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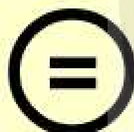

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A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

ECOLOGICAL AND ECO-PHYSIOLOGICAL STUDIES AND APPLICATION OF MICROALGAE ON THE WEST COAST OF JEJU ISLAND

MD. ABU AFFAN

DEPARTMENT OF OCEANOGRAPHY

GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

2006. 12

ECOLOGICAL AND ECO-PHYSIOLOGICAL STUDIES AND APPLICATION OF MICROALGAE ON THE WEST COAST OF JEJU ISLAND

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A thesis submitted in partial fulfillment of the requirement for the Degree of

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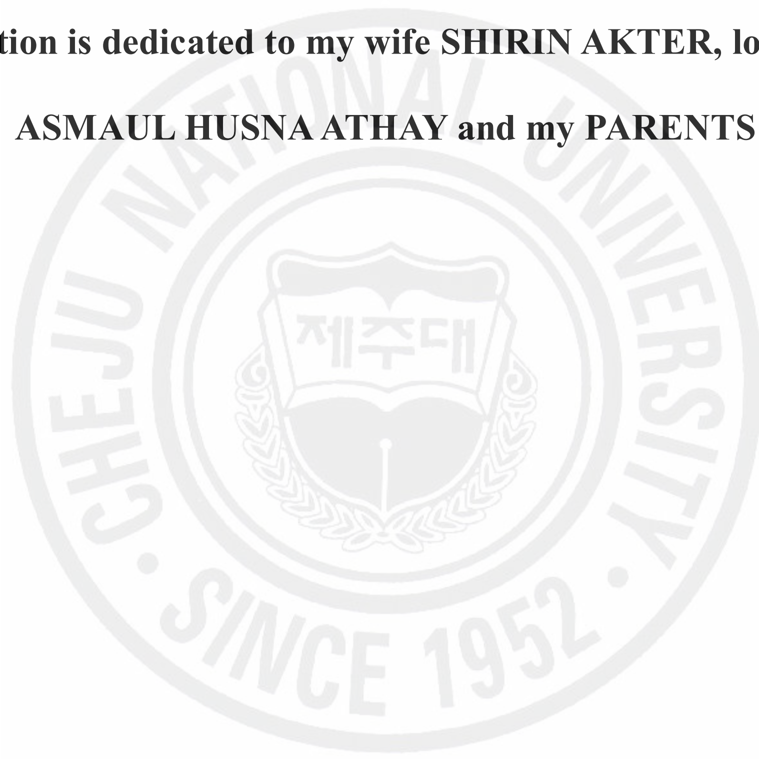
This dissertation is dedicated to my wife SHIRIN AKTER, lovely daughter

ASMAUL HUSNA ATHAY and my PARENTS



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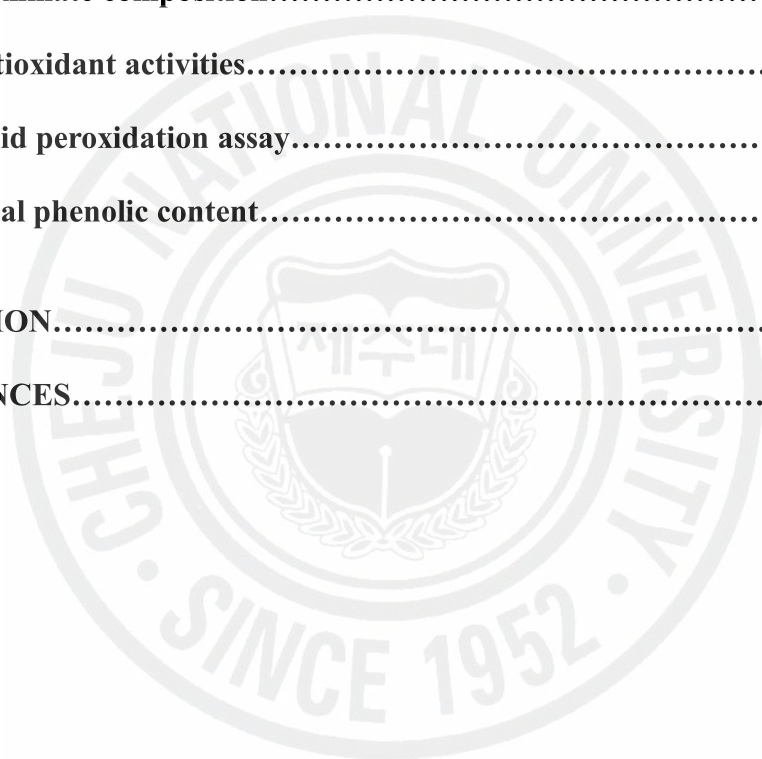

Summary

The west coast of Jeju Island Island is affected by different water mass such as Yellow Sea Warm Current, Yellow Sea Cold Water, and Tsushima Warm Current according to season and especially by overflows from Changjiang River in summer. So the hydrograph seem to be very complicated throughout the year. The changeable and strong wind may also change a hydrographic situation in this area. Thus microalgal occurrence in the area shows seasonal characteristics. Among them benthic diatoms which inhabit on the surface of rocky shore are floated periodically and found in phytoplankton community as dominant species. The benthic diatoms are very important as a live feed for larval and juvenile abalone in gastropod aquaculture.

This thesis involve ecological and eco-physiological study, and potentiality of industrial application of microalgae occurred in the west coast of Jeju Island, where has been recently designated as a marine ranching area and being developed at the moment. First of all, seasonal dynamics of phytoplankton community are investigated in the area in terms of species composition and dynamics of standing crops with relation to environmental factors. For the purpose of application study of the benthic diatoms, several species were isolated from natural seawater and wavy plates (called 'papan') used in aquaculture farm and characterized to find out an optimal culture condition, so that a mass culture of the species was carried out to get sufficient samples for the further study of functional activity. A comparison study of efficiency for early settlement and specific growth rate of larval abalone was done using mono-strain wavy plates and wild mixed-strain wavy plates of the benthic diatoms in tanks of aquaculture farm. Finally antioxidant activities of the benthic diatoms were also analyzed by various extracts from freeze-dried sample to estimate the potentiality as useful bio-resources.

For ecological study, sampling was done at 10 stations in the marine ranching area from September 2004 to November 2005. Water temperature was 12.1-28.9° C (average of 18.8° C), and salinity was 28.9-34.9 psu (average of 33.7 psu). Chlorophyll *a* concentration was 0.02-2.05 μg L^{-1} (average of 0.70 μg L^{-1}). A total of 294 phytoplankton species were identified, among them 182 belong to diatom (Bacillariophyceae), 52 to dinoflagellate (Dinophyceae), and 60 to phytoflagellates. Standing crops was 2.21-48.69×10^4 cells L^{-1} (average of 9.23×10^4 cells L^{-1}), and the maximum was in April. Spring and autumn phytoplankton blooms occurred with a peak in April and with a peak in November, respectively. The spring bloom was represented by 3 *Chaetoceros* spp., *Skeletonema costatum* and *Hillea* sp., while autumn bloom was in association of dinoflagellates, diatoms and phytoflagellates. Among them dinoflagellates were predominant by the assemblage of *Gymnodinium conicum, Prorocentrum micans* and *P. triestinum*. Spring bloom might be related to increasing water temperature and sufficient nutrient in April, whereas autumn bloom seems to be related to low salinity water from Changjiang River in fall season.

For eco-physiological study, several benthic diatoms were investigated from isolation in nature and in aquaculture farm with respect to axenic culture after treatment of antibiotics for making a bacteria free sample. Among them 2 species, *Navicula incerta* and *Cylindrotheca closterium*, were selected for mass culture and investigated to find out an optimum culture condition in 3 different temperatures, 3 salinities, and 3 different nutrient concentrations. The highest cell density of *Navicula incerta* occurred in 20˚ C water with the maximum specific growth rate of 0.88 d^{-1}, 30 psu salinity, and F/2 (100%) nutrient concentration. The highest cell density (7.20 × 10^4 cells mL^{-1}) of *Cylindrotheca closterium* occurred with the maximum specific growth rate of 0.82 d^{-1} in a condition of 20˚ C water with salinity of 30 psu and nutrients concentration of F (200%) nutrient concentration. Using the optimal culture condition the mass culture of *Navicula* and *Cylindrotheca* was done and biomass was harvested as much as needed for further functional analyses.

To compare efficiency of live feed for larval and juvenile abalone between mono-strain wavy plates and wild mixed-strain wavy plates, *Navicula incerta* and *Grammatophora marina* mono-strain on wavy plates produced in the laboratory and wild mixed-stains from natural seawater were applied with larval abalone in aquaculture tanks. The average number of settled juvenile abalone was 1000, 1080 and 640 in the tank of *N. incerta*, *G. marina* mono-strain and wild mixed-strain, respectively, and the survival rate was 2.00, 2.16 and 1.28% in each kind of tank, respectively. The specific growth rate of juvenile abalone was 3.28, 3.07, and 2.92% on the plates of *N. incerta*, *G. marina* mono-strain and wild mixed-strain, respectively. In conclusion the plates of mono-strain showed better settlement and growth of abalone larvae than that of wild mixed-strain. Thus further application of mono-strain wavy plates of benthic diatoms for abalone aquaculture will be available to enhance the efficiency of settlement and growth at the early stage of abalone aquaculture.

To estimate the functional activity of benthic diatoms, antioxidant activities were determined from *N. incerta* and *C. closterium* with the extract of methanol, enzymes and water. The rates of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging for Neutrase and methanol extracts were 81.6% and 62.8% from the extract of *N. incerta*, respectively. The *C. closterium* showed 72.5% and 69.4% DPPH scavenging activity from the extract of Viscozyme and methanol. Flavourzyme extract of *N. incerta* had a superoxide scavenging rate of 57.7%. Kojizyme and Ultraflo extracts had nitric oxide scavenging rates of 42.2% and 40.6%, respectively, significantly higher than α-tocopherol and BHT. The metal-chelating activities of the methanol, Neutrase, and Termamyl extracts of *N. incerta* were 68.5%, 45.2%, and 41%, respectively. The Kojizyme, Alcalase, methanol, Viscozyme and Neutrase extracts from *C. closterium* were 67.1%, 53.9%, 53.2% and 50.2% for metal chelating, respectively. The Termamyl extract of *N. incerta* and AGM, Viscozyme and Neutrase extracts of *C. closterium* showed the highest linoleic acid peroxidation inhibition, exceeding α-tocopherol and on par with BHT. Thus if massive cultivation and harvest of the

useful benthic diatoms could be industrialized with appropriate economic benefit, those species would be of importance as one of candidates for the potential antioxidants.

국문 요약

제주도 서부 연안은 주변 해역에 영향을 미치는 황해난류수, 황해저층수, 쓰시마난류수의 계절적인 영향과 여름철 양자강에서 흘러 들어오는 담수의 영향을 받으면서 연중 복잡한 양상을 보이고 있다. 또한 계절에 따라 바뀌는 바람의 영향은 주변 해역의 수리적인 특성을 변화시키기도 한다. 따라서 이 해역에 출현하는 미세조류는 계절적 특성을 잘 나타내고 있으며, 특히 해안선에 잘 발달되어 있는 암반을 기반으로 서식하는 저서성 규조류는 강한 바람의 영향으로 일시 부유성으로 출현하여 식물플랑크톤 군집에서도 매우 중요한 우점종으로 출현한다. 또 이들 저서성 규조류는 제주도 패류 양식장에서 전복과 소라의 초기 먹이 생물로 이용되고 있다.

본 논문은 최근 제주도 바다목장 해역으로 지정되어 개발되고 있는 제주도 서부연안을 중심으로 미세조류의 생태적 특성, 생리생태적 특성, 그리고 산업적 응용의 잠재성을 연구하였다. 먼저 조사해역의 식물플랑크톤 군집의 계절적 동태를 파악하여 군집의 종조성과 현존량의 변화를 환경 요인과의 관계에서 분석하였다. 또 저서성 규조류를 보다 효율적으로 응용 연구에 이용할 목적으로 자연 상태와 양식장의 치패 사육용 파판에서 분리한 몇 종의 저서성 규조류의 증식 특성을 파악하였고 이를 이용한 대량 배양으로 수확한 시료로 기능성 연구에 필요한 건조 표본을 생산하였다. 또 본 연구에서 특별히 제작된 단일종에 의한 파판과 기존에 사용되어 왔던 혼합종에 의한 파판을 이용하여 전복 유생의 초기 정착과 성장율을 비교하였다. 또 대량 배양에서 수확된 2종류의 단일종 건조 표본을 이용하여 항산화성 기능을 분석하여 앞으로 저서성 규조류가 기능성 바이오 소재로서의 잠재력을 평가하였다.

생태적인 연구를 위해 2004년 9월부터 2005년 11월까지 제주도 바다목장 해역의 선정된 10개 정점에서 매월 채집 및 관측을 실시하였다. 연중 수온은 12.1-28.8 ℃ (평균 18.7 ℃), 염분은 28.8-34.9 psu (평균 33.7 psu)의 범위를 보였다. 출현한 식물플랑크톤은 총 294종으로, 규조류 182종, 와편모조류 52종, 식물편모조류 60종이 동정되어 규조류의 출현종이 가장 많았다. 연중 현존량 범위는 2.2×10^4 - 4.9×10^5 cells/L (평균 9.2×10^4) 로써, 계절적인 대발생은 봄철(4월)과 가을철(11월)에 발생하였으며, 이중 봄철의 대발생이 훨씬 높은 현존량을 보였다. 봄철 대발생은 *Chaetoceros*속의 3종류와 *Skeletonema costatum*등 우점 규조류의 혼합에 의해 발생하였고, 가을철 대발생은 *Gymnodinium conicum*, *Prorocentrum micans*, *Prorocentrum triestinum* 등 와편모조류가 가장 우점하였고 그 외 규조류와 식물편모조류가 혼합된 상태에서 발생하였다. 이와 같은 봄철과 가을철의 대발생은 온대 해역의 일반적인 식물플랑크톤 동태의 특성을 보이고 있으며, 봄철 대발생은 수온 상승과 영양염의 증가에 의해 발생하였고, 가을철 대발생은 저염분성 양자강 혼합수의 영향으로 발생하였다고 생각된다.

생리생태적인 연구는 자연상태와 패류 양식장에서 순수 분리한 몇 종의 저서성 규조류을 대상으로 이루어졌으며, 우선 박테리아가 제거를 위한 순수 배양 조건을 탐색하였다. 이 중 가장 성장이 좋은 2종의 규조류, 즉 *Navicula incerta*와 *Cylindrotheca closterium*을 대상으로 수온, 염분, 영양분 농도의 조건을 달리하여 모두 27개 배양조건에서 증식 특성을 파악하여 최적의 증식조건을 찾아 대량 배양을 시도하였다. 그 결과 *Navicula*의 최대 성장율은 0.81-1.04 day^{-1}였으며, 그때 최대 생물량은 7.99×10^5 cells mL^{-1}으로 최적 배양조건은 20 ℃, 30 psu, F/2(100%) 영양염 농도였다. 또 *Cylindrotheca closterium*의 최대 성장율은 0.63-0.97 day^{-1}의 범위를 보였고, 최대 생물량은 7.20×10^4 cells mL^{-1} 이었고, 최적 배양조건은 20 ℃, 30 psu, F(200%) 영양염

농도 조건이었다. 이들 최적 배양조건을 이용하여 대량배양을 시도하였으며 기능성 연구를 위해 필요한 건조 표본을 수확하였다.

패류 양식장에서 치패의 초기 먹이로 이용되는 저서성 규조류를 단일종으로 만들었을 때의 효율성을 파악하기 위해 실험실에서 생산한 단일종 파판과 자연적으로 양식 수조에서 생산한 혼합종 파판을 양식장 수조를 이용하여 전복 유생의 착생과 성장율을 비교하였다. 저서성 규조류인 *Navicula* 단일종 파판과 *Grammatophora* 단일종 파판, 그리고 혼합종 파판의 전복 유생의 착생 개체수는 각 파판당 10.0, 10.8, 6.4 개체를 보여, 단일종 파판이 혼합정 파판보다 월등히 높았으며, 유생 전복의 생존률도 각 파판당 2.00, 2.16, 1.28%를 보여 *Grammatophora* 단일종 파판에서 가장 높았다. 그러나 유생 전복의 비성장율은 각 파판당 3.28, 3.07, 2.92%를 보여 큰 차이를 보이지 않았으며, 평균 먹이소화율은 0.17, 0.15, 0.26%를 보여 오히려 혼합종 파판이 높았다. 따라서 단일종 파판을 이용하면 전복 유생의 착생율과 전복 치패의 초기 성장율을 높일 수 있다고 생각되며, 본 연구의 결과는 앞으로 저서성 규조류의 단일종 파판 생산에 활용될 수 있을 것으로 생각된다.

저서성 규조류의 생리활성 물질의 기능성을 파악하기 위해 대량 배양이 가능하고 가장 성장조건이 좋은 *Navicula incerta*와 *Cylindrotheca closterium*을 대상으로 항산화특성 연구를 수행하였다. *Navicular incerta*의 항산화 특성을 보면, DPPH 자유라디칼 소거가 Neutrase와 methanol 추출물에서 81.6%와 62.8%를 각기 보였다. 이외 Flavouzyme 추출물은 DPPH 소거율이 57.7%, Kojizyme과 Ultraflo 추출물은 42.2%와 40.6%를 보여 상용으로 이용되는 α-tocopheral과 BHT보다 높게 나타났다. 또 methanol, Neutrase, Termamyl 추출물의 metal-chelating 활성은 68.5, 45.2, 41.0% 각각 나타내어 상용되는 항산화제보도 4-6배 높았다. 한편 *Cylindrotheca closterium*의 DPPH 자유라디칼 소거 조건은 Viscozyme 과 methanol 추출물에서 72.5%와 69.2%를 각기

나타내었다. Kojizyme, Alcalase, methanol, Viscozyme 과 Neutrase 추출물의 metal-chelating 활성은 67.1%, 53.9%, 53.2%, 52.1%, 50.2%을 보였으며 상용되는 항산화제보다 5-6배 높은 활성을 보였다. 따라서 이 들 저서성 규조류가 산업적으로 이용 가능할 수 있게 대량 배양될 수 있고, 경제성이 확보된다면 항산화제로 개발될 수 있는 잠재력을 보이고 있다고 평가된다.

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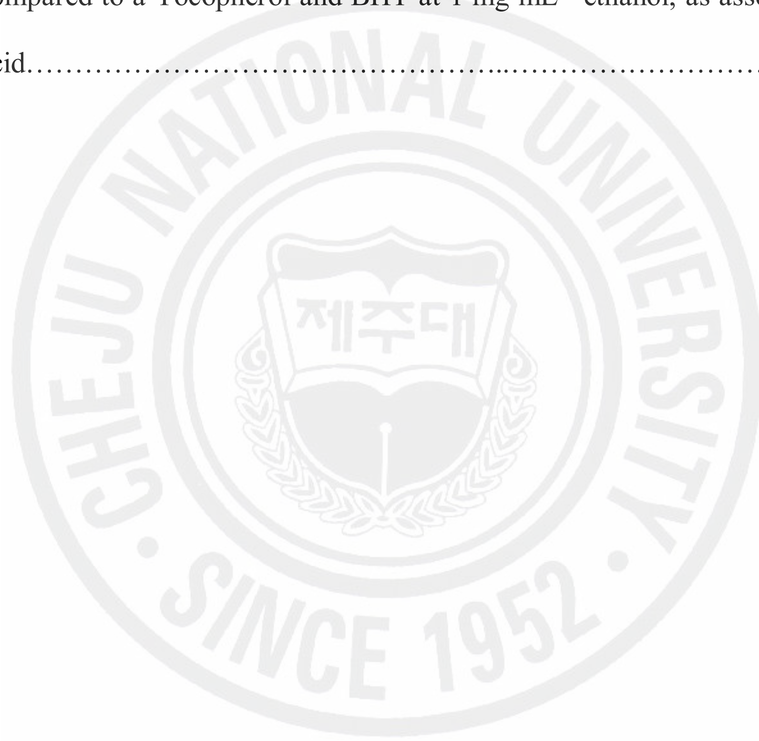

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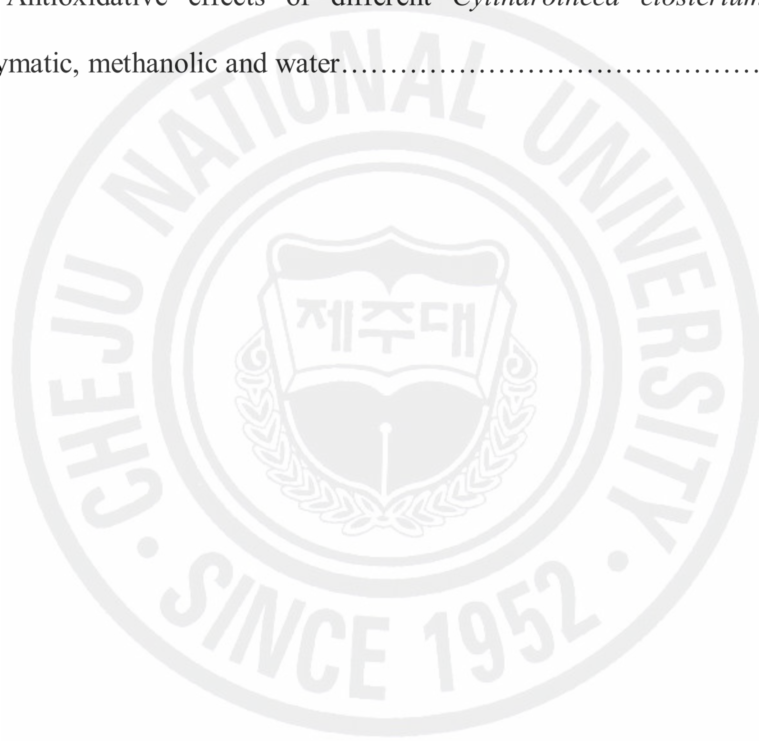


GENERAL INTRODUCTION

Algae (singular *alga*) encompass several different groups of living organisms that capture light energy through photosynthesis converting inorganic substances into simple sugars using the trapped energy. Algae have been traditionally regarded as simple plants, and some are closely related to the higher plants. All algae have photosynthetic machinery and produce oxygen as a by-product of photosynthesis. Algae are usually found in damp places or water-bodies and thus are common in terrestrial as well as aquatic environments.

The name "plankton" was derived from the Greek term phyavktov, meaning "wanderer" or "drifter" (Thurman 1997). While some forms of phytoplankton are capable of independent movement and can swim up to several hundred meters vertically in a single day, in contrast their horizontal position is primarily determined by the water currents. Most phytoplankton is too small to see individually with the unaided eye. During very high densities (so-called algal blooms) these algae may discolor the water and outcompete or poison other life forms. Phytoplankton, like plants, obtain energy through a process called photosynthesis and so must live in the well-lit surface layer (termed the euphotic zone) of an ocean, sea, lake or pond. Their cumulative energy fixation in the form of carbon compounds (primary production) is the basis for the vast majority of oceanic and some freshwater food chain (chemosynthesis is a notable exception). Marine algae contribute a major part of primary production, being responsible for 46 % of global productivity (Field et al. 1998). They are the chief producer organisms supporting aquatic food webs from pond to oceans. Aquatic environments are subjected to high temporal variability due to the interactions among physical, chemical and biological factors, resulting in noticeable changes in relative abundance and species composition of phytoplankton (Reynolds et al. 2000). Environmental disturbances in aquatic systems alter phytoplankton community structure, diversity and biomass (Hutchinson 1961). For example, laboratory experiments and field studies have shown that episodic flushing and nutrient loading can result in enhanced phytoplankton

species diversity (Padisak 1993, Hambright and Zohary, 2000, Lovejoy et al. 2002). Because of the close coupling of physical forcing and biota, environmental fluctuations are expected to affect communities in aquatic systems significantly (Steele 1985).

Jeju Island is located at the southern coast of Republic of Korea, characterized by volcanic rocky inter-tidal and sub-tidal zones that are subjected to strong wave action. The waters off the west coast of Jeju Island is influenced by Yellow Sea Warm Current from winter to spring, whereas the waters receive runoff from Changjiang River during summer (Pang et al. 1996; Hyun et al. 1997). Especially, during the summer the Changjiang River overflows result in devastating effect on fisheries by causing mass mortality of shellfish and fish due to the considerable dilution of the seawater around the coast of Jeju Island (Suh et al. 1998, Lee et al. 1999). At this moment, the reports about seasonal changes and structure in phytoplankton community along the west coast of Jeju Island are scarce. Affan and Lee (2004) monitored the phytoplankton dynamics on a single site on the west coast of Jeju Island and they reported 101 species of phytoplankton in different groups and among them the benthic diatoms were the most dominant group but they did not mention the actual mechanisms of phytoplankton assemblages with seasonal changes and environmental condition throughout the west coast. As a part of the marine ranching program, it was attempted to know the spatial and temporal variation in the assemblage of phytoplankton in relation to environmental dynamics for the proper management of aquatic resources in this area.

Benthic diatoms are the most important food source for the abalone postlarvae (Kawamura et al. 1998). Biofilms of mixed benthic diatoms have been used traditionally as settlement substrata for abalone postlarvae in worldwide hatcheries (Hahn 1989, Roberts 2001). Several problems have been recognized in regards to the current raising method of abalone on diatom biofilms, as it is difficult to continue the supply of readily ingestible and digestible food (diatom biofilms) for growing postlarvae (Kawamura et al. 1998). Apart from the monitoring of phytoplankton dynamics in natural environemnts, *Isochrysis*, *Pavlova*,

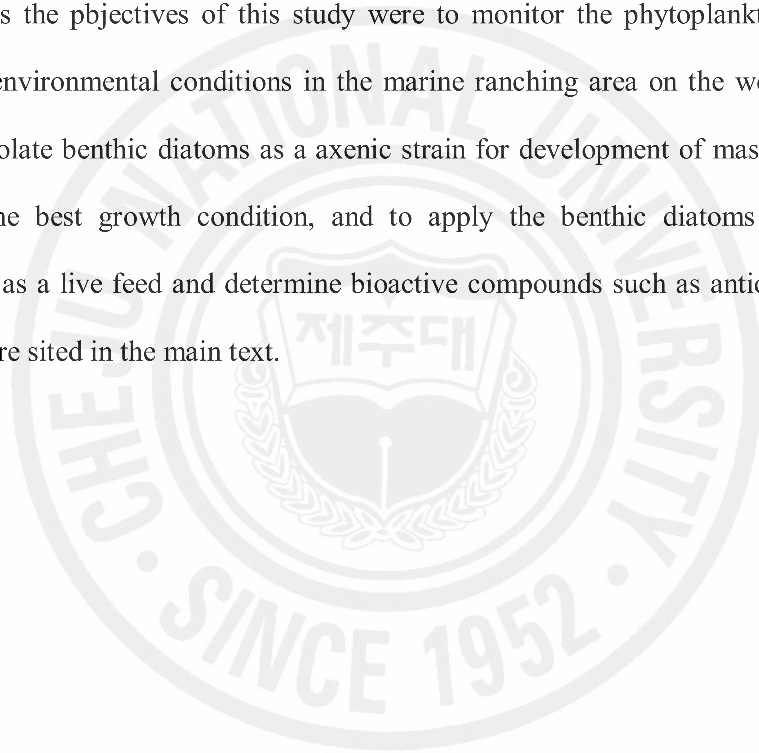
Chaetoceros, *Thalassiosira*, *Nannochloris* and *Tetraselmis* are used as a live feed for the shellfish seedling production in Korea. Due to technical problem in benthic diatom mass culture system, the abalone seedling production from the aquaculture farms is still very low in relation to its increasing demand in Korea (Hur 1997).

Wild mixed benthic diatoms on wavy plates are traditionally used as live feed in commercial shellfish hatcheries, in Jeju Island, Korea. At present, benthic diatoms are cultured based on the natural with the addition of fertilizer for aquaculture purposes. But this traditional production of benthic diatoms can not fulfill the demands year-round for rapidly expanding aquaculture sector due to population fluctuations resulted from natural environmental dynamics. The development of sustainable and suitable technique for the mass culture of benthic diatoms is necessary to meet up aquaculture demand. For mass culture, the most important task is the selection of species which depends on- i) the availability of the species year-round, ii) their optimal environmental conditions, and iii) their suitability as food for the targeted shellfish. Therefore, isolation of single species, find out their best growth condition with different environmental factors and finally the development of low-cost mass culture technique is utmost important to fulfill demand for live feed in the shellfish aquaculture. For the settlement and growth of abalone larvae we used *N. incerta* and *G. marina* monostrains which showed stronger attachment habit than others, and exhibited thick, dark brown mats on the wavy plates. Finally the efficiency of abalone larval settlement was compared between the mono-strain benthic diatoms and wild mixed benthic diatom species.

On the other hand, microalgae are also increasingly being promoted in the human diet as nutraceutical and health food products. Much effort has been expended to search new compounds of therapeutic potential, and it was demonstrated that in microalgae of all classes possess antibacterial, antifungal and anticancer ability (Tredici 2004). Extract of *Chlorella* sp. and *Spirulina* sp. are being proposed to use with noodles, bread, green tea, beer and candy (Liang et al. 2004). Several microalgae such as *Chlorella* spp. *Spirulina* spp. and *Dunaliella*

spp. are grown commercially and algal products such as β-carotene and phycocyanin are available. The antioxidative activity of phycocyanobilin from *Spirulina platensis* was evaluated against oxidation of methyl linoleate in a hydrophobic system or with phosphatidylcholine liposomes. *Aphanizomenon flos-aquae* (AFA), a blue green-alga is rich in phycocyanin (PC), a photosynthetic pigment with antioxidant and anti-inflammatory properties (Benedetti, 2004). Apart from the use of benthic diatoms as a live feed in aquaculture, we determined bio-active compounds from benthic diatoms.

Thus the pbjectives of this study were to monitor the phytoplankton dynamics in relation to environmental conditions in the marine ranching area on the west coast of Jeju Island, to isolate benthic diatoms as a axenic strain for development of mass culture system based on the best growth condition, and to apply the benthic diatoms in the abalone aquaculture as a live feed and determine bioactive compounds such as antioxidants. All the references are sited in the main text.



Part I

Seasonal dynamics of phytoplankton in the marine ranching area on the west coast of Jeju Island

1. Abstract

Dynamics of phytoplankton abundance with seasonal variation of physico-chemical condition were investigated monthly about 10 stations, including inshore, middle shore and offshore in the marine ranching area on the west coast of Jeju Island. Water temperature varied from 12.1 to 28.9° C (average of 18.8° C), and salinity from 28.9 to 34.9 psu (average of 33.7 psu). Chlorophyll *a* concentration was 0.02-2.05 μg L^{-1} (average of 0.70 μg L^{-1}), and the maximum concentration was at the bottom layer in April. A total of 294 phytoplankton species were identified, among them 182 belong to Bacillariophyceae, 52 Dinophyceae, 9 Chlorophyceae, 12 Cryptophyceae, 6 Chrysophyceae, 4 Dictyophyceae, 13 Euglenophyceae, 6 Prymnesiophyceae, 5 Prasinophyceae, 5 Raphidophyceae. Standing crops was 2.21 to 48.69×10^{4} cells L^{-1} (average of 9.23×10^{4} cells L^{-1}), and the maximum was in April at the bottom layer. Diatoms were most abundant throughout the year, followed by dinoflagellates and phytoflagellates. Phytoplankton bloom occurred in two times; one in spring with a peak in April and the other one in autumn with a peak in November. The spring bloom was represented by 4 *Chaetoceros* spp. and *Skeletonema costatum*, which contributed 10-20% to total phytoplankton abundance as a single species. Autumn bloom was in association of dinoflagellates, diatoms and phytoflagellates. Among them dinoflagellates were predominant, and *Gymnodinium conicum, Prorocentrum micans* and *P. triestinum* represented above 10% abundance of total phytoplankton as a single species.

2. Introduction

Marine algae perform a major part of primary production, being responsible for 46 % of global productivity (Field et al. 1998) with supporting food webs in waters from pond to oceans. Temporal variability in the structure and function of a phytoplankton community is of fundamental importance to aquatic system. Aquatic environments are subject to high temporal variability, with frequent reorganization of relative abundance and species composition of phytoplankton, as a result of interaction between physical, chemical and biological variables (Reynolds et al. 2000). The seasonal dynamics and succession of phytoplankton populations are often associated with water temperature, salinity and nutrients concentrations. Water temperature is an important factor controlling the algal growth in natural environments (Lund 1949, Talling 1955) and growth response to water temperature may be essential in regulating the predominance of phytoplankton species (Harris 1986). Wide ranges of salinity and water temperature may play the important roles for the frequent appearance of phytoplankton throughout the year in the ocean (Hoshiai et al. 2003).

There is growing evidence that human activities are changing the distribution and movement of nutrient elements, resulting in nutrient's increase and loading to receiving waters, and changes in nutrients can alter the species composition of primary producers (Reolke et al. 1999). A pulsed supply of nutrients, for example, has been shown to increase phytoplankton diversity to values far above the number of limiting nutrients (Grover 1989). For another example, laboratory experiments and field studies have shown that episodic flushing and nutrient loading can result in enhanced phytoplankton species diversity (Padisak 1993, Hambright and Zohary 2000, Lovejoy et al. 2002). From experimental studies with temporal changes in nutrient supply, there is an evidence that the impact on species diversity of phytoplankton is most evident when nutrient supply fluctuates at intervals of 3–7 days, corresponding to two to four generation times (Sommer 1995, Sommer and Floder 1999). Competitive abilities of phytoplankton species vary as a function of the physicochemical environment. Environmental factors and population densities fluctuate in time and in space

(Litchman and Klausmeyer 2001). Because of the close coupling of physical forcing and biota, environmental fluctuations are expected to affect communities in aquatic systems significantly (Steele 1985).

Jeju Island is located at the southern part of Republic of Korea, characterized by volcanic rocky inter-tidal and sub-tidal zones that are subjected to strong wave action. The west coast of Jeju Island is influenced by Yellow Sea Warm Current, a branch of Tsushima Warm Current, from winter to spring, and by a huge freshwater runoff from Changjiang River in summer (Pang et al. 1996, Hyun et al. 1997). Especially, during the summer the Changjiang River overflows result sometimes in devastating effect on fisheries by causing mass mortality of shellfish and fish due to the considerable dilution of the seawater around the west coast of Jeju Island (Suh et al. 1998, Lee et al. 1999). At this moment, the reports about seasonal changes and structure of phytoplankton community along the west coast of Jeju Island are scarce. Affan and Lee (2004) monitored the phytoplankton dynamics on a single site on the west coast of Jeju Island and they reported 101 species of phytoplankton in different groups. Among them the benthic diatoms were the most dominant group, but they did not mention the actual mechanisms of phytoplankton assemblages with seasonal changes and environmental condition throughout the west coast. As a part of the marine ranching program, it was attempted to know the spatial and temporal variation in the assemblage of phytoplankton in relation to environmental factors for the proper management of aquatic resources in this area.

3. Materials and methods

3.1. *Study area and sampling*

This study was conducted at the west coast of Jeju Island Island where the marine ranching program has been going since 2004. A total of 10 sampling stations along four transects (A, B, C and D lines) belong to inshore, middle shore and offshore of the coast (Fig.

1). Water samples were collected monthly at the surface layer as well as the bottom layer from September 2004 to November 2005 using water sampler. The samples were divided into two bottles from each site; one sample was used for the analysis of chlorophyll *a* and chemical factors such as nutrients concentration, and another sample for quantitative and qualitative analyses of phytoplankton.

3.2. *Environmental factosr analyses*

A CTD (conductivity-temperature-depth) unit (Sea-Bird Electronics SBE 16, USA) was used for a measurement of water temperature and salinity. The concentration of NO_3–N, NO_2–N, NH_4-N and PO_4-P were determined with a spectrophotometer (Shimadzu UV-1201, Japan) according to Parsons et al. (1984).

3.3. *Chlorophyll* a *analysis*

After water samples were filtered through glass fiber filter (Whatman GF/C 47 mm, UK), the filters were kept into a 15-ml test tube with 90% acetone and placed in refrigerator overnight. The test tube was centrifuged at 3500 rpm for 15 mins and the supernatant was used for chlorophyll *a* analysis by using the spectrophotometer (Shimadzu UV-1201, Japan). The concentration was calculated according to Parsons et al. (1984).

3.4. *Phytoplankton analysis*

Water samples were fixed with Lugol's iodine solution at a final concentration of 2%, and the fixed sample was transferred into a one-liter volume graduated cylinder which was designed with siphon at the bottom of 60 ml. The sample was kept for 2-3 days and the supernatant was removed. For quantitative study, a 1 ml sample was taken after mixing of the concentrated sample and counted on a Sedgewick-Rafter (S-R) counter chamber using light microscope. Counted results were summarized as cells per liter. For species identification, the sample was observed under the phase-contrast microscope (Zeiss Axioplan,

Germany) at 400x magnification. Species diversity index (H) was calculated according to the formula of Shannon and Weaner (Washington 1984).

3.5. *Nutrients analyses*

Nutrient concentrations such as NH_4-N, NO_3-N, NO_2-N and PO_4-P were measured at 10 stations every two months interval from November 2004 to November 2005 by standard method (Parsons et al. 1984) and each value was averaged at the surface and bottom layer in inshore, middle shore, and offshore.

4. Results

4.1. *Dynamics of hydrological factors*

4.1.1 *Water temperature*

During this study, water temperature at the surface layer went through a seasonal cycle characterized by a minimum of 14.2° C (March) and a maximum of 28.9° C (August) with the average of 20.1° C (Fig. 2 A), not showing significant variation among the stations. At the bottom layer, the water temperature varied from 12.1 to 27.0° C with average of 20.3° C, showing the minimum and maximum in August and September 2005, respectively. From November to May the bottom water temperature also did not show any significant difference between stations, but from June to September it showed significant difference. In the middle shore and offshore, the temperature was much lower than inshore (Fig. 2 B). The bottom water temperature of inshore was higher from June to September than that of offshore and middle shore (Fig. 2 B). The water temperature did not show any difference between the surface and bottom layer from winter to spring (Fig. 2 A and B).

4.1.2. *Salinity*

Salinity varied from 29.8 to 35.0 psu with the average of 33.8 psu during the study period. The lowest salinity was recorded at surface layer of inshore in July, whereas the highest was in the bottom layer of offshore in November 2005 (Fig. 3 A and B). The average salinity at the surface and bottom layer was 33.0 and 34.0 psu, respectively. The salinity at the surface layer was significantly lower than that at the bottom layer from June to September 2005. The salinity did not show any significant difference in the all station at the surface layer throughout the study period (Fig. 3 A). The variation of salinity at the bottom layer was also same from November 2004 to May 2005, i.e., it did not show any significant differences between stations. However the salinity of inshore was much lower than that of middle shore and offshore from June to October 2005 (Fig. 3 B).

At the surface layer, the salinity showed highly significant negative correlation with water temperature in inshore ($r = -0.79$), middle shore ($r = -0.80$) and offshore ($r = -0.82$), but the salinity did not show any significant relation with water temperature at the bottom layer.

4.1.3. *Chlorophyll* **a**

Chlorophyll *a* concentration varied from 0.06 to 1.81 $\mu g\ L^{-1}$ with the average of 0.60 $\mu g\ L^{-1}$ in all stations. The chlorophyll *a* concentration was higher in April in the all stations during the study, and the highest concentration was in the bottom layer of inshore (Fig. 4 A and B).

4.1.4. *Nutrient dynamics*

NH_4-N concentration varied from 0.08 to 5.85 μg-at L^{-1} with the average of 1.63 μg-at L^{-1}, and the lowest and the highest concentrations were in the surface layer of offshore in November 2004 and February 2005, respectively. In February the NH_4-N was higher both at the surface and bottom layer in offshore than in middle shore, followed in inshore. Among

the all stations, the highest (2.24 μg-at L^{-1}) average concentration of NH_4-N was at the surface layer of middle shore and the lowest (0.86 μg-at L^{-1}) was at the bottom layer of inshore (Table 1). NO_3-N concentration fluctuated from 0.09 to 9.95 μg-at L^{-1} with the average of 3.93 μg-at L^{-1} among the all stations. The highest and the lowest concentrations were at the bottom layer of middle shore in August and November. The highest average concentration of NO_3-N was 4.35 μg-at L^{-1} at the surface layer of inshore (Table 1). NO_2-N concentration varied 0.09 - 8.68 μg-at L^{-1} in the all stations. The lowest was at the surface layer of inshore in November 2005 and the highest at the bottom layer of middle shore in the same time. The lowest mean value of NO_2-N concentration was 0.14 μg-at L^{-1} at the surface layer of inshore, whereas the highest mean value was 1.92 μg-at L^{-1} at the bottom layer of middle shore (Table 1). PO_4-P concentration was 0.06 to 0.66 μg-at L^{-1} with the average of 0.26 μg-at L^{-1} in the all stations. The lowest value was at the surface and bottom layer of inshore and middle shore in May 2005, whereas the highest concentration was in the bottom layer of offshore in November 2005 (Table 1).

NH_4-N showed negative correlation with water temperature in the middle shore ($r = -0.51$) and offshore ($r = -0.79$) at the surface layer, whereas it showed negative correlation with salinity at the bottom layer of inshore ($r = -0.65$). NO_3-N showed highly positive correlation with water temperature in the surface layer of inshore ($r = 0.89$), middle shore ($r = 0.81$). The concentration of NO_3-N showed negative correlation with salinity at the surface layer of inshore ($r = -0.73$) and middle shore ($r = -0.81$), and at the bottom layer of inshore ($r = -0.88$), middle shore ($r = -0.95$), offshore ($r = -0.96$). NO_2-N showed strong positive correlation with standing crops production at the bottom layer of inshore ($r = 0.94$), middle shore ($r = 0.96$) and offshore ($r = 0.95$). PO_4-P exhibited the positive correlation with water temperature at the bottom layer of inshore ($r = 0.57$) and it also showed negative correlation with salinity at the bottom layer of middle shore ($r = -0.51$).

4.2. *Dynamics of phytoplankton*

4.2.1. Species composition and diversification

During the monitoring period, a total of 294 phytoplankton species were identified, among them 182 belong to Bacillariophyceae, 52 Dinophyceae, 9 Chlorophyceae, 12 Cryptophyceae, 6 Chrysophyceae, 4 Dictyophyceae, 13 Euglenophyceae, 6 Prymnesiophyceae, 5 Prasinophyceae and 5 Raphidophyceae (Table 2). The variation in species diversity index (H) ranged from 1.88 to 3.18 with the average of 2.50, and the lowest and highest diversity were at the surface layer of middle shore in April and November 2005, respectively (Fig. 5 A and B).

4.2.2. *Standing crops*

The phytoplankton abundance fluctuated from 2.21 to 48.69 $\times 10^4$ cells L^{-1} with the average of 9.23 $\times 10^4$ cells L^{-1} among the all stations throughout the study period. The lowest abundance was at the surface layer of offshore in February and the highest at the bottom layer of inshore in April 2005 (Fig. 6 A and B). The phytoplankton abundance was significantly high at the bottom layer of inshore, followed by at the surface layer of inshore and at the surface and bottom of middle shore. During the study period, phytoplankton blooms occurred two times; one was in spring (March to May) with a peak in April and another in autumn (October to November) with a peak in November 2005 (Fig. 6 A and B). Among the phytoplankton communities, diatoms were dominant throughout the study period except in November 2005 when dinoflagellates were dominant (Fig. 7 A, B and C). Dinoflagellates also showed higher abundance in the all stations in July 2005. The contribution of dinoflagellates to the total phytoplankton abundance varied from 32.2 to 48.1% and the highest was in offshore (Fig. 7 C). The other phytoflagellates except dinoflagellates showed their maximum presence in all stations in September 2004 and ranged in 18.4-31.8% with the average of 9.7%. The highest abundance percentage to the

total phytoplankton was 25.9%, 18.3% and 31.3% in inshore, middle shore and offshore, respectively (Fig. 7 A, B and C).

The spring bloom occurred by diatoms and the autumn bloom occurred in association of dinoflagellates, diatoms, and phytoflagellates. Dinoflagellates were the most dominant among the phytoplankton (Fig. 8 A). During the spring bloom, the highest abundance (47.50 $\times 10^4$ cells L^{-1}) of diatoms was at the bottom layer of inshore (Fig. 8 A). The contribution of diatoms among the phytoplankton varied from 93.5 to 97.6 % with the highest at the bottom layer of inshore. The most dominant species was *Chaetoceros lorenzianus*, *C. pseudocrinitus*, *C. socialis*, *Skeletonema costatum* of diatoms, and *Hillea* sp. of phytoflagellates. The *C. socialis* and *S. costatum* played an important role of spring bloom with the highest abundance. The percent contribution of *C. socialis* varied from 19.7 to 31.5% with the average of 24.6%, and from 29.7 to 43.3% with the average of 35.8% to the total phytoplankton as a single species at the surface and bottom layer, respectively (Table 3). The percent abundance of *S. costatum* varied from 12.5 to 28.1% with the average of 18.5% and from 10.0 to 30.4% with the average of 19.0% to the total phytoplankton at the surface and bottom layer, respectively (Table 3).

During the autumn bloom the abundance ranges of diatoms, dinoflagellates and phytoflagellates were 4.04-8.02 $\times 10^4$ cells L^{-1}, 5.63-9.83 $\times 10^4$ cells L^{-1}, and 1.04-2.27 $\times 10^4$ cells L^{-1}, respectively (Fig. 8 B). At the surface 16 species of phytoplankton showed above 10% dominance to total phytoplankton abundance, of which 10 species belonged to Bacillariophyceae, 3 to Dinophyceae, 1 to Chlorophyceae, 1 to Cryptophyceae and 1 to Euglenophyceae. Whereas 14 species were dominant at the bottom layer, including 9 species of Bacillariophyceae, 3 of Dinophyceae, 1 of Cryptophyceae and 1 of Dictyophyceae (Table 4). The contribution of dinoflagellates varied from 46.9 to 58.4% with the average of 51.2% and the highest abundance was at the bottom layer of offshore (Fig. 8, B). The most dominant species were *Gymnodinium conicum*, *Prorocentrum micans* and *Prorocentrum*

triestinum at the surface and bottom layer. The average contribution of *G. conicum*, *P. micans* and *P. triestinum* was 10.4%, 11.3% and 10.5 % at the surface layer, and 11.1%, 12.2% and 11.8 % at the bottom layer, respectively (Table 4). Whereas the dominant species of phytoflagellates was *Hillea fusiformis* (13.1%), *Dunaliella martimum* (11.9%) and *Eutreptia viridis* (12.1%) at the surface layer, and *H. fusiformis* (11.6%) and *Pesudopedinella pyriforme* (10.7%) at the bottom layer (Table 4).

5. Discussion

5.1. *Dynamics of environmental factors*

The physical characteristics, especially the changing pattern of water temperature and salinity was same from the beginning of winter (January) to the end of spring (May) and there were no significant difference from the both parameters of one station to that of another station. The reason might be the well vertical mixing of surface and bottom water. The water temperature was high from the beginning of summer (June) to the end of autumn (November) at the surface layer and there was no significant difference among the all stations. The water temperature at the bottom layer of offshore and middle shore was much lower than that of inshore. This indicates that a distinct thermocline might occur in summer by very weak vertical mixing as well as the Yellow Sea Cold Water forms which flows southward under the thermocline (Mask and O'Brien 1998, Park 1986).

Like water temperature, the salinity from winter to spring was almost same in the surface and bottom layer due to well vertical mixing, whereas the salinity in summer was higher at the bottom layer than at the surface layer. The heavy freshwater flows from Changjiang River in summer decrease the surface water salinity through dilution. The low salinity in summer are frequently observed in the west coast of Jeju Island Island and sometimes caused a severe damage to fishery industry such as fish and gastropod

aquaculture (Suh et al. 1998). The inshore salinity was lower than middle shore and offshore during summer both at the surface and bottom. The huge surface runoff from the adjacent land due to heavy rainfall during the summer may the possible cause for that phenomenon.

5.2. *Dynamics of phytoplankton in relation to environmental factors*

The abundance and species composition of phytoplankton varied strongly with season. The spring and autumn phytoplankton bloom occurred in the study area and this was also confirmed from the Chl *a* values which were higher during the season. The species diversity index was also the lowest during the spring bloom. The spring bloom was higher than the autumn bloom in terms of phytoplankton abundance. The rising temperature, increasing irradiance, longer day length and availability of nutrients from the bottom layer might be favorable for growth of dominant species during the spring bloom. Additionally the NH_4-N concentration, the most preferable nutrient by phytoplankton, might increase in winter and be available in spring during the bloom.

The centric diatoms were most dominant during the spring bloom. The smaller chain forming diatoms such as *C. socialis*, *C. lorenzianus*, *C. pseudocrinitus* and *S. costatum* and *Hillea* sp. of phytoflagellates mainly represented in the spring bloom. The smaller size of phytoplankton may be more efficient for the utilization of nutrients for their growth. Chisholm (1992) found that the smaller phytoplankton could be able to minimize its sinking velocity and help them a higher surface area to volume ratio, thereby optimizing light and nutrients absorption efficiencies. Diatoms may also have another advantage because of their high fucoxanthin content. In coastal waters, where particulate and dissolved organic matter are in high concentrations, blue light is rapidly attenuated with preferential transmission of the green-to yellow wavelengths for photosynthesis (Gin et al. 2003). Fucoxanthin is the most efficient photosynthesis carotenoid absorbing light in the green waveband (Ondrusek et al. 1991).

Dinoflagellates were dominant in autumn bloom and abundant rather than diatom in the bottom layer of the all stations with the highest (51.2%) in offshore. The high NO_2-N concentration and moderate temperature might contribute for rapid growth of dominant dinoflagellates in autumn. Dinoflagellates were also higher in mid summer (July), and *P. triestinum* was the most dominant specie with the contribution of 30.1% and 15.3% at the surface and bottom layer. Low salinity, high water temperature and some nutrients were likely to increase the species. Such low salinity and inputs of various nutrients might be affected by Changjiang River overflows in summer. Hyun and Pang (1998) reported that massive water of the Changjiang River has frequently reached the west coast of Jeju Island in summer season.

In conclusion, the water temperature dynamics at the surface layer is quite similar with atmospheric temperature, but the low water temperature occurred at the bottom layer, especially in summer. Diatoms were dominant in spring, and dinoflagellates and phytoflagellates in autumn. The most important factors for the phytoplankton bloom dynamics mechanism are temperature and nutrients, especially nitrogen source.

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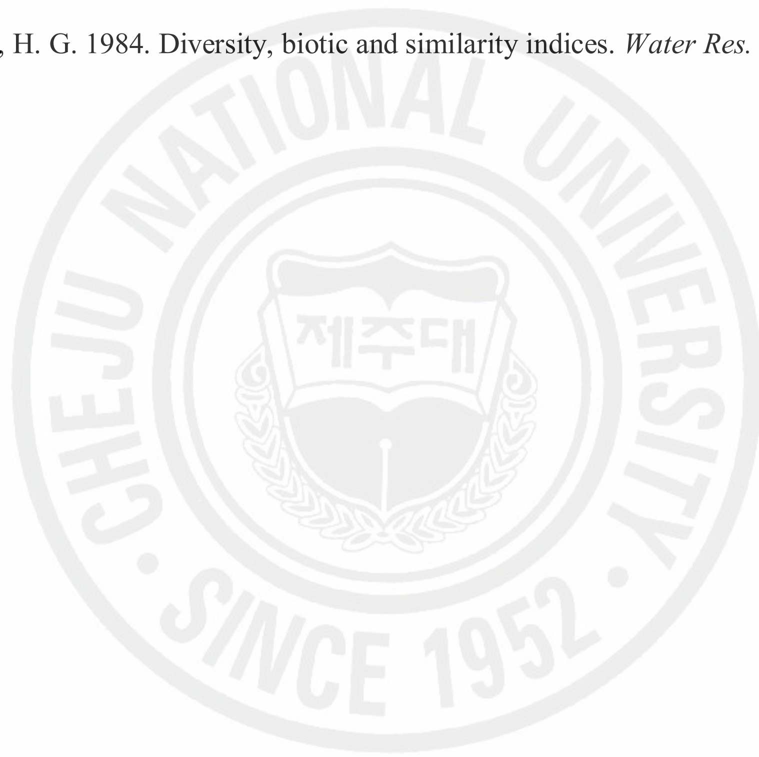
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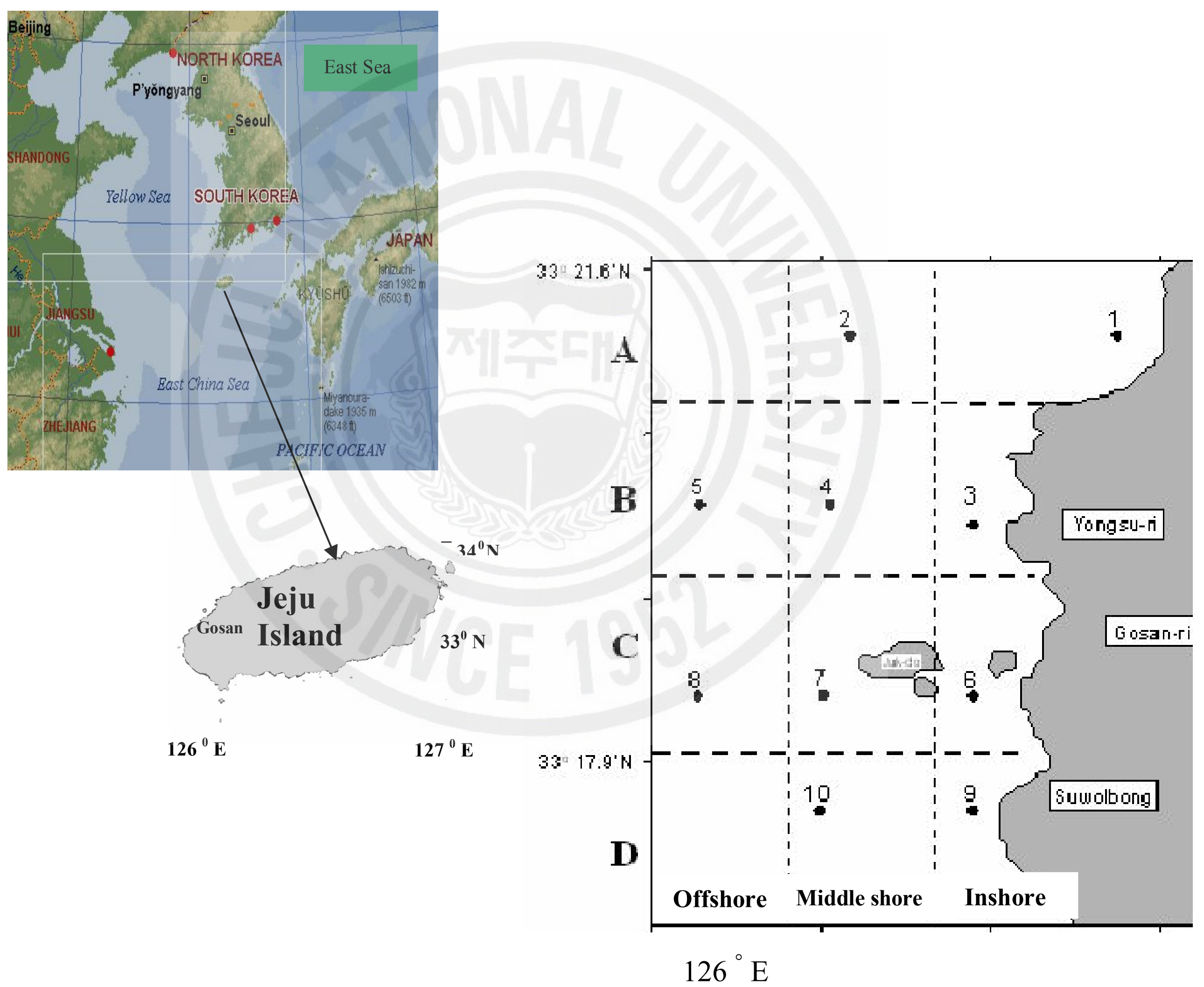

Fig. 1. The map of marine ranching area on the west coast of Jeju Island Island.

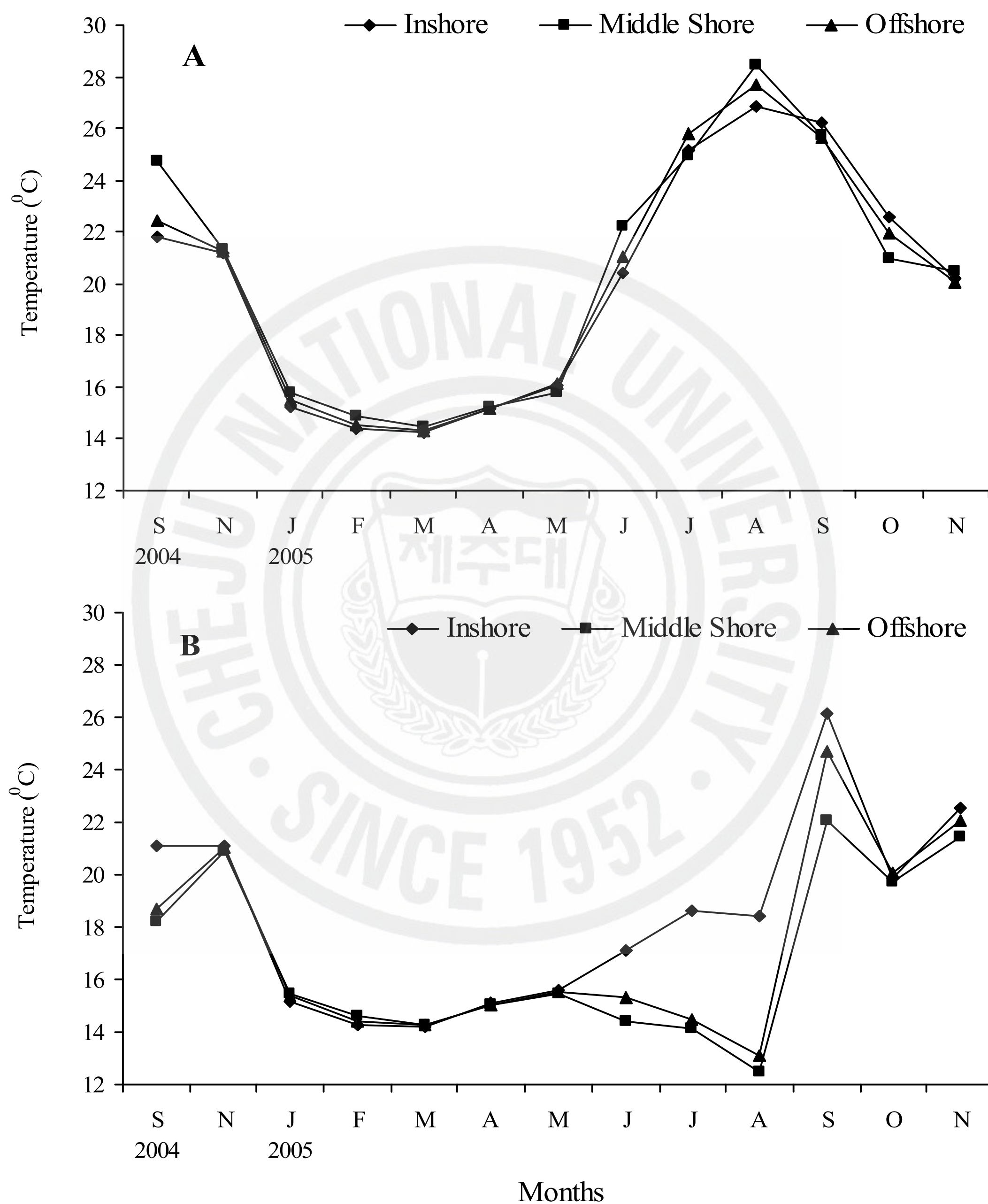

Fig. 2. Variation of water temperature in inshore, middle shore and offshore at the surface layer (A) and at the bottom layer (B) in the marine ranching area on the west coast of Jeju Island Island from September 2004 to November 2005.

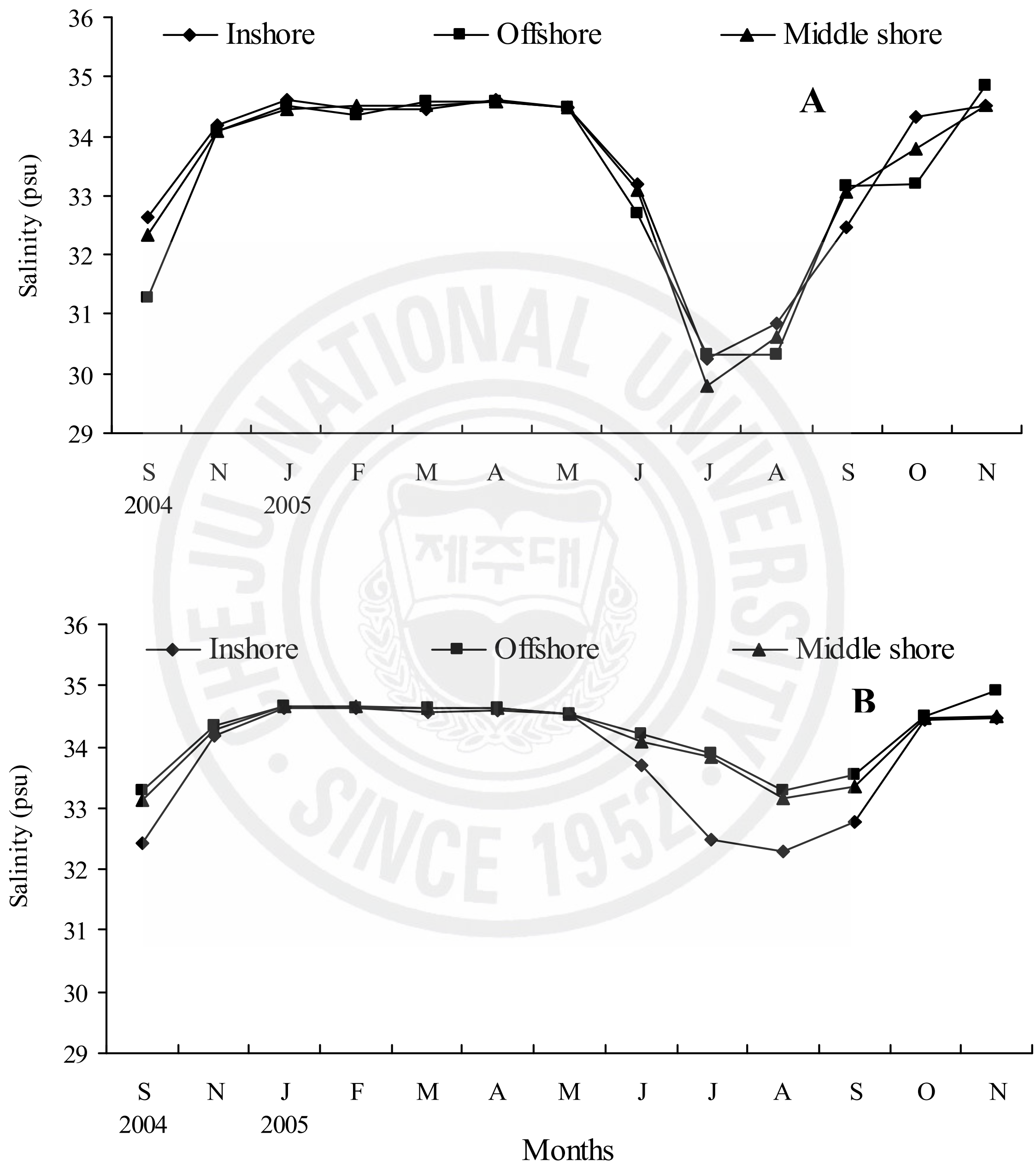

Fig. 3. Variation of salinity in inshore, middle shore and offshore at the surface layer (A) and at the bottom layer (B) in the marine ranching area on the west coast of Jeju Island Island from September 2004 to November 2005.

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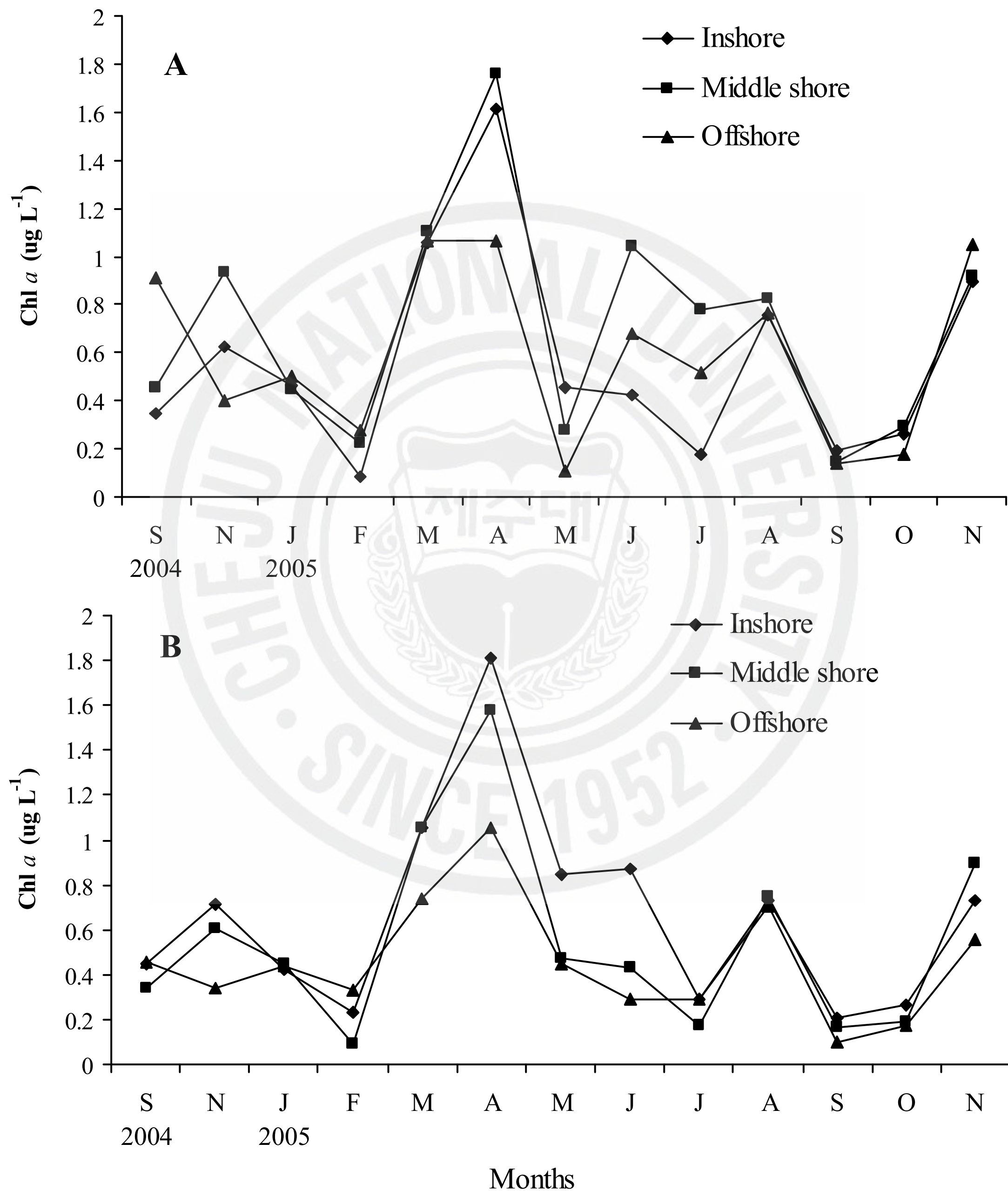

Fig. 4. The fluctuation of chlorophyll *a* concentration in inshore, middle shore and offshore at the surface layer (A) and at the bottom layer (B) in the marine ranching area on the west coast of Jeju Island Island from September 2004 to November 2005.

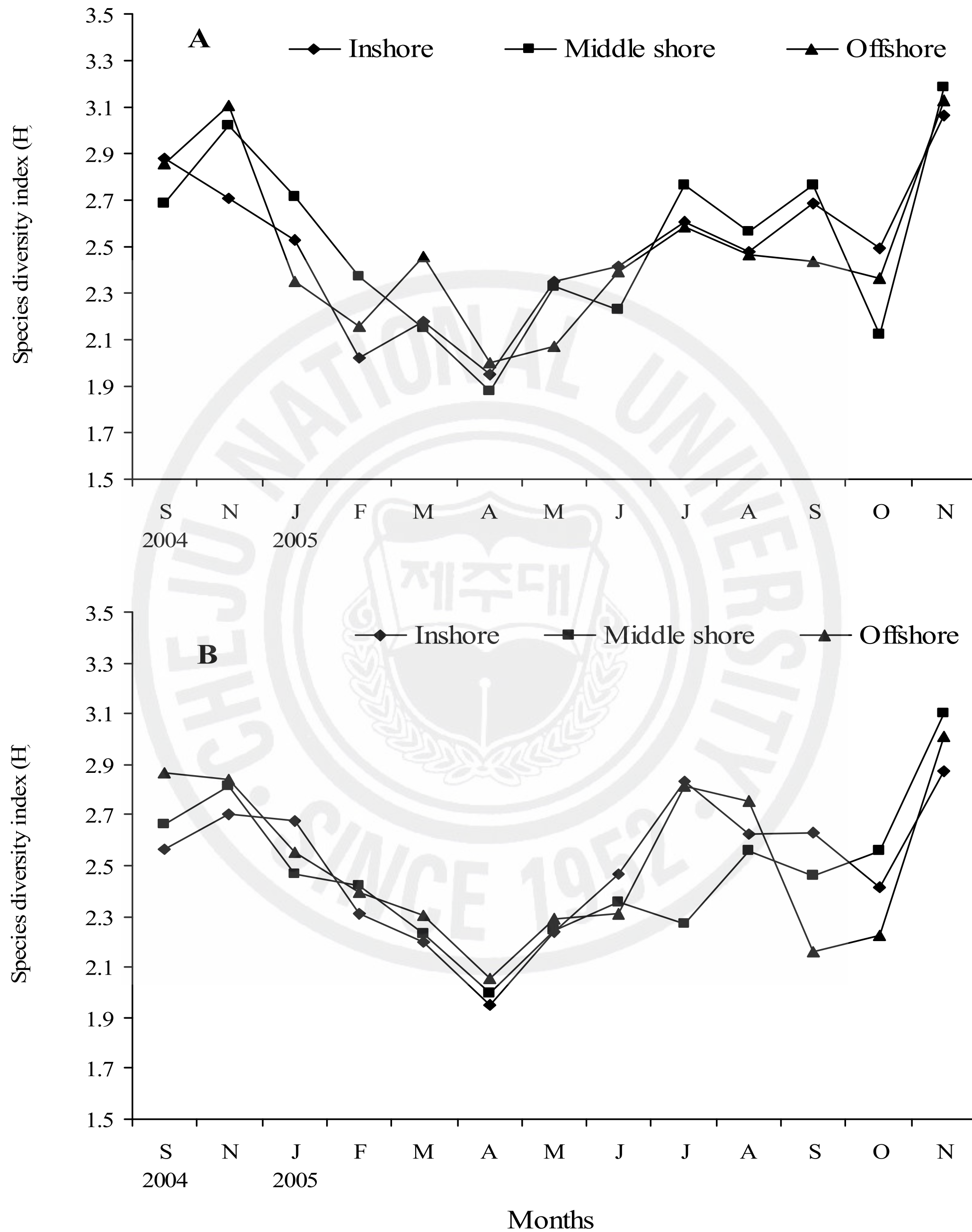

Fig. 5. Variation of phytoplankton species diversity index (H) in inshore, middle shore and offshore at the surface layer (A) and at the bottom layer (B) in the marine ranching area on the west coast of Jeju Island Island from September 2004 to November 2005.

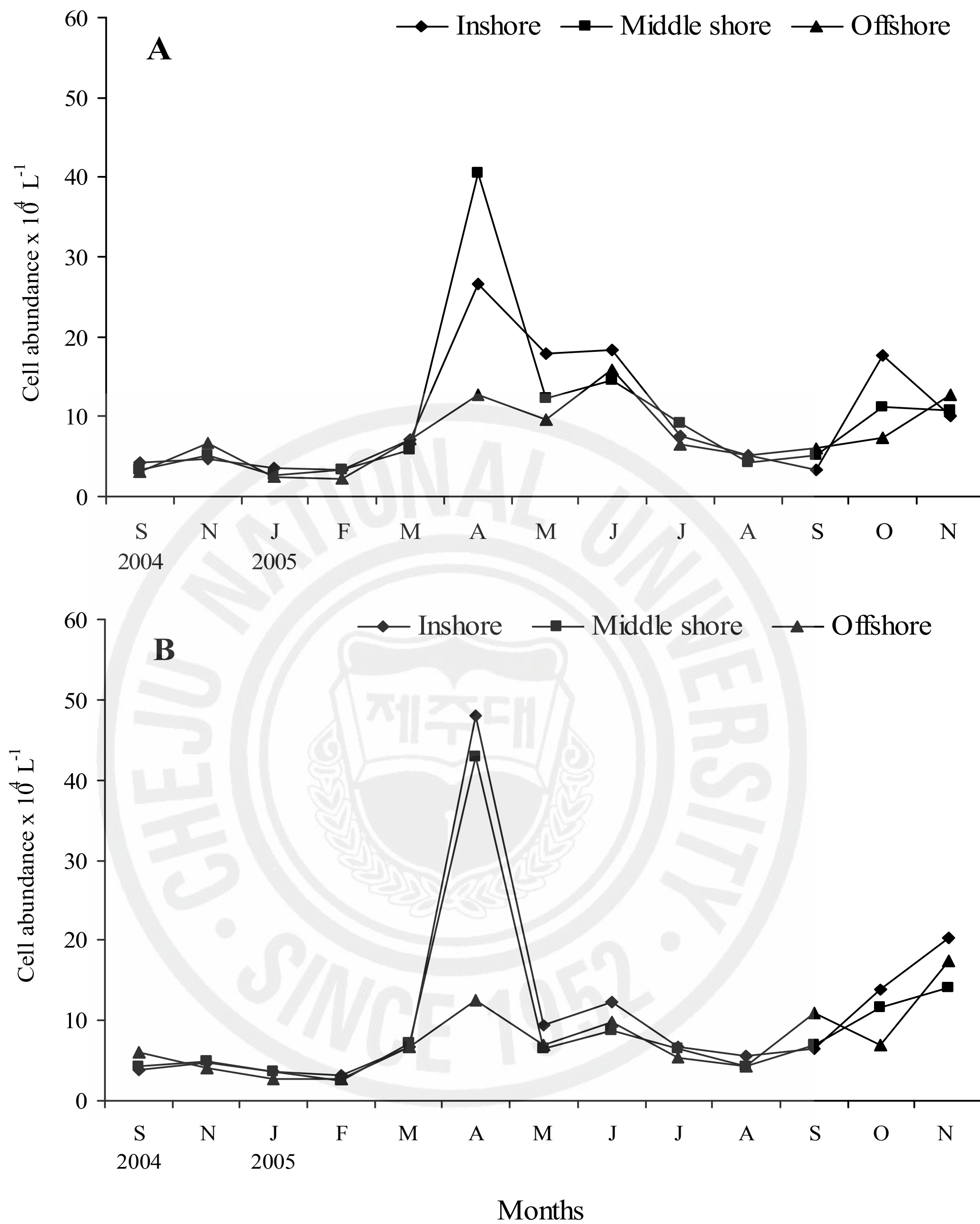

Fig. 6. The dynamics of phytoplankton cell abundance in inshore, middle shore and offshore at the surface layer (A), and at the bottom layer (B) in the marine ranching area on the west coast of Jeju Island Island from September 2004 to November 2005.

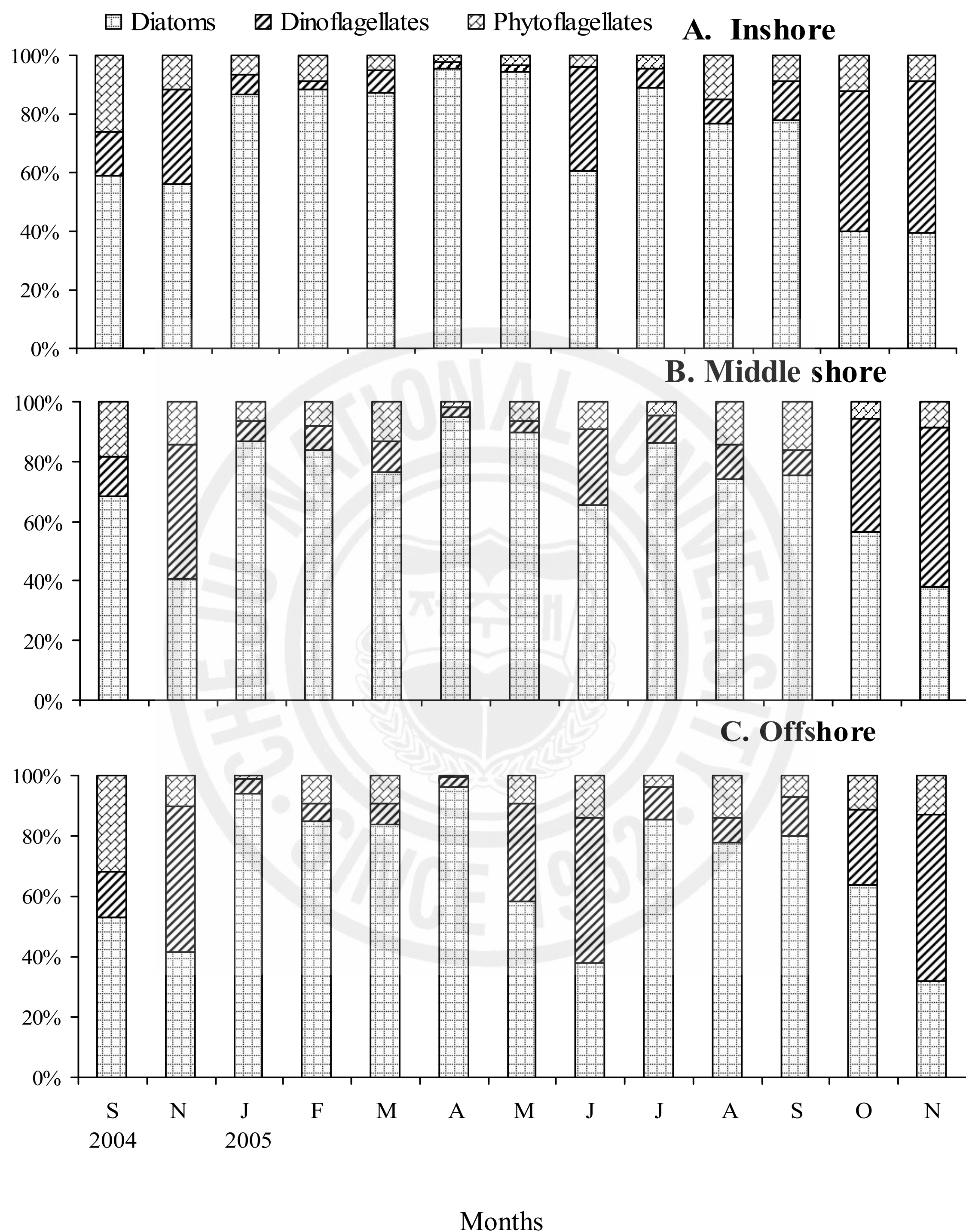

Fig. 7. The percent of abundance composition of diatoms, dinoflagellates and phytoflagellates in inshore (A), middle shore (B) and offshore (C) in the marine ranching are on the west coast of Jeju Island Island from September 2004 to November 2005.

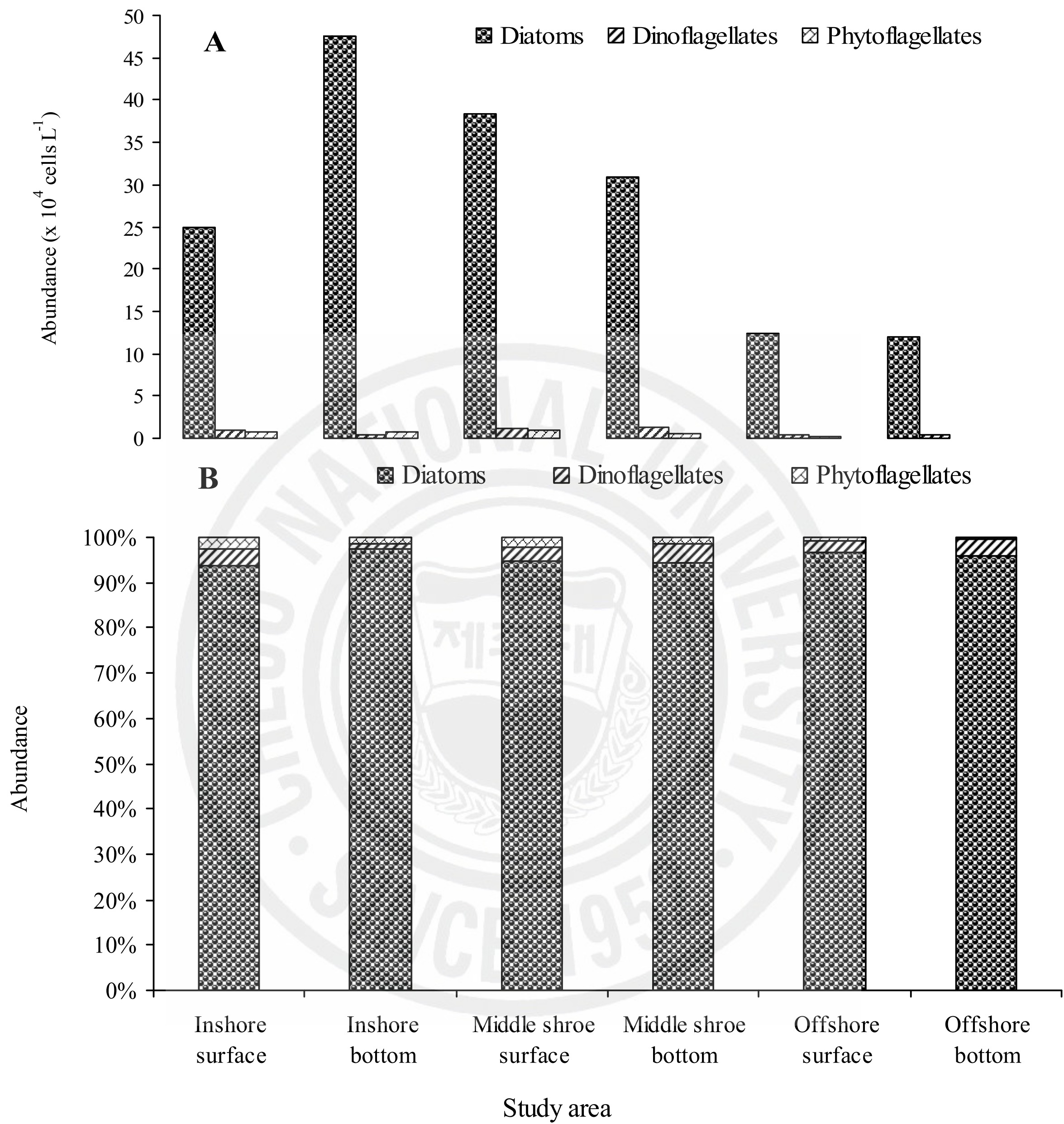

Fig. 8. The cell abundance of diatoms, dinoflagellates and phytoflagellates (A) and the percent of abundance composition (B) during the peak of the spring bloom in the marine ranching area on the west coast of Jeju Island Island.

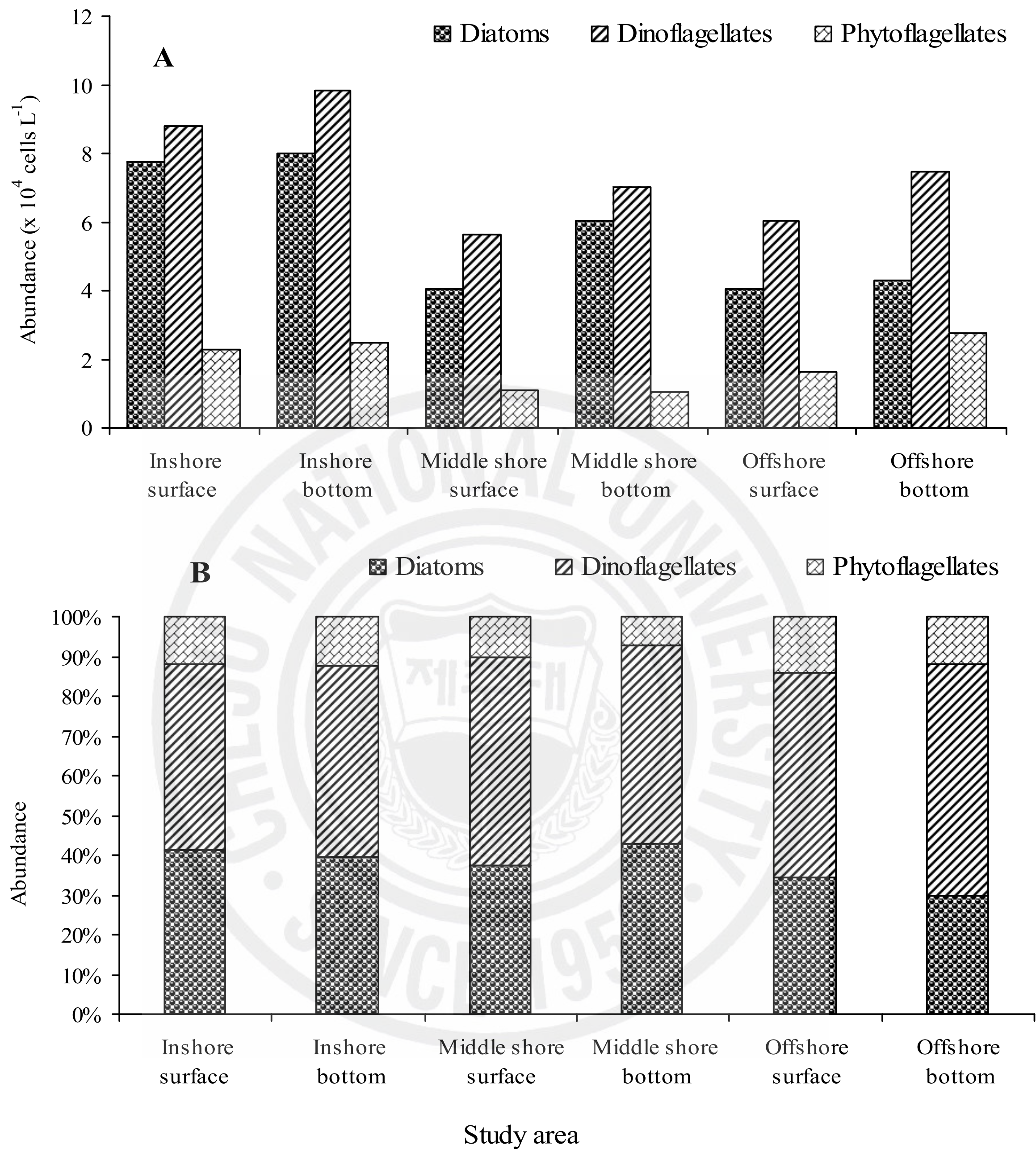

Fig. 9. The cell abundance of diatoms, dinoflagellates and phytoflagellates (A) and the percent of abundance composition (B) during the peak of the autumn bloom in the marine ranching area on the west coast of Jeju Island Island.

Table 1. Seasonal dynamics of NH_4-N, NO_3-N, NO_2-N and PO_4-P in inshore, middle shore and offshore at the surface and bottom layer of the marine ranching area on the west coast of Jeju Island Island

	Inshore Surface				Middle shore Surface				Offshore Surface			
	NH_4+N (µg-at/L)	NO_3-N (µg-at/L)	NO_2-N (µg-at/L)	PO_4-P (µg-at/L)	NH_4+N (µg-at/L)	NO_3-N (µg-at/L)	NO_2-N (µg-at/L)	PO_4-P (µg-at/L)	NH_4+N (µg-at/L)	NO_3-N (µg-at/L)	NO_2-N (µg-at/L)	PO_4-P (µg-at/L)
NOV 2004	0.63±0.27	4.53±0.63	0.32±1.81	0.45±0.09	0.13±0.06	3.58±0.07	0.26±0.61	0.47±0.04	0.08±0.12	4.07±0.02	0.26±1.15	0.25±0.03
FEB 2005	1.68±0.03	3.33±0.39	0.11±0.84	0.21±0.03	5.56±0.09	3.66±0.63	0.11±1.02	0.34±0.01	5.85±0.01	3.96±0.19	0.12±1.71	0.23±0.03
MAY 2005	0.67±0.05	1.94±0.36	0.17±1.22	0.07±0.17	1.00±0.03	1.53±0.26	0.20±1.25	0.06±0.14	2.65±0.02	0.51±0.05	0.02±0.43	0.08±0.01
AUG 2005	2.28±0.05	6.65±0.10	0.18±0.59	0.15±0.10	1.44±0.20	6.57±0.85	0.18±1.23	0.19±0.08	0.53±0.08	5.37±0.37	0.15±0.58	0.11±0.01
NOV 2005	1.89±0.18	5.32±1.12	0.09±1.58	0.24±0.03	1.52±0.05	3.99±0.99	0.16±1.18	0.13±0.01	2.10±0.04	4.51±1.92	0.10±0.49	0.13±0.01
Average	1.43±0.05	4.35±0.79	0.17±0.05	0.22±0.05	2.24±1.43	3.68±0.04	0.18±0.02	0.16±0.04	1.93±2.05	3.87±0.88	0.14±0.01	0.24±0.06
	Inshore Bottom				Middle shore Bottom				Offshore Bottom			
NOV 2004	0.45±0.71	4.29±1.27	0.29±0.09	0.47±0.25	0.11±0.11	4.29±0.68	0.26±0.03	0.60±0.13	0.39±0.41	5.05±1.00	0.31±0.05	0.66±0.01
FEB 2005	0.41±0.32	3.44±0.41	0.16±0.05	0.23±0.01	3.54±4.04	2.89±0.44	0.17±0.03	0.30±0.13	4.89±0.42	3.08±0.01	0.15±0.07	0.31±0.04
MAY 2005	0.67±0.69	2.50±0.10	0.18±0.04	0.06±0.09	1.34±0.25	2.65±2.24	0.25±0.06	0.13±0.05	1.86±0.34	2.86±0.18	0.23±0.01	0.18±0.02
AUG 2005	1.83±1.38	8.01±2.04	0.18±0.03	0.13±0.17	0.79±0.54	9.95±2.33	0.14±0.02	0.37±0.10	0.44±0.06	9.17±0.15	0.17±0.01	0.37±0.08
NOV 2005	0.97±0.90	0.12±0.04	6.75±0.95	0.23±0.01	2.11±1.22	0.09±0.03	8.27±2.13	0.29±0.05	1.03±0.30	0.13±0.04	8.68±2.17	0.30±0.01
Average	0.86±0.57	3.67±0.42	1.52±0.20	0.22±0.07	1.72±1.36	4.06±1.07	1.92±0.57	0.27±0.02	1.58±0.94	3.97±0.99	1.82±0.43	0.34±0.04

Table 2. List and occurrence frequency of phytoplankton taxa in marine ranching area on the west coast of Jeju Island Island from September 2004 to November 2005

Species	2004		2005									2005	
	S	N	J	F	M	A	M	J	J	A	S	O	N
Bacillariophyceae													
Centrales ditoms													
Actinocyclus octonarius Ehrenberg		X	X		X								
Actinoptychus senarius Ehrenberg			X	R	C	X	F		X	X	X	X	R
Actinoptychus splendens (Shadbolt) Ralfs			X						R	X			
Asteriomphalus heptactis (Brebisson) Ralfs									R	X			
Aulacodiscus kittoni Arnott ex Ralfs									R	X	X		
Bacteriastrum comosum Pavillard		X				X		X			X	X	R
Bacteriastrum hyalinum Lauder													
Bacteriastrum minus Karsten													X
Biddulphia granulata Roper										X			
Biddulphia longicruris Greville		X				X						X	
Biddulphia pulchella Gray	X								X	X			
Biddulphia reticulata Boyer					X	X			X	X			
Biddulphia tuomeyi Balley			X	X			X						
Campylodiscus undulatus Schmidt									R	X			
Chaetoceros affinis Lauder			X									X	
Chaetoceros brevis Schütt													X
Chaetoceros compressus Lauder												X	
Chaetoceros convolutus Castracane	R							X			X		R
Chaetoceros costatus Pavillard	R	X						C			R	X	C
Chaetoceros danicus Cleve	R	C	F	R	C	H	X	C			R	C	C
Chaetoceros decipiens Cleve		X						X					X
Chaetoceros denticulatus Ostenfeld	X							X	C		X		
Chaetoceros didymus Ehrenberg	X	X				F	X				X		R
Chaetoceros laciniosus Schütt		X			X				C				
Chaetoceros lorenzianus Grunow	X	R	X	X		H	F	C			C	C	C
Chaetoceros messanensis Castracane			X										
Chaetoceros pendulus Karsten	X	X	R	X	X	X	X				X	X	
Chaetoceros peruvianus Brightwell								X			X	C	R
Chaetoceros pseudocrinitus Ostenfeld	X	C	X	R	R	H	C	F	F		X	F	F
Chaetoceros socialis Lauder			X	C	H	H	H	H	H	X	C	F	C
Chaetoceros willei Gran		X											
Corethron criophilum Castracane		X	X									X	
Coscinodiscus centralis Ehrenberg	X	X			X								
Coscinodiscus concinnus Ehrenberg		X											
Coscinodiscus curvatulus Grunow	X												
Coscinodiscus granii Gough		X											X
Coscinodiscus marginatus Ehrenberg	X		X							X	X		
Coscinodiscus megalomma Schmidt			X										
Coscinodiscus nobilis Grunow	X	X	X	X						X	X		
Coscinodiscus nodulifer Schmidt	X	X	X	X				X		X	X	X	X
Coscinodiscus radiatus Ehrenberg			X	X	X	X	C			X			
Coscinodiscus rothii (Ehrenberg) Grunow	X		X					X		X			X
Coscinodiscus stellaris Roper	X		X	R	R	R	X	X	C	C	R	X	X
Cyclotella choctawhatcheeana Prasad	X												
Detonula pumila (Castracane) Schütt						C							
Guinardia flaccida (Castracane) Peragallo									F	R	X	X	
Hemiaulus hauckii Grunow ex Van Heurck		X					X					X	X
Hemiaulus membranaceus Cleve		X				X							
Hyalodiscus scoticus (Kützing) Grunow	X								X	X	X		X
Lauderia borealis Cleve									X	X			
Leptocylindrus danicus Cleve	C	C	F		R	X	C	C	C	X	C	C	C

Species	2004		2005									2005	
	S	N	J	F	M	A	M	J	J	A	S	O	N
Leptocylindrus mediterraneus Peragallo		X										X	X
Melosira juergensii Agardh											X		
Melosira moniliformis (Muller) Agardh	R	C	R	R	X	C	X	X	X	X	X		R
Melosira nummuloides Agardh													
Odontella obtusa (Kützing) Ralfs		X				X							
Odontella rhombus (Ehrenberg) Kützing													
Odontella sinensis (Greville) Grunow		X						X					R
Paralia sulcata (Ehrenberg) Cleve	X	X	X				X	X		X	X		R
Podosira stelliger (Bailey) Mann		X									X		
Rhizosolenia alata Gran		F	R	X				R	H	F	X	H	F
Rhizosolenia bergonii Peragallo						X							
Rhizosolenia delicatula Ostenfeld	X	X	R			X			C	R		X	
Rhizosolenia fragilissima Bergon			X		X	X				R		X	
Rhizosolenia hebetata (Bailey) Gran			X										
Rhizosolenia pungens Cleve								X			X	C	X
Rhizosolenia robusta Norman ex Pritchard			X										
Rhizosolenia setigera Brightwell	X	X										X	
Rhizosolenia stolterforthii Peragallo	X	X						C		X	R	R	C
Skeletonema costatum (Greville) Cleve	X	X	R	R	F	H	H	C	H	X	X	F	C
Stephanodiscus rotula (Kutzing) Hendey	X												
Stephanopyxis nipponica Gran &Yendo	X	X		X	X					X			X
Stephanopyxis palmeriana (Greville) Grunow		X				X							X
Stephanopyxis turris (Greville) Ralfs										X			
Surirella armoricana Peragallo								X					
Surirella fastuosa Ehrenberg										X			
Thalassiosira aestivalis Gran & Angst								X					
Thalassiosira baltica (Grunow) Ostenfeld						X						X	
Thalassiosira eccentrica (Ehrenberg) Cleve			X	X									
Thalassiosira hyalina (Grunow) Gran	X	X								X	X		X
Thalassiosira pacifica Gran & Agnst	X									X			
Thalassiosira punctigera (Gran) Hasle		X	X										
Thalassiosira rotula Meunier		R	X	X		H	R	X	C		X	X	F
Thalassiosira subtilis (Ostenfeld) Gran	X		X			X				R	X	C	
Triceratium alternans Balley	X												
Triceratium favus Ehrenberg	X												
Triceratium reticulum Ehrenberg													
Pennales diatoms													
Achnanthes brevipes Agardh	X	R	X			X		X	X	X			X
Achnanthes delicatula (Kutzing) Grunow											X		
Achnanthes longipes Agardh	X			X		X	X	R	R	R	R		X
Amphiprora alata (Ehrenberg) Kutzing												X	
Amphiprora angustata Hendey				X						X	X		
Amphiprora gigantea (O' Meara) Cleve	X	X											
Amphora coffeaeformis Kutzing								X	R	X	R		X
Amphora decussata Grunow		X	X	X					R	X	X	X	X
Amphora laevis Gregory			X			X			X	X			
Amphora lineata Gregory									X		X		
Amphora lineolata Ehrenberg				X	X	X				X			
Amphora ovalis Kutzing				X	X		R						
Asterionella glacialis Castracane								X	R	X			X
Asterionella japonica Cleve					X	X	X				X		
Asterionella kariana Grunow								X					
Bacillaria paxillifer (O. F. MUller) Hendey	C	C	C	F	C	R	R	C	F	C	F	F	F
Caloneis bacillum (Grunow) Mereschkowsky			X										
Caloneis crassa (Gregory) Ross	X	X	X		X	X	X		C	R	X	X	R
Caloneis linearis (Grunow) Boyer										X			
Caloneis schroederi Hustedt	X												
Cistula lorenziana (Grunow) Cleve			X										
Cocconeis pellucida Grunow	X			X				X		R	X		

Species	2004		2005										2005
	S	N	J	F	M	A	M	J	J	A	S	O	N
Cocconeis pseudomarginata Gregory	X					X							
Cocconeis quarnerensis Grunow				X									
Cocconeis scutellum Ehrenberg	X	X	X		X	X			R		X		
Cylindrotheca closterium (Ehrenberg) Reimann & Lewin	H	C	R	R	X		X	X			C	C	F
Diploneis crabro Ehrenberg ex Cleve	X		X		X	X				X			
Diploneis lineata (Donkin) Cleve						X					X		
Diploneis litoralis (Donkin) Cleve								X		X			
Diploneis splendida (Gregory) Cleve								X			X		
Ditylum brightwellii Grunow		C		X	R	R	R			R			C
Ditylum sol Grunow		X							C	X			X
Fragilaria capucina Desmazieres		X							R	X			X
Fragilaria ica Grunow ex Van Heurck			X							X			
Fragilaria pinnata Ehrenberg	X												
Fragilaria striatula Lyngbye	X	X						X				X	X
Grammatophora angulosa (Lyngbye) Kutzing	X	X				R		X	R	X	X		
Grammatophora marina Ehrenberg		F	C	C	C		R		C	R	X	X	C
Gyrosigma balticum (Ehrenberg) Cleve	X												
Gyrosigma fasciola (Ehrenberg) Cleve	X		X					X					
Gyrosigma strigile (W. Smith) Cleve													
Licmophora abbreviata Agardh		R	X	X			X				X		X
Licmophora dalmatica (Lyngbye) Agardh												X	
Licmophora flabellate (Greville) Agardh		R					X						
Licmophora gracilis (Ehrenberg) Grunow			X										
Licmophora paradoxa (Lyngbye) Agardh	X	R	X		X	X	X	X			X	X	R
Mastogolia minuta Greville			X										
Navicula annexa Hustedt	X	X											
Navicula arenaria Donkin			X							X			
Navicula cancellata Donkin	X	X											
Navicula distans (W. Smith) Schmidt			X										
Navicula elegans W. Smith	X										X		
Navicula flanatica Grunow	X												
Navicula fortis (Gregory) Donkin								X					
Navicula incerta Grunow	R	X											C
Navicula ica Østrup											X		
Navicula marina Ralfs								X			X		
Navicula mutica Kutzing	X		X							X	X		
Navicula oblonga Kutzing		R	F	X	X	R	X	X	R	X	R		R
Navicula ramosissima (Agardh) Cleve	X												
Navicula salinarum Grunow			X								X		
Navicula viridula (Kutzing) Ehrenberg	R	R	C	R	X	X	X	R	X	X	X	X	R
Navicula vula J. R. Carter	X	R	C	R	R	X	X	R	C	X	C	X	X
Nitzschia acicularis (Kutzing) W. Smith			X					X			X		X
Nitzschia angularis W. Smith	X												
Nitzschia delicatissima Cleve	X	X										X	X
Nitzschia longissima (BrEbisson ex Kutzing) Ralfs	H	H	H	H	H	F	C	C	R	X	R	C	F
Nitzschia pacifica Cupp								X			X		X
Nitzschia panduriformis Gregory	X	X						X			X		X
Nitzschia pseudonana Hasle	X	R											
Nitzschia seriata Cleve	R	R									X	X	
Nitzschia sigma Grunow	H	R	R	X	X		X	X			X	C	R
Nitzschia socialis Gregory	X		F	R	C	R	C	F	H	C	C	F	R
Pleurosigma angulatum (Quekett) W. Smith	X	R	X			X				X			X
Pleurosigma elongatum W. Smith	X		R	C	R		X					X	
Pleurosigma formosum W. Smith	X		X										
Pleurosigma longum Cleve	X											X	
Pleurosigma naviculaceum BrEbisson			X										
Pleurosigma normanii Ralfs	X		C									X	X
Pleurosigma strigosum W. Smith			X										
Pseudo-nitzschia pungens Grunow	F	H	F	H	F	H	F	H	H	F	H	H	H

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Species	2004		2005										2005
	S	N	J	F	M	A	M	J	J	A	S	O	N
Rhaphoneis amphiceros Ehrenberg									C	X			
Rhaphoneis surirella (Ehrenberg) Grunow			X					X				X	
Stauroneis amphioxys (Ehrenberg) Ralfs	X	X											
Stauroneis membranacea (Cleve) Hustedt	X		X			X				X			X
Streptotheca indica Karsten		X						C					X
Synedra affinis Kutzing		X								X			
Synedra gaillonii (Bory) Ehrenberg				X									
Synedra pulchella Kutzing			X										
Synedra tabulata (Agardh) Kutzing			R	R	R	X	R	X		X	R	X	X
Thalassionema nitzschiodes Hustedt	R	F	C	R	R	X	X	X	C	C	R	X	C
Thalassiothrix frauenfeldii (Grunow) Castracane		X		X					X	X		X	X
Thalassiothrix longissima Cleve & Grunow	R	X	R	X	X		X	R	R	R	X	R	R
Torpidoneis antarctica Cleve								X					
Torpidoneis lepidoptera (Gregory) Cleve							X	X					
Dinophyceae													
Alexandrium catenella (Whedon et Kofoid) Balech	X										X		
Alexandrium tamarensis (Lebour) Balech						X							
Amphidinium crassum Lohmann		X	X										X
Amphidinium lacustre Stein		X											
Amphidinium longum Lohmann	X	X											X
Ceratium extensum (Gourret) Cleve									R	X		X	
Ceratium furca (Ehrenberg) Claparede et Lachmann	R		X		X		X			X			
Ceratium fusus (Ehrenberg) Dujardin										X			
Ceratium tripos (O. F. Muller) Nitzsch			X							X			
Cochlodinium catenatum Okamura													
Dinophysis rotundata Calparede & Lachmann	X												
Dissodinium pseudolunula Swift					X								
Gonyaulax digitale (Pouchet) Kofoid		X				X			R	X			X
Gonyaulax grindleyi Reinecke		X	X										X
Gonyaulax sp.		X											
Gonyaulax spinifera (Claparede & Lachmann) Dissing	X							X			X		F
Gonyaulax verior Sourina			X										
Gymnodinium conicum Kofoid et Swezy	R	R			X	C	F	H	X			F	H
Gymnodinium lohmanii Paulsen		R											X
Gymnodinium sanguineum Hirasaka											X		
Gymnodium galaucum (lebour) Kofoid & Swezy								X	X	C			X
Gymnodium sp.								F	X	R	F	X	C
Gyrodinium sp.			X	X	X	X	R					X	
Gyrodinium spirale (Bergh) Kofoid & Swezy		X	R	X	X	X	X		R	X	R		X
Heteraulacus sphaericus (Murry et Whitting) Loeblich	X		X										
Heterocapsa triquetera (Ehrenberg) Stein		X											
Noctiluca scintillans (Macartney) Kofoid & Swezy	X						X						
Oxyphysis oxytoxoides Kofoid													X
Podolampas spinefera Okamura	X	X						X					
Polykrikos kofoidii Chatton		X						X			R		X
Polykrikos schwarzii Butschi			X								X		X
Prorocentrum balticum (Lohmann) Loeblich	C	X	X			X		X		R	X	X	X
Prorocentrum compressum (Bailey) Abe ex dodge	X	C		C	R	X	R	X		X	X	X	R
Prorocentrum lima (Ehrenberg) Dodge		C	X		X					X			R
Prorocentrum micans Ehrenberg	R	X	X	X	X		X	H			X	F	H
Prorocentrum minimum Dodge & Bibby								H	X			X	F
Prorocentrum triestinum Schiller	C	X	X					H	F	X		F	H
Protoceratium reticulatum Butschli			X										
Protoperidinium breve Schiller	X	X						X		X			X
Protoperidinium brochii (Kofoid et Swezy) Balech	X	R	X		X	X	X	R	R	C	C	X	C
Protoperidinium claudicans (Paulsen) Balech											X		
Protoperidinium conicoides (Paulsen) Balech	X												
Protoperidinium depressum (Bailey) Balech	X	X									X	X	
Protoperidinium leonis (Paulsen) Balech	X												

Species	2004		2005										2005
	S	N	J	F	M	A	M	J	J	A	S	O	N
Protoperidinium minutum (Kofoid) Leoblich		X											X
Protoperidinium nipponicum Abe	X	X		X	X		X			X			X
Protoperidinium ovatum Pouchet		X	X			X							
Protoperidinium pentagonum (Gran) Balech				X	X	H	X					X	
Protoperidinium pyriforme (Paulsen) Balech				X									
Protoperidinium soma Matzenhauer	X				X					C			
Pyrophacus steinii (Schiller) Wall & Dale											X		
Zygabikodinium lenticulatum (Paulsen) Loeblich		C											X
Chlorophyceae													
Chlamydomonas coccoides Butcher	X				X			R					
Chlamydomonas pulsatilla Wollenweber			X							X			
Chlamydomonas quardrilobata N. Carter	X				X				X				
Dunaliella martima Massjuk	X	X								X		X	
Dunaliella primolecta Butcher	X												
Dunaliella salina Teodoresco									X	X			
Dunaliella tertiolecta Butcher			X										
Phyllomitus yorkeensis Ruinen	X												
Pleurostomum gracile Namyslovski		X											
Cryptophyceae													
Chroomonas marina Butcher	X								R	X			X
Hemiselmis rufescens Parke	X												X
Hemiselmis simplex Butcher										X	X		
Hillea fusifomis (Schiller) Schiller	H	F	R	R	R	R	C	R	C	C	R	X	C
Hillea marina Butcher	R			X		X			X	X	X		
Hillea sp. Schiller	R			X	C		R	F	R	R	C	R	C
Rohdomonas balticum Karsten									X				C
Rohdomonas salina (Wislouch) Hill & Wetherbee									X				X
Rohdomonas abbreviate Hill & Wetherbee			X		X						X		
Rohdomonas abbreviate Hill & Wetherbee			X				X						X
Rohdomonas maculate Hill & Wetherbee			X					X					
Teleaulax acuta (Butcher) Hill	X	X								X			
Chrysophyceae													
Ochromonas bourrellyi Magne			X										
Ochromonas oblonga Carter										X			X
Pseudobodo minimus Ruinen	X												
Pseudobodo tremulans Griessmann	X												
Sarcinochrysis marina Geitler		X											
Sphaleromantis marina Piennar		X											
Dictyphyceae													
Dictyocha fibula Ehrenberg		X										X	X
Dictyocha speculum Ehrenberg								X					
Pseudopedinella pyriforme Carter	X												
Pseudopedinella tricostata Thmsen		X											
Euglenophyceae													
Euglena acusformis Schiller	X	R	X	R	X	X		X					C
Euglena sp. Ehrenberg	X	X						X					
Euglena viridis Ehrenberg			C	R		X			X	X		X	
Eutreptia lanowii Steur										X			
Eutreptia pertyi Pringsheim			X			X	X	X			X		X
Eutreptia sp. Perty	R	X									X		
Eutreptia viridis Perty	F	F			X		R	R	X	X	X	X	C
Eutreptiella braarudii Throndsen	X												
Eutreptiella cornubiense Butcher	X												
Eutreptiella eupharyngea Moestrup & Norris			X										
Eutreptiella gymnastica Throndsen													X
Eutreptiella hirudoidea Butcher		X											
Eutreptiella marina Cunha	X	R	X	X	X	X	R	X		X	X		X
Prymnesiophyceae													
Acanthoica aculeate Kamptner											X		

Species	2004		2005									2005	
	S	N	J	F	M	A	M	J	J	A	S	O	N
Acanthoica quattrospina Lohmann											X		
Chrysochromulina alifera Parke & Manton			X										
Diacronema vlkianum Prauser			X										
Imantonia rotunda Reynolds										X			
Isochrysis galbana Parke								X		X		X	X
Prasinophyceae													
Mamiella gilva Park & Rayns	X							X					X
Mantoniella squamata Manton & Parke										X			
Metromonas simples (Griessmann) Larsen & Patterson		X									X		
Mircromonas pusilla (Butcher) Manton & Parke	X	X	X					X	R				R
Pseudoscourfieldia marina (Thronndsen) Manton						X				X			
Raphidophyceae													
Fibriocapsa japonica Toriumi & Takno											X		
Heterosigma akashiwo Hada			X									X	X
Heterosigma inlandica Hada									X	X			X
Olisthodiscus luteus Carter	X	X											X
Oltmannsia viridis Schiller		X	X										X

Legend of occurrence frequency : Sporadically (X=1- 20%), Rarely (R= 21- 40%),

Commonly (C = 41-60%), frequently (F = 61-80%) and High frequently (H = 81-100%)

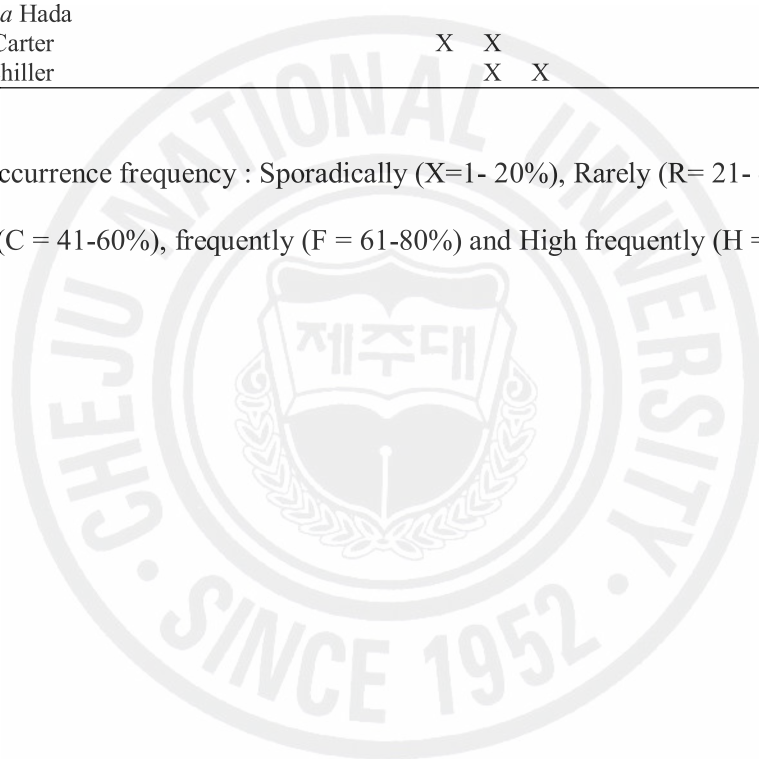

Table 3. List of dominant phytoplankton species (above 10% abundance) during the spring bloom in the marine ranching area on the west coast of Jeju Island Island

Species name	Inshore surface			Middle shore surface			Offshore surface			Average
	March	April	May	March	April	May	March	April	May	
Diatoms										
Chaetoceros lorenzianus		11.33			10.11			10.04	10.85	10.58
Chaetoceros pseudocrinitus		16.66			15.01			13.35		15.01
Chaetoceros socialis	19.95	22.59		26.16	19.70	15.95	16.58	31.45	12.42	20.60
Skeletonema costatum	11.03	10.85	22.91	14.61	15.34	18.81	16.78	12.46	17.85	15.63
Phytoflagellates										
Hillea sp.							10.33			10.33

Species name	Inshore bottom			Middle shore bottom			Offshore bottom			Average
	March	April	May	March	April	May	March	April	May	
Diatoms										
Chaetoceros lorenzianus		11.05			13.34			10.38		11.59
Chaetoceros pseudocrinitus		14.08	12.52		11.50	11.54		12.90	17.65	13.37
Chaetoceros socialis	15.40	29.69	25.66	22.64	43.26	17.51	16.26	34.53	19.85	24.98
Skeletonema costatum	30.39	12.98	22.60	10.12	15.23	16.87	18.67	16.57	14.71	17.57

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Table 4. List of dominant phytoplankton species (above 10% abundance) during the autumn bloom in the marine ranching area on the west coast of Jeju Island Island

Species name	Inshore surface		Middle shore surface		Offshore surface		Average
	October	November	October	November	October	November	
Diatoms							
Achnanthes longipes			10.29				10.29
Chaetoceros convolutus		11.89		10.02			10.96
Chaetoceros costatus		12.03				10.16	10.11
Chaetoceros lorenzianus				11.21		18.28	14.15
Chaetoceros pseudocrinitus		14.74				11.00	12.87
Leptocylindrus danicus			11.86				11.86
Licmophora paradoxa	10.00						10.00
Nitzschia sigma	12.00						12.00
Paralia sulcata			21.50				21.50
Skeletonema costatum			13.24		11.94		12.59
Dinoflagellates							
Gymnodinium conicum	11.03	10.21	10.05	10.16	10.01	12.02	10.41
Prorocentrum micans	11.67	10.00	11.29	10.45	11.04	11.60	11.34
Prorocentrum triestinum		10.42		10.14		11.01	10.52
Phytoflagellates							
Hillea fursiformis	10.34				11.84		11.09
Dunaliella martimum					10.08		10.08
Eutreptia viridis	12.07						12.07
Species name	Inshore bottom		Middle shore bottom		Offshore bottom		Average
	October	November	October	November	October	November	
Diatoms							
Chaetoceros convolutes		17.85	10.36	11.00		10.20	12.35
Chaetoceros costatus		16.74		12.01		11.03	13.26
Chaetoceros pseudocrinitus				10.39			10.39
Leptocylindurs danicus	10.10		18.16		11.58		13.28
Nitzschia longissima		10.75					10.75
Skeletonema costatum			11.11		11.43		11.27
Nitzschia sigma	11.65		12.12		10.98		11.58
Paralia sulcata	14.38						14.38
Stauroneis membranacea	10.10						10.10
Dinoflagellates							
Gymnodinium conicum	10.01	10.25	12.13	10.02	13.00	10.26	11.12
Prorocentrum micans	13.65	13.00	15.79	12.50	10.02	10.00	12.16
Prorocentrum triestinum		11.52		13.67		10.11	11.77
Phytoflagellates							
Hillea fusiformis	11.67				11.43		11.55
Pseudopedinella pyriforme			10.71				10.71

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Part II

Ecophysiological characteristics and biochemical compositions of several benthic diatoms from Jeju Island coast

1. Abstract

Axenic strain is very important for the advanced study of benthic diatoms as bacteria can play vital role by inserting their cellular materials to the chemical compositions of each benthic diatom. The antibiotic cocktail 200 units penicillin mL^{-1}, 200 μg streptomycin mL^{-1}, and 400 μg neomycin mL^{-1} made the axenic diatoms strain. Determination of the best growth condition would be the significant information for the mass culture of benthic diatom. The highest cell density of *Navicula incerta* was in 20˚ C water on day 12 after being inoculated in the media of 30 psu salinity, and F/2 (100%) nutrient concentration. *Cylindrotheca closterium* showed the highest cell density (7.20×10^4 cells mL^{-1}) in a culture condition of 20˚ C water with salinity of 30 psu and nutrients concentration of 'F' (200%) the biomass production was 1.1 mg L^{-1} of *Achnanthes longipes* and *Nitzschia longissima.* The protein, lipid and carbohydrate content were higher from *Navicula* sp. (cf. *viridula*) than from other diatoms examined here which were followed by *Amphora coffeaeformis* and *Nitzschia longissima*, respectively. The ash content was over than 50% total dry biomass for the all benthic diatoms.

2. Introduction

The marine algae perform a major part of primary production, being responsible for 46 % of global productivity (Field et al. 1998) and supporting food webs in waters from pond to oceans. Diatoms are the major component of phytoplankton, one of the major groups f primary producers in aquatic ecosystems and an essential food source for aquaculture (St. John et al. 2001) which have seldom been found a source of ecological problems in nature

(Officer and Ryther 1980). Affan and Lee (2004) monitored the dynamics of abundance of phytoplankton around the coastal water of Jeju Island, Korea. And they also reported the presence of 101 species of phytoplankton and among them benthic diatoms were the most dominant group throughout the year.

Apart from the monitoring of phytoplankton dynamics in natural environments, *Isochrysis*, *Pavlova*, *Chaetoceros*, *Thalassiosira*, *Nannochloris* and *Tetraselmis* are used as a live feed for the shellfish seedling production in Korea. Due to technical problem in benthic diatom mass culture system, the abalone seedling production from the aquaculture farms is still very low in relation to its increasing demand in Korea (Hur 1997). Traditionally cultured wild mixed benthic diatoms on wavy plates are used as live feed in commercial shellfish hatcheries in Jeju Island. At present, benthic diatoms are cultured based on the natural conditions with the addition of fertilizer for aquaculture purposes. But this traditional production of benthic diatoms can not fulfill the demands year-round for rapidly expanding aquaculture sector due to phytoplanmkton population fluctuations resulted from natural environmental dynamics. The development of sustainable and suitable technique for the mass culture of benthic diatoms is necessary to meet up aquaculture demand. For mass culture, the most important task is the selection of species which depends on i) the availability of the species year-round, ii) their optimal environmental conditions, and iii) their suitability as food for the targeted shellfish. Therefore, isolation of single species, finding out their best growth condition with different environmental factors and finally the development of low-cost mass culture technique are utmost important to fulfill demand for live feed in the shellfish aquaculture.

On the other hand, microalgae are also increasingly being promoted in the human diet as nutraceutical and health food products. Much effort has been expended to search new compounds of therapeutic potential, and it was demonstrated that all taxa of micoralgae possess antibacterial, antifungal and anticancer ability (Tredici 2004). Extract of *Chlorella* sp. and *Spirulina* sp. are being proposed to use with noodles, bread, green tea, beer and candy

(Liang et al. 2004). Among the microalgae, *Spirulina platensis*, *Botryococcus braunii* and *Dunaliella salina* are important sources of antioxidants for human consumption (Herrero et al. 2006, Herrero et al. 2005, Rao et al. 2006) and there are no published reports about the benthic diatoms.

In this study we isolated eight benthic diatoms such as *Navicula incerta* Grunow, *Grammatophora marina* (Lyngbye) Kutzing and *Cylindrotheca closterium* (Ehrenberg) Reimann & Lewin, *Achnanthes longipe*s Agardh, *Amphora coffeaeformis* Kutzing, *Navicula* sp. (cf. *viridula*), *Nitzschia* sp. (cf. *socialis*), and *Nitzschia longissima* Ralfs. The objectives of this study were to isolate the benthic diatoms as a axenic strain, determine the best growth condition, develop mass culture system and determine the biochemical compositions of several species.

3. Materials and methods

3.1. *Sampling and isolation of benthic diatoms*

Natural sample of benthic diatoms was collected from aquaculture tanks at National Fisheries Research and Development Institute (NFRDI) of Jeju Island, where the benthic diatoms on wavy plastic plate are being used for rearing of larval abalone. During the sampling, the environmental conditions of the sampling spot, especially temperature, pH and salinity were 25 to 30°C, 7.61 to 8.52, and 25 to 33 psu, respectively. The attached benthic diatoms were removed with brush and diluted with seawater, and a 1-mL diluted sample was transferred to the S-R chamber. Selected single cell of benthic diatoms was picked up from the S-R chamber by using micropipette under an inverted microscope (Olympus IX71, Japan). Selected single cell was transferred into the well of multi-well plate cultue for subculture. Subculture of the isolated species was done with autoclaved seawater which was filtered through 0.4-μm membrane filter (Millipore Co., Bedford, MA, USA) and enriched

with F/2 nutrients media, trace metal solutions (Aquacenter Inc., Leland, MS, USA) and metasilicate anhydrous crystals (Na_2SiO_3). The isolation process was carried out until to get the mono-strain of each species of the benthic diatoms.

3.2. ***Axenic strain of benthic diatoms***

The isolation of benthic monostrain diatoms was done by following the protocol which was described by Affan et al. (2006). Benthic diatoms on a wavy plastic plate were collected from the abalone culture hatchery of the National Fisheries Research and Development Institute (NFRDI) of Jeju Island. The diatoms were removed from the wavy plastic plate with a brush, and diluted with seawater. One mL of diluted sample was transferred to a Sedgewick Rafter (S–R) counting chamber, and single diatom cells were micropipetted from the counting chamber under an inverted microscope (Olympus IX71). Thereafter, each cell was transferred to a multi-well plate for subculture with autoclaved seawater, which was filtered through 0.45-μm nitrocellulose membrane filters (Millipore MF, Bellerica, MA, USA) and enriched with F/2 medium nutrients (Aquacenter Inc., Leland, MS, USA), trace metals (Aquacenter Inc., Leland, MS, USA), and metasilicate anhydrous crystals (Na_2SiO_3).

The isolation process was done until obtaining a monostrain. The identification of the subcultured benthic diatom sample was done under a phase-contrast microscope (Carl Zeiss, Oberkochen, Germany) at 400x magnification based on the description by Shim (1994). The identified monostrain sample was again streaked onto an agar plate with 2% agar (w/v), 0.04% F/2 (v/v) nutrient medium and autoclaved seawater. The monostrain colony was then transferred to a steriliazed 250-mL flask containing 100 mL F/2 enriched culture medium and antibiotics. Seven different dosages of antibiotic cocktail were used (penicillin 100–250 unit/mL, streptomycin 100–250 μg/mL, and neomycin 200–500 μg/mL). Doses were increased by 25 units penicillin/mL, 25 μg streptomycin/mL, and 50 μg

neomycin/mL (P 4083; Sigma Aldrich Corp, St. Louis, MO, USA). About 10 mL each monostrain sample was transferred from the antibiotic medium to a 250-mL flask with 100 mL of culture medium. The cultured sample was again streaked on the bacto-agar media to observe for bacterial presence.

In this way, we obtained eight axenic strain of *Navicula incerta* Grunow, *Grammatophora marina* (Lyngbye) Kutzing, *Cylindrotheca closterium* (Ehrenberg) Reimann & Lewin, *Achnanthes longipe*s Agardh, *Amphora coffeaeformis* Kutzing, *Navicula* sp. (cf. *viridula*), *Nitzschia* sp. (cf. *socialis*), and *Nitzschia longissima* Ralfs for further study. Bacterial colonies count results from agar plate were kept for *N. incerta*, *C. closterium* and *G. marina*, and for other benthic diatoms such as *A. longipe*s, *A. coffeaeformis*, *Navicula* sp. (cf. *viridula*), *Nitzschia* sp. (cf. *socialis*) and *N. longissima* the same experiment was done to get the axenic species, but data about bacterial colonies was ignored.

3.3. *Growth characteristics study*

The best growth condition was determined for *N. incerta*, *G. marina* and *C. closterium* among 27 different culture conditions. For growth characteristics study, artificial seawater was made by adding synthetic sea salt (Energy Savers Unlimited, Inc. Carson, CA USA) with distilled water to make and three different salinities; 25, 30 and 35 psu. To determine the optimal growth conditions of *N. incerta*, *C. closterium* and *G. marnia* we investigated three culture parameters: water temperature (15˚ C, 20˚ C, 25˚ C), salinity (25, 30, 35 psu), and nutrient concentrations (F/4 = 50%, F/2 = 100% and F = 200%), with each condition categorized as low, medium or high. For nutrient enrichment culture media, each of the A and B solution of F/2 media (Aquacenter Inc., Leland, MS, USA) added at 1 mL per 7.75 L artificial seawater, plus 13 μL L^{-1} trace metal solution (Aquacenter Inc., Leland, MS, USA) and 13 μg L^{-1} metasilicate anhydrous crystal.

The artificial seawater was enriched with F/2 nutrients and used for the growth characteristics experiments and mass culture. Each of 1-L flasks with 250 mL culture media was inoculated with approximately 20 cells mL^{-1} of *N. incerta* and of *C. closterium*. The cultures were grown under fluorescent lights (180 μ mol photons m^{-2} s^{-1}) on a 12:12 light: dark cycle for two weeks. Each treatment was replicated twice. A 1-mL sample was collected from each culture flask every 2 days and fixed with Lugol's iodine solution. The fixed sample was diluted, and the cells were counted using a S-R counting chamber under an inverted microscope at 400x magnification.

The specific growth rate μ_{max} is defined as the increase in cell density per unit time (Pirt 1975), and formulated as follows:

$$\mu\ (day^{-1}) = \frac{\ln (X_1/X_0)}{t_1 - t_0}$$

, where X_0 and X_1 are cell density at the beginning (t_0) and end (t_1) of a selected time interval between inoculation and maximum cell density, respectively. For the growth curve of each sample, I counted the replicates and used the mean value. In this way, we determined the best growth conditions for *G. marina*, *N. incerta* and *C. closterium*. The result about *G. marina* has already been published (Affan et al. 2006).

3.4. ***Mass culture and biomass collection***

N. incerta and *C. closterium* were grown in a 20-L polycarbonate transparent bottles (Nalgene bottle, Rochester, NY, USA). The mass culture of *N. incerta* was done under the best maximum specific growth conditions i.e. B5 culture condition (Fig. 3B) and *C. closterium* was also grown at B6 culture conditions (Table 5) with artificial seawater enriched with F/2 nutrients, as above. Distilled water was added in the mass culture bottles

regularly to maintain the salinity and continuous aeration was provided from the beginning to harvesting day.

The mass culture of another five species namely, *A. longipes*, *A. coffeaeformis, Navicula* sp. (cf. *viridula*), *Nitzschia* sp. (cf. *socialis*), *N. longissima*, was done in salinity 30 psu, temperature 25˚ C, pH 8, a 12:12 dark: light cycle and 180 μ mol photon m^{-2} s^{-1} since those species were available in those kind of natural environment. The biomass was filtered from the culture medium with Advantec filter paper (Tokyo, Japan). The biomass from both bottles and glass tanks was collected together and was transferred to a Petri dish and stored in a freezer at -70˚ C for 24 h. The sample was then freeze-dried at -50˚ C at 5 m Torr for biochemical analysis.

3.5. *Biochemical composition analyses*

Biochemical composition of the freeze-dried microalgal sample was determined according to the AOAC (Association of Official Analytical Chemistry) methods (AOAC, 1995). Crude lipid content was determined by Soxhlet method; crude protein by Kjeldhal method; ash content calcinations in furnace at 550˚ C; and moisture content by heating to 105˚ C for 24 h.

3.6. *Statistical analysis*

All the experiments to determine growth characteristics were conducted in duplicate (n = 2). The experiments to estimate antioxidant activity were conducted in triplicate (n = 3). The mean values of each treatment were compared using one-way analysis of variance (ANOVA) followed by Tukey's tests. P-value of less than 0.05 was considered as significant. All statistical analyses were performed using SPSS Statistical Software, version 11.5 (Edinburgh, Scotland

4. Results

4.1. *Axenic strain*

The mono-strain stock was observed under inverted microscope and a few bacterial cells were observed. After one week, the mono-strain stock was again checked, and a lot of bacterial cells were observed around broken *N. incerta*, *G. marina* and *C. closterium* cells. Thereafter, the presence of bacteria in the mono-strain stock was again confirmed by streaking on agar plates at the same culture conditions. The antibiotic penicillin, streptomycin and neomycin were used individually to eradicate the bacteria from the mono-strain stock, but failed to result in an axenic strain of these three species *N. incerta*, *G. marina* and *C. closterium*, even though the dose was lethal to *N. incerta*. Dosages above 250 units penicillin mL^{-1}, 250 μg streptomycin mL^{-1}, and 500 μg neomycin mL^{-1} were lethal to *N. incerta*, *G. marina* and *C. closterium*. A cocktail of antibiotics was tested at different dosages and I successfully obtained the axenic strain using 200 units penicillin mL^{-1}, 200 μg streptomycin mL^{-1}, and 400 μg neomycin mL^{-1} added to the culture medium (Fig. 10 and 11).

4.2. *Growth characteristics study of* Navicula incerta Grunow

The *N. incerta* grew under all conditions with good sigmoid growth pattern, but the cell abundance differed. The cell density was high in the 20˚ C water temperature condition, followed by 15˚ C and 25˚ C. The maximum cell density occurred either on 10 or 12 day after being inoculated in the culture medium in the all culture experiments (Fig. 12). In 15˚ C water, according to various salinities and nutrient concentrations, the maximum specific growth rate varied from 0.81 to 1.03 d^{-1}, averaging 0.91 d^{-1} (Fig. 13A), and the maximum cell density (6.13×10^5 cells mL^{-1}) occurred at 0.86 d^{-1} on day 12 after inoculation in medium with 35 psu salinity and F/2 nutrient concentration (Fig. 14A).

In 20˚ C water, the maximum specific growth rate was 0.85–1.04 d^{-1}, averaging 0.90 d^{-1} among several salinities and nutrient concentrations (Fig. 13B). The highest cell density

(7.99×10^5 cells mL^{-1}) occurred at 0.88 d^{-1} on day 12 after being inoculated in medium with 30 psu salinity and F/2 nutrient concentration, i.e. the B5 culture condition (Fig. 14B). The cell abundance showed highly significant difference than that of all other culture conditions ($P<0.01$).

In 25˚ C water, the maximum specific growth rate was 0.83–1.01 d^{-1}, averaging 0.95 d^{-1} among various salinities and nutrient concentrations (Fig. 13C). The maximum cell density (4.88×10^5 cells mL^{-1}) occurred on day 10 after being inoculated, when the maximum specific growth rate was 1.01 d^{-1} in medium with 30 psu salinity and F nutrient concentration (Fig. 14C).

Thus, among the various conditions, the highest standing crop was harvested in 20˚ C water on day 12 after inoculation, at the maximum specific growth rate of 0.88 d^{-1} with 30 psu salinity and F/2 nutrient concentration. Biomass production showed highly positive correlations with salinity ($r = 0.85$) and nutrients ($r = 0.87$) at low water temperature (15˚ C), but correlated positively only with salinity ($r = 0.54$) at intermediate temperature (20˚ C). Neither salinity nor nutrients correlated with biomass production at the high temperature (25˚ C).

4.3. *Growth characteristics study of* Cylindrotheca closterium Ehrenberg

C. closterium grew under the all conditions with different cell abundance. In 15˚ C water, with various salinities and nutrient concentrations, the maximum specific growth rate varied from 0.63 to 0.79 d^{-1} with the averaging of 0.66 d^{-1}. The maximums specific growth rate occurred on 12 day after inoculation in the all culture conditions, except A9 culture condition where maximum specific growth rate occurred on 10 day after inoculation (Table 5). The maximum cell density (6.59×10^4 cells mL^{-1}) occurred at 0.68 d^{-1} on day 12 after inoculation in the culture condition of 30 psu salinity and F nutrient concentration (Table 5). The maximum biomass production showed positive correlation with nutrients ($r = 0.94$).

In 20° C water, the maximum specific growth rate was 0.65–0.82 d^{-1}, averaging 0.75 d^{-1} among several salinities and nutrient concentrations of culture conditions. The maximum specific growth rate occurred lately among those mediums where the nutrient concentration was F/4 (Table 5). The highest cell density (7.20×10^4 cells mL^{-1}) occurred at the highest specific growth rate of 0.82 d^{-1} on day 10 after inoculation in medium with 30 psu salinity and F nutrient concentrations (Table 5). The maximum biomass production showed positive correlation with nutrients (r = 0.75) in 20° C water temperature.

In 25° C water, the maximum specific growth rate was 0.73–0.97 d^{-1} with the averaging of 0.86 d^{-1} among various salinities and nutrient concentrations. In 25° C culture conditions, the maximum specific growth rate occurred faster than 15° C and 20° C culture conditions (Table 5). The maximum standing crop (4.54×10^4 cells mL^{-1}) occurred on day 8 after inoculation, when the maximum specific growth rate was 0.97 d^{-1} and the medium was composed of 25 psu salinity and F nutrient concentration (Table 5). The maximum biomass production showed positive correlation with nutrient concentrations (r = 0.91) in 25° C water temperature.

Thus, among the 27 culture conditions of *C. closterium*, the highest (7.20×10^4 cells mL^{-1}) standing crop at the maximum specific growth rate of 0.82 d^{-1} on 10 day after inoculation in the culture condition of 20° C water temperature with salinity of 30 psu and nutrients concentration of F (Table 5).

4.4. *Biomass production of benthic diatoms*

The highest biomass production was 1.1 g L^{-1} (freeze dried biomass from one liter of culture media) obtained from *A. longipes* and *N. longissima* which was followed by *N. incerta* (1.0 mg L^{-1}), *A. coffeaeformis* and *C. closterium* (Table 6).

4.5. *Biochemical compositions*

Chemical composition was determined from seven species of benthic diatoms. Protein content among the benthic diatoms varied from 6.48 to 16.92 % with the highest from the *N. viridula* sp. (cf. *viridula*), followed by *A. coffeaeformis* (15.6%), *N. longissima* (11.17%). The highest (6.90%) lipid content was found from *A. coffeaeformis*, followed by *N. viridula* sp. (cf. *viridula*). The carbohydrate content varied from 13.50 to 18.79% with the highest from *N. longissima*. Ash content was 55.80-68.40% with the highest from *N. incerta*. The moisture content was the lowest (3.55%) in *Navicula* sp. (cf. *viridula*) and the highest (8.88%) was in *Nitzschia* sp. (cf. *socialis*) among the benthic diatoms (Table 7).

5. Discussion

5.1. *Axenic strain of benthic diatoms*

Bacteria grew with benthic diatoms in the culture media and decomposed diatom cells. Because the bacteria could have influenced the biochemical compositions of each benthic diatom by releasing cellular compounds, I used antibiotics to obtain the axenic strain of *N. incerta*, *G. marina, C. closterium, A. longipe*s, *A. coffeaeformis, N.* sp. (cf. *viridula*), *N.* sp (cf. *socialis*), and *N. longissima***.** The successful antibiotic dosages were lower than those (gentamycin 0.05 mg L^{-1}, penicillin-G 16 mg mL^{-1}, and streptomycin 0.8 mg mL^{-1}) used by Kotaki et al. (2000) to obtain the axenic culture of a domoicacid producing *Nitzschia* species. Neomycin is principally active against gram-positive bacteria, whereas streptomycin is mostly active against gram-negative bacteria, and the penicillin family is active against both (Guillard 2005). Thus, an antibiotic cocktail is effective in suppressing the survival and growth of bacteria under benthic diatom culture conditions. The optimal antibiotic dosages may be dependent on the bacterial load or the presence of specific bacteria, such as gram-negative or gram-positive species. So, I suggest to use antiobiotic cocktails rather than single antibiotics for making axenic strain of benthic diatoms.

5. 2. ***Growth characteristics of*** **Navicula incerta Grunow**

Significant biological information for the mass culture of algae can be obtained by determining growth characteristics under controlled, measurable conditions, and then used to create a high-density mass culture system. I determined the limitations of *N. incerta* growth characteristics by using different temperature, salinity, and nutrient concentrations. F/2 medium was used as a nutrient source, as it is commercially available, easy to use, and suited to our goal of producing this alga commercially for industrial and aquaculture use. The cell density of *N. incerta* was greater at the low and medium water temperatures than at the high temperature, with cell densities being greatest at 20° C. The growth pattern of this species suggests that it is eurythermal, but moderate temperatures may create the most favorable conditions for growth.

In terms of salinity, *N. incerta* appears to be euryhaline, because it grew well, achieving different maximum cell abundances, over a range of salinities (25-35 psu). Affan and Lee (2004) monitored the seasonal dynamics of phytoplankton and environmental factors in Jeju Island coastal waters and found that *Navicula* spp. were abundant at intermediate temperatures and salinity. Thus, I suggest that the maximum standing crop of *N. incerta* in commercial aquaculture can be obtained at an intermediate water temperature (20° C) with moderate salinity and nutrient concentrations, as maximum production can only be achieved under optimal growth conditions.

5.3. ***Growth characteristics*** **of Cylindrotheca closterium Ehrenberg**

A combination of moderate temperature, salinity, and higher nutrient concentrations may create favorable circumstances for blooms of this species, indicating that *C. closterium* is a eutrophic benthic diatom. Affan and Lee (2004) monitored the seasonal dynamics of phytoplankton and environmental factors in Jeju Island coastal waters and found that *C. closterium* was more than 10% abundant to the total phytoplankton in the winter when the water temperature and salinity were 16° C and 34 psu, with highly available nutrient

concentrations. Thus, I suggest that the maximum standing crop of *C. closterium* in commercial aquaculture can be obtained at an intermediate water temperature (20˚ C) with higher salinity (35 psu) and nutrient concentrations (F).

5.4. *Biomass production*

In our mass culture system the highest benthic diatoms production was about 1 g dry weight from one liter of culture media. The biomass production may be increased by using the CO_2 gas as I did not supply the CO_2. Lalucat et al. (1984) found that the photosynthesis activity of *Chlorella* sp. was increased sevenfold after adding CO_2.

5.5. *Biochemical composition*

The protein and lipid content was found less in the benthic diatoms from Jeju Island coast. However, Brown and Jeffrey (1995) reported the biochemical composition of several diatoms, including *Skeletonema costatum* and *Nitzschia closterium*, for which the protein, carbohydrate, and ash content were 31-38%, 4.9-6.5%, and 9.4-18% of dry weight, respectively. The other two species, *Cylindrotheca fusiformis* and *Lauderia annulata*, contained only 16% protein, but had nearly twice the total carbohydrate (11-12%) and 2-3 times the ash (29-35%). In comparison with those diatoms, it was found that *N. viridula* showed higher protein and *C. closterium*, *A. coffeaformis* and *N. longissima* also showed 1 to 2% less protein content. In our culture system, most of the diatoms showed higher ash content. Sicko-Goad et al. (1984) found 60% silica content of diatoms from natural assemblages. However, in our mass culture system I found the salt attached on the wall of the Nalgene bottles due to aeration, though distilled water was added everyday to maintain the salinity. Thereafter, during collection of the biomass I removed the benthic diatoms with brush as they were also attached to the wall of the bottles. The salt attached to wall of the empty part of the bottle was mixed without fully dissolve to the culture media, and become precipitated. So, the salt was also gathered with the biomass when the biomass was collected

by filtering. Thus, a part from silicon, salts also might have played role to increase the ash content of our cultured benthic diatoms.

In conclusion, most of the benthic diatoms grew well with range temperature of 20-25° C, salinity of 25-30 psu and nutrients concentration of F/2-F (100-200%). The highest biomass production was about 1 g L^{-1} (freeze dried biomass from one liter of culture media). *N. viridula* sp. (cf. *viridula*) contains more protein and *A. coffeaeformis* contains more lipid than other benthic diatoms. So I suggest that the mass culture of the above mentioned diatoms can give helpful for the fill up the demand of live feed in aquaculture and for other advanced study.

6. References

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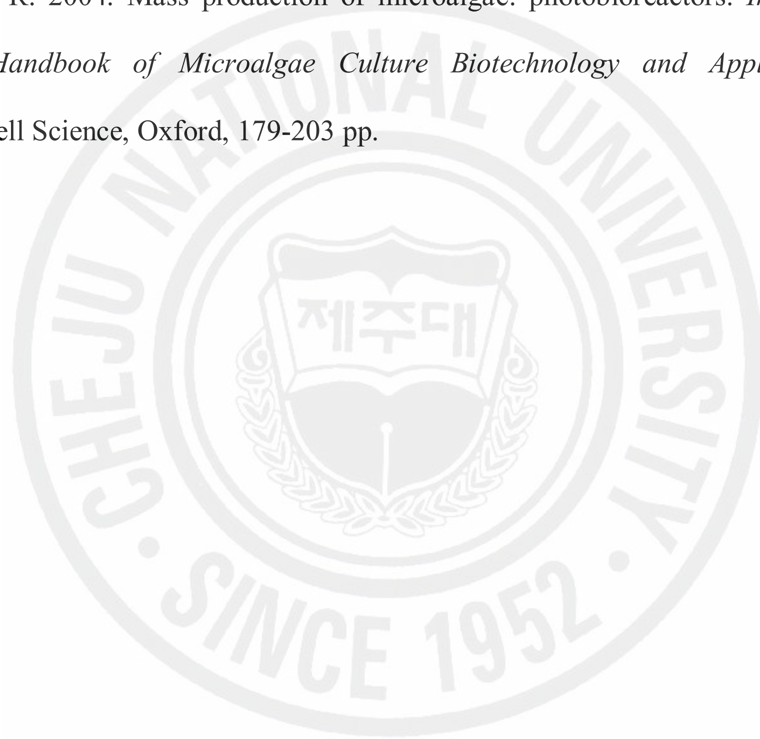

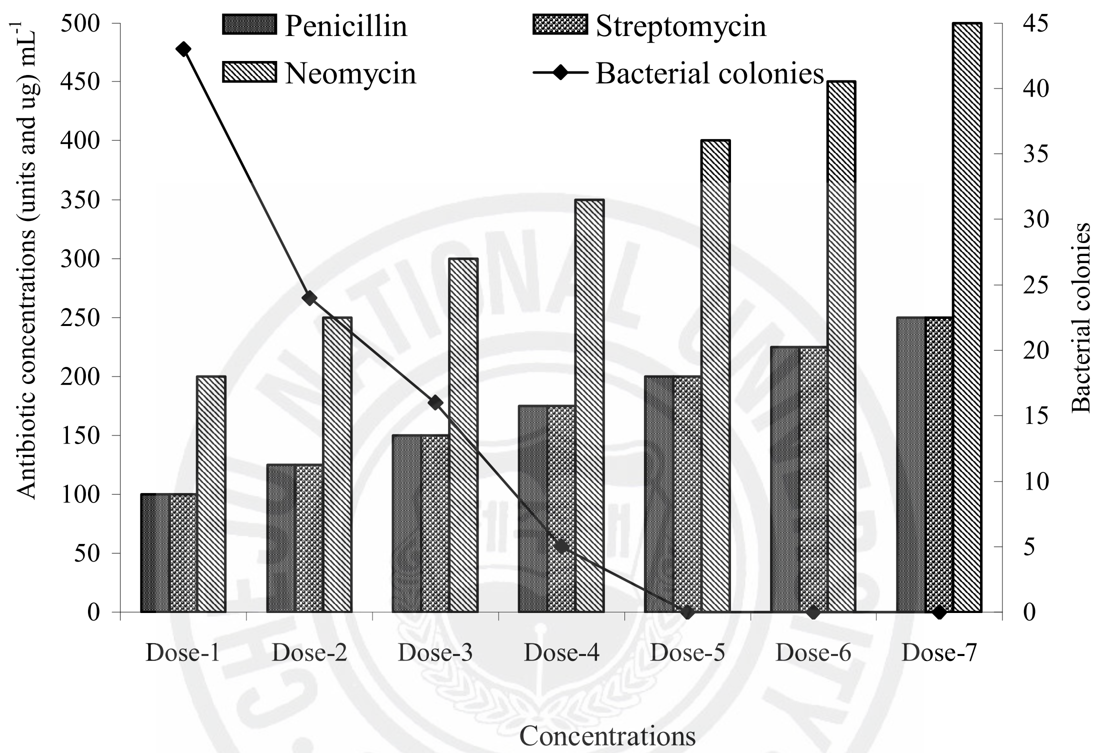

Fig. 10. Reduction of bacterial colonies during *Navicula incerta* isolation with the increase of antibiotic concentrations over Dose 1 (penicillin 100 units mL^{-1}, streptomycin 100 μg mL^{-1}, and neomycin 200 μg mL^{-1}). Each succeeding dose was increased by 25 units penicillin, 25 μg streptomycin, and 50 μg neomycin per mL.

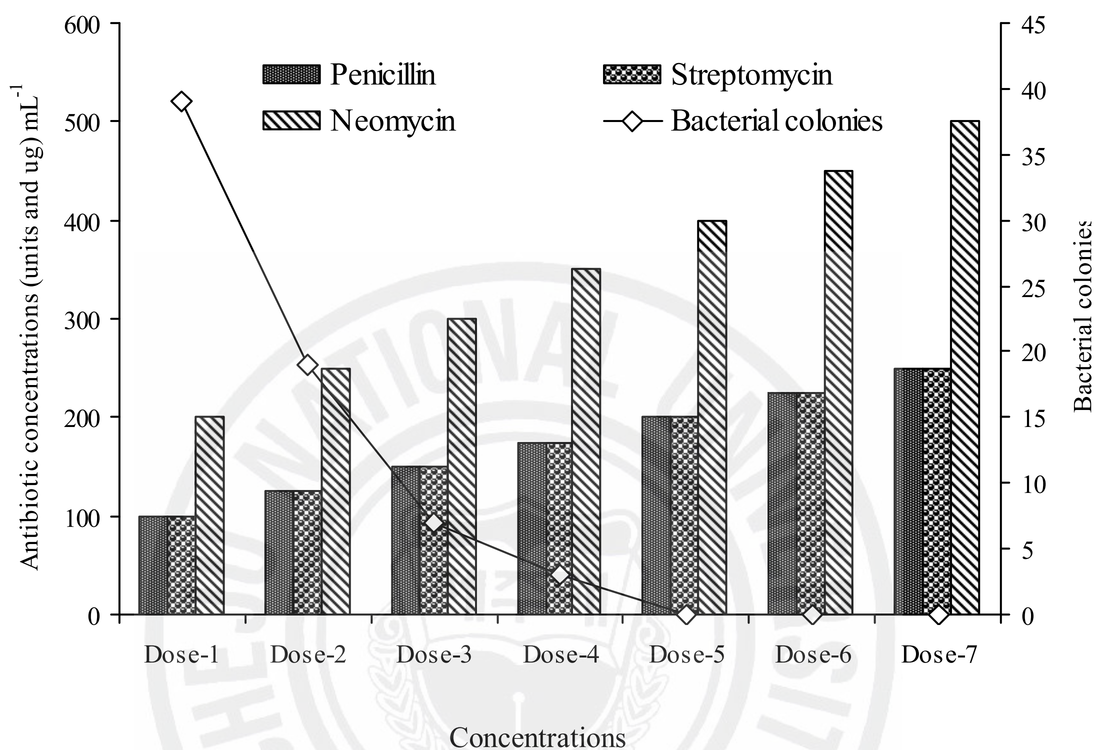

Fig. 11. Reduction of bacterial colonies during *Cylindrotheca closterium* isolation with the increase of antibiotic concentrations over Dose 1 (penicillin 100 units mL^{-1}, streptomycin 100 µg mL^{-1}, and neomycin 200 µg mL^{-1}). Each succeeding dose was increased by 25 units penicillin, 25 µg streptomycin, and 50 µg neomycin per mL.

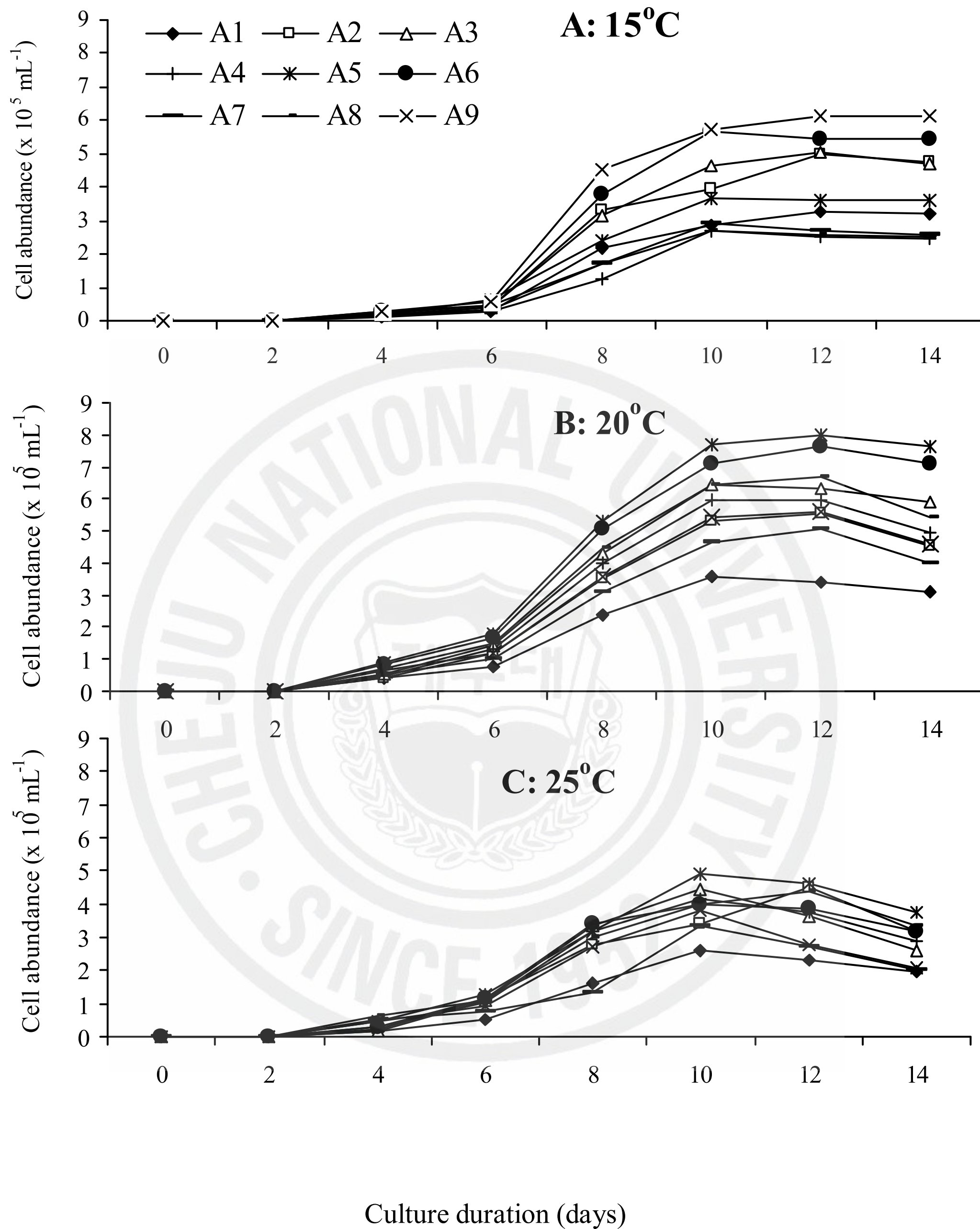

Fig.12. Growth curves of *Navicula incerta* at 15˚ C, 20˚ C, and 25˚ C water temperature, with F/4 (50%), F/2 (100%), and F (200%) nutrient concentrations and 25, 30, and 35 psu salinity. Culture conditions: A1 (25 psu, F/4 nutrients), A2 (25 psu, F/2 nutrients), A3 (25 psu, F nutrients), A4 (30 psu, F/4 nutrients), A5 (30 psu, F/2 nutrients), A6 (30 psu, F nutrients), A7 (35 psu, F/4 nutrients), A8 (35 psu, F/2 nutrients), and A9 (35 psu, F nutrients) in 15˚ C water. Conditions B (20˚ C water) and C (25˚ C water) repeated A as regards salinity and nutrient concentrations.

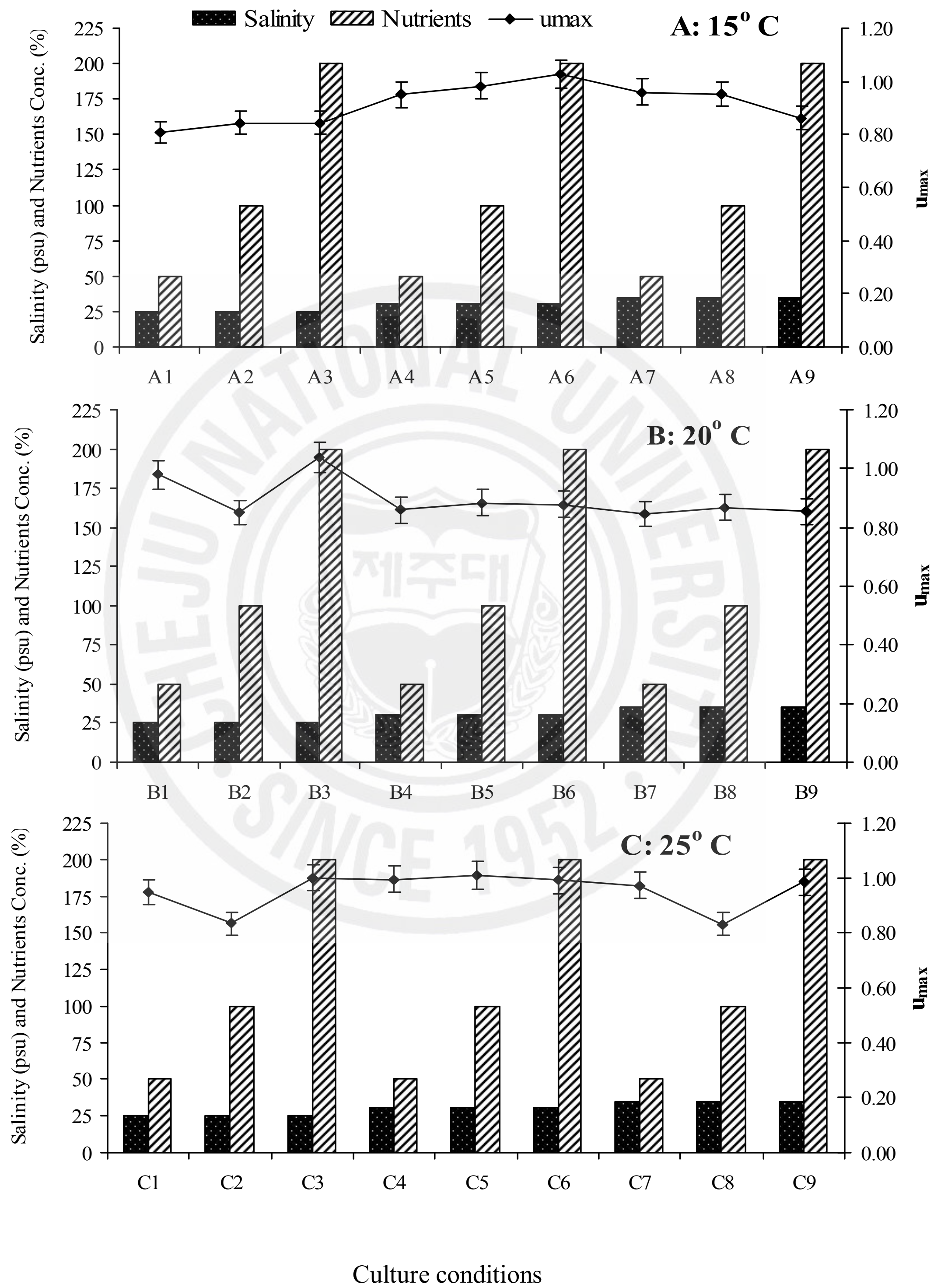

Fig. 13. Maximum specific growth rate (μ_{max}) of *Navicula incerta* at different salinities and nutrient concentrations (see Fig.12 for culture conditions).

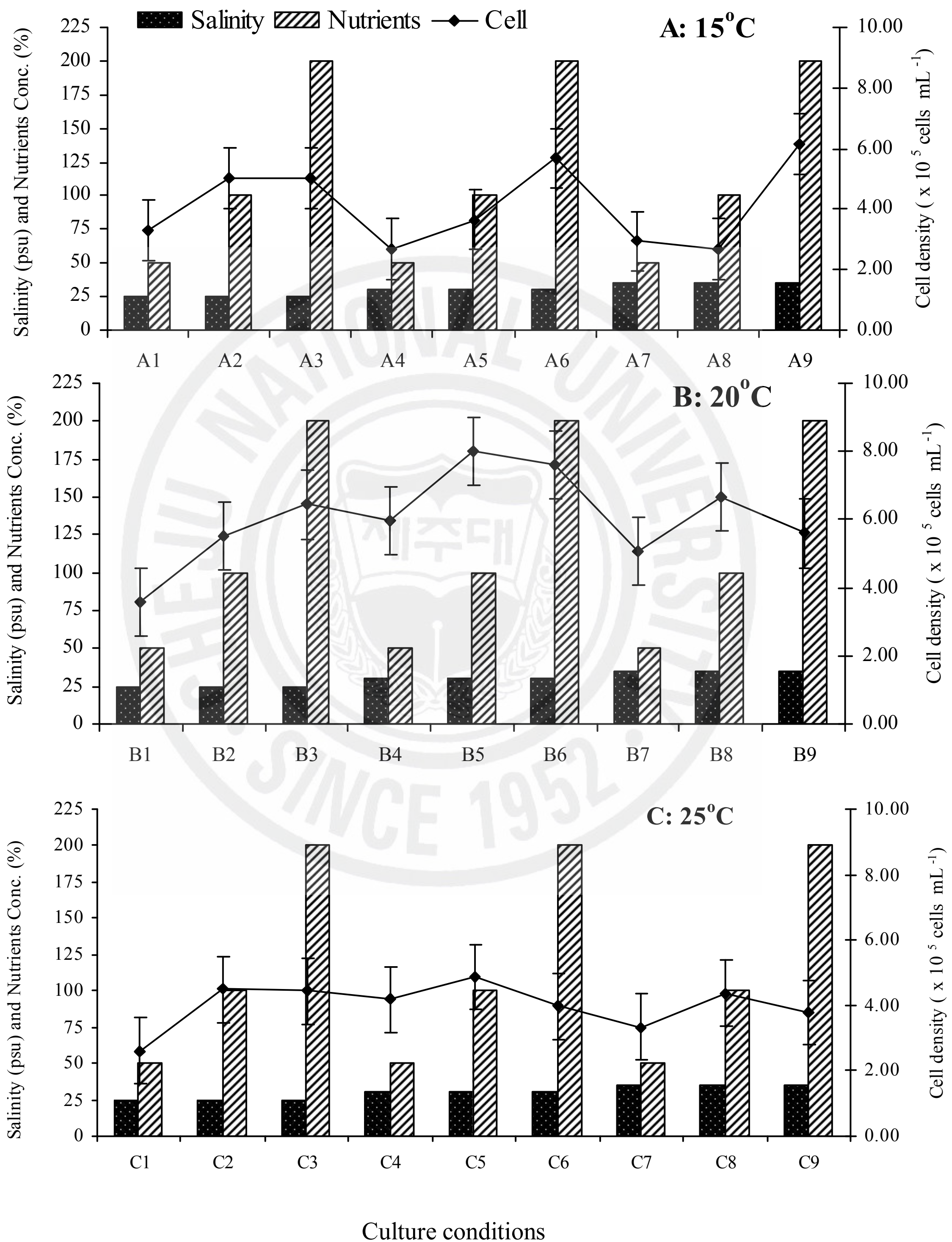

Fig. 14. Maximum cell density occurrence of *Navicula incerta* at different salinities and nutrient concentrations (see Fig. 12 for culture conditions).

Table 5. The maximum specific growth rate (μ_{max}) of *Cylindrotheca closterium* and maximum cells abundance (cells × 10^4) at different temperature (° C), salinity (psu) and nutrient concentrations (%), and maximum growth occurred duration (day)

Culture conditions				μ_{max}	Cells × 10^4	Day
Temperature	Experiments	(psu)	(%)			
15 ° C	A1	25	50	$0.63^{f} \pm 0.01$	$3.83^{de} \pm 0.02$	12
	A2	25	100	$0.64^{f} \pm 0.01$	$4.52^{cd} \pm 0.02$	12
	A3	25	200	$0.66^{ef} \pm 0.02$	$5.62^{b} \pm 0.03$	12
	A4	30	50	$0.63^{f} \pm 0.01$	$3.93^{de} \pm 0.01$	12
	A5	30	100	$0.65^{ef} \pm 0.01$	$4.70^{cd} \pm 0.02$	12
	A6	30	200	$0.68^{e} \pm 0.02$	$6.59^{b} \pm 0.02$	12
	A7	35	50	$0.64^{f} \pm 0.02$	$4.19^{d} \pm 0.01$	12
	A8	35	100	$0.65^{ef} \pm 0.2$	$4.87^{cd} \pm 0.01$	12
	A9	35	200	$0.79^{bc} \pm 0.03$	$5.59^{b} \pm 0.03$	10
20 ° C	B1	25	50	$0.65^{ef} \pm 0.01$	$4.92^{cd} \pm 0.03$	12
	B2	25	100	$0.79^{bc} \pm 0.02$	$5.32^{bc} \pm 0.02$	10
	B3	25	200	$0.80^{b} \pm 0.01$	$5.99^{b} \pm 0.02$	10
	B4	30	50	$0.65^{ef} \pm 0.02$	$5.13^{bc} \pm 0.02$	12
	B5	30	100	$0.79^{bc} \pm 0.01$	$5.60^{b} \pm 0.02$	10
	B6	30	200	$0.82^{b} \pm 0.02$	$7.20^{a} \pm 0.02$	10
	B7	35	50	$0.66^{ef} \pm 0.03$	$5.22^{c} \pm 0.01$	12
	B8	35	100	$0.80^{b} \pm 0.01$	$5.69^{b} \pm 0.03$	10
	B9	35	200	$0.79^{bc} \pm 0.01$	$5.55^{c} \pm 0.01$	10
25 ° C	C1	25	50	$0.73^{d} \pm 0.01$	$2.84^{e} \pm 0.01$	10
	C2	25	100	$0.75^{d} \pm 0.03$	$3.64^{de} \pm 0.01$	10
	C3	25	200	$0.97^{a} \pm 0.03$	$4.54^{cd} \pm 0.02$	8
	C4	30	50	$0.93^{a} \pm 0.01$	$3.33^{de} \pm 0.02$	8
	C5	30	100	$0.93^{a} \pm 0.02$	$3.49^{de} \pm 0.02$	10
	C6	30	200	$0.96^{a} \pm 0.01$	$4.28^{cd} \pm 0.01$	8
	C7	35	50	$0.74^{d} \pm 0.01$	$3.39^{de} \pm 0.03$	10
	C8	35	100	$0.76^{cd} \pm 0.03$	$3.81^{de} \pm 0.03$	10
	C9	35	200	$0.95^{a} \pm 0.02$	$4.04^{d} \pm 0.02$	8

The values represent the mean ± SD from triplicate samples. Values followed by the superscript letters a, b, c, d, e, f, g, h, and i indicate a significant difference ($p<0.05$).

Table 6. Culture conditions, culture duration (days) and biomass production in dry weight (g L^{-1}) of 7 benthic diatoms from one liter of culture media

Species name	Temperature °C	Salinity psu	pH	L:D cycle	(days)	(g L^{-1})
Achnanthes longipes	25	30	8.00	10:14	14	$1.1^{a} \pm 0.20$
Amphora coffeaeformis	25	30	8.00	10:14	14	$0.9^{b} \pm 0.31$
Cylindrotheca closterium	20	30	8.00	10:14	14	$0.9^{b} \pm 0.05$
Navicula incerta	20	35	8.00	10:14	14	$1.0^{ab} \pm 0.20$
Navicula sp. (cf. *viridula*)	25	30	8.00	10:14	14	$0.7^{c} \pm 0.25$
Nitzschia longissima	25	30	8.00	10:14	14	$1.1^{a} \pm 0.09$
Nitzschia sp. (cf. *socialis*)	25	30	8.00	10:14	14	$0.8^{bc} \pm 0.12$

The values represent the mean ± SD from triplicate samples. Values followed by the superscript letters a, b and c indicate a significant difference ($p<0.05$).

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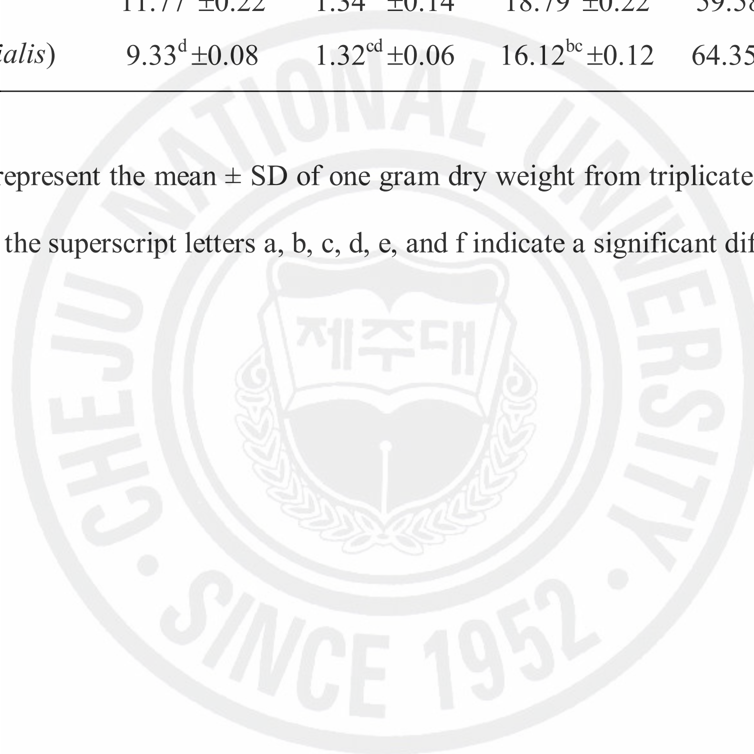
Table 7. Biochemical compositions of 7 mono-strain cultured phytoplankton on dry weight basis

Species name	Protein	Lipid	Carbohydrate	Ash	Moisture
Achnanthes longipes	6.48^{ef} ±0.13	1.08^{ef} ±0.32	16.46^{bc} ±0.11	67.89^{a} ±0.15	8.09^{bc} ±0.10
Amphora coffeaeformis	15.6^{b} ±0.22	6.90^{a} ±0.29	15.80^{cd} ±0.20	55.80^{cd} ±0.47	5.90^{d} ±0.43
Cylindrotheca closterium	11.11^{c} ±0.12	1.18^{d} ±0.10	18.51^{a} ±0.09	61.86^{bc} ±0.11	7.34^{c} ±0.10
Navicula incerta	7.00^{e} ±0.12	1.70^{c} ±0.10	12.80^{e} ±0.11	68.40^{a} ±0.03	10.00^{a} ±0.13
Navicula sp. (cf. *viridula*)	16.92^{a} ±0.26	2.08^{b} ±0.28	13.50^{de} ±0.22	63.95^{b} ±0.49	3.55^{e} ±0.49
Nitzschia longissima	11.77^{c} ±0.22	1.34^{cd} ±0.14	18.79^{a} ±0.22	59.58^{c} ±0.04	8.52^{b} ±0.58
Nitzschia sp. (cf. *socialis*)	9.33^{d} ±0.08	1.32^{cd} ±0.06	16.12^{bc} ±0.12	64.35^{ab} ±0.13	8.88^{ab} ±0.15

The values represent the mean ± SD of one gram dry weight from triplicate samples. Values followed by the superscript letters a, b, c, d, e, and f indicate a significant difference ($p<0.05$).



Part III

Comparison of benthic diatom efficiency for larval and juvenile abalone aquaculture between mono-strain and wild mixed-strain on wavy plates

1. Abstract

Mono-strain of *Navicula incerta* Grunow and *Grammatophora marina* Ehrenberg benthic diatoms were cultured on wavy plates in the laboratory. The benthic diatoms were used as a life feed for settlement of larval abalone, and growth abalone spat. The abundance of benthic diatom on the plates of *N. incerta* mono-strain, *G. marina* mono-strain and wild mixed-strain was 6.71×10^5, 7.01×10^5 and 7.56×10^5 cells $(\text{surf.cm}^2)^{-1}$ at the beginning of the study, and at the end of the study, the abundance was 2.75×10^3, 2.84×10^3 and 3.31×10^3 cells $(\text{surf.cm}^2)^{-1}$, respectively. At the beginning of the study *N. incerta* and *G. marina* mono-strain occupied 95.39% and 96.79% which reduced to 69.82% and 65.49% among the benthic diatoms community. The average number of settled juvenile abalone was 1000, 1080 and 640 in the tank of *N. incerta*, *G. marina* mono-strain and wild mixed-strain, and the survival rate was 2.00, 2.16 and 1.28% in each kind of tank, respectively. The specific growth rate of juvenile abalone was 3.28, 3.07, and 2.92% on the plates of *N. incerta*, *G. marina* mono-strain and wild mixed-strain, respectively. The average live feed consumption rate by each abalone per day was 0.17, 0.15 and 0.26% of total standing crops of benthic diatoms on the plates of *N. incerta*, *G. marina* mono-strain and wild mixed-strain, respectively. In conclusion, the plates with mono-strain of benthic diatom showed better settlement and growth of abalone larvae than that of wild mixed-strain.

2. Introduction

Abalone production from wild fisheries is continuing to decline in the recent decades throughout the world, with the high commercial value of the product and the collapse of many fisheries, it is likely that the remaining abalone stocks will be put under greater pressure (Gordon and Cook 2001). The abalone farming industry is increasing to reduce its dependency on wild abalone stocks. Therefore, commercial abalone farms throughout the world are now intensively producing abalone to meet increasing market demand. There have been extensive studies on commercially important abalone species for aquaculture. Every country has cultivated its own native species because of ecological consideration, and it is simpler to deal with the animals in relation to water quality (temperature and salinity) and suitable natural food. However, *Haliotis discus discus* (Reeve) is a commercially important shellfish species in Korea, China and Japan. Aquaculture of *H. discus discus* has been started since 1970s and aquaculture production is increasing rapidly in recent years. In Korea, the abalone production has been increased from 20 to 1260 Metric ton from 2000 to 2004 (FAO 2004).

Biofilms of mixed benthic diatoms growing on the plates have been used traditionally as settlement substrata for abalone postlarval in worldwide hatcheries (Hahn 1989, Roberts 2001). After metamorphosis, the abalone larvae start to feed benthic diatoms and high mortality occurs from the settling time to first two months (Seki and Kanno 1981, Kawamura et al. 1995, Martinez-Ponce and Searcy-Bernal 1998). The reason may the abalone larvae are not getting sufficient nutrients from the diatoms plates since the diatoms attach on the traditionally culture plates are unpredictable especially for the diversity of the benthic diatoms. However, benthic diatoms are the most important food source for the abalone postlarvae (Kawamura 1996, Kawamura et al. 1998) but there several problems have been recognized in regards to the current raising method of abalone on diatom biofilms, as it is difficult to continue the supply of readily ingestible and digestible food (diatom biofilms) for growing postlarvae (Kawamura et al. 1998). In Korea, *Isochrysis*, *Pavlova*, *Chaetoceros*,

Thalassiosira, *Nannochloris* and *Tetraselmis* are used as a live feed for the shellfish seedling production. Due to technical problem in benthic diatom mass culture system, the abalone seedling production from the aquaculture farms is still very low in relation to its increasing demand in Korea (Hur 1997).

Wild mixed benthic diatoms on plates are traditionally used as live feed in commercial shellfish hatcheries, in Jeju Island, Korea. At present, benthic diatoms are cultured based on the natural condition with the addition of fertilizer for aquaculture purposes. But this traditional production of benthic diatoms can not fulfill the demands year-round for rapidly expanding aquaculture sector due to population fluctuations resulted from natural environmental dynamics. The development of sustainable and suitable technique for the mass culture of benthic diatoms is necessary to meet up aquaculture demand. For mass culture, the most important task is the selection of species which depends on- i) the availability of the species year-round, ii) their optimal environmental conditions, and iii) their suitability as food for the targeted shellfish. Therefore, isolation of single species, find out their best growth condition with different environmental factors and finally the development of low-cost mass culture technique is utmost important to fulfill demand for live feed in the shellfish aquaculture.

In this study, for the settlement and growth of abalone larvae we used *Navicula incerta* and *Grammatophora marina* monostrain growing on plate, since *Navicula* spp. and *G. marina* were more than 15% abundant among the phytoplankton community throughout the year in the coastal water of Jeju Island (Affan and Lee 2004). These two benthic diatoms showed stronger attachment on the growing plate than others, and can make thick, dark brown mats on the plates. However, the objectives of this study were to find out the efficiency of abalone larval settlement between the mono-strain benthic diatoms and wild mixed benthic diatoms species, and also to know the community change of diatoms in both kinds of plates.

3. Materials and methods

3.1. *Isolation and axenic strain of* **Navicula incerta and Grammatophora marina**

The isolation procedure was performed as described in Affan et al. (2006). In summary, benthic diatoms attached to plates were collected from the abalone culture hatchery of the National Fisheries Research and Development Institute (NFRDI), Jeju Island, Korea. The diatoms were removed from the plates with a brush, and diluted with seawater. Then, a 1-mL diluted sample was transferred to a Sedgewick Rafter (S–R) counting chamber, and single diatom cells were micropipetted from the counting chamber under an inverted microscope (Olympus IX71). Thereafter, each cell was transferred to a multi-well plate for subculture in autoclaved seawater, which was filtered through 0.45-μm millipore filter paper and enriched with F/2 medium nutrients trace metals, and metasilicate anhydrous crystals (Na_2SiO_3). The isolation process was performed until obtaining a monostrain. To identify the subcultured benthic diatom, samples were observed under a phase-contrast Zeiss Axioplan microscope (Carl Zeiss, Germany) at 400x magnification. The monostrain sample identified as *N. incerta* and *G. marina* was again treated with antibiotics cocktail of penicillin, streptomycin and neomycin (P 4083; Sigma Aldrich Corp, St. Louis, MO, USA) in same F/2 medium. In this way, we obtained an axenic strain of *N. incerta* and *G. marina* for further study.

3.2. *Growth characteristics experiments*

The growth characteristics of each axenic strain were determined as described by Affan et al. (2006). To determine the optimal growth conditions of *N. incerta* and *G. marina*, we studied three culture parameters: water temperature (15˚ C, 20˚ C, 25˚ C), salinity (25, 30, 35 psu), and nutrient concentration (F/4 = 50%, F/2 = 100%, and F = 200%), with each condition categorized as low, medium, or high. The 100% nutrient concentration was F/2 medium, with the addition of 1 mL per 7.75 L distilled water each of the A and B solution of

F/2 media, plus 13 $\mu L\ L^{-1}$ trace element solution, and 13 $\mu g\ L^{-1}$ metasilicate anhydrous crystal. The artificial seawater was enriched with F/2 nutrients and used for the growth characteristics experiments and mass culture. Each kind of different flask was inoculated with approximately 20 cells mL^{-1} *N. incerta* and *G. marina* individually. The cultures were grown under fluorescent lights (180 μ photons $m^{-2}\ s^{-1}$) on a 12:12 light: dark cycle for two weeks. Each treatment was replicated twice. A 1-mL sample was collected from each culture flask every two days and fixed with Lugol's iodine solution. The fixed sample was diluted, and the cells were counted using a S-R counting chamber under an inverted microscope at 400x magnification. Finally we estimated the maximum specific growth condition of *N. incerta* and *G. marina*.

3.3. *Mass culture of mono-strain benthic diatoms on wavy plastic plates*

The mass culture of *N. incerta* and *G. marina* was done at their maximum growth condition with the same F/2 enriched seawater medium on the wavy plates (37 × 28 cm) in a glass tank at the laboratory and the aeration was provided 24 hours gently and it was carried out for two weeks. Five-cm^2 diatom attached plates was cut and collected all the cells, and preserved with lugol's solutions. The fixed sample was counted directly with phase contrast microscope and finally the cell abundance cm^{-2} of each surface (cells cm^{-2}.surf)$^{-1}$ of plates was calculated. The mono-strain diatoms on plates were transferred from the laboratory to the abalone culture tank. We kept the mono-strain diatoms in the abalone culture tanks for two days for acclimatization in that natural environment. The plates of wild mixed-strain diatoms were taken from the hatchery and those plates were used as control. Both kinds of plates were placed horizontally into the abalone culture tank. Each kind of plates was placed into the separate tank, as our one of the hypothesis was to protect the mono-strain diatom plates with heavy contamination of wild mixed-strain. Water carrying capacity of each abalone culture tank was 4000 L and the inlet-outlet flow was maintained to change the

whole water 2 times within 24 hours. Filtered seawater was provided to the abalone culture tank by using the polypropylene filter cartridge (Hydro-pure, USA).

For continuous growth of the benthic diatoms, F/2 medium nutrients, metasilicate anhydrous and trace metal solution were added weekly to the all kinds of culture tank, and the dosages were same as mentioned in the laboratory culture system, and at the same time temperature, salinity and pH were also checked. The abundance and the species composition of benthic diatom on the plates were monitored two times during this study; first time before releasing the abalone larvae and second time at the end of the experiment. The cell abundance and the community changing on the plates were monitored by following the same procedure which was done to estimate the cell abundance of diatom on the plates at the laboratory.

3.4. *Abalone culture condition*

A large-scale settlement experiment was conducted to test the suitability of different algal species as settlement inducer for *H. discus discus* larvae. After completion of metamorphosis, abalone larvae were released in the culture tank for settlement and growth. The average size of the abalone larvae was 0.35 mm and 50,000 abalone larvae were released in each tank, and 100 plates were kept in each tank with live benthic diatoms. Three tanks were used for this experiment, one for *N. incerta*, one for *G. marina* and one for control. After two months, 20 plates were taken randomly from each tank, and the number and size of the spats were estimated. The cell abundance and community changes of benthic diatoms on the plates were also monitored from those plates. Settled juvenile abalone was counted directly on the plates. The length of the juvenile abalone was measured with slide calipers. The live feed consumption rate (LFC) per day by each abalone in each tank was estimated.

Survival rate of abalone larvae was determined according to following way;

$SR\ \% = (R_2 / R_1) / 100$, Where, SR= survival rate, R_1 = number of released larvae into the tank at the beginning of the study, R_2 = harvested number of juvenile larvae at the end of the study.

The length of all settled juvenile larvae was measured from 20 plates and the specific growth rate (SGR) % was calculated according to the equation;

$SGR\% = \{\ln (L_1) - \ln (L_0)/t\} \times 100$, Where L_1 = the mean final length (mm), L_0 = the mean initial length, and t = the growth period

The estimation of the live feed consumption rate (LFC %) by individual juvenile abalone was calculated as follows;

$$LFC\ \%\ (day^{-1}) = \frac{(P_1 - P_2) \times (\text{Wavy plastic plate}) \times 100}{P_1 \times (t_1 - t_0) \times \text{No. of juvenile abalone on each Wavy plastic plate}}$$

Where LFC = live feed consumption, P_1 = initial abundance of benthic diatoms on the Wavy plastic plate, P_2 = final abundance of benthic diatoms on the Wavy plastic plate, t_0 = beginning day of the study, t_1 = ending day of the experiment.

4. Results

4.1. *Axenic strains, the best growth condition and mass culture*

Many bacteria were present during the isolation and monostrain subculture of *N. incerta* and *G. marina*. The numbers of bacterial colonies were different on the agar plate of each monostrain diatoms but the successful doze was same which were 200 units penicillin mL^{-1}, 200 μg streptomycin mL^{-1}, and 400 μg neomycin mL-1 for both strain of diatoms (Affan et al. 2006).

Both *N. incerta* and *G. marina* grew under all conditions, but the cell abundance was differed. The maximum specific growth rate of *N. incerta* varied from 0.85 to 1.04 d^{-1} with the average of 0.90 d^{-1} in 20° C water among 27 culture conditions. The highest standing crop (7.99 × 10^5 cells mL^{-1}) occurred at 0.88 d^{-1} on day 12 post-inoculation in medium with 30 psu salinity and 100% nutrient concentration (the data is shown in Chapter II). The mass culture of *N. incerta* was done in glass tanks with plastic plates under maximum specific growth conditions. The average cell abundance varied from 5.60 to 7.25 × 10^5 cells $(surf.cm^2)^{-1}$ from bottom to with the average of 6.43 × 10^5 cells $(surf.cm^2)^{-1}$ of the plates after two weeks of culture (Table 8).

The best specific growth rate (1.68 d^{-1}) of *G. marina* was found at the temperature of 25° C with salinity of 35 psu and nutrient concentration of 200% on 6th day during the culture period among the all culture conditions. Maximum biomass was also observed 4.89 × 10^5 cells mL^{-1} on the same condition (Data has been published) (Affan et al. 2006). In the culture tanks this species was found to grow well on the plates with strong attachment by producing thick mats. The indoor mass culture system of benthic diatoms and settled abalone spats on the plates are shown in Fig. 1 A, C & D. The cell density varied from 6.60 to 6.96×10^5 cells $(surf.cm^2)^{-1}$ from bottom to surface of the plates with the average of 6.78×10^5 cells $(surf.cm^2)^{-1}$ (Table 8).

The plates were kept for two days in the abalone rearing tanks for acclimatization and then the abalone larvae were released to the tanks. Before releasing the abalone larvae, the benthic phytoplankton cell abundance was estimated from both mono-strain and wild mixed strain plates. The cell abundance was 6.43×10^5, 7.06×10^5 and 7.18×10^5 cells $(surf.cm^2)^{-1}$ of the plates of *N. incerta, G. marina* mono-strain and wild-mixed strains, respectively. *N. incerta* and *G. marina* occupied 99.66 and 99.51% to the phytoplankton population as a single species (Table 1). At the end of the study, the abundance of phytoplankton was again estimated which was 2.75×10^3, 2.84×10^3 and 3.31×10^3 cells

$(surf.cm^2)^{-1}$ on the plates of *N. incerta*, *G. marina* mono-strain and mixed wild-strain, respectively. However, at the end of the study the abundance of *N. incerta* and *G marina* on each plate reduced to 69.82% and 65.49% among the phytoplankton community (Table 8).

4.2. *Abalone settlement, growth and live feed consumption*

The number of settled juvenile abalone was 1000, 1080 and 640 in the tank of *N. incerta*, *G. marina* mono-strain and wild mixed-strain, respectively. The survival rate of abalone larvae was 2.00, 2.16 and 1.28%, and the average length was 2.51, 2.21 and 2.02 mm on the plates of *N. incerta, G. marina* mono-strain and wild mixed-strain tanks, respectively (Table 9). The settlement rate of larval abalone was 68.7% and 56.2% more on the plates of *G. marina* and *N. incerta* mono-strain than that of wild mixed-strain. The average size of juvenile abalone was 9.4% and 24.2% bigger on the plates of *G. marina* and *N. incerta* mono-strain than that of wild mixed-strain plates. The specific growth rate of juvenile abalone larvae was 3.28, 3.07 and 2.92% on the plates of *N. incerta, G. marina* and wild mixed-strain, respectively (Table 9). The mean specific growth rate of juvenile abalone was 0.15 and 0.36% more on the plates of *G. marina* and *N. incerta* than those of wild mixed-strain plates. The specific growth rate of juvenile abalone on plastic of the *N. incerta* was 0.21% higher than that of *G. marina* plates. The average benthic diatoms consumption rate by each abalone per day was 0.26%, 0.17% and 0.16% on the plates of wild mixed-strain, *N. incerta* and *G. marina* mono-strain, respectively (Table 9).

4.3. *Benthic diatoms species composition*

At the beginning of the study we found 4 benthic diatoms species namely, *Amphora decussate*, *Cylindrotheca closterium*, *Nitzschia longissima*, *Nitzschia socialis* on the on the plate of *N. incerta* as well as on the plate of *G. marina*. And we also found *N. incerta* on the plate of *G. marina* and vice versa (Table 8). On the wild mixed-strain benthic diatoms we found 14 benthic diatoms including *N. incerta* and *G. marina* (Table 8). At the end of the

study same kind of 9 species of benthic diatoms were found on the plate of *N. incerta* and *G. marina* but on the plate of wild mixed strain one more benthic diatom (*Thalassiothrix longissima*) was found and that was absent on the both kinds of mono-strain plate. At the end of the study, the cell abundance of *C. closterium* and *N. longissima* was more on the all kinds of plate (Table 8).

5. Discussion

5.1. *Axenic species*

Bacteria grew with *N. incerta* and *G. marina* mono-strain in the culture media and decomposed diatom cells. Because the bacteria could have destroyed the stock strain of these benthic diatoms, we used antibiotics cocktail to obtain the axenic species. The eradication of bacteria from mono-strain of benthic diatoms may be dependent on the bacterial load or the presence of specific bacteria, such as gram-negative or gram-positive species. The antibiotic cocktail of penicillin, streptomycin and neomycin exhibited the successful result of making axenic strain of *N. incerta* and *G. marina*. Guillard (2005) has described that Neomycin is principally active against gram-positive bacteria, whereas streptomycin is mostly active against gram-negative bacteria, and the penicillin family is active against both.

5.2. *Growth characteristics and proximate composition*

Measuring growth rates is informative about the activity of microbial populations, which can increase at exponential rates. Significant biological information for the mass culture of algae can be obtained by determining growth characteristics under controlled and measurable conditions, and then used to create a high-density mass culture system.

We determined the limitations of *N. incerta* and *G. marina* growth characteristics by using different temperature, salinity, and nutrient concentrations. F/2 medium was used as a nutrient source, as it is commercially available, easy to use, and suited to our goal of

producing these algae commercially for gastropod aquaculture use. The growth pattern of *N. incerta* species suggests that it is eurythermal and euryhaline, but moderate temperatures may create the most favorable conditions for blooming. A combination of moderate temperature, salinity, and nutrient concentrations may create favorable circumstances for blooms of this species, indicating that *N. incerta* is a mesotrophic benthic diatom.

The growth pattern of *G. marina* also seems to be eurythermal and euryhaline with eutrophic habitat as the maximum specific growth was found to be occurred in a condition with high temperature (25° C), higher salinity (35 psu) and maximum nutrient concentrations (F = 200%), indicating that this is an eutrophic benthic diatom. Affan and Lee (2004) reported that *G. marina* was abundant about 20% among the phytoplankton population in the Jeju Island coastal water at higher temperature and high salinity with available nutrient concentrations.

Thus, we suggest that the maximum standing crop of *N. incerta* and *G. marina* in commercial aquaculture can be obtained from the water temperature 20 to 25°C with moderate to higher salinity and with sufficient nutrient concentrations. It is well known that maximum productivity can only be achieved under optimal growth conditions. With suitable climatic conditions and sufficient nutrients, the microalgae grow profusely.

However, the growth rate of *N. incerta* and *G. marina* seems to be faster in the euryhaline and eurythermal environment with available of nutrient concentrations (Affan et al. 2006). The attachment of the mono-strain benthic diatoms film on the plate seemed to be strong and thick with dark brown colour even though after a gently washing, as it was washed with seawater which was providing to the abalone culture tank from the sea through tap. These diatoms can be considered as a suitable live feed for abalone aquaculture, since good diatoms are defined as ones that produce a large amount of secretion and from sheets (Hahn 1989).

5.3. *Abalone settlement, growth, live feed consumption and Benthic diatoms species composition*

The mono-strain benthic diatoms live feeds were superior to mixed wild strain diatoms in terms of survival rate and specific growth rate of abalone larvae. The survival rate was more in the tanks of mono-strains benthic diatoms plate than that of the wild mixed-strain diatoms. The larval rearing for the settlement in wild mixed-stain diatoms tanks may have experienced higher mortality due to the inability of getting appropriate size of diatoms as live feed as the wild mixed strains plate having bigger and heterogeneous size of diatoms. The big size benthic diatoms were 62.60% among the total benthic diatoms on the wild mixed-strain benthic diatoms plate. The abalone larvae on wild mixed-strain benthic diatoms plate might have feed the rest of 37.4% (*N. incerta* 13.38%, *G. marina* 13.55% and *N. viridula* 10.47%) smaller size benthic diatoms which was not sufficient for them or they had to spend more energy to search those smaller size diatoms or they might have got nutrients from the extracellular substances rather than the absorption of the cellular contents and may be that was not sufficient for them. This may be the one of the reason for the lower number of abalone larval settlement on the wild mixed-strain benthic diatoms plate.

At the end of the study, the abundance of relatively bigger size benthic diatoms such as *C. closterium* and *N. incerta* increased. Kawamura et al. (1998) reported that it is a difficult task to maintain a supply of readily ingestible and digestible food (diatoms) for growing abalone postlarvae. Several problems have been identified and documented in regards of raising abalone on diatom biofilms as the management of these diatom biofilms is often challenging and Hahn (1989). Control of the initial food supply is still one of the most critical problems in hatchery seed production Seki (1997). In our experiments with wild mixed-strains diatoms tanks, may be the abalone larvae also ingested the small size diatoms, as we found that the abundance of small size diatoms such as *N. incerta, N. viridula, A. decussata* decreased where as the bigger size such as *C. closterium* and *N. longissima* increased. Weekly supplied F/2 medium and metasilicate to the abalone culture tanks may

have created favourable condition for the continuous growth of diatoms but that was for both sizes of diatoms, small and large. The reducing of small size diatoms abundance is indicating that those diatoms are suitable for abalone larval settlement and growth.

On the other hand, the higher survival and growth rate of abalone larvae in the tanks of mono-strain diatoms than in the tanks of wild mixed-strains is indicating that small structure diatoms with homogeneous size might have created good circumstance as a suitable feed for their settlement and growth. *N. incerta* and *G. marina* were dominant 69.82 and 65.49% as a single species in total benthic diatoms of mono-strain tank of *N. incerta* and *G. marina* at the end of the experiment. In the mono-strain benthic diatoms tanks abalone larvae might have got nutrition from the whole cell of diatoms after feeding as well as from the extracellular substances. However, the abalone spat in the tank with *N. incerta* showed best growth than *G. marina* and wild-mixed strain. Naturally recruited diatoms films have long been used to induce larval settlement in abalone hatcheries around the world, but with a limited awareness or management of suitable settlement-inductive species and the properties that make them attractive to abalone larvae (Roberts 2001). Understanding the factors that regulate effective settlement, post larval growth, and the selection of cultivable diatoms that promote it, are therefore essential (Roberts 2001). Therefore, it is suggested small size diatoms with thick mats on the substrate or plate can be the good food item for the abalone larval settlement and growth. Further experiment can be conducted by using several benthic diatoms mono-strain of smaller and big size, and mixed of both smaller and big size diatoms as a live feed to find out the feed preference of abalone larvae for the settlement and growth.

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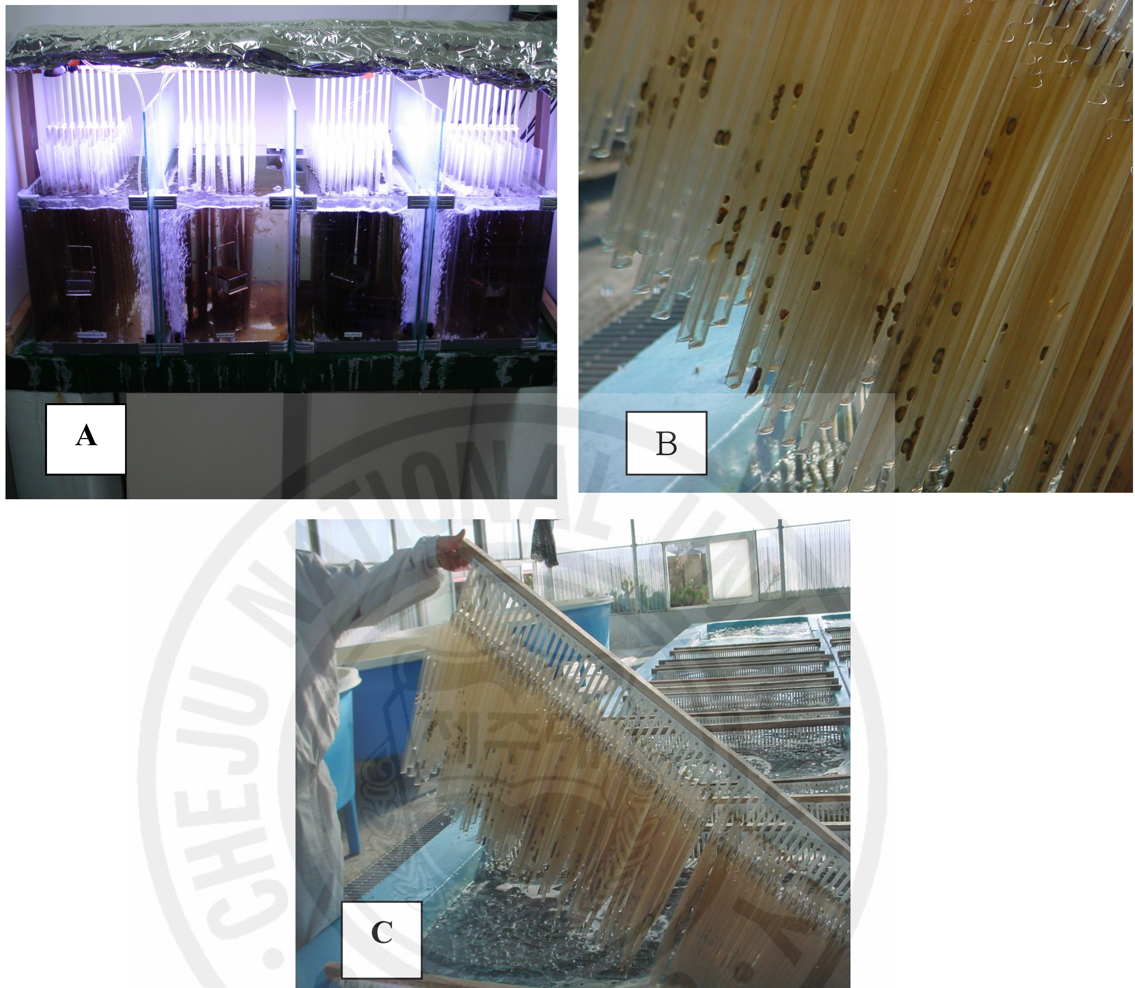

Fig.15. The indoor mass culture of benthic diatoms (A), settled abalone spats on the mono-strain plates of *G. marina* and *N. incerta* (B, C).

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Table 8. Fluctuation of benthic diatoms abundance [$\times10^5$ cells (surf.cm^2)$^{-1}$] and species composition rate (%) during the study period in the abalone larval rearing tank of *N. incerta* (Treatment 1), *G. marina* (Treatment 2) mono-strain and wild mixed-strain benthic diatoms (control)

Date	Identified benthic diatoms	Treatment 1 (*N. incerta*)		Treatment 2 (*G. marina*)		Control	
	Species	$\times10^5$ cells (surf.cm^2)$^{-1}$	(%)	$\times10^5$ cells (surf.cm^2)$^{-1}$	(%)	$\times10^5$ cells (surf.cm^2)$^{-1}$	(%)
May 16, 2005	*Navicula incerta*	6.43 ± 0.25	100				
	Grammatophora marina			6.78 ± 0.39	100		
May 18, 2005	*Amphora coffeaeformis*					5.12 ± 0.11	7.26 ± 0.33
	Amphora decussate	0.07 ± 0.02	1.03 ± 0.02			5.33 ± 0.22	7.55 ± 0.43
	Bacillaria paxillifer			0.01 ± 0.02	0.14 ± 0.01	4.59 ± 0.11	6.51 ± 0.51
	Cocconeis scutellum					3.69 ± 0.35	5.23 ± 0.12
	Cylindrotheca closterium	0.09 ± 0.01	1.32 ± 0.04	0.10 ± 0.12	1.39 ± 0.04	4.39 ± 0.16	6.22 ± 0.36
	Grammatophora marina	0.05 ± 0.03	0.73 ± 0.05	6.81 ± 0.39	96.79 ± 0.07	9.56 ± 0.08	13.55 ± 0.25
	Gyrosigma balticum					4.44 ± 0.09	6.29 ± 0.60
	Licmophora paradoxa			0.05 ± 0.06	0.84 ± 0.03	3.97 ± 0.09	5.63 ± 0.29
	Navicula incerta	6.49 ± 0.26	95.30 ± 0.07	0.04 ± 0.02		9.44 ± 0.08	13.38 ± 0.25
	Navicula viridula					7.39± 0.67	10.47 ± 0.92
	Nitzschia longissima	0.08 ± 0.02	1.17 ± 0.04			1.37 ± 0.20	1.94 ± 0.16
	Nitzschia socialis	0.03 ± 0.01	0.44 ± 0.02	0.06 ± 0.26	0.84 ± 0.03	5.03 ± 0.11	7.13 ± 0.51
	Pleurosigma normanii					2.45 ± 0.22	3.47 ± 0.30
	Synedra tabulate					3.79 ± 0.11	5.37 ± 0.22
July 15, 2005	*Amphora coffeaeformis*					0.22 ± 0.09	6.65 ± 0.09
	Amphora decussate	0.12 ± 0.01	4.36 ± 0.03	0.09 ± 0.02	3.71 ± 0.01	0.10 ± 0.07	3.02 ± 0.11
	Bacillaria paxillifer	0.08 ± 0.05	2.91 ± 0.04	0.05 ± 0.05	1.76 ± 0.02	0.22 ± 0.05	6.65 ±0.10
	Cocconeis scutellum	0.07 ± 0.02	2.55 ± 0.02	0.07 ± 0.03	2.46 ± 0.01	0.13 ± 0.08	3.93 ± 0.12
	Cylindrotheca closterium	0.14 ± 0.01	5.09 ± 0.05	0.15 ± 0.01	5.28 ± 0.03	0.64 ± 0.12	19.34 ± 0.06
	Grammatophora marina	0.13 ± 0.04	4.73 ± 0.07	1.86 ± 0.07	65.49 ± 0.05	0.24 ± 0.07	7.25 ± 0.08
	Gyrosigma balticum					0.15 ± 0.09	4.53 ± 0.05
	Licmophora paradoxa	0.08 ± 0.02	2.91 ± 0.06	0.12 ± 0.03	4.23 ± 0.01	0.24 ± 0.05	7.25 ± 0.09
	Navicula incerta	1.92 ± 0.01	69.82 ± 0.04	0.10 ± 0.02	3.52 ± 0.04	0.21 ± 0.02	6.34 ± 0.12
	Navicula viridula			0.09 ± 0.03	3.17 ± 0.03	0.24 ± 0.06	7.25 ± 0.03
	Nitzschia longissima	0.12 ± 0.03	4.36 ± 0.05	0.12 ± 0.04	4.23 ± 0.02	0.33 ± 0.11	9.97 ± 0.11
	Nitzschia socialis	0.09 ±0.02	3.27 ± 0.06	0.08 ± 0.01	2.82 ± 0.02	0.23 ± 0.03	6.95 ± 0.09
	Pleurosigma normanii			0.05 ± 0.02	1.76 ± 0.01	0.08 ± 0.07	2.42 ± 0.07
	Synedra tabulate			0.06 ± 0.01	2.11 ± 0.04	0.12 ± 0.02	3.63 ± 0.06
	Thalassiothrix longissima					0.16 ± 0.10	4.83 ± 0.04
	Total	**2.75 ± 0.09**	**100**	**2.84 ± 0.11**	**100**	**3.31 ± 0.09**	**100**

Table 9. Number of plates to each tank, amount of released abalone larvae to each tank, life feed consumption rate by each abalone day^{-1} (%) and average number of settled abalone spats in each tank, survival rate of abalone in each tank, initial length and final length, and specific growth rate of abalones in the tank of *N. incerta* (Treatment 1), *G. marina* (Treatment 2) mono-strain and wild mixed-strain benthic diatoms (control) during the study period

	No. plates	Initial No. of abalone larvae	Final No. of abalone larvae	Survival rate (%)	LFC (%)	Initial length mm	Final length mm	Specific growth rate (SGR) %
Treatment 1	100	50,000	1000	2.00^{b}	0.17	0.35 ± 0.01	2.51^{a}±0.04	3.28^{a} ± 0.02
Treatment 2	100	50,000	1080	2.16^{a}	0.15	0.35 ± 0.01	2.21^{b}±0.06	3.07^{b} ± 0.04
Control	100	50,000	640	1.28^{c}	0.26	0.35 ± 0.02	2.02^{c}±0.02	2.92^{c}± 0.02

Values followed by the superscript letters a, b, and c indicate a significant difference (One way ANOVA and Tukey's test, $p<0.05$).

Part IV

Estimation of biochemical composition and antioxidant properties of the benthic diatom *Navicula incerta* and *Cylindrotheca closterium* (Bacillariophyceae) from Jeju Island

1. Abstract

Benthic diatoms are a commonly used as a live feed in shellfish aquaculture. Apart from the aquaculture use, the biochemical composition and antioxidant activities were determined from *N. incerta* and *C. closterium*. The proximate composition was as follows: protein 7.0% and 11.11%, lipid 1.7% and 1.18%, carbohydrate 12.8% and 18.51%, ash 68.4% and 61.86%, and moisture 10.0% and 7.34%, respectively. The antioxidant properties of *N. incerta* and *C. closterium* were determined for various enzymatic and methanol extracts which were prepared by using digestive enzymes and methanol, respectively, as tools for extractions. The scavenging rates of Neutrase and methanol extracts against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical were 81.6% and 62.8% from the extract of *N. incerta*, respectively. The *C. closterium* showed 72.5% and 69.4% DPPH scavenging activity from the extract of Viscozyme and methanol. Flavourzyme extract of *N. incerta* had a superoxide scavenging rate of 57.7%. Kojizyme and Ultraflo extracts had nitric oxide scavenging rates of 42.2% and 40.6%, respectively that were significantly higher than the commercial antioxidants such as α-tocopherol and BHT. The metal-chelating activities of the methanol, Neutrase, and Termamyl extracts of *N. incerta* were 68.5%, 45.2%, and 41%, respectively, four to six times higher than the commercial antioxidants. The Kojizyme, Alcalase, methanol, Viscozyme and Neutrase extracts from *C. closterium* were 67.1%, 53.9%, 53.2% and 50.2% for metal chelating, respectively. The Termamyl extract of *N. incerta* and AGM, Viscozyme and Neutrase extracts of *C. closterium* showed the highest linoleic acid peroxidation inhibition, exceeding α-tocopherol and on par with BHT.

2. Introduction

Algae make up roughly half of the global primary productivity (Field et al. 1998) and provide the foundation of aquatic food webs from ponds to oceans. Among the marine algae, diatoms are the major component of phytoplankton, and also serve as an essential food source in aquaculture (St. John et al. 2001). Compared to other groups of microalgae, diatoms seldom cause ecological problems (Officer and Ryther 1980). However, microalgae are also increasingly being promoted in the human diet as nutraceutical and health food products. The use of *Chlorella* and *Spirulina* spp. extracts as supplements in noodles, bread, green tea, beer, and candy has been proposed (Liang et al. 2004). Several microalgae, such as *Chlorella*, *Spirulina*, and *Dunaliella* spp., are grown commercially for their pigments, such as β-carotene and phycocyanin. In the search for new compounds with therapeutic potential, microalgae in all classes have demonstrated antibacterial, antifungal, and anticancer properties (Metting 1996, Tredici 2004).

Reactive oxygen species (ROS), such as superoxide, hydroxyl, peroxyl, and nitric oxide radicals, attack biological molecules, including lipids, proteins, enzymes, DNA, and RNA, resulting in cell or tissue injuries associated with atherosclerosis and carcinogenesis. In addition, oxidation is a major cause of food quality deterioration, which destroys fat-soluble vitamins and fosters the development of toxicants and discoloring (Yan et al. 1999, Ukeda et al. 2002). Antioxidants scavenge free radicals and reactive oxygen species, and catalyze metal chelation (Shahidi and Wanasundara 1992). Commercial antioxidant supplements, such as α-tocopherol, butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ) are used to increase the food storage life by reducing oxidation (Sherwin 1990, Gülçin et al. 2002). However, some synthetic antioxidants have negative side effects, and attention on development of antioxidants from natural sources is being increased. Natural antioxidants have been isolated from various plants, but microalgae have received less attention as a

source of natural antioxidants. *C*-phycocyanin from the blue-green alga *Arthrospira maxima* has shown high antioxidant activity in scavenging alkoxyl and hydroxyl radicals (Romay et al. 1998). Carotenoid of *Dunaliella salina* showed beneficial effect compared to synthetic carotene as antioxidant (Murthy et al. 2005).

On Jeju Island, Republic of Korea, benthic diatoms are used as live food in commercial shellfish hatcheries. Affan and Lee (2004) found available abundance of *Navicula* spp. and *Cylindrotheca closterium* benthic diatom throughout the year in the coastal water of Jeju Island. Apart from the aquaculture used of benthic diatoms, we determined the biochemical composition and antioxidant activities of the two benthic diatoms with the extracts of enzymes and methanol. Thus, we isolated an axenic strain of *Navicula incerta* and *Cylindrotheca closterium*, determined the optimal environmental conditions for growth, did mass culture based on the maximum specific growth condition (details in Part II). The objectives of this study were to determine the biochemical composition and antioxidant activities of benthic diatoms; *N. incerta* and *C. closterium*.

3. Materials and methods

3.1. *Biochemical composition*

The biochemical composition of *N. incerta* and *C. closterium* was determined following AOAC guidelines (AOAC 1995). Crude lipid content was determined by Soxhlet extraction; crude protein, by the Kjeldahl method; ash content, by calcination in a furnace at 550˚ C; and moisture content, by heating to 105˚ C for 24 h.

3.2. *Chemicals for aantioxidant assays*

The digestive enzymes; Viscozyme L, Celluclast 1.5 L FG, AMG 300 L, Termamyl 120 L, Ultraflo L, Protamex, Kojizyme 500 MG, Neutrase 0.8 L, Flavourzyme 500 MG, and Alcalase 2.4 L FG were purchased from Novo Nordisk, (Bagsvaerd, Denmark). The 1,1-

diphenyl-2-picrylhydrazyl (DPPH), sodium nitroprusside, xanthine, xanthine oxidase from buttermilk, nitro blue tetrazolium salt (NBT), BHT, α-tocopherol, 3-(2-pyridyl)-5,6-di (p-sulfophenyl)-1,2,4-triazinedisodium salt (ferrozine), potassium ferricyanide [$K_3Fe(CN)_6$], Folin-Ciocalteu reagent, and linoleic acid were purchased from Sigma. The 2, 2-azinobis (3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) and 2-deoxyribose were purchased from Fluka Chemie AG, (Buchs, Switzerland). All other chemicals used were analytical grade, supplied by Fluka or Sigma.

3.3. *Preparation of 80% methanolic and enzymatic extract from* N. incerta and C. closterium

The freeze-dried microalgal sample was ground to a fine powder. For the methanol extraction, the powdered sample (5 g) was extracted with 500 mL of 80% methanol at 25˚ C for 24 h. The methanolic extract was collected by filtering, and concentrated by vaccum evaporator. For the enzyme extracts, 1 g of freeze-dried sample was mixed with 100 mL of distilled water. The pH of each reaction mixture for optimal hydrolysis of each enzyme was adjusted with 1 M HCl/NaOH. The enzymatic extracts were obtained according to the method described by Heo at al (2005). The optimum pH and temperature for the respective enzymes were similar to those reported by Heo et al. (2003) (Table 10). Each enzyme was added to microalgal sample at a 1% ratio and incubated for 24 h. These mixtures were filtered, and the enzyme activity of the hydrolysates was inactivated by heating (100˚ C for 10 min). Finally, the pH of each hydrolysate was adjusted to 7.0 with HCl/NaOH. Both the methanolic and enzymatic extracts were adjusted to a final concentration of 2 mg mL^{-1}. The activity of the extracts was compared to the commercial antioxidants such as BHT and α-tocopherol dissolved in ethanol (2 mg mL^{-1}).

3.4. ***DPPH free radical scavenging assay***

This assay involved the scavenging of stable DPPH radical by the radical scavenging components of *N. incerta* extracts, using a modified method of Brand-Williams et al. (1995). A 2-mL fraction of extract was mixed thoroughly with 2 mL freshly prepared DPPH solution 3×10^{-5} M in dimethyl sulfoxide (DMSO). The reaction mixture was incubated for 1 h, and the absorbance of the supernatant was measured at 517 nm using a UV–VIS spectrophotometer (Opron 3000; Hanson Tech. Co. Ltd., Seoul, Republic of Korea).

3.5. ***Superoxide anion scavenging assay***

The superoxide anion scavenging effect was tested following Nagai et al. (2003). The reaction mixture consisted of 0.48 mL of 0.05 M sodium carbonate buffer (pH 10.5), 0.02 mL 3 mM xanthine, 0.02 mL of 3 mM EDTA, 0.02 mL of 0.15% bovine serum albumin, 0.02 mL of 0.75 mM NBT, and 0.02 mL extract. After incubation at 25˚ C for 10 min, the reaction was started by adding 6 mU xanthine oxidase, and maintained at 25˚ C for 20 min. The reaction was stopped by adding 0.02 mL of 6 mM $CuCl_2$. The absorbance was measured in a Sunrise microplate reader (Tecan Co. Ltd., Salzburg, Austria) at 560 nm.

3.6. ***Hydrogen peroxide scavenging assay***

Hydrogen peroxide scavenging activity was determined following Rice-Evans et al. (1995). A sample was prepared in a 96-microwell plate by mixing 80 μL extract, 100 μL phosphate buffer (0.1 M, pH 5.0), and 20 μL mM H_2O_2, and incubated at 37˚ C for 5 min. Then, 30 μL ABTS (1.25 mM) and 30 μL peroxidase (1unit mL^{-1}) were added, and the mixture was incubated at 37˚ C for 10 min. The absorbance was recorded at 405 nm.

3.7. ***Hydroxyl radical scavenging assay***

The hydroxyl radical scavenging effect was determined according to Chung et al. (1997). A Fenton reaction mixture (200 μL of 10 mM $FeSO_4.7H_2O$, 200 μL of 10 mM EDTA, and 200 μL of10 mM 2-deoxyribose) was mixed with 1.2 mL of 0.1 M phosphate buffer (pH 7.4) and 200 μL extract. Then, 200 μL of 10 mM H_2O_2 was added, and the mixture was incubated at 37˚ C for 4 h, after adding 1 mL of 2.8% TCA and 1 mL of 1% TBA, and the mixture was kept in a bath of boiling water for 10 min. After cooling, the mixture was centrifuged for 5 min at 395 × g, and the absorbance was measured at 532 nm.

3.8. ***Nitric oxide radical inhibition assay***

The nitric oxide radical inhibition effect was determined following Garrat (1964). A mixture of 2 mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) and 0.5 mL extract was incubated at 25˚ C for 150 min. From this, 0.5 mL was removed and added to 1.0 mL sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 mL naphthylethylenediamine dihydrochloride (0.1% w/v) was added, and the mixture was incubated at room temperature for 30 min. The absorbance was measured at 540 nm.

3.9. ***Metal-chelating assay***

Metal-chelating ability was determined according to Decker and Welch (1990). The mixture sample was prepared by adding 5 mL extract to a solution of 0.1 mL of 2 mM $FeCl_2$. The reaction was started by the addition of 0.2 mL of 5 mM ferrozine solution. The reaction mixture was incubated, with shaking, at room temperature for 10 min. The absorbance was measured at 562 nm.

3.10. ***Determination of antioxidant activity using the ferric thiocyanate (FTC) method***

This assay was performed following Kikuzaki and Nakatani (1993). The mixture consisted of 2 mL (100 μg L^{-1}), 2 mL of 2.51% linoleic acid in ethanol, 4 mL of 0.05 M phosphate buffer (pH 7.0), and 2 mL distilled water, was kept in the dark at 40˚ C. From this mixture, 0.1 mL was added to 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate. After 5 min, 0.1 mL of 0.02 M ferrous chloride in 3.5% HCl was added. The absorbance was measured every 24 h for 7 days.

3.11. ***Determination of total phenolic content***

The total phenolic content was determined using a protocol similar to that of Chandler and Dodds (1993). The mixture consisted of 1 mL *N. incerta* extract, 1 mL 95% ethanol, 5 mL distilled water, and 0.5 mL 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min, and then 1 mL 5% Na_2CO_3 was added. The sample was thoroughly mixed and placed in the dark for 1 h. The absorbance was measured at 725 nm.

3.12. ***Scavenging activity/chelating ability calculation***

The radical scavenging activity/chelating ability was calculated using the following equation:

$[1-(A_i - A_j)/Ac] \times 100$

,where A_i is the absorbance of the extract mixed with reactive oxygen or nitrogen compounds, A_j is the absorbance of the extract without the reactive compounds, and A_c is the absorbance of the control.

3.13. ***Statistical analysis***

All the experiments were conducted in triplicate (n = 3). Statistical analyses were performed using SPSS Statistical Software, version 11.5 (Edinburgh, Scotland). The mean

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values of each treatment were compared using one-way analysis of variance (ANOVA) followed by Tukey's tests. P-value of less than 0.05 was considered as significant.

l4. Results

4.1. *Proximate composition*

The proximate composition of *N. incerta* was as follows: protein 7.0%, lipid 1.7%, carbohydrate 12.8%, ash 68.4%, and moisture 10.0%, on a dry-weight basis. The protein, lipid, carbohydrate, ash and moisture content of *C. closterium* was 11.11%, 1.18%,18.51%, 61.86% and 7.34%, respectively.

4.2. *Antioxidant activities*

The DPPH free radical scavenging rate was 27.3%–81.6% among the methanolic and enzymatic extracts (five proteases and five carbohydrases) of *N. incerta*. The Neutrase extract showed the highest activity (81.6%), followed by the methanol (62.8%), Ultraflo (49.6%), and Protamex (47.3%) extracts. The remaining extracts had less scavenging activity (Table 12). The DPPH free radical scavenging rate of *C. closterium* varied from 31.1 to 72.5% among the methanolic, water and enzymatic *extracts*. The Viscozyme extract of *C. closterium* showed the highest activity (72.5%), followed by the methanol (69.4%), Ultraflo (63.8%), Neutrase (60.8%), water (59.3%) and Protamex (58.2%) extracts.. The remaining extracts had less activity (Table 12). In H_2O_2 scavenging activity, the methanolic extract of *N. incerta* exhibited a significantly higher scavenging effect (27.6%) than the other extracts, but it was significantly lower than the commercial antioxidants (Table 11). All kinds of extract from *C. closterium* also showed significantly lower activities comparing to the commercial antioxidants (Table 12). The superoxide scavenging rate was 16.8%–57.3% among the different extracts of *N. incerta*, with Flavourzyme (57.3%) showing the highest, followed by Celluclast (51.3%), AMG (44.5%), and Termamyl (43.2%). The Flavourzyme extract

activity was statistically similar to the commercial antioxidants (Table 11). The superoxide scavenging activity of *C. closterium* varied from 29.1 to 53.0% among the extracts, with methanolic extract (53.0%) showing the highest, followed by water (50.0%), AMG (47.8%), and Kojizyme (42.8%). The Viscozyme extract of *N. incerta* exhibited the highest hydroxyl radical scavenging activity (21.6%). The hydroxyl scavenging rate of *C. closterium* was 10.5 to 58.3% among the all extracts with the methanolic extract showing the highest (58.3%), followed by water extract (49.5%) (Table 12). All extracts of *N. incerta*, except the methanolic and Protamex extracts, had significantly higher nitric oxide scavenging effects than the commercial antioxidants, and the Kojizyme extract exhibited the highest scavenging activity (42.16%), followed by Ultraflo (40.6%) and Flavourzyme (39.5%) (Table 11). The nitric oxide activity of *C. closterium* was 31.5-45.8% among the all extracts; and AGM (45.8%) and Viscozyme (45.5%) extract showed higher nitric oxide scavenging activities among the all enzymatic extracts, followed by methanolic (44.8%) and water (44.4%) extracts. The scavenging rate with AGM and Viscozyme was significantly higher than the BHT and lower than α-tocopherol, and the activity from methanolic and water extracts of *C. closterium* was statistically similar to BHT (Table 12).

All of the extracts of *N. incerta* had strong metal-chelating effects that were significantly higher ($P<0.05$) than those of α-tocopherol and BHT (11.5% and 10.3%, respectively). The methanolic extract exhibited a higher metal-chelating effect (68.5%) than the enzymatic extracts. Among the enzymatic extracts, Neutrase showed the highest scavenging activity (45.2%), followed by Termamyl (41.2%), and Ultraflo (33.3%) (Table 11). All the extracts of *C. closterium* showed 2~6 times higher scavenging activity than the commercial antioxidants. The activity rate was 23.5-67.1% among all the enzymatic, methanolic and water extracts. The highest activity was observed from Kojizyme (67.1%), followed by Alcalase (53.9%), methanolic (53.2%) and Viscozyme (52.1%) (Table 12).

4.3. *Lipid peroxidation inhibiting activity*

Antioxidant activity with ferric thiocyanate was determined lipid peroxidation inhibiting effect of all the extract from microalgae at 40˚ C for 7 days. As shown in Fig. 16, the linoleic acid emulsion without *N. incerta* extract (the control) resulted in an increase in lipid peroxidation, and the absorbance increased significantly ($P<0.05$). Among all the extracts, the Termamyl, Flavourzyme, AMG, and Ultraflo extracts exhibited notable antioxidant activity, which exceeded that of α-tocopherol. No significant difference was observed between the activity of BHT and the Termamyl extract ($P<0.05$). None of the remaining extracts showed notable antioxidant activity; compared to the commercial antioxidants. Among all the extracts of *C. closterium*, the AMG, Viscozyme and Neutrase extract exhibited notable antioxidant activity, which exceeded that of α-tocopherol (Fig. 17).

4.4. *Total phenolic content*

The total phenolic content of the various extracts varied with the solvents and enzymes used. For *N. incerta*, the Neutrase extract exhibited the highest polyphenol content (951.2 mg/100 g), followed by Kojizyme (886.1 mg/100 g), and Ultraflo (742.1 mg/100 g; Table 1). The total phenolic content varied from 358 to 642 mg/100g among the various enzymatic, methanolic and water extract of *C. closterium* with the average of 517 mg/100g, and the Neutrase extract exhibited the highest polyphenol content (642.8 mg/100 g), followed by Ultraflo (623.9 mg/100 g) and Kojizyme (553.1 mg/100 g; Table 2).

5. Discussion

The DPPH free-radical scavenging assay is widely used to evaluate the antioxidant efficacy of plant extracts. Free radicals are continuously produced in cells, either as metabolic by-products or protectors against phagocytic pathogens (Cheeseman and Slater 1993). In our study, the methanolic extract of *N. incerta*, and some enzymatic extracts,

especially Neutrase, exhibited higher free-radical scavenging activity than commercial antioxidants. For *C. closterium*, the methanolic and Viscozyme extracts exhibited higher free-radical scavenging activity than the commercial antioxidants. Free-radical scavenging activity may increase with phenolic content (Oki et al. 2002). Our results agree, as the total polyphenolic content, extracted by the hydrolytic action of carbohydrases and proteases (*N. incerta*, showed a high correlation r = 0.76 and 0.87, and from C. closterium showed r = 0.79 and 0.82, respectively) with free-radical scavenging activity. We assume that these enzymes might be useful toold in extracting bioactive compounds from *N. incerta* and *C. closterium* which are responsible for the free radical scavenging effect.

The H_2O_2 scavenging compounds in these extracts obviously have both hydrophilic and hydrophobic properties. H_2O_2, a reactive non-radical compound, can penetrate biological membranes. Although H_2O_2 itself is not very reactive, it may convert into more reactive species, such as singlet oxygen and hydroxyl radicals (Halliweill 1991). Thus, removing H_2O_2 is very important for the protection of living systems. The hydroxyl radical is the most reactive oxygen species, because of its strong affinity for various biomolecules. It can extract hydrogen atoms from phospholipid membranes and perform peroxidic reactions with lipids (Kitada et al. 1979). Although carbohydrase hydrolysates of *N. incerta* showed very high correlations (r = 0.83 and 0.89) with polyphenolic compounds, the hydrogen peroxide and hydroxyl radical scavenging by this species; the activities were lower in comparison to the commercial antioxidants. Thus, the bioactive compounds extracted with carbohydrases may be less effective in scavenging hydrogen peroxide and hydroxyl radical. Among all the extracts of *C. closterium* the methanolic extract showed the higher H_2O_2 scavenging activity, but that was inferior to standard antioxidants. Thus, the activity observed in *C. closterium* from methanolic extract that contains methanolic soluble antioxidants posseses a potential to decrease the pro-oxidants like H_2O_2.

The effects of the superoxide anion can be exaggerated, as it produces other kinds of cell-damaging free radicals and oxidizing agents (Liu and Ng 2000). Superoxide anion has

been implicated in several pathophysiological processes, because of its conversion into more reactive species, such as hydrogen peroxide, singlet oxygen, and the hydroxyl radical, which initiate oxidative damage in lipids, proteins, and DNA (Pietta 2000). The superoxide anion is an oxygen-centered, selectively reactive radical produced by a number of enzyme systems in autooxidation reactions and by non-enzymatic electron transfers that reduce molecular oxygen univalently. It can also reduce certain iron complexes, such as cytochrome *c*. The superoxide anion scavenging potential of *N. incerta* and *C. closterium* enzymatic extracts was greater than that of the methanolic extract, but inferior to α-tocopherol and BHT. In this assay, even the methanol extract indicated a good scavenging activity against superoxide radicals. Superoxide radical is not only formed in the body but also the early products of protein glycation, such as the Schiff base and Amadori compound which may be the key structural components involved in the generation of superoxide radicals (Ukeda et al. 2002). Therefore, *N. incerta* and *C. closterium* can be an important source for superoxide anion scavenging in the medicinal and food fields.

Nitric oxide is a gaseous free radical, which has important functions in physiological and pathological conditions, such as renal injuries. Marcocci et al. (1994) reported that nitric oxide scavengers compete with oxygen, resulting in a lower production of nitric oxide. Both the carbohydrase and protease *N. incerta* extracts showed high nitric oxide scavenging activity, and were significantly higher than standard commericial antioxidants. The Kojizyme and Ultraflo extracts of *N. incerta* had nearly two times higher nitric oxide scavenging ability than the commercial antioxidants. The methanolic, water and AGM extracts of *C. closterium* showed statistically similar nitric oxide scavenging activity to BHT, but inferior to α-tocopherol. The metabolite ONOO-(peroxynitrite) is extremely reactive, directly inducing toxic reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation, and DNA modifications (Moncada et al. 1991, Radi et al. 1991). Thus, *N. incerta* may be a good source of bioactive compounds that can scavenge nitric oxides.

Metal-chelating activity can reduce the concentration of catalyzing transition metals in lipid peroxidation (Duh et al. 1999). Ferrozine complexes with ferrous ions can initiate lipid peroxidation by the Fenton reaction, and accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals (Halliweill 1991, Fridovich 1995). Furthermore, chelating agents that form σ-bonds with a metal are effective as secondary antioxidants, because they reduce the redox potential and thereby stabilize the oxidized form of the metal ion (Gordon 1990). The methanolic *N. incerta* and *C. closterium* extract and the enzymatic extracts had a six-fold and 2~4 folds higher metal chelating activity than the commercial antioxidants. These results suggest that this species has a high ability to bind iron.

To evaluate the antioxidant effects of *N. incerta* and *C. closterium* extracts, we compared their lipid peroxidation inhibitiory activity with that of the commercial antioxidants, using the ferric thiocyanate (FTC) method to determine the amount of peroxide-formed emulsion during incubation period. A high absorbance indicates a high concentration of the formed peroxides. Termamyl, Flavozyme, AMG, and Ultraflo extracts had the highest antioxidant effects among the extracts of *N. incerta*, and *C. closterium* extracted with AGM, Viscozyme and Neutrase showed the higher antioxidants scavenging rate, demonstrating that these extracts reduced peroxide formation. This suggests that digestion by different enzymes releases different antioxidant components from microalgal cells. These extracts contained high levels of polysaccharides, proteins, and polyphenols, and we suggest that these components may influence antioxidant effects.

The total phenolic content in the extracts depended on the polarity of the solvent used. Although the Neutrase extract had the highest polyphenol content, it did not show the highest activity in every assay, indicating that both the content and properties of polyphenolic compounds in different extracts could contribute to different activities. In addition, polyphenolic compounds have inhibitory effects on mutagenesis and

carcinogenesis in humans (Tanaka et al. 1998). Phenols are major plant constituents, owing to the scavenging ability of their hydroxyl groups (Hatano 1995). Many studies have reported a highly positive correlation between total phenol content and antioxidant activity in various plant species (Vinson et al. 1998, Oktay et al. 2003), though we did not find this correlation.

The cells of *N. incerta* and *C. closterium* contain a large amount of soluble polysaccharides and insoluble fibers, such as cellulose. Both insoluble and soluble fibers, together with other cell wall materials, act as a physical barrier to the extraction of bioactive materials. Bioactive gel compounds in gel matrices also interfere with the extraction of bioactive compounds and lead to poor solubility in aqueous media. Surmounting such barriers could allow desirable compounds to be extracted in high yields. The enzymatic hydrolysis of tissues or cells used as raw material has led to significant yields of certain compounds and industrial approaches in extraction and purification (Jeon et al. 2000, Nagai and Suzuki 2000). Also, the breakdown and release of high molecular weight polysaccharides and proteins may contribute to enhancing antioxidative activity (Ramos and Xiong 2002, Ruperez et al. 2002). Moreover, enzymatic extraction possesses innovative advantages over conventional extraction procedures.

Except in aquaculture, diatom culture for biotechnological applications is still at an early stage of development. Further progress will depend on future needs and uses, along with advances in cultivation, including lower costs and sustainable techniques. *N. incerta* and *C. closterium* showed more antioxidant activity in DPPH, superoxide, nitric oxide, and the metal-chelating assays than the commercial antioxidants. These results indicate that the microalgae may be good candidates for natural antioxidant sources, and could be applied in the functional food field. Further studies are necessary to isolate and purify the biochemical compounds responsible for antioxidant effects, and to characterize their *in vivo* antioxidant activity and related antioxidant mechanisms.

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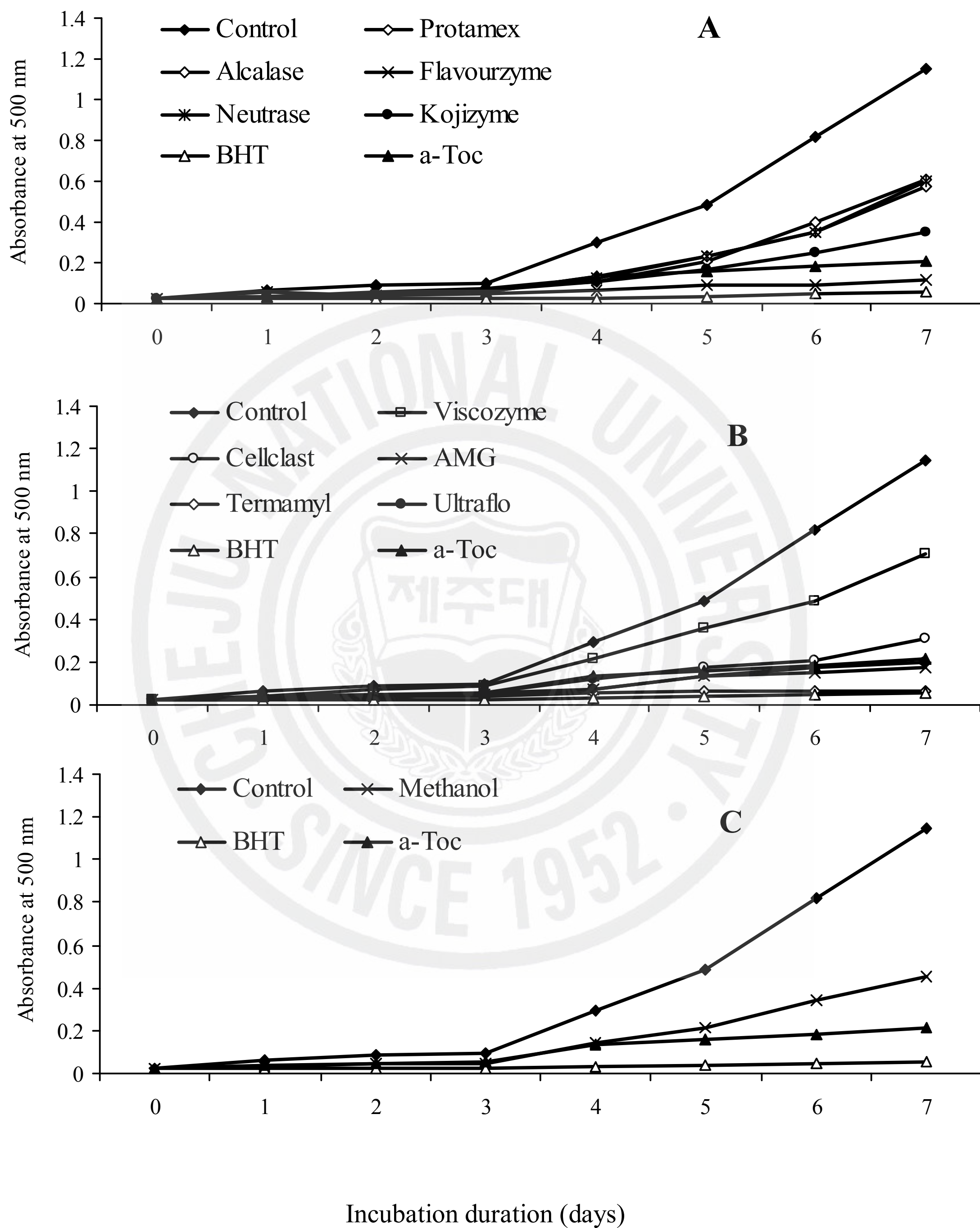

Fig.16. Antioxidant activities of protease (A), carbohydrase (B) and 80% methanolic (C) extracts of *Navicula incerta*, compared to a-Tocopherol and BHT at 1 mg/mL ethanol, as assessed by linoleic acid.

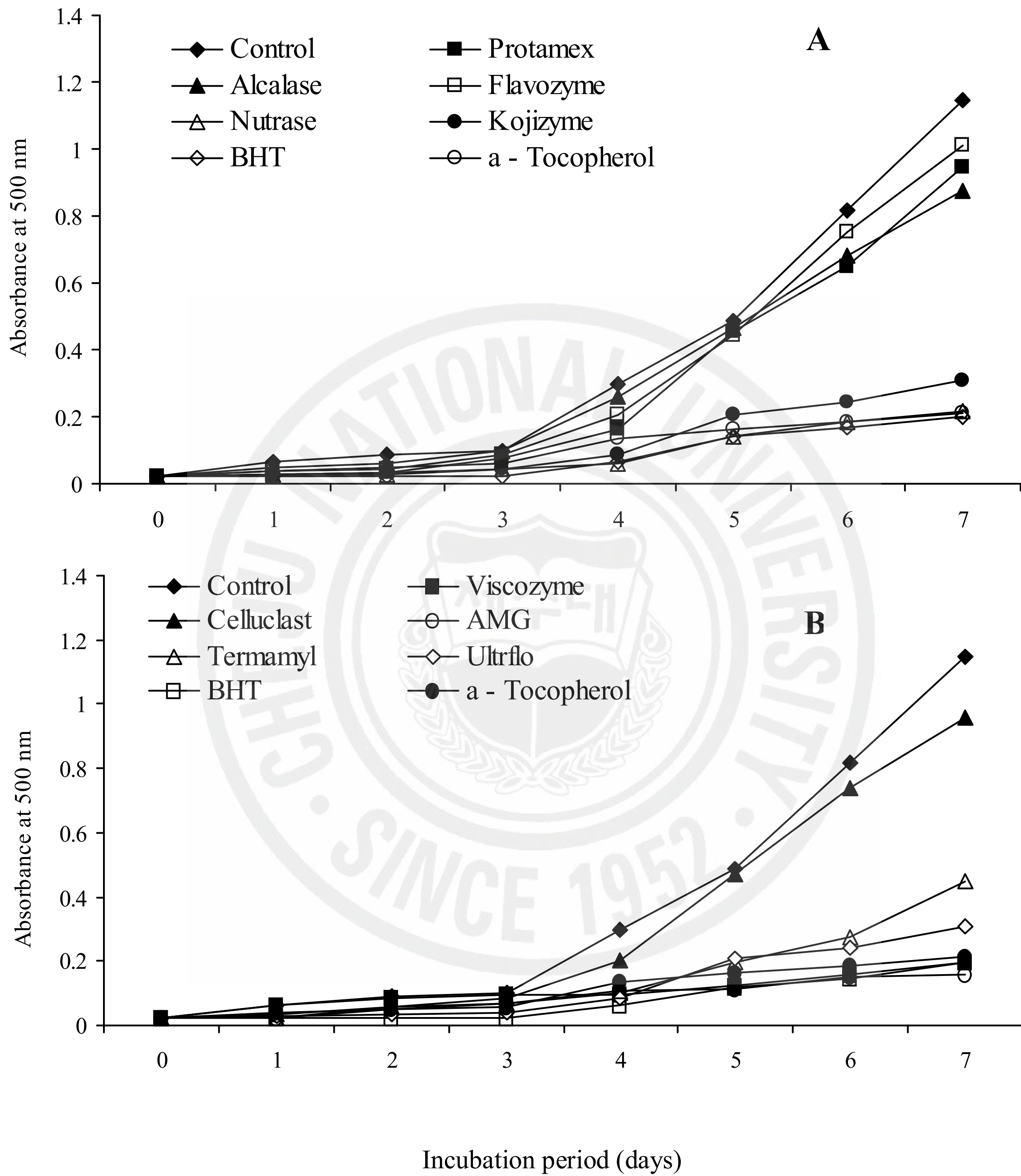

Fig. 17. Antioxidant activities of protease (A) and carbohydrase (B) extracts of *C. closterium*, compared to a-Tocopherol and BHT at 1 mg/ml ethanol, as assessed by linoleic acid.

Table 10. Characterization and optimum hydrolysis conditions of particular enzymes

Enzyme	Enzyme characterization	Optimum conditions	
Carbohydrases		pH	Temp. °C
Viscozyme	Arabanase, celluiase, β-glucanase, hemi-cellulase and xyianase	4.5	50
Celluclast	Catalyzing the breakdown of cellulose into glucose cellobiose and higher glucose polymer	4.5	50
AMG	An exo-1, 4-α-D-glucosidase	4.5	60
Termamyl	A heat stable α-amylase	6.0	60
Ultraflo	A heat stable multi-active β-glucanase	7.0	60
Proteases			
Protamex	Hydrolysis of food protein	6.0	40
Kojizyme	Boosting of the soya sauce fermentation	6.0	40
Neutrase	An endoprotease	6.0	50
Flavourzyme	Containing both endoprotease and exopeptodase activities	7.0	50
Alcalase	A endo protease	8.0	50

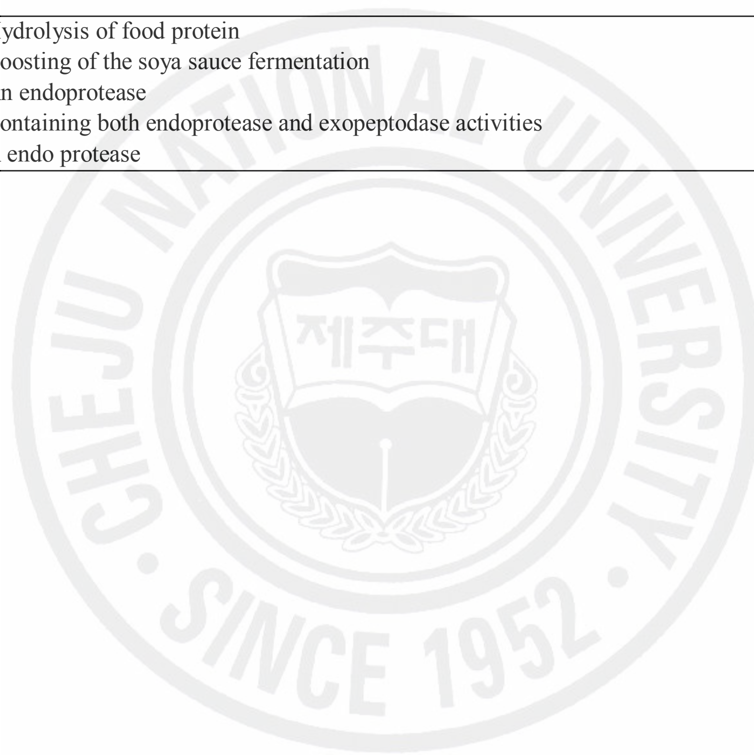

Table 11. Antioxidative effects of different *Navicula incerta* extracts from enzymatic, methanolic and water

Extract	Polyphenol ^{a} (mg /100 g)^{d}	DPPH scavenging activity (%)	Hydrogen peroxide scavenging activity %	Superoxide anion scavenging activity %	Hydroxyl radical activity %	Nitric oxide scavenging activity %	Metal-chelating activity %
Viscozyme	614.5±22	36.1^{h}±1.32	15.7^{fg}±0.68	44.3^{d}±1.22	21.6^{c} ±0.55	26.7^{d}±2.12	20.5^{f}±1.19
Celluclast	467.8±24	38.5^{g}±1.11	17.1^{ef}±1.50	51.3^{c}±1.65	4.4^{h}±0.68	27.5^{d}±1.45	20.1^{f}±2.18
AMG	367.8±18	27.3^{i}±1.18	19.8^{e}±1.11	44.5^{d}±2.22	5.3^{h}±1.11	32.7^{c}±1.32	25.3^{e}±1.76
Termamyl	500.7±21	38.8^{fg}±1.63	14.2^{g}±1.13	43.2^{d}±1.95	1.4^{i}±1.65	27.4^{d}±1.63	41.2^{c}±0.67
Ultraflo	742.1±32	49.6^{d}±1.19	23.4^{d}±0.75	35.2^{f}±0.75	14.6^{de}±2.24	40.6^{ab}±0.80	33.3^{d}±0.85
Protamex	652.7±18	47.3^{de}±0.89	11.4^{h}±0.95	38.1^{e}±0.90	11.1^{f}±1.54	21.4^{e}±0.79	24.6^{e}±0.65
Alcalase	592.3±29	44.9^{e}±0.45	17.3^{ef}±1.30	32.1^{g}±1.26	11.7^{f}±2.10	33.0^{c}±1.14	26.3^{e}±0.98
Flavourzyme	581.4±21	41.1^{f}±1.25	11.6^{h}±1.20	57.7^{b}±2.05	8.2^{g}±1.19	39.5^{b}±0.85	20.7^{f}±1.25
Neutrase	951.2±24	81.6^{a}±0.93	22.4^{d}±1.32	25.2^{h}±1.17	16.6^{d}±1.45	32.8^{c}±0.76	45.2^{b}±2.31
Kojizyme	886.1±29	46.7^{e}±1.80	19.5^{e}±0.80	23.2^{h} ±0.85	13.3^{ef}±1.95	42.2^{a}±1.11	26.9^{e}±0.86
Methanol	426.5±24	62.8^{c}±1.50	27.6^{c}±0.89	16.8^{i}±0.85	7.8^{g}±2.3	9.2^{f}±1.15	68.5^{a}±1.32
α-Tocopherol		72.6^{b}±1.18	76.8^{a}±1.95	61.5^{a}±1.71	79.5^{a}±220	26.1^{d}±1.50	9.7^{g} ±0.85
BHT		70.8^{b}±1.84	69.2^{b}±2.44	63.2^{a}±1.50	76.6^{b}±2.80	25.2^{d}±2.00	10.7^{g}±0.93

The values represent the mean ± SD of 2 mg/mL determined from triplicate samples. Values followed by the superscript letters a, b, c, d, e, f, g, h, and i indicate a significant difference ($p<0.05$).

Table 12. Antioxidative effects of different *Cylindrotheca closterium* extracts from enzymatic, methanolic and water

Extract	Polyphenol [a] (mg /100 g)[e]	Flavonoid [b] (mg/100 g)[e]	DPPH scavenging activity (%)	Hydrogen peroxide scavenging activity %	Superoxide anion scavenging activity %	Hydroxyl radical activity %	Nitric oxide scavenging activity %	Metal-chelating activity %
Viscozyme	357.7±22	32.1±1.1	$72.5^{bc}\pm0.21$	$4.6^{m}\pm0.11$	$42.3^{g}\pm0.11$	$20.7^{h}\pm0.15$	$45.5^{c}\pm0.02$	$52.1^{d}\pm0.11$
Celluclast	455.3±24	31.7±1.1	$37.7^{g}\pm0.19$	$14.6^{i}\pm0.12$	$34.5^{i}\pm0.05$	$10.6^{m}\pm0.09$	$42.8^{f}\pm0.03$	$24.3^{k}\pm0.11$
AMG	304.7±18	24.7±1.6	$52.7^{ef}\pm0.15$	$24.3^{e}\pm0.11$	$47.8^{e}\pm0.6$	$14.1^{k}\pm0.21$	$45.8^{b}\pm0.02$	$33.7^{i}\pm0.10$
Termamyl	391.4±21	29.2±1.7	$54.9^{ef}\pm0.19$	$17.9^{h}\pm0.11$	$25.5^{m}\pm0.5$	$10.5^{n}\pm0.19$	$42.4^{g}\pm0.05$	$35.6^{h}\pm0.09$
Ultraflo	623.9±32	51.2±1.5	$63.8^{cd}\pm0.13$	$23.5^{f}\pm0.10$	$31.8^{j}\pm0.15$	$19.1^{i}\pm0.13$	$37.1^{i}\pm0.5$	$23.50^{l}\pm0.15$
Protamex	358.6±18	36.6±1.8	$58.2^{def}\pm0.28$	$7.0^{l}\pm0.18$	$29.1^{l}\pm0.08$	$12.5^{l}\pm0.11$	$32.5^{k}\pm0.03$	$32.4^{j}\pm0.11$
Alcalase	534.2±29	52.6±2.1	$38.2^{g}\pm0.11$	$4.6^{m}\pm0.15$	$34.5^{i}\pm0.09$	$24.3^{g}\pm0.18$	$31.8^{m}\pm0.02$	$53.9^{b}\pm0.12$
Flavourzyme	434.1±21	41.2±1.2	$31.1^{g}\pm0.18$	$12.5^{k}\pm0.13$	$39.3^{h}\pm0.21$	$17.9^{j}\pm0.15$	$34.4^{j}\pm0.02$	$47.4^{f}\pm0.11$
Neutrase	642.8±24	60.3±2.6	$60.8^{de}\pm0.14$	$25.00^{d}\pm0.11$	$31.2^{k}\pm0.11$	$30.8^{e}\pm0.11$	$31.5^{n}\pm0.08$	$50.3^{e}\pm0.09$
Kojizyme	553.1±29	43.4±2.1	$49.9^{f}\pm0.14$	$13.0^{j}\pm0.10$	$42.8^{f}\pm0.07$	$27.9^{f}\pm0.10$	$32.1^{l}\pm0.06$	$67.1^{a}\pm0.10$
Methanol	532.5±27	39.3±2.2	$69.4^{bc}\pm0.19$	$48.4^{c}\pm0.11$	$53.0^{c}\pm0.10$	$58.3^{c}\pm0.21$	$44.8^{d}\pm0.03$	$53.2^{c}\pm011$
Water	517.8±21	34.8±1.6	$59.3^{de}\pm0.17$	$20.2^{g}\pm0.11$	$50.0^{d}\pm0.11$	$49.5^{d}\pm0.12$	$44.6^{e}\pm0.05$	$40.9^{g}\pm0.11$
α-Tocopherol			$96.6^{a}\pm0.13$	$60.1^{b}\pm0.13$	$61.5^{b}\pm0.10$	$76.6^{b}\pm0.11$	$47.9^{a}\pm0.05$	$10.3^{n}\pm0.12$
BHT			$97.6^{a}\pm0.14$	$62.5^{a}\pm0.18$	$63.2^{a}\pm0.10$	$79.7^{a}\pm0.10$	$44.4^{e}\pm0.06$	$11.5^{m}\pm0.09$

The values represent the mean ± SD of 2 mg/ml determined from triplicate samples. Values followed by the superscript letters a, b, c, d, e, f, g, h, and i indicate a significant difference ($p<0.05$).