes in algal, plankton and fish abundance in high-latitude oceans; increases in algal and zooplankton abundance in high-latitude and high-altitude areas; and range changes and earlier fish migrations are also observed (Bernstein *et al.*, 2007, Tortell *et al.*, 2008). There is increasing evidence of climate change impacts on coral reefs, separating the impacts of climate-related stresses from other stresses. The following events can greatly affect marine phytoplankton growth and ecology:

- Rising water temperature and water heat content. The oceans are warming. Over the period 1961 to 2003, global ocean temperature has risen by 0.10°C from the surface to a depth of 700 m (Bindoff et al., 2007).
- Changing in salinity. Large-scale, coherent trends of salinity are observed for 1955 to 1998, and are characterized by a global freshening in sub polar latitudes (pronounced in the Pacific) and a salinification of shallower parts of the tropical and subtropical oceans (pronounced in the Atlantic and Indian Oceans) (Bindoff *et al.*, 2007).
- Decreasing of ice cover and increasing of sea level.
- Changing in oxygen level and inorganic carbon content
- Changing in oceanic water circulation, thermal expansion and water masses
- Changing in ocean biogeochemistry
- Ocean acidification (mixing with excess CO_2). The uptake of anthropogenic carbon since 1750 has led to the ocean becoming more acidic with an

average decrease in pH of 0.1 units. Revelle and Suess (1957) explained how CO_2 can be mixed rapidly into the upper layers of the ocean while the time to mix with the deep ocean may take many centuries. IPCC projection gives a reduction in average global surface ocean pH of between 0.14 and 0.35 units over the 21st century (Fig. 4) (Bernstein et al., 2007).

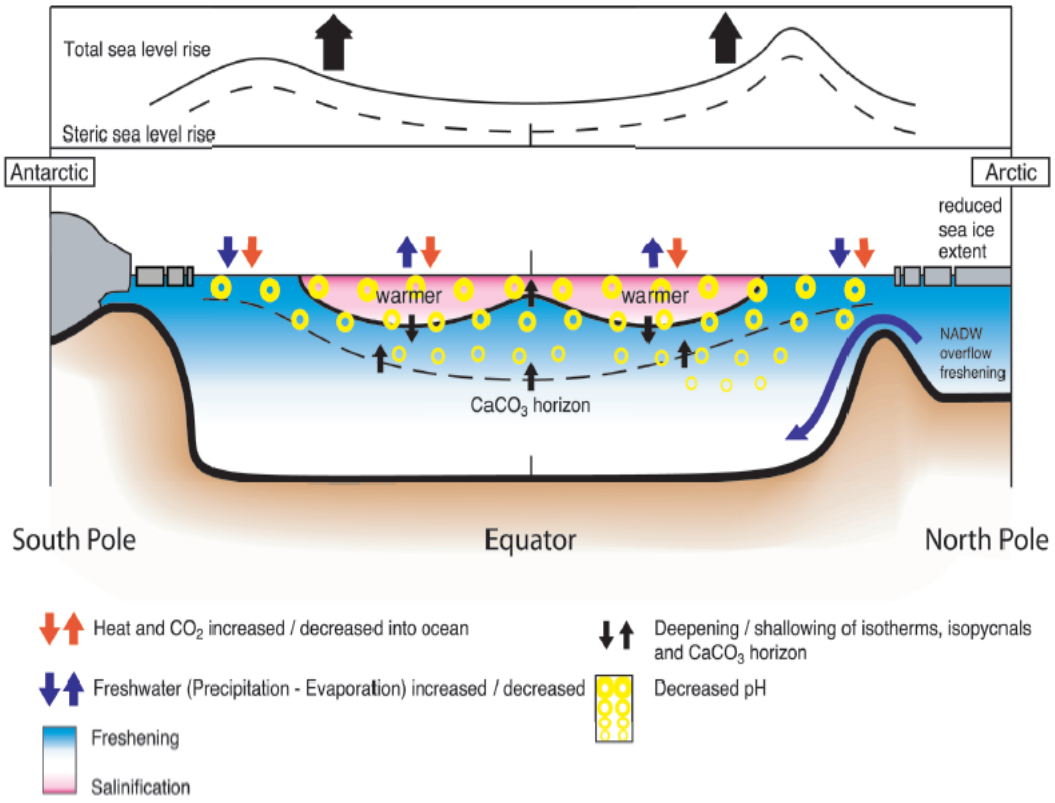

Fig. 2.4: Schematic diagram of the observed changes in the ocean state. The legend identifies the direction of the changes in the variables (Bindoff et al., 2007).

2.4. Climate Change is Mainly Due to CO_2 Emission

Changes in the atmospheric concentrations of GHGs and aerosols, land cover and solar radiation alter the energy balance of the climate system and are drivers of climate change (Bernstein *et al.*, 2007). In 1895, Arrhenius (1896) followed with a climate prediction based on greenhouse gases, suggesting that a 40% increase or decrease in the atmospheric abundance of the trace gas CO_2 might trigger the glacial advances and retreats. Callendar (1938) solved a set of equations linking greenhouse

gases and climate change. He found that a doubling of atmospheric CO_2 concentration resulted in an increase in the mean global temperature of 2°C, with considerably more warming at the poles, and linked increasing fossil fuel combustion with a rise in CO_2 and its greenhouse effects. In 1947, it was proposed that 1.3°C warming in the North Atlantic sector of the Arctic since the 19th century could be explained entirely by greenhouse gas warming (Le Treut *et al.*, 2007). Revelle and Suess (1957) explained why part of the emitted CO_2 was observed to accumulate in the atmosphere rather than being completely absorbed by the oceans. GHGs affect the absorption, scattering and emission of radiation within the atmosphere and at the Earth's surface which is used to compare warming or cooling influences on global climate (Bernstein *et al.*, 2007). Relative proportion of increase of GHGs is shown in Fig. 6. GHG includes:

- Carbon di oxide (CO_2). From 10 kyr before present up to the year 1750, CO_2 abundances stayed within the range 280 ± 20 ppm (Indermühle et al., 1999). During the industrial era, CO_2 abundance rose roughly exponentially to 367 ppm in 1999 and to 379 ppm in 2005, 384 ppm in 2007 and at the end of 2100 the concentration may reach to 1000 ppm (Bernstein et al., 2007).
- Methane (CH_4). The global atmospheric concentration of CH_4 has increased from a pre-industrial value of about 715 ppb to 1732 ppb in the early 1990s, and was 1774 ppb in 2005. Methane abundances were initially increasing at a rate of about 1% per year (Fraser et al., 1981, Graedel & Mcrae, 1980) but then slowed to an average increase of 0.4% per year over the 1990s (Dlugokencky et al., 1998) with the possible stabilisation of CH_4 abundance.
- Nitrous oxide (N_2O). The global atmospheric N_2O concentration increased from a pre-industrial value of about 270 ppb (Flückiger et al., 1999) to 314 ppb in 1998 (IPCC,2001), and 319 ppb in 2005 (Bernstein et al., 2007).
- Halocarbons (a group of gases containing fluorine, chlorine or bromine) and synthetic halocarbons (chlorofluorocarbons; CFCs, hydrofluorocarbons, perfluorocarbons, halons and sulphur hexafl uoride) are greenhouse gases with large global warming potentials. Halocarbons have increased from a

near-zero pre-industrial background concentration, primarily due to human activities (Forster et al., 2007).

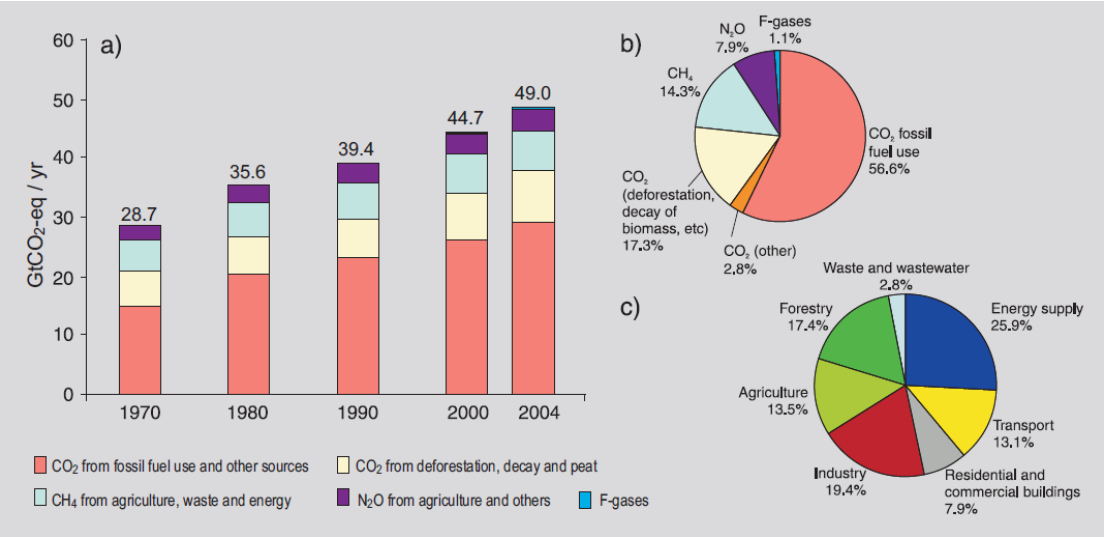

Fig. 2.5: (a) Global annual emissions of anthropogenic GHGs from 1970 to 2004. **(b)** Share of different anthropogenic GHGs in total emissions in 2004 in terms of CO_2-eq. **(c)** Share of different sectors in total anthropogenic GHG emissions in 2004 in terms of CO_2-eq. (Bernstein *et al.*, 2007).

Annual emissions of CO_2, the most important anthropogenic GHG, have grown between 1970 and 2004 by about 80%, from 21 to 38 gigatonnes (Gt), and represented 77% of total anthropogenic GHG emissions in 2004 (Le Treut *et al.*, 2007). The rate of growth of CO_2-eq emissions was much higher during the 10-year period of 1995-2004 (Bernstein *et al.*, 2007). CO_2 emissions are thought to be increased during the recent years in consistent with the projection given in Fig. 7.

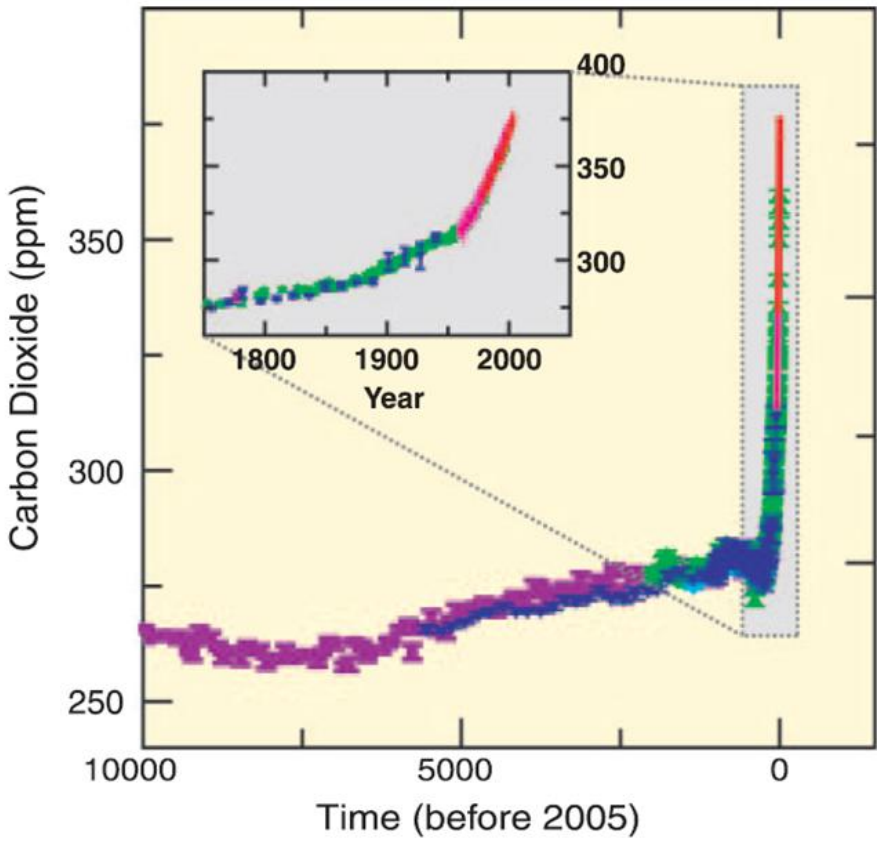

Fig. 2.6: Atmospheric CO_2 concentration over the last 10,000 years (large panel) and since 1750 (inset panel). Measurements are shown from ice cores (symbols with different colors for different studies) and atmospheric samples (red lines).

2.5. Atmospheric CO_2 Increase and Fe Chemistry in the Sea

Some environmental events of climate changes such as dissolution of atmospheric CO_2 in the ocean will likely acidify the ocean leads to the changes in the chemistry of seawater, affect the physiology, growth and species composition of phytoplankton assemblages (Tortell *et al.*, 2008). The increase in total inorganic carbon caused a decrease in the depth at which calcium carbonate dissolves, and also caused a decrease in surface ocean pH by an average of 0.1 units since 1750. Direct observations of pH at available time series stations for the last 20 years also show trends of decreasing pH at a rate of 0.02 pH units per decade (Bindoff *et al.*, 2007). Doney *et al.* (2009) also reported rising atmospheric CO_2 reduces ocean pH and causes wholesale shifts in seawater carbonate chemistry. The process of ocean acidification is well documented in field data, and the rate will accelerate over this

century unless future CO_2 emissions are curbed dramatically. Acidification alters seawater chemical speciation and biogeochemical cycles of many elements and compounds. One well-known effect is the lowering of calcium carbonate saturation states, which impacts shell-forming marine organisms from plankton to benthic molluscs, echinoderms, and corals. Many calcifying species exhibit reduced calcification and growth rates in laboratory experiments under high CO_2 conditions. Moreover, 'ocean acidification' almost certainly create Fe-limited conditions for phytoplankton by decreasing the bioavailability of dissolved Fe and thus reduce algal productivity (Shi et al., 2010). Ocean acidification also causes an increase in carbon fixation rates in some photosynthetic organisms (both calcifying and noncalcifying).Consequently, much effort should be given to predict how climate change will affect iron availability and phytoplankton growth, and how altered phytoplankton growth will itself affect climate change. It showed be studied extensively on the possible fate of artificial Fe fertilization on total oceanic ecosystem and phytoplankton community.

2.6. CO_2 Mitigation and Stabilization of Atmospheric GHGs

The CO_2 mitigation strategies can be generally classified into two categories:

(1) Chemical reaction-based approaches
(2) Biological CO_2 mitigation.

2.6.1. Chemical reaction-based approaches

IPCC proposed CO_2 Capture and Storage (CCS) as a process consisting of the separation of CO_2 from industrial and energy-related sources, transport to a storage location and long-term isolation from the atmosphere (IPCC, 2005). The widespread application of CCS would depend on technical maturity, costs, overall potential, diffusion and transfer of the technology to developing countries and their capacity to apply the technology, regulatory aspects, environmental issues and public perception. CCS also suggested other options including energy efficiency

improvements, the switch to less carbon-intensive fuels, nuclear power, renewable energy sources, enhancement of biological sinks, and reduction of non-CO_2 greenhouse gas emissions.

A popular chemical reaction-based CO_2 mitigation approach is achieved by cyclic carbonation/de-carbonation reactions in which gaseous CO_2 reacts with solid metal oxide (represented by MO) to yield metal carbonate (MCO_3) as presented in the first equation below (Gupta & Fan, 2002). Then the metal carbonate can be thermally regenerated to metal oxide and CO_2 by heating the metal carbonate beyond its calcination temperature.

$$MO + CO_2 \rightarrow MCO_3$$

$$MCO_3 + MO \rightarrow CO_2$$

The calcination reaction based CO_2 separation process in a fossil-fuel-fired utility would consist of a carbonation reactor and a regeneration reactor. Lime, with CaO being the primary component, is probably the most popular solid adsorbent for chemical reaction-based CO_2 mitigation due to its relatively low costs (Grasa & Abanades, 2006, Lu *et al.*, 2006). Another gas-absorption process for CO_2, SO_2 and NO_x separation from a gas mixture is washing with aqueous amine solution (Resnik *et al.*, 2004), among which monoethanolamine is the most widely employed for CO_2 removal (Blauwhoff *et al.*, 1984). CO_2 desorption and, therefore, the regeneration of monoethanolamine could be performed by heating the product solution to facilitate the reverse reaction. Water vapor in the regenerated CO_2 could be easily separated by condensation. CO_2 fixation and desorption reactions can be represented by the following reactions:

$$CO_2 + 2C_2H_4OH–NH_2 \rightarrow C_2H_4OH–NHCO_2^- + C_2H_4OH–NH_3^+$$

$$C_2H_4OH–NHCO_2^- + C_2H_4OH–NH_3^+ \rightarrow CO_2 + 2C_2\ H_4OH–NH_2$$

The chemical reaction-based CO_2 fixation schemes mentioned above typically consist of three procedures: separation, transportation, and sequestration. The cost of CO_2 separation and compression to 110 bars (for transportation) is

estimated to be $30–50 per ton of CO_2, and transportation and sequestration are estimated to cost about $1–3 per ton per 100 km and $1–3 per ton of CO_2, respectively (Gupta & Fan, 2002, Shi & Shen, 2003). Many chemical approaches are reported to be effective for CO_2 mitigation and all of these follow more or less similar strategies (D'alessandro *et al.*, 2010, Liang *et al.*, 2004, Sakakura *et al.*, 2007, Yang *et al.*, 2008). As these methods for capturing CO_2 are relatively costly and energy-consuming, the mitigation benefits are not so appreciating and therefore necessary to develop cost-effective and sustainable alternatives to curb the soaring emission (Wang *et al.*, 2008). The whole process is presented in Fig. 6.

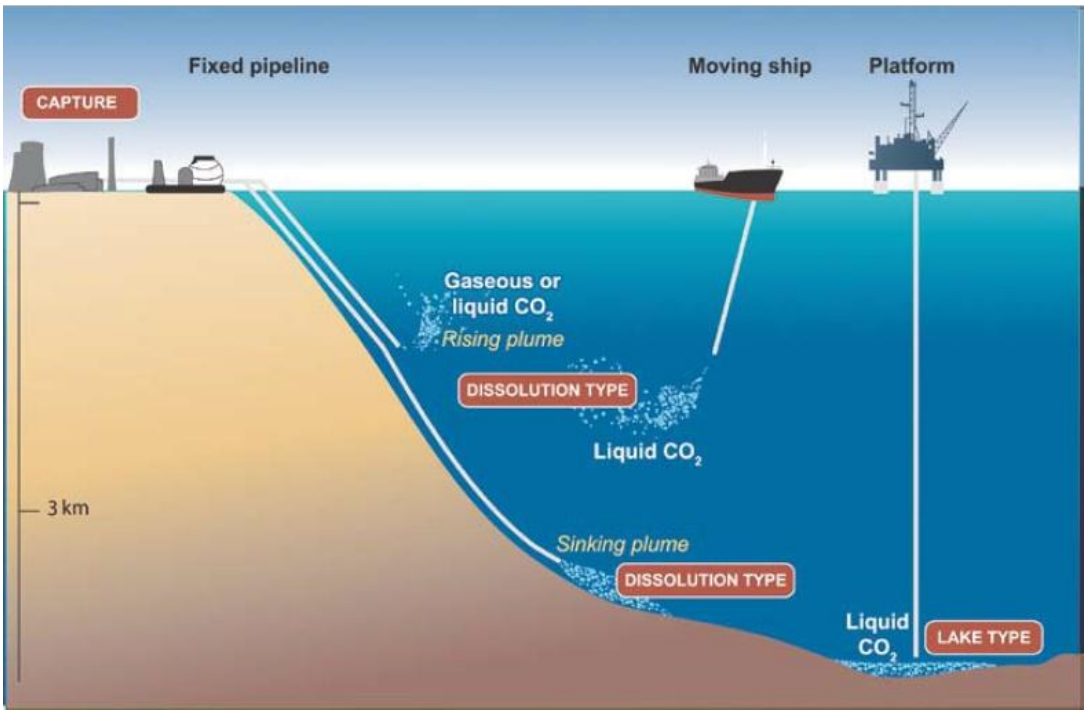

Fig. 2.7: Overview of ocean storage concepts. In "dissolution type" ocean storage, the CO_2 rapidly dissolves in the ocean water, whereas in "lake type" ocean storage, the CO_2 is initially a liquid on the sea floor (Bernstein *et al.*, 2007).

2.6.2. Biological CO_2 mitigation

It is estimated that 1.4±0.7 Gt-carbon is captured by terrestrial systems from atmosphere via photosynthesis (Yamasaki, 2003) which can be increased by forestation, reforestation of arid lands and greening of deserts. A high amount of

atmospheric CO_2 can be removed by the photosynthetic process of terrestrial plants (Brown, 1996, Kohlmaier *et al.*, 1998). The potential capacity for carbon sequestration in terrestrial systems is estimated to be 5-10 Gt-carbon annually (Yamasaki, 2003). Though a significant amount of CO_2 is fixed annually by plants, but based on the slow rate of higher plants, the fresh water requirement, and the high cost of land for growing these plants, this appears not to be a feasible option for removing CO_2.

Ocean stores more CO_2 than terrestrial vegetation. The ocean contains about 38,000 Gt-carbon, and about 1.7±0.5 Gt is taken up annually from the atmosphere (Yamasaki, 2003). The potential of phytoplankton to sequestration of 50–100 Gt-carbon annually is much higher than that of terrestrial vegetation (Yamasaki, 2003). Among many attempts to reduce the quantity of CO_2 in the atmosphere, biotechnology of using microalgae in a photobioreactor has extensively been studied since the beginning of the 1990's (Jeong *et al.*, 2003). Conventional terrestrial plants are not very efficient in capturing solar energy. On the other hand, phytoplankton have high growth rates and photosynthetic efficiencies. It is estimated that the biomass productivity of phytoplankton could be 50 times more than that of switch grass, which is the fastest growing terrestrial plant (Demirbaş, 2006, Li *et al.*, 2008). Biofuel production using phytoplankton farming could be a good alternative for efficient atmospheric CO_2 mitigation (Hu *et al.*, 2008, Jeong *et al.*, 2003, Li *et al.*, 2008, Righelato & Spracklen, 2007, Wang *et al.*, 2008). This offers the following advantages:

- The high growth rate of microalgae makes it possible to satisfy the massive demand on biofuels using limited resources.
- The tolerance of microalgae to high CO_2 content in gas streams allows high-efficiency CO_2 mitigation.
- Nitrous oxide release could be minimized.
- Microalgal farming could be potentially more cost effective.

2.7. CO_2 Mitigation by Marine Phytoplankton

2.7.1. Overview of present status

Phytoplankton are photosynthesizing microscopic organisms that inhabit the upper sunlit layer of almost all oceans and bodies of fresh water. More than two third surface of the earth is covered by ocean where phytoplankton is responsible for the primary productivity, the creation of organic compounds from carbon dioxide dissolved in the water, a process that sustains the aquatic food web (Lalli & Parsons, 1997). The total inorganic carbon content of the oceans has increased by 118 ± 19 GtC between the end of the pre-industrial period (about 1750) and 1994, and continues to increase (Bindoff *et al.*, 2007). It is more likely that the fraction of emitted carbon dioxide that was taken up by the oceans has decreased, from 42 ± 7% during 1750 to 1994 to 37 ± 7% during 1980 to 2005. This would be consistent with the expected rate at which the oceans can absorb carbon, but the uncertainty in this estimate does not allow firm conclusions (Bindoff *et al.*, 2007).

2.7.2. Iron hypothesis and CO_2 removal by Fe fertilization

The idea that iron may be limiting phytoplankton was suggested and investigated by Gran (1931). Many studies were carried out in this regard in the last century. Martin et al. (1991) observed dissolved iron concentrations in surface waters in some ocean habitats are as low as 20-30 pM, that are unlikely to support high phytoplankton biomass. Then he hypothesize that the decrease of global atmospheric CO_2 level from around 280 ppm (during the preindustrial and last interglacial periods) to around 200 ppm during 18,000 years B.P. (during the glacials) (Barnola *et al.*, 1987) was due to photosynthetic utilization of CO_2 by marine phytoplankton (Martin, 1990). The Fe availability at that time was thought to be high enough for phytoplankton bloom which reduced the atmospheric CO_2 level. The controversy pronounced when Martin and his colleagues suggested Fe fertilization of the ocean for atmospheric CO_2 removal (Martin, 1990, Martin, 1992, Martin *et al.*, 1994, Martin *et al.*, 1991). Most of the iron debate centers on the

remote high nutrients low chlorophyll (HNLC) regions of the subarctic Pacific, the equatorial Pacific and the Southern Ocean. These regions are characterized by a persistence of excess major nutrients (N, P) and low biomass relative to coastal systems having similar major nutrient concentrations. Because the root cause(s) for this situation may have important ramification to the ocean-atmosphere exchange of CO_2 and global climate cycles (Martin, 1990), a special symposium was held (February 1991) to address what controls phytoplankton production in nutrient-rich areas of the open sea. John Martin and his colleagues argued that an inadequate iron supply was the major factor (Martin *et al.*, 1991). Results from bottle and on spot Fe enrichment studies of theirs and others along with some natural phytoplankton bloom incidence support this hypothesis (Coale *et al.*, 1996, De Baar *et al.*, 1990, Greene *et al.*, 1994, Martin *et al.*, 1994, Martin & Fitzwater, 1988, Martin *et al.*, 1990, Price *et al.*, 1994), though alternate interpretations have suggested that other factors also play an important role (Banse, 1991, Buma *et al.*, 1991, Mitchell *et al.*, 1991, Nelson & Smith, 1991). Still, limited iron availability impairs phytoplankton growth in as much as 40% of the ocean, notably in the Southern Ocean, equatorial Pacific Ocean, and north Pacific Ocean equatorial Pacific Ocean, and north Pacific Ocean (Moore *et al.*, 2001). This iron limitation has been evidenced by iron fertilization experiments of HNLC waters, which can produce rapidly growing algal blooms (Blain *et al.*, 2007, Boyd *et al.*, 2007), and ultimately, speculation that these blooms could be utilized to capture and sequester carbon from the atmosphere (Chisholm *et al.*, 2001, Huntley & Redalje, 2007).

2.7.3. Observation of artificial Fe fertilization

The work of John Martin (Martin, 1990, Martin *et al.*, 1991) sharply focused attention on the role of Fe in ocean productivity, biogeochemical cycles, and global climate by proposing that “phytoplankton growth in major nutrient-rich waters is limited by iron deficiency”. The candidate mechanism of Martin points to the importance of changes, over geological time, in the magnitude of macronutrient uptake by phytoplankton in waters where macronutrient concentrations are perennially high. Specifically, Fe supply to the ocean was much higher during

glacial maxima than at present, and it is estimated that the increase in Fe induced productivity contributed to the 80-ppm drawdown in atmospheric CO_2 observed during glacial maxima by enhancing the ocean's biological pump. Since then it is thought to have a significant effect of Fe fertilization on concentrations of atmospheric CO_2 by altering rates of carbon sequestration, a theory known as the 'iron hypothesis'. Many artificial Fe fertilization experiment were carried out to study this hypothesis. Few important studies are presented below:

In the year of 1994, Martin and his colleagues tested the idea that iron might limit phytoplankton growth in large HNLC regions of ocean (Martin *et al.*, 1994). They fertilized an area of 64 km^2 in the open equatorial Pacific Ocean with Fe. Phytoplankton biomass was doubled with threefold increase in chlorophyll content with fourfold increase in plant production. Similar result was found in another experimental site near the Galapagos Island.

Coale *et al.* (1996) observed massive phytoplankton bloom in the equatorial Pacific Ocean which consumed large amount of CO_2 and nitrate after low increase of Fe concentration by artificial fertilization. The observations provide unequivocal support for the hypothesis that phytoplankton growth in this oceanic region is limited by iron bioavailability and artificial Fe fertilization could sequester anthropogenic CO_2 from the atmosphere.

A phytoplankton bloom was reported by Boyd *et al.* (2000) followed by artificial Fe fertilization experiment in 61°S and 140°E by increasing 3 nM Fe concentration in 50 km^2 area. Increased iron supply led to elevated phytoplankton biomass and rates of photosynthesis in surface waters, causing a large drawdown of carbon dioxide and macronutrients after 13 days. Chlorophyll content of phytoplankton was significantly increased. The potential to sequester iron elevated algal carbon was spectacular and greatest of all experiments done before. This drawdown was mostly due to the proliferation of diatom stocks. Satellite observations of this massive bloom 30 days later, suggest that a sufficient proportion of the added iron was retained in surface waters.

A whole-ecosystem test of Fe fertilization experiment was done to test iron hypothesis and induced carbon sequestration by Watson and his colleagues in the Southern Ocean (Watson *et al.*, 2000). They reported strong influence of atmospheric CO_2 uptake and uptake ratios of silica to carbon by phytoplankton at nanomolar increases of Fe concentration. Furthermore, they presented a model of global carbon sequestration during glacial periods where Southern Ocean biota was the cause of around 40 ppm reduction of glacial-interglacial CO_2 change.

Two experiments in the Southern Ocean in low and high silicic acid were conducted in the year of 2004 by Coale *et al.* (2004). They found that Fe play pivotal role in controlling CO_2 uptake and regulation of atmospheric pressure of CO_2. The result demonstrated increased primary production decrease atmospheric CO_2 concentration which suggests that the Southern Ocean exported more carbon during the last glacial maximum.

2.7.4. Some natural Fe fertilization and CO_2 sequestration

Artificial Fe fertilization has been proved effective in phytoplankton bloom and atmospheric CO_2 sequestration. Here some naturally Fe fertilization incidence and induced phytoplankton bloom and there CO_2 sequestrations are presented.

Boyd and his colleagues reported a phytoplankton bloom in the Gulf of Alaska by natural Fe fertilization and studied the fate of CO_2 sequestrated by phytoplankton bloom (Boyd *et al.*, 2004). The bloom sustained for 18 days and then was terminated following the depletion of Fe and then silicic acid. Bacterial remineralization and mesozooplankton grazing was reported for more than half of the particulate organic carbon. They reported a concurrent supply of silicic acid to persist Fe stimulated phytoplankton bloom. They proposed a geo-engineering scheme for oceanic iron fertilization for sequestration of anthropogenic increases in atmospheric CO_2.

Blain and his colleagues report an observation of a phytoplankton bloom induced by natural Fe fertilization (Blain *et al.*, 2007). They found a large

phytoplankton bloom over the Kerguelen Plateau in the Southern Ocean was sustained by the supply of iron and major nutrients to surface waters from Fe-rich deep water below. The efficiency of fertilization, defined as the ratio of the carbon export to the amount of iron supplied, was at least ten times higher than previous estimates from short-term blooms induced by iron-addition experiments. This result sheds new light on the effect of long-term fertilization by iron and macronutrients on carbon sequestration, suggesting that changes in Fe supply from below as invoked in some palaeoclimatic and future climate change scenarios may have a more significant effect on atmospheric carbon dioxide concentrations than previously thought.

Natural Fe fertilization near the Kerguelen Islands (Kerguelen Plateau, Southern Ocean) was studied (Timmermans *et al.*, 2008). The cell numbers, chlorophyll auto fluorescence, photosynthetic efficiency of photosystem II, chlorophyll a and phytoplankton carbon concentrations increased especially after translocation into Plateau deep water. The response was most pronounced in terms of increase in carbon assimilation in the larger-sized phytoplankton (48 mm in cell diameter), mainly diatoms. Experiments with single-species cultures of large diatoms (*Fragilariopsis kerguelensis*, *Thalassiosira sp.*, *Chaetoceros dichaeta*), which have high iron requirements, confirmed the observations. The experiments provide evidence that this water contains the growth-stimulating factor, most likely iron, responsible for the formation of a phytoplankton bloom as is observed over the Kerguelen Plateau.

Pollard *et al.* (2009) reported a naturally Fe fertilization in the sub-Antarctic Southern Ocean where carbon fluxes were two to three times larger than the adjacent HNLC area not fertilized by Fe. The findings support the hypothesis that increased iron supply to the glacial sub-Antarctic may have directly enhanced carbon export to the deep ocean.

2.7.5. Some other aspects of Fe fertilization

Though artificial and natural Fe fertilization has proved to be effective for atmospheric CO_2 sequestration, it has many opposite opinions too. Many other studies reported that multiple factors are responsible for phytoplankton bloom induced atmospheric CO_2 sequestration such as silicic acid (Boyd *et al.*, 2004), increased bacterial activity (Suzuki *et al.*, 2005, West *et al.*, 2008) and bacterial mineralization (Obernosterer *et al.*, 2008), and some factors may affect negatively such as increased grazing of phytoplankton (Sarthou *et al.*, 2008), harmful algal blooms (Anderson *et al.*, 2002, Roelke *et al.*, 2007) etc. It may also disrupt the predator-prey control in ocean ecosystem (Irigoien *et al.*, 2005). Harmful algal blooms due to Fe fertilization has also been reported (Heisler *et al.*, 2008, Sunda, 2006, Trick *et al.*, 2010) and this showed be emphasized (Moore *et al.*, 2008) for consideration. The ongoing debate about Fe limitation of phytoplankton in the open ocean and Fe fertilization for atmospheric CO_2 sequestration has highlighted how little we know about the marine chemistry of iron and its relationship to iron uptake by microorganisms. Therefore, our study centered to know the molecular responses and adaptation of phytoplankton to Fe limitation.

2.8. Selection of *Prymnesium parvum* for Present Study

Prymnesium parvum (commonly known as golden algae) is an ichthyotoxic phytoplankton (Haptophyte) grown in geographically widespread areas of the open oceans including temperate waters, both in the northern and southern hemispheres (Fistarol *et al.*, 2003), vast areas of temperate and tropical open seas (Edvardsen & Imai, 2006), Baltic Sea (Edler *et al.*, 1984) and also of vast coastal plankton communites all over the world (Carvalho & Granéli, 2010, Edvardsen & Imai, 2006). It is a regular component of the coastal plankton community in many parts of the world. It was first identified in North America in 1985, and is characterized by 2 flagella and a haptonema (Fig. 7). A typical cell contains two large deeply bifid plastids (Fig. 7, d). Toxin production mainly kills fish and appears to have little

effect on cattle or humans. This distinguishes it from red tide, which are algal bloom whose toxins lead to harmful effects to people.

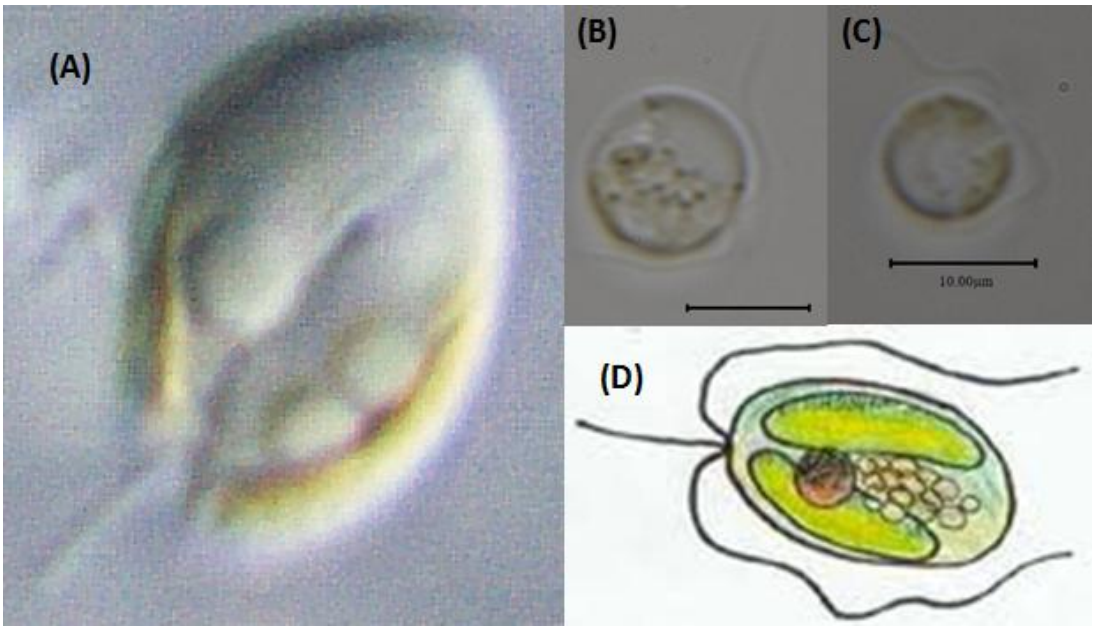

Fig. 2.8: (A) Cellular organization of *Prymnesium parvum* cell. *P. parvum* cell under Fe limited **(B)** and Fe rich **(C)** conditions. **(D)** A hypothetical cell.

Since a first report in the 1920s (Liebert & Deerns, 1920), toxic blooms of *Prymnesium parvum*, have frequently been reported from coastal marine and brackish waters around the world (Kaartvedt *et al.*, 1991, Lindholm & Virtanen, 1992, Michaloudi *et al.*, 2009, Otterstrøm & Nielsen, 1939). Blooms of this microalgae have often been associated with massive fish mortalities (Cortes-Altamirano & Carrillo-Ovalle, 2004, Guo *et al.*, 1996, Hallegraeff, 1993, Kaartvedt *et al.*, 1991, Roelke *et al.*, 2007, Roelke *et al.*, 2011), resulting in negative impacts on the marine ecosystem and large economic consequences for commercial aquaculture. This organism produces a set of highly potent exotoxins (with ichthyotoxic, cytotoxic and haemolytic activity) commonly called prymnesins (Igarashi *et al.*, 1996, Shilo, 1981), indicating a complex nature of the toxins. It has allelopathic effect to other phytoplankton which may adversely affect plankton community (Fistarol *et al.*, 2003). Fish mortality by *P. parvum* due to nutrient enrichment experiment has been reported by Valenti Jr *et al.* (2010). Toxin

production mainly kills fish and appears to have little or no effect on cattle or humans. This distinguishes it from red tide toxins lead to harmful effects in people.

Because of its geographical widespread and have a major effect on natural communities, it has been the focus of considerable research during the last decade (Carvalho & Granéli, 2010, Fistarol *et al.*, 2003, La Claire, 2006, Maki *et al.*, 2008, Susan *et al.*, 2010). Except some coastal areas, most of the marine areas where *P. parvum* is naturally grown are low in Fe content. In addition, in many HNLC areas it is common in the phytoplankton community (Edler *et al.*, 1984, Edvardsen & Imai, 2006, Fistarol *et al.*, 2003). Therefore, we think *P. parvum* as a good model phytoplankton to study molecular responses of Fe stress condition to the phytoplankton community. Though different phytoplankton response differently to Fe stress condition (Allen *et al.*, 2008, Sunda & Huntsman, 1995), but we hope the result using this model phytoplankton would represent a wide range phytoplankton including toxic algal species. Considering above facts, *P. parvum* is suitable to study molecular responses against Fe stress and to know its cellular adaptation under Fe limited condition.

On the other hand, this is very effective phytoplankton for blooms under favorable environment (Manton & Leedale, 1963). Reduction of pH increase the toxic production of *P. parvum* which may affect the plankton community due to ocean acidification (Valenti Jr *et al.*, 2010). Therefore, artificial Fe fertilization may create suitable environment for *P. parvum* to bloom. This may sequester considerable atmospheric CO_2 too. But it can cause high adverse effect by killing fishes and affecting the other components of natural oceanic ecosystem including phytoplankton community by producing toxic compound and allelopathic chemicals (Fistarol *et al.*, 2003, Igarashi *et al.*, 1996, Shilo, 1981). In that case study on this phytoplankton is also required.

2.9. Tools Used for Molecular Studies

Proteomics has a central role in molecular biology about biological systems because it generates knowledge about the concentrations, interactions, functions, and

catalytic activities of proteins, which are the major structural and functional determinants of cells (Baginsky, 2009). The bases for molecular study are Deoxyribo Nucleic acid (DNA), Ribo Nucleic Acid (RNA), proteins and metabolites. It is presented in Fig. 9. At DNA level genetic constituents or genetic potentiality can be assessed. Since different regulatory levels of gene expression exist, predicting cellular protein concentrations from the levels of mRNAs is difficult. Though it is possible in many cases but it can't be used for the proteins in different functional categories (Baginsky, 2009). Especially if the accumulation of proteins involved in signal transduction, for example is also controlled by posttranscriptional and posttranslational mechanisms (Hack, 2004). Transcription in the intergenic regions and non-coding regions of the genome also suggests the process as erroneous even for well characterized model organism (Stolc *et al.*, 2005). Since peptides are considered the final proof for gene expression, the proteomic analysis by proteomics is ideal for molecular studies. Furthermore, with the development of new data analysis tools and MS equipment with higher mass accuracy, proteomics has now become the method of choice for cataloguing full proteomes to refine gene structure prediction and genome annotation (Deutsch, 2010). The considerable denominator of proteomics approaches is the comprehensiveness of protein characterization. Now a days, protein profiling is very much rich compared to other molecular approaches and with the application of quantitative mass spectrometric technologies as well as the development of new strategies for data analysis can also provide the quantitative information about the identified proteins (Baginsky, 2009, Heal & Tate, 2012). Proteomics also integrates the sub-disciplines protein profiling, protein quantification, posttranslational modifications, and protein/protein interactions (Fig. 9).

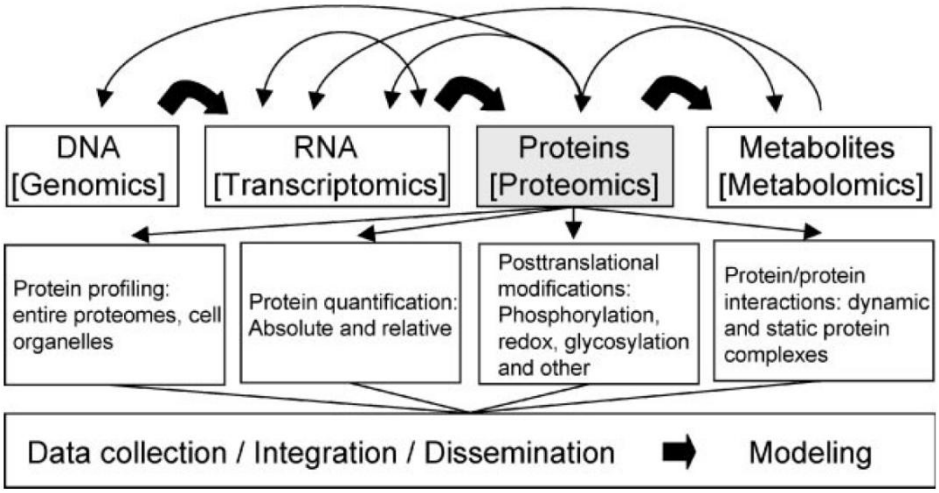

Fig. 2.9: Interactions between the basic molecular elements of biological systems. Several complex interactions between the system's elements DNA, RNA, proteins and metabolites exist that go beyond the central dogma of molecular biology. Bold arrows represent transcription (from DNA to RNA), translation (from RNA to proteins) and catalysis/enzyme activity (from proteins to metabolites). Thin arrows indicate additional interactions. Proteins and proteomics have a central position in this scheme and several approaches are employed to analyze and characterize a biological system at the proteome level (Baginsky, 2009).

2.9.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is the preliminary stage for proteomic studies. It is a technique widely used in biochemistry, forensics, genetics, molecular biology and biotechnology to separate proteins according to their electrophoretic mobility (a function of the length of a polypeptide chain and its charge) and no other physical or chemical features. Based on the findings of this technique further analysis is to be selected.

2.9.2. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The two dimensions that proteins are separated into using this technique can be isoelectric point, protein complex mass in the native state, and protein mass. This

process begins with 1-D electrophoresis to separate the proteins by isoelectric point (pH) is called isoelectric focusing (IEF). In this process a gradient of pH is applied to a gel and an electric potential is applied across the gel, making one end more positive than the other. At all pH values other than their isoelectric point, proteins will be charged. If they are positively charged, they will be pulled towards the more negative end of the gel and if they are negatively charged they will be pulled to the more positive end of the gel. The proteins applied in the first dimension will move along the gel and will accumulate at their isoelectric point; that is, the point at which the overall charge on the protein is 0 (a neutral charge).

In the second dimension, an electric potential is again applied, but at a 90 degree angle from the first field. The proteins will be attracted to the more positive side of the gel proportionally to their mass-to-charge ratio. In the gel proteins are separated on the basis of their molecular weight with larger proteins being retained higher in the gel and smaller proteins being able to pass through the gel and reach lower regions of the gel. Thus in 2-D PAGE, proteins are separated based on molecular weight and their isoelectric point. In our study we have used two-dimensional differential gel electrophoresis (2-D DIGE) technique where proteins are labeled by using CyDyes DIGE Fluors for efficient comparison and efficient quantification of the proteins (Asano *et al.*, 2012).

2.9.3. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS)

In the last few years significant technology development has taken place both at the level of data analysis software and mass spectrometry hardware. Conceptual progress in proteomics has made possible the analysis of entire proteomes at previously unprecedented density and accuracy. New concepts have emerged that comprise quantitative analyses of full proteomes, database-independent protein identification strategies, targeted quantitative proteomics approaches with proteotypic peptides and the systematic analysis of an increasing number of posttranslational modifications at high temporal and spatial resolution. The basic principle of MALDI-TOF MS is presented in Fig. 10.

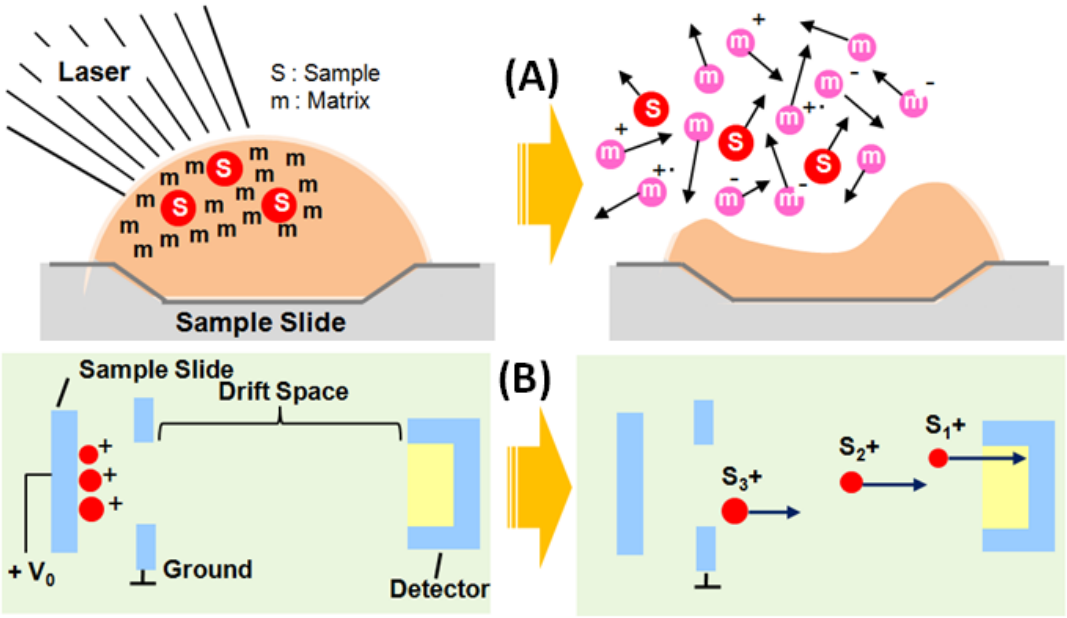

Fig. 2.10: Figure shows the basic principle of MALDI-TOF MS analysis. **(A):** Matrix-assisted laser desorption/ionization (MALDI) process. **(B):** time-of-flight mass spectrometry (TOF MS).

In principle, two different approaches are applicable for the identification of MS/MS spectra without relying on existing protein databases. First, MS/MS data are searched against a protein database and those spectra that were left unassigned despite originating from a true peptide must be identified and subjected to a modified database search. This could be a genome database or a protein database search with relaxed search parameters, for example, allowing a variety of different posttranslational modifications or SNPs. This strategy requires a database-independent spectrum scoring scheme to identify putative peptide-derived spectra and to distinguish them from low quality noise fragmentation or nonprotein derived contaminants. Ms/Ms data analysis can be represented by Fig. 11. In our study the proteins were identified by comparing MS/MS data against the NCBI and Uniprot database using the Paragon algorithm of ProteinPilot. Here we have searched Ms/Ms data against closely related complete database of *Emiliania huxleyi*, then other phytoplankton species and lastly to general organisms.

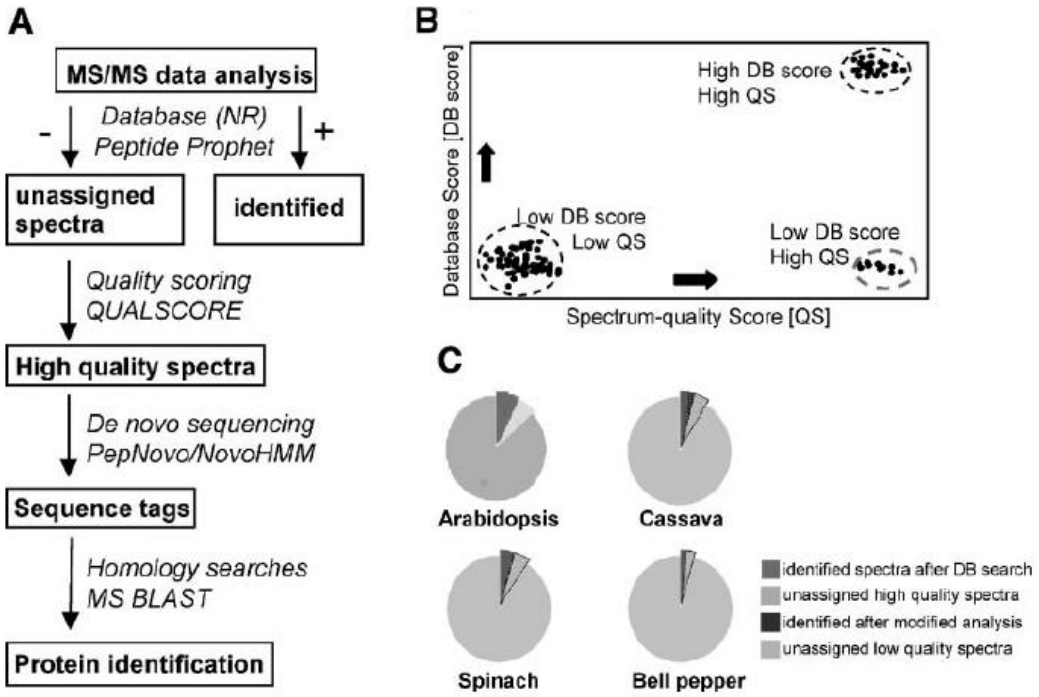

Fig. 2.11: Protein database-independent identification of peptides and proteins from high-throughput MS/MS data. **A:** MS/MS data are first subjected to a standard database search. Unidentified spectra are scored by their quality and high quality spectra are then subjected to de novo peptide sequencing. Peptide de novo sequencing results are only accepted if they exceed a user-defined reliability score. Reliable sequences are subsequently submitted to software for identifying a peptide by homology to peptides in a protein database from a related species. **B:** The principle is based on a 2-dimensional evaluation of MS/MS spectra by database score and spectrum quality score. **C:** Fraction of high quality unassigned spectra in proteomics data from three unsequenced plant species, and *Arabidopsis thaliana* as reference (Baginsky, 2009).

References:

Allen, A. E., Laroche, J., Maheswari, U., Lommer, M., Schauer, N., Lopez, P. J., Finazzi, G., Fernie, A. R. and Bowler, C. (2008) Whole-cell response of the pennate diatom *Phaeodactylum tricornutum* to iron starvation. *Proc. Natl. Acad. Sci.,* **105,** 10438.

Anderson, D. M., Glibert, P. M. and Burkholder, J. M. (2002) Harmful algal blooms and eutrophication: Nutrient sources, composition, and consequences. *Estuaries,* **25,** 704-726.

Arrhenius, S. (1896) On the influence of carbonic acid in the air upon the temperature of the ground. *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science,* **41,** 237-276.

Asano, T., Kimura, M. and Nishiuchi, T. (2012) The defense response in *Arabidopsis thaliana* against *Fusarium sporotrichioides*. *Proteome Sci.,* **10,** 61.

Baginsky, S. (2009) Plant proteomics: Concepts, applications, and novel strategies for data interpretation. *Mass Spectrom. Rev.,* **28,** 93-120.

Banse, K. (1991) Rates of phytoplankton cell division in the field and in iron enrichment experiments. *Limnol. Oceanogr.,* **36,** 1886-1898.

Barnola, J. M., Raynaud, D., Korotkevich, Y. S. and Lorius, C. (1987) Vostok ice core provides 160,000-year record of atmospheric CO_2. *Nature,* **329,** 408-414.

Bernstein, L., Bosch, P., Canziani, O., Chen, Z., Christ, R., Davidson, O., Hare, W., Huq, S., Karoly, D., Kattsov, V., Kundzewicz, Z., Liu, J., Lohmann, U., Manning, M., Matsuno, T., Menne, B., Metz, B., Mirza, M., Nicholls, N., Nurse, L., Pachauri, R., Palutikof, J., Parry, M., Qin, D., Ravindranath, N., Reisinger, A., Ren, J., Riahi, K., Rosenzweig, C., Rusticucci, M., Schneider, S., Sokona, Y., Solomon, S., Stott, P., Stouffer, R., Sugiyama, T., Swart, R., Tirpak, D., Vogel, C. and Yohe, G. (2007) Climate Change 2007: Synthesis Report: An Assessment of the Intergovernmental Panel on Climate Change. Intergovernmental Panel on Climate Change (IPCC), Valencia, Spain., pp. 1-52.

Bindoff, N. L., Willebrand, J., Artale, V., Cazenave, A., Gregory, J. M., Gulev, S., Hanawa, K., Le Quere, C., Levitus, S., Nojiri, Y., Shum, C. K., Talley, L. D. and Unnikrishnan, A. (2007) Observations: Oceanic climate change and sea level. In: S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M. Tignor and H. L. Miller (eds) *Climate change 2007: The physical science basis. Contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change.* Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Blain, S., Quéguiner, B., Armand, L., Belviso, S., Bombled, B., Bopp, L., Bowie, A., Brunet, C., Brussaard, C. and Carlotti, F. (2007) Effect of natural iron fertilization on carbon sequestration in the Southern Ocean. *Nature,* **446,** 1070-1074.

Blauwhoff, P. M. M., Versteeg, G. F. and Van Swaaij, W. P. M. (1984) A study on the reaction between CO_2 and alkanolamines in aqueous solutions. *Chem. Eng. Sci.*, **39,** 207-225.

Boyd, P. W., Jickells, T., Law, C. S., Blain, S., Boyle, E. A., Buesseler, K. O., Coale, K. H., Cullen, J. J., De Baar, H. J. W. and Follows, M. (2007) Mesoscale iron enrichment experiments 1993-2005: Synthesis and future directions. *Science,* **315,** 612-617.

Boyd, P. W., Law, C. S., Wong, C. S., Nojiri, Y., Tsuda, A., Levasseur, M., Takeda, S., Rivkin, R., Harrison, P. J., Strzepek, R., Gower, J., Mckay, R. M., Abraham, E., Arychuk, M., Barwell-Clarke, J., Crawford, W., Crawford, D., Hale, M., Harada, K., Johnson, K., Kiyosawa, H., Kudo, I., Marchetti, A., Miller, W., Needoba, J., Nishioka, J., Ogawa, H., Page, J., Robert, M., Saito, H., Sastri, A., Sherry, N., Soutar, T., Sutherland, N., Taira, Y., Whitney, F., Wong, S. K. E. and Yoshimura, T. (2004) The decline and fate of an iron-induced subarctic phytoplankton bloom. *Nature,* **428,** 549-553.

Boyd, P. W., Watson, A. J., Law, C. S., Abraham, E. R., Trull, T., Murdoch, R., Bakker, D. C. E., Bowie, A. R., Buesseler, K. O., Chang, H., Charette, M., Croot, P., Downing, K., Frew, R., Gall, M., Hadfield, M., Hall, J., Harvey, M., Jameson, G., Laroche, J., Liddicoat, M., Ling, R., Maldonado, M. T., Mckay, R. M., Nodder, S., Pickmere, S., Pridmore, R., Rintoul, S., Safi, K., Sutton, P., Strzepek, R., Tanneberger, K., Turner, S., Waite, A. and Zeldis, J. (2000) A mesoscale phytoplankton bloom in the polar Southern Ocean stimulated by iron fertilization. *Nature,* **407,** 695-702.

Brown, S. (1996) Mitigation of carbon dioxide emissions by management of forests in Asia. *Ambio,* **25,** 273-278.

Buma, A. G. J., Baar, H. J. W., Nolting, R. F. and Bennekom, A. (1991) Metal enrichment experiments in the Weddell-Scotia Seas: Effects of iron and manganese on various plankton communities. *Limnol. Oceanogr.,* **36,** 1865-1878.

Callendar, G. S. (1938) The artificial production of carbon dioxide and its influence on temperature. *Quarterly Journal of the Royal Meteorological Society,* **64,** 223-240.

Carvalho, W. F. and Granéli, E. (2010) Contribution of phagotrophy versus autotrophy to *Prymnesium parvum* growth under nitrogen and phosphorus sufficiency and deficiency. *Harmful Algae,* **9,** 105-115.

Chisholm, S. W., Falkowski, P. G. and Cullen, J. J. (2001) Dis-crediting ocean fertilization. *Science,* **294,** 309-310.

Coale, K. H., Johnson, K. S., Chavez, F. P., Buesseler, K. O., Barber, R. T., Brzezinski, M. A., Cochlan, W. P., Millero, F. J., Paul, G., Falkowski, P. G., Bauer, J. E., Wanninkhof, R. H., Kudela, R. M., Altabet, M. A., Hales, B. E., Takahashi, T., Landry, M. R., Bidigare, R. R., Wang, X., Chase, Z., Strutton, P. G., Friederich, G. E., Gorbunov, M. Y., Lance, V. P., Hilting, A. K., Hiscock, M. R., Demarest, M., Hiscock, W. T., Sullivan, K. F., Tanner, S. J., Gordon, R. M., Hunter, C. N., Elrod, V. A., Fitzwater, S. E., Jones, J. L., Tozzi, S., Koblizek, M., Roberts, A. E., Herndon, J., Brewster, J., Ladizinsky, N., Smith, G., Cooper, D., Timothy, D., Brown, S. L., Selph, K. E., Sheridan, C. C., Twining, B. S. and Johnson, Z. I. (2004) Southern Ocean Iron

Enrichment Experiment: Carbon Cycling in High- and Low-Si Waters. *Science,* **304,** 408-414.

Coale, K. H., Johnson, K. S., Fitzwater, S. E., Gordon, R. M., Tanner, S., Chavez, F. P., Ferioli, L., Sakamoto, C., Rogers, P., Fillero, F., Steinberg, P., Nightingale, P., Cooper, D., Cochlanl, W. P., Landry, M. R., Constantinou, J., Rollwagen, G., Trasvina, A. and Kudela, R. (1996) A massive phytoplankton bloom induced by an ecosystem-scale iron fertilization experiment in the equatorial Pacific Ocean. *Nature,* **383,** 10.

Cortes-Altamirano, R. and Carrillo-Ovalle, L. (2004) The coincidence of a Prymnesium parvum bloom and the mass kill of birds and fish in Lake Koronia. *Harmful algae news,* 1-3.

D'alessandro, D. M., Smit, B. and Long, J. R. (2010) Carbon dioxide capture: prospects for new materials. *Angewandte Chemie International Edition,* **49,** 6058-6082.

De Baar, H. J. W., Buma, A. G. J., Nolting, R. F., Cadee, G. C., Jacques, G. and Treguer, P. J. (1990) On iron limitation of the Southern Ocean: Experimental observations in the Weddell and Scotia Seas. *Mar. Ecol. Prog. Ser,* **65,** 105-122.

Demirbaş, A. (2006) Oily products from mosses and algae via pyrolysis. *Energy Sources, Part A,* **28,** 933-940.

Deutsch, E. W. (2010) The peptide atlas project*Proteome Bioinformatics.* Springer, pp. 285-296.

Dlugokencky, E. J., Masarie, K. A., Lang, P. M. and Tans, P. P. (1998) Continuing decline in the growth rate of the atmospheric methane burden. *Nature,* **393,** 447-450.

Doney, S. C., Fabry, V. J., Feely, R. A. and Kleypas, J. A. (2009) Ocean acidification: The other CO_2 problem. *Marine Science,* **1**.

Edler, L., Hällfors, G., Niemi, Å. and Tiedeakatemia, S. (1984) A preliminary check-list of the phytoplankton of the Baltic Sea. *Acta Bot. Fenn.,* **128,** 1-26.

Edvardsen, B. and Imai, I. (2006) The ecology of harmful flagellates within Prymnesiophyceae and Raphidophyceae*Ecology of Harmful Algae.* Springer, pp. 67-79.

Fistarol, G. O., Legrand, C. and Granéli, E. (2003) Allelopathic effect of *Prymnesium parvum* on a natural plankton community. *Mar. Ecol. Prog. Ser.,* **255,** 115-125.

Flückiger, J., Dällenbach, A., Blunier, T., Stauffer, B., Stocker, T. F., Raynaud, D. and Barnola, J. M. (1999) Variations in atmospheric N_2O concentration during abrupt climatic changes. *Science,* **285,** 227-230.

Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., Fahey, D. W., Haywood, J., Lean, J., Lowe, D. C., Myhre, G., Nganga, J., Prinn, R., Raga, G., Schulz, M. and Van Dorland, R. (2007) Changes in atmospheric constituents and in radiative forcing. In: S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M. Tignor and H. L. Miller (eds) *Climate change 2007: The physical science basis. Contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change.* Vol. 20. Cambridge University Press, Canbridge, United Kingdom and New York, NY, USA.

Fraser, P. J., Khalil, M. A. K., Rasmussen, R. A. and Crawford, A. J. (1981) Trends of atmospheric methane in the southern hemisphere. *Geophys. Res. Lett.,* **8,** 1063-1066.

Graedel, T. E. and Mcrae, J. E. (1980) On the possible increase of the atmospheric methane and carbon monoxide concentrations during the last decade. *Geophys. Res. Lett.,* **7,** 977-979.

Gran, H. H. (1931) On the conditions for the production of plankton in the sea. *Rapports et Proces-verbaux des Réunions. Conseil International pour l'Éxploration de la Mer,* **75,** 37-46.

Grasa, G. S. and Abanades, J. C. (2006) CO_2 capture capacity of CaO in long series of carbonation/calcination cycles. *Ind. Eng. Chem. Res.,* **45,** 8846-8851.

Greene, R. M., Kolber, Z. S., Swift, D. G., Tindale, N. W. and Falkowski, P. G. (1994) Physiological limitation of phytoplankton photosynthesis in the eastern equatorial Pacific determined from variability in the quantum yield of fluorescence. *Limnol. Oceanogr.,* 1061-1074.

Guo, M., Harrison, P. J. and Taylor, F. J. R. (1996) Fish kills related to *Prymnesium parvum* N. Carter (Haptophyta) in the People's Republic of China. *J. Appl. Phycol.,* **8,** 111-117.

Gupta, H. and Fan, L. S. (2002) Carbonation-calcination cycle using high reactivity calcium oxide for carbon dioxide separation from flue gas. *Ind. Eng. Chem. Res.,* **41,** 4035-4042.

Hack, C. J. (2004) Integrated transcriptome and proteome data: The challenges ahead. *Brief. Funct. Genomics Proteomics,* **3,** 212-219.

Hallegraeff, G. M. (1993) A review of harmful algal blooms and their apparent global increase. *Phycologia,* **32,** 79-99.

Heal, W. P. and Tate, E. W. (2012) Application of activity-based protein profiling to the study of microbial pathogenesis. In: S. A. Sieber (ed) *Activity-Based Protein Profiling.* Vol. 324. Springer Berlin Heidelberg, pp. 115-135.

Heisler, J., Glibert, P. M., Burkholder, J. M., Anderson, D. M., Cochlan, W., Dennison, W. C., Dortch, Q., Gobler, C. J., Heil, C. A. and Humphries, E. (2008) Eutrophication and harmful algal blooms: A scientific consensus. *Harmful Algae,* **8,** 3-13.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M. and Darzins, A. (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J.,* **54,** 621-639.

Huntley, M. E. and Redalje, D. G. (2007) CO_2 mitigation and renewable oil from photosynthetic microbes: A new appraisal. *Mitigation and adaptation strategies for global change,* **12,** 573-608.

Igarashi, T., Satake, M. and Yasumoto, T. (1996) Prymnesin-2: A potent ichthyotoxic and hemolytic glycoside isolated from the red tide alga *Prymnesium parvum*. *J. Am. Chem. Soc.,* **118,** 479-480.

Indermühle, A., Stocker, T. F., Joos, F., Fischer, H., Smith, H. J., Wahlen, M., Deck, B., Mastroianni, D., Tschumi, J. and Blunier, T. (1999) Holocene carbon-cycle dynamics based on CO_2 trapped in ice at Taylor Dome, Antarctica. *Nature,* **398,** 121-126.

Ipcc (2001) *Climate change 2001: The scientific basis. Contribution of working group I to the third assessment report of the intergovernmental panel on*

climate change. Vol., Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Ipcc (2005) *IPCC special report on carbon dioxide capture and storage. Prepared by working group III of the Intergovernmental Panel on Climate Change.* Vol., Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Irigoien, X., Flynn, K. J. and Harris, R. P. (2005) Phytoplankton blooms: A 'loophole'in microzooplankton grazing impact? *J. Plankton Res.,* **27,** 313-321.

Jeong, M. L., Gillis, J. M. and Hwang, J. Y. (2003) Carbon dioxide mitigation by microalgal photosynthesis. *Bulletin of Korean Chemical Society,* **24,** 1763-1766.

Kaartvedt, S., Johnsen, T. M., Aksnes, D. L., Lie, U. and Svendsen, H. (1991) Occurrence of the toxic phytoflagellate *Prymnesium parvum* and associated fish mortality in a Norwegian fjord system. *Can. J. Fish. Aquat. Sci.,* **48,** 2316-2323.

Kohlmaier, G. H., Weber, M. and Houghton, R. A. (1998) Carbon dioxide mitigation in forestry and wood industry*Carbon dioxide mitigation in forestry and wood industry*. Springer-Verlag, Berlin, Germany, pp. 375.

La Claire, J. (2006) Analysis of expressed sequence tags from the harmful alga, Prymnesium parvum (Prymnesiophyceae, Haptophyta). *Mar. Biotechnol.,* **8,** 534-546.

Lalli, C. and Parsons, T. (1997) *Biological oceanography: An introduction.* Vol., Butterworth-Heinemann Ltd, Linacre House, Jordan Hill, Oxford OX2 8DP.

Le Treut, H., Somerville, R., Cubasch, U., Ding, Y., Mauritzen, C., Mokssit, A., Peterson, T. and Prather, M. (2007) Historical overview of climate change. In: S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M. Tignor and H. L. Miller (eds) *Climate change 2007: the physical science basis. Contribution of working group I to the fourth assessment report of the Intergovernmental Panel on Climate Change.* Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Li, Y., Horsman, M., Wu, N., Lan, C. Q. and Dubois-Calero, N. (2008) Biofuels from microalgae. *Biotechnol. Prog.,* **24,** 815-820.

Liang, Y., Harrison, D. P., Gupta, R. P., Green, D. A. and Mcmichael, W. J. (2004) Carbon dioxide capture using dry sodium-based sorbents. *Energy Fuels,* **18,** 569-575.

Liebert, F. and Deerns, W. M. (1920) Onderzoek naar de oorzak van een Vischsterfte in den Polder Workumer Nieuwland, nabij Workum. *Verhandungen en Rapporten uitgegeven door Rijkinstituten voor Visscherijonderzoek,* **1,** 81-93.

Lindholm, T. and Virtanen, T. (1992) A bloom of *Prymnesium parvum* Carter in a small coastal inlet in Dragsfjärd, southwestern Finland. *Environmental Toxicology and Water Quality,* **7,** 165-170.

Lu, H., Reddy, E. P. and Smirniotis, P. G. (2006) Calcium oxide based sorbents for capture of carbon dioxide at high temperatures. *Ind. Eng. Chem. Res.,* **45,** 3944-3949.

Maki, T., Suzuki, T., Kido, K., Nakahara, A., Higashi, T., Hasegawa, H., Ueda, K. and Saijoh, K. (2008) Effect of Iron Stress on Gene Expression in Harmful Microalga *Prymnesium Parvum*. *J. Ecotechnol. Res.,* **14,** 13-16.

Manton, I. and Leedale, G. F. (1963) Observations on the fine structure of prymnesium parvum carter. *Arch. Mikrobiol.,* **45,** 285-303.

Martin, J. H. (1990) Glacial-interglacial CO_2 change: The iron hypothesis. *Paleoceanography,* **5,** 1-13.

Martin, J. H. (1992) *Iron as a limiting factor in oceanic productivity.* Vol. 137, Plenum Press, New York.

Martin, J. H., Coale, K. H., Johnson, K. S., Fitzwater, S. E., Gordon, R. M., Tanner, S. J., Hunter, C. N., Elrod, V. A., Nowicki, J. L., Coley, T. L., Barber, R. T., Lindley, S., Watson, A. J., Van Scoy, K., Law, C. S., Liddicoat, M. I., Ling, R., Stanton, T., Stockel, J., Collins, C., Anderson, A., Bidigare, R., Ondrusek, M., Latasa, M., Millero, F. J., Lee, K., Yao, W., Zhang, J. Z., Frederich, G., Sakamoto, C., Chavez, F., Buck, K., Kolber, Z., Yungel, J., Turner, S., Nightingale, P., Hatton, A., Liss, P. and Tindale, N. W. (1994) Testing the iron hypothesis in ecosystems of the equatorial Pacific Ocean. *Nature,* **371,** 123.

Martin, J. H. and Fitzwater, S. E. (1988) Iron deficiency limits phytoplankton growth in the north-east Pacific subarctic. *Nature,* **331,** 341-343.

Martin, J. H., Fitzwater, S. E. and Gordon, R. M. (1990) Iron deficiency limits phytoplankton growth in Antarctic waters. *Global Biogeochem. Cycles,* **4,** 5-12.

Martin, J. H., Gordon, R. M. and Fitzwater, S. E. (1991) The case for iron. *Limnol. Oceanogr.,* 1793-1802.

Michaloudi, E., Moustaka-Gouni, M., Gkelis, S. and Pantelidakis, K. (2009) Plankton community structure during an ecosystem disruptive algal bloom of *Prymnesium parvum*. *J. Plankton Res.,* **31,** 301-309.

Mitchell, B. G., Brody, E. A., Holm-Hansen, O., Mcclain, C. and Bishop, J. (1991) Light limitation of phytoplankton biomass and macronutrient utilization in the Southern Ocean. *Limnol. Oceanogr.,* **36,** 1662-1677.

Moore, J. K., Doney, S. C., Glover, D. M. and Fung, I. Y. (2001) Iron cycling and nutrient-limitation patterns in surface waters of the World Ocean. *Deep Sea Research Part II: Topical Studies in Oceanography,* **49,** 463-507.

Moore, S. K., Trainer, V. L., Mantua, N. J., Parker, M. S., Laws, E. A., Backer, L. C. and Fleming, L. E. (2008) Impacts of climate variability and future climate change on harmful algal blooms and human health. *Environ. Health,* **7,** S4.

Nelson, D. M. and Smith, W. O. (1991) The role of light and major nutrients. *Limnol. Oceanog,* **36,** 1650-1661.

Obernosterer, I., Christaki, U., Lefèvre, D., Catala, P., Van Wambeke, F. and Lebaron, P. (2008) Rapid bacterial mineralization of organic carbon produced during a phytoplankton bloom induced by natural iron fertilization in the Southern Ocean. *Deep Sea Research Part II: Topical Studies in Oceanography,* **55,** 777-789.

Otterstrøm, C. V. and Nielsen, S. (1939) Two cases of extensive mortality in fishes caused by the flagellate *Prymnesium parvum*. *Report of the Danish Biological Station,* **44,** 5-24.

Pollard, R. T., Salter, I., Sanders, R. J., Lucas, M. I., Moore, C. M., Mills, R. A., Statham, P. J., Allen, J. T., Baker, A. R., Bakker, D. C. E., Charette, M. A., Fielding, S., Fones, G. R., French, M., Hickman, A. E., Holland, R. J., Hughes, J. A., Jickells, T. D., Lampitt, R. S., Morris, P. J., Nedelec, F. H., Nielsdottir, M., Planquette, H., Popova, E. E., Poulton, A. J., Read, J. F., Seeyave, S., Smith, T., Stinchcombe, M., Taylor, S., Thomalla, S., Venables, H. J., Williamson, R. and Zubkov, M. V. (2009) Southern Ocean deep-water carbon export enhanced by natural iron fertilization. *Nature,* **457,** 577-580.

Price, N. M., Ahner, B. A. and Morel, F. M. M. (1994) The equatorial Pacific Ocean: Grazer-controlled phytoplankton populations in an iron-limited ecosystem. *Limnol. Oceanogr.,* **39,** 520-534.

Resnik, K. P., Yeh, J. T. and Pennline, H. W. (2004) Aqua ammonia process for simultaneous removal of CO_2, SO_2 and NOx. *International journal of environmental technology and management,* **4,** 89-104.

Revelle, R. and Suess, H. E. (1957) Carbon dioxide exchange between atmosphere and ocean and the question of an increase of atmospheric CO_2 during the past decades. *Tellus,* **9,** 18-27.

Righelato, R. and Spracklen, D. V. (2007) Carbon mitigation by biofuels or by saving and restoring forests? *Sci. Sports,* **317,** 902.

Roelke, D. L., Errera, R. M., Kiesling, R., Brooks, B. W., Grover, J. P., Schwierzke, L., Ureña-Boeck, F., Baker, J. and Pinckney, J. L. (2007) Effects of nutrient enrichment on *Prymnesium parvum* population dynamics and toxicity: Results from field experiments, Lake Possum Kingdom, USA. *Aquat. Microb. Ecol.,* **46,** 125.

Roelke, D. L., Grover, J. P., Brooks, B. W., Glass, J., Buzan, D., Southard, G. M., Fries, L., Gable, G. M., Schwierzke-Wade, L. and Byrd, M. (2011) A decade of fish-killing *Prymnesium parvum* blooms in Texas: Roles of inflow and salinity. *J. Plankton Res.,* **33,** 243-253.

Sakakura, T., Choi, J. C. and Yasuda, H. (2007) Transformation of carbon dioxide. *Chem. Rev.,* **107,** 2365-2387.

Sarthou, G., Vincent, D., Christaki, U., Obernosterer, I., Timmermans, K. R. and Brussaard, C. P. D. (2008) The fate of biogenic iron during a phytoplankton bloom induced by natural fertilisation: Impact of copepod grazing. *Deep Sea Research Part II: Topical Studies in Oceanography,* **55,** 734-751.

Shi, D., Xu, Y., Hopkinson, B. M. and Morel, F. M. M. (2010) Effect of ocean acidification on iron availability to marine phytoplankton. *Science,* **327,** 676-679.

Shi, M. and Shen, Y. M. (2003) Recent progresses on the fixation of carbon dioxide. *Curr. Org. Chem.,* **7,** 737-745.

Shilo, M. (1981) The Toxic Principles of Prymnesium Parvum. In: W. Carmichael (ed) *The Water Environment.* Springer US, pp. 37-47.

Stolc, V., Samanta, M. P., Tongprasit, W., Sethi, H., Liang, S., Nelson, D. C., Hegeman, A., Nelson, C., Rancour, D. and Bednarek, S. (2005) Identification of transcribed sequences in *Arabidopsis thaliana* by using high-resolution genome tiling arrays. *Proc. Natl. Acad. Sci. U. S. A.,* **102,** 4453-4458.

Sunda, W. G. (2006) Trace Metals and Harmful Algal Blooms. In: E. Granéli and J. T. Turner (eds) *Ecology of Harmful Algae.* Vol. 189. Springer Berlin Heidelberg, pp. 203-214.

Sunda, W. G. and Huntsman, S. A. (1995) Iron uptake and growth limitation in oceanic and coastal phytoplankton. *Mar. Chem.,* **50,** 189-206.

Susan, V. J., Theodore, W. V., Daniel, L. R., James, P. G. and Bryan, W. B. (2010) Probabilistic ecological hazard assessment of microcystin-LR allelopathy to *Prymnesium parvum*. *J. Plankton Res.,* **00,** 1-14.

Suzuki, K., Hinuma, A., Saito, H., Kiyosawa, H., Liu, H., Saino, T. and Tsuda, A. (2005) Responses of phytoplankton and heterotrophic bacteria in the northwest subarctic Pacific to in situ iron fertilization as estimated by HPLC pigment analysis and flow cytometry. *Progress in Oceanography,* **64,** 167-187.

Timmermans, K. R., Veldhuis, M. J. W., Laan, P. and Brussaard, C. P. D. (2008) Probing natural iron fertilization near the Kerguelen (Southern Ocean) using natural phytoplankton assemblages and diatom cultures. *Deep Sea Research Part II: Topical Studies in Oceanography,* **55,** 693-705.

Tortell, P. D., Payne, C. D., Li, Y., Trimborn, S., Rost, B., Smith, W. O., Riesselman, C., Dunbar, R. B., Sedwick, P. and Ditullio, G. R. (2008) CO_2 sensitivity of Southern Ocean phytoplankton. *Geophys. Res. Lett.,* **35,** L04605.

Trick, C. G., Bill, B. D., Cochlan, W. P., Wells, M. L., Trainer, V. L. and Pickell, L. D. (2010) Iron enrichment stimulates toxic diatom production in high-nitrate, low-chlorophyll areas. *Proc. Natl. Acad. Sci.,* **107,** 5887-5892.

Valenti Jr, T. W., James, S. V., Lahousse, M. J., Schug, K. A., Roelke, D. L., Grover, J. P. and Brooks, B. W. (2010) A mechanistic explanation for pH-dependent ambient aquatic toxicity of *Prymnesium parvum* carter. *Toxicon,* **55,** 990-998.

Wang, B., Li, Y., Wu, N. and Lan, C. Q. (2008) CO_2 bio-mitigation using microalgae. *Appl. Microbiol. Biotechnol.,* **79,** 707-718.

Watson, A. J., Bakker, D. C. E., Ridgwell, A. J., Boyd, P. W. and Law, C. S. (2000) Effect of iron supply on southern ocean CO_2 uptake and implications for glacial atmospheric CO_2. *Nature,* **407,** 730-733.

West, N. J., Obernosterer, I., Zemb, O. and Lebaron, P. (2008) Major differences of bacterial diversity and activity inside and outside of a natural iron-fertilized phytoplankton bloom in the Southern Ocean. *Environ. Microbiol.,* **10,** 738-756.

Yamasaki, A. (2003) An overview of CO_2 mitigation options for global warming-Emphasizing CO_2 sequestration options. *J. Chem. Eng. Jpn.,* **36,** 361-375.

Yang, H., Xu, Z., Fan, M., Gupta, R., Slimane, R. B., Bland, A. E. and Wright, I. (2008) Progress in carbon dioxide separation and capture: A review. *J. Environ. Sci.,* **20,** 14-27.

3

RESULTS AND DISCUSSION

GROWTH AND PROTEOMIC RESPONSES OF MARINE PHYTOPLANKTON TO LIGAND-INDUCED IRON LIMITATION

3.1 Introduction

Iron (Fe) deficiency is limiting phytoplankton growth in major nutrient-rich open seas. Phytoplankton needs Fe for photosynthetic and respiratory electron transport (Morrissey & Bowler, 2012), and is directly involved in nitrate and nitrite reduction (Martin & Fitzwater, 1988), sulfate reduction, nitrogen fixation, chlorophyll biosynthesis, and a number of other biosynthetic and degradative reactions, including those involved in detoxification of O_2 radicals (Geider & Roche, 1994). Marine phytoplankton, namely photosynthetic microalgae and cyanobacteria, account for half of the global primary production, and play a major role in regulating global climate and sequestering carbon dioxide (CO_2) from the atmosphere (Benner, 2011, Field *et al.*, 1998). However, the dissolution of atmospheric CO_2 in the ocean

leads to the changes in the chemistry of seawater, commonly described by the term 'ocean acidification' that may create Fe-limited conditions for phytoplankton by decreasing the bioavailability of dissolved Fe (Shi et al., 2010). The oxidized form of Fe, Fe(III), that prevails in the ocean is scarcely soluble in oxygenated seawater (Benner, 2011), and therefore, occurs at extremely low concentrations in the oceans (Wells et al., 1995). Considering the significance of Fe for phytoplankton growth, physiology and ecology, we must understand the relationship between uptake mechanisms of phytoplankton and the concentration, bioavailability and speciation of Fe in seawater.

In order to understand the effects of Fe on phytoplankton productivity, it is essential to identify Fe transport systems and uptake strategies of the organism under different Fe conditions in seawater. Several uptake pathways for free inorganic iron (Fe′) and organically bound iron (Fe-L) have been described amongst aquatic phytoplankton. The conventional model of Fe´ uptake by marine eukaryotic phytoplankton suggests a dependency of uptake rate on the concentration of unchelated Fe species (Fe´) (Hudson & Morel, 1990, Sunda & Huntsman, 1995). According to this model, phytoplankton uptake Fe via binding of Fe(III)´ or Fe(II)´, which is rapidly oxidized to Fe(III)´, to a cell surface ligand and subsequent transfer across the plasma membrane. Therefore, the rate of uptake is controlled by the rate of ligand exchange between the Fe species and a membrane transporter (Hudson & Morel, 1990). However, more recent electrochemical measurements have shown that most of the Fe in seawater is chelated by natural organic ligands (Gledhill *et al.*, 1994, Rue & Bruland, 1995, Wu & Luther, 1995) that buffer such low Fe´ concentrations that may not be sufficient to support phytoplankton growth. Other studies have also reported that synthetic complexing ligands such as desferrioxamine (DFO), diethylenetriamine-N,N,N',N",N"-pentaacetate (DTPA) and ethylenediaminetetraacetic acid (EDTA) form stable Fe(III)-ligand [Fe(III)-L] complexes at high ligand concentration and results in the decrease of Fe bioavailability to marine phytoplankton (Boye *et al.*, 2000, Hutchins *et al.*, 1999a). In a recent study, for example, Shi et al. (2010) reported that excess Ethylenediaminetetraacetic acid (EDTA) creates Fe-limited condition for

phytoplankton, and Fe uptake rate depends on the unchelated Fe concentration. Addition of strong Fe(II) chelators was also found to reduce Fe(III) in growing medium and inhibit Fe uptake in phytoplankton (Maldonado & Price, 2000, Soria-Dengg & Horstmann, 1995). Thus, ligand-mediated Fe uptake in marine phytoplankton is an important concern in the scientific community.

To date, two major Fe-L uptake pathways have been suggested for phytoplankton; i) siderophore mediated Fe acquisition (Goldman *et al.*, 1983, Soria-Dengg *et al.*, 2001), and ii) the reductive Fe uptake pathway (Eckhardt & Buckhout, 1998, Maldonado & Price, 2001, Shaked *et al.*, 2005). Prokaryotic phytoplankton adopt siderophore-mediated Fe uptake systems while the eukaryotic phytoplankton utilize a reductive strategy (Hutchins et al., 1999b). It has been reported that certain phytoplankton produce compounds to facilitate Fe uptake from seawater under Fe limitation (Macrellis et al., 2001). For example, under Fe-limited condition, marine cyanobacteria produce siderophores of low molecular mass, which solubilize Fe-(hydro)oxdies or remove Fe from other organic complexes, and make Fe bioavailable to the organism (Anderson & Morel, 1982, Hopkinson & Morel, 2009). Some ealier reports have shown that phytoplankton also produce siderophores under Fe-limiting conditions to assist Fe uptake (Naito *et al.*, 2001, Trick *et al.*, 1983). Laboratory studies have also shown that phytoplankton can uptake Fe and grow in the presence of the model siderophores desferrioxamine B and E, which, like the oceanic organic ligands, maintain very low Fe´ concentrations (Maldonado & Price, 2000, Soria-Dengg & Horstmann, 1995). The Fe(III)-siderophore complexes are recognized at the cell surface by high-affinity siderophore receptors and enter the cell through these chelate-specific outer membrane transport proteins (Dhungana & Crumbliss, 2005). However, genetic evidence (Hopkinson & Morel, 2009, Webb *et al.*, 2001) as well as short term Fe uptake experiments (Fujii *et al.*, 2010, Kranzler *et al.*, 2011, Lis & Shaked, 2009, Rose & Waite, 2005) challenge this model. In a recent review, Shaked and Lis (2012) proposed reductive Fe uptake a prevalent Fe acquisition strategy of phytoplankton. However, it is not clear whether marine phytoplankton express similar proteins for Fe uptake under low total Fe conditions that occur due to low absolute Fe concentration, or under induced Fe-limited

conditions that occur due to the formation of stable Fe(III)-L complexes with commonly observed organic ligands in seawaters.

The present study was carried out to investigate the protein expressions of three eukaryotic and photosynthetic marine phytoplankton strains (*Pleurochrysis roscoffensis, Prymnesium parvum* and *Skeletonema marinoi-dohrnii complex*) under low total Fe and ligand-induced Fe-limited conditions to understand Fe uptake mechanisms under Fe-limited conditions. *P. roscoffensis* and *P. parvum* belong to the division "haptophyta" and are characterized by two slightly unequal and smooth flagella, a unique organelle called "haptonema", and mitochondria having tubular cristae. Haptophyte algae are important primary producers in aquatic habitats, and they are probably the primary carbon source for petroleum products (Andersen, 2004). A fungal siderophore DFO-B (Kraemer et al., 1999) and DTPA were used in this study to create Fe-limited conditions in the phytoplankton growth medium.

3.2 Materials and Methods

3.2.1 Phytoplankton culture and maintenance

Three strains of marine phytoplankton, *Prymnesium parvum* (Prymnesiophyceae), *Skeletonema marinoi-dohrnii complex* (Bacillariophyceae, NIES-323) and *Pleurochrysis roscoffensis* (Prymnesiophyceae, NIES-8), were used in this study. *P. parvum* was from Hokkaido University, Japan, which was originally collected from Fukuyama Bay, Hiroshima, Japan. The other two phytoplankton strains (*P. roscoffensis* and *S. marinoi-dohrnii complex*) were collected from National Institute of Environmental Studies (NIES), Japan. All the phytoplankton strains were axenic. The axenicity was assessed and monitored by 4′ 6-diamidino-2-phenylindole (DAPI) test.

The phytoplankton strains were grown in 1-L polycarbonate bottles containing modified f/2 growing medium in artificial seawater (Table 3.1) (Guillard & Ryther, 1962, Lyman & Fleming, 1940). One-litter polycarbonate bottles containing modified f/2 growing medium in artificial seawater was sterilized by placed in a autoclave machine (Sanyo Labo, Model: MLS-3780). $FeCl_3$ (10^{-5} M) solution (in 1

M HCl) was placed in the autoclave machine in a separate bottle to avoid Fe contamination. Then $FeCl_3$ solution was mixed with the modified f/2 growing medium. The tips and micropipettes used in the test were also sterilized by autoclaving (wrapping with aluminum foil). Sterilization was done at 121 °C for 30 min. After sterilization, the materials were placed in a clean bench (SANYO, MCV-710ATS, Japan) and was kept under ultra violet ray for 20 min.

Table 3.1: Composition of the f/2 growing medium in artificial seawater used for growing phytoplankton

Nutrient	Concentration (mol L^{-1})
$NaNO_3$	8.82×10^{-4}
$NaH_2PO_4\cdot2H_2O$	3.85×10^{-5}
$Na_2SiO_3\cdot9H_2O$	3.52×10^{-5}
SeO_2	1.00×10^{-8}
$CoSO_4\cdot7H_2O$	4.27×10^{-8}
$ZnSO_4\cdot7H_2O$	7.30×10^{-8}
$MnCl_2\cdot4H_2O$	9.09×10^{-7}
$CuSO_4\cdot5H_2O$	2.80×10^{-8}
$Na_2MoO_4\cdot2H_2O$	2.89×10^{-8}
Vitamin B12	3.69×10^{-10}
Biotin	2.05×10^{-9}
Thiamin HCl	2.97×10^{-7}
HEPES buffer	5.04×10^{-6}

There was limited scope of Fe contamination during the sterilization process. On the other hand, all chemicals and their method/source of preparation were carefully selected to avoid Fe contamination. Before adding Fe (10^{-5} M as $FeCl_3$) to the sterilized growing medium, Fe concentration of the medium was measured and was found below 0.05 µM in every measurement. EDTA (a source of Fe contamination) was not used in this study. Other chemicals were also carefully selected to avoid any possible Fe contamination. Since Fe (10^{-5} M as $FeCl_3$) was added to the growing medium after sterilization, and the phytoplankton were grown

only 15 d, the possibility of precipitation of added $FeCl_3$ was minimal, and EDTA was not necessarily required.

A 4 mL solution of phytoplankton strains (2×10^3 cells mL^{-1} phytoplankton in logarithmic growth phase) was inoculated in 1 L of modified f/2 growing medium under laminar air flow condition. *P. parvum* and *P. roscoffensis* were grown at 20 °C, while *S. marinoi-dohrnii complex* was grown at 5 °C. Light and dark schedule in the growth chamber was 12:12 h with light intensity of 188 μE m^{-2} S^{-1}.

3.2.2 Chemical treatments and growth measurement

The Fe concentration of control treatments (Fe-rich conditions) for all the three phytoplankton strains was 1 μM. The Fe concentration in low total Fe treatments was 0.07 μM for *P. roscoffensis* and *S. marinoi-dohrnii complex* and 0.03 μM for *P. parvum*. Ligand induced Fe-limited condition was achieved by adding either DFO-B or DTPA to the phytoplankton growing medium containing 1 μM Fe concentration. A 5 mL of DFO-B or DTPA solution (20 mM) was added to the growing medium 10 days after inoculation with *P. roscoffensis* and *S. marinoi-dohrnii complex* inoculation, and 8 days after inoculation with *P. parvum*. Phytoplankton growth was measured on the basis of optical density using a spectrophotometer (U-2000, Hitachi High Technologies, Japan) at a wavelength of 540 nm (OD_{540}) according to the method of Sorokin (1973). The experiment was replicated for three times.

3.2.3 Protein extraction

After reaching logarithmic growth phase, the phytoplankton growth solution was transferred to a 50-mL centrifuge tube and centrifuged at 1800 rpm (relative centrifugal force; g = 450) for 10 min at 4 °C. The supernatant was removed and the phytoplankton pellet was washed twice using 2 mL of 10 mM Tris-HCl (pH 7.2). Then 1.5 mL of 10 mM Tris-HCl was added to the sample to make a phytoplankton cell suspension. One mL of the phytoplankton cell suspension was transferred to a 1.5-mL micro-tube and sonicated for 20 seconds using an ultrasonic homogenizer (UH-50, Surface Mount Technology (SMT), Japan) at output 5 under ice-cold

condition. The phytoplankton cell suspension was then centrifuged at 15000 rpm (*g* = 14000) for 10 min at 4 °C. The supernatant was then transferred to centrifugal filter unite (Amicon Ultra-4, MWCO of 5000 Da, Millipore). The proteins were recovered on the filters after being concentrated by centrifugation at 2000 rpm (*g* = 550) for 60 min at 4 °C. Protein quantification was performed by the Bradford assay (Bio-Rad, Hercules, CA, USA) (Bradford, 1976). The protein concentrations of the samples were then made more or less equal and were subjected to further analysis.

Ultrasonic homogenization method was used in this study for protein extraction. The protein extraction efficiency of this method was compared with that of freeze-thaw homogenization method. Results showed that the ultrasonic homogenization method was better than freeze-thaw homogenization method. Protein concentrations were also compared for these two methods, and the results were found to be approximately identical for both methods (Table 3.2).

Table 3.2: Comparison of protein concentrations estimated by ultrasonic and freeze-thaw homogenization methods

Ultrasonic homogenization method		Freeze-thaw homogenization method	
Wet weight (g)	Protein concentration (mg/ml)	Wet weight (g)	Protein concentration (mg/ml)
0.0067	15.9	0.0067	14.3
0.0050	12.3	0.0060	14.8
0.0091	17.9	0.0093	14.2

3.2.4 Gel electrophoresis of protein

The protein samples were incubated in ice for 60 min after adding 3.75 µL of 20% Triton X-100 [$C_{14}H_{22}O(C_2H_4O)_n$] and 3.75 µL of 50 mM $MgCl_2$ to 30 µL of protein sample for each treatment. Equal volumes of sample buffer solution (125 mM Tris-HCl; pH 6.8, 4% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 10% sucrose, 0.004% bromophenol blue (BPB)) were then added, and the samples were incubated for 3 min at 95 °C. A molecular weight marker was diluted by 20-

fold and an equal volume of sample buffer was added and incubated for 3 min at 95 °C. A 20 µL protein sample was added to 14% and 10% polyacrylamide gels along with the molecular weight markers. The gel was then run for 100 min using buffer solution prepared with 25 mM Tris-HCl, 0.1% SDS, 192 mM glycine at 40 mA current. After electrophoresis the gel was stained by shaking for 60 min in coomassie brilliant blue (CBB) solution (0.25% CBB R-250, 5% methanol, 7.5% acetic acid). The stained gel was then shaken with de-staining solution (25% methanol, 7.5% acetic acid) until the background color was removed and protein bands were clearly seen. The molecular weights of protein bands were then measured by Alpha-Ease FC (v 4.0) software.

3.2.5 Statistics

One-way ANOVA of the growth and protein expression data was performed with GraphPad InStat (GraphPad Software, USA) to assess the significant differences among the mean values of the nutrient treatments.

3.3 Results and Discussion

3.3.1 Growth response of phytoplankton under different Fe conditions

The three species of marine phytoplankton (*P. roscoffensis, P. parvum* and *S. marinoi-dohrnii complex*) showed different growth responses under different Fe conditions. The growth of the phytoplankton was significantly higher at Fe-rich (1 µM) conditions than in Fe-limited conditions (Fig. 3.1). In low total Fe (0.07 µM) medium, the growth of *P. roscoffensis* and *S. marinoi-dohrnii complex* increased gradually up to 10 days, and then slowed down (Figs. 3.1 A and B). The growth of *P. parvum* stopped after 9 days of incubation in low total Fe (0.03 µM) medium (Fig. 3.1C). The Fe concentrations in growth medium of low total Fe(artificial seawater) were higher than the usual range of dissolved Fe concentrations in seawater (10^{-3}-10^{-2} µM) (Geider, 1999). The different growth response of the phytoplankton strains to different Fe conditions reflects their differential Fe requirement. It was reported that

the Fe requirement for phytoplankton depends on the species and phenotype and on the environmental conditions (Geider & Roche, 1994, Wells *et al.*, 1995).

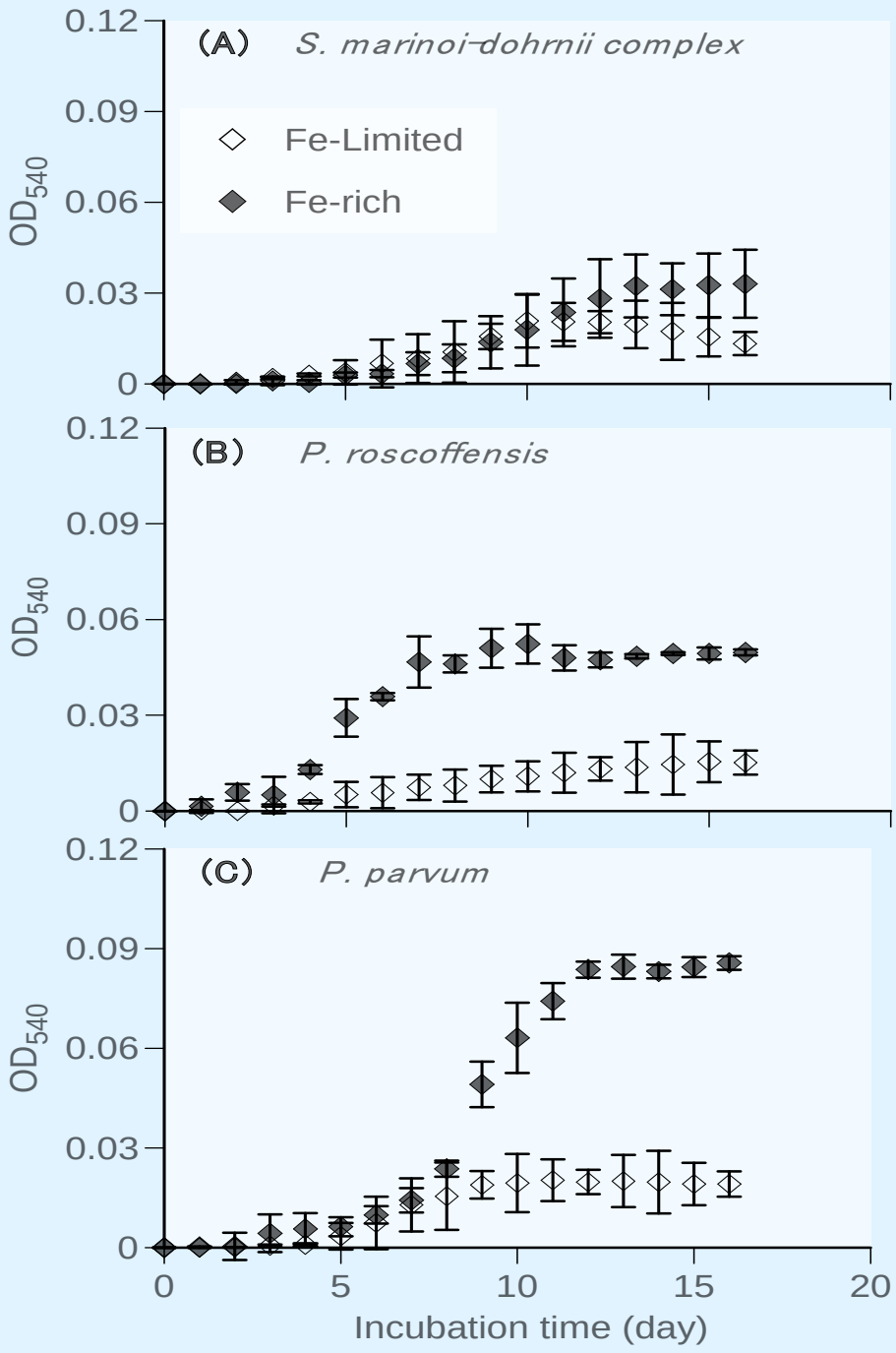

Fig. 3.1: Growth response of marine phytoplankton (*P. roscoffensis*, *S. marinoi-dohrnii complex* and *P. parvum*) to Fe-rich (1 µM) and Fe-limited (for *P. roscoffensis* and *S. marinoi-dohrnii complex* was 0.07 µM, while for *P. parvum* was 0.03 µM) conditions. Data are mean ± SD (n = 3).

The growth of the phytoplankton decreased steadily for the addition of 100 µM DFO-B or DTPA (ligand-induced Fe-limited conditions) to the growing medium after 10 days (Fig. 3.2). This was because the ligands form stable Fe(III)-L complexes that decrease Fe bioavailability to phytoplankton (Boye *et al.*, 2000, Hutchins *et al.*, 1999a), and creates an Fe-limited condition in the growing medium. As a result, the phytoplankton could not obtain required amount of Fe for normal growth.

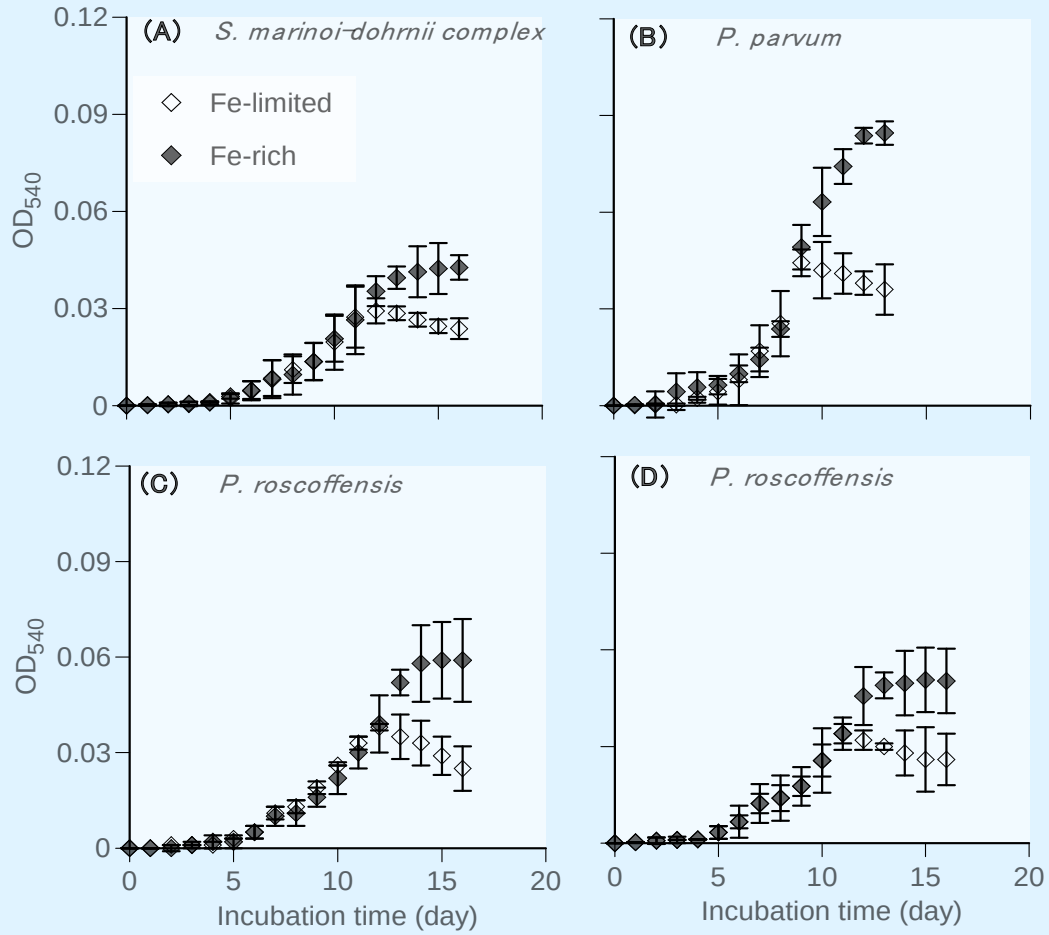

Fig. 3.2: Growth response of marine phytoplankton (*P. roscoffensis*, *S. marinoi-dohrnii complex* and *P. parvum*) to ligand-induced Fe-limited condition. Fe concentration in Fe-rich growing medium for all phytoplankton was 1 µM, and Fe-limited condition was induced by adding 100 µM DFO-B (A – C) or 100 µM DTPA (D) in the growing medium after 10 days of inoculation. The growth of phytoplankton was measured on the basis of optical density at a wavelength of 540 nm (OD_{540}). Data are mean ± SD (n = 3).

3.3.2 Protein expression in response to low total Fe condition

The phytoplankton expressed different types of proteins under Fe-limited and Fe-replete conditions, and the protein expression differed among them (Fig. 3.3). *Pleurochrysis roscoffensis* expressed three types of proteins with molecular weights of 19, 33 and 42 kDa under low total Fe (0.07 µM) conditions (Fig. 3.3a). *Prymnesium parvum* also expressed similar proteins (Fig. 3.3b); while a new protein of 55 kDa molecular weight was found to be expressed by *S. marinoi-dohrnii complex* under the Fe-limited conditions (Fig. 3.3c). Both *P. parvum* and *P. roscoffensis* expressed a protein of 42 kDa molecular weight under low total Fe conditions in this study. Many prokaryotic and eukaryotic organisms under Fe-limited conditions have also been reported to express a similar protein (42 kDa), which is characterized as a cytoplasmic carotenoprotein of the organisms (Reddy et al., 1988). From a marine cyanobacteria, Webb et al. (2001) isolated a 42 kDa *idiA* protein, which is characterized as an outer membrane protein.

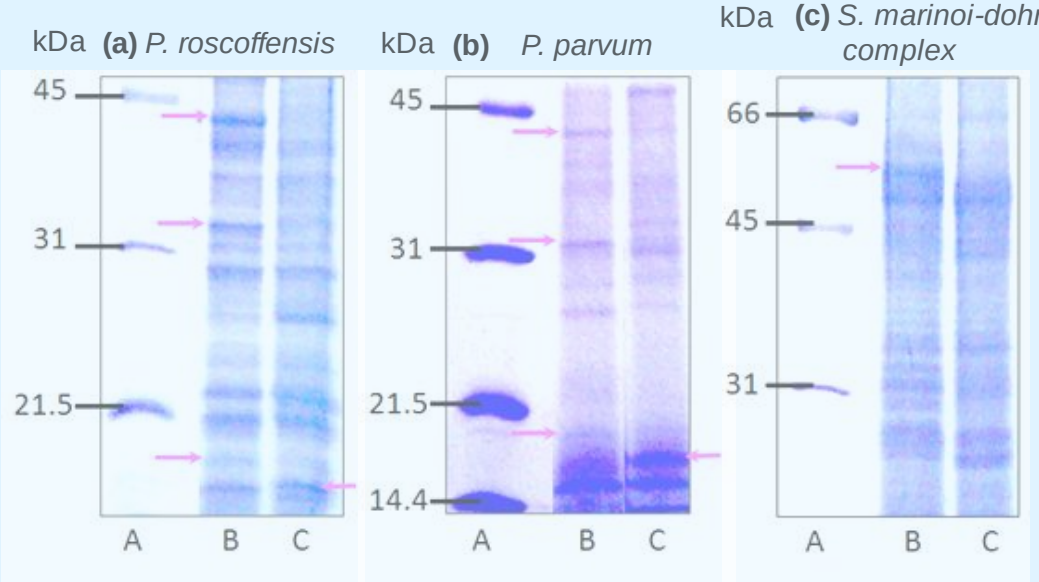

Fig. 3.3: Protein expression of marine phytoplankton (*P. roscoffensis*, *S. marinoi-dohrnii complex* and *P. parvum*) under Fe-rich (1 µM) and Fe-limited (for *P. roscoffensis* and *S. marinoi-dohrnii complex* was 0.07 µM, while for *P. parvum* was 0.03 µM) conditions. Lane A = Marker, Lane B = F-limited (normal), and Lane C = Fe-rich. Arrows indicate bands of proteins that are up regulated in Fe-limited conditions compared with Fe-rich condition.

In *P. roscoffensis* and *P. parvum*, two different proteins of similar molecular weights (32 and 33 kDa, respectively) were expressed under low total Fe conditions (Fig. 3.3a, b). Under Fe-deficient conditions, an ATP-binding cassette protein and an ABC-transporter protein with molecular weight of 32 kDa (Cockayne *et al.*, 1998, Heinrichs *et al.*, 1999, Menozzi *et al.*, 1991) and 33 kDa (Andreoni *et al.*, 2009, Tong & Guo, 2007) have been identified in bacteria. Therefore, the 32 and 33 kDa proteins in *P. parvum* and *P. roscoffensis*, respectively, are assumed to be components of ATP-binding cassette or ABC transporter proteins, which may play important role in Fe uptake under Fe-depleted conditions. *Skeletonema marinoi-dohrnii complex* expressed a protein of 55 kDa molecular weight under low total Fe conditions (Fig. 3.3c). Li et al. (2003) also reported the expression of a 55 kDa protein in red tide alga (*Heterosigma akashiwo*) and proposed this protein as a molecular marker of Fe-limited condition in the alga. Therefore, this 55 kDa protein is considered to be a Fe transport protein in the phytoplankton membrane, which facilitate Fe uptake in the cell under Fe-deficient conditions.

3.3.3 Proteomic response to Fe-limitation and replacement of ferredoxin by flavodoxin

Under low total Fe conditions, *P. parvum* expressed three proteins with molecular weights of 19, 32, and 42 kDa, which were very similar to those proteins expressed by *P. roscoffensis*. In addition, one protein with a molecular weight of 17 kDa was also expressed by *P. roscoffensis* and *P. parvum* under Fe-replete conditions (Table 3.3). Thus, *P. roscoffensis* and *P. parvum* expressed very similar proteins under low total Fe (0.07 and 0.03 μM for *P. roscoffensis* and *P. parvum*, respectively) and Fe-replete (1 μM) conditions.

Marine microbes (phytoplankton, blue green algae and bacteria) have been observed to employ several strategies to maintain normal Fe acquisition and growth in Fe-limited environments. These strategies include i) increase of the number of Fe transport sites on the cell surface, ii) decrease the cellular Fe requirements by replacing ferredoxin with flavodoxin, and iii) use of a Fe-siderophore uptake system or a transport ligand process involving cell surface complexation and internalization

of Fe (Geider & Roche, 1994, Roche *et al.*, 1999, Wells *et al.*, 1995). Phytoplankton can employ one or a combination of more than one of these strategies under Fe-limited conditions. *Pleurochrysis roscoffensis* and *P. parvum* strongly expressed a protein with a molecular weight of 17 kDa under Fe-replete conditions; whereas they expressed another protein with a molecular weight of 19 kDa under Fe-limited conditions (Table 3.3). We supposed that this was a replacement of ferredoxin (17 kDa) by flavodoxin (19 kDa) in *P. parvum* and *P. roscoffensis.* This would be a strategy of *P. parvum* and *P. roscoffensis* to reduce cellular Fe requirement under Fe-limited conditions.

Table 3.3: Proteins in phytoplankton expressed under low total Fe and chelant-induced Fe-limited conditions and under Fe-rich condition.

Phytoplankton	**Proteins under Fe-rich condition**	**Proteins under Fe-limited conditions**		
		Low total Fe	**DFO-B**	**DTPA**
P. roscoffensis	17 kDa	19 kDa	19 kDa	19 kDa
	-	33 kDa	33 kDa	27 kDa
	-	42 kDa	-	42 kDa
P. parvum	17 kDa	19 kDa	19 kDa	-
	-	32 kDa	-	-
	-	42 kDa	-	-
S. marinoi-dohrnii complex	-	55 kDa	58 kDa	-
	-	-	60 kDa	-

Protein substitution is a successful mechanism commonly observed in microorganisms to compensate for stress induced by deficiency of trace elements (Roche *et al.*, 1999). Under Fe deficient conditions, the low molecular weight protein flavodoxin can successfully replace the Fe-sulfur protein ferredoxin in several cellular metabolic processes of phytoplankton, including photosynthetic electron transport (Geider & Roche, 1994, Razquin *et al.*, 1995, Straus, 2004), and as an electron donor to both nitrite reductase and glutamate synthase (Roche *et al.*, 1999, Roche *et al.*, 1993, Vigara *et al.*, 1995). Anderson (1999) reported that marine

phytoplankton replace the common Fe-sulfur redox protein 'ferredoxin' with a functionally equivalent non-Fe-containing protein 'flavodoxin' under Fe stress condition. Therefore, these two proteins (17 kDa and 19 kDa) have been proposed as indicators of Fe nutritional status in marine phytoplankton (Anderson, 1999). The present study showed that *P. roscoffensis* and *P. parvum* also replace ferredoxin with flavodoxin under Fe-limited conditions, whether the Fe-limitation was due to low concentration of absolute Fe (Fig. 3.3a, b) or to complexation of Fe with chelating ligands (Fig. 3.4a, b).

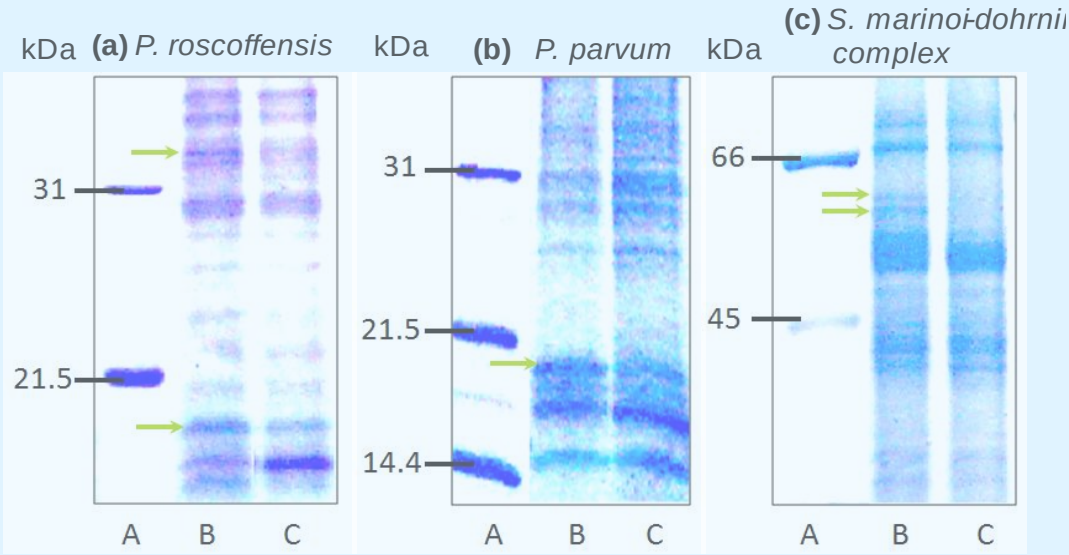

Fig. 3.4: Protein expression of marine phytoplankton (*P. roscoffensis*, *S. marinoi-dohrnii complex* and *P. parvum*) under ligand-induced Fe-limited condition. Lane A = Marker, Lane B = F-limited (1 µM Fe + 100 µM DFO-B), and Lane C = Fe-rich (1 µM Fe). Arrows indicate protein bands that are up regulated in Fe-limited conditions.

3.3.4 Protein expression under ligand-induced Fe-limited condition

When the Fe-limited condition was created by adding 100 µM DFO-B, *P. roscoffensis* and *P. parvum* strongly expressed a 19 kDa protein (Fig. 3.4a, b). In addition, *P. roscoffensis* expressed a 33 kDa protein (Fig. 3.4a), which was not expressed by *P. parvum*. Two proteins (58 kDa and 60 kDa) were expressed by *S. marinoi-dohrnii complex* under DFO-B-induced Fe-limited conditions (Fig. 3.4c),

which are different from those proteins expressed under low Fe conditions (Fig. 3.3c).

It is notable that *P. roscoffensis* expressed two proteins of (19 kDa and 33 kDa) under both low total Fe (due to low absolute Fe concentration) and DFO-B-induced Fe-limited conditions (Fig. 3.4a, 3.5a), while this phytoplankton expressed three proteins (19 kDa, 27 kDa and 42 kDa) under DTPA-induced Fe-limited conditions (Fig. 3.5b). The expression of a new protein (27 kDa) under DTPA-induced Fe-limited conditions, instead of the 33 kDa protein under low total Fe (Fig. 3.3a) and DFO-B-mediated Fe-limited (Fig. 3.4a) conditions, would be a strategy of this phytoplankton for Fe acquisition. It is necessary to characterize the 27 kDa and 33 kDa proteins to understand which of the strategies the phytoplankton used for Fe acquisition under ligand-induced Fe-limited conditions.

It is notable that *P. roscoffensis* expressed two proteins of (19 kDa and 33 kDa) under both low total Fe (due to low absolute Fe concentration) and DFO-B-induced Fe-limited conditions (Fig. 3.4a, 3.5a), while this phytoplankton expressed three proteins (19 kDa, 27 kDa and 42 kDa) under DTPA-induced Fe-limited conditions (Fig. 3.5b). The expression of a new protein (27 kDa) under DTPA-induced Fe-limited conditions, instead of the 33 kDa protein under low total Fe (Fig. 3.3a) and DFO-B-mediated Fe-limited (Fig. 3.4a) conditions, would be a strategy of this phytoplankton for Fe acquisition. It is necessary to characterize the 27 kDa and 33 kDa proteins to understand which of the strategies the phytoplankton used for Fe acquisition under ligand-induced Fe-limited conditions.

Skeletonema marinoi-dohrnii complex expressed two proteins (58 kDa and 60 kDa) under DFO-B-induced Fe-limited conditions (Fig. 3.4c); however, this phytoplankton did not express the 55 kDa protein under these conditions that was expressed under low total Fe conditions (Fig. 3.3c). This differential expression of new proteins against ligand-induced Fe limitation suggests that phytoplankton might alter uptake mechanisms for the ligand-bound Fe, and might use some cell surface proteins to liberate and uptake the ligand-bound Fe. Hutchins et al. (1999a) also

reported that different groups of marine phytoplankton use different uptake mechanisms to acquire Fe that is tightly bound to organic ligends.

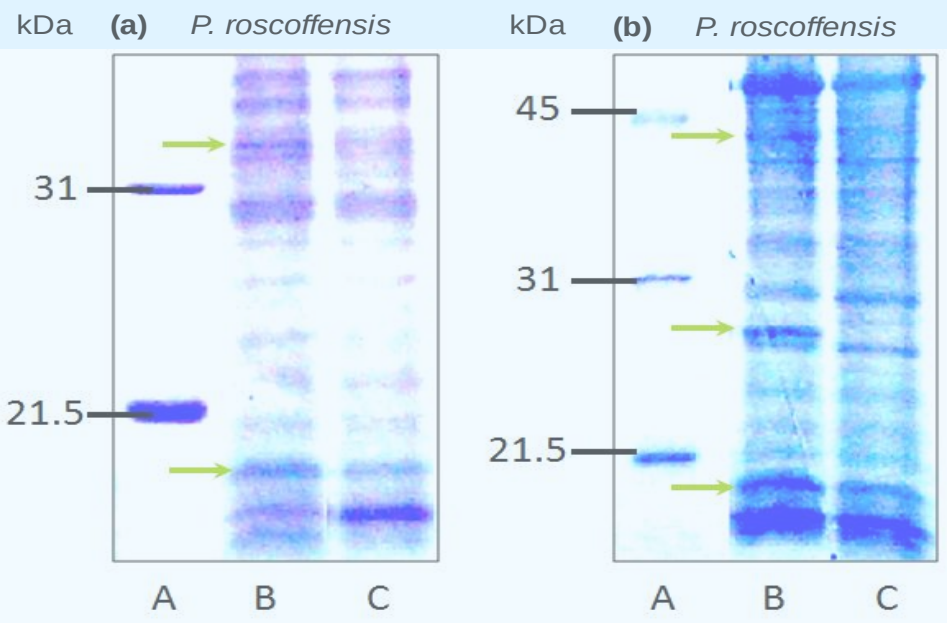

Fig. 3.5: Protein expression of marine phytoplankton (*P. roscoffensis*) under **(a)** DFO-B-induced and **(b)** DTPA-induced Fe-limited conditions. Lane A = Marker, Lane B = Fe-limited (1 µM Fe + 100 µM DPTA or DFO-B), and Lane C = Fe-rich (1 µM Fe). Arrows indicate bands of proteins that are up regulated in Fe-limited conditions compared with Fe-rich condition.

3.4 Conclusion

Marine phytoplankton needs adequate amount of Fe for normal growth and cellular metabolism. However, under Fe-limited conditions, whether the limitation is due to low concentration absolute Fe or to low bioavailability, marine phytoplankton employ several strategies to compensate for Fe stress. These strategies are regulated by the expression of specific proteins in marine phytoplankton. For example, in the present study, we found that *P. roscoffensis* and *P. parvum* expressed a 17 kDa protein under Fe-rich conditions, while they expressed a 19 kDa protein under Fe-limited conditions. We supposed that this was a substitution of 17 kDa (ferredoxin) by 19 kDa (flavodoxin) that was utilized by the phytoplankton to reduce cellular Fe

requirement under Fe-limited conditions. The present study also showed that marine phytoplankton produce different proteins under low total Fe and ligand-induced Fe-limited conditions. Protein expression under different Fe conditions also varies within phytoplankton species. Form the present studies; it was not clear whether the proteins expressed in phytoplankton under different Fe conditions are membrane proteins or cytoplasmic proteins. Further studies are needed to identify the location of the proteins and to characterize the proteins to understand Fe uptake mechanisms and the role of the proteins in Fe acquisition under Fe-limited conditions.

References

Andersen, R. A. (2004) Biology and systematics of heterokont and haptophyte algae. *American Journal of Botany,* **91,** 1508-1522.

Anderson, D. M. (1999) Characterization of ferredoxin and flavodoxin as markers of iron limitation in marine phytoplankton. *Marine Ecology Progress Series,* **184,** 43-53.1999.

Anderson, M. A. and Morel, F. M. M. (1982) The influence of aqueous iron chemistry on the uptake of iron by the coastal diatom *Thalassiosira weissflogii*. *Limnology and Oceanography,* **27,** 789-813.

Andreoni, F., Boiani, R., Serafini, G., Bianconi, I., Dominici, S., Gorini, F. and Magnani, M. (2009) Expression, purification, and characterization of the recombinant putative periplasmic hemin-binding protein (HutB) of *Photobacterium damselae* subsp. *piscicida*. *Bioscience, biotechnology, and biochemistry,* **73,** 1180-1183.

Benner, R. (2011) Loose ligands and available iron in the ocean. *Proceedings of the National Academy of Sciences,* **108,** 893-894.

Boye, M., Berg, V. D. and G., C. M. (2000) Iron availability and the release of iron-complexing ligands by *Emiliania huxleyi*. *Marine Chemistry,* **70,** 277-287.

Cockayne, A., Hill, P. J., Powell, N. B. L., Bishop, K., Sims, C. and Williams, P. (1998) Molecular cloning of a 32-kilodalton lipoprotein component of a novel iron-regulated *Staphylococcus epidermidis* ABC transporter. *Infection and immunity,* **66,** 3767.

Dhungana, S. and Crumbliss, A. L. (2005) Coordination chemistry and redox processes in siderophore-mediated iron transport. *Geomicrobiology Journal,* **22,** 87-98.

Eckhardt, U. and Buckhout, T. J. (1998) Iron assimilation in *Chlamydomonas reinhardtii* involves ferric reduction and is similar to Strategy I higher plants. *Journal of experimental botany,* **49,** 1219-1226.

Field, C. B., Behrenfeld, M. J., Randerson, J. T. and Falkowski, P. (1998) Primary production of the biosphere: Integrating terrestrial and oceanic components. *Science,* **281,** 237-240.

Fujii, M., Rose, A. L., Waite, T. D. and Omura, T. (2010) Oxygen and superoxide-mediated redox kinetics of iron complexed by humic substances in coastal seawater. *Environmental Science & Technology,* **44,** 9337-9342.

Geider, R. J. (1999) Biological oceanography: Complex lessons of iron uptake. *Nature,* **400,** 815-816.

Geider, R. J. and Roche, J. (1994) The role of iron in phytoplankton photosynthesis, and the potential for iron-limitation of primary productivity in the sea. *Photosynthesis research,* **39,** 275-301.

Gledhill, M., Van Den Berg and G., C. M. (1994) Determination of complexation of iron(III) with natural organic complexing ligands in seawater using cathodic stripping voltammetry. *Marine Chemistry,* **47,** 41-54.

Goldman, S. J., Lammers, P. J., Berman, M. S. and Sanders-Loehr, J. (1983) Siderophore-mediated iron uptake in different strains of *Anabaena* sp. *Journal of bacteriology,* **156,** 1144-1150.

Guillard, R. R. L. and Ryther, J. H. (1962) Studies of marine planktonic diatoms: I. Cyclotella Nana Hustedt, and Detonula Confervacea (Cleve) Gran. *Canadian Journal of Microbiology,* **8,** 229-239.

Heinrichs, J. H., Gatlin, L. V. E., Kunsch, C., Choi, G. H. and Hanson, M. S. (1999) Identification and characterization of *Sir*A, an iron-regulated protein from *Staphylococcus aureus*. *Journal of bacteriology,* **181,** 1436.

Hopkinson, B. M. and Morel, F. M. M. (2009) The role of siderophores in iron acquisition by photosynthetic marine microorganisms. *Biometals,* **22,** 659-669.

Hudson, R. J. M. and Morel, F. M. M. (1990) Iron transport in marine phytoplankton: Kinetics of cellular and medium coordination reactions. *Limnology and Oceanography,* **35,** 1002-1020.

Hutchins, D. A., Franck, V. M., Brzezinski, M. A. and Bruland, K. W. (1999a) Inducing phytoplankton iron limitation in iron-replete coastal waters with a strong chelating ligand. *Limnology and Oceanography,* **44,** 1009-1018.

Hutchins, D. A., Witter, A. E., Butler, A. and Luther, G. W. (1999b) Competition among marine phytoplankton for different chelated iron species. *Nature,* **400,** 858-861.

Kraemer, S. M., Cheah, S. F., Zapf, R., Xu, J., Raymond, K. N. and Sposito, G. (1999) Effect of hydroxamate siderophores on Fe release and Pb(II) adsorption by goethite. *Geochimica et Cosmochimica Acta,* **63,** 3003-3008.

Kranzler, C., Lis, H., Shaked, Y. and Keren, N. (2011) The role of reduction in iron uptake processes in a unicellular, planktonic cyanobacterium. *Environmental Microbiology,* **13,** 2990-2999.

Li, D., Cong, W., Cai, Z., Shi, D. and Ouyang, F. (2003) Some physiological and biochemical changes in marine eukaryotic red tide alga *Heterosigma akashiwo*

during the alleviation from iron limitation. *Plant Physiology and Biochemistry,* **41,** 295-301.

Lis, H. and Shaked, Y. (2009) Probing the bioavailability of organically bound iron: A case study in the *Synechococcus*-rich waters of the Gulf of Aqaba. *Aquatic Microbial Ecology,* **56,** 241-253.

Lyman, J. and Fleming, R. H. (1940) Composition of sea water. *Journal of marine Research,* **3,** 134-146.

Macrellis, H. M., Trick, C. G., Rue, E. L., Smith, G. and Bruland, K. W. (2001) Collection and detection of natural iron-binding ligands from seawater. *Marine Chemistry,* **76,** 175-187.

Maldonado, M. T. and Price, N. M. (2000) Nitrate regulation of Fe reduction and transport by Fe-limited *Thalassiosira oceanica*. *Limnology and Oceanography,* 814-826.

Maldonado, M. T. and Price, N. M. (2001) Reduction and transport of organically bound iron by *Thalassiosira oceanica* (Bacillariophyceae). *Journal of Phycology,* **37,** 298-310.

Martin, J. H. and Fitzwater, S. E. (1988) Iron deficiency limits phytoplankton growth in the north-east Pacific subarctic. *Nature,* **331,** 341-343.

Menozzi, F. D., Gantiez, C. and Locht, C. (1991) Identification and purification of transferrin-and lactoferrin-binding proteins of *Bordetella pertussis* and *Bordetella bronchiseptica*. *Infection and immunity,* **59,** 3982.

Morrissey, J. and Bowler, C. (2012) Iron utilization in marine cyanobacteria and eukaryotic algae. *Frontiers in Microbiology,* **3,** 1-13.

Naito, K., Suzuki, M., Mito, S., Hasegawa, H., Imai, I., Sohrin, Y. and Matsui, M. (2001) The pursuit of siderophore secreted by marine phytoplankton *Rhodomonas ovalis*. *Analytical Sciences,* **17,** i817-i819.

Razquin, P., Schmitz, S., Peleato, M. L., Fillat, M. F., Gómez-Moreno, C. and Böhme, H. (1995) Differential activities of heterocyst ferredoxin, vegetative cell ferredoxin, and flavodoxin as electron carriers in nitrogen fixation and photosynthesis in *Anabaena* sp. *Photosynthesis research,* **43,** 35-40.

Reddy, K. J., Bullerjahn, G. S., Sherman, D. M. and Sherman, L. A. (1988) Cloning, nucleotide sequence, and mutagenesis of a gene (*irpA*) involved in iron-deficient growth of the cyanobacterium *Synechococcus sp.* strain PCC7942. *Journal of bacteriology,* **170,** 4466.

Roche, J. L., Mckay, R. and Boyd, P. (1999) Immunological and molecular probes to detect phytoplankton responses to environmental stress in nature. *Hydrobiologia,* **401,** 177-198.

Roche, L. J., Geider, R. J., Graziano, L. M., Murray, H. and Lewis, K. (1993) Induction of specific proteins in eukaryotic algae grown under iron phosphorus or nitrogen deficient conditions. *Journal of Phycology,* **29,** 767-777.

Rose, A. L. and Waite, T. D. (2005) Reduction of organically complexed ferric iron by superoxide in a simulated natural water. *Environmental Science & Technology,* **39,** 2645-2650.

Rue, E. L. and Bruland, K. W. (1995) Complexation of iron(III) by natural organic ligands in the Central North Pacific as determined by a new competitive ligand equilibration/adsorptive cathodic stripping voltammetric method. *Marine Chemistry,* **50,** 117-138.

Shaked, Y., Kustka, A. B. and Morel, F. M. M. (2005) A general kinetic model for iron acquisition by eukaryotic phytoplankton. *Limnology and Oceanography,* **50,** 872-882.

Shaked, Y. and Lis, H. (2012) Disassembling iron availability to phytoplankton. *Frontiers in Microbiology,* **3,** 1-26.

Shi, D., Xu, Y., Hopkinson, B. M. and Morel, F. M. M. (2010) Effect of ocean acidification on iron availability to marine phytoplankton. *Science,* **327,** 676-679.

Soria-Dengg, S. and Horstmann, U. (1995) Ferrioxamines B and E as iron sources for the marine diatom *Phaeodactylum tricornutum*. *Marine Ecology Progress Series,* **127,** 269-277.

Soria-Dengg, S., Reissbrodt, R. and Horstmann, U. (2001) Siderophores in marine coastal waters and their relevance for iron uptake by phytoplankton: Experiments with the diatom Phaeodactylum tricornutum. *Marine Ecology Progress Series,* **220,** 73-82.

Sorokin, C. (1973) Dry weight packed cell volume and optical density. In: S. J. (ed) *Handbook of Phycological Methods: Culture Methods and Growth Measurements.* Cambridge University Press, New York, pp. 321-343.

Straus, N. A. (2004) Iron deprivation: Physiology and gene regulation. In: D. A. Bryant (ed) *The Molecular Biology of Cyanobacteria.* Springer, Netherlands, pp. 731-750.

Sunda, W. G. and Huntsman, S. A. (1995) Iron uptake and growth limitation in oceanic and coastal phytoplankton. *Marine Chemistry,* **50,** 189-206.

Tong, Y. and Guo, M. (2007) Cloning and characterization of a novel periplasmic heme-transport protein from the human pathogen *Pseudomonas aeruginosa*. *Journal of Biological Inorganic Chemistry,* **12,** 735-750.

Trick, C. G., Andersen, R. J., Price, N. M., Gillam, A. and Harrison, P. J. (1983) Examination of hydroxamate-siderophore production by neritic eukaryotic marine phytoplankton. *Marine Biology,* **75,** 9-17.

Vigara, A. J., Gómez-Moreno, C. and Vega, J. M. (1995) The role of flavodoxin in the reaction catalysed by the glutamate synthase from *Monoraphidium braunii*. *Bioelectrochemistry and Bioenergetics,* **38,** 21-24.

Webb, E. A., Moffett, J. W. and Waterbury, J. B. (2001) Iron stress in open-ocean cyanobacteria (*Synechococcus*, *Trichodesmium*, and *Crocosphaera spp*.): identification of the *Idi*A protein. *Applied and environmental microbiology,* **67,** 5444.

Wells, M. L., Price, N. M. and Bruland, K. W. (1995) Iron chemistry in seawater and its relationship to phytoplankton: A workshop report. *Marine Chemistry,* **48,** 157-182.

Wu, J. and Luther, G. W. (1995) Complexation of Fe(III) by natural organic ligands in the Northwest Atlantic Ocean by a competitive ligand equilibration method and a kinetic approach. *Marine Chemistry,* **50,** 159-177.

4

RESULTS AND DISCUSSION

PROTEINS AND THEIR EXPRESSION LEVEL IN MARINE PHYTOPLANKTON (*Prymnesium parvum*) AS BIOMARKER OF NUTRIENT CONDITIONS IN AQUATIC SYSTEMS

4.1 Introduction

Primary productivity of aquatic systems is frequently rate-limited by the availability of nutrients. Principally this involves limitations of nitrogen (N), phosphorus (P) and iron (Fe) (Menzel *et al.*, 1963, Moore & Doney, 2007, Timmermans *et al.*, 2005). Nitrogen is an important nutrients for phytoplankton and is a major element of proteins, nucleic acids, chlorophylls, amino acids, N-containing osmolytes (glycine betaine) and chitin (Geider & La Roche, 2002). Nitrogen has been considered to be the predominant limiting nutrient in many coastal and deep sea systems (Elser *et al.*, 2007, Howarth & Marino, 2006), which is

assumed to be responsible for the decrease of primary production of the aquatic systems (Elser *et al.*, 2007, Tyrrell, 1999). Phosphorus is also an obligate requirement for all organisms, which is used for RNA, phospholipid and DNA biosynthesis (Van Mooy *et al.*, 2009). It is also an important component of many cellular substrates such as glucose phosphate coenzymes, Adenosine triphosphate (ATP), Cyclic adenosine monophosphate (cAMP), Inositol triphosphate (IP3), and inorganic polyphosphate reserves (Geider & La Roche, 2002). Several studies have shown that many marine systems such as the Subtropical Pacific waters (Ammerman *et al.*, 2003), the Mediterranean Sea (Beardall *et al.*, 2001), the Sargasso Sea (Moore *et al.*, 2008), the North Pacific subtropical gyre (Van Mooy *et al.*, 2006), and the Atlantic Ocean (Ammerman *et al.*, 2003) are P limited, which has been an important concern for a sustainable and healthy aquatic environment. A study by Elser *et al.* (2007) showed that P limitation is equally strong and important concern to N limitation across marine and freshwater systems.

Iron comprises the central part of chlorophyll-*a* (Chl-*a*) molecule, and it is involved in many cellular biochemical processes such as photosynthesis, respiration, nitrogen fixation and nitrate, nitrite, and sulfate reductions (Geider & Roche, 1994). Several studies have identified Fe as a limiting nutrient for phytoplankton (Shi *et al.*, 2010, Watson *et al.*, 2000) that controls the primary productivity in many high nutrient and low chlorophyll open seas such as the equatorial Pacific and Southern Ocean (Beardall *et al.*, 2001, Blain *et al.*, 2007). Iron limitation is also a major factor that control phytoplankton growth in the subarctic Pacific and the North Atlantic oceans (Moore *et al.*, 2007).

A bioindicators, which are defined as organisms or biological responses that reveal the presence of chemicals in the environment by producing typical symptoms or measurable responses, have been widely accepted for monitoring ecological risks of environmental pollutants (Bartell, 2006). Usually, biological species or group of species, whose function, population, or status can reveal what degree of ecosystem or environmental integrity is present, are used as bioindicator. However, with the advancement of biotechnology, cellular macromolecules such as phytochelatins

(Gawel *et al.*, 2001, Kawakami *et al.*, 2006, Morelli & Pratesi, 1997), glutathione (Kawakami *et al.*, 2006), phospholipid fatty acid (Kaur *et al.*, 2005), proteins (Haasch *et al.*, 1992, Karsten & Rice, 2004, Lewis *et al.*, 2001, Sánchez *et al.*, 2000, Shpigel *et al.*, 1999), and RNA (Haasch *et al.*, 1992) have also been used as bioindicators of metals, nutrients, and environmental pollutants in order to monitor and assess their ecological risks. In addition, several studies have shown that the expression levels of certain proteins [e.g., circulating reactive protein; CRP (Karsten & Rice, 2004), heat shock protein;Hsp70 (Lewis *et al.*, 2001)] can also be used as bioindicators of exposure to environmental contaminants and stressors. In the present study, the growth responses and protein expressions of unicellular marine phytoplankton *Prymnesium parvum* (Haptophyta) under different conditions of N, P and Fe were investigated under laboratory conditions in order to assess the potential of protein expression levels as bioindicators of nutritional conditions in aquatic systems.

4.2 Materials and Methods

4.2.1 Phytoplankton pre-culture and maintenance

Marine microalga *P. parvum* was collected from Fukuyama Bay, Hiroshima, Japan (This strain is gently provided from Prof. I. Imai, Hokkaido University, Japan). The microalgal strain was axenic (axenicity was assessed and monitored by 4′ 6-diamidino-2-phenylindole (DAPI) test). The axenic microalgal strain was pre-cultured for two weeks in 30-mL polycarbonate bottles with modified f/2 culture solution in artificial seawater without nitrogen (NO_3^-), phosphate (PO_4^{3-}), and Fe ($FeCl_3$) (Guillard & Ryther, 1962, Lyman & Fleming, 1940).

4.2.2 Chemical treatments and growth measurement

Nitrogen concentrations (prepared from $NaNO_3$; Kanto Chemicals, Tokyo, Japan) of the culture medium were 5, 20, 30, and 50 μM. Phosphate solution was prepared from $NaH_2PO_4 \cdot 2H_2O$ (Wako Pure Chemicals, Osaka, Japan), and treatment concentrations were 10, 5, 1, 0.5 and 0.3 μM. The Fe concentrations (prepared from

$FeCl_3$; Kanto Chemicals, Tokyo, Japan) in the culture medium were 1, 0.7, 0.3, 0.1 and 0.01 µM. All other chemicals and reagents used in this study were of analytical grade.

Phytoplankton growth was measured on the basis of optical density using a spectrophotometer (U-2000, Hitachi High Technologies, Japan) at a wavelength of 540 nm (OD_{540}) following the same method of Maki *et al.* (2008). The experiment was replicated for three times.

4.2.3 Growing of phytoplankton

The same procedure was followed as described under the subsection 2.2.1.

4.2.4 Protein extraction

The same procedure was followed as described under the subsection 2.2.3.

4.2.5 Gel electrophoresis of protein

After adding 3.75 µL of 20% Triton X-100 [$C_{14}H_{22}O(C_2H_4O)_n$] and 3.75 µL of 50 mM $MgCl_2$ to 30 µL of protein sample of Fe-deplete and Fe-rich treatments, the samples were incubated in ice for 60 min. Equal volume (37.5 µL) of the sample and buffer solution (125 mM Tris-HCl, 4% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 10% sucrose, 0.004% bromophenol blue (BPB), pH 6.8) were then mixed together and then incubated for 3 min at 95 °C. A molecular weight marker (MWM) was diluted by 20-times and an equal volume (30 µL) of MWM and buffer were mixed together and then incubated for 3 min at 95 °C. A 20 µL protein sample and MWM were loaded in 14% polyacrylamide gel. The gel was then run in a mini pageRun system (AE-6531, ATTO bioscience & Biotechnology, Japan) for 100 min at 40 mA current using buffer solution prepared with 25 mM Tris-HCl, 0.1% SDS, 192 mM glycine. After electrophoresis, the gel was stained by $AgNO_3$ staining following the procedure of Gromova and Celis (2006). The molecular weights of protein bands were then measured by Alpha-Ease FC (v 4.0) software.

4.2.6 Statistics

One-way ANOVA of the growth and protein expression data was performed with GraphPad InStat (GraphPad Software, USA) to assess the significant differences among the mean values of the nutrient treatments.

4.3 Results and discussion

4.3.1 Growth of phytoplankton under different N, P, and Fe conditions

The growth of marine phytoplankton *P. parvum* was largely affected by N, P and Fe concentrations in culture medium (Fig.4.1A). Phytoplankton growth was the highest at 100 µM nitrate concentration and the lowest in N starvation (0 – 5 µM nitrate concentrations). In fact, phytoplankton growth was gradually increased with the increase of nitrate concentration in the culture medium. However, the growth of the organism decreased gradually after day 12, which may be because of gradual decrease of N concentration in the culture medium with exposure time due to uptake by the organism. The phytoplankton did not grow when phosphate was not added to the culture medium. Compared to 0.3 – 1.0 µM phosphate treatments, the growth of *P. parvum* was significantly ($p < 0.05$) higher under 5 – 10 µM phosphate treatments (Fig.4.1B). P and N limitation of phytoplankton growth has been well documented in previous studies (Colijn & Cadée, 2003, Elser *et al.*, 1990) due to the fact that these nutrients are involved in many biochemical reactions including proteins, nucleic acids, chlorophylls and amino acids biosynthesis in photosynthetic organisms (Geider & La Roche, 2002, Van Mooy *et al.*, 2009).

The marine phytoplankton *P. parvum* showed normal growth at Fe concentration of 0.1 µM or above, and growth was significantly reduced ($p < 0.05$) at 0.01 µM Fe concentration (Fig.4.1C). In marine systems, Fe has been identified as a limiting nutrient for phytoplankton (Shi *et al.*, 2010, Watson *et al.*, 2000) that reduce phytoplankton grown and primary productivity in many open seas such as the equatorial Pacific and Southern Ocean (Beardall *et al.*, 2001, Blain *et al.*, 2007) and

the subarctic Pacific and the North Atlantic oceans (Moore *et al.*, 2007). The growth reduction under Fe-trace condition may associated with the fact that this nutrient play important roles in many cellular biochemical processes such as photosynthesis, respiration, nitrogen fixation, and nitrate, nitrite, and sulfate reductions (Geider & Roche, 1994).

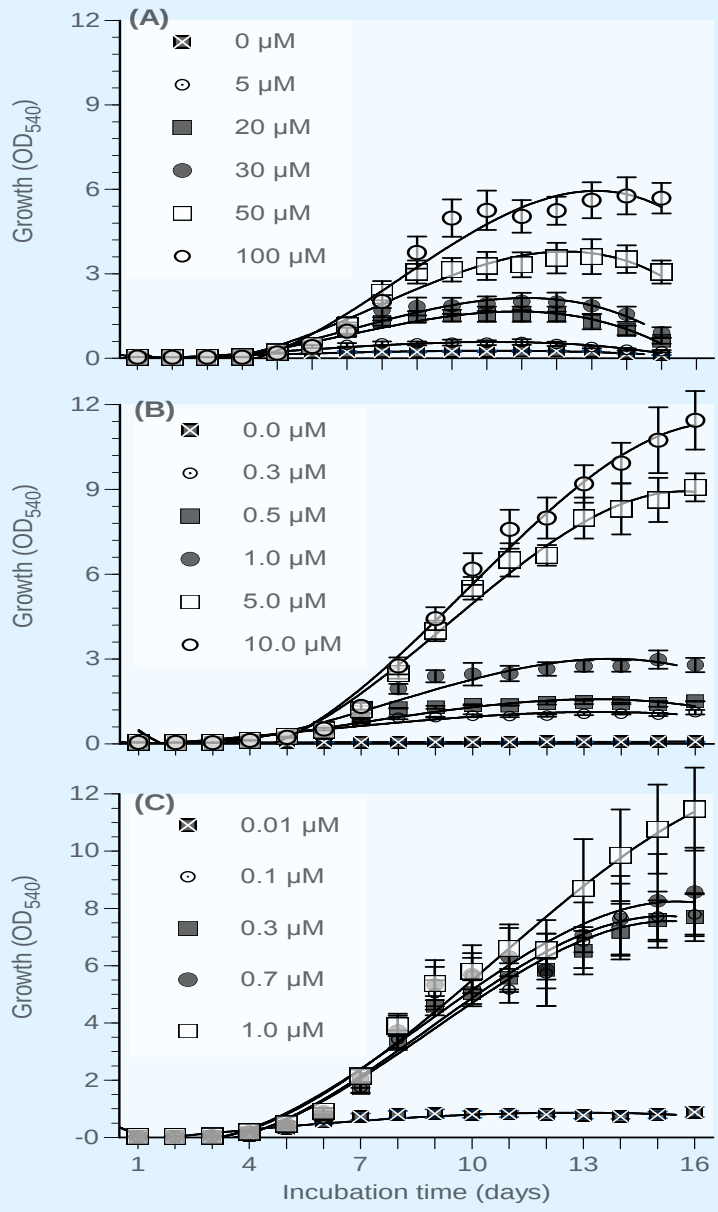

Fig.4.1: Effect of nitrate (A), phosphate (B) and iron (C) concentrations on the growth of *Prymnesium parvum*. The growth measurement was performed based on the optical density at a wavelength of 540 nm (OD_{540}). Data are mean ± SD (n = 3).

4.3.2 Proteomic expression of P. parvum in response to different N, P, and Fe conditions

Protein expression of the marine phytoplankton *P. parvum* was studied under different conditions of nitrate, phosphate and iron in the culture medium. Results showed that *P. parvum* changes the expression levels of certain proteins in response to different concentrations of the nutrients. For example, an 83 kDa protein was highly expressed in *P. parvum* at 5 µM nitrate treatment, and it's expression was significantly down-regulated ($p < 0.001$) at higher nitrate treatments (20 – 100 µM) (Fig.4.2). The expression level of this protein at 0 µM nitrate treatment was also significantly lower ($p < 0.001$) than that at 5 – 100 µM nitrate treatments (Fig.4.1B). In the present study, we did not identify and characterize the 83 kDa protein, however, Palenik and Koke (1995) reported the expression of a N-regulated cell membrane or cell wall bound protein (*nrp1*) of similar molecular weight (82 kDa) under N-limitation in two strains of a single-celled planktonic alga *Emiliania huxleyi*. Although Palenik and Koke (1995) reported the expression of 82 kDa protein under N-deplete condition, the 83 kDa protein was expressed under both N-deplete and N-replete conditions, however, the expression levels differed for N-deplete and N-replete conditions (Fig.4.2B). Therefore, the expression levels of the 83 kDa protein can be a useful biomarker for N conditions in an aquatic systems.

The levels of protein expression in *P. parvum* also differed significantly for different phosphate concentrations in the culture medium. *Prymnesium parvum* expressed a new protein of 121 kDa at phosphate concentrations of ≤ 1 µM, and this protein was not expressed at phosphate concentrations ≥ 5 µM (Fig.4.3). It is notable that *P. parvum* showed significant growth reduction ($p < 0.05$) at phosphate concentrations of ≤ 1 µM (Fig.4.1B) in which the 121 kDa protein was upregualted. Therefore, this protein in *P. parvum* can be used as a biomarker of P-deplete condition of an aquatic systems. Further studies on the identification and characterization of the 121 kDa protein are needed in order to better understand the molecular functions of this protein under P-deplete condition.

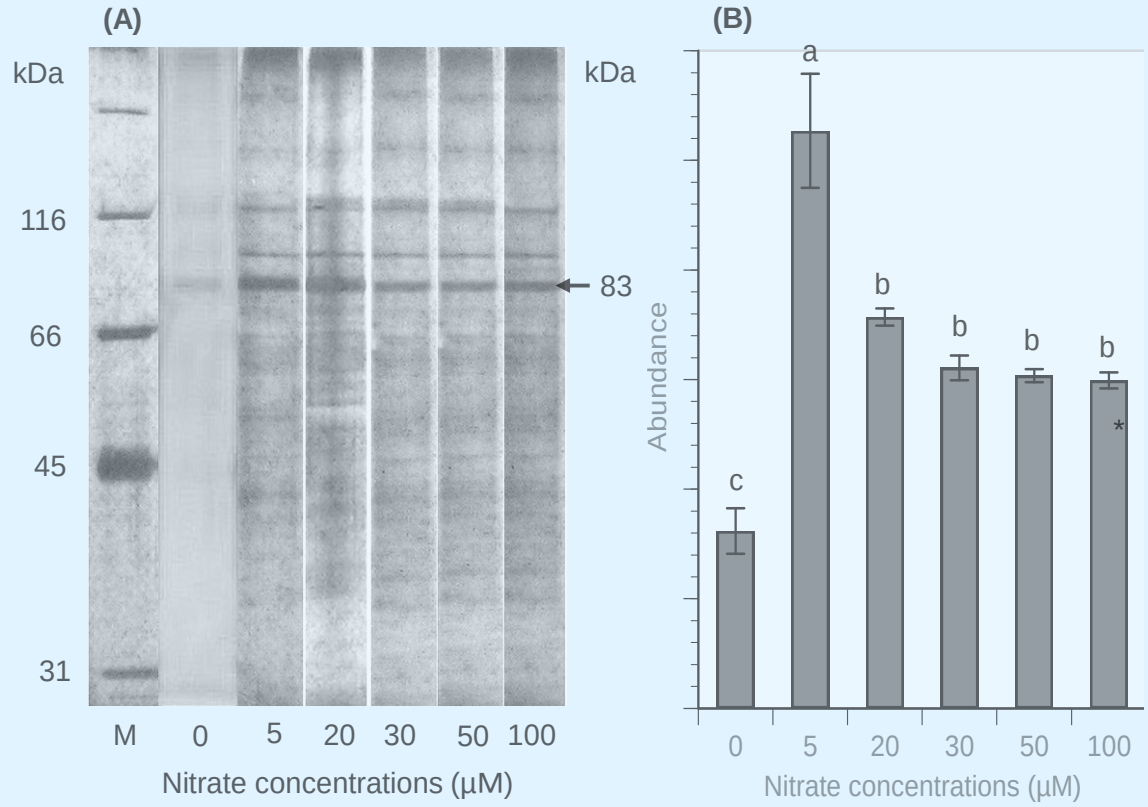

Fig.4.2: **(A)** Protein expression of marine phytoplankton *Prymnesium parvum* in sodium dodecyl sulfate (SDS) gel electrophoresis under different nitrate concentrations. Lane M is the molecular marker. Arrow indicates protein band that was differentially expressed under different nitrate concentrations. **(B)** Expression levels of 83 kDa protein under different nitrate concentrations. Different letters on the bars indicate significant difference ($p < 0.001$) between the treatments.

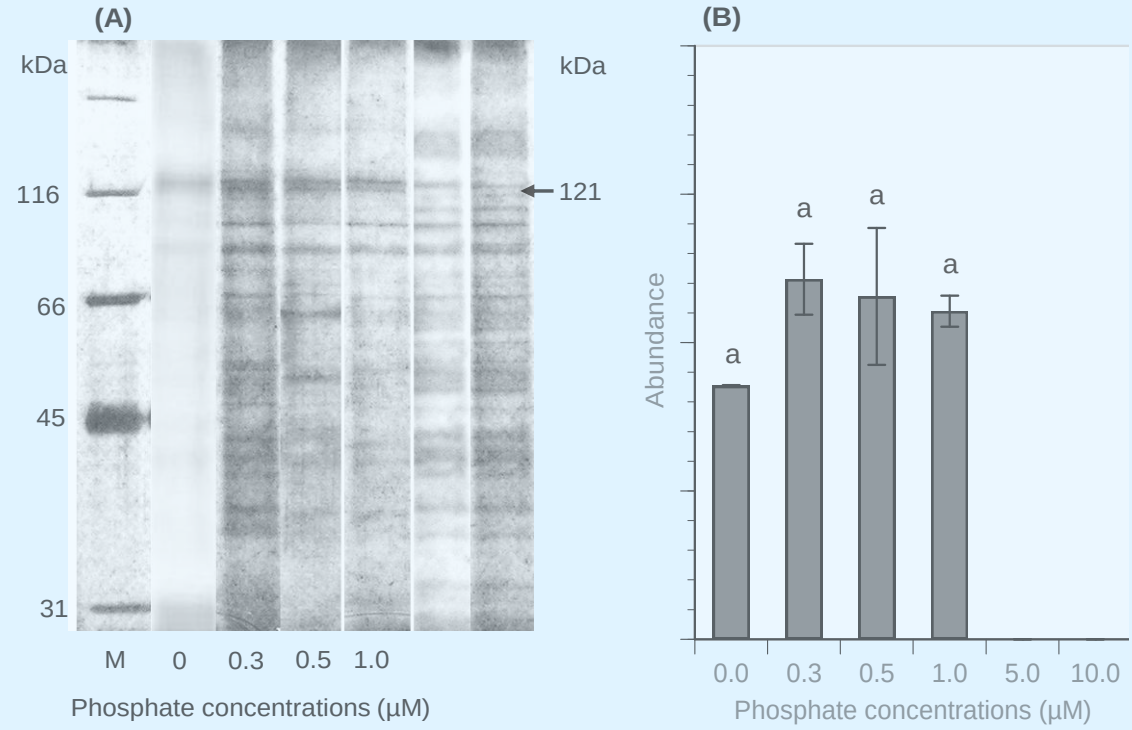

Fig.4.3: **(A)** Protein expression of marine phytoplankton Prymnesium parvum in sodium dodecyl sulfate (SDS) gel electrophoresis under different phosphate concentrations. Lane M is the molecular marker. Arrow indicates protein band that was differentially expressed under different phosphate concentrations. **(B)** Expression levels of 121 kDa protein under different phosphate concentrations. Same letter on the bars indicate no significant difference ($p > 0.05$) between the treatments.

Compared to Fe-replete conditions ([Fe] ≥ 0.1 µM), the expression of a 42 kDa protein increased significantly ($p < 0.01$) and a new protein of 103 kDa was expressed under Fe-deplete condition ([Fe] = 0.01 µM) (Fig.4.4A). The up-regulation of 42 kDa protein has also been reported in cyanobacteria [*Synechococcus, Trichodesmium*, and *Crocosphaera* spp. (Webb *et al.*, 2001) and *Synechococcus sp.* (Reddy *et al.*, 1988)] under Fe-depleted condition. This protein has been characterized as an cytoplasmic membrane carotenoprotein of the cyanobacterium *Synechococcus* sp. strain PCC7942 by Reddy et al. (1989) which is involved in Fe-deficient growth of the cyanobacterium. In a latter study, Webb et al. (2001) identified the 42 kDa protein as membrane-bound iron deficiency-induced protein A (IdiA) homologue. Webb et al. (2001) proposed this protein as an

excellent biomarker for Fe stress in open-ocean cyanobacterial field populations. The present study showed that marine phytoplankton *P. parvum* also expressed the 42 kDa protein in response to Fe-stress condition, which can be used as a biomarker of Fe-stress condition of marine systems. However, further study is needed to characterize this protein in order to better understand the cellular functions in *P. parvum*.

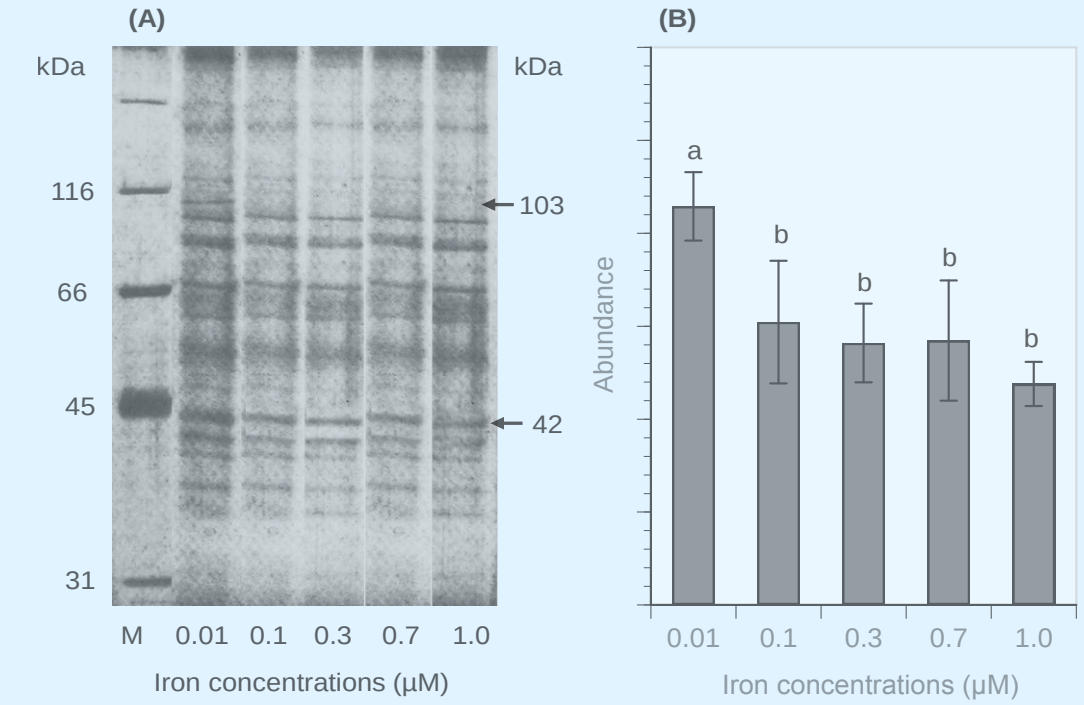

Fig.4.4: **(A)** Protein expression of marine phytoplankton *P. parvum* in SDS-PAGE under different iron concentrations. Lane M is the molecular marker. The 103 kDa protein only expressed under iron-deplete condition, while a 42 kDa differentially expressed under different iron concentrations. **(B)** Expression levels of 42 kDa protein under different Fe concentrations. Different letters on the bars indicate significant difference ($p < 0.01$) between the treatments.

A new protein of 103 kDa was expressed only under Fe-deplete condition (Fig.4.4A). Prinz and Tommassen (2000) reported the expression of a 103 kDa protein in a bacterium (*Neisseria meningitidis*), and characterized as a Fe-regulated outer membrane protein of the organism. The present study reports the expression of 103 kDa protein in marine phytoplankton *P. parvum* in response to Fe-limitation,

and we propose this protein as a potential biomarker of Fe-deplete condition of marine systems.

4.3.3 Proteins and their expression levels as biomarker of N, P and Fe status in aquatic systems

A biomarker, an indicator of a biological state, has been widely used in many scientific fields such as medicine, cell biology, geology, astrobiology and ecotoxicology. The nature of biomarkers and the objectives of their use differ for scientific fields. For example, in cell biology biomarker is a molecule that facilitates the characterization of a cell type, their identification, and eventually their isolation. In ecotoxicology, biomarkers are used to indicate an exposure to or the effect of xenobiotics which are present in the environment and in organisms.

Biomarkers have been used in order to monitor the health of an environment or ecosystem and the ecological risks of pollutants (Bartell, 2006). Biomarkers can be measured at molecular, biochemical, cellular, or physiological levels of biological organization (Ricketts *et al.*, 2004). The use of proteins (Shepard & Bradley, 2000, Shepard *et al.*, 2000, Shpigel *et al.*, 1999) and their expression level (Karsten & Rice, 2004, Lewis *et al.*, 2001) in microorganisms have been proposed as biomarkers of nutrient and heavy metal conditions in aquatic systems. In the present study, we also have found several proteins to be differentially expressed in marine phytoplankton *P. parvum* in response to different exposure levels of nitrate, phosphate and iron. We proposed for the present study that the expression levels of an 83 kDa protein in *P. parvum* can be used as biomarker of N-status, while a 121 kDa protein can be used as a biomarker of P-deplete condition in aquatic systems. In addition, a 103 kDa protein expression in *P. parvum* can be the indication of Fe-limitation, while the expression level of a 42 kDa protein can be used as biomarker of Fe-status (deplete or replete conditions) in aquatic systems. In the present study, we could not characterized and identified these proteins. Further studies are needed to identify the location of the proteins and to characterize the proteins to understand the role of the proteins in cellular metabolic pathways of *P. parvum*.

References

Ammerman, J. W., Hood, R. R., Case, D. A. and Cotner, J. B. (2003) Phosphorus deficiency in the Atlantic: An emerging paradigm in oceanography. *Eos, Transactions American Geophysical Union,* **84,** 165.

Bartell, S. M. (2006) Biomarkers, bioindicators, and ecological risk assessment- A brief review and evaluation. *Environmental Bioindicators,* **1,** 60-73.

Beardall, J., Berman, T., Heraud, P., Omo Kadiri, M., Light, B. R., Patterson, G., Roberts, S., Sulzberger, B., Sahan, E. and Uehlinger, U. (2001) A comparison of methods for detection of phosphate limitation in microalgae. *Aquatic Sciences-Research Across Boundaries,* **63,** 107-121.

Blain, S., Quéguiner, B., Armand, L., Belviso, S., Bombled, B., Bopp, L., Bowie, A., Brunet, C., Brussaard, C. and Carlotti, F. (2007) Effect of natural iron fertilization on carbon sequestration in the Southern Ocean. *Nature,* **446,** 1070-1074.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248-254.

Colijn, F. and Cadée, G. C. (2003) Is phytoplankton growth in the Wadden Sea light or nitrogen limited? *Journal of Sea Research,* **49,** 83-93.

Elser, J. J., Bracken, M. E. S., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., Ngai, J. T., Seabloom, E. W., Shurin, J. B. and Smith, J. E. (2007) Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecology letters,* **10,** 1135-1142.

Elser, J. J., Marzolf, E. R. and Goldman, C. R. (1990) Phosphorus and nitrogen limitation of phytoplankton growth in the freshwaters of North America: A review and critique of experimental enrichments. *Canadian Journal of Fisheries and Aquatic Sciences,* **47,** 1468-1477.

Gawel, J. E., Trick, C. G. and Morel, F. M. M. (2001) Phytochelatins are bioindicators of atmospheric metal exposure via direct foliar uptake in trees near Sudbury, Ontario, Canada. *Environmental Science & Technology,* **35,** 2108-2113.

Geider, R. and La Roche, J. (2002) Redfield revisited: Variability of C: N: P in marine microalgae and its biochemical basis. *European Journal of Phycology,* **37,** 1-17.

Geider, R. J. and Roche, J. (1994) The role of iron in phytoplankton photosynthesis, and the potential for iron-limitation of primary productivity in the sea. *Photosynth. Res.,* **39,** 275-301.

Gromova, I. and Celis, J. E. (2006) Protein detection in gels by silver staining: A procedure compatible with mass-spectrometry. *Cell biology: A laboratory Handbook,* **4,** 421-429.

Guillard, R. R. L. and Ryther, J. H. (1962) Studies of marine planktonic diatoms: I. *Cyclotella Nana* Hustedt, and *Detonula Confervacea* (Cleve) Gran. *Canadian Journal of Microbiology,* **8,** 229-239.

Haasch, M. L., Quardokus, E. M., Sutherland, L. A., Goodrich, M. S., Prince, R., Cooper, K. R. and Lech, J. J. (1992) CYP1A1 protein and mRNA in teleosts as an environmental bioindicator: Laboratory and environmental studies. *Marine Environmental Research,* **34,** 139-145.

Howarth, R. W. and Marino, R. (2006) Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: Evolving views over three decades. *Limnology and Oceanography,* **51,** 364-376.

Karsten, A. H. and Rice, C. D. (2004) c-Reactive protein levels as a biomarker of inflammation and stress in the Atlantic sharpnose shark (Rhizoprionodon terraenovae) from three southeastern USA estuaries. *Marine Environmental Research,* **58,** 747-751.

Kaur, A., Chaudhary, A., Kaur, A., Choudhary, R. and Kaushik, R. (2005) Phospholipid fatty acid- A bioindicator of environment monitoring and assessment in soil ecosystem. *Current Science,* **89,** 1103-1112.

Kawakami, S. K., Gledhill, M. and Achterberg, E. P. (2006) Production of phytochelatins and glutathione by marine phytoplankton in response to metal stress. *Journal of Phycology,* **42,** 975-989.

Lewis, S., Donkin, M. E. and Depledge, M. H. (2001) Hsp70 expression in *Enteromorpha intestinalis* (Chlorophyta) exposed to environmental stressors. *Aquatic Toxicology,* **51,** 277-291.

Lyman, J. and Fleming, R. H. (1940) Composition of sea water. *Journal of marine Research,* **3,** 134-146.

Maki, T., Suzuki, T., Kido, K., Nakahara, A., Higashi, T., Hasegawa, H., Ueda, K. and Saijoh, K. (2008) Effect of Iron Stress on Gene Expression in Harmful Microalga *Prymnesium Parvum*. *Journal of Ecotechnology Research,* **14,** 13-16.

Menzel, D. W., Hulburt, E. M. and Tyther, J. H. (1963) The effects of enriching Sargasso sea water on the production and species composition of the phytoplankton. *Deep Sea Research and Oceanographic Abstracts,* **10,** 209-219.

Moore, C. M., Hickman, A. E., Poulton, A. J., Seeyave, S. and Lucas, M. I. (2007) Iron-light interactions during the CROZet natural iron bloom and EXport experiment (CROZEX): II-Taxonomic responses and elemental stoichiometry. *Deep Sea Research Part II: Topical Studies in Oceanography,* **54,** 2066-2084.

Moore, C. M., Mills, M. M., Langlois, R., Milne, A., Achterberg, E. P., La Roche, J. and Geider, R. J. (2008) Relative influence of nitrogen and phosphorous availability on phytoplankton physiology and productivity in the oligotrophic sub-tropical North Atlantic Ocean. *Limnology and Oceanography,* **53,** 291.

Moore, J. K. and Doney, S. C. (2007) Iron availability limits the ocean nitrogen inventory stabilizing feedbacks between marine denitrification and nitrogen fixation. *Global Biogeochemical Cycles,* **21,** GB2001.

Morelli, E. and Pratesi, E. (1997) Production of phytochelatins in the marine diatom *Phaeodactylum tricornutum* in response to copper and cadmium exposure. *Bull. Environ. Contam. Toxicol.,* **59,** 657-664.

Palenik, B. and Koke, J. A. (1995) Characterization of a nitrogen-regulated protein identified by cell surface biotinylation of a marine phytoplankton. *Applied and environmental microbiology,* **61,** 3311-3315.

Prinz, T. and Tommassen, J. (2000) Association of iron-regulated outer membrane proteins of *Neisseria meningitidis* with the RmpM (class 4) protein. *FEMS Microbiology Letters,* **183,** 49-53.

Reddy, K. J., Bullerjahn, G. S., Sherman, D. M. and Sherman, L. A. (1988) Cloning, nucleotide sequence, and mutagenesis of a gene (*irpA*) involved in iron-deficient growth of the cyanobacterium *Synechococcus sp.* strain PCC7942. *Journal of bacteriology,* **170,** 4466-4476.

Reddy, K. J., Masamoto, K., Sherman, D. M. and Sherman, L. A. (1989) DNA sequence and regulation of the gene (cbpA) encoding the 42-kilodalton cytoplasmic membrane carotenoprotein of the cyanobacterium *Synechococcus* sp. strain PCC 7942. *Journal of Bacteriology,* **171,** 3486-3493.

Ricketts, H. J., Morgan, A. J., Spurgeon, D. J. and Kille, P. (2004) Measurement of annetocin gene expression: a new reproductive biomarker in earthworm ecotoxicology. *Ecotoxicology and Environmental Safety,* **57,** 4-10.

Sánchez, E., Soto, J. M., García, P. C., López-Lefebre, L. R., Rivero, R. M., Ruiz, J. M. and Romero, L. (2000) Phenolic and oxidative metabolism as bioindicators of nitrogen deficiency in French bean plants (*Phaseolus vulgaris* L. cv. Strike). *Plant Biology,* **2,** 272-277.

Shepard, J. L. and Bradley, B. P. (2000) Protein expression signatures and lysosomal stability in *Mytilus edulis* exposed to graded copper concentrations. *Marine Environmental Research,* **50,** 457-463.

Shepard, J. L., Olsson, B., Tedengren, M. and Bradley, B. P. (2000) Protein expression signatures identified in *Mytilus edulis* exposed to PCBs, copper and salinity stress. *Marine Environmental Research,* **50,** 337-340.

Shi, D., Xu, Y., Hopkinson, B. M. and Morel, F. M. M. (2010) Effect of ocean acidification on iron availability to marine phytoplankton. *Science,* **327,** 676-679.

Shpigel, M., Ragg, N. L., Lupatsch, I. and Neori, A. (1999) Protein content determines the nutritional value of the seaweed *Ulva lactuca* L. for the abalone *Haliotis tuberculata* L. and H. discus hannai Ino. *Journal of Shellfish Research,* **18,** 227-234.

Timmermans, K. R., Van Der Wagt, B., Veldhuis, M. J. W., Maatman, A. and De Baar, H. J. W. (2005) Physiological responses of three species of marine pico-phytoplankton to

ammonium, phosphate, iron and light limitation. *Journal of sea research,* **53,** 109-120.

Tyrrell, T. (1999) The relative influences of nitrogen and phosphorus on oceanic primary production. *Nature,* **400,** 525-531.

Van Mooy, B. A. S., Fredricks, H. F., Pedler, B. E., Dyhrman, S. T., Karl, D. M., Koblí and Zcaron, M. (2009) Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature,* **458,** 69-72.

Van Mooy, B. A. S., Rocap, G., Fredricks, H. F., Evans, C. T. and Devol, A. H. (2006) Sulfolipids dramatically decrease phosphorus demand by picocyanobacteria in oligotrophic marine environments. *Proceedings of the National Academy of Sciences,* **103,** 8607-8612.

Watson, A. J., Bakker, D. C. E., Ridgwell, A. J., Boyd, P. W. and Law, C. S. (2000) Effect of iron supply on Southern Ocean CO_2 uptake and implications for glacial atmospheric CO_2. *Nature,* **407,** 730-733.

Webb, E. A., Moffett, J. W. and Waterbury, J. B. (2001) Iron stress in open-ocean Cyanobacteria (*Synechococcus, Trichodesmium, and Crocosphaera spp.*): Identification of the *IdiA* Protein. *Applied and environmental microbiology,* **67,** 5444-5452.

5

RESULTS AND DISCUSSION

PROTEOMIC RESPONSE OF MARINE PHYTOPLANKTON *Prymnesium parvum* TO IRON LIMITATION

5.1. Introduction

Iron is an essential nutrient for photosynthetic organisms including phytoplankton due to its involvement in chlorophyll-*a* biosynthesis, respiration, photosynthesis, nitrate and nitrite reduction, sulfate reduction, nitrogen fixation and a number of other biosynthetic and degradative reactions including those involved in detoxification of O_2 radicals (Geider & Roche, 1994). Marine phytoplankton and cyanobacteria account for half of the global primary production, and they play a major role in regulating global climate by sequestering carbon dioxide from the atmosphere (Benner, 2011). Iron is an important limiting factor for marine phytoplankton and cyanobacteria, and many environmental events, (for example,

ocean acidification) may result in Fe limitation to phytoplankton by decreasing dissolved Fe concentration in seawater (Shi *et al.*, 2010).

Iron may also greatly influence the ecology and physiology of phytoplankton in open oceans and upwelling regimes (Shaked *et al.*, 2005). The oxidized form of Fe, Fe(III), is sparingly soluble in oxygenated open oceans, and therefore occurs at extremely low concentration (Wells *et al.*, 1995). Marine diatoms and other eukaryotic autotrophs usually acquire Fe as dissolved inorganic species (Maranger *et al.*, 1998), however, low concentration of Fe in seawaters and slow rates of water loss from the cells limit the reaction kinetics of Fe with transport ligands on the surface of phytoplankton (Hudson & Morel, 1993). In addition, sub-nanomolar concentration of dissolved Fe in open oceans confines phytoplankton growth (Marchetti *et al.*, 2006). Under Fe-starvation conditions, phytoplankton alter Fe acquisition strategy by up-regulating Fe transport competence (Urzica *et al.*, 2012) , by reducing Fe(III) chelates, and even by ingesting insoluble Fe (Maranger *et al.*, 1998) to meet the cellar Fe requirements. Phytoplankton are also thought to reduce cellular Fe requirements by changing cellular biochemical processes such as reducing nitrogen fixation and photosynthetic metalloenzyme inventory (Saito *et al.*, 2011), remobilization and utilization of stored Fe in cell vacuoles (Urzica *et al.*, 2012), prioritizing biosynthesis of Fe containing proteins and synthesising manganese superoxide dismutases instead of Fe superoxide dismutases etc (Page *et al.*, 2012).

Prymnesium parvum is an ichthyotoxic phytoplankton (Haptophyte) grown in geographically widespread areas of the open oceans including temperate waters, both in the northern and southern hemispheres (Fistarol *et al.*, 2003), vast areas of temperate and tropical open seas (Edvardsen & Imai, 2006), Baltic Sea (Edler *et al.*, 1984) and also of vast coastal plankton communites (Carvalho & Granéli, 2010, Edvardsen & Imai, 2006). Because of its geographical widespread and have a major effect on natural communities, it has been the focus of considerable research during the last decade (Carvalho & Granéli, 2010, Fistarol *et al.*, 2003, La Claire, 2006, Maki *et al.*, 2008, Susan *et al.*, 2010). Except some coastal areas, most of the above

areas where *P. parvum* is naturally grown are low in Fe content (Allen *et al.*, 2008, Mills *et al.*, 2004, Vuorio *et al.*, 2005, Wells *et al.*, 1995). Therefore, in the present study, proteomic responses of marine phytoplankton *Prymnesium parvum* under Fe-deplete condition were investigated. However, it is important to ascertain the biosynthesis of proteins responsible for Fe acquisition and cellular mechanisms in phytoplankton to understand the adaptation strategy of the organism to the Fe-deplete condition. In addition, proteins that were highly expressed under Fe-deplete condition were identified and characterized to understand their roles in cellular biochemical processes and Fe acquisition mechanisms under Fe-deplete conditions.

5.2. Materials and Methods

5.2.1. Phytoplankton pre-culture and maintenance

Already described in subsection 2.2.1.

5.2.2. Growing phytoplankton in test solutions and growth measurement

A 2 mL pre-culture solution of phytoplankton (2×10^3 mL^{-1} cells of logarithmic growth phase) was inoculated in 1 L of Fe-deplete and Fe-rich f/2 nutrient solution in artificial seawater under laminar air flow condition. Iron concentrations in Fe-deplete and Fe-rich medium were 0.0025 and 0.05 µM, respectively. In order to extract sufficient amount of proteins from the cells, the phytoplankton was cultured in 14 (for Fe-deplete) and 8 (for Fe-rich) one-L volume polycarbonate bottles in a growth chamber. Light and dark schedule in the growth chamber was 12:12 h with 188 µE m^{-2} S^{-1} light intensity. Temperature in the growth chamber was 20±2 °C.

Phytoplankton growth was measured on the basis of optical density using a spectrophotometer (U-2000, Hitachi High Technologies, Japan) at a wavelength of 540 nm (OD_{540}) following the same method of Maki *et al.* (2008). The experiment was replicated for three times.

5.2.3. Extraction of soluble proteins

Already described in subsection 2.2.3.

5.2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Already described in subsection 2.2.4.

5.2.5. Two-dimensional differential gel electrophoresis (2-D DIGE)

A 50 µL Trichloroacetic acid was mixed with 950 µL of protein sample of each treatment, and was centrifuged at 14,000 rpm (g = 10,000) for 15 min at 4 °C. The supernatant was removed and pellet was dried after washing 3 times with acetone. A 400 µL swelling buffer (urea 7.0 M, thiourea 2.0 M urea, 4% CHAPS, 0.5% IPG buffer, 0.2% DTT, 0.002% BPB) was mixed with the samples and was centrifuged at 14,000 rpm (g = 10,000) for 15 min at 4 °C. Then protein concentrations in the samples were measured using RC DC Protein Assay Kit (Bio-Rad, USA). Approximately 50 µg of protein of each treatment was labeled by using CyDye (Cy2 and Cy3) DIGE Fluors (GE Healthcare, Tokyo, Japan), and was applied on an IPG strip (pH 3-10) in-gel rehydration protocol (Asano *et al.*, 2012). Protein extracts were applied to the IPG dry strips pH 3-10 (18 cm, GE Healthcare, Tokyo, Japan) during the rehydration step followed by focusing for increasing of voltage linearly from 300 to 3,500 V during 1.5 h, followed by additional 5 h at 3,500 V using an IPGphor (GE Healthcare, Tokyo, Japan). After IEF, the IPG strips were equilibrated at room temperature (22±2 °C) for 15 min in an equilibration buffer (50 mM Tris-HCl, pH 8.8, 6.0 M urea, 30% v/v glycerol, 2% w/v SDS, 1% DTT and 0.002% BPB) followed by 15 min in an equilibration buffer containing 25 mg mL^{-1} of iodoacetamide. SDS-PAGE in the second dimension was carried out, using 12% polyacrylamide gel. After electrophoresis, fluorescent signals were detected by a variable Typhoon™ 9400 imager (GE healthcare, Tokyo, Japan). The 2-D gel images were analyzed with the PDQuest Advanced software (Bio-Rad, CA, USA) (Asano & Nishiuchi, 2011). A 0.5 mg protein sample was used for

identification purpose. Automated spot detection and matching was applied, followed by a manual spot editing to achieve a sufficient correlation between the gels. An analysis set was created to find the spots with a minimum of around 2-fold increase or decrease between the two replicates of the Fe-deplete and Fe-rich treatments. Only spots that differed significantly ($p < 0.05$) in abundance according to the Student's *t*-test were further investigated for the identification of the proteins.

5.2.6. Purification and identification of the proteins

The excised spots were subjected to in-gel digestion with trypsin, and the peptides extracted as described elsewhere (Asano & Nishiuchi, 2011). Briefly, excised spots from 2-D gels were washed sequentially by acetonitrile (100%), DTT (10.0 mM) and iodoacetamide (33.0 mM). Then the gels were washed 3 times sequentially by 100 mM NH_4HCO_3 and CH_3CN and then vacuum-dried. A 20 µL trypsin (12.5 ng mL^{-1}) was added to the gels and heated overnight at 37 ℃. The samples were mixed with 200 µL solution of 50% acetonitrile and 5% formic acid. The samples were then evaporated using a centrifuge, and concentrated to a volume of 10-20 µL.

The peptide mixture was analyzed using 4800 plus MALDI-TOF/TOF™ Analyzer (Applied Bioscience, Carlsbad, CA). Samples were prepared for matrix-assisted laser desorption-ionization-time of flight-mass spectrometer (MALDI-TOF-MS) analysis by mixing 1 mL of the peptide sample with 0.5 µL a-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA, and spotting the mixture on a MALDI target (Asano & Nishiuchi, 2011). For protein identification, MS/MS data were evaluated by considering amino acid substitution and modification against the NCBI and UniProt database using the Paragon algorithm of ProteinPilot™ v 2.0 software (AB Sciex, California, USA).

5.2.7. Statistics

The intensities of the differentially expressed protein spots were statistically analyzed to determine significant differences between their expression levels by

Student's *t*-test using SPSS (v17 for windows). A *p*-value of ≤ 0.05 was considered statistically significant.

5.3. Results

5.3.1. Effect of Fe-limitation on microalgal growth

The growth of *P. parvum* did not differ up to 8 days of incubation for Fe-rich and Fe-limit conditions. However, their growth slowed down from 9 day and almost stunted after 11 days of incubation under Fe-limit condition (Fig. 5.5.1). On the other hand, the microalga showed steady and drastic growth up to 12 days of incubation and then the growth of the microalga slowed down gradually up to 16 day (Fig. 5.5.1). Thus, Fe-limitation showed direct and spectacular effect on the growth of *P. parvum*.

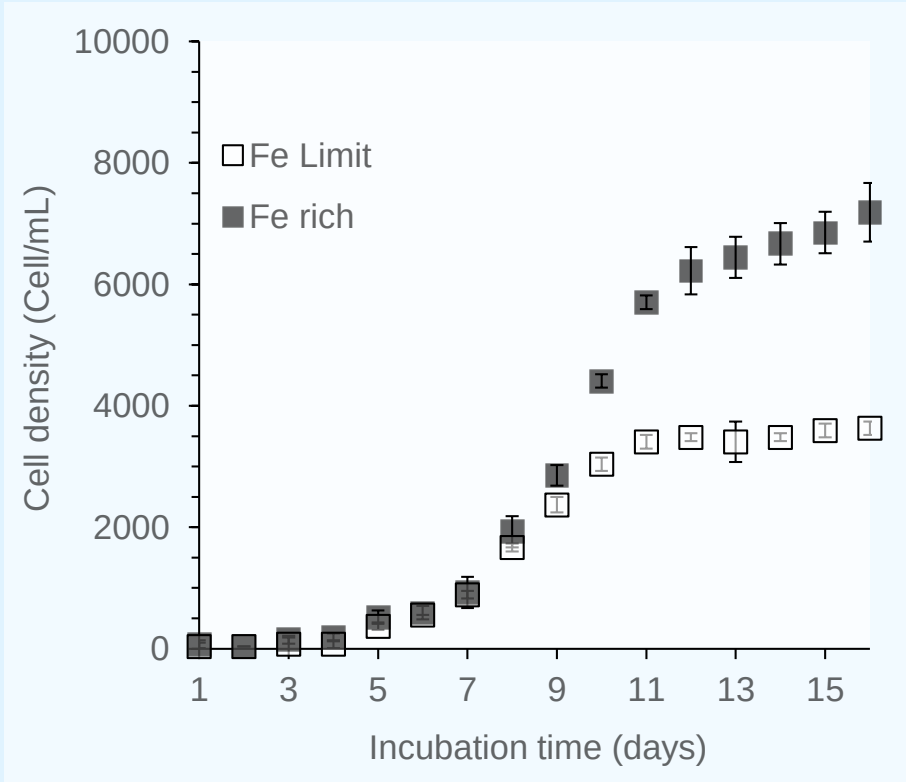

Fig. 5.1: Growth of marine microalga *Prymnesium parvum* to Fe-limited and Fe-rich conditions. Fe concentrations in Fe-limited and Fe-rich culture medium were 0.0025 and 0.05 µM, respectively. The growth of phytoplankton was calculated based on the optical density (OD) at 540 nm wavelength (OD_{540}).

5.3.2. Protein expression determined by SDS-PAGE

In SDS gel electrophoresis, seven protein bands of 16, 18, 32, 34, 75, 82 and 114 kDa were highly expressed under Fe-limited condition and one protein band of 23 kDa was highly expressed under Fe-rich condition (Fig. 5.5.1(a)). Relative abundance of the protein bands is presented in Figure 1(b) which revealed that the phytoplankton *P. parvum* differentially synthesized some proteins of these molecular weights. Biosynthesis of these differentially expressed proteins may help the phytoplankton to change their Fe acquisition strategy under Fe-limited condition to adapt with Fe-limitation. Among significantly up regulated protein bands, the protein of 114 kDa was the most differentially expressed protein band under Fe limited condition followed by 82 kDa protein band.

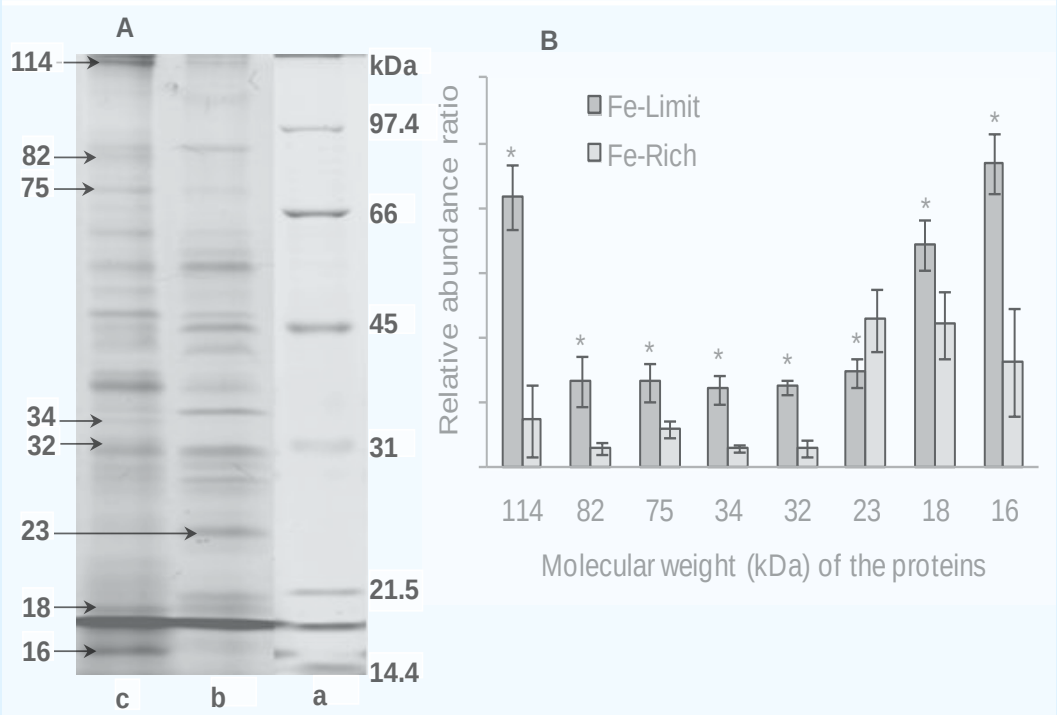

Fig. 5.2: (A) Protein expression of marine phytoplankton *Prymnesium parvum* in SDS-PAGE under Fe-rich (Lane b; Fe = 0.05 µM) and Fe-deplete (Lane c; Fe = 0.0025 µM) conditions. Lane (a) is the molecular marker. Arrows indicate bands of proteins that were up-regulated in Fe-deplete or Fe-rich conditions. **(B)** Relative abundance ratio of protein bands under Fe-rich and Fe-limited conditions. Error bars represent standard deviations of three biological replicates. '*' above the bars indicate significant difference ($p \leq 0.05$) according to the Student's *t*-test.

5.3.3. Differentially expressed proteins under Fe-limited condition

The proteins were subjected to 2-D DIGE and then analyzed with the PDQuest Advanced software to characterize the differentially expressed proteins. In an iso-electric focusing (IEF) between 3 and 10, 13 spots of significant difference ($p < 0.05$) in expression level were identified on the gels. The spots are numbered as shown in Table5.1 and in Figure 2. Out of 13 spots, 10 showed an increase while 3 showed a decrease in abundance under Fe-limited condition (Table5.1 and Fig. 5.5.3). The differentially expressed proteins were then identified using MALDI-TOF-TOF-MS analysis. However, some spots could not be identified on the gels because they were not well resolved from neighboring spots.

5.3.4. Up-regulated proteins under Fe-limited condition

Ten proteins were significantly up-regulated in their expression when the phytoplankton was grown under Fe-limited condition (Fig. 5.5.3). Among the up-regulated proteins, ABC transporters and ATP synthase were the most differentially expressed proteins (114 kDa and 82 kDa, respectively). In the present study it was revealed that the phytoplankton *P. parvum* increased ABC transporter sites and a flagellar associated protein under Fe-limited condition (Table5.1). It was found that phosphoribosylaminoimidazole-succinocarboxamide synthase (spot ID 4, Fig. 5.5.2), a key enzyme for purine metabolism was expressed 3-times higher under Fe-limitated condition than Fe-rich condition (Table5.1).

Biosynthesis of ribuluse biphosphate carboxylase (RuBisCO) and/or pyruvate dehydrogenase E1, alpha and beta subunits, was increased more than 3-fold under iron limited condition (Table5.1). *Prymnesium parvum* up regulated biosynthesis of two oxidative stress response proteins. They were: i) manganese superoxide dismutase (MnSOD) (spot ID 5, Fig. 5.5.2), and ii) Serine threonine kinase protein (STK) (spot ID 6, Fig. 5.5.2). Serine threonine kinase (STK), an oxidative stress response protein, was up-regulated 2-fold under Fe-limited condition (spot ID 6, Fig. 5.5.2) compared to Fe-replete condition (Table5.1). Glycosyl hydrolase protein, a protein responsible for carbohydrate degradation was upregulated more than 2 times

under Fe limited condition compared to Fe replete condition. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme for glycolysis was also up regulated under Fe stress condition.

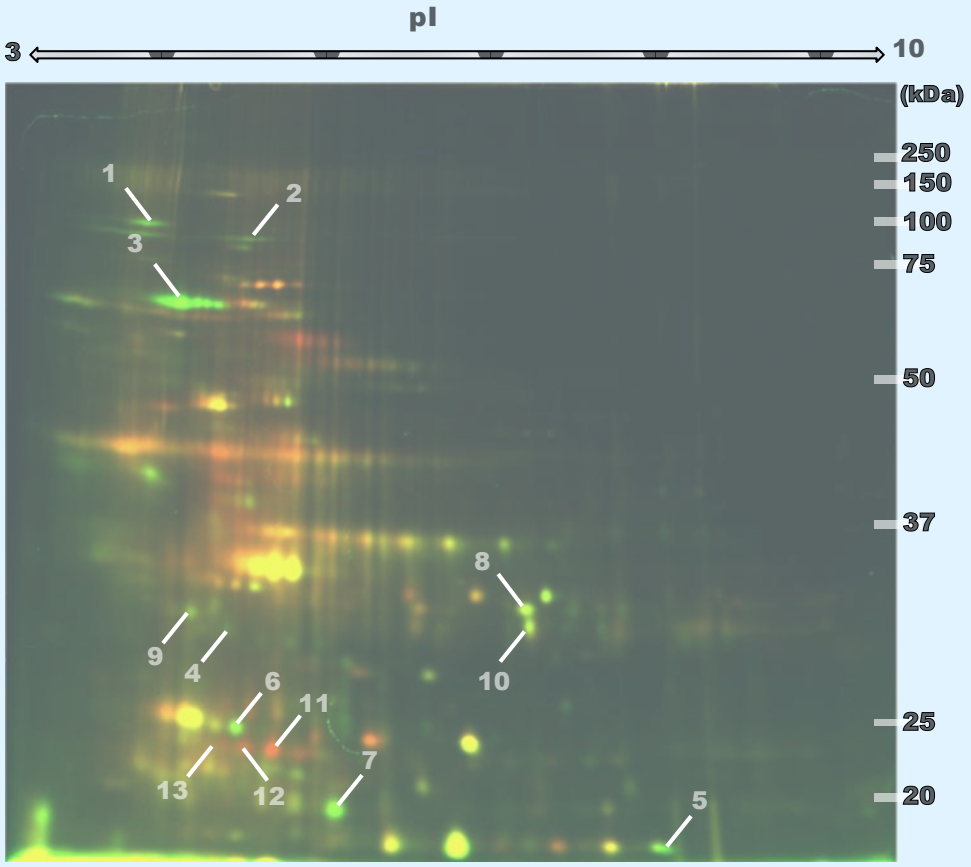

Fig. 5.3: Two-dimensional differential gel electrophoresis (2D-DIGE) analysis of the soluble proteins of marine phytoplankton *Prymnesium parvum* under Fe-rich and Fe-deplete conditions labeled by Cy3 (red) and Cy2 (green), respectively. Selected spots (1 to 13) represent proteins that were differentially expressed under Fe-rich (red) and Fe-deplete (green) conditions.

5.3.5. Down-regulated proteins under Fe-limited condition

Three protein spots were down-regulated in *P. parvum* under Fe-limited condition (Fig. 5.5.2). Among these three spots, two spots (spot ID 9 and spot ID 10, fig 2) were found to be oxygen evolving enhancer 1 precursor (Table5.1). Another down regulated protein was identified as Ribose-5-phosphate isomerase.

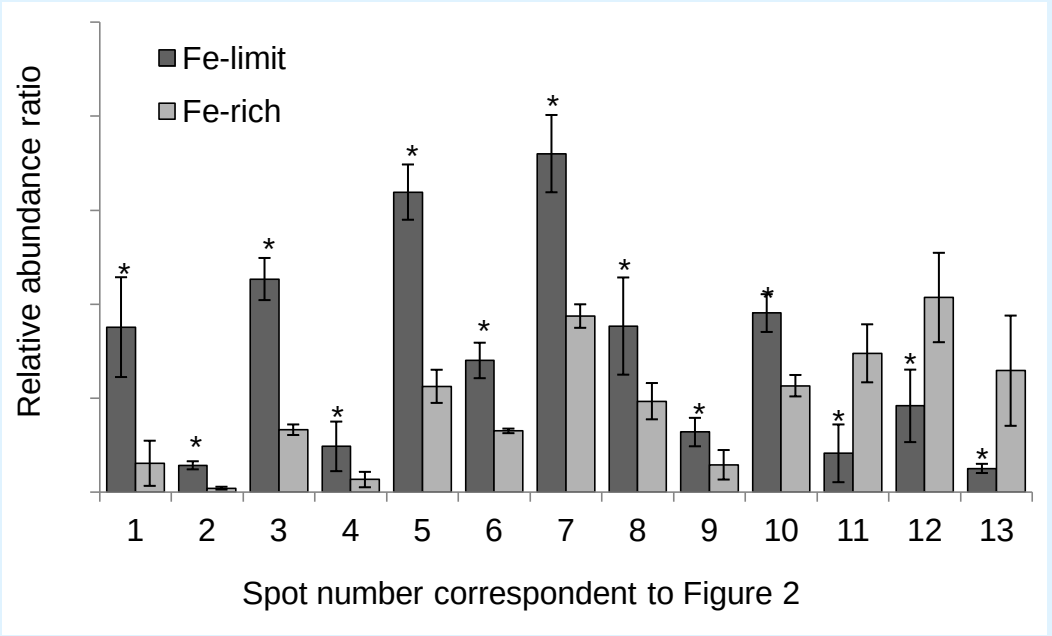

Fig. 5.4: Graphical representation of relative abundance ratio of differentially expressed proteins in *Prymnesium parvum* under Fe-rich and Fe-limited conditions as separated by two-dimensional differentials in gel electrophoresis (2D-DIGE) analysis. Error bars represent standard deviations of three biological replicates. '*' above the bars indicate significant difference ($p \leq 0.05$) according to the Student's *t*-test.

5.4. Discussion

5.4.1. Growth reduction of phytoplankton under Fe limited condition

Marine micro alga *P. parvum* showed significantly different growth under Fe rich and Fe limited conditions. Low Fe concentration (0.0025 µM) in the growing medium limits the growth of microalgae substantially, which is assumed to be related to the cellular biochemical responses due to Fe limitation condition. Growth of phytoplankton was stunted after 11^{th} day of inoculation under Fe limited condition, but phytoplankton showed gradual increase during these days under Fe rich condition. Previous studies have also reported similar decreased growth of

microalgae as an indicator of Fe-limit growing condition (He *et al.*, 2010, Maki *et al.*, 2008).

5.4.2. Proteins that increase Fe uptake

In the present study, 2 proteins (spot ID 1and 7, Fig. 5.5.2) have been found to be highly expressed under Fe-limited condition that may involve in cellular biochemical processes for increasing Fe uptake in the cell. These proteins were identified as flagella-associated protein and ABC transporters (Table5.1). Although flagella has been reported to play important roles in sensory functions (Rosenbaum *et al.*, 1999, Silflow & Lefebvre, 2001) and in changing swimming direction (Silflow & Lefebvre, 2001) of phytoplankton, the involvement of flagella in Fe uptake is unclear. The single-celled green alga *Chlamydomonas reinhardtii* has been reported to have a receptor signal in its flagella, whcih is used for changing swimming direction (Silflow & Lefebvre, 2001). The highly expressed (about 2-fold) flagellar-associated protein (spot ID 7, Fig. 5.5.2) in *P. parvum* may help in Fe acquisition of the phytoplankton under Fe-deplet condition (Fig. 5.5.4). No morphological changes were also observved in the length or number or form of flagella between Fe rich and Fe limit conditions (data is not shown). The specific function of this protein is not clear in our study, and also not reported in previous studies. But it is clear from the present study that this protein in significantly upregulated under Fe limited condition.

ABC transporter proteins are membrane-bound proteins for Fe acquisition and inter organelle Fe transport in phytoplankton (Hanikenne *et al.*, 2005, Wells *et al.*, 1995). It has also been reported in a number of studies that ABC transporters are the key acquisition site for metals including Fe in bacteria and cyanobacteria (Braun & Hantke, 2011, Hanikenne *et al.*, 2005, Krämer *et al.*, 2007, Rangachari *et al.*, 2002). Over 6-fold increase in the abundance of ABC transporters on the gels (spot ID 1, Fig. 5.5.2) indicates that this protein may be involved in Fe acquisition under Fe-limited condition (Fig. 5.5.4). Prokaryotes and bacteria primarily dependent on ABC transporters for Fe acquisition (Braun & Hantke, 2011, Rocap *et al.*, 2003) and some eukaryotes are also known to possess ABC transporter for Fe acquisition (Hutchins

et al., 1999, Rees *et al.*, 2009, Worms *et al.*, 2006). From our study its mechanism in Fe acquisition in *P. parvum* under Fe limited condition is not clear.

5.4.3. Proteins involve in photorespiration

Ribuluse biphosphate carboxylase (RuBisCo) protien participates in the Calvin cycle (CO2 fixation) as well as in the oxidative fragmentation of the pentose substrate during the photorespiration process (Garcia et al., 2006). The photorespiration is assumed to be enhenced by Fe-limitation, and under Fe stress condition phytoplankton up-regulate the biosynthesis of RuBisCo (Allen et al., 2008). Bertrand et al (2012) reported a similar finding for *Phaeodactylum tricornutum*, but the opposite response for *Thalassiosira pseudonana*. Photorespiration is a wasteful process and it increases ATP and NADPH requirements (3 ATP and 2 NADPH), and thylakoid reactions have been thought to satisfy this requirements by producing the ATP and NADPH (Foyer et al., 2009). Findings of the present study suggest that under Fe limited condition phytoplankton produce significantly higher amounts of ATP synthase (spot ID 2, Fig. 5.5.2) for ATP production. The higher abndance of ATP synthase documented here could be, in part, due to the increase in photorespiration and ABC transporter use that we hypothesize occurs under Fe limitation. Increased biosynthesis of malate dehydrogenase under Fe limited condition (spot ID 8, Fig. 5.5.2) may also involve in maintaining specific proportion of ATP and NADPH required for photorespiration (Scheibe, 2004). During photorespiration under Fe-limited condition, malate dehydrogenase may also play an important role in directing photosynthetic electrons into the mitochondrial electron transport chain through the Malate:aspartate shuttle system (Fig. 5.5.4) (Allen et al., 2008). It also converts malate into oxalo acetate in TCA cycle in photorespiration. On the other hand, increased pyruvate dehydrogenase E1, alpha and beta subunits are the key enzyme for Acetyl CoA biosynthesis to start TCA cycle (Peterhansel & Maurino, 2011, Tovar-Méndez et al., 2003).

Table 5.1: Differentially expressed proteins in *P. parvum* under Fe-limited and Fe-rich conditions identified by MALDI-TOF-MS. The proteins were identified by comparing MS/MS data against NCBI and Uniprot database using Paragon algorithm of ProteinPilot.

Spot ID [a]	Protein abundance (Fe-rich/Fe-limit) [b]	Protein's wt. (kDa)	pI value	Name of the proteins [c]	Organisms	Acc. no. [d]	Total ProtSore [e]	% Coverage [f]	No of matched peptides
1↑	0.16	114	4.1	ABC transporter	*Emiliania huxleyi*	jgi|Emihu1|271323|estExt_fgeneshEH_pg.C_1180033	1.7	17.9	8
2↑	0.14	82	4.9	ATP synthase subunit alpha, chloroplastic	*Emiliania huxleyi*	jgi|Emihu1|351045|fgenesh_newKGs_kg.9_64_2695689:3	2.00	2.6	13
3↑	0.29	75	4.4	Ribulose bisphosphate carboxylase	*Emiliania huxleyi*	tr|L7QI74	8.35	21.4	34
			4.4	Pyruvate dehydrogenase	*Phaeodactylum tricornutum CCAP 1055/1*	gi|219119135	6.06	8.7	11
4↑	0.37	32	4.7	Phosphoribosylaminoimidazole-succinocarboxamide synthase	*Sideroxydans lithotrophicus ES-1*	gi|255259484	4.00	7.5	3
5↑	0.36	16	8.2	Manganese superoxide dismutase	*Arabidopsis thaliana*	gi|79313181	1.70	3.5	1
6↑	0.47	24	4.8	Serine threonine protein kinase	*Paramecium tetraurelia*	gi|145486541	1.52	6.7	1
7↑	0.53	18	5.6	Flagellar associated protein	*Thalassiosira pseudonana CCMP1335*	gi|224004678	2.68	13.7	2
8↑	0.60	34	7.1	Malate dehydrogenase	*Thalassiosira pseudonana CCMP1335*	gi|223992865	5.52	13.1	5
9↑	0.44	34	4.5	Glycosyl hydrolase family 17	*Arabidopsis thaliana*	gi|15238256	1.1	1.9	1
10↑	0.59	32	7.1	Glyceraldehyde-3-phosphate dehydrogenase	*Prymnesium parvum*	Q6WEV1	14	40.8	12
11↓	4.59	23	5.1	Oxygen evolving enhancer 1 precursor	*Emiliania huxleyi*	jgi|Emihu1|88271|estExt_Genewise1.C_710038	16.75	63	26
12↓	2.36	23	4.9	Oxygen evolving enhancer 1 precursor	*Isochrysis galbana*	jgi|Emihu1|88271|estExt_Genewise1.C_710038	15.9	58.2	16
13↓	5.02	23	4.7	Ribose-5-phosphate isomerase	*Phaeodactylum tricornutum CCAP 1055/1*	gi|219120917	6.88	22	5

[a] Numbers correspond to the 2-D electrophoresis gel in Fig. 5.5.2. Proteins up (↑) and down (↓) regulated under Fe-limit condition.
[b] Protein abundance was calculated based on the protein concentration/abundance in 2-D gel electrophoresis.
[c] All proteins were identified comparing MS/MS data against the NCBI and Uniprot database using the Paragon algorithm of ProteinPilot.
[d] Accession number in NCBI and Uniprot database.
[e] A measure of evidence of an identified protein calculated from the confidence level of all peptides detected. ProtSore 2 indicates confidence level > 99%.
[f] The percent coverage of all amino acids from a valid peptide matches to the total number of amino acids in the protein.

5.4.4. Proteins to reduce oxidative stresses

Marine phytoplankton may produce reactive oxygen species (ROS) through photosynthesis under Fe-limited condition (Thamatrakoln *et al.*, 2012, Wolfe-Simon *et al.*, 2005). The ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, and organohydroperoxides, many of which are enzymatically detoxified (Halliwell, 1982, Peers & Price, 2004). The photorespiratory pathway is assumed to be a major source of H_2O_2 in photosynthetic cells (Allen *et al.*, 2008). Superoxide is highly reactive and destructive because it cannot diffuse across the cell membranes, and therefore, must be destroyed at the site of production (Wolfe-Simon *et al.*, 2006). Superoxide dismutases (SODs) are enzymes of polyphyletic family that protect cells from destructive reaction of superoxides, and phytoplankton appear to rely primarily on MnSOD for SOD (Fig. 5.5.4) (Peers & Price, 2004). In the present study, the higher occurrence of MnSOD (2.8-fold) under Fe-limited condition presumably facilitates the rapid destruction of ROS, which is inevitably photochemically generated from the reaction centers in photosystems (Wolfe-Simon *et al.*, 2006) and in photorespiration (Allen *et al.*, 2008, Kitayama *et al.*, 1999). Previous study also reported upregulation of MnSOD in coastal and oceanic phytoplankton as a defence mechanism against Fe-limited condition induced oxidative stress (Bertrand *et al.*, 2012, Page *et al.*, 2012, Palenik *et al.*, 2007, Peers & Price, 2004, Urzica *et al.*, 2012).

The STK production has been reported to be induced by Fe-limitation (Cheng *et al.*, 2006), and a STK gene (*pkn*22) expression has been confirmed in cyanobacteria (Xu *et al.*, 2003, Zhang *et al.*, 2007) under Fe-deficient condition. It is likely that under Fe-limited condition, light-generated electrons in cyanobacteria cells are not sufficiently consumed, and ultimately cause oxidative stress in photosystems (mainly in PSI) (Straus, 1994, Xu *et al.*, 2003). The STK may help the micro-organisms to adapt with Fe limitation and also to the oxidative stress condition inactivating the access electrons (Xu *et al.*, 2003). Up-regulation of these proteins (MsSOD and STK) under Fe-limited condition was of importance for *P.*

parvum and possibly other phytoplankton against Fe-limited condition to adopt with Fe stress.

The ROS produced during Fe limitation induced oxidative stress can couse numerous lesions or damage in DNA, and phytoplankton cells have DNA repair mechanism to defend this DNA damage (Latifi *et al.*, 2009, Lesser, 2006, Rivers *et al.*, 2009). Allen *et al.*, (2008) reported 2-phosphoglycolate phosphatase for the repair of damageed DNA under Fe limitation oxidative stress. phosphoribosylaminoimidazole-succinocarboxamide synthase is an enzyme belongs to the family of ligases which is invovled in purine biosynthesis for DNA synthesis (Brown *et al.*, 2011). In our study phosphoribosylaminoimidazole-succinocarboxamide synthase was upregulated under Fe stress condition which might be invoved in purine biosynthesis for reparing of oxidative stress resulted DNA damage.

5.4.5. Increase of degradative and glycolytic activity

Upregulation in the expression of glycosyl hydrolase family 17 protein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) under Fe limited condition indicate the increased degradation of carbohydrates compared to Fe replete conditions. Glycosyl hydrolase hydrolase family 17 protein hydrolyse the glycosidic bond of carbohydrates to produce glucose and GAPDH involved in glycolysis of glucose to produce pyruvate, ATP and NADH (Davies & Henrissat, 1995). Allen *et al.*, (2008) also reported increased carbohydrate degradation by glycosyl hydrolase family 17 protein (endo-1,3-beta-glucanase) and increase of glycolytic activity by upregulating GAPDH under Fe limitation in diatom *Phaeodactylum tricornutum*. Our finding suggests increased conversion of polysaccharides to glucose fuels (carbohydrate degradation) and increased glycolytic activity under Fe limitation in *P. parvum*.

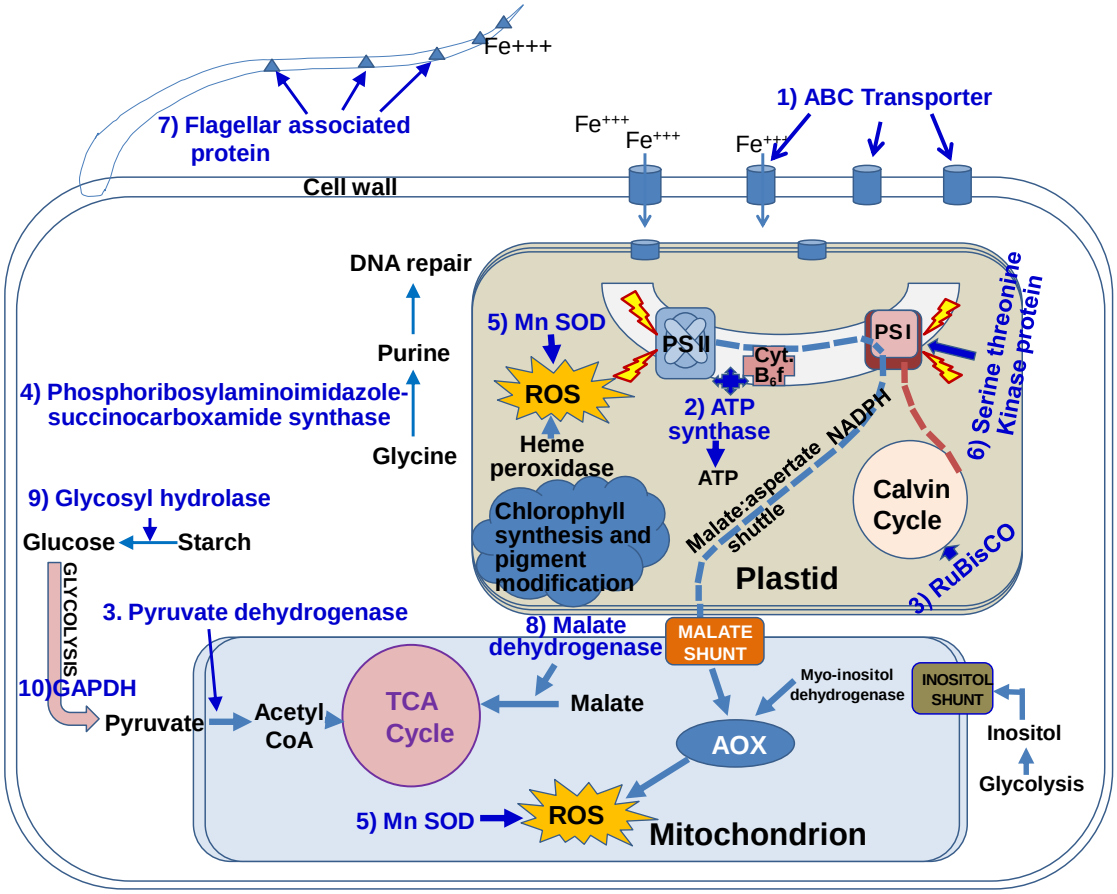

Fig. 5.5: Biochemical pathways and processes in *Prymnesium parvum* cells under Fe limitation (hypothetical). The figure shows how different proteins (in blue color), up-regulated under Fe-limited conditions, involve in biochemical pathways in *P. parvum* cells. The phytoplankton has two flagella, and the flagellar associated protein (spot ID 7) is provably linked with the flagellar activity of finding iron molecules under Fe-limited condition. ABC transporters (spot ID 1) help in Fe acquisition. Phosphoribosylaminoimidazole-succinocarboxamide synthase (spot ID 4) is associated with the synthesis of purines for DNA repair. Oxidative stresses induced by photosynthesis (Xu *et al.*, 2003) are supposed to be minimized by Serine threonine kinase protein (spot ID 6). Manganese superoxide dismutase (MnSOD; spot ID 5) may involve in destruction of reactive oxygen species (ROS), which is highly produced in the cells under Fe-limited condition (Wolfe-Simon *et al.*, 2005). RuBisCO takes part in Calvin cycle in photorespiration under Fe-deplete condition (Allen *et al.*, 2008). Under Fe-limited condition, increased malate dehydrogenase (spot ID 8) may help in electron transfer through Malate:aspertale shuttle (Yoshida *et al.*, 2007). Pyruvate dehydrogenase increased the Acetyl CoA biosynthesis during photorespiration (Peterhansel & Maurino, 2011). Glycosyl hydrolase and GAPDH is used for carbohydrate degradation and glycolysis, respectively under Fe limited condition.

5.5. **Conclusions**

Under Fe-limited condition, marine phytoplankton *P. parvum* alters some of its cellular biochemical processes by up-regulating proteins that are assumed to be involved in Fe uptake, photorespiration, and reduction of oxidative stress in the cells. *Prymnesium parvum* may increase Fe uptake efficiency by increasing Fe acquisition sites (mediated by ABC transporters) when they are grown under Fe-limited condition. Under Fe-limited condition, *P. parvum* may also increase photorespiration which needs high metabolic energy. The phytoplankton may satisfy the demand of high metabolic energy by increasing ATP synthase. Fe-limitation induces oxidative stresses in phytoplankton. *P. parvum* is assumed to up-regulate oxidative stress response proteins MnSOD and STK to minimize the oxidative stresses by inactivating the access electrons in the cells. However, the occurrences and action sites of up-regulated proteins phytoplankton under Fe-limited condition are to be determined to be acquainted with the detail cellular biochemical mechanisms.

References

Allen, A. E., Laroche, J., Maheswari, U., Lommer, M., Schauer, N., Lopez, P. J., Finazzi, G., Fernie, A. R. and Bowler, C. (2008) Whole-cell response of the pennate diatom *Phaeodactylum tricornutum* to iron starvation. *Proc. Natl. Acad. Sci.,* **105,** 10438.

Asano, T., Kimura, M. and Nishiuchi, T. (2012) The defense response in *Arabidopsis thaliana* against *Fusarium sporotrichioides*. *Proteome Sci.,* **10,** 61.

Asano, T. and Nishiuchi, T. (2011) Comparative analysis of phosphoprotein expression using 2D-DIGE. *Methods Mol. Biol.,* **744,** 225-233.

Benner, R. (2011) Loose ligands and available iron in the ocean. *Proc. Natl. Acad. Sci.,* **108,** 893-894.

Bertrand, E. M., Allen, A. E., Dupont, C. L., Norden-Krichmar, T. M., Bai, J., Valas, R. E. and Saito, M. A. (2012) Influence of cobalamin scarcity on diatom molecular physiology and identification of a cobalamin acquisition protein. *Proc. Natl. Acad. Sci.,* **109,** 1762-1771.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248-254.

Braun, V. and Hantke, K. (2011) Recent insights into iron import by bacteria. *Curr. Opin. Chem. Biol.*, **15,** 328-334.

Brown, A. M., Hoopes, S. L., White, R. H. and Sarisky, C. A. (2011) Purine biosynthesis in archaea: Variations on a theme. *Biol. Direct,* **6,** 1-21.

Carvalho, W. F. and Granéli, E. (2010) Contribution of phagotrophy versus autotrophy to *Prymnesium parvum* growth under nitrogen and phosphorus sufficiency and deficiency. *Harmful Algae,* **9,** 105-115.

Cheng, Y., Li, J. H., Shi, L., Wang, L., Latifi, A. and Zhang, C. C. (2006) A pair of iron-responsive genes encoding protein kinases with a Ser/Thr kinase domain and a His kinase domain are regulated by NtcA in the cyanobacterium *Anabaena sp.* strain PCC 7120. *J. Bacteriol.*, **188,** 4822-4829.

Davies, G. and Henrissat, B. (1995) Structures and mechanisms of glycosyl hydrolases. *Structure,* **3,** 853-859.

Edler, L., Hällfors, G., Niemi, Å. and Tiedeakatemia, S. (1984) A preliminary check-list of the phytoplankton of the Baltic Sea. *Acta Bot. Fenn.,* **128,** 1-26.

Edvardsen, B. and Imai, I. (2006) The ecology of harmful flagellates within Prymnesiophyceae and Raphidophyceae*Ecology of Harmful Algae.* Springer, pp. 67-79.

Fistarol, G. O., Legrand, C. and Granéli, E. (2003) Allelopathic effect of *Prymnesium parvum* on a natural plankton community. *Mar. Ecol. Prog. Ser.,* **255,** 115-125.

Foyer, C. H., Bloom, A. J., Queval, G. and Noctor, G. (2009) Photorespiratory metabolism: genes, mutants, energetics, and redox signaling. *Annu. Rev. Plant Biol.,* **60,** 455-484.

Garcia, J. S., Gratão, P. L., Azevedo, R. A. and Arruda, M. A. Z. (2006) Metal contamination effects on sunflower (*Helianthus annuus* L.) growth and protein expression in leaves during development. *J. Agric. Food Chem.,* **54,** 8623-8630.

Geider, R. J. and Roche, J. (1994) The role of iron in phytoplankton photosynthesis, and the potential for iron-limitation of primary productivity in the sea. *Photosynth. Res.,* **39,** 275-301.

Halliwell, B. (1982) *The toxic effects of oxygen on plant tissues.* Vol. 1, CRC Press, Boca Raton, FL.

Hanikenne, M., Krämer, U., Demoulin, V. and Baurain, D. (2005) A comparative inventory of metal transporters in the green alga *Chlamydomonas reinhardtii* and the red alga *Cyanidioschizon merolae*. *Plant Physiol.,* **137,** 428-446.

He, H., Chen, F., Li, H., Xiang, W., Li, Y. and Jiang, Y. (2010) Effect of iron on growth, biochemical composition and paralytic shellfish poisoning toxins production of *Alexandrium tamarense*. *Harmful Algae,* **9,** 98-104.

Hudson, R. J. and Morel, F. M. (1993) Trace metal transport by marine microorganisms: implications of metal coordination kinetics. *Deep-Sea Research Part I: Oceanographic Research Papers,* **40,** 129-150.

Hutchins, D. A., Franck, V. M., Brzezinski, M. A. and Bruland, K. W. (1999) Inducing phytoplankton iron limitation in iron-replete coastal waters with a strong chelating ligand. *Limnol. Oceanogr.,* **44,** 1009-1018.

Kitayama, K., Kitayama, M., Osafune, T. and Togasaki, R. K. (1999) Subcellular localization of iron and manganese superoxide dismutase in *Chlamydomonas reinhardtii* (Chlorophyceae). *J. Phycol.,* **35,** 136-142.

Krämer, U., Talke, I. N. and Hanikenne, M. (2007) Transition metal transport. *FEBS Lett.*, **581,** 2263-2272.

La Claire, J. (2006) Analysis of expressed sequence tags from the harmful alga, Prymnesium parvum (Prymnesiophyceae, Haptophyta). *Mar. Biotechnol.,* **8,** 534-546.

Latifi, A., Ruiz, M. and Zhang, C. C. (2009) Oxidative stress in cyanobacteria. *FEMS Microbiol. Rev.,* **33,** 258-278.

Lesser, M. P. (2006) Oxidative stress in marine environments: Biochemistry and physiological ecology. *Annu. Rev. Physiol.,* **68,** 253-278.

Lyman, J. and Fleming, R. H. (1940) Composition of sea water. *J. Mar. Res.,* **3,** 134-146.

Maki, T., Suzuki, T., Kido, K., Nakahara, A., Higashi, T., Hasegawa, H., Ueda, K. and Saijoh, K. (2008) Effect of Iron Stress on Gene Expression in Harmful Microalga *Prymnesium Parvum*. *J. Ecotechnol. Res.,* **14,** 13-16.

Maranger, R., Bird, D. and Price, N. (1998) Iron acquisition by photosynthetic marine phytoplankton from ingested bacteria. *Nature,* **396,** 248-251.

Marchetti, A., Sherry, N. D., Kiyosawa, H., Tsuda, A. and Harrison, P. L. J. (2006) Phytoplankton processes during a mesoscale iron enrichment in the NE subarctic Pacific: Part I-biomass and assemblage. *Deep Sea Research Part II: Topical Studies in Oceanography,* **53,** 2095-2113.

Mills, M. M., Ridame, C., Davey, M., La Roche, J. and Geider, R. J. (2004) Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. *Nature,* **429,** 292-294.

Page, M. D., Allen, M. D., Kropat, J., Urzica, E. I., Karpowicz, S. J., Hsieh, S. I., Loo, J. A. and Merchant, S. S. (2012) Fe sparing and Fe recycling contribute to increased superoxide dismutase capacity in iron-starved *Chlamydomonas reinhardtii*. *The Plant Cell Online,* **24,** 2649-2665.

Palenik, B., Grimwood, J., Aerts, A., Rouzé, P., Salamov, A., Putnam, N., Dupont, C. L., Jorgensen, R., Derelle, E., Rombauts, S., Zhou, K., Otillar, R., Merchant, S. S., Podell, S., Gaasterland, T., Napoli, C., Gendler, K., Manuell, A., Tai, V., Vallon, O., Piganeau, G., Jancek, S., Heijde, M., Jabbari, K., Bowler, C., Lohr, M., Robbens, S., Werner, G., Dubchak, I., Pazour, G. J., Ren, Q., Paulsen, I., Delwiche, C., Schmutz, J., Rokhsar, D., Van De Peer, Y., Moreau, H. and Grigoriev, I. V. (2007) The tiny eukaryote *Ostreococcus* provides genomic insights into the paradox of plankton speciation. *Proc. Natl. Acad. Sci.,* **104,** 7705-7710.

Peers, G. and Price, N. M. (2004) A role for managanese in superoxide dismutases and growth of iron-deficient diatoms. *Limnol. Oceanogr.,* **49,** 1774-1783.

Peterhansel, C. and Maurino, V. G. (2011) Photorespiration redesigned. *Plant Physiol.,* **155,** 49-55.

Rangachari, K., Davis, C. T., Eccleston, J. F., Hirst, E. M. A., Saldanha, J. W., Strath, M. and Wilson, R. J. M. (2002) SufC hydrolyzes ATP and interacts with SufB from *Thermotoga maritima*. *FEBS Lett.,* **514,** 225-228.

Rees, D. C., Johnson, E. and Lewinson, O. (2009) ABC transporters: The power to change. *Nat. Rev. Mol. Cell Biol.,* **10,** 218-227.

Rivers, A. R., Jakuba, R. W. and Webb, E. A. (2009) Iron stress genes in marine *Synechococcus* and the development of a flow cytometric iron stress assay. *Environ. Microbiol.,* **11,** 382-396.

Rocap, G., Larimer, F. W., Lamerdin, J., Malfatti, S., Chain, P., Ahlgren, N. A., Arellano, A., Coleman, M., Hauser, L. and Hess, W. R. (2003) Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. *Nature,* **424,** 1042-1047.

Rosenbaum, J. L., Cole, D. G. and Diener, D. R. (1999) Intraflagellar transport: The eyes have it. *The J. Cell Biol.,* **144,** 385-388.

Saito, M. A., Bertrand, E. M., Dutkiewicz, S., Bulygin, V. V., Moran, D. M., Monteiro, F. M., Follows, M. J., Valois, F. W. and Waterbury, J. B. (2011) Iron conservation by reduction of metalloenzyme inventories in the marine diazotroph *Crocosphaera watsonii*. *Proc. Natl. Acad. Sci.,* **108,** 2184-2189.

Scheibe, R. (2004) Malate valves to balance cellular energy supply. *Physiol. Plant,* **120,** 21-26.

Shaked, Y., Kustka, A. B. and Morel, F. M. M. (2005) A general kinetic model for iron acquisition by eukaryotic phytoplankton. *Limnology and Oceanography,* **50,** 872-882.

Shi, D., Xu, Y., Hopkinson, B. M. and Morel, F. M. M. (2010) Effect of ocean acidification on iron availability to marine phytoplankton. *Science,* **327,** 676-679.

Silflow, C. D. and Lefebvre, P. A. (2001) Assembly and motility of eukaryotic cilia and flagella. Lessons from *Chlamydomonas reinhardtii*. *Plant Physiol.,* **127,** 1500-1507.

Straus, N. A. (1994) Iron deprivation: Physiology and gene regulation. In: D. A. Bryant (ed) *The molecular biology of cyanobacteria.* Vol. 1. Kluwer Academic Publishers, Dordrecht, pp. 731-750.

Susan, V. J., Theodore, W. V., Daniel, L. R., James, P. G. and Bryan, W. B. (2010) Probabilistic ecological hazard assessment of microcystin-LR allelopathy to *Prymnesium parvum*. *J. Plankton Res.,* **00,** 1-14.

Thamatrakoln, K., Korenovska, O., Niheu, A. K. and Bidle, K. D. (2012) Whole-genome expression analysis reveals a role for death-related genes in stress acclimation of the diatom *Thalassiosira pseudonana*. *Environ. Microbiol.,* **14,** 67-81.

Tovar-Méndez, A., Miernyk, J. A. and Randall, D. D. (2003) Regulation of pyruvate dehydrogenase complex activity in plant cells. *Eur. J. Biochem.,* **270,** 1043-1049.

Urzica, E. I., Casero, D., Yamasaki, H., Hsieh, S. I., Adler, L. N., Karpowicz, S. J., Blaby-Haas, C. E., Clarke, S. G., Loo, J. A. and Pellegrini, M. (2012) Systems and trans-system level analysis identifies conserved iron deficiency responses in the plant lineage. *The Plant Cell Online,* **24,** 3921-3948.

Vuorio, K., Lagus, A., Lehtimäki, J. M., Suomela, J. and Helminen, H. (2005) Phytoplankton community responses to nutrient and iron enrichment under different nitrogen to phosphorus ratios in the northern Baltic Sea. *J. Exp. Mar. Biol. Ecol.*, **322,** 39-52.

Wells, M. L., Price, N. M. and Bruland, K. W. (1995) Iron chemistry in seawater and its relationship to phytoplankton: A workshop report. *Mar. Chem.,* **48,** 157-182.

Wolfe-Simon, F., Grzebyk, D., Schofield, O. and Falkowski, P. G. (2005) The role and evolution of superoxide dismutases in algae. *J. Phycol.*, **41,** 453-465.

Wolfe-Simon, F., Starovoytov, V., Reinfelder, J. R., Schofield, O. and Falkowski, P. G. (2006) Localization and role of manganese superoxide dismutase in a marine diatom. *Plant Physiol.,* **142,** 1701-1709.

Worms, I., Simon, D. F., Hassler, C. S. and Wilkinson, K. J. (2006) Bioavailability of trace metals to aquatic microorganisms: importance of chemical, biological and physical processes on biouptake. *Biochimie,* **88,** 1721-1731.

Xu, W. L., Jeanjean, R., Liu, Y. D. and Zhang, C. C. (2003) *pkn22* (*alr2502*) encoding a putative Ser/Thr kinase in the cyanobacterium *Anabaena sp.* PCC 7120 is induced by both iron starvation and oxidative stress and regulates the expression of *isiA*. *FEBS Lett.*, **553,** 179-182.

Yoshida, K., Terashima, I. and Noguchi, K. (2007) Up-regulation of mitochondrial alternative oxidase concomitant with chloroplast over-reduction by excess light. *Plant Cell Physiol.*, **48,** 606-614.

Zhang, X., Zhao, F., Guan, X., Yang, Y., Liang, C. and Qin, S. (2007) Genome-wide survey of putative Serine/Threonine protein kinases in cyanobacteria. *BMC Genomics,* **8,** 395.

6

RESULTS AND DISCUSSION

PHOTOSYNTHETIC ACTIVITY OF MARINE MICROALGA *Prymnesium parvum* AS AFFECTED BY IRON LIMITATION

6.1. Introduction

Iron (Fe) is an essential nutrient element for almost all autotrophic organisms. It is the most abundant transition metal in the earth crust, and because of its key role in the redox reactions of electron transport chains in respiratory systems and photosynthesis, Fe is an absolute requirement for all the photosynthetic organisms including microalgae (Chen *et al.*, 2005). Fe comprises the central part of chlorophyll-*a (Chl-a)* molecule, and is contained in many redox enzymes of the intermediary metabolism and membrane-bound electron transport chains (Naumann *et al.*, 2007). Fe is also involved in many other cellular biochemical processes such

as respiration, nitrate and nitrite reduction, sulfate reduction, nitrogen fixation, and a number of other biosynthetic and derivative reactions (Geider & Roche, 1994). Thus, Fe greatly influences the ecology, physiology and productivity of microalgae.

Fe deficiency is one of the common nutritional disorder in natural systems. This is due to the fact that, despite the high abundance of Fe on earth, its bioavailability is restricted (Naumann *et al.*, 2007). The global impact of Fe deficiency on photosynthetic productivity has also been shown in vast areas of marine systems where Fe bioavailability is extremely low. Autotrophic organisms have been reported to change their morpho-physology (e.g., the chloroplast structure, photosynthetic capacity and the composition of thylakoid membranes) in response to cope with Fe limitation (Naumann *et al.*, 2007).

The tolerance of microalgae to Fe limitation varies widely between species (Strzepek & Harrison, 2004). Generally, sub-nanomolar concentration of dissolved Fe in open oceans confines the growth of microalgae (Marchetti *et al.*, 2006), however, few microalgae are highly tolerant to Fe limitation and can grow in steady-state at Fe levels in the range of 10-30 pmol, where Fe is considered as the sum of all unchelated Fe species (Allen *et al.*, 2008, Strzepek & Harrison, 2004). Microalgae and other autotrophic microorganisms have evolved a number of physiological responses to cope with the frequently occurring condition of Fe deficiency in the aquatic ecosystems where Fe concentration is sufficiently low to limit photosynthetic activity (Chen *et al.*, 2005). However, the molecular mechanisms of microalgal adaptations to Fe-limit condition has not been well understood. In the present study, proteomic responses of microalgae to Fe-limitation were investigated in order to understand physiological and biochemical adaptation mechanisms of the autotrophic marine microalgae using a unicellular microalga *Prymnesium parvum*. In addition, proteins that were highly expressed under Fe-limit condition were identified and characterized to understand their roles in cellular biochemical processes.

6.2. Materials and Methods

6.2.1. Microalgae pre-culture and maintenance

Already described in subsection 2.2.1.

6.2.2. Growing microalgae in test solutions and Growth measurement

Already described in subsection 4.2.2.

6.2.3. Extraction of membrane proteins

After reaching logarithmic growth phase, the microalgal growth solution was transferred to 50-mL centrifuge tubes and centrifuged at 1,800 rpm (relative centrifugal force; g = 380) for 10 min at 4 °C. The supernatant was removed and the microalga pellet was washed for two times using 2 mL of 10 mM Tris-HCl (pH 7.2). Then 1.5 mL of 10 mM Tris-HCl was added to the sample to make microalgal cell suspension of which 1 mL was transferred to a 1.5-mL micro-tube and was sonicated for 20 seconds using an ultrasonic homogenizer (UH-50, Surface Mount Technology (SMT), Japan) at output 5 under ice-cold condition. The membrane protein was separated by the method described by (Enami *et al.*, 1995). Briefly, the microalgae cell suspension was then centrifuged at 15,000 rpm (g = 10,000) for 10 min at 4 °C. The supernatant was then centrifuged at 35, 000 rpm (g = 105,000) for 30 min at 4 °C and the protein pellates were washed two times by phosphate buffer saline (PBS). The pellates were then mixed with PBS containing 1% Triton X-100x detergent. Then the membrane protein was collected from the supernatant after centrifugation at 35, 000 rpm (g = 105,000) for 30 min at 4 °C. Protein quantification was performed by the Bradford assay (Bio-Rad, Hercules, CA, USA) (Bradford, 1976). The protein samples were then made equal concentration and were subjected to further analysis.

6.2.4. Two-dimensional differential gel electrophoresis (2-D DIGE)

Already described in subsection 4.2.5.

6.2.5. Purification and identification of the proteins

Already described in subsection 4.2.6.

6.2.6. Statistics

Already described in subsection 4.2.7.

6.3. Results and Discussion

6.3.1. Effect of Fe-limitation on microalgal growth

The growth of *P. parvum* did not differ up to 8 days of incubation for Fe-rich and Fe-limit conditions. However, their growth slowed down from 9 day and almost stunted after 11 days of incubation under Fe-limit condition (Fig. 6.1). On the other hand, the microalga showed steady and drastic growth up to 12 days of incubation and then the growth of the microalga slowed down gradually up to 16 day (Fig. 6.1). Thus, Fe-limitation showed direct and spectacular effect on the growth of *P. parvum*. Low Fe condition in the growing medium limits the growth of microalgae substantially, which is assumed to be related to the altered biochemistry of photosynthesis. Previous studies have also reported decrease growth of microalgae under Fe-limit condition (Imai *et al.*, 1999, Liu *et al.*, 2008). In order to better understand the altered biochemical reactions in photosynthesis, soluble proteins extracted from the algal cells were subjected to 2-D DIGE for protein characterization and MALDI-TOF-TOF-MS analysis for their identification.

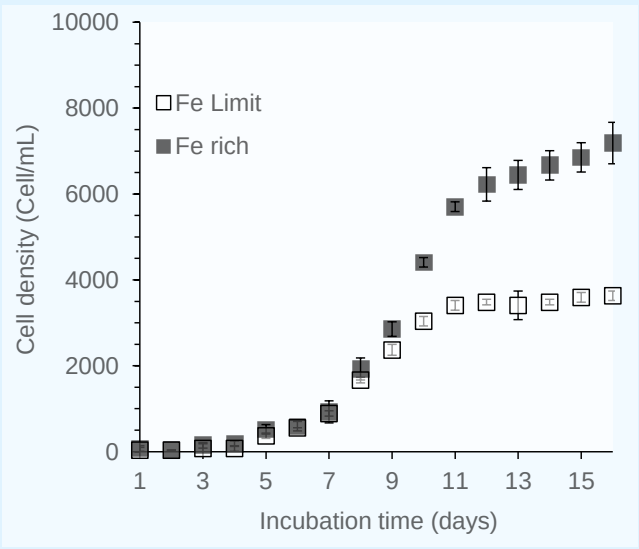

Fig. 6.1: Growth of marine microalga *Prymnesium parvum* to Fe-limited (0.0025 µM) and Fe-rich (0.05 µM) conditions. Data represent mean ±SD (n=3).

6.3.2. Differentially expressed proteins under Fe-limit condition

The proteins extracted from microalgal cells of Fe-limit and Fe-rich medium were subjected to 2-D DIGE and then analyzed with the PDQuest Advanced software to characterize the differentially expressed proteins. In an iso-electric focusing (IEF) between 3 and 10, 11 spots of significant difference ($p \leq 0.05$) in expression level were identified on the gels. The spots are numbered as shown in Table 6.1 and in Figure 2. The differentially expressed proteins were then identified using MALDI-TOF-TOF-MS analysis. However, some spots could not be identified on the gels because they were not well resolved from neighboring spots.

6.3.3. Proteins involved in photosynthesis

Photosynthesis, a process used by photoautotrophic organisms to convert light energy into chemical energy, is a complex process where several proteins are involved. Microalgae use Fe mainly for photosynthetic electron transport, and it is an essential component of the cytochrome (Cyt) and Fe-sulfur protein cofactors of the major photosynthetic complexes in PSII, the Cyt b_6f complex, and PSI (Raven *et al.*, 1999, Strzepek & Harrison, 2004). In the present study, several protiens associated with photosynthesis (spots 1, 3, 4, 7, 8 and 10; Fig. 6.2) were found to be up-regulated more than 2.5 fold under Fe-limit condition (Table 6.1). These up-regulated proteins are the component proteins of photosystem (PS) II.

The light harvesting complex (LHC) protein (spot 7, Fig. 6.2) was up-regulated about 2.42 fold under Fe limitation (Table 6.1). LHC of PSII comprises of chlorophylls (Chl-*a* and Chl-*b*) and carotinoides (Fig. 6.3), the primary reaction center of light harvesting in PSII (Liu *et al.*, 2004). Naumann *et al.* (2007) reported that under Fe-limit condition the microalga *Chlamydomonas reinhardtii* increases LHC protein biosynthesis along with the size of PSII. Photosystem Q_B protein (spot 1, Fig. 6.2), an electron transport chain protein known as plastoquinone, was up-regulated by more than 2 fold under Fe-limit condition (Table 6.1). It is an important protein of PSII (Fig. 6.3) that undergoes two steps of reduction and protonation and

leaves the Q_B site to carry electrons for Cyt b_6f and PSI (Kern & Renger, 2007, Loll *et al.*, 2005).

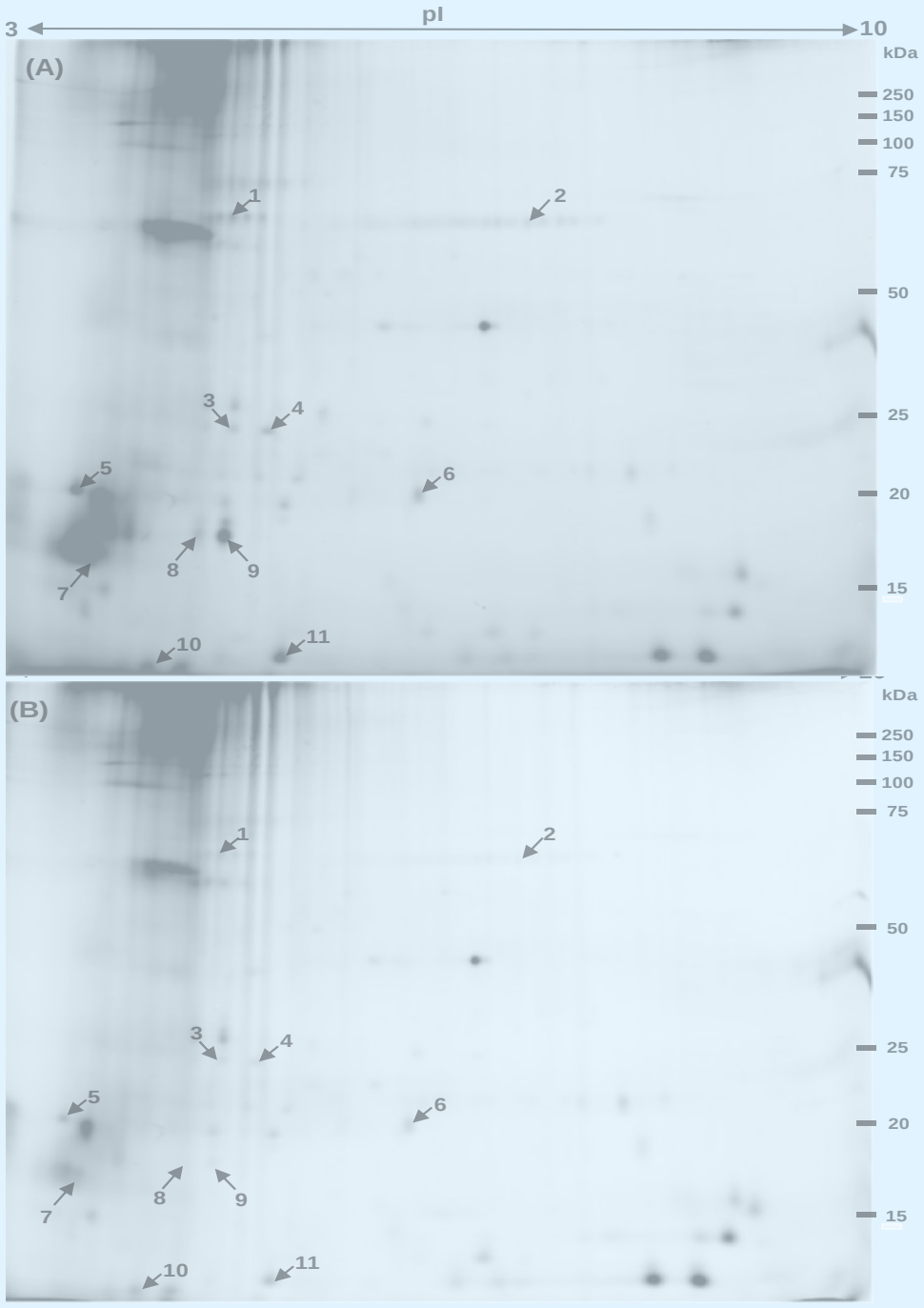

Fig. 6.2: Two-dimensional differentials gel electrophoresis (2D-DIGE) analysis of the membrane proteins of marine microalga *Prymnesium parvum* under Fe-limit **(A)** and Fe-rich **(B)** conditions. Selected spots (1 to 11) represent the proteins that were differentially expressed ($p \leq 0.05$, according to the Student's *t*-test) under Fe-limit and Fe-rich conditions.

Table 6.1: Differentially expressed proteins in *P. parvum* under Fe-limited and Fe-rich conditions identified by MALDI-TOF-MS. The proteins were identified by comparing MS/MS data against the NCBI and Uniprot database using the Paragon algorithm of ProteinPilot.

Spot ID [a]	Protein abundance (Fe-limit/Fe-rich) [b]	Protein wt. (kDa)	pI value	Name of the proteins [c]	Organisms	Accession number [d]	Total ProtSore [e]	% Coverage [f]
1↑	2.16	72.4	4.8	Photosystem Q_B protein	*Cyanidium caldarium*	tr|Q6JAL3|Q6JAL3_CYACA	1.54	12.9
2↑	2.26	70.9	7.2	ATP synthase subunit alpha, chloroplastic	*Ectocarpus siliculosus*	tr|D1J797|D1J797_ECTSI	2.17	10.6
3↑	1.22	26.7	4.8	Oxygen-evolving enhancer protein	*Rhodosorus marinus*	tr|E5RPB6|E5RPB6_9RHOD	1.74	21.3
4↑	1.70	26.9	5.1	33kDa oxygen evolving protein of photosystem II	*Pyramimonas parkeae*	gi|161728803	2.82	11.2
5↑	13.15	20.3	3.5	hypothetical protein Msip34_0367	*Methylovorus* sp. SIP3-4	gi|253998079	1.3	4.9
6↑	2.75	19.7	6.4	Putative uncharacterized protein	*Ectocarpus siliculosus*	tr|D8LB97|D8LB97_ECTSI	0.74	5.2
7↑	2.42	14.7	3.6	Light harvesting complex protein	*Ectocarpus siliculosus*	tr|D8LBT8|D8LBT8_ECTSI	2.43	10.5
8↑	2.62	16.4	4.5	Photosystem II D2 protein	*prymnesiophyte* C19847	tr|D9MYL1|D9MYL1_9EUKA	2.92	6.6
9↑	6.58	16.4	4.8	ATP synthase subunit b, chloroplastic	*Ochrosphaera neapolitana*	gi|2493071	2	6.2
10↑	2.75	8.9	4.1	Photosystem II D2 protein	*prymnesiophyte* C19847	tr|D9MYL1|D9MYL1_9EUKA	6	12.5
11↑	2.41	9.0	5.2	ATP synthase subunit b, chloroplastic	*Ochrosphaera neapolitana*	gi|2493071	6	14

[g] Numbers correspond to the 2-D electrophoresis gel in Fig. 6.1. Proteins up regulated under Fe-limit conditions (↑).

[h] Protein abundance was calculated based on the protein concentration/abundance in 2-D gel electrophoresis.

[i] All proteins were identified comparing MS/MS data against the NCBI and Uniprot database using the Paragon algorithm of ProteinPilot.

[j] Accession number in NCBI and Uniprot database.

[k] A measure of evidence of an identified protein calculated from the confidence level of all peptides detected. ProtSore 2 indicates confidence level > 99%.

[l] The percent coverage of all amino acids from a valid peptide matches to the total number of amino acids in the protein.

Photosystem II D2 protein (spots 8 and 10, Fig. 6.2) was up-regulated by more than 2.5 fold when the microalga was grown under Fe-limit condition (Table 6.1). However, the expression of PSII D1 protein, which is also a core subunit of PSII, was not altered under Fe-limit or Fe-rich conditions. Naumann *et al.* (2007) reported 4 fold up-regulation of PSII D2 protein and dowun-regulation of PSII D1 protein under Fe-limit condition. Komenda *et al.* (2004) reported up-regulation of PSII D2 protein in a cyanobacterial strain *Synechocystis* PCC 6803 lacking PSII D1 protein, and the PSII D2 protein was reported to be the key regulatory protein of PSII D1 accumulation and consecutive assembly of the PSII reaction center complex.

Phytoplankton produce reactive oxygen species (ROS) under Fe-limited condition and cause photo-oxidative damage (Naumann *et al.*, 2007, Oda *et al.*, 1997, Wolfe-Simon *et al.*, 2005). Since PSII D1 protein is susceptible to oxidative damage, enhanced photo-oxidative damage and ROS production may results in rapid degradation of PSII D1 protein under Fe-limited condition (Baroli *et al.*, 2004, Naumann *et al.*, 2007, Yamamoto, 2001, Yoshioka *et al.*, 2010). PSII D1 protein degradation functionally disabled PSII, and the photoinactivation of PSII is regarded as an efficient protective mechanism under Fe stress in order to circumvent photo-oxidative damage and control linear photosynthetic electron transfer (2007). The photoinactivated PSII centers are assumed to be strong quenchers of excitation energy, thereby protecting neighboring PSII centers from damage by acting as effective energy sinks (Chow *et al.*, 2005, Naumann *et al.*, 2007, Peers *et al.*, 2009). Similarly, the down-regulation of PSII D1 under Fe-limit condition (2007) can be explained by the inactivation of some PSII centers resulted from PSII D1 protein degradation in *P. parvum*. The microalga may also minimize electron input by inactivating PSII and avoid over-reduction of the electron transfer chain to protect the system from photo-oxidative damage.

Oxygen evolving enhancer (OEE) protein (spots 3 and 4; Fig. 6.2), also knoown as 33 kDa oxygen evelving protein, were slightly upregulated under Fe-limitation (Table 6.1). These proteins are located on the lumenal side of PSII (Fig. 6.3), and are thought to optimize the manganese cluster during water oxidation

(Heide *et al.*, 2004). Moreover, its extended structure may protect the reaction centre D1 protein in PSII from oxygen radicals (Yamamoto, 2001). Such a dual role of OEE protein is only possible in algal photosynthesis in aquatic environment, but not in terrestril plant leaves (Heide *et al.*, 2004).

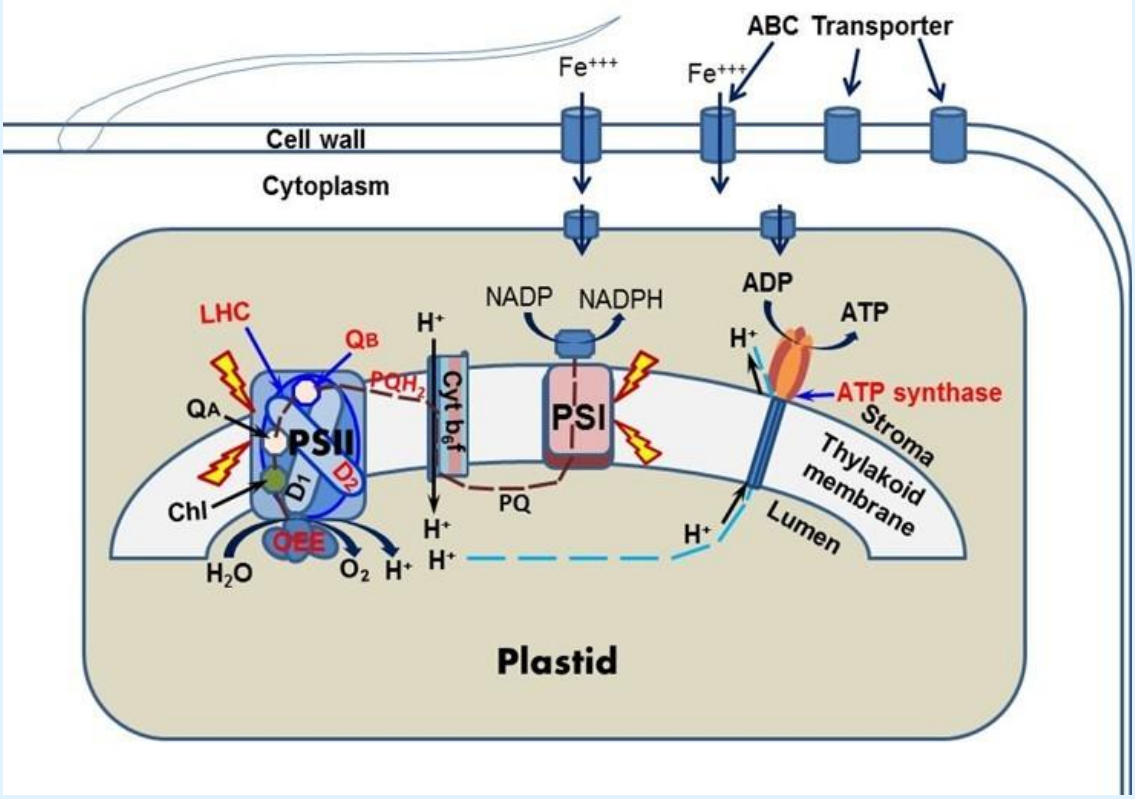

Fig. 6.3: Biochemical pathways and processes of photosynthesis in *Prymnesium parvum* (hypothetical) under Fe limitation. The figure shows how up-regulated proteins (in red color) involve in photosynthetic activities of the microalga under Fe-limitation. Brown dotted line is the electron transport pathway in photosynthesis. Compared to the photosystem I (PSI) and cytochrome b_6f, the biosynthesis of PSII increased under Fe-limitation. Increased PSII/PSI ratio is assumed to be a strategy of the microalga in order to cope with the Fe-limitation condition.

Fe requirement is not similar for PSI and PSII of microalgae (Raven *et al.*, 1999). The relative abundance of PSI and PSII complexes is the key determinant of Fe requirement for microalgae since Cyt b_6f complex (6 atoms of Fe per complex) and PSI (12 atoms of Fe per complex) are disproportionately Fe-rich compared to PSII (2 atoms of Fe per complex) (Strzepek & Harrison, 2004). Since PSI and Cyt

b_6f require higher amount of Fe than PSII, microalgae may up-regulate several component proteins of PSII, as was found in the present study, and thereby the abundance of PSII, in order to maintain photosynthesis under Fe-limit condition. Previous studies have been reported that the ratio of PSII to PSI is 1:1 for terrestrial plants (Raven *et al.*, 1999), while it is 2:1 for microalgae of Fe-rich coastal areas and 10:1 for microalgae of nutrient poor Fe-limit oceanic waters (Strzepek & Harrison, 2004). Since PSI and the Cyt b_6f complex contain considerably more Fe than PSII, these biochemical alterations are indicative of an adaptive strategy of marine microalgae to minimize their Fe requirements (Geider & Roche, 1994, Strzepek & Harrison, 2004). In response to Fe-limitation, marine cyanobacteria and microalgae exhibit reduced efficiency of photochemistry in PSII (Geider & Roche, 1994). However, compared to PSI and Cyt b_6f, high abundance of PSII is assumed to be able to effectively balance excitation energy and electron flow in the photosynthetic reactions. Marine microalgae like *P. parvum* that can grow under very low Fe concentration may have such strategies (Strzepek & Harrison, 2004).

6.3.4. Increased biosynthesis of ATP synthase

Biosynthesis of chloroplast ATP synthase (spots 2, 9 and 11; Fig. 6.2) was up-regulated under Fe-limit condition (Table 6.1). Compared to Fe-rich condition, chloroplast ATP synthase subunit alpha and subunit b were up-regulated by 2.26 and 6.58 fold, respectively, under Fe-limit condition (Table 6.1). Some unrecognized spots in line with subunit alpha are assumed to be the same protein or other subunits of the same protein. Fe-limitation has been reported to enhance photorespiration (2008), a wasteful process that increases ATP and NADPH requirements (3 ATP and 2 NADPH) which have been thought to be satisfied by thylakoid reactions that produce the excess ATP and NADPH (Foyer *et al.*, 2009). Under Fe-limit condition, the increase of chloroplastic ATP synthase in *P. parvum* may be to satisfy the increased need of ATP for photorespiration and other metabolic activities in the cell.

6.4. Conclusions

Under Fe-limit condition, marine microalga *P. parvum* alters its cellular biochemical processes by up-regulating several proteins involved in photosynthesis. Under Fe-limit condition, *P. parvum* may undergo photorespiration which needs high metabolic energy (ATP and NADPH). Microalgae may satisfy the demand of high metabolic energy by increasing ATP synthase in chloroplast. Fe-limitation may also induce oxidative stresses to microalgae that may results in degradation of photosynthetic protein like PSII D1. One hypothetical protein (spot 5, Fig. 6.2) of 20.3 kDa was highly up-regulated (about 13 fold) under Fe-limit condition. A putative uncharacterized protein (spot 6, Fig. 6.2) of 19.7 kDa was also up-regulated under Fe-limit condition. The active sites and functions of these proteins in microalgae have not been reported before. Further studies are needed to know more about these proteins.

References

Allen, A. E., Laroche, J., Maheswari, U., Lommer, M., Schauer, N., Lopez, P. J., Finazzi, G., Fernie, A. R. and Bowler, C. (2008) Whole-cell response of the pennate diatom *Phaeodactylum tricornutum* to iron starvation. *Proceedings of the National Academy of Sciences*, **105,** 10438.

Asano, T., Kimura, M. and Nishiuchi, T. (2012) The defense response in *Arabidopsis thaliana* against *Fusarium sporotrichioides*. *Proteome Science*, **10,** 61.

Asano, T. and Nishiuchi, T. (2011) Comparative analysis of phosphoprotein expression using 2D-DIGE. *Methods in Molecular Biology*, **744,** 225-233.

Baroli, I., Gutman, B. L., Ledford, H. K., Shin, J. W., Chin, B. L., Havaux, M. and Niyogi, K. K. (2004) Photo-oxidative stress in a xanthophyll-deficient mutant of *Chlamydomonas*. *Journal of Biological Chemistry*, **279,** 6337-6344.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72,** 248-254.

Chen, M., Bibby, T. S., Nield, J., Larkum, A. and Barber, J. (2005) Iron deficiency induces a chlorophyll *d*-binding Pcb antenna system around Photosystem I in *Acaryochloris marina*. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, **1708,** 367-374.

Chow, W. S., Lee, H. Y., He, J., Hendrickson, L., Hong, Y. N. and Matsubara, S. (2005) Photoinactivation of photosystem II in leaves. *Photosynthesis research,* **84,** 35-41.

Enami, I., Murayama, H., Ohta, H., Kamo, M., Nakazato, K. and Shen, J. R. (1995) Isolation and characterization of a Photosystem II complex from the red alga *Cyanidium caldarium*: Association of cytochrome c-550 and a 12 kDa protein with the complex. *Biochimica et Biophysica Acta (BBA)-Bioenergetics,* **1232,** 208-216.

Foyer, C. H., Bloom, A. J., Queval, G. and Noctor, G. (2009) Photorespiratory metabolism: genes, mutants, energetics, and redox signaling. *Annual Review of Plant Biology,* **60,** 455-484.

Geider, R. J. (1999) Biological oceanography: Complex lessons of iron uptake. *Nature,* **400,** 815-816.

Geider, R. J. and Roche, J. (1994) The role of iron in phytoplankton photosynthesis, and the potential for iron-limitation of primary productivity in the sea. *Photosynthesis Research,* **39,** 275-301.

Guillard, R. R. L. and Ryther, J. H. (1962) Studies of Marine Planktonic Diatoms: I. Cyclotella Nana Hustedt, and Detonula Confervacea (Cleve) Gran. *Can. J. Microbiol.,* **8,** 229-239.

Heide, H., Kalisz, H. M. and Follmann, H. (2004) The oxygen evolving enhancer protein 1 (OEE) of photosystem II in green algae exhibits thioredoxin activity. *Journal of plant physiology,* **161,** 139-149.

Imai, A., Fukushima, T. and Matsushige, K. (1999) Effects of iron limitation and aquatic humic substances on the growth of *Microcystis aeruginosa*. *Canadian Journal of Fisheries and Aquatic Sciences,* **56,** 1929-1937.

Kern, J. and Renger, G. (2007) Photosystem II: Structure and mechanism of the water: Plastoquinone oxidoreductase. *Photosynthesis research,* **94,** 183-202.

Komenda, J., Reisinger, V., Müller, B. C., Dobáková, M., Granvogl, B. and Eichacker, L. A. (2004) Accumulation of the D2 protein is a key regulatory step for assembly of the photosystem II reaction center complex in *Synechocystis* PCC 6803. *Journal of Biological Chemistry,* **279,** 48620-48629.

Liu, Z.-Y., Wang, G.-C. and Zhou, B.-C. (2008) Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. *Bioresource Technology,* **99,** 4717-4722.

Liu, Z., Yan, H., Wang, K., Kuang, T., Zhang, J., Gui, L., An, X. and Chang, W. (2004) Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution. *Nature,* **428,** 287-292.

Loll, B., Kern, J., Saenger, W., Zouni, A. and Biesiadka, J. (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II. *Nature,* **438,** 1040-1044.

Lyman, J. and Fleming, R. H. (1940) Composition of sea water. *Journal of Marine Research,* **3,** 134-146.

Maki, T., Suzuki, T., Kido, K., Nakahara, A., Higashi, T., Hasegawa, H., Ueda, K. and Saijoh, K. (2008) Effect of Iron Stress on Gene Expression in Harmful Microalga *Prymnesium Parvum*. *Journal of Ecotechnology Research,* **14,** 13-16.

Marchetti, A., Sherry, N. D., Kiyosawa, H., Tsuda, A. and Harrison, P. L. J. (2006) Phytoplankton processes during a mesoscale iron enrichment in the NE subarctic Pacific: Part I-biomass and assemblage. *Deep Sea Research Part II: Topical Studies in Oceanography,* **53,** 2095-2113.

Naumann, B., Busch, A., Allmer, J., Ostendorf, E., Zeller, M., Kirchhoff, H. and Hippler, M. (2007) Comparative quantitative proteomics to investigate the remodeling of bioenergetic pathways under iron deficiency in *Chlamydomonas reinhardtii*. *Proteomics,* **7,** 3964-3979.

Oda, T., Nakamura, A., Shikayama, M., Kawano, I., Ishimatsu, A. and Muramatsu, T. (1997) Generation of reactive oxygen species by raphidophycean phytoplankton. *Biosci. Biotechnol. Biochem.,* **61,** 1658.

Peers, G., Truong, T. B., Ostendorf, E., Busch, A., Elrad, D., Grossman, A. R., Hippler, M. and Niyogi, K. K. (2009) An ancient light-harvesting protein is critical for the regulation of algal photosynthesis. *Nature,* **462,** 518-521.

Raven, J. A., Evans, M. C. W. and Korb, R. E. (1999) The role of trace metals in photosynthetic electron transport in O_2-evolving organisms. *Photosynthesis research,* **60,** 111-150.

Strzepek, R. F. and Harrison, P. J. (2004) Photosynthetic architecture differs in coastal and oceanic diatoms. *Nature,* **431,** 689-692.

Wolfe-Simon, F., Grzebyk, D., Schofield, O. and Falkowski, P. G. (2005) The role and evolution of superoxide dismutases in algae. *Journal of Phycology,* **41,** 453-465.

Yamamoto, Y. (2001) Quality control of photosystem II. *Plant and Cell Physiology,* **42,** 121-128.

Yoshioka, M., Nakayama, Y., Yoshida, M., Ohashi, K., Morita, N., Kobayashi, H. and Yamamoto, Y. (2010) Quality Control of Photosystem II. *Journal of Biological Chemistry,* **285,** 41972-41981.

7

SUMMARY AND CONCLUSIONS

The present study showed that marine phytoplankton *Prymnesium parvum* need adequate amount of Fe for normal growth and cellular metabolism. Fe requirements also varied in different phytoplanktons species. Marine phytoplankton employ different strategies to compensate Fe stress whether it is due to low total Fe or low Fe availability. In the first experiment of the present study, we found that marine phytoplankton produce different proteins under low total Fe and ligand-induced Fe-limited conditions. This result indicates that marine phytoplankton may employ different Fe acquisition strategies under low total Fe and ligand-induced Fe-limited conditions. Protein expression under different Fe conditions also varied within phytoplankton species (*Prymnesium parvum*, *Skeletonema marinoi-dohrnii complex* and *Pleurochrysis roscoffensis*). It indicates that different phytoplankton species may employ different strategies to cope with Fe limited condition.

In the present study, we also have found several proteins to be differentially expressed in marine phytoplankton *P. parvum* in response to different exposure levels of nitrate, phosphate and iron. Biological markers for nutrient stress (such as glutamate/glutamine ratios for nitrogen limitation) have been proposed to evaluate the nutrient condition of an ecosystem. This approach potentially has the advantage of being applicable at the single-cell and species levels. An 83 kDa protein was highly expressed in *P. parvum* at 5 µM nitrate treatment, and its expression was

significantly down-regulated ($p < 0.001$) at higher nitrate treatments (20 – 100 μM). Based on the expression appearance and level of expression, we proposed that the expression levels of an 83 kDa protein in *P. parvum* can be used as biomarker of N-status. The phytoplankton also expressed a new protein of 121 kDa at phosphate concentrations of ≤ 1 μM, and this protein was not expressed at high phosphate concentrations (≥ 5 μM). Based on the observation of 121 kDa protein, it was found that expression of this protein can be used as a biomarker of P-deplete condition in aquatic systems. In addition, two protein can be used as biomarker of Fe-status (deplete or replete conditions) in aquatic systems.

Under Fe-limited condition, marine phytoplankton *P. parvum* alters some of its cellular biochemical processes by up-regulating proteins that are assumed to be involved in Fe uptake, photorespiration, degradation of polysaccharides, glycolysis, and reduction of oxidative stress in the cells. *Prymnesium parvum* may increase Fe uptake efficiency by increasing Fe acquisition sites (mediated by ABC transporters) when they are grown under Fe-limited condition. Under Fe-limited condition, *P. parvum* may also increase photorespiration which needs high metabolic energy. The phytoplankton may satisfy the demand of high metabolic energy by increasing ATP synthase. Fe-limitation induces oxidative stresses in phytoplankton which can damage DNA and proteins of the cell. One ligase enzyme, phosphoribosylaminoimidazole-succinocarboxamide synthase, was found to be up regulated 4 times under Fe limited condition which might be involved in repair of DNA and protein damaged by oxidative stress. In addition, *P. parvum* is assumed to up-regulate oxidative stress response proteins MnSOD and STK to minimize the oxidative stresses by inactivating the access electrons in the cells. It was also found that carbohydrate degradation and glycolytic activity was increased under Fe-limited conditions. Since phytoplankton showed photorespiration under Fe-limited condition, CO_2 mitigation capacity of phytoplankton might be adversely affected by Fe-limited condition in the sea.

Marine microalga *P. parvum* also alters its cellular biochemical processes by up-regulating several proteins involved in photosynthesis. Under Fe-limit condition, *P. parvum* may undergo photorespiration which needs high metabolic energy.

Microalgae may satisfy the demand of high metabolic energy by increasing ATP synthase in chloroplast. Fe-limitation may also induce oxidative stresses to microalgae that may results in degradation of photosynthetic protein like PSII D1. In addition, the phytoplankton increased biosynthesis of PSII component proteins, such as, light harvesting complex protein, PSII D2 protein, photosystem Q_B proteins etc. under Fe-limited conditions. Findings of the study suggest that marine phytoplankton *P. parvum* increase PSII under Fe limited condition. The summery of the molecular responses of phytoplankton under Fe-limited condition could be presented by Fig. 8.1.

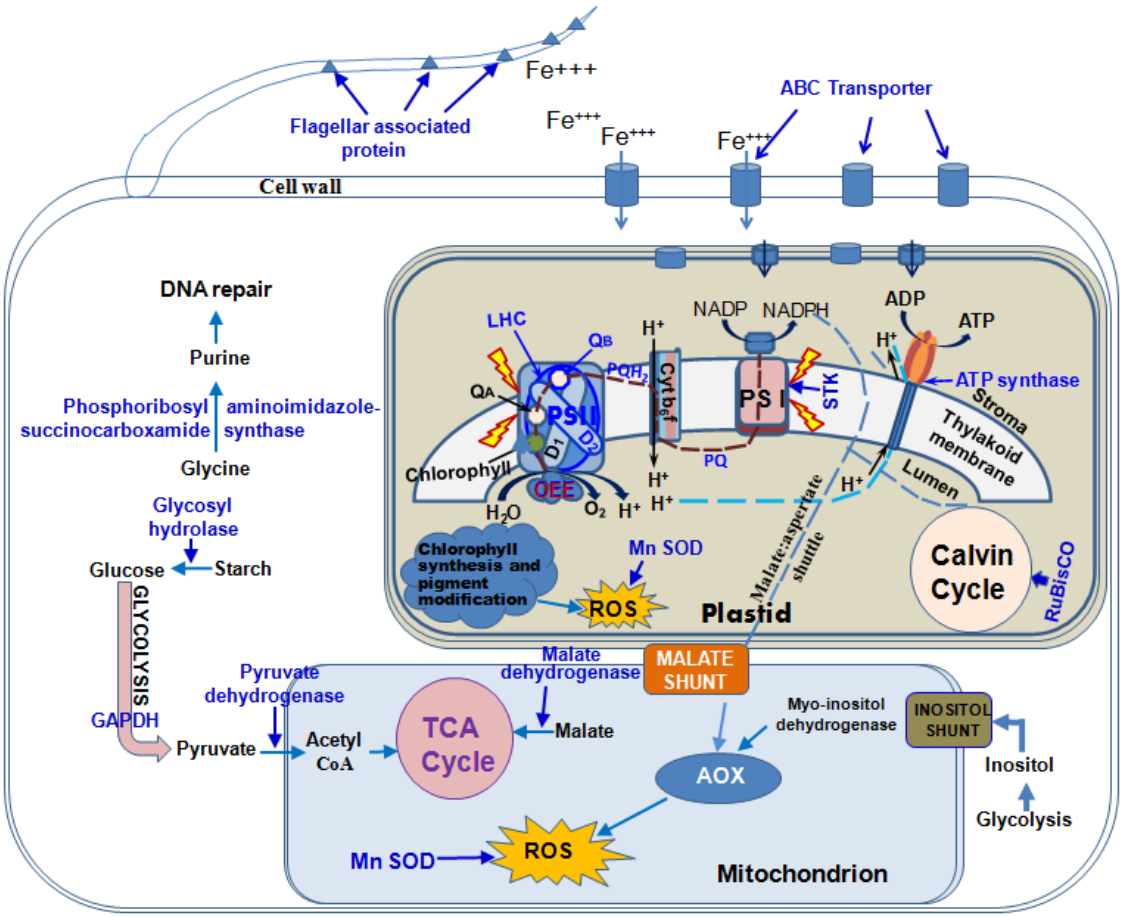

Fig. 7.1: Biochemical pathways and processes in *Prymnesium parvum* cells under Fe limitation (hypothetical). The figure shows how different proteins (in blue color), up-regulated under Fe-limited conditions, involve in biochemical pathways in *P. parvum* cells. The flagellar associated protein is provably linked with the flagellar

activity of finding iron molecules under Fe-limited condition. ABC transporters help in Fe acquisition. PSII component proteins were upregulated under Fe-limited condition. Phosphoribosylaminoimidazole-succinocarboxamide synthase is associated with the synthesis of purines for DNA repair. Oxidative stresses are supposed to be minimized by serine threonine kinase protein and manganese superoxide dismutase. RuBisCO takes part in Calvin cycle in photorespiration under Fe-deplete condition. Increased malate dehydrogenase, and pyruvate dehydrogenase may help in photorespiration. Glycosyl hydrolase and GAPDH is used for carbohydrate degradation and glycolysis, respectively under Fe limited condition.

SCOPE OF FURTHER RESEARCH

a) The specific site of up-regulated proteins in cell organelles and their specific function is to be investigated along with mRNA expression level for confirmation and more clear understanding of molecular response of phytoplankton to Fe-limited condition.
b) It was assumed that photorespiration occurs under Fe-limited condition in marine phytoplankton. Therefore, CO_2 uptake and utilization in photosynthesis under Fe rich and Fe-limited condition can be investigated.
c) Proteins expressed under Fe-limited condition by low total Fe can be identified and compared with the proteins expressed by ligand induced Fe-limitation condition to know Fe acquisition strategies of phytoplankton under these two conditions.

The End

BIOGRAPHICAL SKETCH

I am sixth in a family of seven kids. My education started at my very early age in a heavenly village of Kaligonj upozilla under Gazipur district in Bangladesh. I had had my primary education from my village school, secondary education from Harinal High School, and higher secondary education from Gazipur Cantonment Board Collage in the district town. I completed Bachelor of Science in Agriculture (Honours) degree from Patuakhali Science and Technology University, Patuakhali, Bangladesh, in 2006 and completed a Master of Science degree in Genetics and Plant Breeding in 2010 from Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh. During my graduation, I joined to Bangladesh Rice Research Institute (BRRI), a reputed research organization of Bangladesh as a scientific officer. Immediately after the completion of my graduation, I got final selection from Kanazawa University, Japan to pursue Ph. D. in Environmental Science and Technology as a Japan Government scholarship (MONBUKAGAKUSHO) student in 2010. I leaved Bangladesh for Japan in October 2010, and admitted to Kanazawa University as a Doctor Course student in fall 2010.