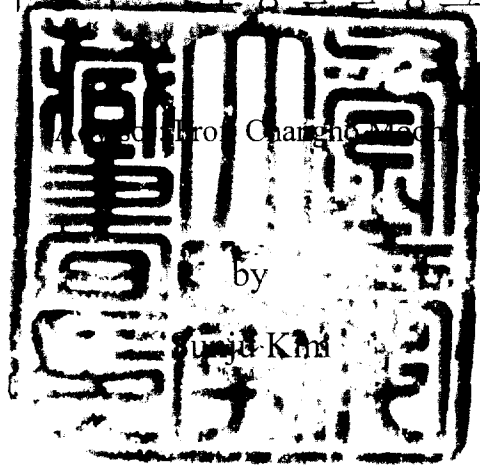


Factors regulating phytoplankton populations,
with special emphasis on parasitism and
microzooplankton grazing

식물플랑크톤 개체군을 조절하는 생물학적
요인: 기생과 미소동물플랑크톤 섭식



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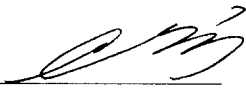
Factors regulating phytoplankton populations, with special emphasis on
parasitism and microzooplankton grazing

A dissertation


by

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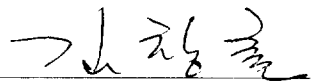
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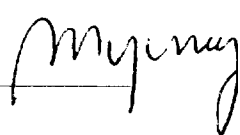
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식물플랑크톤 개체군을 조절하는 생물학적 요인 :기생과 미소동물플랑크톤 섭식

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국 문 요 약

연안 생태계에서 식물플랑크톤 개체군을 조절하는 생물학적 요인으로서 미소동물플랑크톤 섭식(microzooplankton grazing)과 기생(parasitism)에 대해 연구하였다. 연안 식물플랑크톤 개체군을 조절하는데 있어서 미소동물플랑크톤의 섭식이 얼마나 중요한지를 이해하기 위해서 진해만의 한 고정 정점에서 2002년 5월부터 2003년 4월까지 1년동안 dilution method를 이용하여 현장 섭식률을 측정하였다. 현장 식물플랑크톤 성장률과 미소동물플랑크톤 섭식률은 각각 $-0.63 \sim 2.08 \text{ d}^{-1}$ 과 $0 \sim 3.86 \text{ d}^{-1}$ 의 범위였으며, 둘 다 춘계에서 하계로 갈수록 높고 추계에서 동계로 갈수록 낮은 형태의 뚜렷한 계절변동 양상을 보였다. 식물플랑크톤 군집조성과 현존량 ($0.29 \sim 127.42 \mu\text{g Chl } a \text{ l}^{-1}$)이 연간 크게 변화하는 본 연구지역에서 미소동물플랑크톤은 식물플랑크톤 현존량과 일차생산량의 연평균 약 63%와 398%를 각각 소비하는 것으로 추정되었으며, 이러한 결과는 연안 생태계에서도 미소동물플랑크톤의 역할이 중요함을 의미한다. 한편, 식물플랑크톤의 성장률과 미소동물플랑크톤의 섭식률간 일시적인 decoupling현상이 년중 빈번하게 관측된 점으로부터 판단할 때 환경변화가 극심한 연안생태계에서 단발적인 조사는 미소동물플랑크톤의 역할이 과소평가될 수 있음을 확인하였으며, 연안 미소생물 먹이망내 에너지 흐름과 물질순환을 정확하게 이해하기 위해서는 본 연구에서와 같은 장주기(long-term) 조사의 필요성을 입증하였다.

한편, 연안생태계에서 식물플랑크톤 개체군을 조절하는 또 하나의 중요한 생물학적 요인으로서 기생을 들 수 있으나, 이는 연안 식물플랑크톤 동태와 적조연구 분야에서 그간 다소 간과되어온 분야이기도 하다. 본 연구에서는 한국 연안에서 종종 적조를 유발하는 유각 와편모류 *Alexandrium affine*와 *Gonyaulax polygramma*로부터 각각 이들을 감염시키는 기생성 와편모류인 *Amoebophrya* strain을 세계 최초로 실험실 배양체로 확립하는데 성공하였으며, 이 두 strain들의 감염특성 및 세대기간, 숙주특이성 및 계통학적 분석, 기생생물 감염으로 인한 유독성 와편모류의 독소에 대한 영향 등을 연구하였다. 지

지금까지 약 40여종의 외편모류 숙주들로부터 이 기생성 외편모류에 의한 감염이 보고되어 왔고 그 중 약 3/4이 유각성 외편모류임에도 불구하고 이전의 연구는 대부분 무각외편모류에 대해 치증된 경향을 보였다. 실험실 배양체로 확립된 두 *Amoebophrya* strain들을 이용하여 각각의 숙주내부에서 발달 단계 특성을 연구한 결과, *A. affine*를 감염시키는 *Amoebophrya* strain은 그들 숙주의 세포질을 감염시킨 반면 *G. polygramma*에서 기생하는 *Amoebophrya* strain의 경우 숙주 핵을 감염시키는 차이를 나타냈으나, 숙주내부에서 전반적인 발달 양상은 이전의 다른 외편모류 숙주들에서 보고된 것과 유사하였다. 자연상태에서 *Amoebophrya*에 의해 감염된 *A. affine*의 두드러진 형태적 특징 중의 하나는 비감염 세포에 비해 크기가 매우 큰 세포 (giant cells)를 형성한다는 점이며, *G. polygramma*를 감염시키는 *Amoebophrya*와 달리 감염 말기상태에 이르러서야 형광현미경의 청색파장하에서 초록색 자가형광(green autofluorescence)을 내는 특징을 가지고 있다. 두 유각외편모류를 감염시키는 *Amoebophrya* strain들의 세대기간(generation time)은 각각 53시간과 55시간으로 추정되었으며, 이는 무각외편모류를 감염시키는 strain들에 비해 상대적으로 짧다. 따라서 각각의 숙주 외편모류-기생생물 배양체로부터 정확한 세대기간을 추정하는 것은 현장에서 숙주의 개체군을 제거하는 속도를 추정하는 데 중요한 변수로 이용되며 따라서 생태계내의 *Amoebophrya* 감염의 중요성을 평가하는데 있어서 의미를 가진다.

또한 두 종의 숙주인 외편모류 *A. affine*와 *G. polygramma*를 감염시키는 기생생물 *Amoebophrya* strain들의 숙주 특이성(host specificity)에 대해 연구한 결과, 두개의 strains들이 모두 숙주 특이성이 결여되어 있음을 최초로 발견하였다. *A. affine*로부터의 *Amoebophrya*는 *Alexandrium* 속(genus)에 속하는 종들만을 감염시킨 반면에, *G. polygramma*로부터의 *Amoebophrya*는 5개의 속(*Alexandrium*, *Gonyaulax*, *Prorocentrum*, *Heterocapsa*, *Scropsiella*)에 해당하는 종들을 감염시키는 특성을 나타내었다. 이전에 몇몇 숙주종에서 보고된 결과와 종합해 볼 때, *Amoebophrya*의 숙주 특이성은 매우 강한 것부터 약한 것에 이르기까지 strain에 따라서 크게 변화할 뿐만 아니라, 유각보다는 무각성 숙주로부터 분리된 strain들이 더 강한 숙주 특이성을 나타내는 경향이 있다.

본 연구에서 분리한 두 strain들의 SSU rDNA 염기서열 분석과 Genbank로부터 8개 strain들의 자료를 이용하여 최근까지 모든 외편모류에서 감염이 보고된 *Amoebophrya*가 단일종 *Amoebophrya ceratii*인것으로 간주되어 왔던 점을 재평가하였다. 사용된 총 10개의 strain들은 계통학적으로 외편모류 분류군에서 모두 하나의 단일그룹(monophyletic group)을 형성하였으나, 세부적으로는 크게 세 그룹으로 나누어지는 경향이 있었다. 그중 *A. affine*, *Gymnodinium*

instriatum, *Ceratium tripos*, *Prorocentrum micans*를 감염시키는 네 개 *Amoebophrya* strain들은 염기서열만으로 평가할 때 거의 단일종으로 여겨지나 이를 결정적으로 확인하기 위해서는 추후 이 기생생물의 생물학적 특성 및 형태 등의 자료가 보완되어야 할 것으로 판단된다. 그 이외의 strain들의 염기서열은 유사도가 서로 매우 다르게 나타났다.

마비성 패독을 생산하는 유독성 외편모류 *Alexandrium tamarense*가 기생성 외편모류 *Amoebophrya*에 의해 감염된 후 숙주생물의 독소 함량 및 독조성변화 유무 및 숙주 생물의 독소가 기생생물에 전달될 가능성을 연구한 결과, *Amoebophrya*에 감염된 세포의 독소함량 및 독조성은 감염이 안된 세포와 크게 차이가 나지 않았다. 또한, *Amoebophrya*의 생활사 중 감염단계에 속하는 dinospores에서도 독소가 검출되지 않았다. 이러한 결과는 기생성 외편모류 *Amoebophrya*에 의한 감염이 숙주생물의 마비성 패류독소가 상위 영양단계로 전달되는 전달자(vector)로서 역할을 담당하지 않는다는 것을 의미하며, 미세 먹이망내에서 독소를 빠르게 분산시킬 수 있음을 시사한다.

본 연구의 결과들은 연안 식물플랑크톤 생태학과 적조연구 분야에 있어서 미소동물플랑크톤 섭식 및 기생생물의 감염에 대한 좀 더 폭넓은 이해와 지식을 제공하는 중요한 자료로서 활용될 것으로 판단된다.

I. General introduction

Phytoplankton in coastal ecosystems play a key role in maintaining the organisms of higher trophic level in marine food webs and in material cycling and energy flows. Especially, phytoplankton dynamics in temperate coastal ecosystems is generally believed to be highly variable and complex relative to the other marine environments, because a variety of physicochemical and biological factors having influences on phytoplankton fluctuate greatly over a yearly time scale.

Phytoplankton production (PP) can be expressed as a function of two variables, i.e. the specific growth rate of phytoplankton (μ) and phytoplankton biomass (B);

$$PP = \mu \times B$$

Phytoplankton production is thus constrained by all factors that directly or indirectly influence μ , B, or both. While physicochemical factors such as light, temperature, and nutrients limit μ , and thus ultimately PP, loss factors regulate the overall pool size of B (Banse, 1992). Various mechanisms can be responsible for the losses of phytoplankton (Fig. 1), e.g. sinking, vertical mixing, advection, grazing, and mortality due to microparasites (Proctor and Fuhrman, 1990; Banse, 1994; Landry *et al.*, 1997; Calbet, 2001; Calbet and Landry, 2004; Park *et al.*, 2004). While the relative importance of those loss factors is poorly understood, grazing, due particularly to microzooplankton, is generally considered to be one of the most important (Sherr and Sherr, 1992, 2002; Calbet and Landry, 2004).

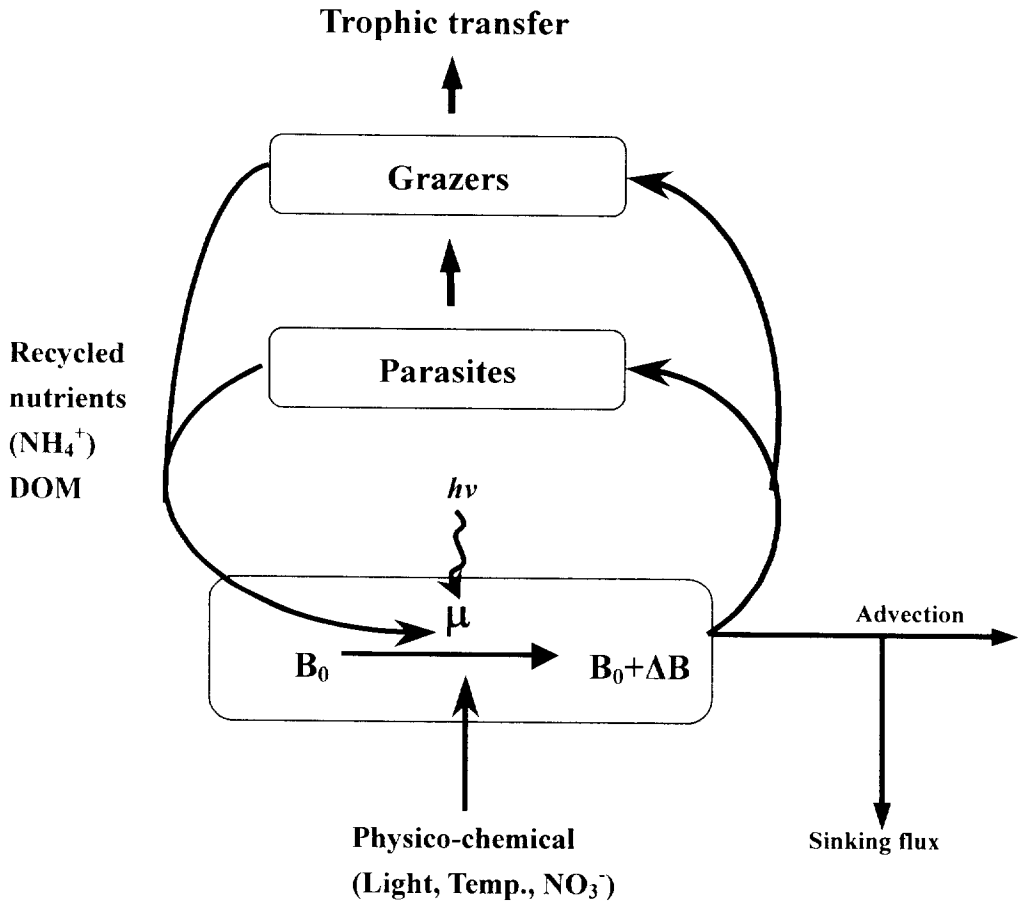


Fig. 1. Conceptual relationships between top-down (grazers and parasites) and bottom-up (nutrients) controls of phytoplankton biomass and primary production.

Nonetheless, most previous studies about the role of microzooplankton grazing tended to focus on a certain season or event such as algal blooms, although a few recent studies have addressed the influences of microzooplankton grazing on phytoplankton over the annual cycle (e.g. Gallegos *et al.* 1996; Strom *et al.*, 2001; Odate and Imai, 2003). Available data concerning the impact of microzooplankton grazing on phytoplankton over a time scale of a year are still limited, particularly in temperate coastal waters, where the levels of phytoplankton biomass and nutrients concentrations are expected to be highly variable in time and space, and when the relative importance of top-down (grazing) and bottom-up (nutrients) controls of phytoplankton vary greatly even on short-term scales (e.g. days).

On the other hand, despite the potential in regulating phytoplankton biomass, another biological factor, parasitism, tends to be more or less neglected in phytoplankton ecology and harmful algal bloom dynamics (Elbrächter and Schnepf, 1998). Nonetheless, eukaryotic parasites, in particular endoparasitic dinoflagellates of the genus *Amoebophrya*, have received some attention in relation to bloom dynamics of red-tide dinoflagellates (Coats, 1999; Park *et al.*, 2004). Especially, *Amoebophrya ceratii* is known to be a species complex capable of infecting roughly 40 different free-living dinoflagellates, including toxic and harmful algal bloom species and is broadly distributed in coastal waters of the world (Fig. 2) (Coats *et al.*, 1996; Park *et al.*, 2004). Although three-quarters of these dinoflagellate hosts are thecate taxa, our knowledge of *Amoebophrya* biology (e.g., life cycle, generation time, and impact on host



Fig. 2. Global distribution of *Amoebophrya* spp. in free-living dinoflagellates. Filled circles indicate sites where infections have been noted in photosynthetic and/or heterotrophic host species. (From Park *et al.*, 2004)

physiology) is largely derived from the study of *Amoebophrya* spp. that have been cultured using athecate hosts (Coats and Bockstahler, 1994; Coats and Park, 2002; Park *et al.*, 2002a,b). In subsequent chapters (III-VI), I expanded the current knowledge of *Amoebophrya* biology by addressing hot issues (e.g., species complex hypothesis and host specificity) in debate and by evaluating parasitic impacts on hosts (e.g. the fate of toxin in toxic dinoflagellate hosts) using two thecate dinoflagellate hosts-*Amoebophrya* parasites systems (*Amoebophrya* spp. from *Alexandrium affine* and *Gonyaulax polygramma*). Below are the brief statements of the goal of this research and summaries from each of the chapters.

Chapter II reports the results of dilution experiments conducted at a fixed

site within Jinhae Bay approximately every two weeks from May 2002 to April 2003 to better understand the role of microzooplankton grazing on coastal phytoplankton and to quantify the extent to which microzooplankton grazing varies over the annual cycle. I found that while frequent decoupling between microzooplankton grazing and phytoplankton growth occurred evenly over the year, microzooplankton grazing constituted a major loss process for phytoplankton in the study site. Further, I observed a pronounced seasonal variation in microzooplankton grazing, with the highest grazing rates in spring to autumn and the lowest rates in winter. The results indicate that the role of microzooplankton as consumers of phytoplankton could be under- and/or overestimated in temperate coastal waters, depending on when sampling was made during the year, raising the need for intensive, long-term measurements of microzooplankton grazing to better understand the cycling of materials and the flow of energy through the microbial food webs of highly dynamic coastal ecosystem.

In **Chapter III**, I report the occurrence of *Amoebophrya* parasites and generation times from two thecate dinoflagellate hosts. In September and October 2002, *Amoebophrya* spp. infected populations of the bloom forming, thecate dinoflagellates *Alexandrium affine* (Inoue et Fukuyo) Balech and *Gonyaulax polygramma* Stein on the south and west coasts of Korea, respectively. The two host-parasite systems were successfully isolated from field samples and have been maintained as stock cultures in the lab. In this paper, I present the first

documentation of *Amoebophrya* infections and parasite developmental stages in *A. affine* and *G. polygramma*. Further, I estimated total parasite generation times (i.e. time required for infection of hosts, intracellular development, vermiform emergence, and extracellular maturation) for the two thecate host-parasite systems. These data will provide important information necessary to assess the impact of parasitism by *Amoebophrya* sp. as a significant loss factor for thecate dinoflagellate populations in Korean coastal waters.

In **Chapter IV**, I addressed host specificity of two *Amoebophrya* strains infecting thecate dinoflagellate hosts. Using two thecate host-parasite systems (*Amoebophrya* spp. from *Alexandrium affine* and *Gonyaulax polygramma*), I tested the hypothesis that the two strains of *Amoebophrya* have a high degree of host specificity. To test this hypothesis, I conducted a series of cross infection experiments, with 10 thecate and 3 athecate dinoflagellate species as potential hosts. Surprisingly, the two strains of *Amoebophrya* exhibited partial host specificity, with relatively broad host range. While *Amoebophrya* sp. from *A. affine* was capable of infecting only the species (*A. affine*, *A. catenella*, and *A. tamarensis*) of the genus *Alexandrium* among host species tested, the parasite from *G. polygramma* could infect species covering 5 genera (*Alexandrium*, *Gonyaulax*, *Prorocentrum*, *Heterocapsa*, and *Scropsiella*). My results, along with previous reports suggest that host specificity of the *Amoebophrya* parasites not only varies from extremely species-specific to rather unspecific, but also tends to be stronger in strains isolated from athecate hosts. This information on host specificity will

be helpful in assessing the possibility of using these parasites as biological control agents for harmful algal blooms, as well as in defining species of the parasite in the future.

In **Chapter V**, I sequenced the SSU rDNA of *Amoebophrya* strains infecting *A. affine* and *G. polygramma* from Korean coastal waters and compared those with previously reported sequences for the *Amoebophrya* strains isolated from several other hosts from different geographic areas. My goal was to determine if available sequences for *Amoebophrya* strains are different from each other. I report that some *Amoebophrya* strains may be the same species despite having different geographic origins and host species, while others are obviously different species when based solely on SSU rDNA sequence data.

In **Chapter VI**, I tested the hypothesis that parasitism by *Amoebophrya* spp. alters cellular toxin content and profiles (relative toxin composition) of paralytic shellfish poisoning (PSP) toxin-producing dinoflagellate hosts. Further, I tested the hypothesis that dinoflagellate toxins are transferred to infective, dispersal dinospore stage of the parasite. To test these hypotheses, I determined cellular toxin content and composition over the infection cycle for infected and uninfected cultures of the PSP toxin-producing dinoflagellate *Alexandrium tamarense* and also measured those variables for *Amoebophrya* dinospores. Cellular toxin contents and profiles of infected and uninfected *A. tamarense* cultures were not significantly different. In addition, no toxins were detected in dinospores. My

results suggest that parasitism by endoparasitic dinoflagellate *Amoebophrya* do not function as vector for transport of PSP toxins to higher trophic levels and instead plays a role to dissipate rapidly the toxins in marine food webs.

My results are believed to contribute greatly to the fields of microzooplankton grazing and parasitism in coastal phytoplankton ecology and harmful algal bloom research.

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II. Seasonal variations in phytoplankton growth and microzooplankton grazing in a temperate coastal embayment, Korea

Abstract

Microzooplankton grazing on coastal phytoplankton was determined by the dilution method from May 2002 to April 2003 at a fixed site within Jinhae Bay of Korea. During the dilution experiments, our study site exhibited a wide range of chlorophyll a concentrations (0.29 to 127.42 $\mu\text{g l}^{-1}$) and the species composition of phytoplankton community changed dramatically over a year. Nutrients enriched (μ_n), in situ phytoplankton growth rates (μ_0), microzooplankton grazing rate (g) all showed the pronounced seasonal variations over a year, ranging from 0.11 to 2.87 d^{-1} , from -0.63 to 2.08 d^{-1} , and from 0 to 3.86 d^{-1} , respectively, all of them with the lowest values in late fall and winter and highest values in spring and summer. The ratio of μ_0/μ_n averaged 0.92 (SE= 0.06), indicating that phytoplankton growth in the study site was not nutrient-limited. Non-significant and/or zero grazing (about 62% of a total of 29 dilution experiments) did frequently occur during this study and distributed evenly over a time scale of the year, suggesting that our study site may be highly dynamic in terms of predator-prey interaction, i.e. tight coupling and transient uncoupling. Nonetheless, phytoplankton populations in our study site appeared to be strongly regulated by microzooplankton grazing over the time scale of year, accounting on average for 63% and 398% of initial Chl a standing stock and potential phytoplankton production, respectively. Our results indicate that the role of microzooplankton as the consumers of phytoplankton could be under-and/or overestimated in temperate coastal waters, depending on when sampling was made of the year, raising the need in intensive, long-term measurements of microzooplankton

grazing to better understand the cycling of materials and the energy flow through microbial food webs in highly dynamic coastal ecosystem.

INTRODUCTION

The level of phytoplankton biomass at any given time and space can be expressed as the difference between their growth and removal processes. The accumulation of phytoplankton biomass and further algal blooms occur when phytoplankton growth rate becomes larger than removal rate or exceed it. While physicochemical factors such as light, temperature, and nutrients limit phytoplankton growth rate and biomass production, loss factors regulate the size of their biomass (Banse, 1992). The losses of phytoplankton occur by various mechanisms, including sinking, vertical mixing, advection, grazing, and mortality due to viruses or parasites (Proctor and Fuhrman, 1990; Banse, 1994; Landry *et al.*, 1997; Calbet, 2001; Calbet and Landry, 2004; Park *et al.*, 2004). While the relative importance of those loss factors is poorly understood, grazing, due particularly to microzooplankton, is generally considered to be one of the most important (Sherr and Sherr, 1992, 2002; Calbet and Landry, 2004).

Microzooplankton usually refer to heterotrophic protists from 20 to 200 μm in size and include taxonomically diverse groups, including ciliates, flagellates, dinoflagellates, and small metazoans such as copepod nauplii and rotifer (Capriulo *et al.*, 1991). They are known to play several important roles in marine pelagic food webs, e.g. as effective consumers of prey ranging in size from bacteria to organisms nearly as large as themselves (Lessard, 1991; Sherr and Sherr, 1994; Strom and Strom, 1996), as an important trophic link between small prey and larger predators (Sherr *et al.*, 1986), and as a major contributor to both nutrient regeneration and dissolved organic material production (Goldman and

Caron, 1985; Nagata, 2000).

The role of microzooplankton as consumers of phytoplankton has been extensively investigated using the dilution technique (Landry and Hassett, 1982) over the past two decades across various marine habitats (estuarine to open ocean environments) (e.g. Landry *et al.*, 1995; Ruiz *et al.*, 1998; Strom *et al.*, 2001; Juhl and Murrell, 2005) and geographic regions (polar to tropical waters) (e.g. Strom and Strom, 1996; Tsuda and Kawaguchi, 1997; Landry *et al.*, 1998), demonstrating the general importance of microzooplankton grazing in the oceans, with accounting on average for 67% and 60-75% of daily phytoplankton growth and production, respectively (Calbet and Landry, 2004; Landry and Calbet, 2004). Nonetheless, most previous studies tended to focus on during a certain season or the event such as algal blooms of the year, although a few recent studies addressed the influences of microzooplankton grazing on phytoplankton over the annual cycle (e.g. Gallegos *et al.*, 1996; Strom *et al.*, 2001; Odate and Imai, 2003). Available data concerning the impact of microzooplankton grazing on phytoplankton over a time scale of year are still limited, particularly in temperate coastal waters, where the levels of phytoplankton biomass and nutrients concentrations are expected to be highly variable in time and space, and further the relative importance of top-down (grazing) and bottom-up (nutrients) controls of phytoplankton could greatly vary even on the short-term scale (e.g. days).

Jinhae Bay is a temperate shallow embayment located in the southern coast of Korea, where is characterized by large seasonal variations in phytoplankton biomass and species composition. Algal blooms by chain-forming diatom

Skeletonema costatum, dinoflagellates *Alexandrium* spp. and *Prorocentrum* spp., and raphidophyte *Heterosigma akashiwo* do frequently occur between spring and autumn every year due to the increased nutrient availability originated from adjacent lands and cities (Lee *et al.*, 1981; Yang, 1989). To better understand the role of microzooplankton grazing on coastal phytoplankton and to quantify the extent to which microzooplankton grazing varies over the annual cycle, we conducted a total of 29 dilution experiments at a fixed site within Bay approximately every two weeks from May 2002 to April 2003. We found that while frequent decoupling between microzooplankton grazing and phytoplankton growth did occur evenly over a year, microzooplankton grazing constituted a major loss process for phytoplankton in our study site. Further, we observed the pronounced seasonal variation in microzooplankton grazing, with the highest grazing rates in spring-autumn season and the lowest rates in winter season. Our results indicate that the role of microzooplankton as the consumers of phytoplankton could be under- and/or overestimated in temperate coastal waters, depending on when sampling was made of the year, raising the need in intensive, long-term measurements of microzooplankton grazing to better understand the cycling of materials and the energy flow through microbial food webs in highly dynamic coastal ecosystem.

METHOD

Study site and sampling

Jinhae Bay is a shallow embayment surrounded by many islands, located in the southern coast of Korea and has increasingly inputs of allochthonous organic matters from adjacent lands and cities (Lee *et al.*, 1981; Yang, 1989; Lee and Kwon, 1994). A fixed site (35°N, 128°40'E, mean depth ca. 8 m) within Bay, where was located in the South Sea Institute dock, Korea Ocean Research and Development Institute (KORDI), was chosen for this study and sampling was made nearly every two weeks over a year from May 2002 to April 2003. The tidal range in the site is less than 1.5 m. Surface seawater samples for analyses of dissolved inorganic nutrients (NO_3^- , NO_2^- , PO_4^{3-} , and SiO_2) and phytoplankton and microzooplankton variables, and for dilution experiments were collected with either a high density polyethylene bucket or a Niskin sampler on the flood tide and were transferred with minimal bubbling to six 20-L polycarbonate carboys (Nalgene) that were transported to the laboratory for further process. Surface water temperature and salinity were measured with a DO meter (YSI, Model 58) and a conductivity meter (Istek, Model 43C), respectively. Aliquots of seawater samples were filtered through Whatman GF/F glass-fiber filters (47 mm diameter), and then the filtrates were stored frozen (-20°C) for analyses of dissolved inorganic nutrients using a Flow Injection Analyzer (FIA, Quickchem 8000, Lachat Co.) according to Parsons *et al.* (Parsons *et al.*, 1984).

Phytoplankton and microzooplankton community structure

Acid Lugol's-preserved samples (1% final concentration) were used for analyses of phytoplankton species composition and abundance. Each 300 mL

sample was first settled for 1 week and then concentrated to 50 mL by gently aspirating the supernatant fluid. Phytoplankton abundance was determined with 1 mL of the concentrated samples using a Sedgewick-Rafter chamber under a light microscopy (Olympus Model BX51).

To determine the community structure of microzooplankton in the initial undiluted seawater, 300 mL seawaters were fixed in acid Lugol's solution (1% final concentration) and stored in the dark until analysis by inverted settlement microscopy (Utermöhl, 1958). Microzooplankton samples in 10 mL settling chambers were identified and enumerated at 100–400× magnification using an inverted light microscope (Zeiss Model Axiovert 25). The microzooplankton fraction was divided into 2 major groups, i.e. heterotrophic dinoflagellates and ciliates. The ciliates were identified as either oligotrichs or tintinnids and further categorized by size. Heterotrophic nanoflagellates (<20 µm) were excluded in analysis of microzooplankton community, whereas 15-20 µm oligotrich ciliates were included. Metazoan nauplii and phototrophic ciliate *Mesodinium rubrum* were occasionally or frequently, respectively, encountered in a few samples but were not included in total biomass and abundance estimates of microzooplankton community. Identifications for ciliates were based primarily on Kofoid and Cambell (Kofoid and Cambell, 1929), Montagness and Lynn (Montagness and Lynn, 1991), and Mitsuo and Masaaki (Mitsuo and Masaaki, 1997). Heterotrophic dinoflagellates were identified based on Dodge (Dodge, 1980), Tomas (Tomas, 1996), and Mitsuo and Masaaki (Mitsuo and Masaaki, 1997). The cell volume was calculated by measuring cell dimension under bright field

microscope according to Edler (Edler, 1979). The carbon biomass of each cell was calculated using carbon:biovolume conversion factors ; 0.19 pg C μm^{-3} for oligotrich ciliates (Putt and Stoecker, 1989), carbon (pg) = 445 + 0.053 \times (lorica volume, μm^3) for tintinnid ciliates (Verity and Langdon, 1984), and carbon (pg) = 0.216 \times (volume, μm^3)^{0.939} for heterotrophic dinoflagellates (Menden-Deuer and Lessard, 2000).

Dilution experiments

Phytoplankton growth and microzooplankton grazing rates were estimated by the dilution method (Landry and Hassett, 1982; Landry *et al.*, 1995). A total of 29 dilution experiments were conducted during the study period. Prior to the experiments, all experimental bottles and carboys were soaked in 10% HCl for more than 24 h until next experiment and rinsed thoroughly with distilled water. Seawater samples for dilution experiment were prescreened through 200 μm Nitex mesh to remove mesozooplankton and then transferred to carboys. However, seawater samples collected in October 2002, when massive blooms by dinoflagellates *Alexandrium* spp. occurred, were not prescreened due to long-chain formation of the cells (up to 70 cells). Dilution series were established by combining prescreened whole seawater with Whatman GF/F filtered seawater in 1-L polycarbonate bottles. Filtered seawater was first added to bottles and then whole seawater was gently poured into bottles. Triplicate 1-L bottles were filled with each of the following fractions, whole seawater (1.0), 0.8, 0.6, 0.4, and 0.2 (total incubation bottles = 15). Nutrients were added to these bottles with final

concentrations of 20 μM NO_3 and 2 μM PO_4 to prevent nutrient limitation of phytoplankton growth during the incubation. Three additional bottles were filled with undiluted whole water without nutrients addition to determine the effect of nutrient on phytoplankton populations. All experimental bottles were incubated *in situ* at a depth of 0.5 m for 24 h. After 24 h incubation, bottles were returned to the laboratory and sampled for chlorophyll *a* (Chl *a*) concentration measurements. Chl *a* concentrations before and after the incubation were determined using a Turner Designs model 10 fluorometer calibrated with pure Chl *a* (Parsons *et al.*, 1984), after 24 h extraction in 90% acetone at 4°C h after filtration onto GF/F filters. In addition, the percent contributions of <3 μm , 3-20 μm , and >20 μm size fractions to total Chl *a* at the start of the dilution experiments were estimated by passing whole seawater through 3.0 and 20 μm pore size polycarbonate filters (Milipore) and then by measuring *in vivo* fluorescence of each fractionated filtrate.

Data analysis

The apparent phytoplankton growth rates (k) in individual bottles were calculated as $1/t \ln (C_t/C_0)$, where C_0 is initial Chl *a* concentration; C_t is Chl *a* concentration at time t . Phytoplankton growth under nutrient enriched conditions (μ_n) and microzooplankton grazing rates (g) were obtained by linear regression of apparent growth rates against diluted fractions of whole seawater: the slope and y-intercept of the regression represent the microzooplankton grazing rate and the phytoplankton growth rate, respectively. Estimates of phytoplankton

growth rate with ambient nutrients (μ_0) were calculated by adding microzooplankton grazing rate (g) to the apparent growth rate in the undiluted bottles without nutrient addition (k_0); i.e. $\mu_0 = k_0 + g$. Regression analysis frequently yielded a positive slope (i.e. negative rates of grazing) or non-significant ($P > 0.05$) estimates of grazing, with each from 9 of 29 experiments, respectively. In the former case, grazing was assumed to be zero. In cases where grazing was treated as zero, growth rates (μ_n and μ_0) were calculated from undiluted (whole water) bottles with and without nutrient addition only, respectively. In the latter case, despite the relatively high r^2 values (usually >0.60), the grazing rates were non-significant, but were included in subsequent analysis. When possible saturation grazing occurred (Gallegos, 1989), apparent growth rates calculated only from bottles with dilution factors < 0.5 were used to obtain μ_n and g . The ratios of phytoplankton growth rates in nutrient unenriched and enriched bottles (μ_0 / μ_n) were used to assess the nutrient limitation on phytoplankton (Landry *et al.*, 1995).

Microzooplankton grazing impact

Chl *a* based-phytoplankton biomass and production grazed by microzooplankton were calculated from μ_0 , g , and initial Chl *a* concentration (C_0) during dilution experiments according to the following equations (Verity *et al.*, 2002).

$$\begin{aligned} &\text{Phytoplankton biomass removed daily, } C_g \text{ (\% day}^{-1}\text{)} \\ &= (1 - e^{-g}) \times 100 \end{aligned}$$

Phytoplankton production grazed daily, P_g (% day⁻¹)

$$= \frac{(C_0 e^{\mu} - C_0) - (C_0 e^{(\mu-g)} - C_0)}{(C_0 e^{\mu} - C_0)} \times 100$$

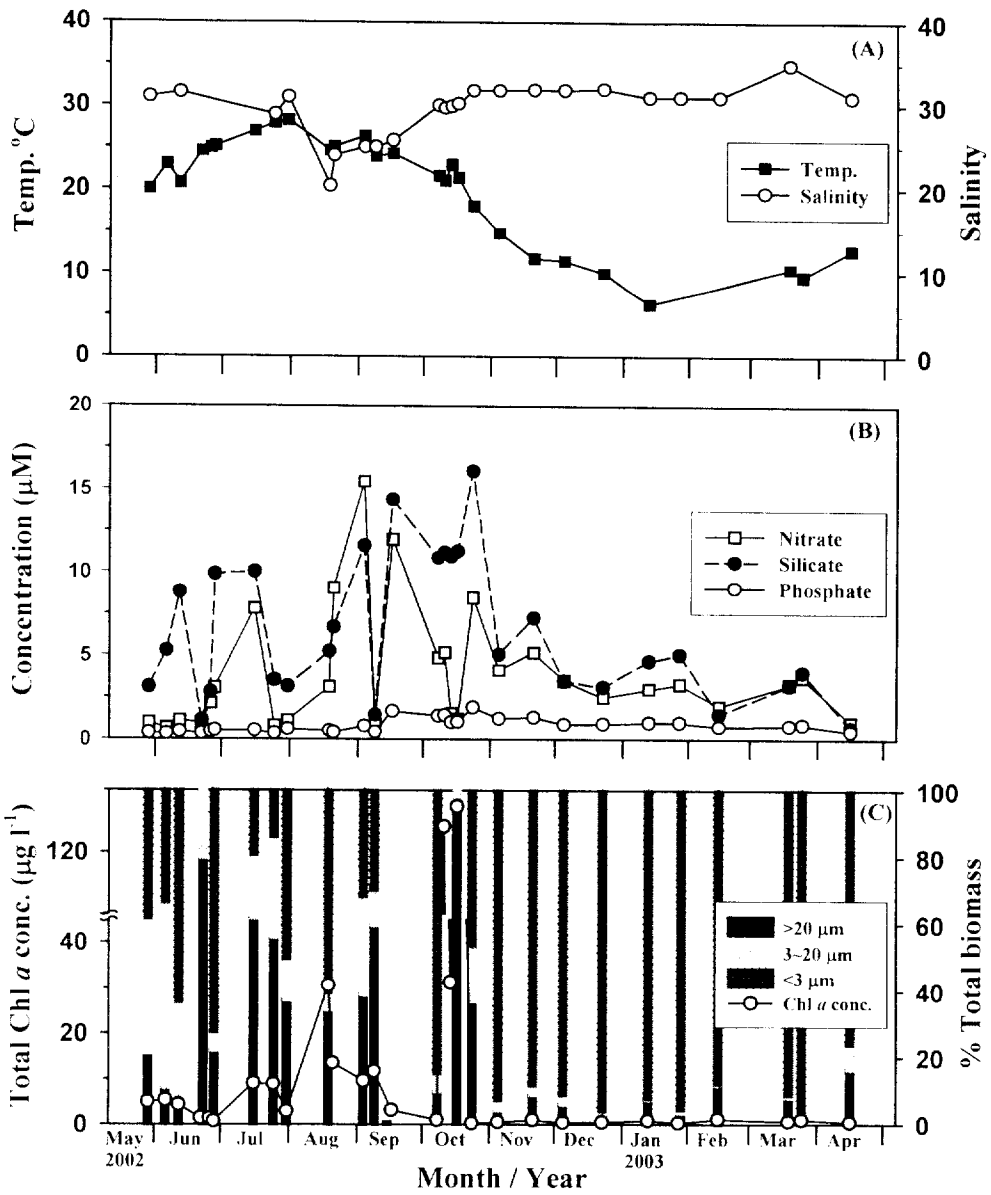
In cases where μ was negative, a value of zero was assigned to the corresponding rate estimate to indicate no growth.

RESULTS

Environmental condition of the study site

Surface water temperature ranged from 6.3°C (in January 2003) to 28.2°C (in August 2002) over a year in the study site, reflecting the typical seasonal variation in temperate coastal waters (Fig. 1A). Salinity was usually around 31 psu throughout the study period, except for the relatively lower salinity (even to 20 psu) due to the rainfalls between August and September 2002 (Fig. 1A). While nitrate ($\text{NO}_3^- + \text{NO}_2^-$) and silicate concentrations remained high over the study period but greatly fluctuated between 0.68 and 15.46 μM and between 0.69 and 16.09 μM , respectively, phosphate concentrations were relatively constant, with a mean value of 0.82 (\pm 0.08) μM (Fig. 1B). Total Chl *a* concentrations ranged from 0.29 to 127.42 $\mu\text{g l}^{-1}$, with three peaks observed in August and October 2002 (Fig. 1C). While the first Chl *a* peak in August resulted from the bloom of chain-forming diatom *Skeletonema costatum*, the other two peaks in October originated from dinoflagellates *Alexandrium* spp. blooms (see below). Total phytoplankton biomass, estimated from the Chl *a* concentration, was represented by the mixed size fractions (<3 μm , 3-20 μm , and >20 μm) between May and

Fig. 1. Seasonal variations in water temperature and salinity (**A**), nutrient concentrations (**B**), and total Chl *a* concentrations (**C**). In (**C**), the percent contributions of <3 μm , 3-20 μm , and >20 μm size fraction to total Chl *a* concentrations were also given together.

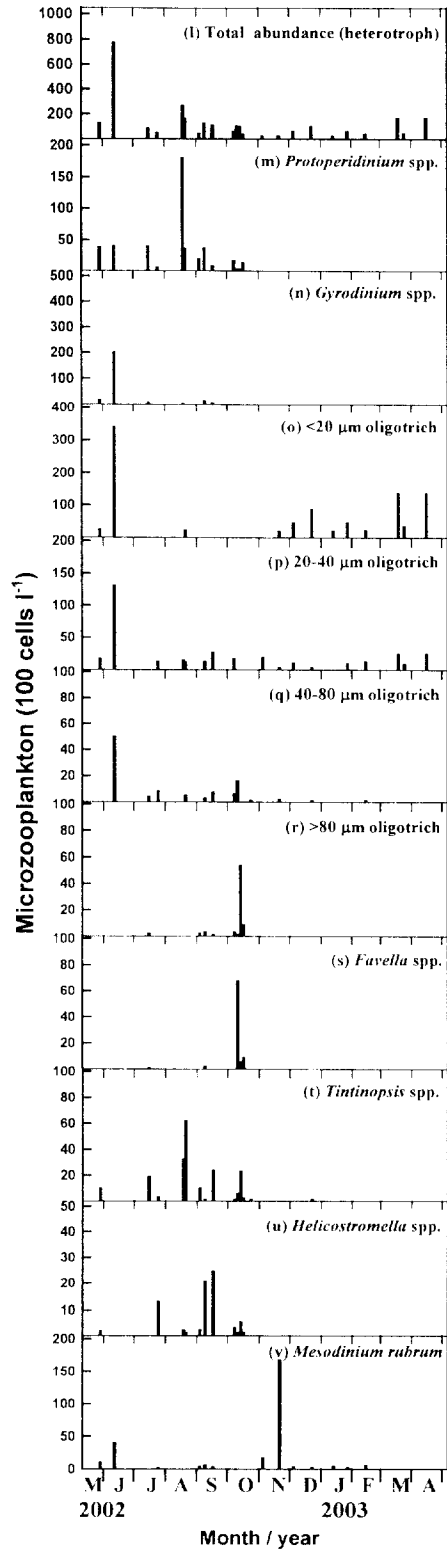
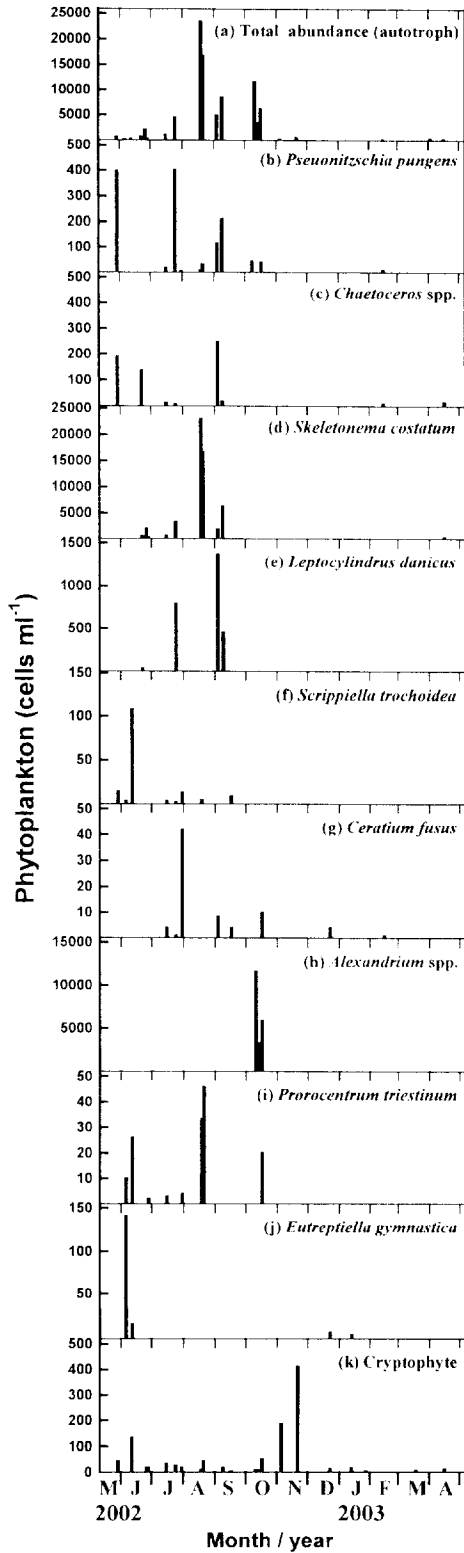


October 2002, but was predominated by $<3 \mu\text{m}$ fraction between November 2002 and April 2003 (Fig. 1C).

Phytoplankton and microzooplankton community dynamics

Phytoplankton abundance showed the large variations over the year, with a maximum of $23,300 \text{ cells mL}^{-1}$ in August 2002 and a minimum of 6 cells mL^{-1} in January and March 2003 (Fig. 2a). The highest cell number was recorded during the summer bloom of chain-forming diatom *Skeletonema costatum* just after the rainfall. The species composition of phytoplankton community during this study changed dramatically (Figs. 2b-k and 3a), shifting from the predominance of chain-forming diatoms, particularly *Chaetoceros* spp., *Leptocylindrus danicus*, *Pseudonitzschia pungens*, and *Skeletonema costatum* (even up to 99% of total abundance in August) between May and September 2002, to the massive bloom by dinoflagellates *Alexandrium* spp. in October 2002, and to predominance of cryptophytes (*Chroomonas* sp.) in November 2002. Unidentified, small-sized ($<3 \mu\text{m}$) picoeukaryotes were abundant between December 2002 and March 2003, and then again diatoms, particularly *Chaetoceros* spp. and *Skeletonema costatum* began to increase toward the end of the experiment. The annual successions of major microzooplankton showed that each species had generally 1 or 2 peaks of occurrence of different amplitude and duration (Fig. 2l-v). Total microzooplankton abundance ranged from 198 in late October 2002 to $78,000 \text{ cells L}^{-1}$ in early June 2002 (Fig. 2l) and total biomass from 0.44 in late October 2002 to $942.61 \text{ mg C L}^{-1}$ in early October 2002. The highest cell number was

Fig. 2. Annual succession of major species and groups of phytoplankton (**left panels; b-k**) and microzooplankton (**right panels; m-u**), with their total abundances (**a** and **l**) given. Abundance of the phototrophic ciliate *Mesodinium rubrum* (**v**) are also shown here, but was not included in total abundance and biomass of microzooplankton (see text).



recorded in early June 2002 when heterotrophic dinoflagellates *Gyrodinium* spp. and small (<20 μm and 20-40 μm) oligotrichs were dominant (Fig. 2n-p). In August 2002, the summer bloom of diatom *Skeletonema costatum* was accompanied by a rapid increase in abundance of heterotrophic dinoflagellates *Protoperidinium* spp. (Fig. 2d, m). Tintinnids *Tintinopsis* spp. and *Helicostromella* spp. both also contributed substantially to abundance and biomass of microzooplankton from the start of this study to October 2002, but since then were of less importance (Figs. 2t, u and 3b, c). Peaks of tintinnids *Favella* spp. having 70 to 100 μm in oral diameter and large (> 80 μm) oligotrichs were found in mid October (Fig. 2r, s) and coincided with the *Alexandrium* spp. peaks (Fig. 2h). At this time, tintinnids *Favella* spp. feeding on many *Alexandrium* cells within the cell were frequently observed (Fig. 4). The phototrophic ciliate *Mesodinium rubrum* had 4 peaks of cell numbers in May-June and November (Fig. 2v), and the peaks all coincided with those of cryptophyte (Fig. 2k). Microzooplankton abundance and biomass were at the lowest levels during winter to early spring, with small (< 20 μm and 20-40 μm) oligotrichs dominating in abundance and biomass until April 2003 (Figs. 2l and 3c).

Phytoplankton growth and microzooplankton grazing

Only 11 (38%) of a total of 29 dilution experiments performed during this study produced statistically significant estimates of phytoplankton growth and microzooplankton grazing rates, and the other experiments produced either non-significant estimates (9 of 29) or exhibited zero-grazing (9 of 29) of

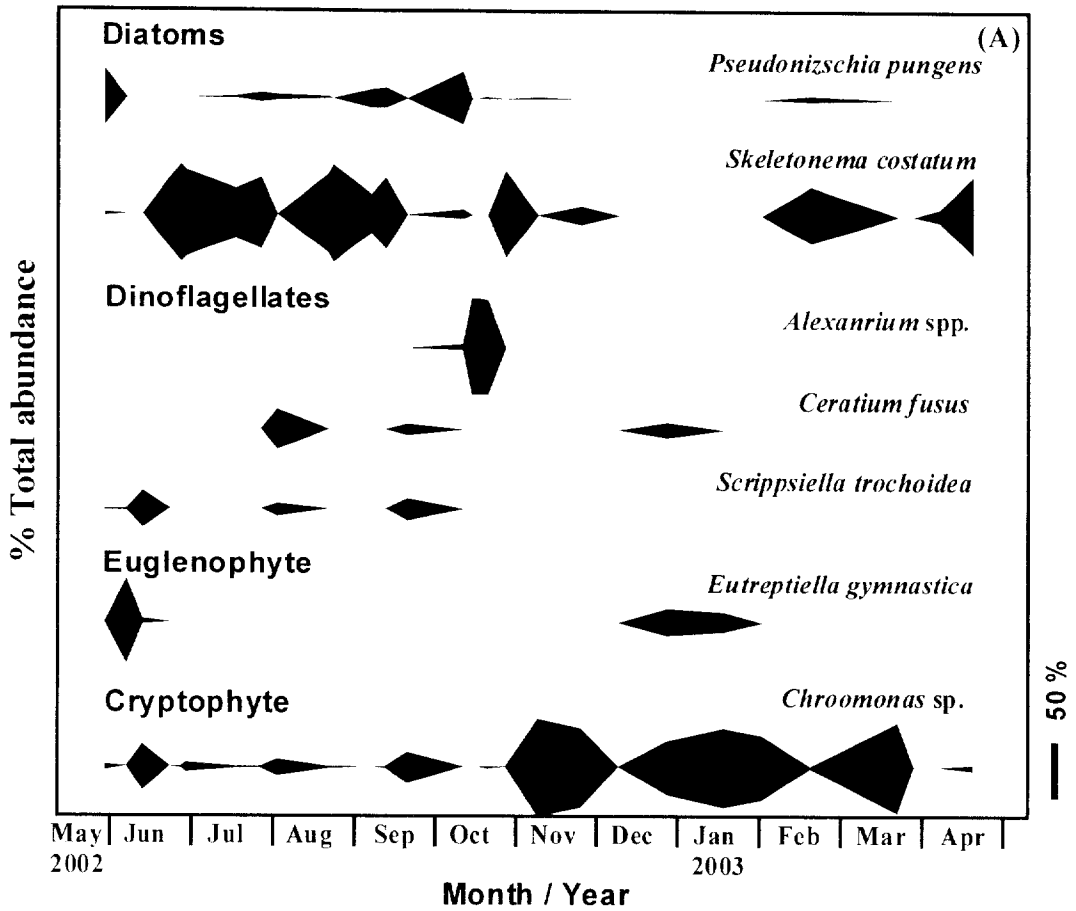


Fig. 3. Seasonal variations in percent compositions of phytoplankton abundance (A), microzooplankton abundance (B), and biomass (C). HDF, heterotrophic dinoflagellates.

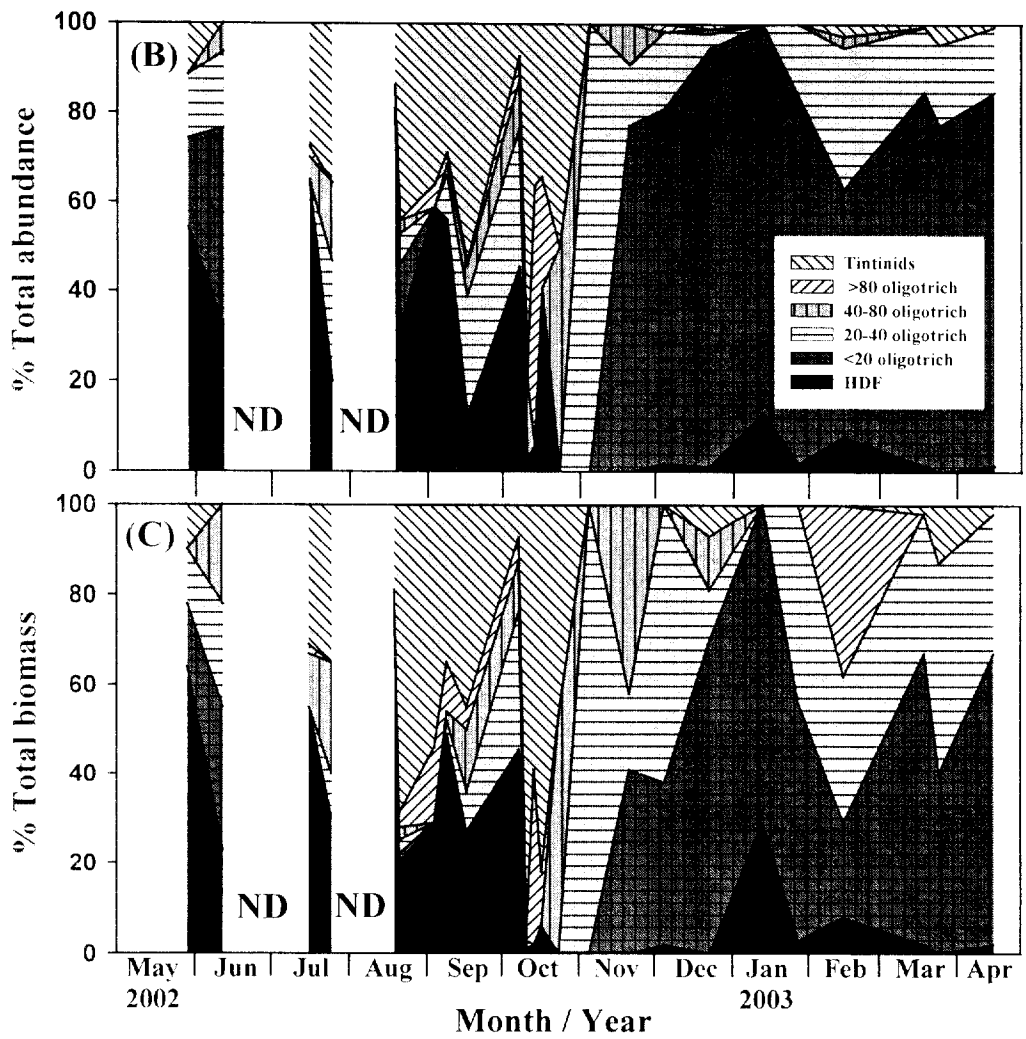


Fig. 3. (To be continued)

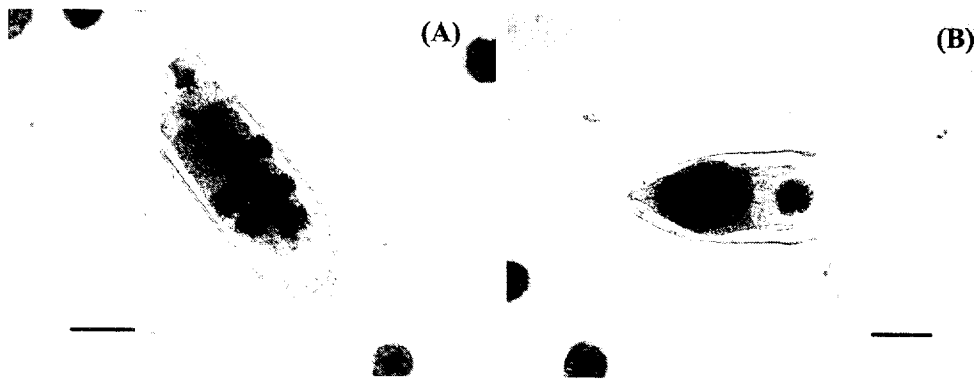
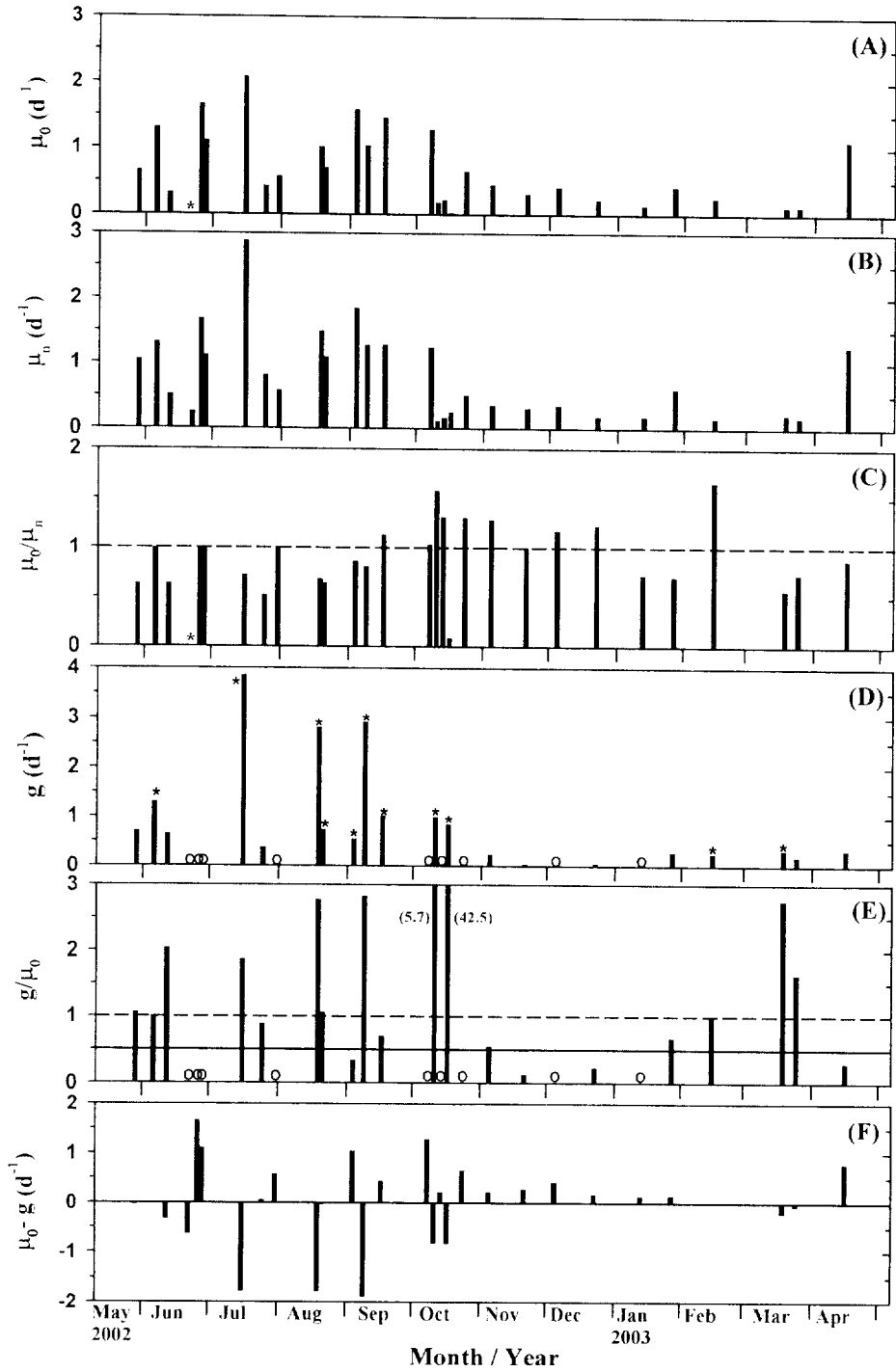


Fig. 4. Photomicrographs of tintinnids *Favella* spp. feeding on many *Alexandrium* cells within the cell in mid October 2002. Scale bars = 50 μm .

microzooplankton. The μ_0 exhibited a pronounced seasonal variation (Fig. 5A). The μ_0 values ranged from -0.63 to 2.08 d^{-1} (mean $0.66 \pm 0.11 \text{ d}^{-1}$) throughout the study period and were generally higher between May and October 2002 (on average $0.82 \pm 0.15 \text{ d}^{-1}$) and lower between November 2002 and April 2003 (on average $0.35 \pm 0.09 \text{ d}^{-1}$) (*t*-test, $P < 0.05$). The highest rate (2.08 d^{-1}), which corresponds to 3 doublings per day, was found in mid July 2002, whereas the lowest rate (-0.63 d^{-1}) was found in late June 2002 when dissolved inorganic nutrients, in particular nitrate ($0.98 \mu\text{M}$) and silicate ($1.12 \mu\text{M}$) concentrations were at the lowest levels during this study. The μ_n showed a similar trend to μ_0 , ranging from 0.11 to 2.87 d^{-1} (Fig. 5B). The μ_0/μ_n ratios averaged 0.92 ± 0.06 (range 0.08 - 1.66) over the study period, excluding an unusual negative ratio of -2.52 in late June 2002 (Fig. 5C), indicating that phytoplankton growth in the study site was not nutrient-limited. The lowest ratio (0.08) was found during the final

Fig. 5. Seasonal variations in phytoplankton growth rates from nutrient unenriched (μ_0 ; **A**) and enriched bottles (μ_n ; **B**), relative growth ratios (μ_0/μ_n ; **C**), microzooplankton grazing rates (g ; **D**), ratios of microzooplankton grazing rate to phytoplankton growth rate (g/μ_0 ; **E**), and differences between growth and grazing rates (μ_0-g ; **F**). The asterisks (*) in (**A** and **C**) represent negative growth rate and ratio of -0.63 d^{-1} and -2.52 , respectively. In (**C**), the dashed line represents the same growth rates (i.e. ratio of 1). In (**E**), the solid and dashed horizontal lines indicate grazing equivalent to half of phytoplankton growth or the same to growth, respectively. In (**D**) and (**E**), zero grazing is indicated above x -axis and significant estimates are indicated as asterisks (*).



stage of *Alexandrium* spp. bloom development in mid October 2002. The g also demonstrated a marked seasonal variation, similar to those of phytoplankton growth rates (Fig. 5D). The g values ranged from 0 to 3.86 d^{-1} , averaged $0.64 \pm 0.18 \text{ d}^{-1}$, and were higher between May and October 2002 (mean $0.88 \pm 0.26 \text{ d}^{-1}$) than between November 2002 and April 2003 (mean $0.17 \pm 0.04 \text{ d}^{-1}$) (t -test, $P < 0.05$). The ratios of g/μ_0 were highly variable (Fig. 5E), varying from 0 to 42.5 during this study, but when the extremely high ratio observed during the *Alexandrium* spp. bloom and zero-grazing rates were not included in this analysis, the ratios averaged $1.45 (\pm 0.31)$. Overall, the g equaled or exceeded μ_0 between May and mid October, was equivalent to $< 50\%$ of μ_0 between late October and January, and since then again equaled or exceeded it. The mean differences between growth and grazing estimates ($\mu_0 - g$) were on average $0.02 (\pm 0.16) \text{ d}^{-1}$, indicating that microzooplankton grazing and phytoplankton growth in the study site was approximately in balance over the long-term scale of year.

Relationships between growth and grazing rates and environmental variables

The μ_0 showed statistically significant positive correlation with water temperatures ($P < 0.05$) and nitrate concentrations ($P < 0.05$), but showed highly significant negative correlation with salinity ($P < 0.01$) (Table I). No significant relationships between μ_0 and other nutrients (PO_4^{3-} and SiO_2) or Chl a were found (all $P > 0.05$). The relative growth ratios (μ_0/μ_n) did not showed the marked dependence on environmental variables including nitrate concentrations (all $P > 0.05$). The g was negatively related to salinity ($P < 0.001$) and positively to μ_0

Table I: Pearson correlation coefficients of environmental variables versus growth (μ_o) and grazing (g) rates or growth ratios (μ_o/μ_n). *: $P < 0.05$; **: $P < 0.01$

Parameter	μ_o	μ_o/μ_n	G
Temperature	0.44*	-0.07	0.40*
Salinity	-0.61**	-0.27	-0.67**
Nitrate	0.44*	0.18	0.21
Silicate	0.27	-0.16	0.20
Phosphate	-0.11	0.06	-0.22
Chl <i>a</i>	-0.22	0.11	0.32
μ_o	-	-	0.49**
G	0.49**	0.45*	-

and temperature ($P < 0.01$ and $P < 0.05$, respectively).

Microzooplankton grazing impact

The daily percentages of Chl *a* biomass grazed by microzooplankton varied from 0% to 97.9% (mean $31.6 \pm 6.0\%$) for all data set (Fig. 6A). When considering only data with significant grazing rates, however, the daily percentages of Chl *a* biomass grazed varied by about a factor of four, averaging $62.5 (\pm 7.8)\%$ (range 22.1-97.9%). By comparison, the daily percentages of phytoplankton production consumed due to microzooplankton grazing ranged from 0% to 2891.7% (mean $176.5 \pm 98.4\%$) for all data set and from 52.5% to 2891.7% (mean $398.4 \pm 251.0\%$) for only significant data (Fig. 6B). The huge grazing impact on phytoplankton production was obtained during the final stage of *Alexandrium* spp. bloom in mid October 2002.

DISCUSSION

Non-significant or zero grazing

Two-thirds of dilution experiments during this study exhibited statistically non-significant or zero grazing rates. Results of dilution experiments that yielded those non-significant or zero grazing rates are not unique to this study only but seem to be common across a wide range of marine ecosystems (Landry and Hassett, 1982; Landry *et al.*, 1984; Campbell and Carpenter, 1986; Paranjape, 1987; Gifford, 1988; McManus and Ederington-Cantrell, 1992; Neuer and Franks,

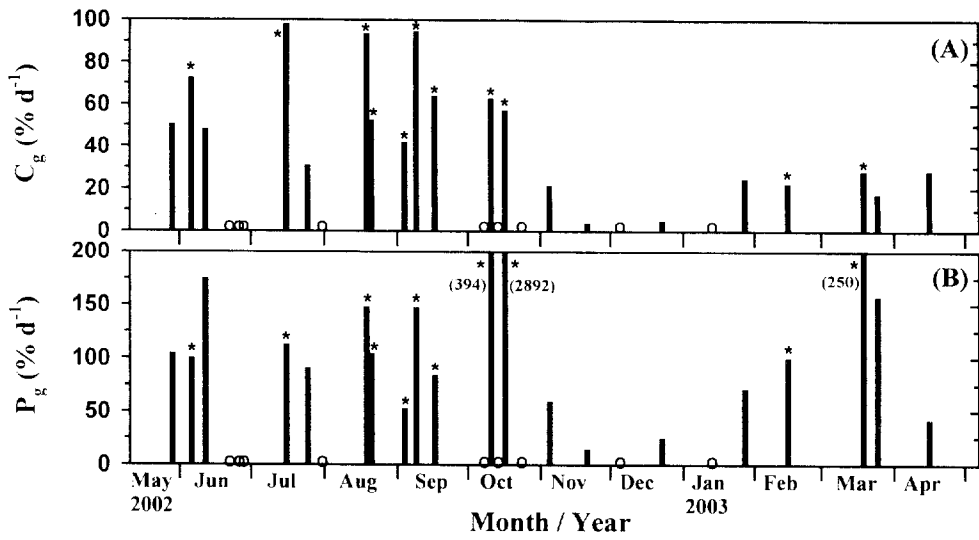


Fig. 6. Seasonal variations in daily Chl *a* biomass grazed (A) and production consumed (B) by microzooplankton in the study site. The characteristics are same to Fig. 5D and E.

1993; Kamiyama, 1994; Strom and Strom, 1996; Murrell and Hollibaugh, 1998; Putland, 2000; Strom *et al.*, 2001; Juhl and Murrell, 2005). For example, Juhl and Murrell (Juhl and Murrell, 2005) recently observed statistically non-significant grazing in 22% of 9 measurements for the bulk fraction in a Gulf of Mexico estuary. The frequency of non-significant or zero grazing is usually between about 13% (Strom *et al.*, 2001) and about 84% (Murrell and Hollibaugh, 1998). Our result (about 62%) falls within the range of frequency of non-significant or zero grazing reported so far. Murrell and Hollibaugh (Murrell and Hollibaugh, 1998) interpreted the frequency of non-significant grazing as an indicator of how frequently predators are decoupled from their prey. Frequent occurrence of non-significant or zero grazing in our study, coupled with its even distributional pattern over a time scale of the year, suggests that our study site may be highly dynamic in terms of predator-prey interaction, i.e. tight coupling and transient uncoupling. Nonetheless, phytoplankton populations in our study site appear to be strongly regulated by microzooplankton grazing over the time scale of year, as indicated by high annual mean ratio (1.45) of g/μ_0 .

Grazing and nutrient controls of phytoplankton

Our results confirm the general importance of microzooplankton grazing, which can remove on average 63% and 398% of phytoplankton biomass and production, respectively, in the study site. Our values fall within the range of grazing impact estimates from other coastal ecosystems (Burkill *et al.*, 1987; Anderson *et al.*, 1991; McManus and Ederington-Cantrell, 1992; Strom and Strom,

1996; Murrell and Hollibaugh, 1998; Gaul and Antia, 2001; Strom *et al.*, 2001; Sherr and Sherr, 2002; Odate and Imai, 2003; Calbet and Landry, 2004; Böttjer and Morales, 2005). Further, the results from this study demonstrate that the impacts of microzooplankton grazing on phytoplankton biomass and production can greatly vary by a factor of 4 and 55, respectively, over a year in temperate coastal waters. These observations, along with frequent uncoupling between phytoplankton growth and microzooplankton grazing, indicate that the role of microzooplankton as the consumers of phytoplankton could be under- and/or overestimated in temperate coastal waters, depending on when sampling was made of the year, raising the need in intensive, long-term measurements of microzooplankton grazing to better understand the cycling of materials and the energy flow through microbial food webs in highly dynamic coastal ecosystem. On the other hand, bottom-up (nutrients) control of phytoplankton appeared to be of less importance in our study site, as indicated by high annual mean ratio (0.92) of μ_0/μ_n and its no nutrient dependency (Fig. 5C and Table I), because dissolved inorganic nutrient concentrations remained relatively high within the water column over a year.

Seasonal variation in microzooplankton grazing

A major finding of our study is that microzooplankton grazing shows the marked seasonal cycle in a temperate coastal water of Korea, with higher rates in spring-autumn seasons (May-October) and lower rates in winter season (November-April). This finding contradict the size-based food web model

prediction (e.g. Sherr and Sherr, 2000) because large size phytoplankton dominated in phytoplankton community in the former period, whereas small-sized cryptophytes and/or unidentified picoeukaryotes less than 3 μm in the latter period. The observed grazing pattern in this study seems to result from (1) the temperature dependence of microzooplankton grazing, (2) differences in the composition and size structure of microzooplankton and phytoplankton communities between two periods, or (3) the combination of both. Water temperature was relatively higher between May and October 2002 (usually $>20^{\circ}\text{C}$) than between November 2002 and April 2003 (usually $<20^{\circ}\text{C}$). Microzooplankton grazing exhibited a positively significant relationship with water temperature during this study. Similarly, the dependence of microzooplankton grazing on water temperature has been reported in previous studies (e.g. Burkill *et al.*, 1993; Verity *et al.*, 2002). Second, the differences in composition and size structure of microzooplankton and phytoplankton communities between two periods was likely to be in part responsible for the observed grazing pattern. While heterotrophic dinoflagellates such as *Gyrodinium* spp. and *Protoberidinium* spp. and tintinnids such as *Favella*, *Tintinopsis*, and *Helicostromella* dominated the microzooplankton community between May and October 2002, small sized oligotrich ciliates (mainly $<20 \mu\text{m}$) did between November 2002 and April 2003. Several previous studies showed that *Protoberidinium* spp. and *Gyrodinium* spp.-like heterotrophic dinoflagellates could graze heavily on large diatoms including chain-forming species (Jacobson and Anderson, 1986; Strom and Strom, 1996; Hansen and Calado, 1999; Stelfox-

Widdicombe *et al.*, 2004). Further, we observed tintinnids *Favella* spp. ingesting many *Alexandrium* cells in October. Paralleling with the increase in chain-forming diatoms or dinoflagellates *Alexandrium* spp., the accompanied rapid increases in abundance (and biomass) of the heterotrophic dinoflagellates and tintinnid ciliates mentioned above were observed between May and October 2002, demonstrating high levels of responsiveness to increases in prey abundance and/or biomass.

On the other hand, despite the facts that small-sized flagellates such as cryptophytes and/or $<3 \mu\text{m}$ picoeukaryotes and $<20 \mu\text{m}$ oligotrich ciliates dominated phytoplankton and microzooplankton communities, respectively, between November 2002 and April 2003, it is interesting that microzooplankton grazing rates were either pretty low or zero during this period. Small sized ($<10 \mu\text{m}$) phytoplankton are known to be primarily grazed by microzooplankton smaller than $20 \mu\text{m}$ (Sherr and Sherr, 1994). Therefore, some possibilities that may account for relatively low or zero grazing rates during this period would be (1) due to low water temperature of winter season, (2) a threshold grazing response, (3) due to the considerable grazing of phototrophic ciliate *Mesodinium rubrum* on cryptophytes, and (4) due to the grazing on bacteria or suspended organic matters as alternative food source. First, as mentioned above, we cannot completely rule out the possibility that low water temperature may limit microzooplankton grazing activity during this period. Second, the ambient phytoplankton biomass during this period might be so low that microzooplankton may cease grazing, thus exhibiting a threshold feeding response (Frost, 1975;

Campbell and Carpenter, 1986) or may weakly graze. In fact, microzooplankton abundance and biomass were at the lowest levels during this period of the year, supporting the possibility. A third possible explanation for low and/or non-significant grazing during the winter season, in particular in November 2002, would be the phototrophic ciliate *Mesodinium rubrum* grazing on cryptophytes. Although the nutritional model of *Mesodinium rubrum* has long been under debate (Sieburth *et al.*, 1978; Smith and Barber, 1979; Lindholm and Mörk, 1989), the feeding of *Mesodinium rubrum* on cryptophytes has been recently documented (Hargraves, 1991; Gustafson *et al.*, 2000; Yih *et al.*, 2004). Nonetheless, the grazing impact of *Mesodinium rubrum* on phytoplankton has been ignored in the field studies or even it has been regarded that *Mesodinium rubrum* does not graze (e.g. Safi *et al.*, 2002). Therefore, high numbers of *Mesodinium rubrum* associated with increase in cryptophytes prey may explain low and/or non-significant grazing rate observed in particular in November because the dilution technique used in this study was based on Chl *a* concentration measurement and thus the extraction of Chl *a* within *Mesodinium rubrum* cell may mask the actual grazing of the ciliate from the regression of dilution experiments. Finally, explanation for low grazing rates during this period is that numerically abundant small oligotrichs <20 µm and 20~40 µm in size were not able to efficiently consume <3 µm sized phytoplankton. In Manukau harbour, New Zealand, recently, Safi *et al.* (Safi *et al.*, 2002) reported that heterotrophic nanoflagellates can play an important intermediary role between small prey such as bacteria and picophytoplankton and the ciliates. Given that the abundance of heterotrophic

nanoflagellates was quite low during the winter season in our study site (W. Lee, Masan, personal communication), however, this seemed not to be the case in our study. Alternatively, it is likely that bacterial biomass or detrital materials suspended due to strong tidal mixing in our shallow study site may be high enough to support the nutritional demands of the small oligotrichs even if their grazing rates were low. Similarly, Gallegos *et al.* (Gallegos *et al.*, 1996) during the winter in a large coastal embayment of New Zealand suggested that the microzooplankton may be supported by alternative food source such as detrital material and bacterioplankton. Recently, Olson and Strom (Olson and Strom, 2002) suggested that nanophytoplankton as well as bacterial biomass could contribute to the diets of small microzooplankton in the southeast Bering Sea.

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**III. Infection of the Bloom-forming, Thecate
Dinoflagellates *Alexandrium affine* and *Gonuaulax
spinifera* (Dinophyceae) by Two Strains of
Amoebophrya (Dinophyta)**

INFECTION OF THE BLOOM-FORMING THECATE DINOFLAGELLATES
ALEXANDRIUM AFFINE AND *GONYAULAX SPINIFERA* BY TWO STRAINS OF
AMOEOBOPHRYA (DINOPHYTA)¹

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In autumn 2002, parasitic dinoflagellates of the genus *Amoebophrya* Koeppen infected populations of the bloom-forming thecate dinoflagellates *Alexandrium affine* (Inoue and Fukuyo) Balech and *Gonyaulax spinifera* (Claparede and Lachmann) Diesing in coastal waters of Korea. Here we present the first documentation of *Amoebophrya* infections in *A. affine* and *G. spinifera* and use host–parasite cultures to provide information on parasite development and total generation times. Parasites of the two dinoflagellate hosts differed in their site of infection, developing in the cytoplasm of *A. affine* but in the nucleus of *G. spinifera*. Developmental stages of the parasite strains were similar to the previous descriptions of *Amoebophrya* spp. infecting other dinoflagellates. A prominent feature of *Amoebophrya* infection in *A. affine* from natural field samples was the presence of abnormal “giant cells” in the long chains formed by this host species. The characteristic green autofluorescence of *Amoebophrya* infections was not evident under blue light excitation until very late in the infection cycle of the *A. affine*–*Amoebophrya* sp. system but was detectable throughout the infection cycle in the *G. spinifera*–*Amoebophrya* sp. system. Despite the relatively long duration (2–10 min) of the emergence process of *Amoebophrya* spp. from these thecate hosts, total parasite generation times were shorter (53–55 h) than those previously reported for athecate host–parasite systems. These observations provide the basis for better assessing the impact of parasitism by *Amoebophrya* sp. as a significant loss factor for thecate dinoflagellate populations in Korean waters.

Key index words: *Alexandrium affine*; *Amoebophrya*; dinoflagellate; *Gonyaulax spinifera*; parasite generation time; parasitism; red tide

Dinoflagellates can act as hosts for a variety of prokaryotic and eukaryotic parasites, as parasites of protists and metazoa, and even as hyperparasites (i.e. a parasite of another parasite) of their parasitic relatives (Cachon and Cachon 1987, Elbrächter and Schnepf 1998, Coats 1999). Parasitic dinoflagellates of the genus *Amoebophrya* Koeppen received considerable attention in recent years and are of particular interest due to their potential value as biological control agents for harmful algal blooms (Taylor 1968). Of the seven species of *Amoebophrya* that have been formally described (Cachon and Cachon 1987), one, *A. ceratii* (Koeppen) Cachon, is now known to be a species complex capable of infecting many free-living photosynthetic and heterotrophic dinoflagellates. Roughly 40 different species representing more than 24 genera of dinoflagellates have been reported to serve as hosts for members of the *Amoebophrya ceratii* complex (Park 2002, Park et al. 2004). Although three-fourths of these host species are thecate taxa, our knowledge of *Amoebophrya* biology (e.g. life cycle, generation time, and impact on host physiology) is largely derived from the study of *Amoebophrya* spp. that have been cultured using athecate hosts (Coats and Bockstahler 1994, Coats and Park 2002, Park et al. 2002a,b). Recently, however, *Amoebophrya* sp. from *Prorocentrum minimum* (Pavillard) Schüller has been established in culture and used to document aspects of the parasite’s developmental stages (Kim et al. 2002).

In September and October 2002, *Amoebophrya* spp. infected populations of the bloom-forming thecate dinoflagellates *Alexandrium affine* (Inoue and Fukuyo)

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Balech and *Gonyaulax spinifera* (Claparede and Lachmann) Diesing on the south and west coasts of Korea, respectively. The two host-parasite systems were successfully isolated from field samples and have been maintained as stock cultures. Here we present the first documentation of *Amoebophrya* infections and parasite developmental stages in *A. affine* and *G. spinifera*. Further, we estimated total parasite generation times (i.e. time required for infection of hosts, intracellular development, vermiform emergence, and extracellular maturation) for the two thecate host-parasite systems. These data will provide important information necessary to assess the impact of parasitism by *Amoebophrya* sp. as a significant loss factor for thecate dinoflagellate populations in Korean coastal waters.

MATERIALS AND METHODS

Sample collection. A bucket was used to gently collect surface seawater samples from coastal waters off Kunsan (35°2'N, 126°25'E) during September 2002 and from Jinhae Bay (35°N, 128°40'E) during October 2002. Aliquots of samples were preserved with acid Lugol's fixative (2% final concentration) and/or modified Bouin's solution (5% final concentration; Coats and Heinbokel 1982). Live field samples were transported to the laboratory to establish host-parasite cultures.

Laboratory cultures. Isolates of *A. affine* and *G. spinifera* were maintained as stock cultures in f/2 S₂ medium (Guillard and Rytner 1962) formulated using 30 psu seawater collected near Kunsan or from Jinhae Bay. Two strains of parasitic dinoflagellates belonging to the genus *Amoebophrya*, one each in the host species *A. affine* and *G. spinifera*, were established in culture using a glass micropipette to transfer individual infected dinoflagellates from field samples to cultures of complementary host species. Parasites were propagated by sequentially transferring aliquots of infected host culture into uninfected host stock at roughly 2-day intervals. Stock cultures were maintained at 20°C under a 14 h light dark cycle with cool white fluorescent light at 50 and 15 μmol photons m⁻² s⁻¹ for *A. affine* and *G. spinifera*, respectively.

Parasite morphology and development. Bright field and/or epifluorescence microscopy of living and protargol-stained specimens was used to document parasite morphology and development inside host cells. For *Amoebophrya* sp. infecting *A. affine*, pictures were taken of live and Lugol-fixed specimens using a microscope (Olympus, Tokyo, Japan) equipped with differential interference contrast. Epifluorescence micrographs (Fluorescence Cube U-MWB2, 450 to 480 nm excitation, 500-nm emission, Olympus) of *Amoebophrya* sp. from *G. spinifera* were obtained for live specimens from culture using a digital camera (PowerShot G5, Canon, Tokyo, Japan) coupled to the microscope and set to the automatic exposure mode. Bouin's preserved field and culture samples were stained using the quantitative protargol technique (Montagnes and Lynn 1993) to characterize the site of infection and developmental stages of *Amoebophrya* spp. within host cells.

Total parasite generation time. For each host species, duplicate 200 mL cultures at initial densities of approximately 1 × 10⁷ cells mL⁻¹ were inoculated with recently formed (< 6 h) dinospores to give a dinospore-to-host ratio of 1:1, incubated under growth conditions described above, and sampled over time to determine the abundance of host cells and parasite dinospores. A relatively low dinospore-to-host ratio was selected for these experiments to avoid possible alteration of parasite generation time resulting from multiple

infections that can occur at high dinospore-to-host ratios (Coats and Park 2002). To obtain recently formed dinospores, stock parasite cultures were gravity filtered through polycarbonate filters of 12 μm pore size (Isopore™, Millipore, Bedford, MA, USA) to produce filtrates containing dinospores of unknown age. These dinospores were added to uninfected host cultures, with resulting infections and subsequent generation of dinospores followed over time to enable harvesting of young (< 6 h old) dinospores using filtration procedures described above.

Host abundance was determined from acid Lugol (for *A. affine*) or Bouin's-fixed (for *G. spinifera*) samples using triplicate Sedgwick-Rafter chambers (Vision Scientific Agency, Gwangju, Korea). In experiments using *G. spinifera*, the abundance of empty host theca that exhibited two-thirds or more of the host cell profile was also determined. Dinospore abundance was determined from samples preserved with CaCO₃ buffered formaldehyde (2% final concentration) using a hemacytometer and an epifluorescence microscope (Olympus microscope, 200 ×). Fluorescence Cube U-MWB2, 450 to 480 nm excitation, 500-nm emission) for each sample, duplicate counts for dinospores were made within 30 min after fixation to avoid fading of the parasite's green autofluorescence (Coats and Bockstahler 1994). Total parasite generation time (i.e. time required for infection of host cells, intracellular development, vermiform emergence, and extracellular maturation) was estimated from the temporal occurrence of new dinospores after infection of host cells, according to the method of Coats and Park (2002). Plot area from the appearance of new dinospores to peak dinospore concentrations was integrated, with elapsed time corresponding to half of the integrated area considered as total parasite generation time. In experiments using *G. spinifera*, dinospore success (i.e. the percent of dinospores that infected host cells) was estimated from parasite prevalence (percent host cells infected) and dinospore abundance following the method of Coats and Park (2002). Data are reported as means ± SE.

RESULTS

Parasite morphology, development, and emergence. A prominent feature of *Amoebophrya* infections in *A. affine* from natural field samples was the presence of abnormal "giant cells" in the long cell chains formed by this host species (Fig. 1, A and B). Up to four giant cells (on average 0.6 ± 0.08 cells per chain, *n* = 100) occurred within the long chains of *A. affine* from natural field samples, with the largest cells reaching 60 μm in width. In culture, however, the significant difference in cell size was not often evident between infected and uninfected host cells. A closer examination of these abnormally large cells revealed that each contained a single fully developed trophont (beehive stage) of *Amoebophrya* sp. (Fig. 1C). These beehive stage trophonts were always oriented perpendicular to the axis of the cell chain, resulting in the apical aperture of the mastigocoel being pressed against the cingulum of the host (Fig. 1D). The green autofluorescence of *Amoebophrya* sp. in *A. affine* was not easily detected until the parasite had developed into a mature trophont. Infections occurred in the cytoplasm of *A. affine*, where they grew in size to eventually produce a multinucleated and multiflagellated trophont with the characteristic "beehive" appearance (Fig. 1, E-G). At maturity, the trophont ruptured



FIG. 1. Bright field micrographs of *Amoebophrya* sp. in *Alexandrium affine*. Scale bars: (A) 40 μ m, (B-J) 20 μ m. (A) Chain of *A. affine* containing four "giant cells" (arrows) resulting from development of mature trophonts of *Amoebophrya* sp. (Lugol-preserved field material). (B) Live chain of *A. affine* containing giant cell (arrow) viewed with differential interference contrast (DIC) microscopy. (C and D) Live *A. affine* cells showing the mature *Amoebophrya* sp. trophonts. In D the mastigocoeles were all orientated in the same direction within the host cells in the chain (DIC). (E-G) Protargol-stained specimens showing early to mid-, mid-, and late (beehive stage) infections, respectively. Pn, parasite nuclei; Hn, host nucleus. (H) The emergence process of *Amoebophrya* sp. vermiform from an *A. affine* cell (DIC). (I and J) Live and protargol-stained vermiforms of *Amoebophrya* sp. from *A. affine*, respectively (DIC).

through the cingulum of the host and transformed into a strongly motile vermiform stage (Fig. 1H). Emergence of *Amoebophrya* sp. from *A. affine* typically required 3 to 10 min and left behind a distorted empty host theca that could not be easily identified by shape. Emergence of a vermiform from an individual host cells within a long chain of *A. affine* fragmented the chain into two or more shorter pieces. Vermiforms were approximately 250 μm long (Fig. 1, I and J) and over a period of about 1 h gradually produced fragments of variable sizes, with each fragment releasing numerous new dinospores.

Amoebophrya sp. infecting *G. spinifera* exhibited green autofluorescence throughout its life cycle (Fig. 2). Fluorescence microscopy was useful in detecting dinospores attached to the surface of host cells (Fig. 2A) and developing inside the nucleus of *G. spinifera* (Fig. 2B). With continued growth (Fig. 2, C and D), the parasite eventually occupied most of the host cell and assumed the characteristic "beehive" appearance of the mature trophont. Emergence of the vermiform stage from *G. spinifera* typically required 2 to 3 min and occurred through an opening produced by dislodging one of the hypothecal plates, leaving behind a relatively intact but empty host theca. Vermiforms of *Amoebophrya* sp. from *G. spinifera* were approximately 200 μm long and persisted for about 30 min before suddenly breaking apart to release numerous dinospores (Fig. 2, E and F).

Total parasite generation time. Densities of *A. affine* and *G. spinifera* in cultures inoculated with dinospores at a 1:1 ratio, respectively, showed a gradual increase or remained relatively constant for the first 40 h, probably due to low growth rates (Fig. 3). Abundance of *A. affine* decreased slightly from 44 to 52 h and then increased toward the end of the experiment (Fig. 3A). By comparison, densities of *G. spinifera* declined gradually to the end of the experiment, accompanied by the accumulation of empty host thecae and the formation of new dinospores (Fig. 3B). Dinospore densities exhibited distinct peaks in both *A. affine* (at 61 h) and *G. spinifera* (at 56 h) cultures, thus permitting calculation of total parasite generation time. Estimates for total parasite generation time averaged 55 ± 1.7 h and 53 ± 1.1 h for *A. affine* and *G. spinifera*, respectively; the two means were not significantly different ($P = 0.409$, *t*-test). In experiments using *G. spinifera*, parasite prevalence averaged $75\% \pm 2.50\%$. The percent of dinospores from *G. spinifera* that successfully established infections (i.e. dinospore success) was $6.9\% \pm 1.81\%$.

DISCUSSION

Several different methods have been used to detect infection of planktonic dinoflagellates by *Amoebophrya* spp. Most of these techniques have relied on examination of preserved samples using cytological staining techniques, DNA-specific fluorochromes, or fluorescent *in situ* hybridization probes (Park et al. 2004). Only two approaches, epifluorescence microscopy to

reveal green autofluorescence of the parasite (Coats and Bockstahler 1994, this study) and detection of "giant" host cells as reported here, have been used to recognize *Amoebophrya* infections in living dinoflagellates. The characteristic green autofluorescence previously noted for *Amoebophrya* spp. from a number of dinoflagellate host species (Coats and Bockstahler 1994, Coats and Park 2002, Salomon et al. 2003) was not evident in infected *A. affine* until very late in the infection cycle. Delayed appearance of green fluorescence has also been reported for *Amoebophrya* spp. that infect *Ceratium furca* (Ehrenberg) Claparede et Lachmann and *Ceratium tripos* (Mueller; Schiller (Park et al. 2004). Whether this condition reflects some attribute of the host cytoplasmic environment or an inherited trait shared by *Amoebophrya* strains that infect *A. affine* and the two *Ceratium* species is uncertain. The development of abnormally giant cells as a consequence of *Amoebophrya* infection may be an uncommon phenomenon. For example, cultures of *Amoebophrya* spp. ex. *Akashiwo sanguinea* (Hirasaka) G. Hansen et Moestrup and *Gymnodinium striatum* (Freudenthal et Lee) Coats showed no noticeable increase in host size during the infection cycle (Coats and Park 2002). Indeed, the only prior report of giant cells being produced by *Amoebophrya* infections comes from Taylor's (1968; examination of formalin-preserved samples of the chain-forming dinoflagellate *Alexandrium catenella* (Whedon and Kofoid) Balech from Sequim Bay, USA. However, significant differences in cell size can be seen in the genus *Alexandrium* depending on the life cycle stages; for example, planozygotes resulting from the fusion of gametes and planomeiocytes from a newly germinated cyst can be much larger than vegetative cells (Anderson and Wall 1978). Thus, although providing simple and rapid means of detecting and isolating some species of *Amoebophrya*, parasite autofluorescence and production of giant cells are not characteristic of all parasite strains and should not be used as the primary means to estimate parasite prevalence.

Although *Amoebophrya* spp. are known to infect both thecate and athecate host species, our understanding of host-parasite biology is largely based on the study of athecate species (Coats and Park 2002, Park et al. 2002a,b). Because completion of the *Amoebophrya* life cycle is dependent on penetration of infective dinospores into the host cell and rupture of the reproductive vermiform through the host pellicle, difference in the surface architecture of thecate and athecate dinoflagellates may pose different constraints on the timing of parasite developmental processes. Cultures of *Amoebophrya* spp. established here for *A. affine* and *G. spinifera* provide the first opportunity to explore that possibility. Estimates of parasite generation time for our two thecate hosts (53–55 h) are shorter than those reported for athecate hosts, including *A. sanguinea*, *G. striatum*, and *Karlodinium micrum* (Leadbeater and Dodge) J. Larsen (59–71 h; Coats and Park 2002). These results suggest that the plates of thecate dinoflagellate are not a major obstacle to infection by

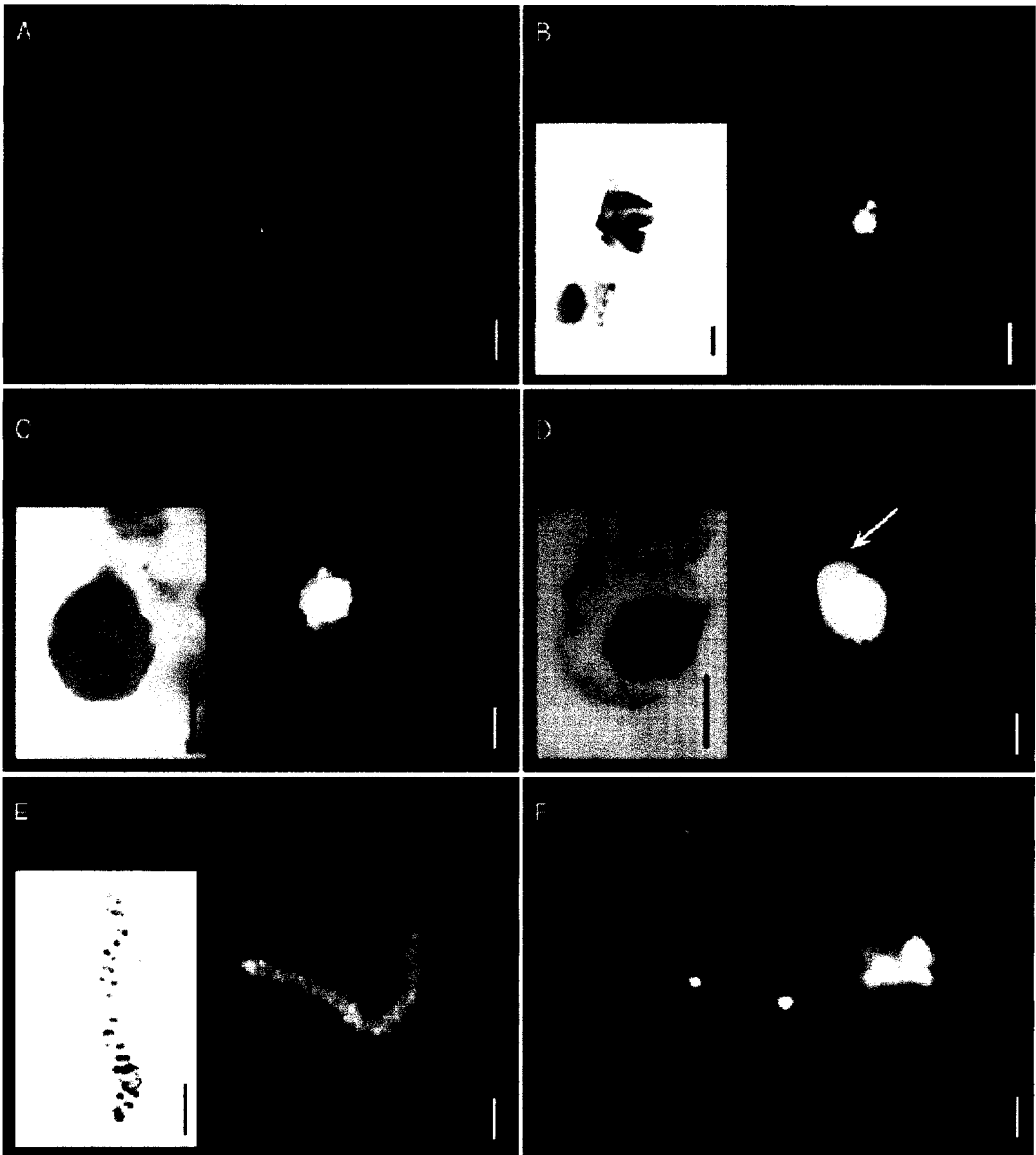


FIG. 2. Epifluorescence and bright field micrographs of *Amoebophrya* sp. in *Gonyaulax spinifera*. Scale bars, 20 μ m. (A) Attachment of dinospore (green fluorescence) of *Amoebophrya* sp. to the surface of *G. spinifera* (red fluorescence) observed under epifluorescence. (B and C) Early and mid-infections where the green autofluorescing parasite is surrounded by red fluorescing chloroplasts of *G. spinifera*. (B, inset) Protargol-stained specimen showing young trophont visible as a lightly staining circle inside the host's nucleoplasm; arrow indicates parasite nucleus. (C, inset) Protargol-stained specimen showing mid-infection with several irregularly arranged parasite nuclei. (D) Epifluorescence of a mature trophont, the remnants of the host's chloroplasts (arrow) are also shown. (Inset) Protargol-stained specimen showing late (beehive stage) infection. (E and F) Vermiform of *Amoebophrya* sp. under epifluorescence and snapshot of the process releasing new dinospores, respectively. (E, inset) Protargol-stained vermiform of *Amoebophrya* sp. from *G. spinifera*.

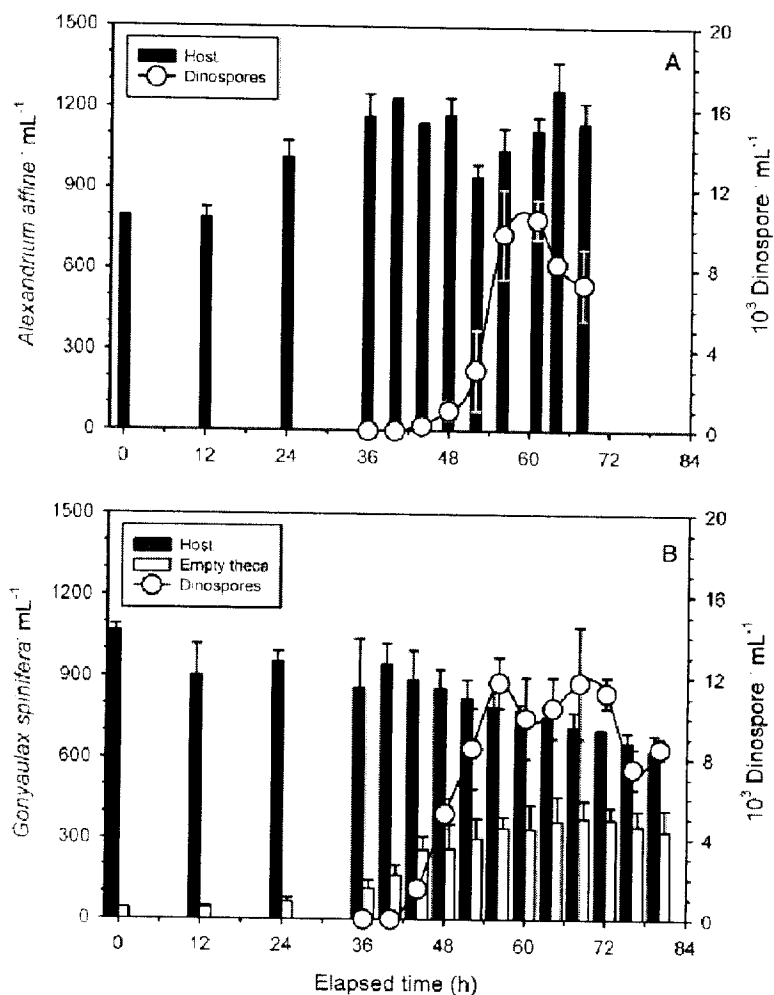


FIG. 3. Time-course studies of *Amoebophrya* spp. infecting (A) *Alexandrium affine* and (B) *Gonyaulax spinifera* inoculated at dinospore-to-host ratios of 1:1. Black bars represent host abundance, and white bars represent the empty theca. Open circles are dinospore abundance. Error bars indicate SE.

Amoebophrya spp. Further, the percent ($6.9\% \pm 1.81\%$) of dinospores from *G. spinifera* that successfully established infections (i.e. dinospore success) was within the range (4.7%–18.0%) reported for athecate hosts (Coats and Park 2002), again suggesting that thecal plates do not act as a barrier to successful penetration of dinospores into host cells and thereby do not reduce parasite success. Although the time required for dinospores to penetrate into host cells is unknown, the duration of vermiform emergence from *A. affine* and *G. spinifera* (2–10 min) is only slightly longer than that recorded for the athecate host *A. sanguinea* (1–2 min;

Coats and Bockstahler 1994). Salomon et al. (2003) also reported a similar emergence time (2–8 min) for *Amoebophrya* sp. from thecate dinoflagellate *Dinophysis norvegica* Claparede and Lachmann. Thus, the emergence process is rapid in both thecate and athecate hosts and represents a relatively short portion of the parasite's life cycle. Whether the timing of dinospore penetration into the host differs among thecate and athecate hosts and is sufficiently long to influence parasite generation time remains an open question.

Parasite generation time may be influenced by a number of factors, including 1) parasite load, 2) host

cell size or biovolume, 3) growth conditions (e.g. temperature, salinity, and light intensity, etc.), and 4) intrinsic host-specific differences in development times among *Amoebophrya* spp. For example, inoculation of *G. instructum* at a high dinospore-to-host ratio (20:1) increased parasite load and shortened parasite generation times, apparently due to more rapid utilization of host resources with growth of multiple parasites (Coats and Park 2002). The short generation times observed here for *Amoebophrya* from *A. affine* and *G. spinifera*, however, are not likely the result of multiple infections, because cultures were inoculated at a low dinospore-to-host ratio (i.e. 1:1). Generation times of *Amoebophrya* spp. that infect athecate dinoflagellates appear positively related to host size ranging from 59 h for *K. micrum* to 71 h for *G. instructum* (Coats and Park 2002). Based on host size, *Amoebophrya* from *A. affine* and *G. spinifera* would be expected to have generation times within this range, yet our estimates (55 h and 53 h for parasites of the two host species, respectively) are less than the shortest parasite generation time reported by Coats and Park (2002). Thus, host cell size (and/or biovolume) may be not the primary factor governing parasite generation time. Our experiments were conducted at the same temperature (20° C) used in the study by Coats and Park (2002), but our cultures were grown at a higher salinity (30 vs. 15 psu) and at lower irradiance (15–50 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ vs. 95–175 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Thus, we cannot exclude the possibility that differences in growth conditions account for the faster generation time observed for *Amoebophrya* for our thecate hosts relative to those of strains infecting athecate dinoflagellates (Coats and Bockstahler 1994, Coats and Park 2002). Nonetheless, intrinsic host-specific differences in intracellular and extracellular development times among *Amoebophrya* species appear the most plausible explanation for observed differences in parasite generation times.

Parasite intracellular development time, representing 60%–80% of total parasite generation time (Coats and Park 2002), has been used along with estimates of parasite prevalence to assess the impact of parasitism by *Amoebophrya* sp. on dinoflagellate populations in the field (Coats and Bockstahler 1994). In some instances, generation times obtained for *Amoebophrya* spp. from athecate hosts have been used to calculate the impact of parasitism on thecate host species (Gisselson et al. 2002, Salomon et al. 2003). Because *Amoebophrya* spp. likely have intrinsic host-specific differences in generation times, applying data for one parasite–host system to estimate the influence of parasitism on a different host species may produce unpredictable biases as recognized by Salomon et al. (2003). Thus, data for intracellular development times and/or total parasite generation times for a variety of athecate and thecate host–parasite systems are needed to better define the significance of parasitism as a loss factor for dinoflagellate populations in nature.

Amoebophrya infections in chain-forming dinoflagellates can facilitate the fragmentation of long cell chains

into multiple short pieces as observed here for *A. affine*. Because swimming speed of chain-forming dinoflagellates is a function of chain length (Fraga et al. 1989), parasitism may reduce host's swimming speed by reducing the length of cell chains. The resulting decrease in host swimming speed may increase the chance of infection by facilitating attachment of parasites to host cells.

Our observations and data for parasite generation times in *A. affine* and *G. spinifera* increase knowledge of thecate dinoflagellate host–parasite systems. Further, our results provide the basis for better assessing the impact of parasitism by *Amoebophrya* sp. as a significant loss factor for thecate dinoflagellate populations in nature.

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**IV. Host range in two strains of *Amoebophyra*
(Dinophyta) infecting the thecate dinoflagellates
Alexandrium affine and *Gonyaulax polygramma***

ABSTRACT

The endoparasitic dinoflagellate *Amoebophrya ceratii* (Koeppen) Cachon uses a number of its free-living relatives as hosts and may represent a species complex composed of several host-specific parasites. Using two thecate host-parasite systems (*Amoebophrya* spp. from *Alexandrium affine* (Inoue and Fukuyo) Balech and *Gonyaulax polygramma* Stein), I tested the hypothesis that two strains of *Amoebophrya* have a high degree of host specificity. To test this hypothesis, I conducted a series of cross infection experiments, with 10 thecate and 3 athecate dinoflagellate species as potential hosts. Surprisingly, the two strains of *Amoebophrya* lack host specificity and have wider host range than previously found. While *Amoebophrya* sp. from *A. affine* was capable of infecting only the species (*A. affine*, *A. catenella*, and *A. tamarense*) of genus *Alexandrium* among host species tested, the parasite from *G. polygramma* infected species covering 5 genera (*Alexandrium*, *Gonyaulax*, *Prorocentrum*, *Heterocapsa*, and *Scropsiella*). My results, along with previous reports suggest that host specificity of the *Amoebophrya* parasites not only varies from extremely species-specific to rather unspecific, but also tends to be stronger in strains isolated from athecate hosts. This information on host specificity of the parasite will be helpful in assessing the possibility of using these parasites as biological control agents for harmful algal blooms, as well as in defining species of the parasite in the future.

INTRODUCTION

Much speculation exists for use of biological methods to control bloom-forming species that occur with globally increasing frequency, magnitude, and impacts of harmful algal blooms (Hallegraeff 1993, Anderson 1997). Among several biological control agents having the potential to influence harmful algal bloom species (Brussaard 2004, Mayali and Azam 2004, Park et al. 2004, Tillmann 2004 for recent reviews), parasitic dinoflagellates have received the most attention. The endoparasitic dinoflagellate *Amoebophrya ceratii* (Koeppen) Cachon is particularly noteworthy, as it is not only widely distributed in coastal environments but also uses as much as 40 free-living dinoflagellates as hosts, including some toxic and harmful algal bloom species (Cachon 1964, Coats 1999, Park et al. 2004). However, the view that all *Amoebophrya* infections in free-living dinoflagellates are attributed to a single species, *A. ceratii* (e.g. Nishitani et al. 1985, Fritz and Nass 1992) has been challenged by Coats et al. (1996). From field observations and laboratory cross-infection experiments, they suggested that *A. ceratii* may represent a species complex composed of several “host-specific” parasites. Subsequently, while molecular data supporting their species complex hypothesis have accumulated over a last decade (Gunderson et al. 1999, 2000, 2002, Janson et al. 2000, Salomon et al. 2003a), our knowledge about host specificity of the parasite is relatively limited, and only a few studies have addressed this issue so far (e.g. Coats and Park 2002, Sengco et al. 2003). The information on host specificity from a variety of host-parasite systems would

not only be useful in sorting species of *Amoebophrya*, but would also be an important in developing biological control agents on harmful algal blooms. In this paper, I report patterns in host specificity for two strains of *Amoebophrya* from the thecate dinoflagellates *Alexandrium affine* (Inoue and Fukuyo) Balech and *Gonyaulax polygramma* Stein, and expanding the known host range of the parasite.

MATERIALS AND METHODS

The two thecate host-parasite systems (*Amoebophrya* spp. ex *Alexandrium affine* and *Gonyaulax polygramma*) were successfully established in cultures during the autumn of 2002 and have been maintained as described in detail elsewhere (Kim et al. 2004). To test host range in the two strains of *Amoebophrya*, the following 13 species were used as potential hosts during this study; 10 thecate dinoflagellates *A. affine* (strain 1: primary host), *A. affine* (strain 2), *A. catenella* (Whedon and Kofoid) Balech, *A. tamarense* (Lebour) Balech, *G. polygramma* (primary host), *Heterocapsa triquetra* (Ehrenberg) Stein, *Prorocentrum dentatum* Stein, *P. micans* Ehrenberg, *P. minimum* (Pavillard) Schiller, *Scripsiella trochoidea* (Stein) Loeblich III, and 3 athecate dinoflagellates *Akashiwo sanguinea* (Hirasaka) G. Hansen et Moestrup, *Cochlodinium polykrikoides* Margelef, and *Gymnodinium catenatum* Graham. All experiments were performed using host cultures in exponential growth and recently formed (≤ 6 h) dinospores of *Amoebophrya* spp. To obtain recently formed dinospores, stock parasite cultures were gravity filtered through polycarbonate filters of 12

µm pore size (Isopore™, MA, USA) to produce filtrates containing dinospores of unknown age. Dinospores were inoculated into uninfected primary host culture, and dinospores produced from the subsequent parasite generation were harvested within 6 h (referred to as recently formed dinospores above) using filtration procedures described above.

To assess the ability of *Amoebophrya* spp. ex *A. affine* (strain 1) and ex *G. polygramma* to infect alternative hosts, sets of triplicate scintillation vials containing 10-mL of host cells at $1 \times 10^3 \text{ mL}^{-1}$ were inoculated with dinospores to give a dinospore:host ratio of 12:1 (for *Amoebophrya* ex *A. affine*) and 10:1 (for *Amoebophrya* ex *G. polygramma*), and incubated for 40-90 h at 20° C under a 14:10 light:dark cycle of cool-white fluorescent light at $50 \text{ µmol photons m}^{-2}\text{s}^{-1}$. After incubation, samples were preserved with CaCO₃ buffered formaldehyde (2% final concentration) and examined by epifluorescence microscopy (Olympus microscope, 200×; Fluorescence Cube U-MWB2, 450- to 480-nm excitation, 500-nm emission) to visualize the distinctive green autofluorescence of the parasite (Coats and Bockstahler 1994, Kim et al. 2004). Parasite prevalence (= percent cells infected) was determined by scoring 100 host cells per sample as infected or uninfected. Data are reported in the text as mean \pm SE.

RESULTS

In the host range test, dinospores of *Amoebophrya* sp. from *A. affine* (strain 1) readily infected the primary host species, with parasite prevalence being $69.3 \pm 2.85\%$ at a dinospore:host ratio of 12:1 (Table 1). Surprisingly, this parasite

TABLE 1. A summary of infection of dinoflagellate species following the exposure to dinospores of *Amoebophrya* spp. from *Alexandrium affine* (strain 1) and *Gonyaulax polygramma*. Values are mean \pm SE (n=3).

Dinoflagellate hosts tested	Parasites (% cells infected)	
	<i>Amoebophrya</i> sp. ex <i>A. affine</i> (strain 1)	<i>Amoebophrya</i> sp. ex <i>G. polygramma</i>
Thecate		
<i>Alexandrium affine</i> (strain 1)	69.3 \pm 2.85	-
<i>Alexandrium affine</i> (strain 2)	51.3 \pm 3.33	-
<i>Alexandrium catenella</i>	1.3 \pm 0.33	1.3 \pm 0.33
<i>Alexandrium tamarense</i>	19.0 \pm 1.00	38.7 \pm 1.20
<i>Gonyaulax polygramma</i>	- ^a	82.3 \pm 1.76
<i>Heterocapsa triquetra</i>	-	0.3 \pm 0.33
<i>Prorocentrum dentatum</i>	nd ^b	-
<i>Prorocentrum micans</i>	-	< 0.1
<i>Prorocentrum minimum</i>	nd	< 0.1
<i>Scipsiella trochoidea</i>	nd	0.7 \pm 0.33
Athebate		
<i>Akashiwo sanguinea</i>	-	-
<i>Cochlodinium polykrikoides</i>	nd	-
<i>Gymnodinium catenatum</i>	-	-

^a Not infected; ^b Not determined

from *A. affine* (strain 1) was also capable of infecting other species within genus *Alexandrium*, with infection levels of $51.3 \pm 3.33\%$ in *A. affine* (strain 2), $1.3 \pm 0.33\%$ in *A. catenella*, and $19.0 \pm 1.00\%$ in *A. tamarense*. However, comparable inoculation using the same dinospore stock produced no infections in thecate species *G. polygramma*, *H. triquetra*, and *P. micans* and athecate species *A. sanguinea* and *G. catenatum*.

Dinospores of *Amoebophrya* sp. from *G. polygramma* also readily infected the primary host species, generating infection level of $82.3 \pm 1.76\%$ at a dinospore:host ratio of 10:1. Interestingly, the same inoculum size produced infections in species covering 4 genera among 12 species tested in this study, but infection levels were much lower than that in the primary host species ($1.3 \pm 0.33\%$ in *A. catenella*, and $< 1\%$ in *H. triquetra*, *P. micans*, and *S. trochoidea*), except for in *A. tamarense* ($38.7 \pm 1.20\%$). Dinospores of *Amoebophrya* sp. from *G. polygramma* failed to infect *A. affine* (strain 1), *A. affine* (strain 2), and all athecate dinoflagellates tested in this study.

DISCUSSION

Amoebophrya ceratii has long been thought to lack host specificity (Cachon 1964, Drebes 1984, Nishitani et al. 1985), but the extent to which this parasite is truly non-host specific has not been fully understood. One reason for this may be due to the absence of available host-parasite systems established in culture to appropriately evaluate host specificity of the parasite. Nonetheless, most

Amoebophrya strains established in laboratory culture have shown a high degree of host specificity; for example, Coats et al. (1996) reported that *Amoebophrya* isolated from *Akashiwo sanguinea* was unable to infect *Ceratium furca*, *Gyrodinium uncatenum*, and *Scrippsiella trochoidea* when incubated in single or mixed host species assemblages. More recently, Coats and Park (2002) showed that each of *Amoebophrya* strains from *A. sanguinea*, *Gymnodinium instriatum*, and *Karlodinium micrum* was unable to successfully cross-infect the alternate host species. By contrast, Sengco et al. (2003) recently reported that *Amoebophrya* isolated from *Alexandrium tamarense* exhibited less host specificity, i.e. a wide host range, with infections also successfully established in other thecate hosts including *Heterocapsa triquetra*, *Prorocentrum minimum*, *P. micans*, and *S. trochoidea*. Our 2 *Amoebophrya* strains also had wide host range, similar to that from *A. tamarense*. These results along with previous reports suggest that *Amoebophrya ceratii* represents a species complex composed of “varying” degrees of host specificity, ranging from extremely species-specific (e.g. *Amoebophrya* spp. from *A. sanguinea*, *G. instriatum*, and *K. micrum*; Coats et al. 1996, Coats and Park 2002), to moderately specific (e.g. *Amoebophrya* sp. from *A. affine*; this study) to rather nonspecific (e.g. *Amoebophrya* spp. from *A. tamarense* and *G. polygramma*; Sengco et al. 2003, this study). Further, host specificity appears stronger in strains isolated from athecate hosts than from thecate hosts, although the reason remains unclear.

Better knowledge of host specificity and/or host range of *Amoebophrya* sp. would have implications in considering the use of parasite as biological control

agents for red tides and in defining species of *Amoebophrya*. In the former case, high levels of host specificity and/or host preference for any particular bloom-forming target species would be necessary, otherwise the parasite would likely have considerable impact on other organisms (i.e. dinoflagellates) within the marine food web. Since Taylor (1968) first suggested using *A. ceratii* as a biological control for red tides, the idea has been considered unacceptable (Nishitani et al. 1985), because data from field samples indicated a lack of host specificity. The increasing data on host specificity of the parasite in a variety of host-parasite systems will be helpful in assessing the possibility of using *Amoebophrya* strains as biological tools for controlling harmful algal blooms. On the other hand, Kim et al. (submitted) reported that some *Amoebophrya* strains from different dinoflagellate hosts can be considered the same species when judged by variation in SSU rDNA sequences, but as different species from the standpoint of host specificity, and further suggested that morphological, biological, and molecular data must be appropriately combined in sorting species of *Amoebophrya*. When based on their sequence data, *Amoebophrya* from *A. affine*, *G. instriatum*, *C. tripos*, and *P. micans* represent a single species. However, while *Amoebophrya* from *G. instriatum* had a high degree of host specificity (Coats and Park 2002), the parasite from *A. affine* appeared to be moderately host-specific (this study), again supporting Kim et al.'s suggestion. My finding on host specificity of the 2 *Amoebophrya* strains from thecate dinoflagellate hosts will provide the basic data for resolving the issue in the future.

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**V. Revisiting the species complex hypothesis for
Amoebophrya ceratii (Dinophyta)**

ABSTRACT

The small-subunit (SSU) rDNA sequences of *Amoebophrya* Köppen strains infecting the thecate dinoflagellates *Alexandrium affine* (Inoue et Fukuyo) Balech and *Gonyaulax polygramma* Stein in Korean coastal waters were determined. By comparing those data with previously reported SSU rDNA sequences of *Amoebophrya* from eight other hosts, the single species hypothesis for the parasitic dinoflagellate *Amoebophrya ceratii* (Köppen) Cachon was revisited. The 10 available *Amoebophrya* sequences clustered together within the dinoflagellates as a monophyletic group with high bootstrap support, although SSU rDNA sequences between the *Amoebophrya* strains differed by 0.6 to 11.8%. The sequences of *Amoebophrya* strains from *A. affine*, *Gymnodinium instriatum* (Freudenthal et Lee) Coats, *Ceratium tripos* (Müller) Nitzsch, and *Prorocentrum micans* Ehrenberg grouped with 100% bootstrap value and showed high similarity (98.5 to 99.4%) despite differences in host species and geographic origin, indicating that these strains may represent a single species. By comparison, sequences of the other strains were sufficiently different from each other in similarity (<95.8%) to warrant separation at the species level. The suggestion, based on SSU rDNA sequence similarity, that *Amoebophrya* strains from *A. affine*, *G. instriatum*, *C. tripos*, and *P. micans* are the same species, however, needs to be confirmed through careful examination of parasite biology and morphology.

INTRODUCTION

Amoebophrya ceratii (Köppen) Cachon is an endoparasitic dinoflagellate that infects several free-living dinoflagellates, including toxin-producing and harmful algal bloom species (Cachon 1964, Taylor 1968, Coats 1999, Park et al. 2004). Cachon (1964) reported the first detailed description of the morphology and life cycle of the parasite while studying *Amoebophrya* infections in a number of dinoflagellate hosts in the Mediterranean Sea. He noted conspicuous developmental differences in parasites among host species, different sites of infection inside hosts, and considerable variation in the morphology of the infective, dispersal “dinospore” stage of the parasite, and recognized the possibility that *Amoebophrya* infections might be caused by more than one species. Nonetheless, subsequent studies attributed all *Amoebophrya* infections in free-living dinoflagellates to a single species, *A. ceratii* (e.g. Nishitani et al. 1985, Fritz and Nass 1992). From field observations and laboratory experiments, however, Coats et al. (1996) suggested that *A. ceratii* may represent a species complex composed of several host-specific parasites. In addition, Coats and Park (2002) reported that *Amoebophrya* strains from *Akashiwo sanguinea* (Hirasaka) G. Hansen et Moestrup, *Gymnodinium instriatum* (Freudenthal et Lee) Coats, and *Karlodinium micrum* (Leadbeater et Dodge) J. Larsen had a high degree of host specificity, along with the marked biological differences (e.g. parasite generation time, dinospore survival, and infectivity) among strains. More recently, Kim et al. (2004b) reported that *Amoebophrya* strains from the

thecate dinoflagellates, *Alexandrium affine* (Inoue et Fukuyo) Balech and *Gonyaulax polygramma* Stein, mistakenly identified as *Gonyaulax spinifera* (Claparede et Lachmann) Diesing, parasitized different sites (i.e. cytoplasm and nucleus, respectively) inside their hosts, and had shorter generation times than those previously reported in athecate hosts. This series of biological observations for a variety of host-parasite systems, along with some molecular studies showing considerable genetic divergence in SSU rDNA sequences among several *Amoebophrya* strains (Gunderson et al. 1999, 2000, 2002, Janson et al. 2000, Salomon et al. 2003a), supports the species complex hypothesis of Coats et al. (1996).

In this study, we sequenced the SSU rDNA of two *Amoebophrya* strains infecting *A. affine* and *G. polygramma* from Korean coastal waters and compared those with previously reported sequences for the *Amoebophrya* strains isolated from several other hosts from different geographic areas. Our molecular data bring the number of complete SSU rDNA sequences available for *Amoebophrya* strains to 10, providing a sufficient database for examining variation among parasite strains.

MATERIALS AND METHODS

Source and culture of cells. Stock cultures of the thecate dinoflagellates *A. affine* and *G. polygramma* were grown in f/2-Si medium (Guillard and Ryther 1962) formulated using 30 psu Korean coastal seawater. In our previous study

(Kim et al. 2004b), the host species *G. polygramma* was misidentified as *G. spinifera* based on its morphology under bright field microscopy. Subsequent observations including scanning electron microscopy and molecular analysis revealed this host species to be *G. polygramma*. Two strains of *Amoebophrya*, one each in the host species *A. affine* and *G. polygramma*, were established in cultures by adding a single infected host cell from field samples to cultures of complementary host species (Kim et al. 2004b). Parasites were subsequently propagated by transferring aliquots of infected host cultures to uninfected host stocks at approximately 2-3 days intervals. All cultures were maintained at 20°C on a 14:10 h light:dark cycle under cool white fluorescent light at 50 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

DNA extraction, PCR amplification, cloning, and sequencing. *A. affine* and *G. polygramma* host cells were harvested from exponentially growing cultures, and their genomic DNA was extracted using a slightly modified LiCl method (Hong et al. 1995). To amplify rDNA directly from *Amoebophrya* strains, vermiforms (i.e. the parasite stage immediately after emergence from the host) were individually captured using a glass micropipette and transferred directly to a PCR tube.

PCR amplification was carried out in MyCycler™ (Bio-Rad, Hercules, CA, USA). The 50- μL reactions contained 1 \times *Ex Taq*™ buffer, 250 μM of each dNTP, 1 μL of genomic DNA (10 $\text{ng}\cdot\mu\text{L}^{-1}$) or single cells, 0.2 μM of forward and reverse primers, and 1.25 unit of *TaKaRa Ex Taq*™ (TaKaRa, Shiga, Japan). The amplification was performed using 18S-0009f as the forward primer and 18S-

1797r as the reverse primer (Kim et al. 2004a; Table 1). The PCR reaction cycles was started with a 3 min initial denaturation at 94°C, followed by 35 cycles of 30 sec denaturation at 94°C, 1 min annealing at 55°C, and 1 min elongation at 72°C. The reaction was completed with a final 7 min elongation at 72°C. The appropriate PCR bands were excised and purified using a QIAquick™ Gel Extraction kit (Qiagen, Hilden, Germany).

The PCR products of hosts *A. affine* and *G. polygramma* were directly sequenced, whereas those of the *Amoebophrya* strains were not due to considerable noise that might have resulted from host cell contamination. Thus, the PCR products of the two *Amoebophrya* strains were ligated into pCR-2.1 vector, which was used to transform *Escherichia coli* (INVαF') with an Original TA Cloning® kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After the color-based selection of transformants using X-gal, white *E. coli* transformant colonies were cultured, and their plasmid DNAs were extracted using a Quantum Prep® Plasmid Miniprep Kit (Bio-Rad, Hercules, CA, USA). Four plasmid DNAs for each *Amoebophrya* strain were pooled and used for sequence analyses.

A cycle-sequencing reaction was performed with the PCR primer set and internal sequencing primers (Table 1) using an ABI Prism BigDye™ Terminator v3.0 Cycle Sequencing kit (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The sequencing reaction was run on an ABI 3100 Sequencer (Perkin-Elmer, Applied Biosystems, Foster City, CA, USA). Sequence data were deposited in GenBank (AY775284 to

TABLE 1. Primers used for PCR amplification and sequencing of SSU rDNA in this study.

Primer ¹	Sequence (5'→3')
18S-0009f ²	GATCCTGCCAGTAGTCATAT
18S-1797r ²	GATCCTTCYGCAGGTTACCTAC
18S-0302f ³	AGTTTCTGACCTATCAG
18S-0437r ³	GCGCCTGCTGCCTTCCTTA
18S-0613f ³	GCGGTAAAAAGCTCGTAGT
18S-0897f ³	AGAGGTGAAATTCTTGGAT
18S-1179f ³	CTTAATTTGACTCAACACG
18S-1435f ³	AACAGGTCTGTGATGCCCTT

¹ Primer nomenclature corresponds to the *Prorocentrum micans* SSU rDNA position (Herzog and Maroteaux 1986); “f” and “r” represent forward and reverse primers, respectively.

² Primers for PCR amplification and sequencing.

³ Primers for sequencing.

AY775287) (Table 2).

Phylogenetic analyses. Our four sequences and other dinoflagellate sequences available in GenBank database were aligned using Clustal W (Thompson et al. 1994) in BioEdit (Hall 1999). The alignment results were examined and errors were corrected manually based on secondary structure of the SSU rDNA molecule (Wuyts et al. 2002).

The SSU rDNAs used for final phylogenetic analyses excluded two primer regions. All phylogenetic analyses were run in PAUP* version 4.0b10 (Swofford 2002) with representative dinoflagellate sequences retrieved from GenBank. Among the SSU rDNA sequences of 13 *Amoebophrya* strains available in GenBank, three were not included in this study because their sequences were incomplete. The unused sequences were for three *Amoebophrya* strains from North Sea, *Amoebophrya* spp. ex *Dinophysis norvegica* Claparède et Lachmann (AY260469), ex *Ceratium tripos* (AY260468), and ex *C. lineatum* (Ehrenberg) Cleve (AY260467). The perkinsozoan *Perkinsus marinus* (AF126013) and the apicomplexan *Toxoplasma gondii* (X75453) were used as outgroup taxa.

Maximum parsimony (MP) analysis was performed using the heuristic search option with random addition of sequences (10 replicates) and tree bisection-reconnection (TBR) branch swapping. The characters were weighted equally and gaps were treated as missing data. Maximum likelihood (ML) analysis was performed using the TrN + I + G model with the following likelihood settings determined by Modeltest 3.06 (Posada and Crandall 1998): base

TABLE 2. Information of isolates sequenced in this study and the GenBank accession numbers for their SSU rDNA sequences.

Species	Strain code	Sampling site	Sampling date	Accession number
<i>Alexandrium affine</i>	JH0210	Jinhae Bay, Korea	Oct. 2002	AY775286
<i>Gonyaulax polygramma</i>	GS0209	Western coastal water, Korea	Sep. 2002	AY775287
<i>Amoebophrya</i> sp. ^a	JH0210a	Jinhae Bay, Korea	Oct. 2002	AY775284
<i>Amoebophrya</i> sp. ^b	GS0209a	Western coastal water, Korea	Sep. 2002	AY775285

¹ The parasite *Amoebophrya* sp. infecting *Alexandrium affine*.

² The parasite *Amoebophrya* sp. infecting *Gonyaulax polygramma*.

frequencies A = 0.2681, C = 0.1821, G = 0.2345; base substitution rates AC = 1.0000, AG = 2.7475, AT = 1.0000, CG = 1.0000, CT = 5.1852; assumed proportion of invariable sites = 0.3584; and gamma distribution shape parameter = 0.5984). Heuristic searches with random sequence addition and TBR branch rearrangements were conducted with 10 replications. Bootstrap analyses were conducted to determine the robustness of the clades (Felsenstein 1985), with 1,000 and 500 replicates for the MP and the ML analyses, respectively.

RESULTS

PCR amplifications of genomic DNA from the two hosts *A. affine* and *G. polygramma* and their parasites *Amoebophrya* strains using primers complementary to the ends of SSU rRNA coding regions yielded a single PCR band for each of the hosts and parasites. PCR products for *A. affine* and *G. polygramma* (1789 and 1787 nucleotides, respectively) were directly sequenced, and nearly complete SSU rDNA sequences were successfully obtained. However, direct sequencing data for the two *Amoebophrya* strains were not obtained due to ambiguous electropherograms that might have resulted from contamination by remnants of host cells. Therefore, the putative SSU rDNA sequences for *Amoebophrya* strains infecting *A. affine* and *G. polygramma* (1790 and 1789 nucleotides, respectively) were identified by sequencing cloned plasmids containing purified PCR products.

The four sequences analyzed in this study clustered together with representative sequences of other dinoflagellates, including those of the eight

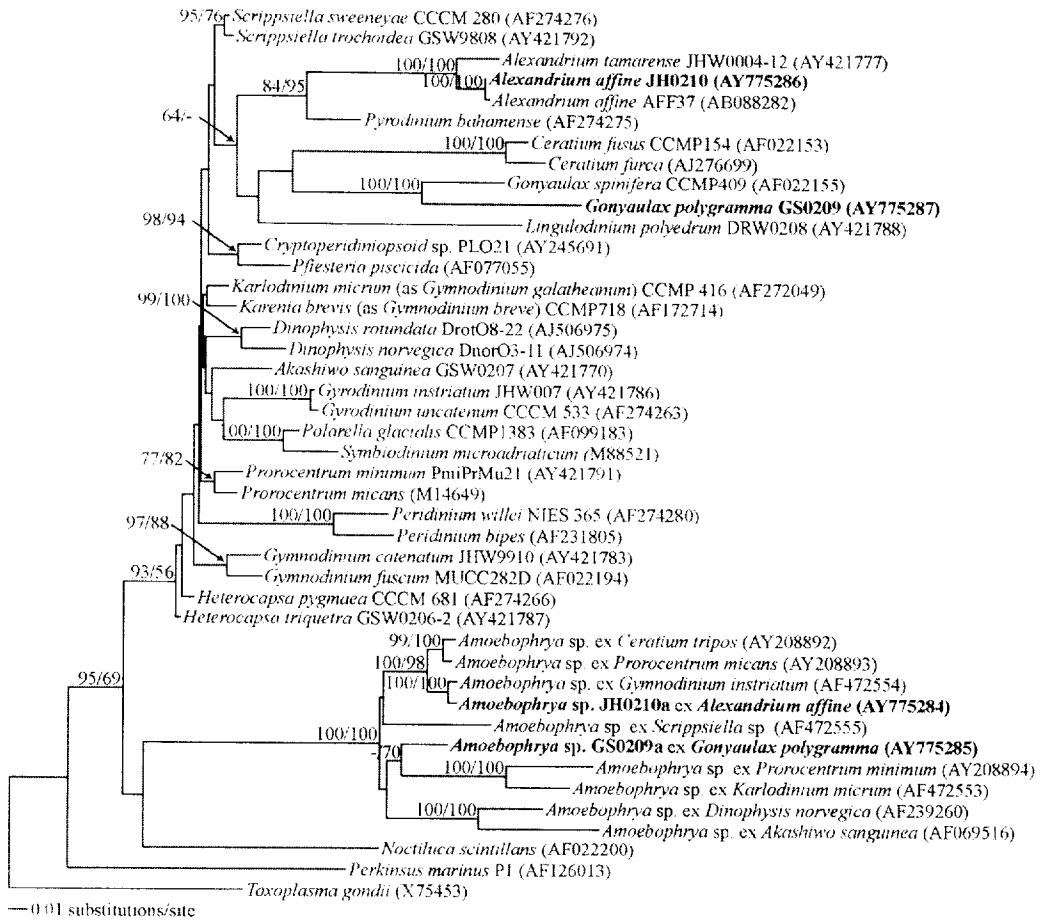
Amoebophrya strains and their host species retrieved from GenBank. The phylogenetic tree constructed with the ML tree constructing method is shown in Figure 1. The same lineages were also observed when neighbor joining (NJ) and MP were used as the tree building methods (data not shown).

Although the 10 *Amoebophrya* strains originated from different dinoflagellate hosts and distant geographic areas, their SSU rDNA sequences consistently formed a monophyletic group with 100/100% (MP/ML) bootstrap supports in both MP and ML trees. In either MP or ML tree, the *Amoebophrya* clade was placed at the base of dinoflagellates together with *Noctiluca scintillans* without significant bootstrap value support. The topology produced with the NJ method showed that the *Amoebophrya* clade did not unite with *N. scintillans* and diverged between *N. scintillans* and *Perkinsus marinus* (data not shown).

The *Amoebophrya* clade was divided into three lineages with 100% bootstrap support (Fig. 1). The first lineage included *Amoebophrya* strains infecting *A. affine*, *G. instriatum*, *C. tripos*, and *Prorocentrum micans*, the second *Amoebophrya* strains infecting *Prorocentrum minimum*, *K. micrum*, and *G. polygramma*, and the third *Amoebophrya* strains ex *D. norvegica* and ex *A. sanguinea*. In NJ and ML trees, *Amoebophrya* sp. ex *Scrippsiella* sp. appeared to unite with the first lineage, while the MP tree placed that strain in a sister position separated from the other parasite sequences (data not shown). However, there was not sufficient bootstrap value support to resolve the position of *Amoebophrya* sp. ex *Scrippsiella* sp..

Within the *Amoebophrya* clade, SSU rDNA sequence similarities ranged

FIG. 1. Maximum likelihood tree of *Amoebophrya* and other representative dinoflagellates including their host species based on the SSU rDNA sequences. *Perkinsus marinus* and *Toxoplasma gondii* were used as outgroup taxa. The ML tree was constructed using the TrN + I + G model in PAUP* 4.0b10. Numbers at each branch node indicate bootstrap values above 50%, which were obtained from MP / ML. The four species analyzed in this study are in bold. Origins of the *Amoebophrya* strains are as follows: *Amoebophrya* spp. ex *Akashiwo sanguinea*, ex *Gymnodinium instriatum*, ex *Karlodinium micrum*, ex *Prorocentrum micans*, ex *Prorocentrum minimum*, and ex *Scrippsiella* sp. from Chesapeak Bay; *Amoebophrya* sp. ex *Ceratium tripos* from Helsingor of Denmark; *Amoebophrya* sp. ex *Dinophysis norvegica* from Baltic Sea.



from roughly 88.2 to 99.4% (Table 3). High similarities of 98.5 to 99.4% were observed for sequences of strains in the first lineage (i.e. *Amoebophrya* sp. ex *A. affine* from Jinhae Bay, Korea, ex *C. tripos* from Helsingør, Denmark, and ex *G. instriatum* and ex *P. micans* from Chesapeake Bay, USA) (Table 3). The SSU rDNA sequence of *Amoebophrya* sp. ex *P. minimum* was 95.5% identical to that of *Amoebophrya* sp. ex *K. micrum*, while the sequence of *Amoebophrya* sp. ex *D. norvergica* showed 93.5% similarity with that of *Amoebophrya* sp. ex *A. sanguinea*. The sequence for *Amoebophrya* sp. ex *G. polygramma* was the most similar (95.8% similarity) to *Amoebophrya* sp. ex *C. tripos*, but those strains emerged in different branches (Fig. 1, Table 3).

DISCUSSION

Parasitic dinoflagellates of the genus *Amoebophrya* have been reported from about 40 different free-living dinoflagellates inhabiting coastal waters of the world (Park et al. 2004). All but one of these parasites were historically classified as *Amoebophrya ceratii*, the single exception being *A. leptodisci* Cachon from the heterotrophic dinoflagellate *Pratjetella medusoides* (Hertwig) Loeblich Jr. et Loeblich III (Coats and Bockstahler 1994). Recently, however, ecological, physiological, and molecular investigations of *Amoebophrya*-host associations have suggested that *A. ceratii* may represent a species complex (Coats et al. 1996, Janson et al. 2000, Coats and Park 2002, Gunderson et al. 2000, 2002, Salomon et al. 2003a). Our molecular analysis of *Amoebophrya* from *Alexandrium affine* and *Gonyaulax polygramma* brings the number of complete

TABLE 3. Similarity percentages of SSU rDNA sequences from *Amoebophrya* spp.

	1	2	3	4	5	6	7	8	9
1 <i>Amoebophrya</i> ex <i>A. sanguinea</i>									
2 <i>Amoebophrya</i> ex <i>G. instriatum</i>	91.6								
3 <i>Amoebophrya</i> ex <i>K. micrum</i>	88.8	93.4							
4 <i>Amoebophrya</i> ex <i>Scripsiella</i> sp.	90.5	95.3	92.7						
5 <i>Amoebophrya</i> ex <i>D. norvegica</i>	93.5	93.2	90.3	91.8					
6 <i>Amoebophrya</i> ex <i>C. tripos</i>	91.8	98.5	92.9	95.2	93.1				
7 <i>Amoebophrya</i> ex <i>P. minimum</i>	88.2	92.3	95.5	92.0	90.1	92.1			
8 <i>Amoebophrya</i> ex <i>P. micans</i>	91.6	98.5	93.0	95.0	92.9	99.4	92.1		
9 <i>Amoebophrya</i> ex <i>G. polygramma</i>	91.3	95.7	94.0	95.0	92.9	95.8	92.9	95.7	
10 <i>Amoebophrya</i> ex <i>A. affine</i>	91.5	99.4	93.6	95.3	93.2	98.5	92.4	98.5	95.7

SSU rDNA sequences available for *Amoebophrya* strains to 10, providing a sufficient database for examining variation among parasite strains.

Construction of a ML tree using host and parasite SSU rDNA sequences placed all 10 *Amoebophrya* sequences within a monophyletic group having 100% bootstrap support, suggesting that *Amoebophrya* species have unique sequences that are not shared with other dinoflagellates. Further, the ML tree split *Amoebophrya* into roughly three lineages, each supported by high bootstrap values. More interestingly, relatively little variation existed among SSU rDNA sequences in the parasite lineage composed of *Amoebophrya* strains from *A. affine*, *G. instriatum*, *C. tripos*, and *P. micans*, as indicated by high similarity values (98.5 to 99.4%). Thus, while these *Amoebophrya* strains were from four different hosts and originated from distant geographic regions (Korea, USA, and Denmark), they may represent a single species.

The level of sequences similarity reported for strains of free-living dinoflagellates from different geographic regions tends to support the idea that *Amoebophrya* ex *A. affine*, *G. instriatum*, *C. tripos*, and *P. micans* are the same species. For example, the SSU rDNA sequences of two *A. sanguinea* strains, one from Korea (Gskor11) and one from USA (CCMP1321), showed 98.49% similarity (Kim et al. 2004a), falling within the range found for *Amoebophrya* from *A. affine*, *G. instriatum*, *C. tripos*, and *P. micans*. Indeed, sequences similarity of *Amoebophrya* from these four hosts appears to be striking.

Amoebophrya infections typically develop either within the host cytoplasm, or inside the host nucleus, depending on the particular dinoflagellate species being

parasitized. Two exceptions to this pattern are *Karlodinium micrum* and *Ceratium tripos*, for which both cytoplasmic and nuclear infections have been reported (Köppen 1899, Coats and Park 2002). Differences in site of infection for *Amoebophrya* ex *K. micrum* likely reflect within species variation, as observations were derived from a host-parasite culture initiated using a single infected host cell (Coats and Park 2002) and yielding only one parasite SSU rDNA sequence (Gunderson et al. 2002). That may not, however, be the case for *C. tripos*, as observations have been based on field samples and may contain more than one parasite species. For example, Elbrächter (1971, 1973) considered nuclear and cytoplasmic infections in *C. tripos* to be different parasites and classified only those that developed in the host nucleus as *Amoebophrya ceratii*. Similarly, two different strains, or species, of *Amoebophrya* have been reported to infect *Dinophysis norvegica* (Salomon et al. 2003a,b). In that case, both parasites appear to infect the host nucleus, but one parasite occurs in North Sea populations of *D. norvegica*, while the other strain infects *D. norvegica* in the Baltic Sea.

In our analysis, all host species known only to have cytoplasmic *Amoebophrya* infections, i.e. *A. affine*, *G. instriatum*, and *P. micans* (Cachon 1964, Coats and Park 2002, Kim et al. 2004b), occupied the same lineage in our ML tree. As mentioned above, *Amoebophrya* may occur in the nucleus or cytoplasm of *C. tripos*, but differences in site of infection in that host species may be attributable to different parasite species. Interestingly, cytological stains of field samples used by Gunderson et al. to obtain SSU rDNA sequence data for *Amoebophrya* ex

C. tripos (GenBank Accession Number AY208892) only revealed the presence of cytoplasmic infections (D.W. Coats, pers. observ.). Thus, the lineage formed by *Amoebophrya* ex *A. affine*, *G. instriatum*, *P. micans*, and *C. tripos* not only had marked SSU rDNA sequence similarity, but also showed similarity in parasite development consistent with the interpretations that these *Amoebophrya* strains represent one species.

Except for *Amoebophrya* ex *K. micrum*, the other parasite strains used in our analysis form infections inside the host nucleus (Coats and Park 2002, Gisselson et al. 2002, Kim et al. 2002, Salomon et al. 2003b), but sorted across two lineages in our ML tree. Four strains from these two lineages (i.e. *Amoebophrya* ex *P. minimum*, *K. micrum*, *D. norvegica*, and *A. sanguinea*) clearly represent different species, as indicated by low sequence similarity (95.5% or less). Thus, knowledge of infection site within the host cells is not sufficient to identify species of *Amoebophrya*. That point is even more obvious when one recognizes that a single strain *Amoebophrya* from *K. micrum* has the ability to produce either nuclear or cytoplasmic infections, as discussed above.

Coats et al. (1996) and later Coats and Park (2002) used host specificity of *Amoebophrya* strains in culture studies to reinforce the suggestion that *A. ceratii* is a species complex. Following their logic, the contention that *Amoebophrya* from *A. affine*, *G. instriatum*, *C. tripos*, and *P. micans* represent a single species draws into questions, as some of these strains show a degree of host specificity. For example, *Amoebophrya* ex *A. affine* exhibited partial host specificity, with relatively broad host range, but failed to infect *G. instriatum*, *C. tripos*, and *P.*

micans in laboratory culture (S. Kim, pers. observ.). By contrast, *Amoebophrya* ex *G. instriatum* appeared to be host specific (Coats and Park 2002), supporting the argument that these strains are separate species.

Molecular data presented here support the hypothesis that *A. ceratii* is a species complex. However, as in the case of the clade containing *Amoebophrya* ex *A. affine*, parasite strains from different dinoflagellate hosts can be considered the same species when judged by variation in SSU rDNA sequences, but as different species from the standpoint of host specificity. This contradiction suggests that morphological, biological, and molecular data must be appropriately combined in sorting species of *Amoebophrya*. Until such correlative data are available for all *Amoebophrya* strains within the *A. affine*, *G. instriatum*, *C. tripos*, and *P. micans* lineage, we feel it premature to merge these parasites into a single species.

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**VI. The influence of parasitism by *Amoebophrya* sp.
on paralytic shellfish poisoning toxins of
*Alexandrium tamarense***

ABSTRACT: Endoparasitic dinoflagellates *Amoebophrya* spp. have been known to parasitize a number of their relatives, including toxic and harmful algal bloom species. This study was performed to test the hypotheses that (1) parasitism by *Amoebophrya* spp. alters cellular toxin content and profiles (relative toxin composition) of toxic dinoflagellates that cause paralytic shellfish poisoning (PSP) and (2) dinoflagellate PSP toxins are transferred to the infective, dispersal dinospore stage of the parasite. To test these hypotheses, we determined cellular toxin content and composition over the infection cycle for infected and uninfected cultures of *Alexandrium tamarense* and measured toxin content of dinospores produced at the end of the infection cycle. Cellular toxin contents and profiles of infected and uninfected *A. tamarense* cultures were not significantly different. In addition, no toxins were detected in dinospores. Our results suggest that parasitism by the endoparasitic dinoflagellate *Amoebophrya* does not function as vector for transport of PSP toxins to higher trophic levels and may play a role in dissipation of the toxins in marine food webs.

INTRODUCTION

Paralytic shellfish poisoning (PSP) toxins consisting of saxitoxin and its derivatives are produced by some marine dinoflagellates including *Alexandrium* spp., *Gymnodinium catenatum*, and *Pyrodinium bahamense* var. *compressum*, freshwater cyanobacteria, and dinoflagellate-associated bacteria (Cembella 1998, Hallegraeff 1993, Kodama 2000, Oshima et al. 1993, Smayda 1989, Shimizu 1996, Usup et al. 1994). Phytochemicals including PSP toxins are either accumulated and transferred to higher trophic levels or dissipated within the food web. In the former case, some grazers such as filter feeding shellfish (Schantz 1986, Shumway 1990, Cembella et al. 1993), metazooplankton (Turner & Anderson 1983, Turriff et al 1995, Teegarden & Cembella 1996), and heterotrophic protists (Jeong et al. 2001a, b, Stoecker et al. 2002) are well known to effectively graze on PSP toxins-producing phytoplankton without apparent harm and to transfer the toxins to higher trophic levels. By comparison, in the latter case, the toxins are reduced following feeding by some consumers such as scallop *Patinopecten yessoensis* (Sekiguchi et al. 2001), Mussels *Mytilus galloprovincialis* (Suzuki et al. 2003), and heterotrophic dinoflagellate *Polykrikos kofoidii* (Jeong et al. 2003), although the detoxification mechanism is not clearly understood.

On the other hand, endoparasitic dinoflagellates *Amoebophrya* spp. have been known to parasitize about 40 different free-living dinoflagellates including toxic or harmful algal bloom-forming species (Park et al. 2004). For example, Talyor (1968) reported that a toxic dinoflagellate *Alexandrium catenella* was

infected by *Amoebophrya* sp. with infection levels as high as 30-40% in Sequim Bay, USA. Nishitani & Chew (1984) and Nishitani et al. (1984, 1985) observed 2-47% prevalence of *Amoebophrya* infections in the toxic dinoflagellate *Alexandrium catenella* in Puget Sound, USA. Despite the numerous reports of *Amoebophrya* infections from toxic dinoflagellates, however, the fate of the toxins has not been addressed.

In this study, we tested the hypotheses that (1) cellular toxin content and profiles (relative toxin composition) of toxic hosts are altered following infection of parasite *Amoebophrya* sp. and (2) host toxins are transferred to infective, dispersal dinospore stage of the parasite. To test these hypotheses, we determined cellular toxin contents and toxin profiles over the infection cycle for infected and uninfected cultures of PSP toxins-producing dinoflagellate *Alexandrium tamarense* and measured toxin content of resulting dinospores. Toxin dynamics following the infection by the parasites will be discussed in the contexts of marine microbial food web and public human health.

MATERIALS AND METHODS

Culture conditions. The non-toxic dinoflagellate *Gonyaulax polygramma* and its parasite *Amoebophrya* sp. were collected from coastal water off Kunsan in Korea and established in culture (Kim et al. 2004). The parasitic dinoflagellate *Amoebophrya* sp. was obtained from field samples by isolating infected *G. polygramma* using epifluorescence microscopy and a glass micropipette. This parasite was propagated by sequentially transferring aliquots of infected host

culture into uninfected host stock at roughly 2-day intervals. A toxic strains of *Alexandrium tamarense* DDPW isolated from Jinhae Bay in the southern coast of Korea, was obtained from Aquaculture laboratory of Pukyong National University, Korea. *A. tamarense* and *G. polygramma* were grown in f/2-Si medium (Guillard & Ryther 1962) formulated using 30 psu seawater. All stock cultures used in the experiment were maintained at 20°C under a 14:10 light:dark cycle of cool-white fluorescent light at 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Experimental design. Experiments were conducted in triplicate using stock culture of *Alexandrium tamarense* in exponential growth. For infected treatments, triplicate flasks containing *A. tamarense* cultures ($\sim 1 \times 10^3 \text{ cells ml}^{-1}$) were inoculated at a dinospore:host ratio of 70:1 using recently formed (≤ 6 h old) dinospores of *Amoebophrya* sp. from *G. polygramma* to yield near 100% infection levels. To obtain the recently formed dinospores, the parasite stock cultures in *G. polygramma* were gravity filtered through 12 μm pore-size Millipore PC filters. Triplicate flasks for uninfected controls were run with addition of equivalent volumes of harvested dinospore filtrate (Millipore PC filters with 1 μm pore size). Subsamples for analyses of host and dinospore abundances, parasite prevalence (i.e. percent cells infected), and toxins were collected at T_0 (start of the experiment), T_{36} , T_{48} , T_{58} , and T_{70} after inoculation. Twenty-ml subsamples from each flask were preserved with modified Bouin's fixative (Coats & Heinbokel 1982) for measuring host abundance and parasite prevalence. Host abundance was determined using Sedgwick-Rafter chambers

under bright field microscopy (Olympus Model BX51). Parasite prevalence was assessed by scoring 100 cells as uninfected or infected hosts using two methods; one by green auto-fluorescence of the parasite under the blue excitation of epifluorescence microscopy (Coats & Bockstahler 1994) and the other by Protargol staining (Montagnes & Lynn 1993). For analysis of nitrate (NO_3^-) and phosphate (PO_4^{3-}), 2 ml aliquots were filtered through syringe filters with 0.22 μm pore-size and then the filtrates were stored at -20°C until analyzed. Nutrient concentrations were determined with a Bran Luebbe AutoAnalyzer (Model TRAACS 2000) according to Parsons et al. (1984).

Toxin analysis. Eighty-ml subsamples were taken from treatments and controls for analysis of PSP toxin amount and toxin profiles. Aliquots of the triplicate samples from each flask were collected by centrifugation (Vision Scientific Co.) at 10,000 rpm for 10 min, rinsed twice using distilled water to remove salts and then re-pelleted. The cells were resuspended in 0.5 ml of 0.5 N acetic acid and stored at -20°C until analyzed. To extract toxin from cells, the pellets were homogenized with three successive sonications in ice and the supernatant was obtained after centrifugation at 12,000 rpm for 10 min. Each 50 μl of these extracts was used for high-performance liquid chromatography (HPLC) analysis of toxins. Analysis of PSP toxins was performed by HPLC with fluorescence detection according to the method of Oschima (1995).

Statistical analyses. Data are presented as mean \pm SE of the mean from

triplicate flasks unless otherwise stated. Statistical analyses between infected treatments and uninfected controls were made by two-way ANOVA test (SigmaStat Ver. 2.0).

RESULTS

Host and dinospore abundance, parasite prevalence, and nutrient concentrations in infected and uninfected *A. tamarensis* cultures

Abundance of *Alexandrium tamarensis* in uninfected controls gradually increased to 3073 ± 327 cells ml⁻¹ during the experiment with growth rate of 0.35 ± 0.03 d⁻¹ (Fig. 1). By contrast, *A. tamarensis* cells in parasitized cultures did not reproduce during the experimental period, with abundance being relatively constant until 48 h, decreasing to 369 ± 51 cells ml⁻¹ by the end of the experiment.

At 36 h, parasite prevalence was $78 \pm 3.28\%$ and infected *A. tamarensis* cells were all in early stage of infection (Fig. 2). Parasite prevalence increased to $97 \pm 0.33\%$ at 48 h, with the early and late stages of infections being 64 ± 4.84 and $33 \pm 5.00\%$, respectively (Fig. 2). After 58h, mature trophonts of the parasite (28% of total host cells) began to emerge from host cells and produced numerous new dinospores. Abundance of newly-formed dinospores at 58 h and 70 h were 22 ± 2 and $36 \pm 4 \times 10^3$ cells ml⁻¹, respectively.

Inorganic nutrients concentration in both infected and uninfected cultures remained relatively stable over time, averaging 533.2 ± 9.48 and 15.44 ± 0.15 μM for NO₃ and PO₄, respectively (Fig. 3). Nitrate and phosphate concentrations for

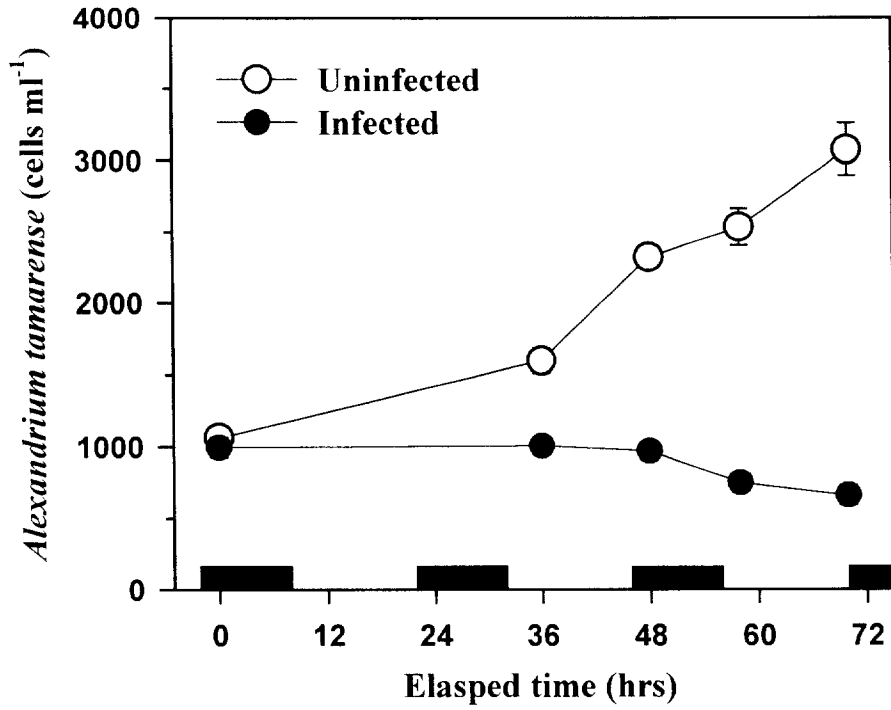


Fig. 1. Changes in cell density for infected and uninfected cultures of *Alexandrium tamarense*. Solid bars on abscissa indicate the dark period. Data points represent mean \pm SE of the mean.

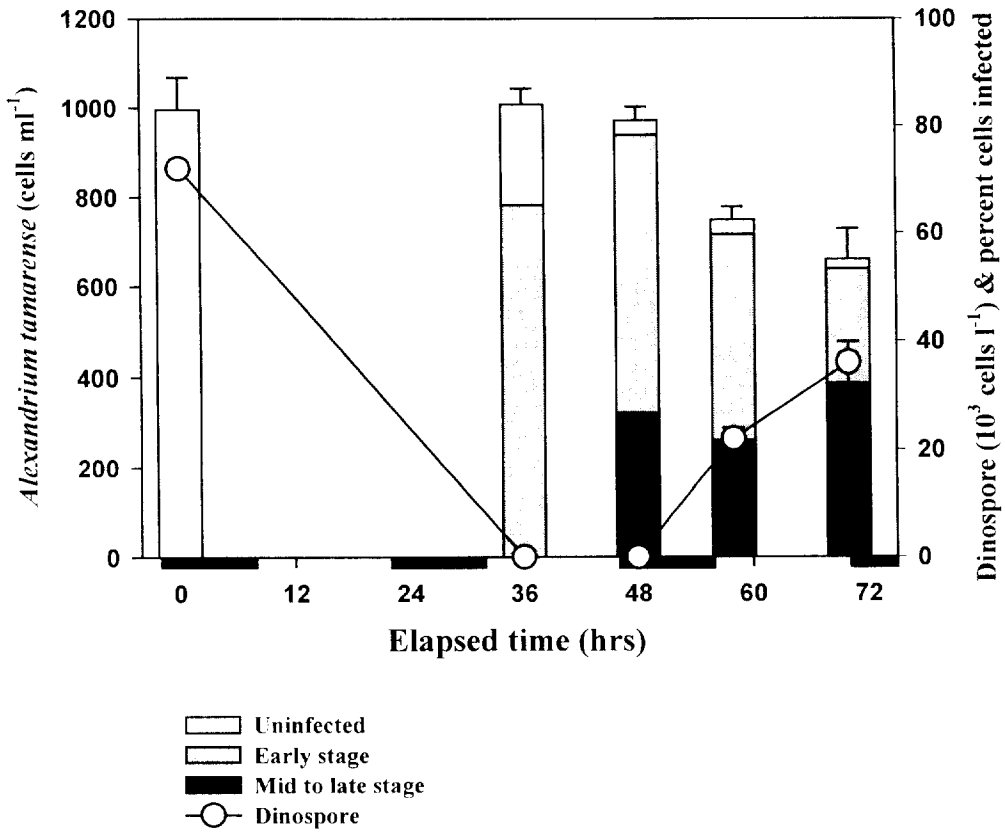


Fig. 2. Variations in dinospore abundance and percent infectivity sorted as early and late infections during this experiment.

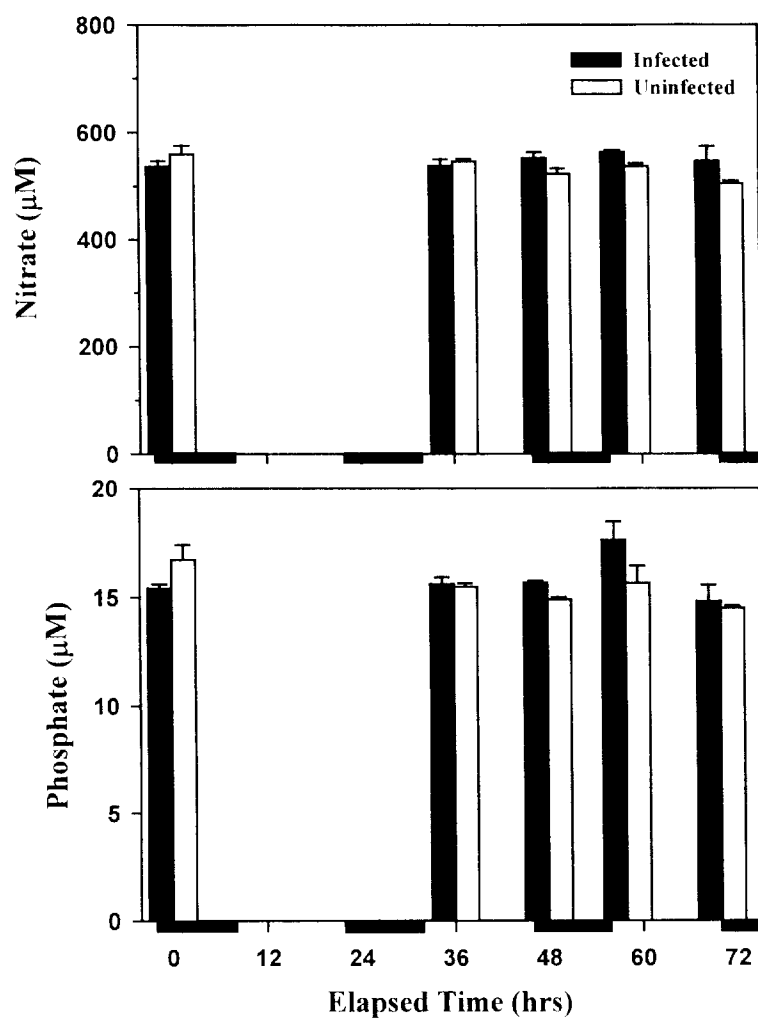


Fig. 3. Variations in nitrate and phosphate concentrations in infected and uninfected cultures of *Alexandrium tamarense*. Data points represent mean \pm SE of the mean.

infected treatments and uninfected controls were not significantly different ($p > 0.05$), although the highest phosphate concentration for infected treatments was observed at 58 h when mature trophonts of the parasites began to rupture from host cells.

Cellular toxin content and toxin profiles in infected and uninfected *A. tamarensis* cultures

Total cellular toxin content for infected and uninfected *A. tamarensis* cultures are shown in Fig. 4. At the start of experiment, toxin content of *A. tamarensis* was 56.68 ± 3.37 and 58.95 ± 2.13 fmol cell⁻¹ for infected and uninfected cultures, respectively. Toxin content of uninfected cultures tended to decline gradually toward the end of experiment, but were not significantly different ($p > 0.05$, one-way ANOVA test) compared to that at T_0 . By contrast, total cellular toxin content for infected cultures increased to 87.13 ± 10.34 fmol cell⁻¹ during the first 36 h of the infection and then showed the apparent diel rhythm although the differences were not statistically significant ($p > 0.05$, ANOVA test). The increase in toxin amounts in media was qualitatively observed from 58 h when the matured trophont began to rupture through the host cells. Any toxin components were not detected in newly formed dinospores.

Uninfected *A. tamarensis* produced saxitoxin (STX), neosaxitoxin (NEO), gonyautoxin (GTX1-5), C1 and C2 toxins (C1 and C2), and decarbamoyl derivatives (dc GTX-3) (Table 1, Fig. 5). Of these, the GTX-4 toxin was a major component with relative molar abundance of 64.8%, and toxin composition was

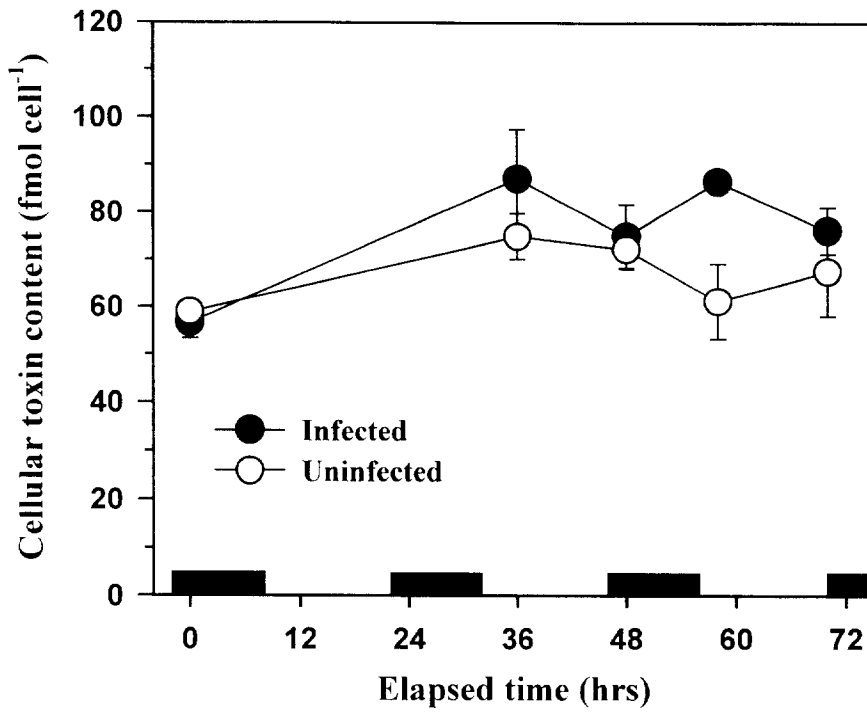


Fig. 4. Variation in cellular toxin contents in infected and uninfected cultures of *Alexandrium tamarense*. Dark bars on abscissa indicate the dark period. Data points represent mean \pm SE of the mean.

Table 1. Cellular PSP toxin contents and toxin compositions (fmol cell⁻¹; mean \pm SE) of *Alexandrium tamarense* DDPW in infected and uninfected cultures

Toxins	Infected (fmol cell ⁻¹)						Uninfected (fmol cell ⁻¹)					
	0	36	48	58	70	70	0	36	48	58	70	
Cl+C2	0.49 (0.068)	0.68 (0.117)	0.60 (0.138)	0.63 (0.103)	0.58 (0.034)	0.58 (0.034)	0.48 (0.024)	0.56 (0.035)	0.58 (0.066)	0.48 (0.080)	0.55 (0.002)	
GTX1+2	0.82 (0.106)	0.78 (0.055)	0.77 (0.104)	2.59 (0.366)	1.07 (0.302)	1.07 (0.302)	1.73 (0.211)	1.43 (0.809)	0.60 (0.085)	1.92 (0.828)	0.62 (0.271)	
GTX 3	5.57 (0.213)	5.40 (0.484)	3.97 (0.136)	3.94 (0.129)	2.88 (0.294)	2.88 (0.294)	6.44 (0.660)	5.61 (0.490)	7.78 (0.519)	6.10 (1.142)	7.82 (1.463)	
GTX 4	39.54 (2.193)	69.94 (6.868)	49.00 (4.552)	57.64 (0.429)	51.98 (5.255)	51.98 (5.255)	42.58 (2.223)	39.16 (3.525)	38.97 (2.927)	29.08 (5.309)	32.31 (6.453)	
GTX 5	1.50 (0.691)	2.15 (0.414)	2.00 (0.265)	3.10 (0.286)	2.34 (1.167)	2.34 (1.167)	1.98 (0.490)	2.25 (0.504)	0.99 (0.534)	1.22 (0.786)	1.44 (0.051)	
dcGTX 3	2.68 (2.012)	4.20 (1.273)	3.51 (0.321)	5.31 (0.436)	2.54 (1.963)	2.54 (1.963)	4.70 (2.133)	7.21 (1.779)	3.44 (2.232)	3.53 (2.525)	4.09 (0.111)	
STX	1.02 (0.051)	0.58 (0.003)	0.27 (0.070)	0.29 (0.052)	0.24 (0.062)	0.24 (0.062)	1.11 (0.006)	2.22 (0.332)	3.49 (0.707)	2.58 (0.234)	2.80 (0.018)	
NEO	7.11 (0.440)	13.68 (1.259)	11.55 (1.855)	12.61 (1.268)	10.29 (0.276)	10.29 (0.276)	7.75 (0.018)	12.20 (1.369)	13.05 (1.985)	10.50 (1.409)	12.42 (0.228)	
Total	56.68 (4.132)	87.13 (12.66)	75.00 (8.237)	86.59 (2.884)	76.27 (6.022)	76.27 (6.022)	55.53 (1.789)	52.87 (7.816)	50.68 (5.011)	40.59 (6.780)	44.80 (9.295)	

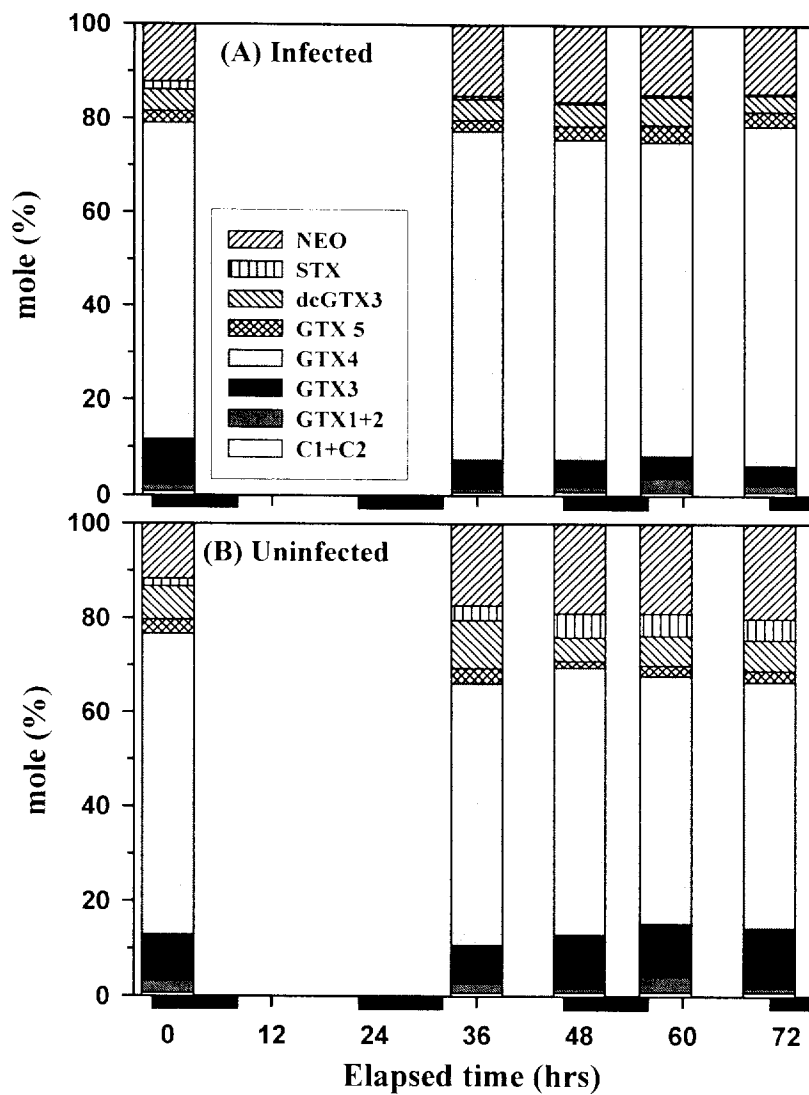


Fig. 5. Variation in PSP toxin profiles presented as percent molar composition of all toxins detected in infected (A) and uninfected cultures (B) of *Alexandrium tamarense*.

followed by order of NEO (12.32%), GTX-3 (9.28%), and dcGTX-3 (5.78%).

There were no remarkable differences in the toxin profile of infected *A. tamarensis* cells over the infection cycle compared to the initial toxin composition, although slight increases in the relative proportion of GTX 4 and NEO and decrease in GTX-3 were observed (Fig. 5). By contrast, in the profiles of uninfected *A. tamarensis* cultures, GTX-4 decreased the relative molar abundance of STX, NEO, and GTX-3 toxins increased during the first 36 h, and then remained relatively constant until the end of experiment.

DISCUSSION

The striking results of this study were that cellular toxin contents and toxin profiles of a toxic dinoflagellate *A. tamarensis* did not change following the *Amoebophrya*, and furthermore the toxins were not transferred to the infective dinospore stage of the parasite. Possible explanations for no toxin variations in infected *A. tamarensis* cells and their ecological implications are discussed below.

PSP toxin and toxin profiles in infected and uninfected *A. tamarensis*

Cellular toxin contents and toxin profiles of toxic phytoplankton in batch cultures are known to be affected by a variety of physical (e.g. temperature, salinity, light, and nutrients; White 1978, Boyer et al. 1987, Ogata et al. 1987, Anderson et al. 1990a,b, Usup et al. 1994, Bechemin et al. 1999, John & Flynn 2000, Hwang & Lu 2001, Hamasaki et al. 2001, Hamasaki et al 2001, Wang & Hsieh 2002) and physiological factors (e.g. growth phase and cell cycle; Prakash 1967, Proctor et

al. 1975, Anderson et al. 1990b, Flynn et al. 1994, Eschbach et al. 2005). In addition, the amounts and profiles of the phytotoxins can be expected to vary in association with endosymbionts such as endoparasitic dinoflagellate *Amoebophrya* in the present study, when nutrition is dependent on host materials. Contrary to our expectation, however, the results from this study showed that toxin content and toxin profiles of *A. tamarensis* were not altered following infection by *Amoebophrya*. Furthermore, toxins were not detected in newly formed dinospores. There are at least 2 possible explanations for these results (Fig. 6): (1) PSP toxins are known to be water soluble (Turner & Tester 1997), and thus the host toxins may not be able to be passively transported through the parasite membrane during growth of the parasite inside the dinoflagellate host; (2) *Amoebophrya* may do not possess specific enzymes capable of utilizing the nitrogen-enriched compound, i.e. PSP toxins. By either or both of the above mechanisms, PSP toxins would remain inside the dinoflagellate hosts, without variation in toxin content and toxin profiles.

Ecological implication in marine food webs

The plausible fate of phytotoxins in marine food webs include (1) accumulation and transport into higher trophic levels (e.g., Turner & Anderson 1983, Schantz 1986, Shumway 1990, Cembella et al. 1993, Turriff et al. 1995, Teegarden & Cembella 1996), (2) dissipation inside the first consumers' bodies (e.g., Jeong et al. 2003), and (3) excretion to the ambient seawater (Sekiguchi et al. 2001). During this study, we found that the toxins were released into the environmental

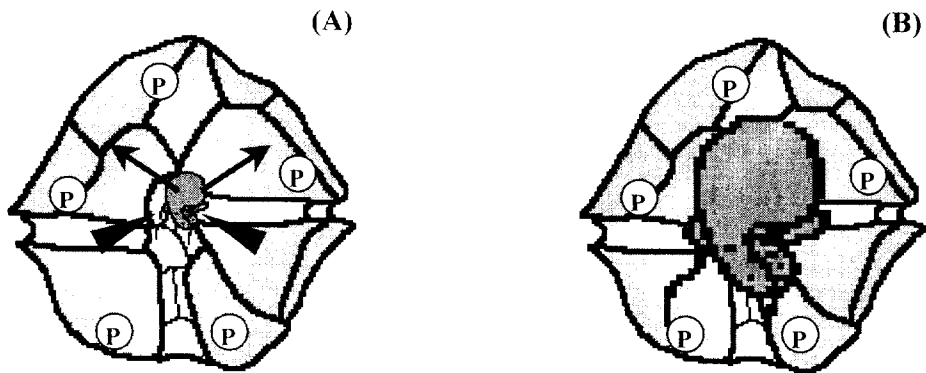


Fig. 6. Conceptual model of the fate of PSP toxins in *Alexandrium tamarense* (A) early stage and (B) late stage of infection after *Amoebophrya* infection. Arrow indicate flow of digestive enzyme (via exocytosis) and of factors which may increase membrane permeability. Arrow head is flow of nutrients, products of digestive processes or flow of inorganic nutrients, ions, CO₂.

medium without accumulation in dinospores, suggesting that the parasitic dinoflagellate *Amoebophrya* sp. do not function as vector for transport of PSP toxins to higher trophic levels. Instead, given the relatively short generation times of about 2 to 3 d (Kim et al. 2004) and widespread occurrence of these parasites in coastal waters (Park et al. 2004), *Amoebophrya* infections appear to rapidly dissipate toxin in marine food webs. On the other hand, it is possible that the PSP toxins released into the water after *Amoebophrya* infections act as allelochemicals on competing phytoplankters or grazers for some times.

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VII. Concluding remarks and future perspectives

The research presented in this dissertation adds to our knowledge of significance of microzooplankton grazing and parasitism in coastal phytoplankton ecology and red-tide research. This research has resulted in discovery that microzooplankton and parasitism play significant roles in regulating phytoplankton populations in temperate coastal environments. Several issues raised by this research are discussed below.

I found that microzooplankton are an important loss factor of phytoplankton biomass and production even in highly dynamic coastal waters (Chapter II). This finding is more or less surprising because the role of microzooplankton grazing is expected to be minor in regulating phytoplankton populations in coastal waters where large phytoplankton usually dominate when based on traditional sized-based food web model (e.g., Sherr & Sherr 2000). Furthermore, I observed frequent transient uncouplings in the interaction between predator and prey, despite the strong impact of microzooplankton on phytoplankton populations over the time scale of a year. My work suggests that the role of microzooplankton as the consumers of phytoplankton could be under- and/or overestimated in temperate coastal waters, depending on when sampling was made of the year, raising the need in intensive, long-term measurements of microzooplankton grazing in future studies to better understand the cycling of materials and the energy flow through microbial food webs in highly dynamic

coastal ecosystem.

My works on parasite biology and ecology using two thecate dinoflagellate hosts-parasites systems (*Amoebophrya* spp. from *Alexandrium affine* and *Gonyaulax polygramma*) did significantly expand our current knowledge of the parasite, but raise several questions about the morphological and biological properties of the parasite. For example, are generation times of the *Amoebophrya* parasites from other thecate dinoflagellate hosts as short as those used in my study? Is it enough having morphological data of the infective, dispersal stage dinospores of the parasite to test Coats et al.(1996)'s hypothesis? On the other hand, my work along with the results from previous studies revealed that *Amoebophrya* parasite has a variety of degree of host specificity, from less species-specific to extremely species-specific. This observation raises several questions as to whether the *Amoebophrya* parasite has different generation times when infecting secondary hosts, not primary hosts, and whether the parasite has host preference when several potential hosts coexist in natural waters. My work on the fate of PSP toxins following *Amoebophrya* infection raises question about whether this parasite has structural differences in sodium channel within its membrane. Future studies need to address several questions raised by these my works.

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VIII. Acknowledgments (in Korean)

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