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ARTICLE

Effects of Zooplankton Extracts on the Production of Paralytic Shellfish Toxins by *Gymnodinium catenatum* and *Alexandrium pacificum*

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Abstract

The presence of zooplankton in marine ecosystems is an important factor affecting toxin production in dinoflagellates. However, whether the production of paralytic shellfish toxins (PSTs) by *Gymnodinium catenatum* and *Alexandrium pacificum* is affected by substances produced by zooplankton is not yet fully understood. This study assessed the effects of zooplankton extracts on cell abundance, pigment concentration and composition, chlorophyll-*a*-specific primary production (Chl-*a* SP), and PST production in *G. catenatum* and *A. pacificum,* isolated from Korean coastal waters. In addition, a novel group of hydroxybenzoate PSTs known as GC toxins, which lack commercial reference standards, was evaluated. No significant differences were observed in cell abundance, pigment composition, or Chl-*a* SP in the experimental group of *G. catenatum* strains exposed to zooplankton extracts compared to the control group. However, the production of PSTs, including GC toxins, was significantly enhanced when the strain was exposed to extracts of *Calanus finmarchicus*. Meanwhile, in the *A. pacificum* strain, some experimental groups showed significant differences in cell abundance and pigment composition, while Chl-*a* SP significantly decreased in all experimental groups. In addition, PST production was stimulated by the addition of *C. finmarchicus* extracts. No GC toxins were detected in the *A. pacificum* strain. This study demonstrated that substances derived from *C. finmarchicus* enhance the production of PSTs in *G. catenatum* and *A. pacificum*. Our findings will improve the current understanding of the occurrence and development mechanisms of PSTs in marine ecosystems and contribute to developing strategies to efficiently secure standard reference toxin products using toxic dinoflagellates.

Keywords *Alexandrium pacificum* · *Gymnodinium catenatum* · *Calanus finmarchicus* · GC toxins · Paralytic shellfish poisoning

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1 Introduction

Massive outbreaks of toxic dinoflagellates in marine ecosystems negatively affect ecosystem health by degrading water quality and disrupting ecosystem balance. They affect marine life and aquaculture, and extend their adverse effects to seabirds and humans, causing economic losses (Lehane and Lewis 2000; Pratchett et al. 2008; Badjeck et al. 2010; Grattan et al. 2016). Considering the public health risks, the Korean government has defined red tide phenomena as fishery disasters since 1990 and continues monitoring their occurrence, detecting shellfish toxins, and providing shellfish warnings when dangerous levels of paralytic shellfish toxins (PSTs) are detected.

The main biotoxins detected in bivalve mollusks include paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), and neurotoxic shellfish poisoning (NSP) (National Institute of Food and Drug Safety Evaluation 2011). Among them, PSP, which occurs periodically along the southern coast of Korea every year, is characterized by its high mortality rate in cases of intoxication and is one of the most infamous and longstanding groups of marine toxins (Osek et al. 2006; Mok et al. 2013). Incidents of intoxication have been reported worldwide since the first documented case of poisoning by PSTs in Alaska in the 1790s. In addition, fatalities attributed to PSTs have been reported in Korea. Therefore, the Korean National Institute of Fisheries Science (NIFS) has been monitoring PSTs to prevent poisoning incidents. However, the current standard analytical method, which is based on the AOAC mouse bioassay, has limitations as it cannot identify the composition of the toxins, raising ethical concerns regarding animal use. Various efforts are ongoing to develop alternative testing methods to overcome these limitations (Mok et al. 2013).

There is an increasing likelihood that toxic algal blooms or novel marine biotoxins, which previously did not pose a threat in certain regions, may become more prevalent and pose new threats owing to various factors such as climate change and transport via ballast water (Lassus et al. 2015). Various analytical methods have been developed globally to pre-emptively respond to these emerging threats. Various analytical methods have been developed globally to pre-emptively respond to these emerging threats. High-performance liquid chromatography (HPLC) equipped with an ultraviolet (UV) detector, diode array detector (DAD), or fluorescence detector (FLD) and liquid chromatography–mass spectrometry (LC–MS)-based analyses are typical examples (Panda et al. 2022 and references therein). These approaches have the advantages of qualitatively and quantitatively identifying the components of toxins and are potential alternatives to traditional animal testing methods. However, accurate instrument analysis requires the availability of standard substances, highlighting the need to develop technologies to create these reference materials, including novel toxins. A significant number of commercially available marine toxin standards are extracted and purified from toxic microalgae. Therefore, understanding the conditions that enhance toxin production in these algae is directly linked to the development of efficient technologies for creating reference materials.

Dinoflagellates are an important group of microalgae used to produce certified reference materials (CRMs) for commercially available marine toxins. Among these, *Alexandrium* sp. and *Gymnodinium* sp. are extensively used for acquiring CRMs from PSTs, and they also occur along the Korean coasts. Identifying the physicochemical and biological factors that enhance toxin production in these microalgae is crucial for developing efficient technologies to create standard materials and understanding the mechanisms underlying the occurrence of PSTs in Korea. The physical, chemical, and biological factors that control toxin production in these species, such as light wavelength, nutrient conditions, bacterial interactions, and predation, have been explored by various researchers (Hold et al. 2001; Selander et al. 2006; Bui et al. 2024). However, the effect of biological interactions on PST production is poorly understood. Selander et al. (2006) provided direct evidence that the substances produced by copepod grazers can induce toxin production in toxic dinoflagellates. Considering that some marine zooplankton can distinguish toxic microalgae and do not consume them (Teegarden 1999), such interactions may serve as a survival strategy for toxic dinoflagellates within marine ecosystems. These findings suggest that the presence of predators could be used as a strategic method to enhance toxin production by toxic microalgae, which may be beneficial for creating CRMs for these toxins. Particularly, since *Gymnodinium catenatum* is a species that produces GC toxins, a type of PST that is not yet commercially available, identifying the environmental conditions that promote the production of these toxins could be vital for developing effective strategies to create novel PST CRMs.

Therefore, in this study, we conducted culture experiments to examine whether zooplankton extracts could enhance PST production in the toxic dinoflagellates *G. catenatum* and *Alexandrium pacificum* isolated from Korean coastal waters. Methanol extracts from zooplankton were used in this study. The investigated PSTs included carbamoyl, N-sulfocarbamoyl, and decarbamoyl toxins, which have commercially available CRMs, and GC toxins, which have no commercially available CRMs. Changes in dinoflagellate abundance, pigment content and composition, and chlorophyll-*a*-specific primary production, induced by zooplankton-derived substances, were observed.

2 Materials and Methods

2.1 Isolation and Culture of Toxic Dinoflagellate Strains

The toxic dinoflagellates used in this experiment, *G. catenatum* (strain KM032) and *A. pacificum* (strain KM02), were isolated directly from the coastal waters of Tongyeong (34°47′45.0"N) and Busan (35°03′24.5"N) in South Korea, respectively. The culture medium was prepared from 30 psu autoclaved 0.2-μm filtered natural seawater enriched with f/2-si (Guillard 1975). All strains were cultured in an incubator at 20 °C with a light intensity of 200–250 μmol E s^{-1} m^{-2}.

2.2 Preparation of Zooplankton Extracts

Commercially available zooplankton (*Cyclops vicinus*, *Calanus finmarchicus*, and *Artemia franciscana*) were used to prepare the zooplankton extracts. Further analysis was conducted to identify the zooplankton species using DNA sequencing when the exact species name was not specified. Zooplankton extracts were prepared according to a previously reported procedure (Trapp et al. 2021) with slight modifications. Briefly, each zooplankton sample was freeze-dried, followed by the extraction of 400 mg of the dry sample with 10 mL of methanol at − 20°C for 24 h. After extraction, the mixture was centrifuged to separate the supernatant, which was concentrated using nitrogen gas. The concentrated extract was re-dissolved in different volumes of methanol (1.5, 1.0, and 0.5 mL) to create gradient levels labeled as "L" (low), "M" (medium), and "H" (high), respectively. Extracts at all three levels were prepared for *C. vicinus* and *C. finmarchicus*. However, for *A. franciscana,* only the "M" level extract was prepared and used. The effects of methanol alone were also tested in these experiments.

2.3 Toxin Induction Experiments Using Zooplankton Extracts

Toxin induction experiments were performed using two strains in the exponential growth phase, with cell abundances of approximately 1,230 cells mL^{-1} for *G. catenatum* and 5,130 cells mL^{-1} for *A. pacificum*. The control groups were set up as follows: Control-a, which contained the strains without any treatments, and Control-b, which included zooplankton exposed to the same amount of methanol (10 μL) used in the experimental groups. The experimental groups comprised seven different setups—one using "M" level extracts from *A. franciscana* to assess the effects of non-copepod zooplankton, and three setups each for *C. vicinus* and *C. finmarchicus* extracts at the "L", "M", and "H" levels to investigate the effect of copepods. Toxin induction experiments were conducted in 50-mL plastic tissue culture flasks (SPL Life Sciences, Korea) for 48 h under the same temperature and light conditions used for strain culture. The control and experimental setups were replicated three to six times. $NaH^{13}CO_3$ was added simultaneously with the zooplankton extract as a tracer to calculate chlorophyll-*a*-specific primary production (Chl-*a* SP).

2.4 Cell Abundance and Pigment Composition

Triplicate aliquots from each flask sample were fixed with Lugol's solution to determine cell abundance at the beginning and end of the toxin induction experiment. All the fixed cells were counted in a Sedgewick–Rafter chamber under a microscope (Nikon, Eclipse TI-U). Samples for pigment analysis were filtered through Whatman GF/F filters at the end of the toxin induction experiment, then immediately wrapped in aluminum foil and stored in a deep freezer (− 80 °C). The pigment concentration and composition were analyzed using HPLC (LC-20a; Shimadzu Corporation, Kyoto, Japan), as described previously (Zapata et al. 2000; Hyun et al. 2022, 2023).

2.5 Carbon Isotopic Composition and Chlorophyll-*a*-Specific Primary Production

The concentration and carbon isotopic composition of particulate organic carbon were determined using an isotope ratio mass spectrometer (Isoprime; GV Instruments, Manchester, UK) interfaced with an elemental analyzer (Euro EA3028; EuroVector, Milan, Italy), following previously reported procedures (Lee et al. 2016, 2022), to calculate chlorophyll-*a* SP. This analysis was performed according to previously reported procedures (Lee et al. 2016, 2022). The analytical precision for the carbon isotopic composition, calculated by the IAEA standard (CH-3), was 0.07‰. The standard reference material for carbon was the Vienna PeeDee Belemnite (VPDB). A Whatman GF/F filter, precombusted at 450 °C for 4 h, was used to collect particulate organic matter. The organic carbon production rate was calculated following the method described by Hama et al. (1983). This value was divided by the chlorophyll-*a* concentration to determine Chl-*a* SP.

2.6 Toxin Profiles of *Gymnodinium catenatum* and *Alexandrium pacificum*

A 40–45 mL volume of cell suspension was harvested by centrifugation (3000 rpm; 20 min), followed by resuspension

Table 1 HPLC conditions for the analysis of paralytic shellfish toxins (PSTs) including GC toxins in toxic dinoflagellates *Gymnodinium catenatum* and *Alexandrium pacificum*

GC toxins	Other PSTs
Target toxins	
GC1, GC2&4, GC5, and GC3	STX, NeoSTX, GTX1&4, GTX2&3, C1&2, dcSTX, dcNeoSTX, and dcGTX2&3
Column	
Alltech Alltima C18, 4.6 × 250 mm, 5 µm	Agilent Zorbax Bonus RP C18, 4.6 × 150 mm, 3.5 µm
Flow rate	
0.8 ml min^{-1}	0.8 mL min^{-1}
Mobile phase A	
10 mM heptafluorobutyric acid	11 mM heptane sulfonate, 5.5 mM phosphoric acid, pH 7.1 with NH_4OH
Mobile phase B	
CH_3CN	11 mM heptane sulfonate, 16.5 mM phosphoric acid, 11.5% CH_3CN, pH 7.1 with NH_4OH
Gradient condition	
0–40 min (sol A:sol B = 82:18, v/v)	0–8 min (sol A), 8–19 min (sol B), 19–30 min (sol A)
Oxidation and acidifying reagent/condition	
Oxidant: 10 mM periodic acid in 550 mM ammonium hydroxide; acidifier: 0.75 N nitric acid; flow rate: 0.4 mL min^{-1}; reactor temperature: 85 °C	Oxidant: 100 mM phosphoric acid, 5 mM periodic acid, pH 7.8; acidifier: 0.75 N nitric acid; flow rate: 0.4 mL min^{-1}; reactor temperature: 85 °C
Detection	
Ex 330 nm and Em 390 nm	Ex 330 nm and Em 390 nm

HPLC, high-performance liquid chromatography; STX, saxitoxin; NeoSTX, neosaxitoxin; GTX1&4, gonyautoxins 1&4; GTX2&3, gonyautoxins 2&3; C1&2, N-sulfocarbamoyl gonyautoxins 2&3; dcSTX, Decarbamoylsaxitoxin; dcNeoSTX, Decarbamoylneosaxitoxin; dcGTX2&3, Decarbamoylgonyautoxins 2&3

of the remaining cell pellet in 0.05 N acetic acid. Toxins were extracted using three cycles of freezing–thawing and ultrasonication (probe type, Branson) in an ice bath. The extraction procedure followed the methods described by Flynn and Flynn (1996) and Negri et al. (2003). Subsequently, the cell suspension was centrifuged again at 3,000 rpm for 20 min and filtered using a 0.2-µm syringe filter. The filtrate was analyzed using an HPLC-FLD (e2695; Waters, Milford, MA) coupled with a post-column reaction module. PSTs, including GC toxins, were identified using methods described by Negri et al. (2003) and Han et al. (2021), with slight modifications (Table 1).

Certificate reference materials of saxitoxin (STX), neosaxitoxin (NeoSTX), gonyautoxins 1&4 (GTX1&4), gonyautoxins 2&3 (GTX2&3), N-sulfocarbamoyl gonyautoxins 2&3 (C1&2), decarbamoylsaxitoxin (dcSTX), decarbamoylneosaxitoxin (dcNeoSTX), and decarbamoylgonyautoxins 2&3 (dcGTX2&3) were purchased from the National Research Council (NRC; Halifax, NS, Canada) and CIFGA (Lugo, Spain). Standard solutions of PSTs were diluted with 0.003 M HCl for NeoSTX, dcNeoSTX, dcSTX, and GTX1&4, 0.5 mM HCl for STX, GTX2&3, and dcGTX2&3, and 20 µM acetic acid for C1&2 to obtain the standard curves. The concentrations of these toxins were expressed in picogram per cell (pg $cell^{-1}$) for both strains, excluding the GC toxins. The GC toxin content was expressed as area per cell, and its peaks were identified based on reference materials obtained by this research team in a previous study (unpublished data) (Fig. S1).

2.7 Statistical Analysis

Statistical analyses were performed using SigmaStat (version 3.5), SigmaPlot (version 11.0), or SPSS v29.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to analyze the differences between the control and treatment groups, specifically the mean values of algal cell abundance, Chl-*a* concentration, Chl-*a* SP, and toxin content. A non-parametric Kruskal–Wallis one-way ANOVA based on ranks was performed for toxin content data that failed the tests for normal distribution and homogeneity of variances. A post-hoc analysis using the Bonferroni *t* test was conducted for all the parameters to determine the differences between Control-b and other experimental groups. Pearson's correlation coefficients were used to assess the correlation between toxin contents and biomass-related components in *G. catenatum* and *A. pacificum*.

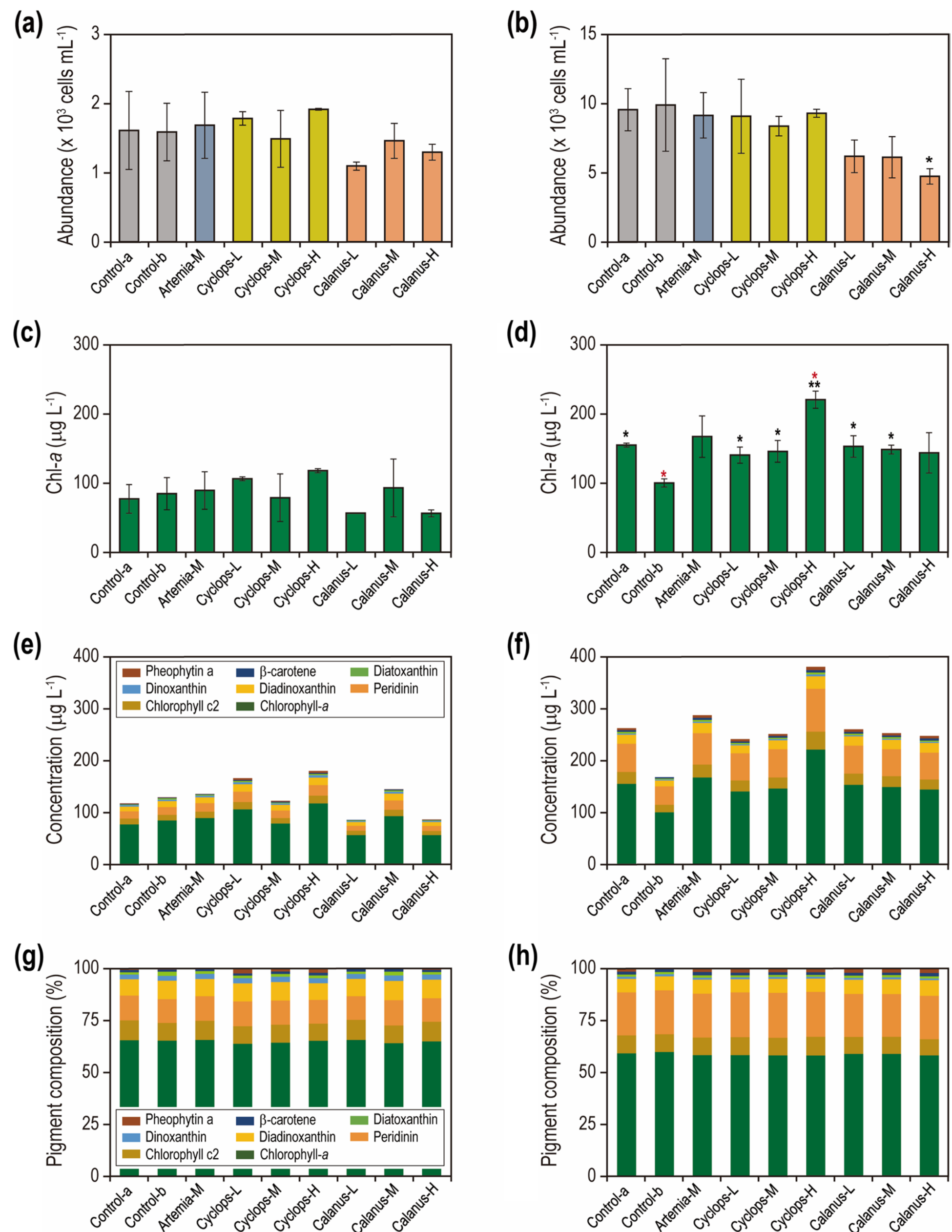

Fig. 1 Cell abundance (**a** and **b**) and chlorophyll-*a* concentration (**c** and **d**) of *Gymnodinium catenatum* (**a** and **c**) and *Alexandrium pacificum* (**b** and **d**) in the different treatments. Values are presented as the mean ± standard deviation (SD). Data were analyzed using one-way ANOVA with Bonferroni's *t* test for multiple comparisons. Black asterisks and red asterisk indicate the level of statistical significance differences between the mean values of experimental groups versus Control-b and Control-a, respectively (**$p < 0.001$, *$p < 0.05$). Pigment concentration and composition of *Gymnodinium catenatum* (**e** and **g**) and *Alexandrium pacificum* (**f** and **h**)

3 Results

3.1 Cell Abundance, Chlorophyll-*a* Concentration, and Pigment Composition

The addition of methanol alone to either strain had no influence on the cell abundance during toxin induction. No significant differences in cell abundance were observed for *G. catenatum* between the control (Control-b) and the experimental groups treated with zooplankton extracts (Fig. 1a). However, for *A. pacificum*, a significantly lower cell abundance ($p < 0.05$) was observed in the experimental group treated with the highest level of *C. finmarchicus* extract (Calanus-H) compared to the Control-b group (Figs. 1b and S2). Changes in the chlorophyll-*a* (Chl-*a*) concentration, an indicator of phytoplankton biomass, showed a pattern similar to that of cell abundance in both strains, even though there were some differences (Fig. 1c, d). No significant differences in Chl-*a* concentrations were observed between the control and experimental groups in *G. catenatum*.

In the case of *A. pacificum*, the Chl-*a* concentration was lower in Control-b than in Control-a. Although the Chl-*a* concentrations in the experimental groups did not show statistically significant differences compared to Control-a, they were significantly higher than those in

Control-b in all experimental groups, except for Cyclops-H. However, no increase in Chl-*a* concentration was observed in *A. pacificum* after the addition of methanol in further experiments (data not shown). The pigment concentrations in the control and experimental groups demonstrated patterns similar to those of Chl-*a*. The pigment compositions of *G. catenatum* and *A. pacificum* were similar (Fig. 1e–h). In addition, no differences in pigment composition were observed between the control and experimental groups for either strain. After Chl-*a*, the most abundant pigment was peridinin, followed by chlorophyll c2, and diadinoxanthin.

3.2 Incorporation of ^{13}C-Labeled Inorganic Carbon and Chlorophyll-*a*-Specific Primary Production

The carbon isotopic composition ($\delta^{13}C$) of the *G. catenatum* samples revealed no statistically significant differences between the control group (Control-b) and all experimental groups with zooplankton extracts after 48 h. In contrast, for *A. pacificum*, the experimental groups treated with copepod extracts demonstrated significantly lower $\delta^{13}C$ values compared to that in the control group (Control-b) (Fig. 2). There was no significant difference in Chl-*a* SP between the control and experimental groups for *G. catenatum*. However, for *A.*

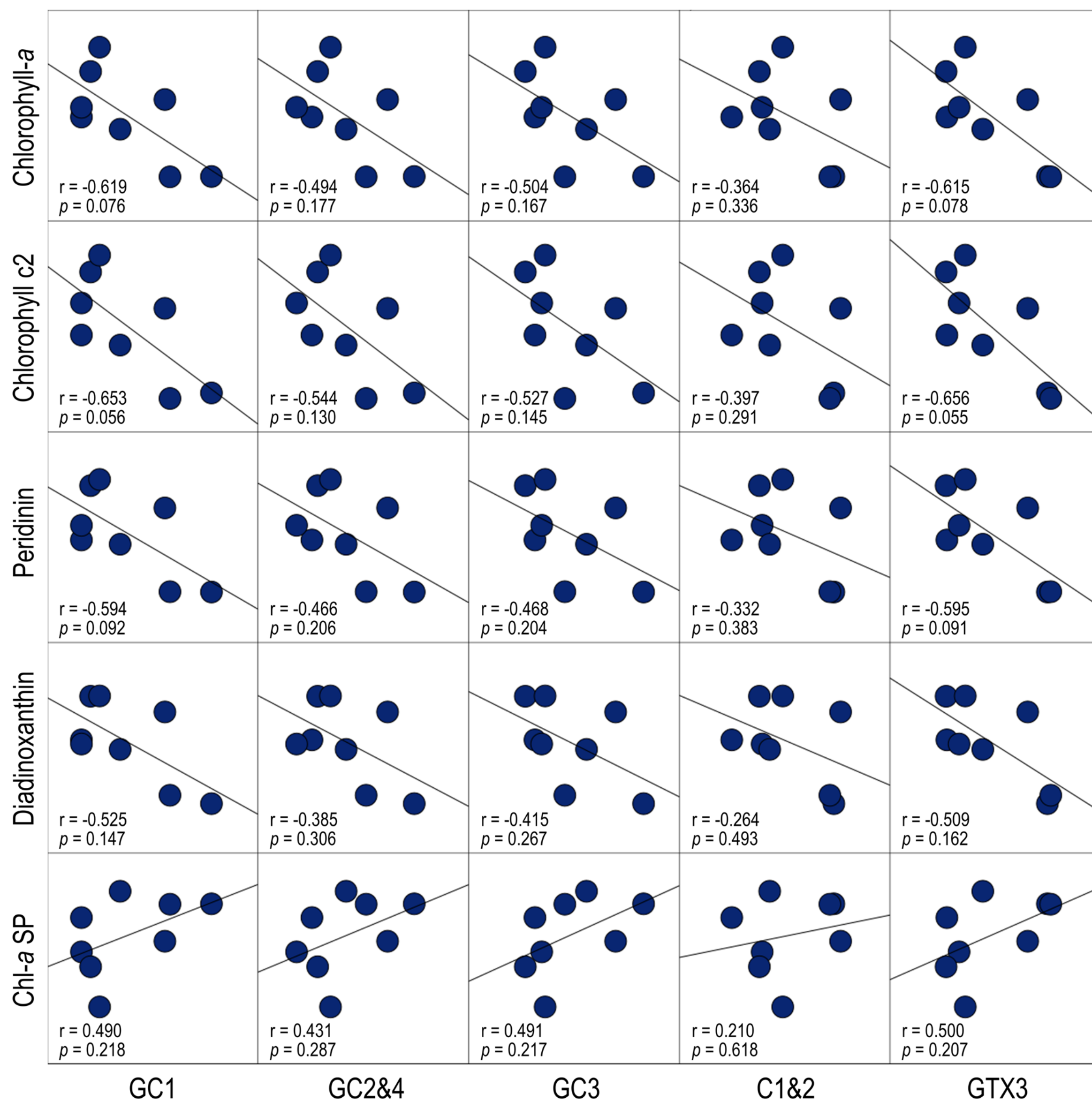

Fig. 2 Carbon isotopic composition ($\delta^{13}C$) (**a** and **b**) and chlorophyll-*a*-specific primary production (**c** and **d**) of *Gymnodinium catenatum* (**a** and **c**) and *Alexandrium pacificum* (**b** and **d**). Data were analyzed using one-way ANOVA with Bonferroni's *t* test for multiple comparisons. Asterisks indicate the level of statistical significance differences between the mean values of experimental groups versus Control-b (**$p < 0.001$, *$p < 0.05$)

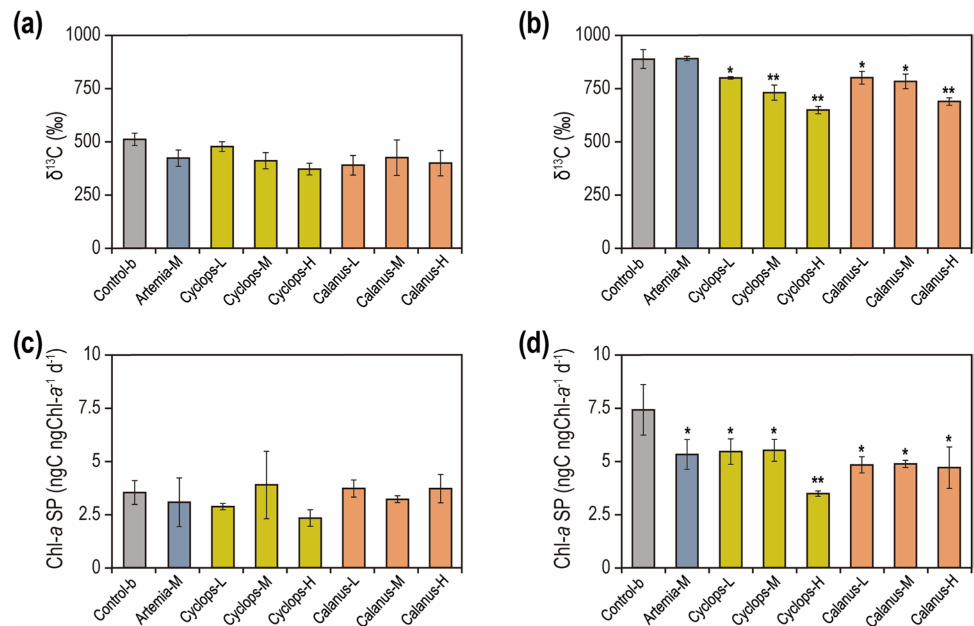

Fig. 3 Toxin content of *Gymnodinium catenatum* exposed to extracts from Artemia (*Artemia franciscana*) and copepods (*Cyclops vicinus* and *Calanus finmarchicus*) from low to high levels (L, low; M, medium; H, high). (**a**–**c**) The GC toxin contents and (**d**, **e**) the concentration of other paralytic shellfish toxins (PSTs). Values are presented as mean ± SD of cellular toxin concentrations. Data were analyzed using one-way ANOVA with Bonferroni's *t* test for multiple comparisons. Asterisks indicate the level of statistical significance differences between the mean values of experimental groups versus Control-b (**$p < 0.001$, *$p < 0.05$)

pacificum, all the experimental groups exhibited lower Chl-*a* SP values than the control group.

3.3 Paralytic Shellfish Toxins of *G. catenatum*

There was no significant difference in the PST composition between the control and experimental groups for the *G. catenatum* strain (Figs. 3 and S3). Among the quantifiable PSTs (NEO, dcNEO, dcSTX, GTX 1&4, STX, GTX 2&3, dcGTX2&3, and C1&2), C1&2 and GTX3 were detected, with C1&2 being the most abundant toxin. For the GC toxins, GC1, GC2&4, and GC3 were detected in the cultured strain.

Compared to the control, the experimental group treated with the copepod extract of *C. finmarchicus* demonstrated a 1.9- to 4.3-fold increase in the peak area per cell for GC1 and GC2&4. Although an increasing trend was observed for GC3 in the experimental groups compared with the control group, the difference was not statistically significant. The C1&2 toxins accounted for the highest proportion of PSTs, demonstrating a 2.2- to 2.3-fold increase in concentration in the experimental groups treated with *C. finmarchicus* extract, and the concentration of GTX3 increased 2.5- to 2.9-fold, compared with the control. However, higher concentrations of Calanus extract did not demonstrate a consistent increase in toxin levels per cell. The toxin content (GC1, GC2&4, GC3, C1&2, and GTX3) of *G. catenatum* was not significantly correlated with biomass-related components such as Chl-*a*, pigments, and Chl-*a* SP (Fig. 4a).

3.4 Paralytic Shellfish Toxins of *A. pacificum*

There was no significant difference in PST composition between the control and experimental groups for *A. pacificum* (Figs. 5 and S3). Among the quantifiable PSTs, C1&2, dcGTX2, and GTX4 were detected. The most abundant toxins were C1&2, similar to those in *G. catenatum*. GC toxins were not detected in any of the strains used in this experiment. The concentrations of C1&2 increased 3.8- to 5.5-fold in the experimental groups treated with *C. finmarchicus* extract compared to those in the control. The dcGTX2 toxin increased 2.4- to 2.6-fold and the GTX4 toxin increased 2.3- to 2.7-fold. Similar to the pattern observed in *G. catenatum*, toxin concentrations in *A. pacificum* did not demonstrate an increasing trend with higher concentrations of *C. finmarchicus* extract. In addition, there was no significant correlation between the toxin content in *A. pacificum* and biomass-related components (Fig. 4b).

4 Discussion

G. catenatum, *A. pacificum*, and *Alexandrium catenella* are the dominant causative species of PSTs along the Korean coast. Although there have been no reports of PST poisoning caused by *G. catenatum* in Korea (Nam et al. 2020), blooms of this species have led to human intoxication in several countries, including New Zealand, Mexico, Venezuela, Spain, and China (MacKenzie 2014; Chen 2018; Band-Schmidt et al. 2019). There is a growing concern that climate change may extend the duration and geographical range of

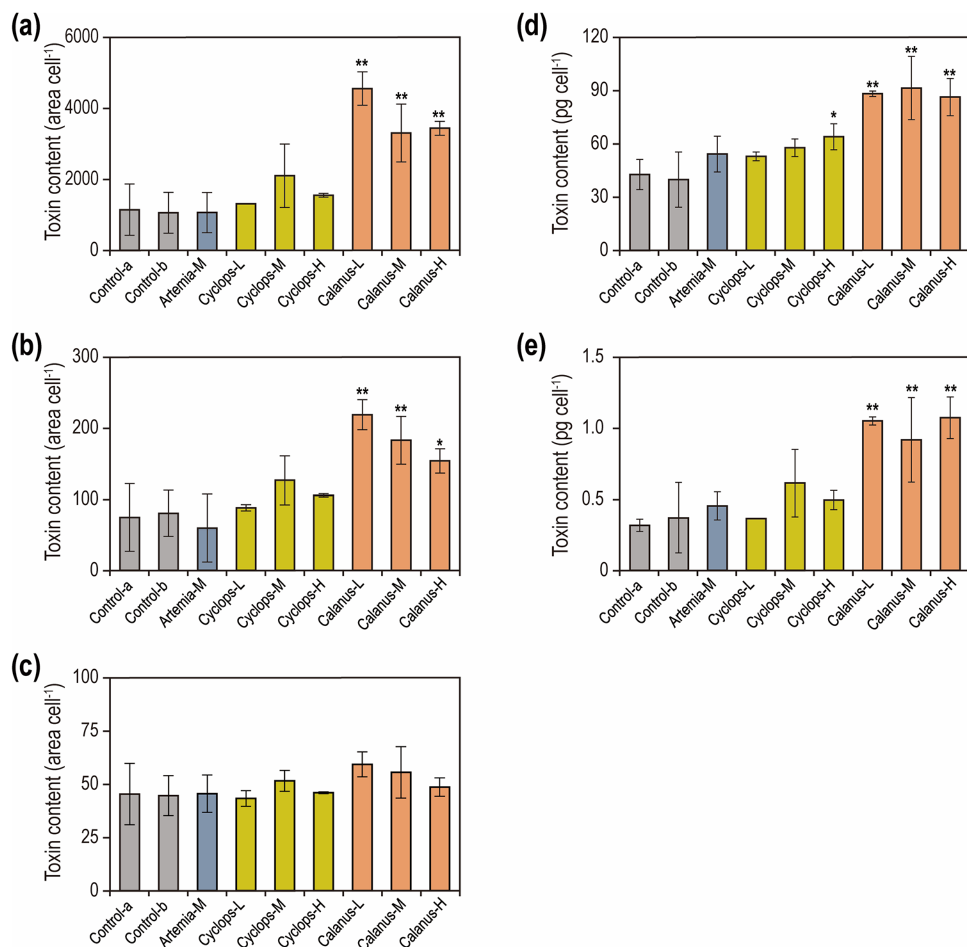

Fig. 4 Scatter plots and Pearson's correlation coefficients (*p*) for toxins versus biomass-related components including chlorophyll-*a*, major pigments (chlorophyll c2, peridinin, and diadinoxanthin), and chlorophyll-*a*-specific primary production (Chl-*a* SP) for (**a**) *Gymnodinium catenatum* and (**b**) *Alexandrium pacificum*

these blooms (Hallegraeff et al. 2012; Wang et al. 2023). In particular, while the genus *Alexandrium* is traditionally the major group causing paralytic shellfish poisoning events along the Chinese coast, there was a significant incident during 2017–2018 where a *G. catenatum* bloom resulted in 36 people being poisoned after consuming contaminated mussels (Yu and Luo 2016; Chen 2018). Given the geographical proximity, this underscores the need for increased awareness and vigilance regarding this species in Korea. In addition, since blooms of these species have been reported in locations such as the Taiwan Strait, Haizhou Bay, and Bohai Bay, it is necessary to anticipate their potential dispersal to the Korean marine ecosystem via water circulation and prepare accordingly (Zhang et al. 2020; Wang et al. 2023).

In the present study, the addition of zooplankton extract did not produce identical changes in cell abundance, pigment concentration and composition, or chlorophyll-*a*-specific primary production in *G. catenatum* and *A. pacificum*. However, the production of PSTs was strongly enhanced in both strains by the addition of the *C. finmarchicus* extract to the experimental groups. Previous studies have shown that photosynthesis is a crucial driver of PST production in toxic strains of *A. catenella*. However, higher levels of PSTs are produced in environments with phosphate deficiency, while the expression of photosynthesis-related proteins is reduced (Tse et al. 2020). This suggests that PST production in these species involves complex regulatory mechanisms involving both light-dependent and light-independent pathways. Flynn et al. (1994) reported that the relationships among pigments, chromophore amounts, and toxins in *Alexandrium minutum* were highly variable. They noted that pigment analysis might not be reliable for estimating the biomass or toxicity of harmful algae. These results are consistent with the findings of the present study; however, although the addition of zooplankton extracts resulted in inconsistent and varied effects on carbon fixation patterns between the two toxic dinoflagellate strains, the PST production was enhanced in both strains. Numerous reports have also indicated that the photosynthesis or growth rates of toxic microalgae are not necessarily correlated with their overall toxicity or toxin content (Etheridge and Roesler 2005; Band-Schmidt et al. 2014). Considering that cells may produce more toxins under stress or less-than-optimal growth conditions (Hamasaki et al. 2001; Navarro et al. 2006), it appears that substances derived from zooplankton may act as stressors for both strains. These results demonstrate the complexity of

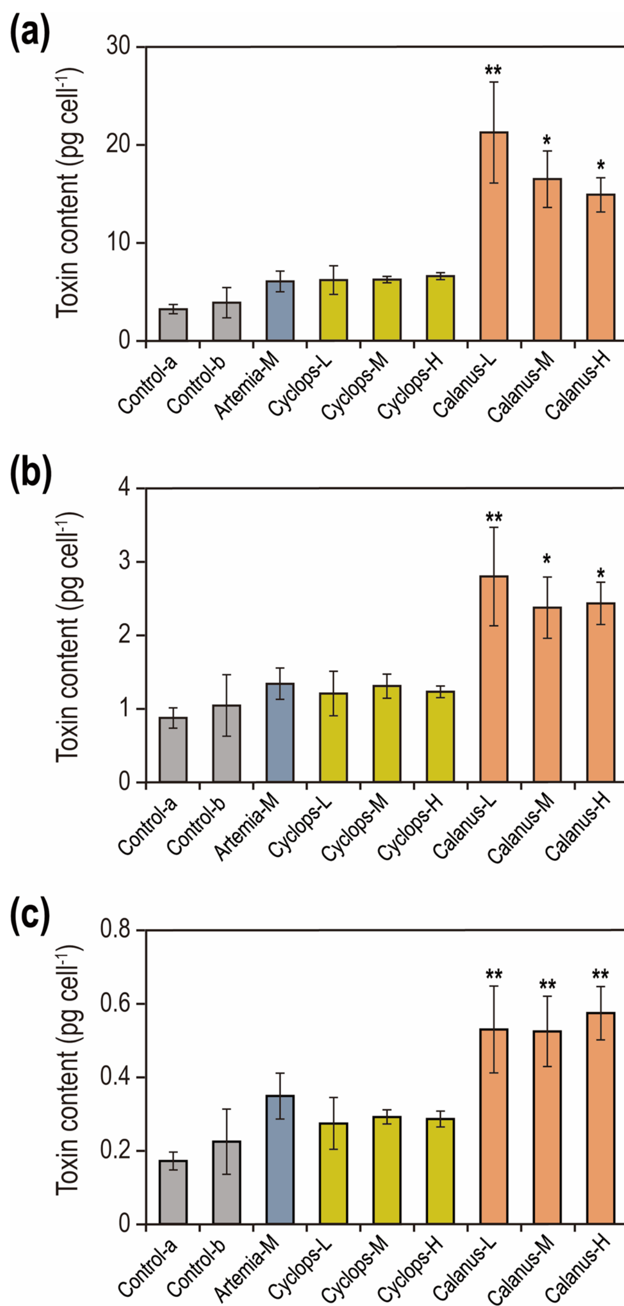

Fig. 5 PST contents of *Alexandrium pacificum* exposed to extracts from Artemia (*Artemia franciscana*) and copepods (*Cyclops vicinus* and *Calanus finmarchicus*) from low to high levels (L, low; M, medium; H, high). Values are presented as mean ± SD of cellular toxin concentrations. Data were analyzed using one-way ANOVA with Bonferroni's *t* test for multiple comparisons. For C1&2 toxins, Dunn's test was used for all pairwise comparisons versus the Control-b group using a rank-based ANOVA. Asterisks indicate the level of statistical significance differences between the mean values of experimental groups versus Control-b (**$p < 0.001$, *$p < 0.05$)

the factors that influence toxin production in harmful algal species.

The highest concentrations in the two strains used in this experiment were of C1&2 toxins among the quantifiable PSTs. These toxins have toxicity equivalency factors (TEFs) of 0.01 and 0.1 (World Health Organization 2016), respectively, which are relatively lower compared to other PSTs. However, these toxins undergo metabolic transformations in dinoflagellates and/or bivalves producing more toxic PSTs, such as GTX2, GTX5, and dcGTX2 (Leal and Cristiano 2022), which have higher TEFs. This indicates that even if these strains primarily contain toxins with lower toxicity, such as C1&2, the toxicity of these dinoflagellates can significantly increase through bioconversion reactions in the organisms. Furthermore, Vale et al. (2008) reported that C1&2 toxins can be transformed into GTX2&3 through treatments, such as pH adjustment and boiling. This suggests that chemical reactions can be utilized to convert the high concentrations of C1&2 toxins present in these strains into other types of PSTs with higher TEFs, potentially enabling the production of higher toxicity PST reference materials from the more prevalent C1&2 toxins. The Canadian NRC and CIFGA, internationally recognized producers of marine toxin CRMs, use C1&2 toxins to create other toxins such as dcSTX, GTX2, GTX3, GTX5, dcGTX2, and dcGTX3. Although C1&2 toxins, which have relatively low TEFs, comprised the highest proportion of detected PSTs in the strains used in the present study, it does not imply that their toxicity is insignificant in ecosystems or unsuitable for use as reference materials.

In the *G. catenatum* strain, C1&2 toxins as well as GC1, GC2&4, and GC3 toxins were detected. These are a new group of PSTs, known as GC toxins, which possess hydroxybenzoate R4 substituents and exhibit lipophilic features (Negri et al. 2003). It was reported that GC toxins could increase the risk of food poisoning associated with the consumption of scallop adductor muscles because GC toxins influence the anatomical distribution and transformation of PSTs in scallops (Lin et al. 2024). These findings suggest that the consideration of GC toxins is necessary when replacing the mouse bioassay method currently used for testing PSTs with instrumental analytical methods. The *G. catenatum* strain used in the present study was isolated directly from the coastal waters of Korea. The detection of GC toxins within this strain suggests that it may contribute to the dynamics of GC toxins in the Korean coastal ecosystem. Thus, this *G. catenatum* strain could be used to produce reference standards to facilitate further research and monitoring of GC toxins in the environment. In particular, the HPLC peak area representing the concentration of GC toxins increased when extracts of the marine copepod *C. finmarchicus* were added, suggesting that a predator-based approach may be beneficial for effectively producing reference standards for GC toxins that are currently commercially

unavailable. To our knowledge, this is the first report of GC toxin production stimulated by predator extracts.

This study used methanol-soluble zooplankton extracts to observe changes in toxin profiles. It was, therefore, not possible to provide specific information regarding the substances that induced the production of PSTs in *G. catenatum* and *A. pacificum*. However, considering that some studies have shown that waterborne chemical cues from copepods enhance toxin production in toxic dinoflagellates (Selander et al. 2006; Bergkvist and Selander 2008), it is plausible that methanol-soluble substances produced by copepods may have induced toxin production in the two dinoflagellates used in this study. Recent studies have reported that copepodamides, which are distinct polar lipids produced by copepods, promote toxin production in toxic dinoflagellates and diatoms (Selander et al. 2015, 2019). The *C. finmarchicus* used in the present study has been reported to produce copepodamides in other studies (Lundholm et al. 2018; Selander et al. 2019; Ryderheim et al. 2021). Based on these findings, we speculate that the copepodamides present in *C. finmarchicus* may be one of the reasons for the enhanced production of GC toxins and other PSTs observed in our study. However, for a more precise understanding, further experiments such as the quantification of copepodamides are required. There is a significant lack of understanding regarding the mechanisms of PST production in toxic dinoflagellates by zooplankton in Korean coastal waters. Further research related to this aspect is, therefore, necessary to understand the mechanisms underlying PST occurrence along the Korean coast.

Dinoflagellates produce secondary metabolites, such as toxins, for various reasons. The reported motives include inhibiting other microalgae to gain a competitive advantage, capturing prey during mixotrophic feeding, and protecting cells from predators. However, the mechanisms by which dinoflagellates produce these compounds are poorly understood (Adolf et al. 2007; Waggett et al. 2008). Dinoflagellates generally exhibit slower growth and less efficient nutrient absorption than other microalgae, such as diatoms, coccolithophores, and chlorophytes (Litchman et al. 2007). This inefficiency may place them at a disadvantage when competing with other microalgae (Verma et al. 2019). However, dinoflagellates can proliferate extensively in marine ecosystems, causing harmful algal blooms and red tides. The biological and/or ecological mechanisms enabling such proliferation remain uncertain; however, toxin production may be considered a strategic adaptation to survive competitive pressures in the environment. Furthermore, previous studies have reported that toxic dinoflagellates are more likely to be rejected by predators than non-toxic ones (Selander et al. 2006; Xu and Kiørboe 2018). This suggests that toxins play a crucial role in dinoflagellate survival. Although toxin production may positively impact the survival of dinoflagellates by reducing predation, these toxins can accumulate in organisms at higher trophic levels that consume them, potentially leading to negative impacts on humans who rely on these organisms as food sources. In some cases, it may result in death. Therefore, it is crucial to thoroughly manage marine toxins and understand their production mechanisms from both ecological and public health perspectives.

5 Conclusion

This study investigated the effects of zooplankton-derived substances on cell abundance, pigment composition, carbon assimilation, and PST production, including GC toxins, in the toxic dinoflagellates *G. catenatum* and *A. pacificum* isolated from the southern coast of Korea. No differences in pigment composition were observed with the addition of zooplankton-derived substances in either strain. However, cell abundance, pigment concentration, and Chl-*a* SP varied between strains and experimental groups. Notably, both strains exhibited a significant increase in PST production following the addition of extracts from the marine copepod *C. finmarchicus*. In particular, *G. catenatum* produced GC toxins, which do not have commercially available CRMs. These toxins were enhanced up to 4.3-fold by substances derived from *C. finmarchicus*, based on peak area. To our knowledge, this is the first study to report the stimulation of GC toxin production in *G. catenatum* by predator-derived substances. These findings indicate that, while the effects of marine copepod-derived substances on cell abundance, pigment quantity, and Chl-*a* SP in *G. catenatum* and *A. pacificum* may be inconsistent, the enhancement of PST production was consistent in both dinoflagellate strains. Understanding the interactions between toxic dinoflagellates and predators is crucial for understanding the toxin production mechanisms of dinoflagellates on the southern coast of South Korea. Moreover, these interactions could serve as valuable technical approaches for creating novel toxin reference materials.

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Data availability The data supporting the findings of this article can be obtained from the corresponding author upon request.

Declarations

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial

interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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Supplementary materials for

Effects of zooplankton extracts on the production of paralytic shellfish toxins by toxic dinoflagellates *Gymnodinium catenatum* and *Alexandrium pacificum*

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This PDF file includes:

Number of pages: 4

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Supplementary Figures

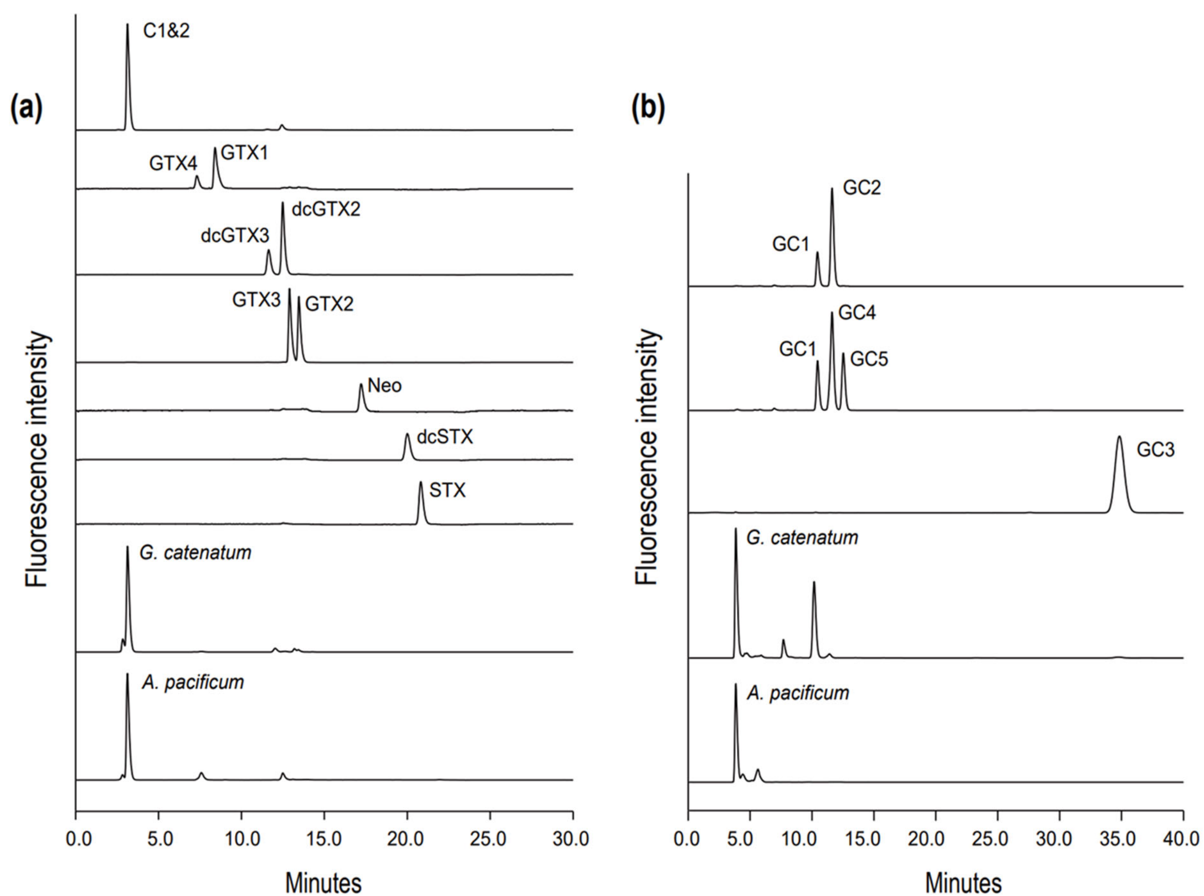

Fig. S1. HPLC-FLD chromatograms of paralytic shellfish toxins included (a) carbamoyl, N-sulfocarbamoyl, and decarbamoyl toxins, and (b) a novel group of hydroxybenzoate (GC toxins) along with reference materials and extracts from *Gymnodinium catenatum* and *Alexandrium pacificum*.

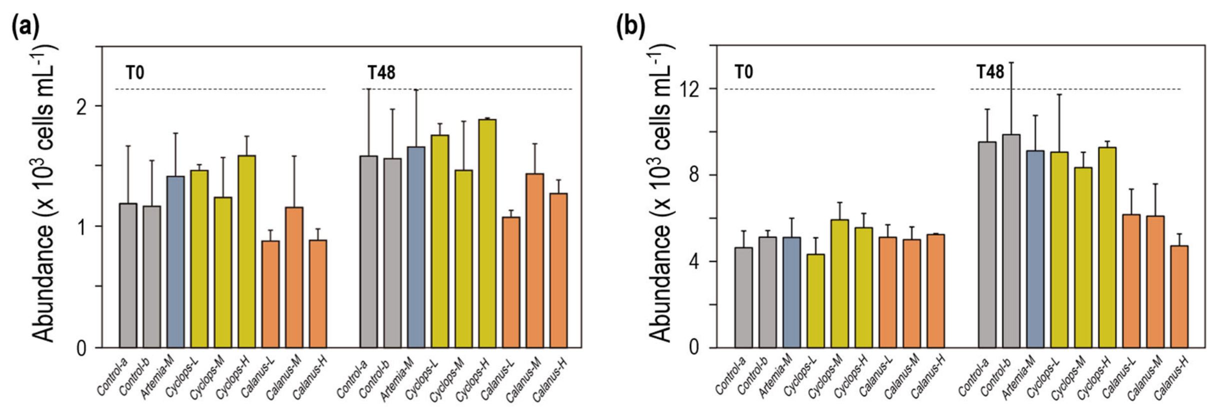

Fig. S2. Cell abundance at the beginning (T0) and end of the toxin induction experiment (T48) of (a) *Gymnodinium catenatum* and (b) *Alexandrium pacificum* in the different treatments. Values represent the mean ± standard deviation.

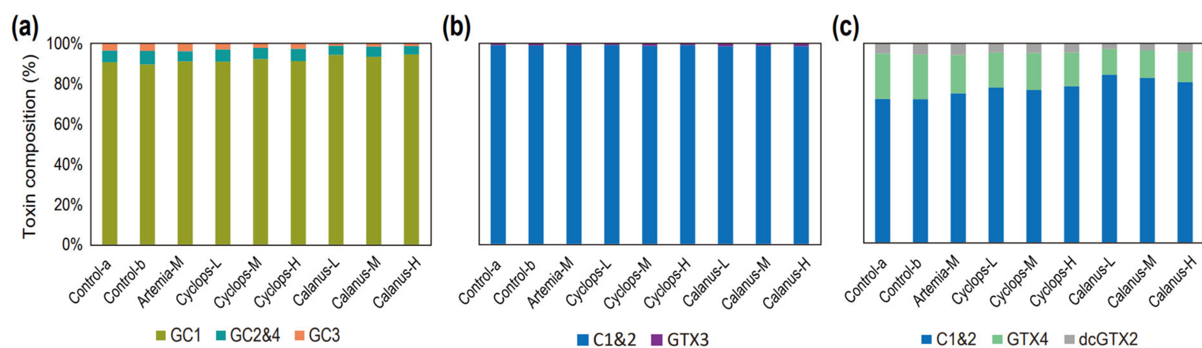

Fig. S3. (a) Toxin composition expressed as the relative HPLC peak area per cell for GC toxins in *Gymnodinium catenatum*. (b) and (c) represent the composition of paralytic shellfish toxins (mol%; carbamoyl, N-sulfocarbamoyl, and decarbamoyl toxins) in *Gymnodinium catenatum* and *Alexandrium pacificum*, respectively.