Thesis for the Degree of Master of Science

Paralytic shellfish toxins (PSTs) toxification of zooplankton in association with appearance of toxic dinoflatellate genus *Alexandrium*, and PSTs production according to temperature and salinity condition of the *A. catenella* and *A. pacificum*

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유독 와편모조류 *Alexandrium*속 출현과 관련된 동물플랑크톤의 마비성 패독(PSTs) 독화와 수온 및 염분 조건에 따른 *A. catenella*와 *A. pacificum*의 PSTs 변화

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by

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Contents

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Abstract

Alexandrium catenella and *A. pacificum* produce Paralytic Shellfish Toxins (PSTs) such as saxitoxin and analogues which can cause paralysis and eventula death. Over the last several decades, PSTs have become more frequent throughout the world due to an increase in water temperatures caused by global warming. In Korea, PST occurs every spring centered on Jinhae Bay. As a result, exceeding the standard value of prohibited shipments of toxins (80 μg/100 g) in bivalves aquareculture around April caused economic damage and raised social concerns. Ingestion of toxic dinoflagellates containing PST toxins by zooplankton leads to the transmission of these toxins by zooplankton to high trophic levels. In this study, growth characteristics and changes in PST by caused *A. catenella* and *A. pacificum* were observed at different water temperatures and salinities in laboratory. The purpose is to provide the necessary basic information for the *A. catenella* and *A. pacificum* emergence forecast system. Moreover PST changes in suspended matter and zooplankton were measured around Jinhae Bay.

To basic data for predicting PSTs of bivalve, this study was conducted to estimate the effects temperature and salinity on the growth and PSTs production of *A. catenella* and *A. pacificum* in the laboratory, and to temporal and spatial variations characteristics of PST of the suspended matter and zooplankton collected from 5 stations of Jinhae Bay.

Based on laboratory culture experiments, the changes in growth rate and PST of toxic dinoflagellates *A. catenella* (JM-1) and *A. pacificum* (LIMS-PS-2611) were investigated under different water temperature and salinity conditions. The maximum growth rate (0.34/day) of *A. catenella* was observed under 15℃ and 30 psu. Optimal growth (≥ 70% of maximum growth rate) was obtained between 10-20℃ and 25-35 psu. Maximum toxin content was observed under 25℃ and 35 psu, and the toxin content increased with the increase of salinity. Among the PSTs of *A. catenella*, the principal toxins were C1+2 in N-sulfocarbamoyl toxin group and neoSTXs and GTX1+4 in the carbamate toxin group. High toxin contents were measured under the temperature and salinity conditions of the maximum growth rate. Also the maximum growth rate (0.38/day) of *A. pacificum* was observed under 25℃ and 30 psu. Optimal growth (≥ 70% of maximum growth rate) was obtained between 20-30℃ and 25-35 psu. Maximum toxin content was observed under 20℃ and 30 psu, and the toxin content increased with the increase of salinity. Among the PSTs of *A. pacificum*, the principal toxins were C1+2 and GTX5 in N-sulfocarbamoyl toxin group, and minor components were characterized as neoSTX in the carbamate toxin group. Low toxin contents were measured under the temperature and salinity conditions of the maximum growth rate.

In addition, zooplankton and suspended matter were collected in Jinhae Bay, a sea area where PST occurs frequently in spring, for PST detection. The water temperature at the time of sample collection was known to be between 8-15℃ and the salinity was between 31-33 psu. Based on the results of the samples collected in the field, as water temperature increased, the toxin content and toxicity of suspended matter increased, but decreased over time. Among the PSTs of suspended matter, the principal toxins were C1+2 in the N-sulfocarbamoyl toxin group. Pearson correlation analysis showed a significant positive correlation between the amount of PST toxin content detected in suspended matter and the cell density of toxic dinoflagellates in Jinhae Bay ($r = 0.75$; $p < 0.05$; $n = 33$). In Korea, this was the first reported of PSP intoxication in zooplankton. There was an increasing trend in toxin content and toxicity among zooplankton as the water temperature increased. Among the PSTs of zooplankton, the principal toxins were GTX1+4 in the carbamate toxin group. Pearson correlation analysis showed a significant positive correlation between the amount of PST toxin content detected in zooplankton and the cell density of toxic dinoflagellates in Jinhae Bay ($r = 0.70$; $p < 0.05$; $n = 35$). There was also a significant positive correlation between the amount of PST toxin content detected in zooplankton and the PST toxin content detected in suspended matter ($r = 0.75$; $p < 0.05$; $n = 35$).

Based on the above experimental results, *A. catenella* and *A. pacificum* are the causative species that lead to the emergence of PST every spring. Zooplankton PST toxification was associated with the appearance of the toxic toxic dinoflagellates *Alexandrium* in spring. Therefore the results of this experiment can provide basic data for the PST prediction system.

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Ⅰ. General Introduction

In marine ecosystems, dinoflagellates are one of the most important primary producers. It is possible that some of these species may adversely affect marine ecosystems and lead to the occurrence of harmful algal blooms (HABs). Toxins often accumulate in bivalve tissue after continuous ingestion of toxic dinoflagellates (Taylor et al., 1995). Toxins produced by bivalve shellfish include paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP) and amnesic shellfish poisoning (ASP), among others. Consumption of poisoned bivalves will cause the same symptoms as each name (Wang, 2008). These include the widespread distribution of PSP, which causes serious damage to the fishing industry, and attracts social concern. Recently, filter-fed bivalve shellfish poisoning has become a global hot spot, along with the proliferation of toxic dinoflagellates that causes paralyzing shellfish poisoning (Hallegraeff, 1993). It is known that paralytic shellfish toxin (PST) blocks sodium channel conduction in nerve cells, resulting in neurological paralysis in muscles (Van Dolah, 2000), and when humans ingest 1－4 mg of STX, they will die (James et al., 2010).

In South Korea, PSP occurred in 1986 in Gamcheon Port, Busan, when 11 people were poisoned by eating mussels grown on the bottom of a waste ship, and two deaths were officially reported (Chang et al., 1987). Furthermore, the occurrence area of paralytic shellfish poisoning is gradually

expanded from the Jinhae Bay to coastal area of Busan such as eastern part of Korean peninsular (NIFS, 2022).

As a group of PST, saxitoxins (STX) are most commonly found in *Alexandrium catenella* (Group I), *A. pacificum*, and *Gymnodinium catenutum* among dinoflagellates. PST in East Asian waters is mostly caused by *Alexandrium catenella* and *A. pacificum* (Anderson et al., 2012). The distribution of *A. catenella* and *A. pacificum* has expanded across seas of the world with the development of industry an anthropogenic marine pollution caused by ships balancing water (Hallegraeff et al., 1990). Moreover, global warming is expected the geographic ranges of toxic dinoflagellates in the mid latitudes due to increasing water temperature of nearshore water (Fischetti et al., 2013).

Several studies have demonstrated that growth rates and PST of *Alexandrium* spp. were affected by changes in various environmental factors as water temperature, salinity, and nutrients etc (Hosoi-Tanabe and Sako, 2006). Water temperature affects enzyme activity during growth, so as the water temperature rises, the activity of cells rises, and the growth rate is accelerated (Goldman and Carpenter, 1974). But at lower water temperatures, the rate of cell division is reduced, resulting in greater intracellular PST, and protein synthesis is reduced, resulting in excess arginine, which also leads to an increase in PST (Anderson et al., 1990b). According to Hamasaki et al. (2001), intracellular PST content was inversely proportional to salinity and growth rate. According to Parkhill and Cembella (1999), salt changes do not directly impact PST content since salinity changes directly affect growth rate,

resulting in PST content changes (Parkhill and Cembella 1999). In marine phytoplankton communities, nitrogen availability can have a significant impact on the abundance and composition of different species. It has been reported that dinoflagellates have a low nitrate concentration and a high ammonium concentration or a high dissolved organic nitrogen (DON) concentration, indicating that some species within this phytoplankton class have a selective preference for organic nitrogen sources (Glibert and Terlizzi 1999).

PST is known to accumulate in various taxa of the marine food chain, including bivalves, zooplankton, crustaceans, and gastropods (Chen and Chou 1998). Bivalves can accumulate high toxicity of PST during a toxic dinoflagellate bloom, and even after the bloom, the toxins are still present for a short period of time in the bivalves (Samsur et al., 2006). South Korean coastal waters were found to toxicity in bivalve exceeding the standard value (80 μg/100 g) at every year, and been occurring at an increasingly earlier and long-term trends (NIFS, 2022). According to Colin and Dam (2002); Dutz (1998); Teegarden and Cembella (1996), there is evidence to suggest that the copepod *Acartia* spp. ingests the toxic dinoflagellate *Alexandrium* spp., and subsequently accumulates PST through their bodies as the result of ingesting it.

Research has increasingly shown that bivalves are capable of filtering zooplankton directly from the surrounding water column and obtaining energy in the process. An analysis of stomach contents from mussels revealed evidence of zooplankton ingestion, with spring and summer having the highest levels of zooplankton ingestion. The size range of ingested zooplankton was 126 μm to 6 mm, but with a much higher proportion of smaller organisms

ingested than larger ones (Lehane and Davenport 2006). There is evidence that *Mytilus edulis* preyed on the copepod *Acartia tonsa* (Davenport et al. 2000, Nielsen & Maar 2007). It is thus evident that bivalves accumulate PST toxins by filter feeding on toxic dinoflagellates, but also by filter feeding on zooplankton that is already poisoned.

As such, if no countermeasures are taken, aquacultures will suffer significant economic losses, as well as health security will be threatened. In the present study, the objectives were to determine the variation in growth rate and PST of the toxic dinoflagellates *A. catenella* and *A. pacificum* under culture conditions of basal water temperature and salinity. To provide basic information for forecasting systems that predict the occurrence of *Alexandrium* spp. in seawater. In addition, samples of suspended matter and zooplankton were regularly collected in Jinhae Bay to analyze the content of PST in nature. Further, it was compared with the PSP detection report from the National Institute of Fisheries Science to understand how PST is passed between organisms. To provide basic information for better improvement of the PSP prediction system.

Ⅱ. Effects of temperature and salinity on the growth and paralytic shellfish toxin (PST) production by dinoflagellate *Alexandrium catenella* and *A. pacificum*

1. Introduction

The dinoflagellates in marine ecosystems play an important role as primary producers, however, they also produce harmful algal blooms (HABs) (Sunda et al. 2006), some of which can poison other organisms owing to their ability to synthesize toxic compounds (Oikawa et al., 2004; Sekiguchi et al., 2001). It should be noted that *Alexandrium pacificum* (formerly known as *A. catenella*) is a toxic dinoflagellate that produces a potent neurotoxin known as paralytic shellfish toxin (PST) within the cells. That Paralytic Shellfish Toxin (PST), which is represented by saxitoxins (STX) and has over 30 isomers, accumulates in filter-feeding bivalves after they consume toxic phytoplankton (Hosoi and Sako, 2006). In several studies, toxic dinoflagellates have been observed to be expanding as a result of global warming, industrial development, ship-balance water migration, and other factors (Hallegraeff et al., 2010). There is a gradual widening trend in the PST occurrence area across the globe. Each spring in Korea, there is a PST problem centered on Jinhae

Bay (Shon et al., 2009). Due to the occurrence of PST, aquaculture and public health worldwide were negatively impacted (Hallegraeff, 1993).

In Korea, the representative PST species is *Alexandrium catenella* (formerly known as *Alexandrium tamarense*), *A. pacificum* (formerly known as *A. catenella*) and *Gymnodinium catenatum* (Shin et al., 2017). *A. catenella* has been reported to be the main species responsible for PST in spring (Mok et al., 2013). It is well known that *A. catenella* was not discovered until 1970. It is often distributed in temperate regions such as North America, Europe, and Japan. Recently, the range of growth has gradually expanded due to anthropogenic pollution such as industrial development and the increase in water temperature caused by global warming (Fischetti et al., 2013). Toxicity in bivalves has also been reported to be positively correlated with the increase or decrease in cells density of *A. catenella* in Korea (Shon et al., 2009). In addition, when the density of *A. catenella* cells in seawater was less than 1,000 cells per liter, it would also poison *Mytilus edulis*, and subsequent continuous supply of *A. catenella* cells to the *M. edulis* would result in toxins in the *M. edulis* exceeding the prohibited shipping criteria values (80μg STX eq/100 g) (Shon et al., 2009; Mok et al., 2013).

The occurrence of PST is also widespread and early with increasing coastal surface water temperatures (Hallegraeff, 2003). It was also known from other studies that *A. pacificum* growth showed a good trend in the presence of the higher water temperature (Oh et al., 2012). In the past, the occurrence of PST was only limited to spring, but in recent years it has gradually extended to early summer (Kim et al., 2002; Oh et al., 2012). In June this year, PST

was reported to exceed the standard near Gijang-gun, Busan, and PST was also detected along the South East Sea coast of Korea, although it was below the standard value (NIFS, 2022). As a result, to safeguard the nation's health, Korea established a station for detecting paralytic shellfish poisoning after 1987 at the National Institute of Fisheries Science (Shon et al., 2009).

Composition of the toxin compositions in *A. tamarense* cells is dominated by N-sulfocarbamoyl group (C1, 2) and carbamate group (GTX1, 4, NEO, and STX) (Persich et al., 2006). It is known that bivalves do not have the ability to selectively absorb and release PST. According to the results, the toxin composition of PST in *Mytilus galloprovincialis* following the eating of *A. tamarense* cells was different from that of *A. tamarense* cells (Suzuki et al., 2003). Therefore, the appearance of some toxin components that are not present in the cells of toxic dinoflagellates may be the result of enzymes or chemical reactions in the tissues of bivalves that are converted into other toxins (Bricelj and Shumway, 1998).

The occurrence of PST is known to be caused by a combination of physical and chemical environmental factors such as water temperature, salinity, nutrients and irradiance (Anderson et al., 1990b; Hamasaki et al., 2001). Several environmental factors can directly or indirectly affect the production of toxin content (Laabir et al., 2013). In the early stages of bloom, water temperature plays an important role in the change of chlorophyll a. As a result, it is confirmed that elevated water temperatures are an important factor in phytoplankton blooms in aquatic ecosystems (Trombetta et al., 2019). It is well known that temperature has a significant effect on the physiological and

metabolic processes of phytoplankton. The uptake of nutrient in seawater by phytoplankton increases with increasing water temperature under non-restricted culture conditions. Therefore, the phytoplankton growth rate increased with increasing water temperature. Especially at relatively low field water temperatures, the growth of phytoplankton is more favorable as the water temperature increases (Trombetta et al., 2019). In addition, it is understood that water temperature has a catalytic effect on the activity of enzymes during growth, so when the water temperature rises, the activity of the cells rises and the growth rate is accelerated (Goldman and Carpenter, 1974). On the other hand, at lower water temperatures, the rate of cell division decreases. The result of this was an increase in the concentration of toxic contents per cell, and since there was a decrease in protein synthesis, excessive amounts of arginine were produced, resulting in increased amounts of toxic contents (Anderson et al., 1990b).

Phytoplankton species show different tolerances to salinity, which can be divided into broad and narrow salinity depending on the range of tolerance (Lim and Ogata, 2005). The blooms of the genus *Alexandrium* often occur in estuaries and coastal areas with terrestrial freshwater inflows (Franks and Anderson, 1992), and some culture experiments suggest that it is a euryhaline species (Franks and Anderson, 1992). Regarding the relationship between PST content and salinity, Hamasaki et al. (2001) reported that intracellular PST content was inversely proportional to changes in salinity and growth rate, but Parkhill and Cembella (1999) also reported that salt changes do not directly affect PST changes due to the fact that salinity changes directly affect growth

rate and thus PST content changes were produced (Parkhill and Cembella 1999). In addition, Laabir et al. (2013) reported that different researchers obtained different results, and changes in salinity did not affect the content of PST.

In this study, laboratory experiments were conducted on this *A. catenella* and *A. paicficum*, which may cause PST problems in spring and summer, to observe the effects of the water temperature and salinity on its growth and changes in PST. As a result of the experimental results, we can gain the first understanding of the factors contributing to the occurrence of toxic dinoflagellates genus *Alexandrium*, and provide the basic data that can be used in the development of a prediction system of PST.

2. Materials and Methods

2.1 Culture conditions

The *Alexandrium catenella* strain JM-1 used in this study was obtained after isolation of a single vegetative cell from a seawater sample that was collected by using a 20 μm phytoplankton net during the toxic bloom event in Jangmok Bay, Geoje, Korea in March 2021 (Figure 1). In the laboratory, vegetative cells of *A. catenella* were isolated from enrichments with Pasteur pipettes. In parallel with the transfer of single cells into 96 well plates, since then, JM-1 cultures have been maintained in Gijang Bay water enriched with f/2 medium (Guillard and Ryther, 1962) without selenium. The strain (LIMS-PS-2611) of *Alexandrium pacificum* was obtained from the Library of Marine Samples at the Korea Institute of Ocean Science and Technology (KIOST). The salinity was 30 psu, at a temperature of 20℃ and light conditions for stock cultures were 150 μmol photons m^{-2} s^{-1}. Cool-white fluorescent lamps with a 12 h:12 h light/dark cycle were used. Although no sterilization was performed on the isolate, all equipment and glassware were washed and thoroughly rinsed with distilled water and then autoclaved (120℃, 200 kpa, 20 min) and dried (90℃, 4 hours) to prevent biological contamination. And all processes of *A. catenella* and *A. pacificum* culture were performed on a clean bench.

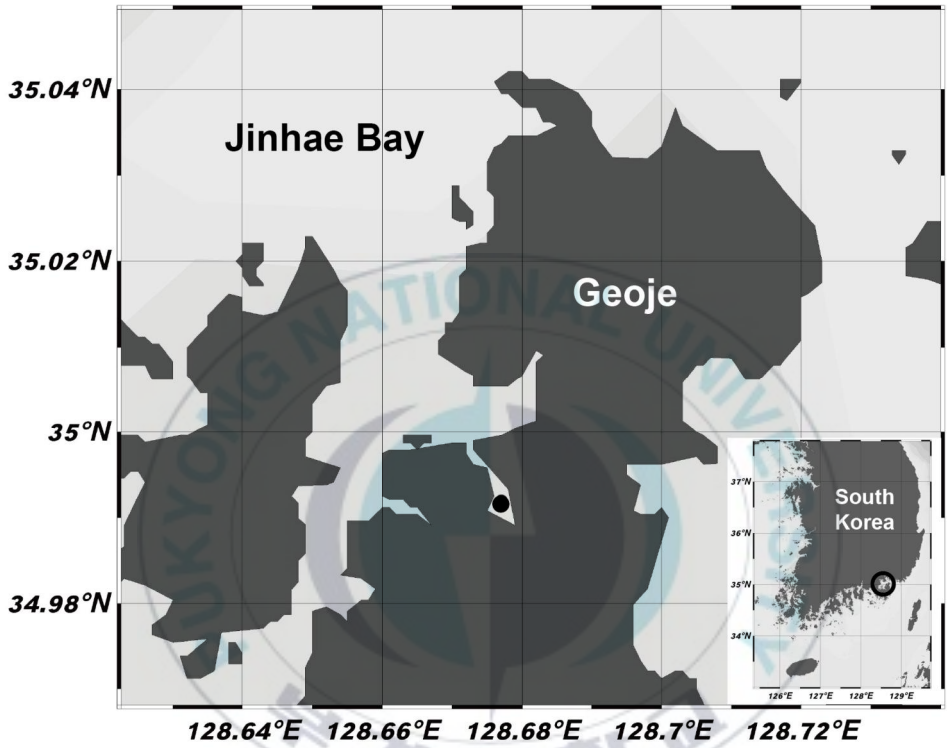

Figure 1. Sampling site for *Alexandrium catenella* vegetative cells at Jangmok, Geoje, Korea.

2.2 Experimental design

In experiments, the growth rates were studied using a crossed factorial design with 25 different conditions, obtained from combining five temperatures 10, 15, 20, 25 and 30℃ and five salinities 15, 20, 25, 30 and 35 psu under an irradiance of 150 μmol photons m^{-2} s^{-1} (12L:12D; cool-white fluorescent lamp). Prior to the experiments, the cells in maintenance stock cultures were inoculated into each experimental medium and cultured under each experimental condition for 5-10 days to acclimate them. After the conditions of temperature of 20°C and salinity of 30 psu have reached the exponential growth phase, raise or lower 1°C per day until each temperature condition is reached. In addition, cells in 30 psu medium were transferred to 25 psu medium after reaching the exponential growth phase to minimize effects due to changes in salinity. (experiment repeated to 15 psu salinity environment) Finally, after each temperature and salinity stage has grown to a stationary phase of the exponential growth phase, the experiments are then carried out. The medium with high salinity content of 35 psu was adjusted by natural evaporation, and the medium with low salinity content of 15-30 psu was adjusted by adding Ultra pure water.

At the beginning of the exponential phase of a culture, the cells were inoculated into glass flasks, and fresh f/2 medium was added to each condition in order to begin measuring the cell growth. And each contained 150 ml of culture medium inoculated with 150-200 cells/ml. In addition, cell density counts were performed under a light microscope once a day until the end of

the exponential growth phase. All of the experiments were carried out in duplicate. All culture experiments, the growth rate, μ, for the exponential growth phase was calculated using the following equation:

$$\mu = \frac{1}{\triangle t} \ln \frac{N_t}{N_0} \qquad (1)$$

μ: growth rate (day)

N_0,N_t: N_t and N_0 is Cell density at the initial stage and after t time in the exponential growth phase (cells/ml)

Δt: Cultivation period of the exponential growth phase (day)

2.3 Toxicity measurements

Sample Pre-treatment for analyzing PST was carried out from water temperature and salinity gradient to late logarithmic growth phase. Aliquots of 100 ml were centrifuged at 3000 × g for 10 min to pellet the cells. Cell pellets were resuspended with of 0.5 N acetic acid. Cells were disrupted by Sonication by using a Powersonic (POWERSONIC 420, Hwashin tech Co., Kwangju) and 3 times at 100 Hz for 180 s each on ice. Sonicated cell samples were subjected to centrifugation (10,000 × g, 10 min), and the supernatant of each sample was placed in an ultrafilter (Ultrafree-MC; MWCO 10,000Da, Millipore, Massachuetts) and centrifuged (10,000 × g, 10 min). A 0.5 ml aliquot of each filtrate was subjected and the filtrate was stored at -20℃ until analysis.

Compositional change of PSP toxin composition during growth was analyzed with the Oshima's post-column derivatization High Performance Liquid Chromatography (HPLC; Thermo Scientific UltiMate 3000, Chromeleon 6.8) and was analyzed after separation with a Reverse Engineering column. The standard toxin used in the analysis process were purchased from Marine biosciences at the National Research Council; (NRC-IMB; Halifax, NS), including N-sulfocarbamoyl toxin 1&2 (C1&2), gonyautoxin 1&4 (GTX 1&4), gonyautoxin 2&3 (GTX 2&3), gonyautoxin 5 (GTX5), neosaxitoxin (neoSTX), saxitoxin (STX), and decarbamoylsaxitoxin (dcSTX).

Toxin contents were calculated from the relative area ratio of the analyzed sample peak and the standard toxin peak and expressed as fmol/cell. Toxicity

in saxitoxin equivalents (STX equiv) was calculated using Toxicity Equivalency Factors on the specific toxicity of each derivative reported by Oshima (1995). and expressed as pg STXeq/cell.

3. Results and Discussion

3.1 Effect on growth rate

3.1.1 *A. catenella*

In laboratory growth experiments, observe the effect of water temperature and salinity on the growth rate of *A. catenella*, so as to understand the optimal growth conditions of *A. catenella*. Combination of five water temperatures 10, 15, 20, 25, 30℃ and five salinities 15, 20, 25, 30, 35 psu, the growth rate changes were observed under the conditions of 25 stages in total.

Water temperature and salinity had a predominant influence on both cell density and growth for *A. catenella* (Figure 2). It is unable to grow in extreme conditions of high water temperature and low salinity. In addition, the maximum cell density of *A. catenella* was 13,750 cells/ml under the conditions of water temperature 15℃ and salinity 35 psu. It was also found that *A. catenella* displayed halotolerance characteristics at low water temperatures of 10℃ to 15℃. However, when the water temperature increased to 20℃, only 25-35 psu salinity ranges could be grown. Secondly, when the water temperature increased to 25℃, only 35 psu salinity ranges could be grown. As the temperature of the water decreased, the exponential growth phase of *A. catenella* also extended. For the purpose of demonstrating the variation in growth rate, a contour plot of growth rate against water temperature and

salinity was produced (Figure 3). The maximum growth rate of 0.34/day was observed under the culture conditions of 15℃ and 30 psu water temperature, based on the contour plot. In addition, 70% of the maximum growth rate occurred in the water temperature range of 10 to 20℃ and salinity range of 25 to 35 psu. As the water temperature increased, the growth rate decreased significantly, and although changes in salinity affected the growth rate, there was no significant effect. Therefore, the experimental results allow us to know that *A. catenella* has physiological characteristics of halotolerance at low water temperature.

The results obtained from this study were compared with those of *A. catenella* isolated from other sea areas, as shown in Table 1. The Japanese isolates were similar to the results of the present study, as were the isolates from Jinhae Bay and Masan Bay in Korea. However, the isolates from Hong Kong were not the same as the results of this experiment. It is reported that even the same species may have different physiological characteristics depending on the separation sea area. In fact, according to the report of the National Institute of Fisheries Science, *Alexandrium* spp. started to appear at the beginning of March when the average water temperature was 10℃ in Masan Bay and Jinhae Bay, and the maximum cell density appeared in April and May when the water temperature was in the range of 14-17℃, and gradually disappeared in July when the water temperature rose above 25℃. Therefore, under environmental conditions that are different from those of competing species, this study found that when temperature and salinity are within 70% of optimal growth, *A. catenella* can completely lead to the

occurrence of PST in coastal waters. The average water temperature in Jinhae Bay in 2009 was between 6.4℃ and 26.1℃. PST was detected from bivalves in April-May when water temperatures ranged from 12.0℃ to 19.0℃. Therefore, changes in PST are closely related to water temperature (Lee et al., 2009).

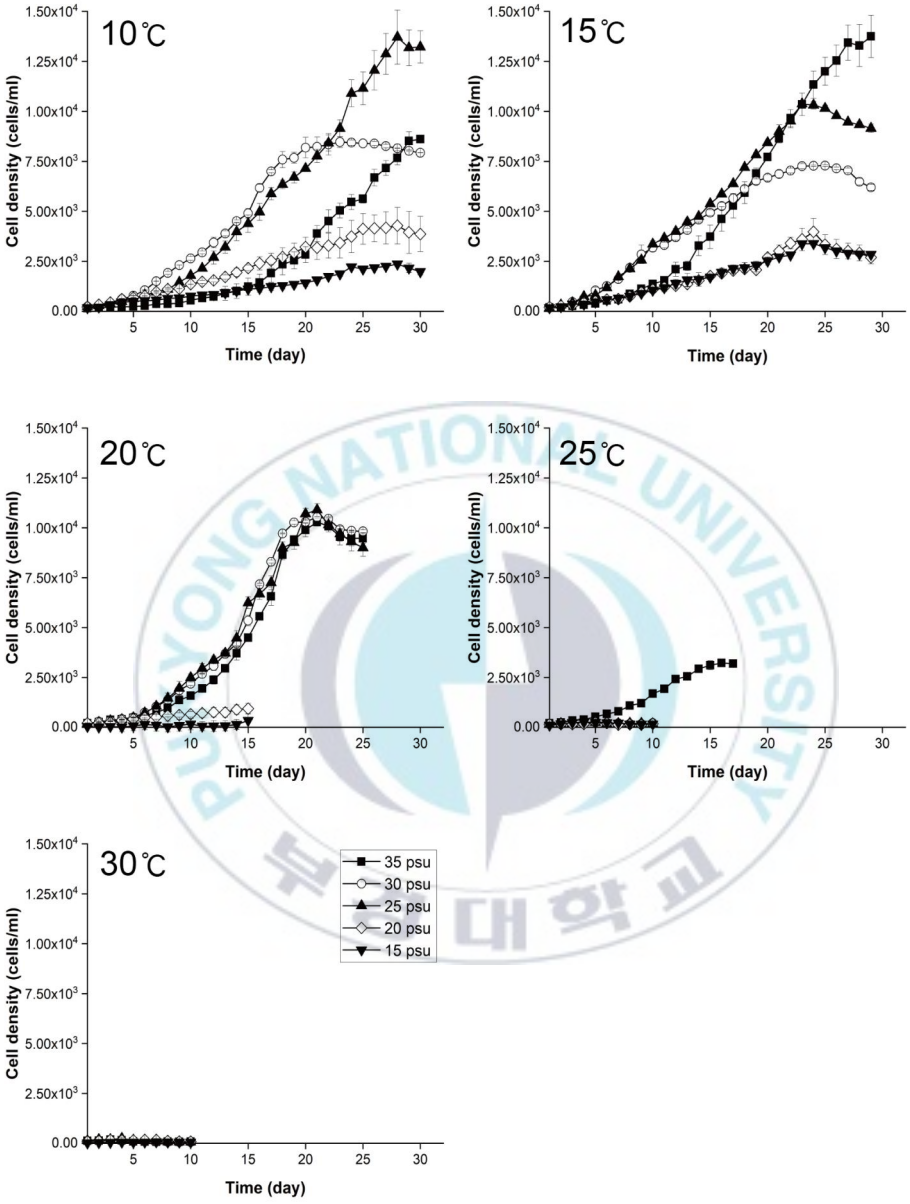

Figure 2. Growth curves of *Alexandrium catenella* grown at various water temperature and salinity conditions. Values represent mean ± SD of two replicates.

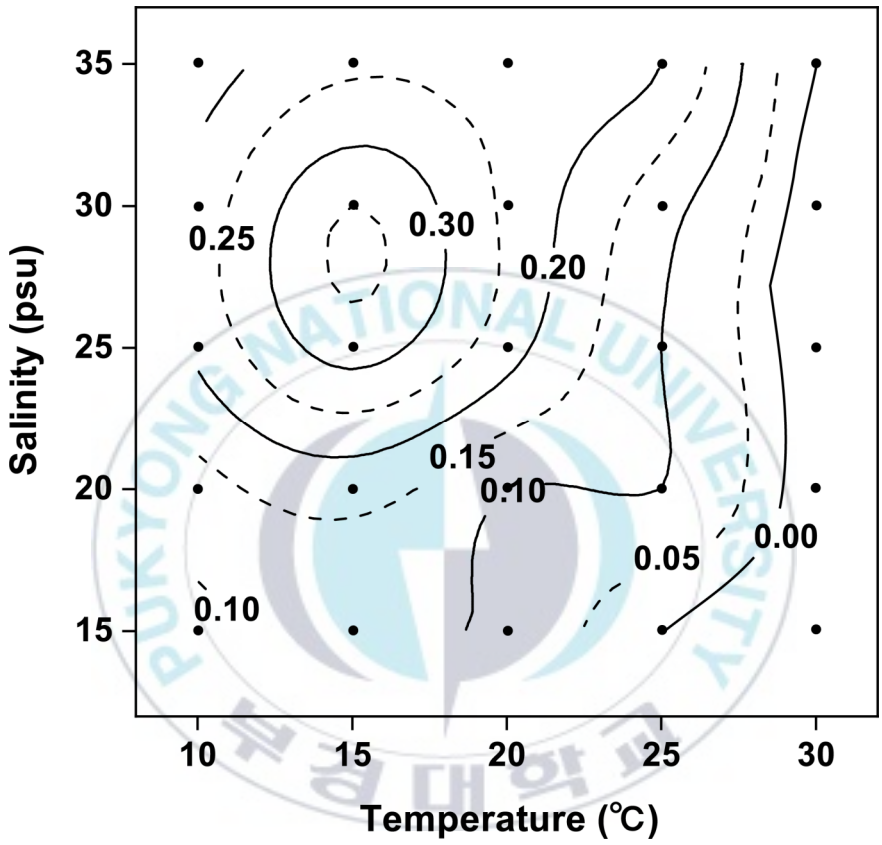

Figure 3. Contour plotting of exponential phase growth of *Alexandrium catenella* at various water temperature and salinity conditions.

Table 1. Comparison of optimal temperature and salinity for growth rates of various *Alexandrium catenella* and *A. pacificum* strains

Species	Isolated area	Growth rate (day^{-1})	Temperature (℃)	Salinity (psu)	References
A. catenella	Geoje, Korea	0.34	15	30	This study
	Masan, Korea	0.31	15	30	Oh et al. (2012)
	Japan	0.42	17	29	Hamasaki et al. (2001)
	Hong Kong, China	0.60	23	25-30	Wang and Hsieh (2005)
A. pacificum	Geoje, Korea	0.38	25	30	This study
	Masan, Korea	0.36	25	30	Oh et al. (2012)
	Japan	0.51	20-25	25-35	Matsuda et al. (2006)
	Hong Kong, China	0.28	20-25	30-35	Siu et al. (1997)

3.1.2 *A. pacificum*

Investigate the effect of water temperature and salinity on the growth rate of *A. pacificum* in a laboratory experiment. Combination of five water temperatures 10, 15, 20, 25, 30℃ and five salinities 15, 20, 25, 30, 35 psu, the growth rate changes were observed under the conditions of 25 stages in total.

From Figure 4, it can be observed that water temperature and salinity are the main factors affecting *A. pacificum* growth. It is unable to grow in extreme conditions of low water temperature and low salinity. In addition, the maximum cell density of *A. pacificum* was 10,750 cells/ml under the conditions of water temperature 25℃ and salinity 30 psu. It was also found that *A. pacificum* displayed halotolerance characteristics at high water temperatures of 20℃ to 25℃. The cell density was generally higher under the high water temperature conditions of 20-25℃, and the cell density decreased when the water temperature dropped to 15°C. For the purpose of demonstrating the variation in growth rate, a contour plot of growth rate against water temperature and salinity was produced (Figure 5). The maximum growth rate of 0.38/day was observed under the culture conditions of 25℃ and 30 psu water temperature, based on the contour plot. In addition, 70% of the maximum growth rate occurred in the water temperature range of 20 to 30℃ and salinity range of 25 to 35 psu. As the water temperature decreased, the growth rate decreased significantly, and although changes in salinity affected the growth rate, there was no significant effect. Therefore, the

experimental results allow us to know that *A. pacificum* has physiological characteristics of halotolerance at high water temperature.

The results obtained from this study were compared with those of *A. catenella* and *A. pacificum* isolated from other sea areas, as shown in Table 1. It can be seen from the table that although the growth rate of different isolates of *A. pacificum* was somewhat different among them, the optimal water temperature and salinity conditions were found to be similar. In addition, *A. pacificum* grew better than *A. catenella* at high water temperatures if Hong Kong isolates were excluded. Oh et al. (2012) reported that *A. catenella* was dominant in April and May when the water temperature was relatively low, *A. catenella* and *A. pacificum* appeared mixedly in June, and *A. pacificum* might be more dominant in July. Kremp et al. (2012) reported that *Alexandrium* has sufficient adaptive capacity to cope with climate change, it is likely that *A. pacificum* adapted to higher water temperatures will emerge earlier and for a longer period of time along with the increase in water temperature in the surrounding seas.

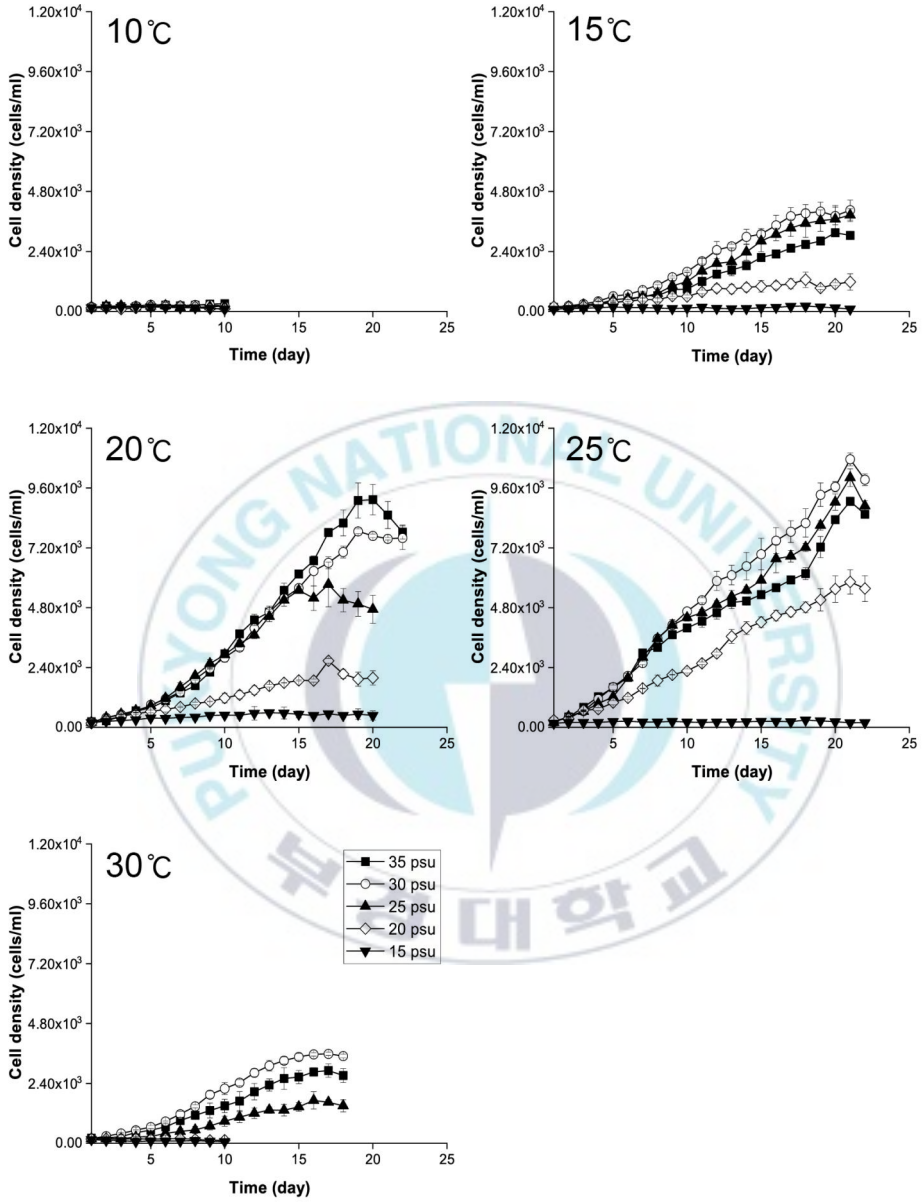

Figure 4. Growth curves of *Alexandrium pacificum* grown at various water temperature and salinity conditions. Values represent mean ± SD of two replicates.

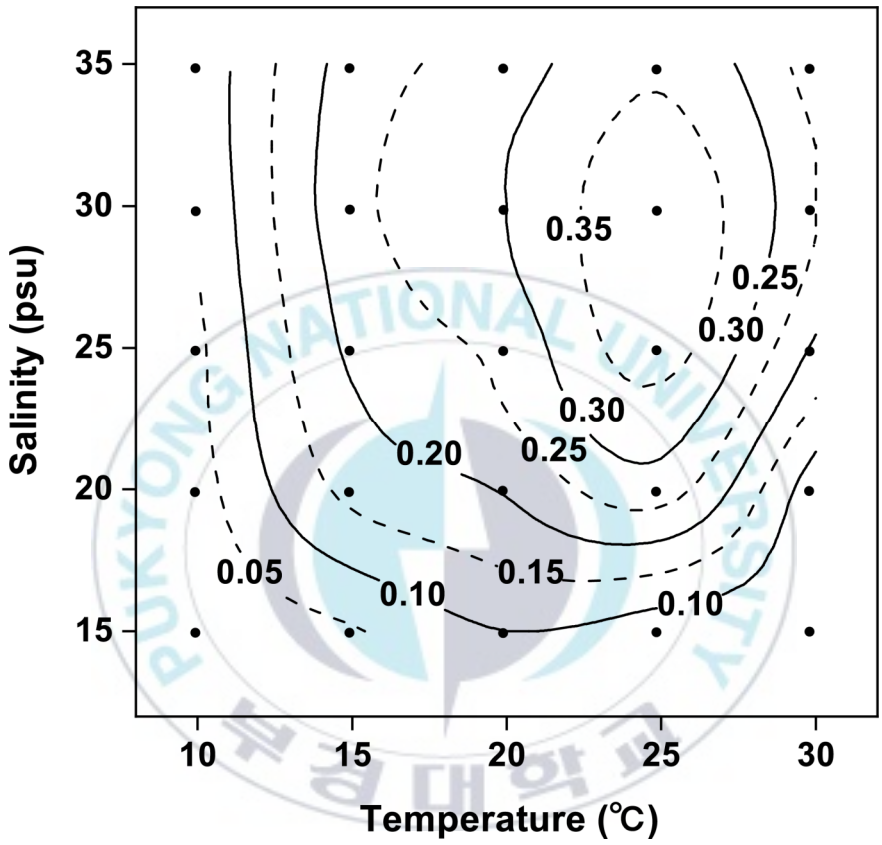

Figure 5. Contour plotting of exponential phase growth of *Alexandrium pacificum* at various water temperature and salinity conditions.

3.2 Effect on toxin content and toxicity

3.2.1 *A. catenella*

According to Figures 6 and 7, there is a significant variation in the toxic content and toxicity of *A. catenella* under every combination of water temperature and salinity. From the figures, it can be learned that the toxic content and toxicity increased with the increase of salinity under the culture conditions from 10℃ to 20℃, with the highest intracellular toxic content (366 fmol/cell) and toxicity (145 pg STXeq/cell) found at 25℃, 35 psu. In addition, the growth rate was greatest at water temperature of 15℃ and salinity of 30 psu, and its toxic content (94 fmol/cell) and toxicity (34 pgSTXeq/cell) were higher than other conditions. For the purpose of demonstrating the variation in toxin contents and toxicities, a contour plot of toxin contents and toxicities against water temperature and salinity was produced (Figure 8; Figure 9). When the water temperature was 25℃ and the salinity was 35 psu, its toxicity and toxin content were the highest. In addition, when the growth rate was highest at 15℃ and 30 psu, its toxic content and toxicity were also higher than under other conditions. The results obtained from this study were compared with those of *A. catenella* isolated from other seas, as shown in Table 2. The Hong Kong isolates differed in terms of water temperature conditions, but the toxic content was similar to the results of this study. However, for the Japanese and Scottish North isolates, the toxic content was lower compared to the present study. There has been research that suggests

even the same species may have different physiological characteristics depending on the separation sea area.

It was found that during *Alexandrium* growth, under nutrient-enriched conditions without environmental stress, toxin contents were low during the lag phase, peaked during the exponential growth phase, and declined again during the stationary phase. Therefore, in this experiment, *A. catenella* was cultured at various water temperatures and salinities until the exponential growth phase, and then it was pro-treated, after which the intracellular PST content was analyzed.

The toxin content reflects the balance between the initial content of toxins in the cell and the synthesis and loss of toxins due to metabolic processes or cell division. Relatively low rates of toxin synthesis may also result in higher toxin contents when cells divide slowly. However, relatively high rates of toxin synthesis may also result in lower toxin contents when cells divide rapidly (Anderson et al., 1990b). According to our experiment, in 25℃, 35 psu salinity conditions, the toxic content is the greatest and the most toxic. This is caused by slow cell division caused by high water temperatures, which accumulates toxins in the cells. It was also observed that high levels of toxic content and toxicity occurred at optimal growth conditions of 15℃ and 30 psu salinity. This is due to the fact that low water temperature leads to a decrease in protein synthesis, which increases the synthesis of arginine, the precursor substance of PST, leading to a large amount of toxin being synthesized (Anderson et al., 1990b).

It is now known that change in salinity affects the response of *A. catenella*

to nutrient intake, cellular activities, and intracellular transport systems. As the salinity increases from 31 psu to 37 psu, the amount of toxin content in the cells doubles (White 1978). High salinity conditions resulted in an increase in intracellular toxicity (Parkhill and Cembella, 1999). In addition, Hwang and Lu (2000) found that the toxin content increased with salinity in the salinity range of 15-30 psu. On the contrary, it has been observed that toxicity do not increase with salinity, but do increase with decreasing salinity (Hamasaki et al., 2001). It was also found that salinity change had no apparent effect on the production of toxin in some *A. catenella* cells (Anderson et al., 1990b). Thus, the relationship between salinity and toxicity varies from *Alexandrium* species to species based on the characteristics of the species. Even for the same species, the characteristics vary in different sea areas (Laabir et al., 2013).

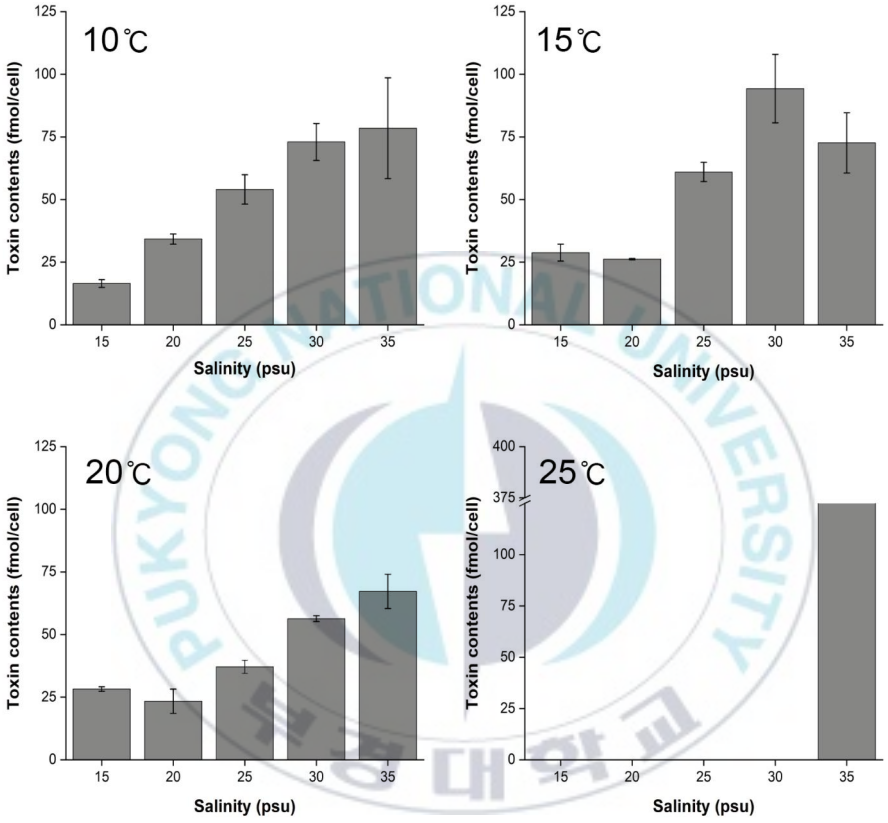

Figure 6. Changes in toxin contents (fmol/cell) of *Alexandrium catenella* grown at different water temperatures and salinities.

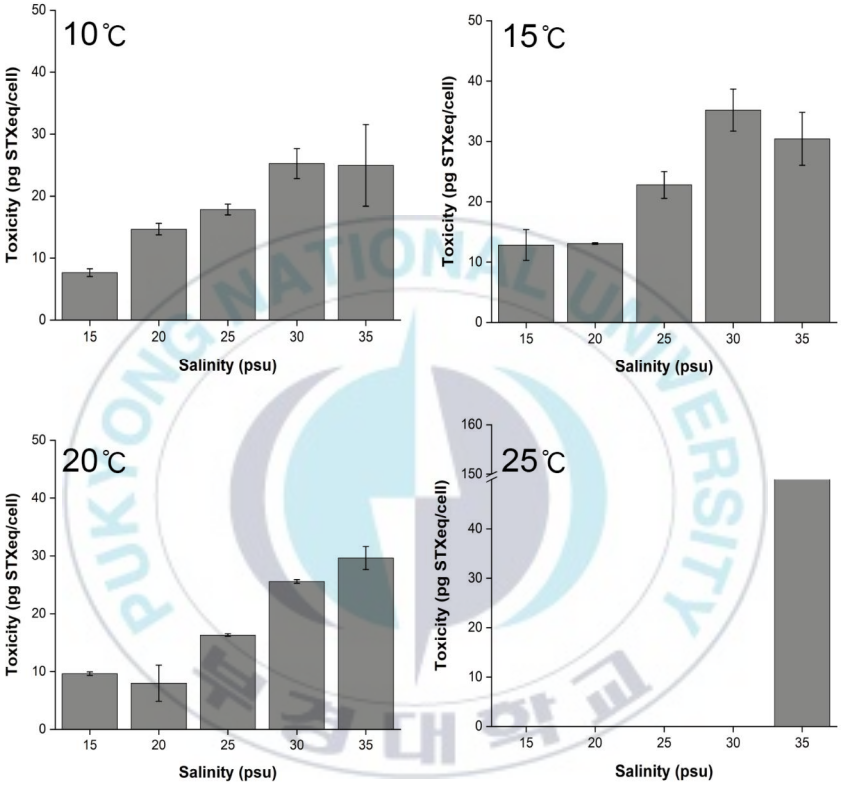

Figure 7. Changes in toxicities (pg STXeq/cell) of *Alexandrium catenella* grown at different water temperatures and salinities.

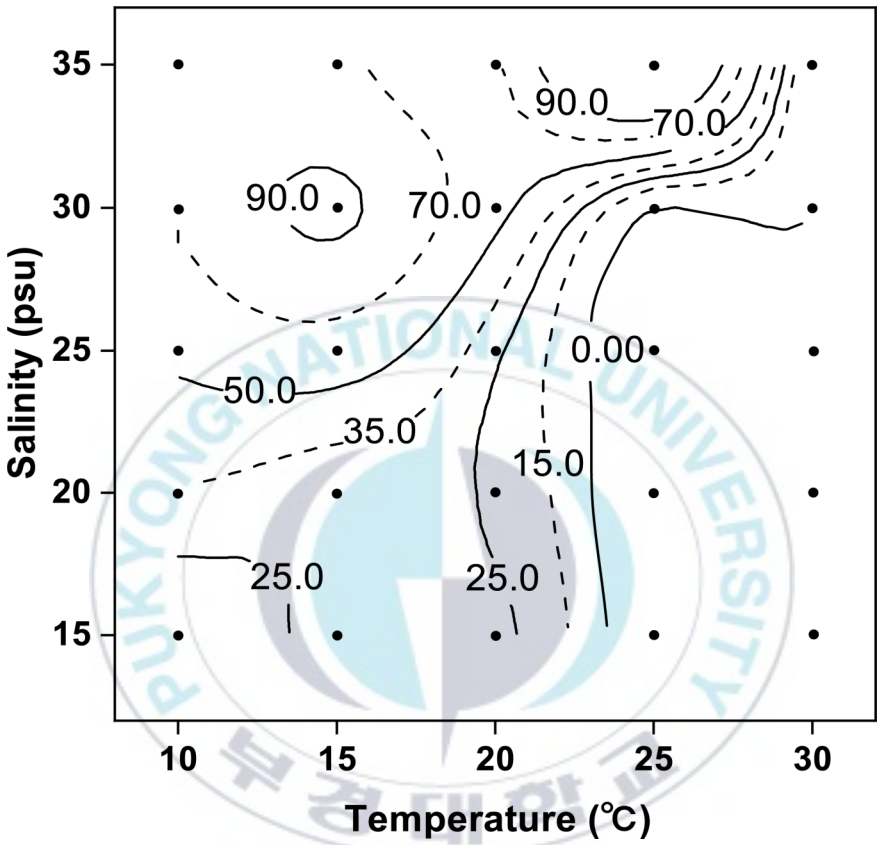

Figure 8. Contour plotting of toxin contents (fmol/cell) of *Alexandrium catenella* growth at various water temperature and salinity conditions.

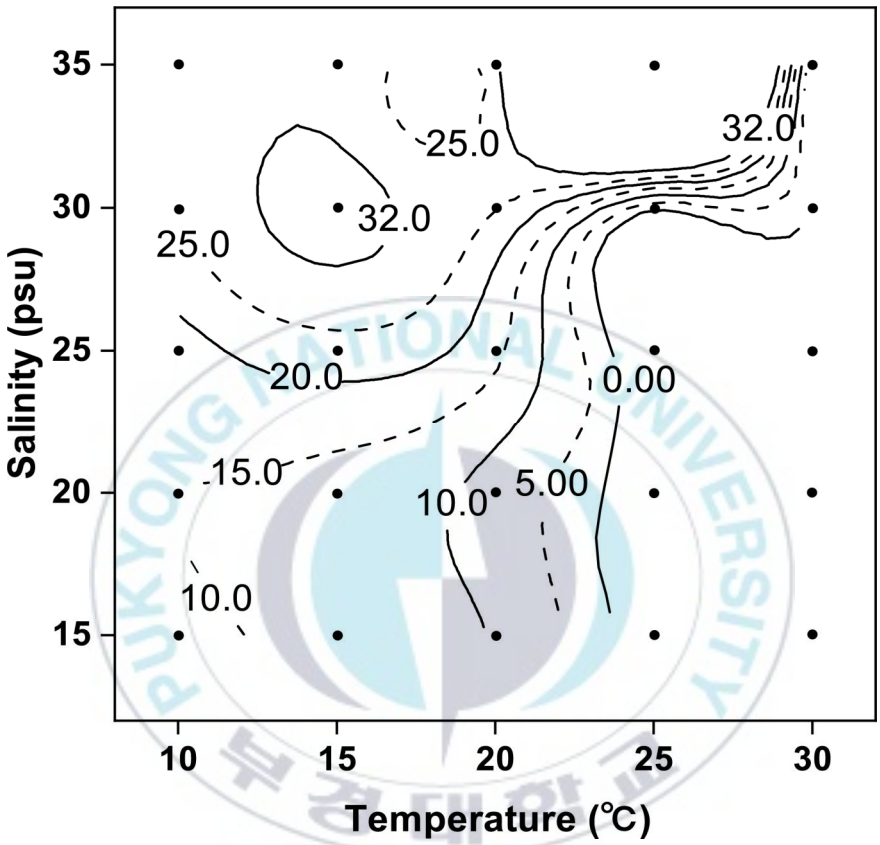

Figure 9. Contour plotting of toxicities (pg STXeq/cell) of *Alexandrium catenella* growth at various water temperature and salinity conditions.

Table 2. Toxin contents and toxicities of *Alexandrium catenella* and *A. pacificum* grown at various combinations of temperature and salinity conditions

Species	Isolated area	Temperature (℃)	Salinity (psu)	Toxin contenys (fmol cell^{-1})	Toxicity (pg STXeq cell^{-1})	References
A. catenella	Geoje, Korea	15	30	94	34	This study
	Japan	15	-	53	20	Yoshida et al. (2001)
	Hong Kong, China	23	30	95	-	Wang and Hsieh (2005)
	Scottish North	15	32	43	-	Tillmann and Hansen (2009)
A. pacificum	Geoje, Korea	20	30	24	3	This study
	Japan	20	-	24	4	Yoshida et al. (2001)
	Japan	20	-	34	2	Yoshida et al. (2001)
	Annaba	20	-	-	3.8	Hadjadji et al. (2020)

3.2.2 *A. pacificum*

According to Figures 10 and 11, there is a significant variation in the toxic content and toxicity of *A. pacificum* under every combination of water temperature and salinity. From the figures, it can be learned that the toxic content and toxicity increased with the increase of salinity under the culture conditions from 15℃ to 25℃, the effect of each salinity on toxin content and toxicity was not significant at 30℃ water temperature, with the highest intracellular toxic content (24 fmol/cell) and toxicity (2.96 pg STXeq/cell) found at 20℃, 30 psu. For the purpose of demonstrating the variation in toxin contents and toxicities, a contour plot of toxin contents and toxicities against water temperature and salinity was produced (Figure 12; Figure 13). As the water temperature was 20℃ and salinity was 30-35 psu, the amount of toxin content and toxicity was highest, but as the water temperature was 25℃ and 30 psu, the toxic content and toxicity were low. Comparison of *A. pacificum* strains isolated from other seas revealed that they also showed high toxin content and toxicity at 20℃ water temperature conditions, which was similar to this study (Table 2).

As a result of many studies, high toxin content does not necessarily mean that the rate of toxin synthesis is fast. In contrast, low toxin content does not necessarily mean that the rate of toxin synthesis is slow (Proctor et al., 1975 and Anderson et al., 1990). Anderson et al. (1990) reported that an inverse relationship between toxin content and growth rate. Moreover, Gedaria et al. (2007) reported that low water temperature conditions affect the activity of

intracellular enzymes, resulting in reduced conversion of constituent substances and increased toxic content in cells. This study also found that the toxin content and toxicity were lower under the optimal growth condition of 25℃, but the toxin content and toxicity were higher under the low water temperature environment of 10-20℃.

Salinity is closely related to the osmotic pressure of cells, and species have different ranges of salinity tolerance, resulting in differences in growth rates and thus affecting the occurrence of PST (Laabir et al., 2013). Lim and Ogata (2005) divided them into three categories based on the relationship between growth rate and poison content. The first category is photohaline species that can adapt to lower salinity. The second category is photohaline species adapted to higher salinity. The third category is narrow salinity species. *A. pacificum* was a species corresponding to halotolerance species adapted to higher salinities, showing higher growth rates at higher salinities, and its toxin content and toxicity were also influenced by changes in salinity. Thus, the toxic contents contained in *Alexandrium* isolated from different sea areas ranged from 7.5-72.8 fmol/cell, indicating a large variation between populations in the same or different sea areas (Yoshida et al. 2001). In view of this, it is necessary to isolate *Alexandrium* grown in different sea areas in the future and study its growth characteristics, after which the establishment of the PST prediction system will be further improved.

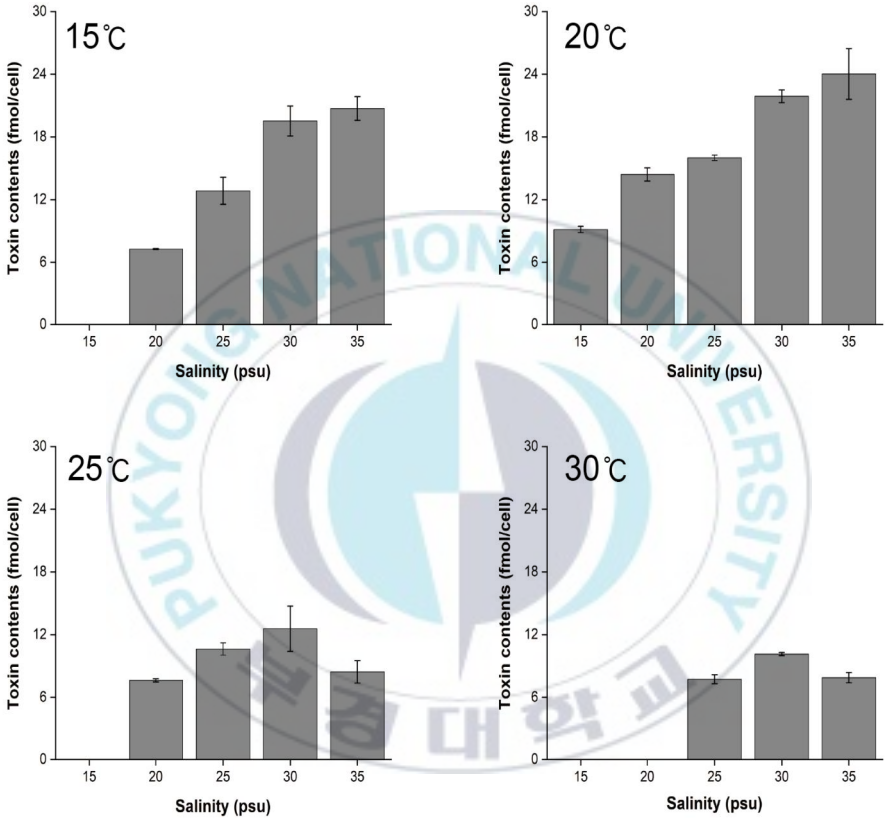

Figure 10. Changes in toxin contents (fmol/cell) of *Alexandrium pacificum* grown at different water temperatures and salinities.

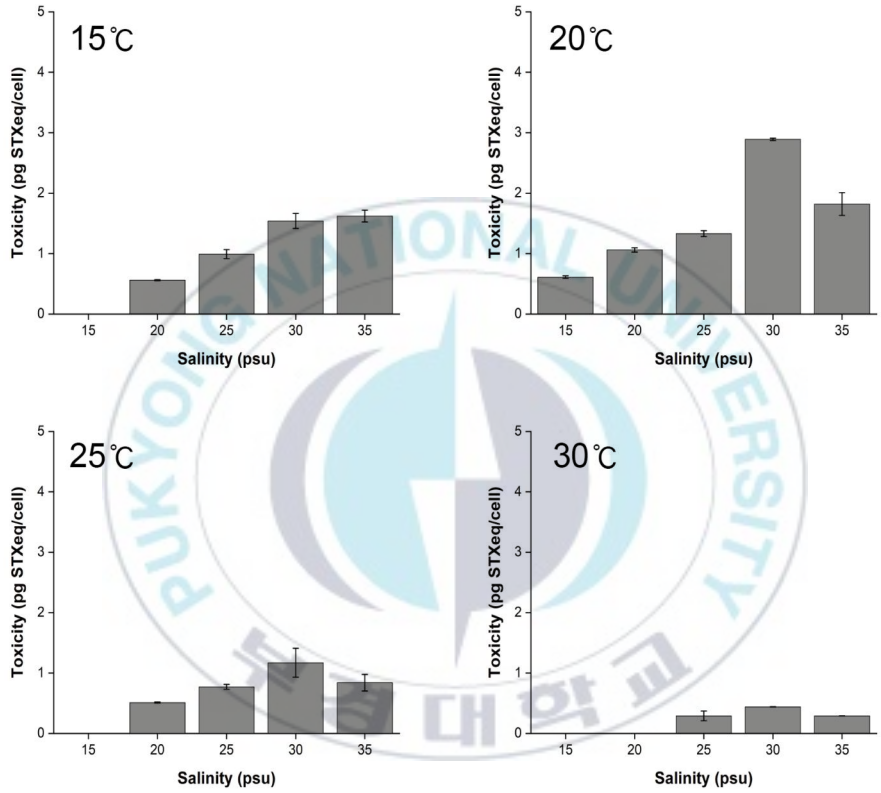

Figure 11. Changes in toxicities (pg STXeq/cell) of *Alexandrium pacificum* grown at different water temperatures and salinities.

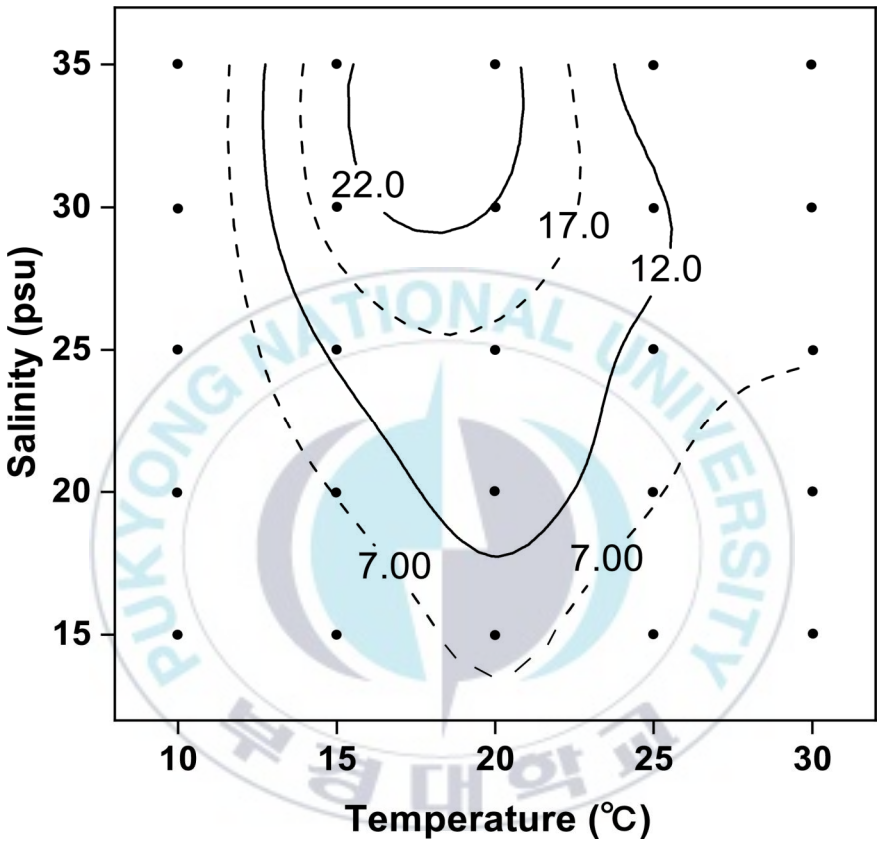

Figure 12. Contour plotting of toxin contents (fmol/cell) of *Alexandrium pacificum* growth at various water temperature and salinity conditions.

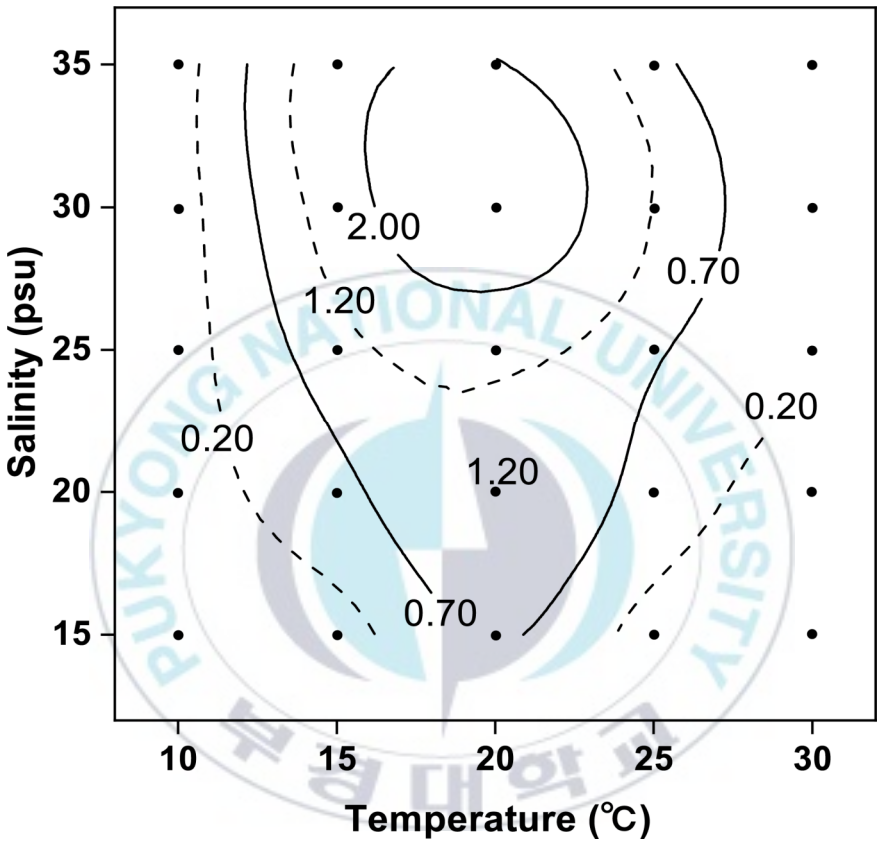

Figure 13. Contour plotting of toxicities (pg STXeq/cell) of *Alexandrium pacificum* growth at various water temperature and salinity conditions.

3.3 Effect on toxin composition

3.3.1 *A. catenella*

The main components of PST in *A. catenella* used in this experiment were the N-sulfocarbamoyl group C1+2, GTX5, and trace components were the carbamate group GTX1+4, GTX2+3 and neoSTX (Table 3). In comparison with isolates from other sea areas, C1+2 was found to be dominant in almost all strains, but there were significant differences in the proportions of other toxic components (Table 3).

The changes in toxic components of water under different temperatures and salinities (Figure 14). When the water temperature was 10-15℃, C1+2 and GTX1+4 were found to be dominant. That C1+2 increases with a salinity increase, but GTX1+4 decreases with a salinity increase when a low water temperature is maintained between 10-15℃. In contrast, at 20℃ water temperature, it was found that neoSTX and GTX1+4 increased with increasing salinity, while C1+2 and GTX5 decreased with increasing salinity. Moreover, it was noted that *A catenella* was capable of growing at a high temperature of 2 5℃ and a high salinity of 35 psu, and its cells contained high concentrations of neoSTX and GTX1+4. As a result of the results of this experiment, it is known that the synthesis rate of carbamate toxins in the cells of *A catenella* is very high under conditions of high water temperature and high salinity.

As a result of many studies, it has been demonstrated that the toxic components of *A. catenella* cells differ based on the growth stage and culture

conditions (Boczar et al., 1988; Anderson et al., 1990b; Hamasaki et al., 2001). When nutrient limitations are present, changes in the composition of the toxin components of *Alexandrium fundynse* are observed. Nitrogen limitation was found to favor the synthesis of C1 and C2 toxins as well as GTX1 and GTX4. Phosphorus limitation favors the synthesis of GTX2 and GTX3 (Anderson et al. 1990a). Wang et al. (2004) reported that in the presence of without sufficient nitrate and phosphate, only C1 and C2 were found in the cells. This suggests that these two nutrients may be the parent compound of the PSTs while providing nutrients.

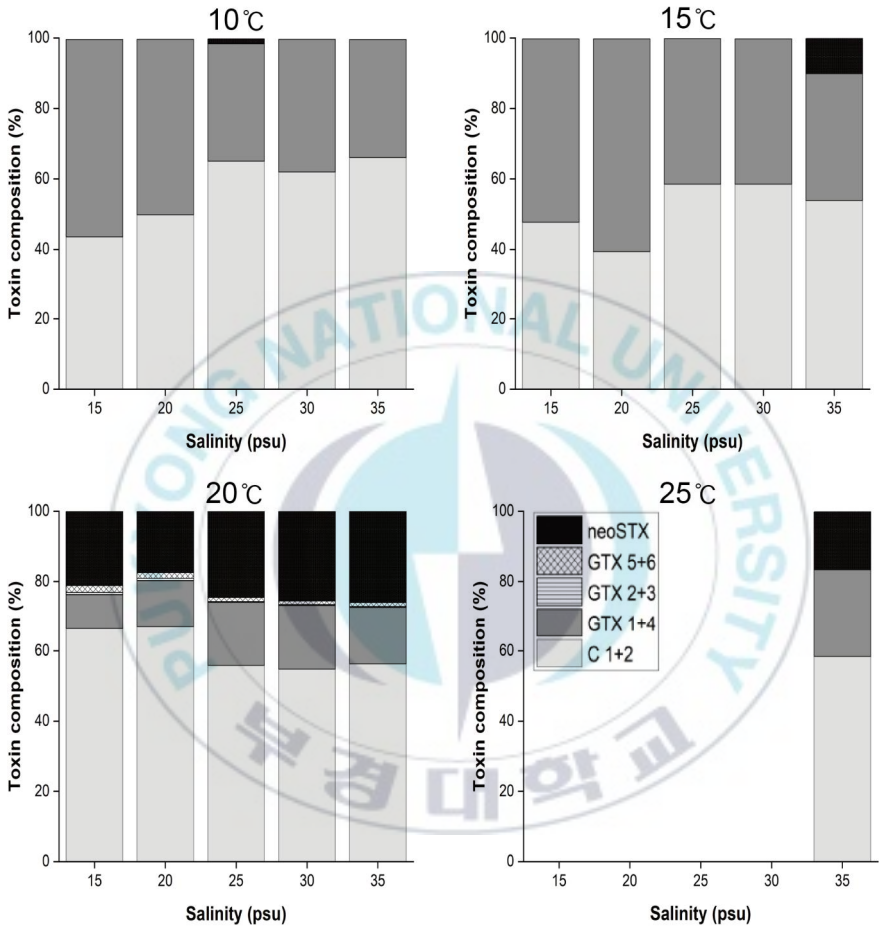

Figure 14. Changes in toxin compositions of *Alexandrium catenella* grown at different water temperatures and salinities.

Table 3. Toxin compositions of various *Alexandrium catenella* and *A. pacificum* at different temperature and salinity conditions

Species	Isolated area	C1+2	C3+4	GTX1+4	GTX2+3	GTX5	neoSTX	STX	References
A. catenella	Geoje, Korea	56	-	16	1	1	26	-	This study
	Japan	26	28	-	-	-	42	4	Yoshida et al. (2001)
	Hong Kong, China	25	5	55	-	5	-	-	Wang and Hsieh (2005)
	Scottish North	30	-	13	-	-	45	12	Tillmann and Hansen (2009)
A. pacificum	Geoje, Korea	44	-	-	-	49	7	-	This study
	Japan	46	19	29	-	-	6	-	Yoshida et al. (2001)
	Japan	72	4	5	1	18	-	-	Yoshida et al. (2001)
	China	80-90	-	5-15	-	-	-	-	Xu et al. (2012)

3.3.2 *A. pacificum*

The main components of PST in *A. pacificum* used in this experiment were the N-sulfocarbamoyl group C1+2, GTX5, and trace components were the carbamate group neoSTX (Table 3). In comparison with isolates from other sea areas, C1+2 was found to be dominant in almost all strains, but there were significant differences in the proportions of other toxic components (Table 3).

The changes in toxic components of water under different temperatures and salinities (Figure 15). That GTX5 increases with a salinity increase, but C1+2 decreases with a salinity increase when a low water temperature is maintained 2 5℃. As a result, the change in salinity has no effect on C1+2 and GTX5 under the conditions of water temperature 15℃, 25℃, and 30℃.

According to Anderson (1990b) and Oshima et al (1993), the generation of PST is genetically determined, so the toxicity cannot change with environmental conditions, so the composition of the toxin is species-specific and can be used as a biochemical marker (Ogata et al., 1987; Boyer et al., 1987). However, many studies have revealed that toxin composition changes with the environment, with increased rates of neoSTX and STX synthesis when cells were in stationary phase, and under nitrogen-rich conditions (Boczar et al., 1988). It was found that the GTX1 in *A. minutum* cells was higher in the low-salinity environment, but the GTX2+3 was higher in the high-salinity environment (Hwang and Lu, 2000).

It is known that toxic dinoflagellates have unstable N-sulfocarbamoyl toxins, so when bivalves ingest them, they are biotransformed in vivo and converted to highly toxic carbamoyl toxins. Shon et al. (2009) reported that even when the

toxin concentration of toxic dinoflagellates in seawater is measured to be low, bivalves may become intoxicated. Thus, determining the relationship between toxic dinoflagellate toxin composition and bivalve feeding is an essential part of predicting and forecasting PSTs.

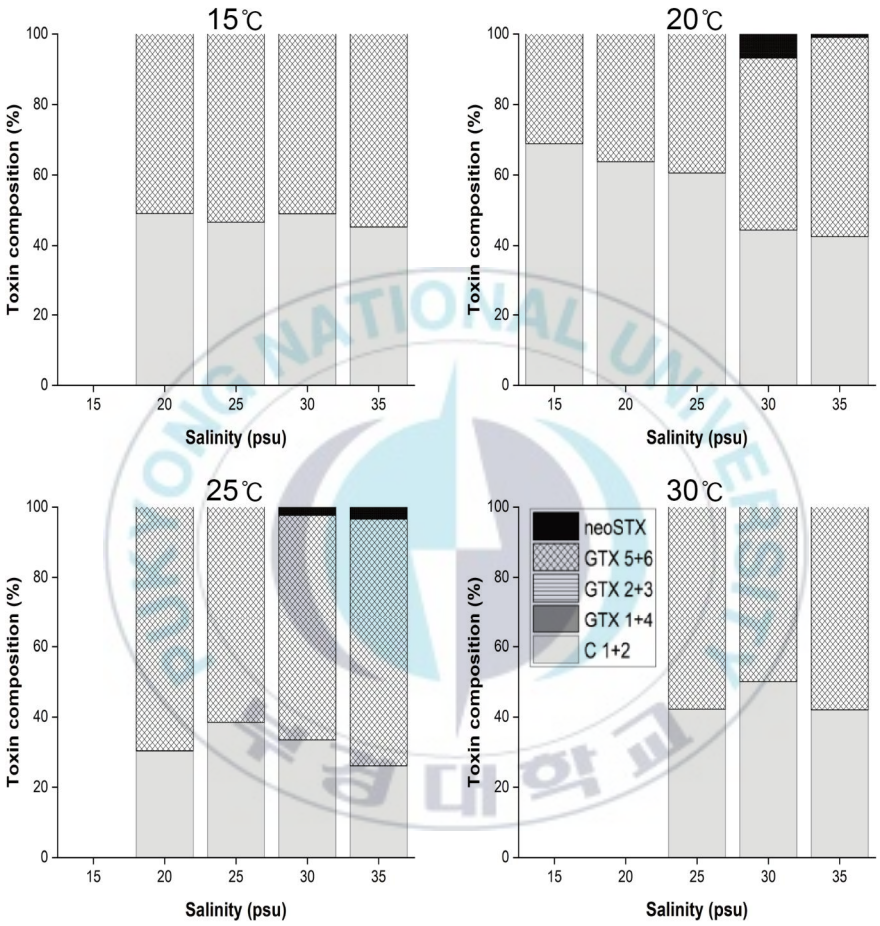

Figure 15. Changes in toxin compositions of *Alexandrium pacificum* grown at different water temperatures and salinities.

Ⅲ. PST poisoning of zooplankton is associated with the appearance of the toxic dinoflagellate *Alexandrium* spp.

1. Introduction

In marine ecosystems, energy transfer between organisms usually begins with photosynthetic organisms, phytoplankton that can photosynthesize and use ocean energy to convert inorganic compounds into organic compounds. Diatoms and dinoflagellates are the most significant phytoplankton species in marine ecosystems, and they are important primary producers in the food web (Lee et al. 2001). Copepods comprise 70% of total zooplankton biomass, and zooplankton play a significant role in energy transfer to higher trophic levels, not only as herbivores that prey on phytoplankton but also as a food source for numerous invertebrates and fishes. Thus, zooplankton play an important role in regulating the abundance of fishes and invertebrates in the marine resources (Lee et al. 2001). In addition, it has a relatively short life history and is sensitive to environmental changes, so it can act as an indicator organism in coastal environments (Gismervik 2006).

Toxins from marine toxic dinoflagellates can cause fish kills and intoxication of bivalve mollusks. When humans, marine mammals and seabirds eat contaminated fish or bivalve molluscs, they can cause poisoning or death.

Considering that White (1980) reported that marine zooplankton contaminated by PSP toxins caused fish kills in the Bay of Fundy, extensive laboratory experiments have been conducted and revealed that many copepod species such as *Acartia hudsonica*, *A. tonsa*, *Calanus finmarchicus*, *Eurytemora herdmani*, *Euterpina acutifrons* and *Tigriopus californicus* can retain PSP toxins after ingestion of toxic dinoflagellates (Teegarden and Cembella 1996). It is imperative to note, however, that while shellfish accumulate PSP toxins by direct ingestion or filter-feeding, the mechanisms by which finfish may be intoxicated or die from PSP toxicity are more complex, since most adult fish cannot feed directly on toxic dinoflagellates. Therefore, copepods and other mesozooplankton are likely to be the most effective transfer organisms of PSP toxins from lower trophic levels to higher trophic levels (White, 1981).

Several physiological and behavioral effects have been reported in marine copepods and bivalves following consumption of PST. That bivalves can accumulate a large amount of toxic PST during a toxic dinoflagellate bloom, and even after the bloom, the toxins are still present for a short period of time in the bivalves for a variety of reasons (Samsur et al., 2006). There is evidence that copepods will graze *Alexandrium* spp. populations, they may serve as vectors for the transfer of toxin to higher trophic levels (White, 1979). Moreover, they were capable of metabolically altering the toxins they ingested, providing a mechanism for modifying the body's toxin load (Robineau et al., 1991). In recent years, research has demonstrated that bivalves are capable of directly filtering zooplankton from the surrounding water column and obtaining energy from the process. In an analysis of

stomach contents from mussels, it was found that there was evidence of ingested zooplankton, and that the size range of the ingested zooplankton was from 126 μm to 6 mm (Lehane and Davenport 2006).

When bivalves consume toxic dinoflagellates or poisoned zooplankton, their responses to toxins are influenced by their nerve sensitivity. This is because resistance to the toxin is caused by a mutation of an amino acid which decreases saxitoxin's affinity for the sodium channel pore of the cell membrane. Therefore, when increased levels of PST are present in the natural environment, this leads to increased resistance of the bivalves to the toxin, with a smaller impact on behavioral and physiological responses (Navarro et al., 2014). This response increases the concentration of toxin in the bivalve, thereby elevating the risk to humans.

White (1981); Boyer et al. (1985) pointed out that zooplankton will die after ingesting toxic dinoflagellates containing PST toxin, and even if there is no fatal effect, there will be inhibition of feeding, or a decrease in the number of eggs laid by copepods. And the feeding process in copepods appears to be highly dependent on specific interactions between producers and predators. It is well known that *Paracalanus parvus* (Huntley et al. 1986) and *Acartia clausii* (White 1981) often prey upon *Alexandrium tamarense*. However, other findings suggest that although toxic dinoflagellates were rejected by *Calanus pacificus* for predation, *C. pacificus* did not reject the toxic dinoflagellates *Alexandrium tamarense* isolated from the Pacific coast of North America (Huntley et al. 1986). Thus, under certain circumstances, zooplankton copepods will selectively prey on toxic algae. As a result,

neurotoxins in toxic dinoflagellates was absorbed by zooplankton copepods and transferred to the upper trophic level through the food web. Nevertheless, few studies have analyzed whether changes in toxin content and toxin composition in zooplankton are affected during periods of large seasonal blooms of *Alexandrium* (Campbell et al., 2005).

This study examines the accumulation process of PST toxins in pelagic copepods by monitoring the toxicity of both suspended matter and copepods in natural environments. In order to monitor the occurrence of PST in coastal waters, it is therefore necessary to have a good understanding of the toxicity content of toxic dinoflagellates and zooplankton.

2. Materials and methods

2.1 Sampling

From December 2021 to May 2022, field observations of PST will be conducted at five locations to gain a better understanding of the occurrence of PST in Jinhae Bay. These surveys were conducted in the following order: the first (12.15.-12.18.), the second (1.4.-1.6.), the third (1.24.-1.26.), the fourth (2.17.-2.19.), the fifth (3.11.-3.15.), the sixth (3.29.-3.31.), the seventh (4.16.-4.20.), the The eighth (5.07.-5.11.). This study collected samples of suspended matter and zooplankton at these five locations and examined them for PST contents (Figure 16).

To measure the cell density and toxin content of the toxic dinoflagellate *Alexandrium* spp. in seawater, 10 L samples were concentrated to approximately 50 ml using a 10 μm net after collecting 10 L of surface seawater. The zooplankton were then collected using a conical net (0.6 m × 2.5 m) of 250 μm mesh. Finally, samples were stored frozen at a temperature of -20℃.

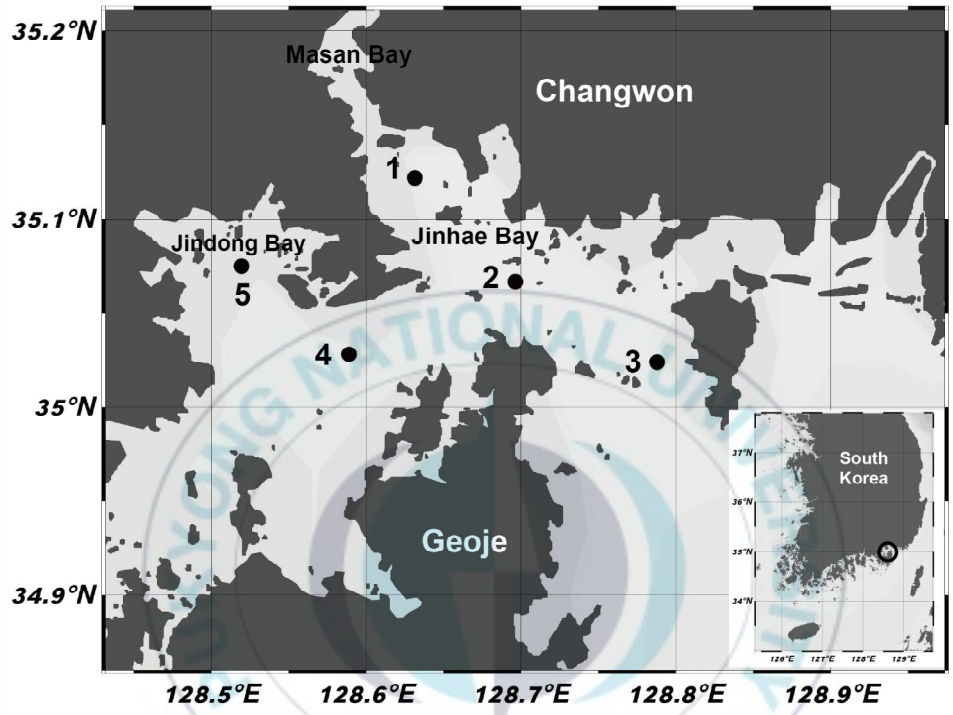

Figure 16. During the spring-summer bloom season of *Alexandrium* spp. in Jinhae Bay in 2022, five stations of constructed in order to monitor the accumulation of paralytic shellfish toxin (PST) in seawater and zooplankton.

2.2 Toxicity measurements

2.2.1 Pre-treatment of suspended matter samples

In order to conduct PST analysis on suspended matter samples containing toxic dinoflagellates, we first thawed those samples that had been stored in a －20℃ freezer. And samples were centrifuged at 3000 × g for 10 min to pellet the cells. Cell pellets were resuspended with of 0.5 N Acetic acid. Cells were disrupted by Sonication by using a Powersonic (POWERSONIC 420, Hwashin tech Co., Kwangju) and 3 times at 100 Hz for 180 s each on ice. Sonicated cell samples were subjected to centrifugation (10,000 × g, 10 min), and the supernatant of each sample was placed in an ultrafilter (Ultrafree-MC; MWCO 10,000Da, Millipore, Massachuetts) and centrifuged (10,000 × g, 10 min). A 0.5 ml aliquot of each filtrate was subjected and the filtrate was stored at -20℃ until analysis.

2.2.2 Pre-treatment of zooplankton samples

Before PST analysis of zooplankton, four pre-treatment methods were carried out.

In the first case, the pre-treatment method is based on the experimental method presented by Turner et al. (2000), prior to analysis, the frozen samples were thawed, after which the samples were concentrated using a 20 μm mesh. The sample was then treated with an equal amount of 0.1 N HCl. The samples were Heat in boiled water for 5-10 minutes, centrifuged (3,000 × g, 10 min), and the supernatants were passed through a Sep-Pak C18 cartridge column for partial purification. And supernatant of each sample was placed in an ultrafilter (Ultrafree-MC; MWCO 10,000Da, Millipore, Massachuetts) and centrifuged (10,000 × g, 10 min). Samples were stored at －20℃ until analysis.

In the second case, the pre-treatment method is based on the experimental method presented by Doucette et al. (2005), prior to analysis, the frozen samples were thawed, filtered onto a 47 μm diamter GF glass-fiber filter (Whatman), which was then placed in a plastic 15 ml Conical tube and added the equal amount of 0.1 N HCl as the sample. The samples were Heat in boiled water for 5-10 minutes, centrifuged (3,000 × g, 10 min), and the supernatants were passed through a Sep-Pak C18 cartridge column for partial purification. And supernatant of each sample was placed in an ultrafilter and centrifuged (10,000 × g, 10 min). Samples were stored at －20℃ until analysis.

In the third case, prior to analysis, the frozen samples were thawed, filtered onto a 47 μm diamter GF glass-fiber filter （Whatman）, dried (60℃, 2 hours) (Campbell et al. 2005), which was then placed in a preweighed plastic 15 ml Conical tube and added the 2 ml of 0.1 N HCl. The samples were Heat in boiled water for 5-10 minutes, centrifuged (3,000 × g, 10 min), and the supernatants were passed through a Sep-Pak C18 cartridge column for partial purification. And supernatant of each sample was placed in an ultrafilter and centrifuged (10,000 × g, 10min). Samples were stored at －20℃ until analysis.

In the fourth case, according to the collected samples, the pre-treatment method refers to the experimental method in the paper of Hamasaki et al. (2003), prior to analysis, the frozen samples were thawed, counting of individuals in the sample was counted using a light microscope， sample was then treated with an equal amount of 0.1 N HCl. The samples were Heat in boiled water for 5-10 minutes, centrifuged (3,000 × g, 10 min), and the supernatants were passed through a Sep-Pak C18 cartridge column for partial purification. And supernatant of each sample was placed in an ultrafilter and centrifuged (10,000 × g, 10min). Samples were stored at －20℃ until analysis.

Toxins were extracted and subsequently determined by High Performance Liquid Chromatography (HPLC; Thermo Scientific UltiMate 3000, Chromeleon 6.8) with fluorescence detector. Hypersil GOLD C8 column (250 mm × 4.6 mm; Thermo Scientific, Massachusetts) was used for toxin composition separation. Toxin contents were calculated from the relative area ratio of the analyzed sample peak and the standard toxin peak and expressed as fmole/cell.

Toxicity in saxitoxin equivalents (STX equiv) was calculated using Toxicity Equivalency Factors on the specific toxicity of each derivative reported by Oshima (1995). and expressed as pg STXeq/cell.

3. Results and Discussion

3.1 PST analysis of suspended matter samples

The suspended matter analyzed in this experiment was collected from Jinhai Bay, Korea. From December 2021 to May 2022, eight batches of samples will be collected, resulting in 35 samples in total. The toxin content and toxicity of suspended matter in the seawater of Jinhae Bay in spring changed with time (Figure 17). The results showed that PST was progressively detected in the samples taken since the third collection (January 24, 2022). Since there was no obvious pattern to follow, a preliminary study was conducted. Initially, PST was detected only at collection sites 1 , 2 and 3 in Jinhae Bay and Masan Bay. Subsequently, PST was also detected at collection sites 4 and 5 in Jindong Bay. After the seventh sample collection (April 20, 2022), PST could no longer be detected. In addition, the figure indicates that the toxin content and toxicity of suspended matter in seawater has decreased over time. The results of this study were similar to those of the sea areas where PST mainly occurs each year in the National Institute of Fisheries Science toxin forecast (NIFS, 2022).

The composition of PST toxic compositions in the collected samples consisted of N-sulfocarbamoyl group C1+2 and carbamate group GTX1+4, GTX2+3 (Figure 18). From the results, it was found that C1+2 was predominant in almost all samples, but there were significant differences in the proportions of other toxic compositions. Shon et al. (2009) reported that it is known that *Alexandrium* have unstable N-sulfocarbamoyl toxins, so when bivalves ingest

them, they are biotransformed in vivo and converted to highly toxic carbamoyl toxins. Therefore, bivalves may become intoxicated even when toxin concentrations of *Alexandrium* in seawater are low.

Correlation between the cell density of toxic dinoflagellates in seawater and of detected toxin content in the spring Jinhae Bay From figure 19, it is known that there is a close correlation between the density of toxic dinoflagellates in seawater and of detected toxin content. There was a significant positive correlation by Pearson's correlation analysis ($r = 0.75$; $p < 0.05$; $n = 33$). In other studies, it has been demonstrated that PST is very toxic and can cause bivalve intoxication even in low concentration environments where *Alexandrium* spp. cells in seawater are less than 100-200 per liter. (Townsend et al., 2001). Therefore, when the cell density of *Alexandrium* spp. in seawater increased, there was a tendency to increase the amount of PST in seawater, and we concluded that the susceptibility to toxification of bivalves was high. In addition, it was judged that zooplankton, primary consumers that consume toxic dinoflagellates, were likely to undergo toxification in vivo.

According to survey data, Jinhae Bay has a water temperature range of 6.04-17.85℃ in spring, and a salinity range of 31-33 psu. Water temperature increased with the seasons, but the difference in salinity was not significant. As can be seen from the figure, *Alexandrium* spp. in seawater showed an increasing trend in toxin content and toxicity with the increase in water temperature. The salinity did not change much, so it did not affect the toxin content and toxicity (Figure 20). Yoo et al. (2000) reported that *A. tamarense* appeared in Jinhae Bay when the water temperature was 12.3-12.7℃ and the salinity was 32.4-32.8 psu.

Moreover, it has been reported in Hiroshima Bay, Japan, that blooms occur every spring when the water temperature is between 10.2-20.2℃ (Itakura et al., 2002). In addition, the results of previous laboratory experiments also confirmed that *A. catenella* grows well under the condition of 10-20℃. Therefore, it is believed that *Alexandrium* is prone to proliferate in the water temperature range of 10-20℃ in Jinhae Bay from February to May, which leads to the toxification of bivalves. Therefore, we believe that the results of previously conducted laboratory experiments compared with the results of field experiments can provide insight into the occurrence of *Alexandrium* in Jinhae Bay in spring.

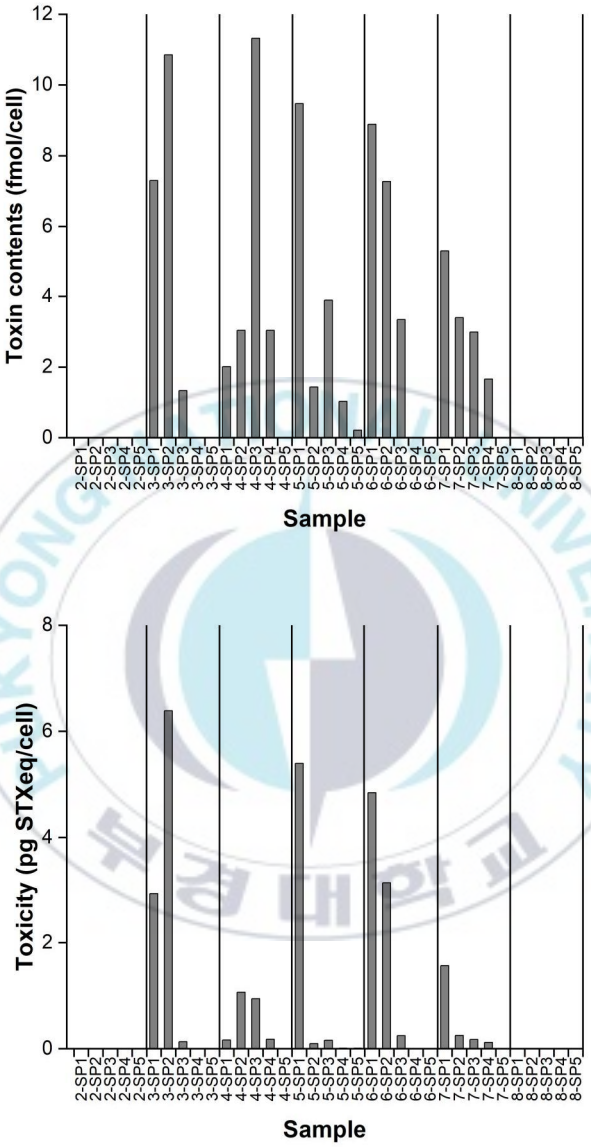

Figure 17. Changes over time in the toxin contents (fmol/cell) and toxicities (pg STXeq/cell) of suspended matter.

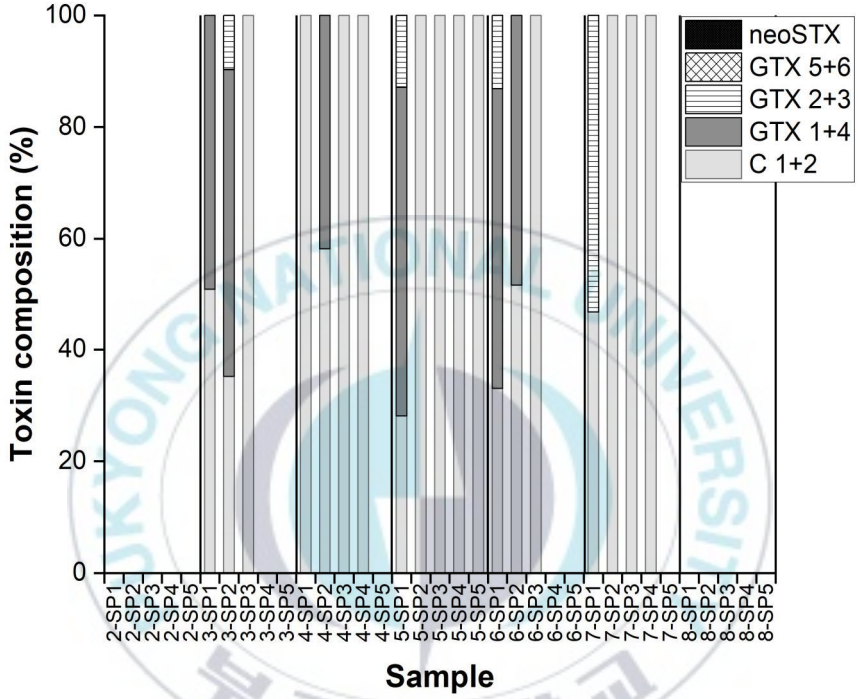

Figure 18. Changes over time in the toxin compositions (%) of suspended matter.

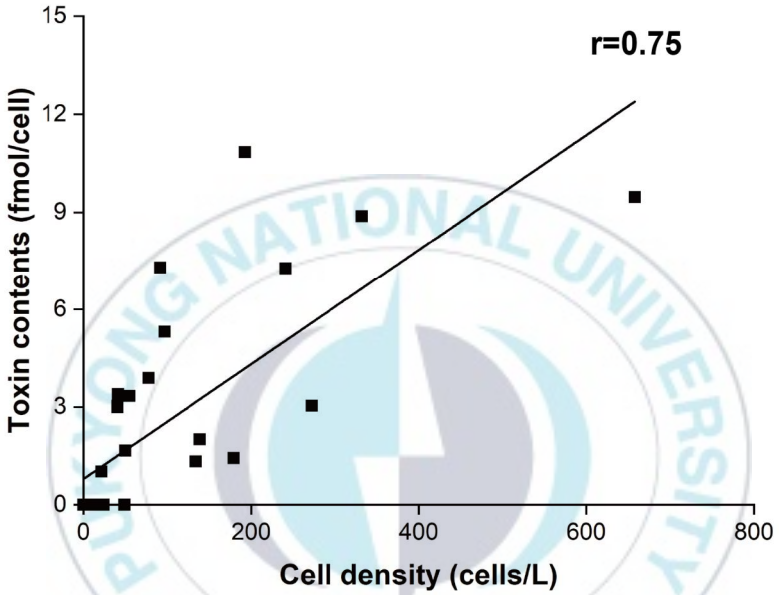

Figure 19. Correlation comparison of cell concentrations of *Alexandrium* spp. and PST toxin contents (fmol/cell) in seawater (r = 0.75; $p < 0.05$; n = 33).

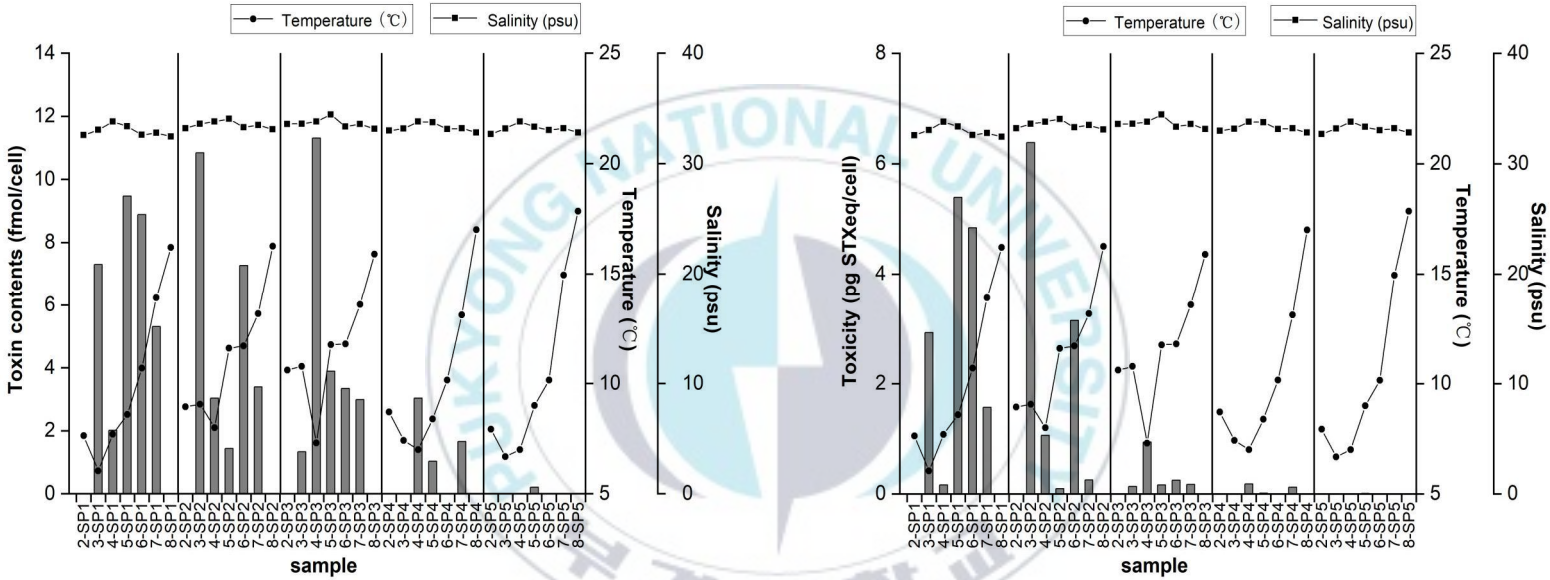

Figure 20. Changes in toxin contents (fmol/cell) and toxicities (pg STXeq/cell) of suspended matter at different water temperatures and salinities over time.

3.2 Pre-treatment analysis of zooplankton samples

Before PST analysis of zooplankton, four pre-treatment methods were carried out. Select the most accurate pre-treatment method among them. The difference between these four pre-treatment methods is that different methods are used to concentrate the collected zooplankton samples.

The first pre-treatment method was to use a 20 μm net in order to concentrate the zooplankton samples. In Figure 21, it can be seen that toxins were detected in both concentrated samples and the filtrate that passed through the grid. The reason is that zooplankton samples should be cryopreserved after collection; however, during the thawing process before PST analysis, the toxins can be easily released, resulting in residual toxins in the filtrate.

The second pre-treatment method is the concentration of zooplankton samples using a filtration unit. When using the filtration unit for pre-treatment, the filter paper will be clogged. In view of the difference in size of Zooplankton in collected samples, the GF/F filter is easy to clog when using it, leading to a decrease in concentrate amount and inability to perform PST analysis.

The third pre-treatment method is similar to the second pre-treatment method, but adds the process of drying the filtered GF/F filter in a dryer. Zooplankton samples vary in size, making the GF/F filter prone to clogging, resulting in a decrease in concentrate amount and inability to perform PST analysis. In addition, the weight of the filter after drying was too light, but subsequent experiments required the addition of 0.1 N HCl equal to the weight

of the filter for toxin extraction. At this time, if an equal amount of 0.1 N HCl is added, the toxin cannot be extracted. Conversely, if an excess of 0.1 N HCl is added the toxin can be extracted, but there will be a dilution of the original toxin content in the sample, leading to errors in subsequent toxin content calculations.

After summarizing the reasons for the failure of the previous experiments, the fourth pre-treatment method was established. In order to accurately detect the content of PST in zooplankton, before pre-treatment, use an optical microscope to count the individual zooplankton in the sample, perform toxin extraction, and then calculate the toxin content contained in each individual. Finally, compared with the above pre-treatment methods, the fourth pre-treatment method could detect the PST content in zooplankton more accurately, so the fourth pre-treatment method was used for PST analysis of zooplankton in this experiment.

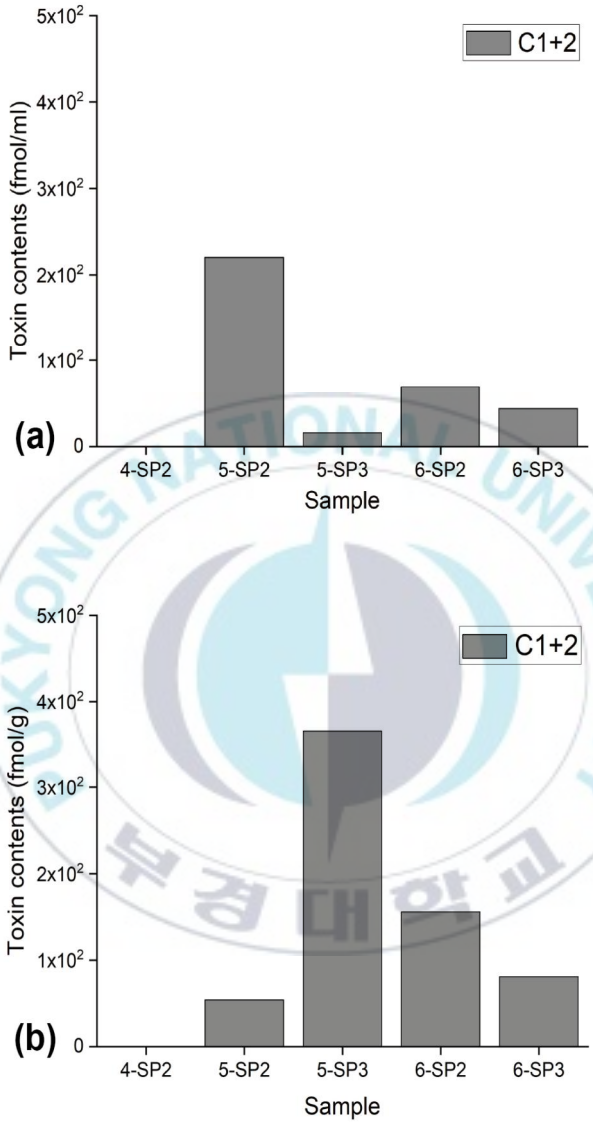

Figure 21. Comparison of the toxin content of (a) filtrate and (b) concentrated samples of the same zooplankton sample after pre-treatment with a Net.

3.3 PST analysis of zooplankton samples

The zooplankton in this experiment was collected from Jinhai Bay, Korea. From December 2021 to May 2022, eight batches of samples will be collected, resulting in 35 samples in total. The toxin content and toxicity of zooplankton in the seawater of Jinhae Bay in spring changed with time (Figure 22). The results showed that PST was progressively detected in the samples taken since the third collection (January 24, 2022). Although the early PST detections were low, over time, high levels of PST were found in the fifth and sixth samples taken during March. After the eighth sample collection (May 11, 2022), PST could still be detected. In addition, it is known from the figure that the toxin content and toxicity of zooplankton organisms show a trend of increasing and then decreasing over time. As reported by the National Institute of Fisheries Science, in the coastal areas of Masan Bay and Jinhae Bay every spring, from March when the water temperature is 10℃, *Alexandrium* spp., which can cause PST, appears one after another, and increases with the increase in water temperature. When the water temperature is between 14-17℃ from April and May, the cell density of *Alexandrium* spp. in seawater is the highest (NIFS, 2022).

The composition of PST toxic compositions in the collected samples consisted of N-sulfocarbamoyl group C1+2, GTX5, and carbamate group GTX1+4, GTX2+3 (Figure 23). The figure shows that the concentration of GTX5 was higher in the zooplankton collected in the third and eighth collections. In addition, the concentrations of GTX1+4 and GTX2+3 were higher in the zooplankton collected in March for the third and sixth collections. When

comparing the PST virulence composition of zooplankton copepod with that of *Alexandrium* spp., changes in the composition of PST toxins can be observed. From the experimental results, it can be seen that GTX1+4 accounts for a significantly higher proportion in animal plankton than C1+2, and copepods will preferentially accumulate GTX than C toxin. Thus, neurotoxins in toxic dinoflagellates can be absorbed and converted into strong toxins by zooplankton copepods, which then transfer the toxins to higher stages of the food wed (White 1981).

Correlation between the cell density of toxic dinoflagellates in seawater and of detected toxin content of zooplankton in the spring Jinhae Bay From figure 24, it is known that there is a close correlation between the density of toxic dinoflagellates in seawater and of detected toxin content of zooplankton. There was a significant positive correlation by Pearson's correlation analysis ($r = 0.70$; $p < 0.05$; $n = 35$). Figure 25 illustrates the correlation between the toxin content of toxic dinoflagellates and the detected toxin content of zooplankton. There was a significant positive correlation by Pearson's correlation analysis ($r = 0.75$; $p < 0.05$; $n = 35$). When copepods are in a low-concentration toxin environment, as the toxin concentration increases, the toxin content in the copepod also increases, but if it continues to be exposed to a high-concentration toxin environment, the toxin content in the copepod will reach saturation. Because when the toxin in the copepod exceeds a certain standard, the activity of the copepod is inhibited, so this mechanism can inhibit the accumulation of toxin in the copepod (Hamasaki et al., 2003).

There has been a detection of PST in the suspended matter collected in

Jinhae Bay. And the water temperature at the time of sample collection was about 8-15℃ and the salinity was 33 psu. Based on laboratory culture results, It was found that *A catenella* had a high growth rate and a higher toxin content at water temperatures of 10-15℃. Therefore, *Alexandrium* grew well in Jinhae Bay, and the toxin content increased as the water temperature increased. Copepod named Acartia clausii has been observed to prey on PST-producing *A. catenella* and then transfers the toxin to higher trophic levels (White 1981). Therefore, the culture results show that as the water temperature increases the toxic dinoflagellates grow rapidly and the toxin content also increases, so the toxin content and toxicity in zooplankton also tend to increase with the increase of water temperature (Figure 26).

PST was determined by measuring the toxin content of bivalves in Masan Bay and Jinhae Bay, according to a report from the National Institute of Fisheries Science. According to the results of this experiment, it was demonstrated that toxic dinoflagellates would accumulate in the bodies of zooplankton when ingested by them. The results were also compared and analyzed with the PST test report of the National Institute of Fisheries Science. It was found that zooplankton had an earlier formation of toxification to PST compared with bivalves. In terms of marine ecology, this indicator is of high significance and can be used to monitor and evaluate the transmission process of PST among organisms. Thus, it provides the basis for the development of a more accurate prediction system for PST.

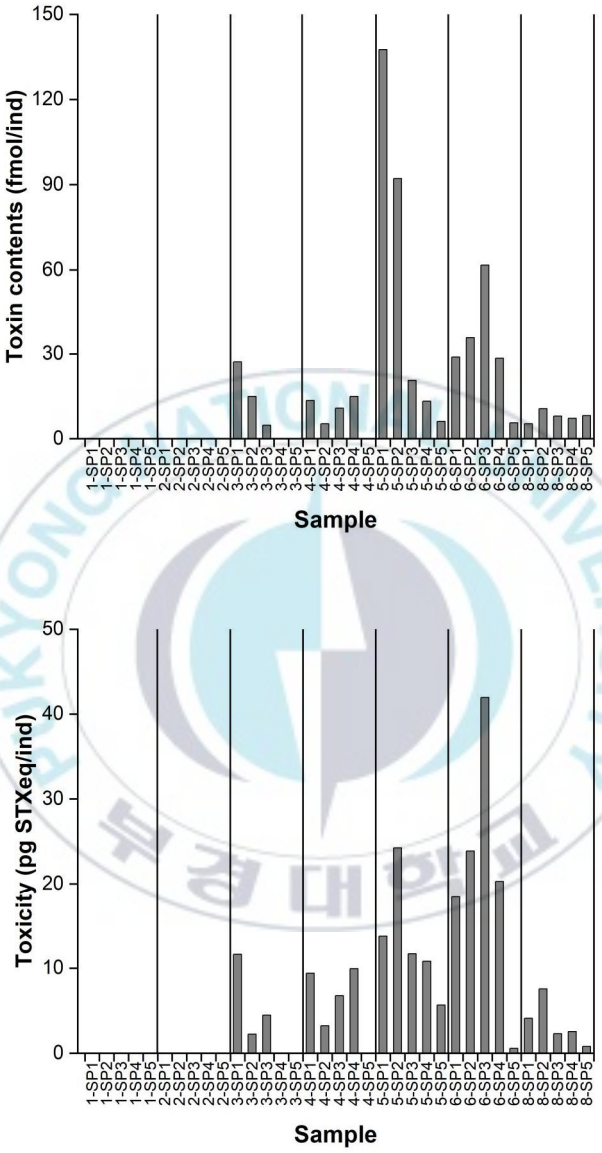

Figure 22. Changes over time in the toxin contents (fmol/ind) and toxicities (pg STXeq/ind) of zooplankton.

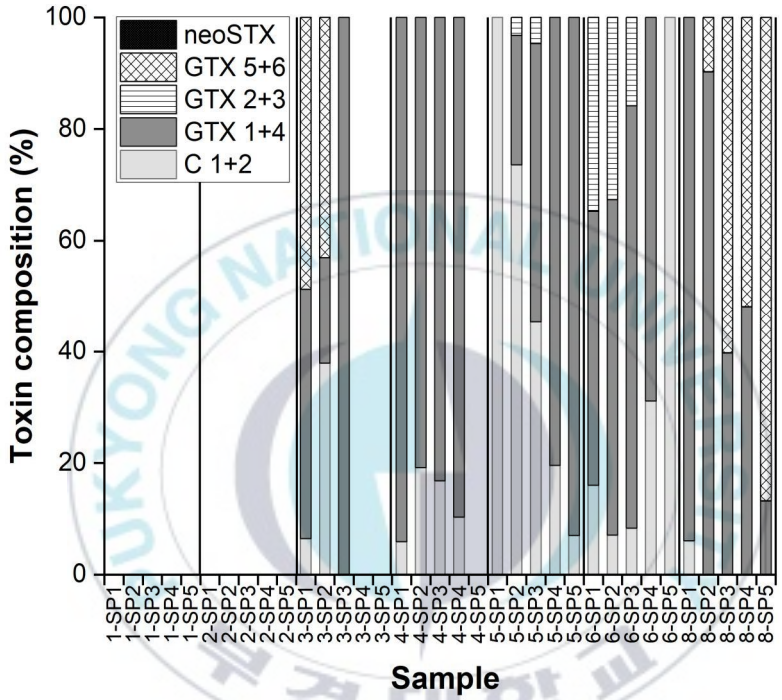

Figure 23. Changes over time in the toxin compositions (%) of zooplankton.

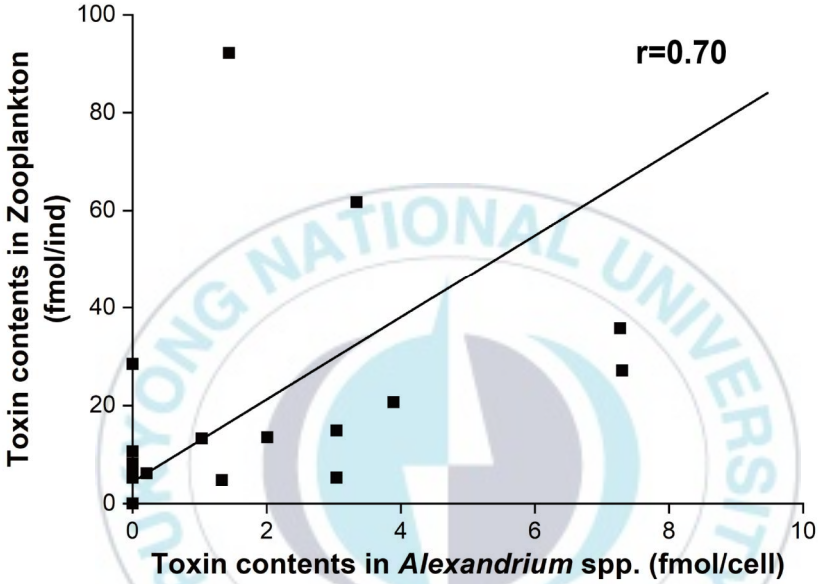

Figure 24. Correlation comparison of PST toxin contents (fmol/ind) accumulated in individuals zooplankton against toxin contents in cells of *Alexandrium* spp. (fmol/cell) (r = 0.70; $p < 0.05$; n = 32).

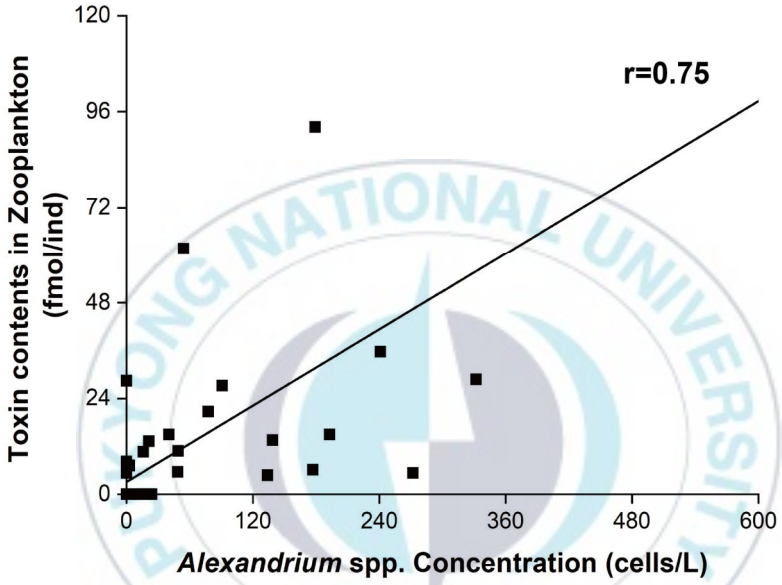

Figure 25. Correlation comparison of PST toxin contents (fmol/ind) accumulated in individuals zooplankton against *Alexandrium* spp. cell concentration (cells/L) (r = 0.75; $p < 0.05$; n = 35).

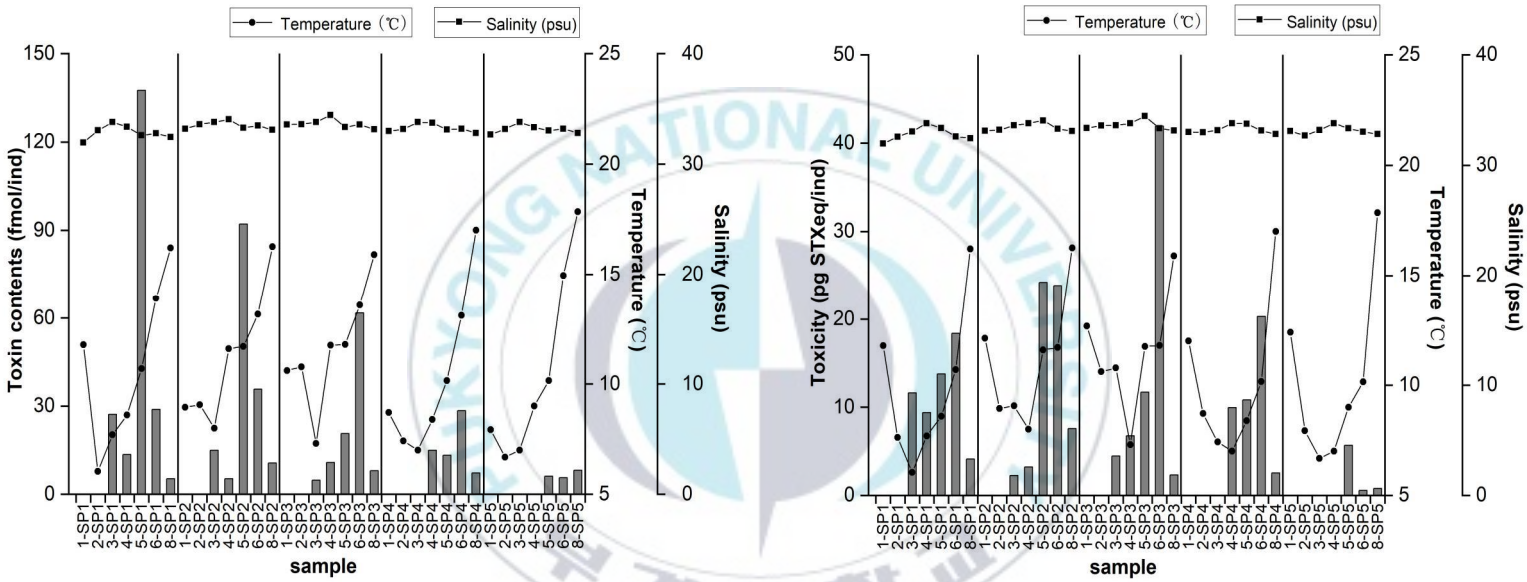

Figure 26. Changes in toxin contents (fmol/ind) and toxicities (pg STXeq/ind) of zooplankton at different water temperatures and salinities over time.

Ⅳ. Conclusion

The occurrence area of PST in the world has gradually become more widespread. In Korea, PST occurs every spring centered on Jinhae Bay. As a result, excessive levels of toxins in bivalves cultured around April have caused damage to the fishing industry and raised social concerns. PST has been detected at an increased rate as a result of the increase in water temperature caused by global warming, the number of occurrences has increased, and the incidence is expected to rise in the future. It is also known that zooplankton ingest toxic dinoflagellates containing PST toxins and then transmit the toxins to upstream trophic stages through the food web. In this study, we used *A. catenella* and *A. pacificum* isolated from the Korean coast to observe the growth characteristics and PST changes in different water temperatures and salinities. We also analyze it with the appearance and change of PST in the suspended matter and zooplankton collected in the field.

Laboratory culture results showed that *A. catenella* grew at an increased rate under water temperature conditions from 10-20℃ and halotolerance characteristics. *A. catenella* was most toxicity at water temperatures of 25℃ and 35 psu, but it was also more toxicity than other intervals of water temperatures and salinities at the maximum growth rate of 15℃ and 30 psu. And the toxicity increased with the increase in salinity. When exposed to high temperatures, *A. pacificum* exhibits physiological characteristics of low toxicity but a very rapid growth rate.

Based on the results of the samples collected in the field, PST was detected in the suspended matter collected in Jinhae Bay this year. The water temperature at the time of sample collection was known to be between 8-15℃ and the salinity was between 31-33 psu. The results of the study can be compared with those of the laboratory culture to demonstrate that there is a high probability that *A. catenella* will be found in the spring Jinhae Bay water temperature and salinity environment. According to the test results, PST toxin has been detected in the zooplankton collected in Jinhae Bay since late January. It is known that zooplankton ingest toxic dinoflagellates containing PST toxins, which can be transferred via the food chain to upstream trophic levels. Accordingly, it is concluded that, depending on the water temperature and salinity conditions in Jinhae Bay in spring, the toxic dinoflagellates *Alexandrium* spp. were prone to blooming. This contributed to the intoxication of zooplankton and bivalves. It should be noted that bivalves and zooplankton are intoxicated even at low concentrations of *Alexandrium* spp. (100-200 cells per liter of seawater). And compare this result with the PST detection report of the National Institute of Fisheries Science. PST was detected in bivalve molluscs about two weeks after PST was detected in zooplankton (>15 d).

Based on the above experimental results, the appearance of bivalve PST in spring each year appears to be as previously known, perhaps because of *A. catenella*, and the recent bivalve PST poisoning event in June was most likely caused by *A. catenella* together with *A. pacificum*. Not to mention that because of global warming, the water temperature in the Korean Peninsula waters continues to rise, and with the early appearance of *A. catenella* and *A.*

pacificum, the problem of PST has the potential to become long-term. Therefore, in order to improve the accuracy of the PST prediction system, the growth characteristics and PST changes of *Alexandrium* spp. in Jinhae Bay and different sea areas will be studied in the future. In addition, considering the physical, chemical and biological environmental factors, it is considered necessary to grasp and understand the emergence of toxic dinoflagellates and the process of PST transfer and toxification among zooplankton.

This study not only provides significant insights into the growth characteristics of toxic dinoflagellates and the changes in PST under water temperature and salinity conditions, as well as demonstrating that PST can cause toxification in bivalves and zooplankton through interorganismal transfer. In order to further improve the PST prediction system, in the future we will comprehensively consider the impact of other environmental factors and conduct research on this, and provide assistance to the coastal aquaculture industry as a result.

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유독 와편모조류 *Alexandrium*속 출현과 관련된 동물플랑크톤의 마비성 패독(PSTs) 독화와 수온 및 염분 조건에 따른 *A. catenella*와 *A. pacificum*의 PSTs 변화

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요약

유독 와편모조류인 *Alexandrium catenella*와 *A. pacificum*은 *Alexandrium*속에서 마비성패독(Paralytic Shellfish Toxin; PST)을 발생시키는 대표적인 원인종으로 알려져 있으며, 최근에는 선박평형수, 그리고 지구온난화로 인한 수온 상승 등에 따라 전 세계적으로 PST 독화의 조기화 및 광역화의 추세를 보였다. 한국의 경우도 매년 춘계 진해만을 중심으로 PST 문제가 발생하며, 4월에는 양식 이매패류의 출하금지 기준치(80 μg/100 g)를 초과하여 사회적으로 문제를 발생시키고 있다. 또한 동물플랑크톤은 PST 독소를 함유한 유독 와편모조류를 섭취하면 먹이망을 통해 상위 영양 단계로 패독 독소를 전달한다는 것으로 알려져 있다. 본 연구는 한국 연안에서 출현하는 유독 와편모조류 *Alexandrium* 분리종을 활용하여, 수온 및 염분 조건의 변화에 따른 생장특성과 PST 변화를 파악하고자 하였다. 또한 춘계 유독 와편모조류 *Alexandrium*의 출현에 관련된 동물플랑크톤의 PST 독화 가능성에 대한 연구를 수행하였다.

배양 실험을 바탕으로 다양한 수온과 염분 조건에서 유독 와편모조류 *Alexandrium catenella* (JM-1)와 *A. pacificum* (LIMS-PS-2611)의 생장속도와 PST의 변화를 조사하였다. *A.*

*catenella*는 저수온 환경에서 생장속도가 높았고 광염성의 생리적인 특성을 보였다. 수온과 염분에 따른 PST 변화를 보면, *A. catenella*는 수온 25℃, 염분 35 psu의 조건에서 가장 높은 독 함량을 보였지만, 최대생장속도를 보였던 수온 15℃, 염분 30 psu에서도 다른 수온과 염분 구간보다 높은 독 함량을 보였다. 또한 염분의 증가에 따라 독 함량이 증가하는 경향을 나타내었다. 반대로 *A. pacificum*은 고수온 환경에서 생장속도가 높았으며 광염성의 생리적 특성을 보였다. 또한 최대생장속도를 보였던 수온과 염분에서 낮은 독함량과 독성을 보였지만, 20℃의 고수온 조건에서 높은 독함량과 독성을 나타내었다.

Alexandrium spp.가 현장에 출현할 때의 수온과 염분 조건이 실내 배양 결과와 일치하는지 확인하기 위해 봄철 PST가 자주 발생하는 해역인 진해만에서 유독 와편모조류를 포함하는 부유물질과 동물플랑크톤을 채집하여 PST 분석을 수행하였다. 진해만에 채집된 유독 와편모조류를 포함하는 부유물질과 동물플랑크톤 체내 PST는 시간의 변화에 따라 독 함량과 독성이 먼저 증가하다가 감소하는 경향을 보였다. 부유물질의 독조성 결과를 비교해 보면 동물플랑크톤의 독조성은 강독인 GTX1+4가 약독인 C1+2보다 더 많은 비율을 차지하였다. 또한 국립수산과학원 패류독소속보를 비교해 보면 해수 중의 동물플랑크톤이 이매패류보다 먼저 PST에 독화되는 양상을 보였다.

본 연구를 토대로 매년 춘계에서 발생하는 이매패류 PST는 기존에 알려진 것과 같이 *A. catenella*에 따른 것으로 보이며, 최근 6월에 발생한 이매패류의 PST 독화는 *A. catenella* 및 *A. pacificum*에 기인했을 가능성이 높을 것으로 판단된다. 이러한 결과를 바탕으로 춘계 유독 와편모조류 *Alexandrium*의 출현과 관련된 동물플랑크톤의 독화 가능성을 검토하고 먹이사슬을 통한 PST 독화 양상을 파악하여 향후 마비성패독 조기화 예측 시스템 구축을 위한 기초 데이터를 제공할 수 있을 것으로 판단된다.

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