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Research Paper

Rising CO_2 will increase toxicity of marine dinoflagellate *Alexandrium minutum*

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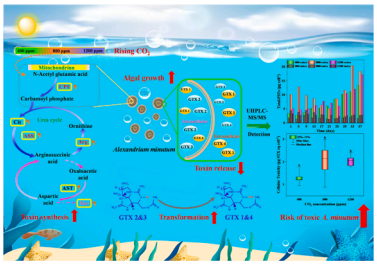
HIGHLIGHTS

- High level CO_2 significantly promoted growth of toxic *Alexandrium minutum*.
- Total yields of paralytic shellfish toxins by *A. minutum* were enhanced by rising CO_2.
- Rising CO_2 promoted transformation from GTX2&3 to more potent GTX1&4.
- High level CO_2 may depress the release of PSTs from inside to outside of the cells.
- All the processes collectively increase the risk of *A. minutum* under CO_2 enrichment.

GRAPHICAL ABSTRACT



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ABSTRACT

Ocean acidification caused by increasing emission of carbon dioxide (CO_2) is expected to have profound impacts on marine ecological processes, including the formation and evolution of harmful algal blooms (HABs). We designed a set of experiments in the laboratory to examine the effects of increasing CO_2 on the growth and toxicity of a toxic dinoflagellate *Alexandrium minutum* producing paralytic shellfish toxins (PSTs). It was found that high levels of CO_2 (800 and 1200 ppm) significantly promoted the growth of *A. minutum* compared to the group (400 ppm) representing the current CO_2 level. The total yields of PSTs by *A. minutum*, including both intracellular and extracellular toxins, were significantly enhanced, probably due to the induction of core enzyme activity and key amino acids synthesis for PST production. More interestingly, high level of CO_2 promoted the transformation from gonyautoxin2&3 to gonyautoxin1&4 and depressed the release of PSTs from inside to outside of the cells. All these processes collectively led to an apparent increase of *A. minutum* toxicity. Our study demonstrated that rising CO_2 would increase the risk of toxic *A. minutum* based on the comprehensive analyses of different processes including algal growth and toxin synthesis, transformation and release.

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1. Introduction

The global atmospheric concentration of CO_2 has increased from roughly 280 ppm in pre-industrial time to the present-day level of 380 ppm, which is mostly attributed to fossil fuel burning, deforestation and industrialization (Moore et al., 2008). Current predictions indicate that atmospheric CO_2 levels might rise beyond 800 ppm and 1200 ppm by 2100 and 2200, respectively (Caldeira and Wickett, 2003). The world's oceans, which represent a major sink of anthropogenic carbon dioxide (CO_2), have absorbed nearly 30% of the total carbon emissions since the industrial revolution, and caused a continuous decrease in seawater pH (Dupont and Portner, 2013; Zeebe et al., 2008). This process, now widely referred to as ocean acidification (OA), is expected to have potential impacts on a variety of marine life, and many laboratory and field experiments have been conducted to examine the responses of marine organisms to OA (Dutkiewicz et al., 2015; Zark et al., 2015). Apparently, OA is not the only driver affecting marine systems. Global warming and excessive nutrient enrichment (i.e., eutrophication) also pose a significant threat to water bodies worldwide (Howarth and Marino, 2006). Several studies have reported that excessive nitrogen loading from terrestrial sources can fuel eutrophication and increase the frequency and intensity of harmful algal blooms (HABs) in estuarine and coastal waters (Lee et al., 2019; Sinha et al., 2017). As one of the most important primary producers, phytoplankton is the foundation of marine food webs. Environmental changes in the sea have potential impacts on the physiological and ecological features of phytoplankton, particularly those of harmful algae capable of producing marine biotoxins (Hallegraeff, 2010).

Currently, more than 300 marine biotoxins from toxic algae have been identified, which seriously affect the health of human beings as well as marine ecosystems (Liu et al., 2021). Among them, paralytic shellfish toxins (PSTs) represent a large group of potent neurotoxic alkaloids including saxitoxin (STX) and its 57 analogs, which are primarily produced by marine dinoflagellates in the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium* (Lian and Wang, 2013). *Alexandrium* is among the most diverse dinoflagellate genera, and *Alexandrium*-associated HABs have increased in recent years. *Alexandrium* species can be found globally and lead to potential negative impacts on aquatic biota, particularly on commercially and ecologically relevant shellfish. Poisoning events or seafood contamination have been reported from arctic to tropical waters around the world (Castrec et al., 2020). Furthermore, changes in environmental conditions will not only affect blooming features of *Alexandrium* species but also their toxicity level (Anderson et al., 2012; Van de Waal et al., 2014; Xie et al., 2020). Many studies have been conducted to examine the responses of toxic *Alexandrium* species to environmental changes, such as acidification, warming and shifts in nutrient availability (Caruana et al., 2020; Hii et al., 2016; Tobin et al., 2019). Recent studies have reported that the incidence of HABs caused by toxic *Alexandrium* species will likely increase in response to increasing temperature coupled with the drop of pH value. However, the toxicity of these algae remains unpredictable (Flores-Moya et al., 2012).

Most photosynthetic organisms in the sea can benefit from an elevated level of CO_2, while increased acidity could alter transmembrane potentials, thus affecting a range of cellular processes including acid-base regulation and nutrient uptake (Riebesell et al., 2018). Dinoflagellates possessing the form II Rubisco are more sensitive to CO_2 restriction. To counteract these limitations, some dinoflagellates compartmentalize Rubisco within the chloroplast to avoid photorespiration (Fu et al., 2012). Therefore, *Alexandrium* species might profit from rising CO_2 concentrations to bloom. High CO_2 concentrations can promote the growth rate of *A. fundyense* (now renamed to *A. catenella*) from 16% to 190% (Hattenrath-Lehmann and Gobler, 2011). Many studies have also indicated that toxic *Alexandrium* species exhibit a positive growth response to increasing CO_2, including *A. minutum* (Flores-Moya et al., 2012), *A. ostenfeldii* (Kremp et al., 2012), *A. catenella* (Fu et al., 2012) and *A. fundyense* (Hattenrath-Lehmann et al., 2015). Nevertheless, increasing CO_2 levels might have minor consequences for the growth of some *Alexandrium* species, such as two *A. tamarense* strains isolated from the North Sea off the Scottish east coast (Eberlein et al., 2014; Van de Waal et al., 2014). Pang et al. (2017) reported that an *A. tamarense* strain ATDH isolated from the East China Sea showed a higher growth rate under elevated levels of pCO_2 (1000 μatm), but its cellular toxin content was reduced by half. Interestingly, the cellular toxicity of ATDH increased significantly under elevated pCO_2 levels due to an increase in the more toxic derivatives GTX1&4. Brandenburg et al. (2019) adopted a meta-analysis approach to evaluate the effects of CO_2 enrichment on the growth and toxicity of *Alexandrium*. The authors reported that CO_2-driven increases in growth rates may present an additional competitive advantage for HAB species compared to other non-HAB phytoplankton species. Toxicity responses across dinoflagellate species were also inconsistent and highly variable. Generally, the content of PSTs produced by *Alexandrium* decreased with elevated CO_2 levels. On the basis of this research, it can be concluded that CO_2-induced acidification in seawater will likely affect the physiological properties of HAB-forming *Alexandrium* species. However, different species or strains may exhibit different responses (e.g., growth and toxin production rates) to rising CO_2 levels.

One possible reason for the aforementioned species- and strain-dependent variations in toxin production in response to CO_2 enrichment may derive from the quantification of only intracellular toxins. Paralytic shellfish toxins produced by toxicogenic dinoflagellates are not only retained within the cells but also released to the environment. Consequently, the extracellular toxins should not be ignored when evaluating the changes in toxin production in response to environmental cues. Nevertheless, studies on extracellular toxins are still quite scarce due to the inherent limitations of current analytical methods. Using an HPLC-based approach, Hsieh et al. (2000) found that the PST level in the culture medium of *A. tamarense* was between 50 and 75 μg L^{-1}. Moreover, Yang et al. (2011) reported a high proportion of extracellular PSTs released from some toxigenic algae. Furthermore, one of our recent studies demonstrated that toxic *Alexandrium* could release a remarkable amount of PSTs into seawater, which varied dramatically at different growth phases (Liu et al., 2020). Extracellular toxins in the culture medium are an important fraction of the total toxins produced by toxic dinoflagellates. Therefore, future studies must account for extracellular toxins to assess the responses of toxigenic dinoflagellates more accurately to rising CO_2 levels.

Dinoflagellate *A. minutum* is among the many harmful algal species that occur naturally around the globe, from the Atlantic to the Mediterranean and subtropical Asia (Yang et al., 2011). There are currently few records of non-toxic *A. minutum* (Lewis et al., 2018). Paralytic shellfish poisoning incidents involving *A. minutum* have been reported in south Australia (Hallegraeff et al., 1988), France (Le Doux et al., 1989), Italy (Montresor et al., 1990) and China (Chou et al., 2004). Given the apparent increase in HABs around the world and the potential impacts of ocean acidification, a series of culture experiments were conducted to assess the growth and toxin production responses of *A. minutum* to rising CO_2 levels (800 ppm and 1200 ppm). In particular, the content and composition of both intracellular and extracellular toxins of *A. minutum* were investigated to explore the effects of ocean acidification on the production of PSTs, in consideration of the potential contributions of extracellular toxins often ignored in previous studies. Meanwhile, core enzyme activity and crucial amino acids for PST production were also examined. The study showcased a comprehensive approach to assess the impacts of rising CO_2 level on toxicity of toxic *Alexandrium* species, considering the effects on both growth and synthesis, transformation and release of PSTs. We found high levels of CO_2 significantly promoted algal growth and enhanced PST yield, suggesting that future ocean acidification may increase the severity of toxic HABs.

2. Materials and methods

2.1. Reagents and chemicals

Certified reference materials of gonyautoxins (GTX1, CAS number 60748-39-2; GTX4, CAS number 64296-26-0; GTX2, CAS number 60508-89-6; GTX3, CAS number 60537-65-7) were purchased from the National Research Council Canada–Institute for Marine Biosciences (Halifax, Nova Scotia, Canada). Stock solutions of GTXs were prepared in 0.1 M acetic acid aqueous solution and stored under refrigeration at 4 °C in darkness. Amino acid reference standards containing methionine (Met, CAS number 63-68-3), citrulline (Cit, CAS number 372–75–8) and arginine (Arg, CAS number 74-79-3) were obtained from Sigma-Aldrich (Darmstadt, Germany). Several enzyme-linked immunosorbent assay (ELISA, RJ21784, 48T) kits for the analysis of argininosuccinate synthetase (ASS), aspartate aminotransferase (AST) and carbamoyl phosphate synthetase (CPS) were purchased from Renjie biology technology limited company (Shanghai, China).

HPLC-grade formic acid (CAS number 64-18-6) and acetate (CAS number 64-19-7) was purchased from Macklin (Shanghai, China). LC-MS grade acetonitrile (CAS number 75-05-8) was obtained from TEDIA (Ohio, USA), and ammonium acetate (CAS number 631-61-8) was from Aladdin (Shanghai, China). Acetone (HPLC grade, CAS number 67-64-1) was supplied by Merck (Darmstadt, Germany). The ultrapure water was prepared using a Millipore purification system (Bedford, MA, USA) with a resistivity of 18.2 MΩ cm^{-1}.

2.2. Culture conditions

The dinoflagellate *A. minutum* (strain AM–1 from the South China Sea) was incubated at 20 °C in 300 mL of autoclaved natural seawater (pH 8.0 ± 0.1, salinity 31 ± 0.1, mean ± standard deviation) filtered through precombusted GF/F glass fiber filters (Whatman). Cells collected at the exponential growth phase were inoculated into 300 mL of medium with an inoculum density of 1000–1500 cells mL^{-1}. Nitrate (NO_3^-) and phosphate (PO_4^{3-}) were used as the sole source of nitrogen and phosphorus, and their initial concentrations were 485.2 ± 0.4 μM and 10.0 ± 0.3 μM (mean ± standard deviation), respectively. Vitamins and microelements were added according to f/2−Si to the culture medium (Elodie et al., 2017). Based on the reported prediction from Caldeira and Wickett (2003), three CO_2 levels were evaluated: 400 ppm (current ambient CO_2 level), 800 ppm (predicted CO_2 concentration by 2100) and 1200 ppm (predicted CO_2 concentration by 2200). These CO_2 concentrations were achieved by gentle bubbling with 0.22 μm filtered ambient air and air/CO_2 mixtures using an ecological simulation incubator (SF-ESS150, Starfish, Qingdao, China). The volume of the incubator was approximately 150 L with a 300 W power and the adjustable concentration of CO_2 was 180–2000 ppm with a variation of less than 5%. All the cultures were maintained at 20 °C under a light intensity of 54 μmol m^{-2} s^{-1} with a light/dark cycle of 12 h/12 h. All treatments were conducted independently in triplicate for 37 days.

2.3. Physiological parameters

To characterize *A. minutum* growth, one milliliter of algal culture was collected from different treatments and fixed with Lugol's solution. Afterward, 100 μL of sample was transferred to a phytoplankton counting board and cells were counted under an optical microscope (Model IX51, Olympus Corporation, Japan) at a 10 × magnification to calculate the cell density. Additionally, chlorophyll-*a* (Chl-*a*) levels were detected with a chlorophyll fluorescence analyzer (Trilogy, Turner Designs, Sunnyvale, USA).

2.4. Analysis of paralytic shellfish toxins

To analyze shellfish toxins, 300 mL of algal culture was filtered through GF/A filters to collect *A. minutum* cells. The membranes were stored at − 20 °C until required for toxin analysis. To extract the toxins, the glass-fiber membranes were cut into pieces and transferred to a centrifugal tube. A total of 10 mL of acetic acid aqueous solution (0.1 M) was then added, and the mixture was placed in an ice bath and treated with a probe sonicator for 10 min. After centrifugation (14,976 *g*, 4 °C) for 5 min, the supernatant was collected and evaporated to dryness using a rotary evaporator (Eyela N-1100, Tokyo, Japan) at 40 °C under vacuum. The residue was re-dissolved in 1 mL 0.1 M acetic acid solution.

Extracellular toxins were extracted from the culture medium using a graphitized carbon black solid-phase extraction cartridge (GCB100–060250–1, 250 mg/6 mL, NanoChrom Technologies, China) and a vacuum solid-phase extraction unit (VISIPREP, Supelco, America) as described by Liu et al. (2020).

2.5. UHPLC-MS analysis of paralytic shellfish toxins and amino acids

Toxin analysis was performed on a 1290 II ultrahigh performance liquid chromatography (UHPLC) system coupled with a G6470 triple quadrupole mass spectrometry (Agilent, USA). The analytical method was modified from a method developed by Costa et al. (2015) and Dell'Aversano et al. (2019). Separation of PSTs was achieved using a hydrophilic interaction liquid chromatography (HILIC) plus RRHD chromatographic column (ZORBAX, 3.0 mm × 100 mm, 1.8 μm, Agilent Technologies, USA), which was maintained at 25 °C. Sample injection volume was 2 μL. Mobile phase A was composed of water, 0.2% (v/v) formic acid and 5 mmol L^{-1} ammonium acetate. Mobile phase B was 90% acetonitrile aqueous solution containing 0.2% formic acid and 5 mmol L^{-1} ammonium acetate. Toxins were eluted at a flow rate of 0.4 mL min^{-1} via a normal phase gradient from 98% B to 60% B within 17 min.

The triple quadrupole MS system includes a jet steam electrospray ionization (ESI) source equipped with Agilent jet steam (AJS), ion optics, mass analyzer and detector. The parameters of the AJS-ESI source were as follows: 300 °C drying gas temperature, 8 L min^{-1} drying gas flow rate, 22 psi nebulizer pressure, 310 °C sheath gas temperature, 10 L min^{-1} sheath gas flow rate; negative: 500 V nozzle voltage and 3500 V capillary voltage; positive: 4000 V capillary voltage. Data acquisition was performed using multiple reaction monitoring mode. The $[M\text{-}H]^-$ and $[M+H]^+$ ions were selected as precursor to detect PSTs and amino acids, respectively. Secondary fragment ion with higher abundance was regarded as qualitative ion and the fragment ion with the highest abundance was chosen for quantification. The MS/MS parameters in MRM model are shown in Table S1 of Supplementary Material.

2.6. Enzyme activity assay

Enzyme activity assays were conducted using algal cells obtained by centrifuging 300 mL of culture (10,400 *g*, 4 °C) for 10 min. The supernatants were discarded, and 10 mL of room temperature phosphate-buffered saline (PBS, pH=7.3 ± 0.3, mean ± standard deviation) solution was added. The mixture was then placed in an ice bath and treated with a probe sonicator for 5 min (400 W). After centrifugation (10,400 *g*, 15 min, 4 °C), the precipitate was extracted again with the PBS solution following the same procedure. The two extracts were then combined and centrifuged again to discard the precipitate and obtain the algal extracts. The enzyme activity of the extracts was then measured following the protocols supplied with the ELISA kit manufacturer. After incubating for 60 min at 37 °C, a chromogenic agent was added and the absorbance was quantified at 450 nm using a multichannel full wavelength continuous spectrum scanning microplate (Model Spectra Max M2, Molecular Devices, USA).

2.7. Data analysis

All the data were analyzed using SPSS 25.0 and all the figures were

prepared using the Origin 2021 software (Origin Corporation, MA, USA). Differences between treatments were assessed via one-way ANOVA or Kruskal-Wallis tests. One-way ANOVA was coupled with the least significant difference (LSD) test for multiple comparative analysis. A p-value ≤ 0.05 was deemed significant and $p \leq 0.01$ was considered extremely significant. The relationship between amino acids and intracellular PSTs was established by regression analysis.

3. Results

3.1. Growth of Alexandrium minutum

The cell density and Chl-*a* content of *A. minutum* cultured under different CO_2 concentrations is shown in Fig. 1. CO_2 significantly affected *A. minutum* growth in the three experimental groups. Statistical analyses were conducted and the results of the normal distribution test and homogeneity test of variance of the cell densities and Chl-*a* are shown in Table S2. The cell density and Chl-*a* LSD results in different sampling points are summarized in Tables S3 and S4. At the two CO_2 levels evaluated herein (400 and 800 ppm), the *A. minutum* cells exhibited a similar growth curve. In contrast, the *A. minutum* cells cultured under 1200 ppm CO_2 had a clear plateau after the exponential phase, and a maximum abundance was not reached until day 30. Additionally, *A. minutum* cultured under 800 ppm CO_2 had an extended exponential growth phase, and the maximum level of algal cells (6×10^4 cells mL^{-1}) was much higher than that of the other two groups. The Chl-*a* per litre of algal culture was primarily consistent with the algal abundance. Semilogarithmic plots on cell abundances of *A. minutum* were shown in Fig. S1. Cellular Chl-*a* content decreased gradually during the growth experiment (Fig. 1-D). The 800 ppm group had a remarkably higher level of Chl-*a* than the other two groups. Besides, NO_3-N and PO_4-P were also determined during the experiments and the results are illustrated in Fig. S2.

3.2. Intracellular toxin quota and composition

The results presented that the total amount of intracellular toxins had a gradually decreasing trend before the *A. minutum* cells reached the maximum density, after which a slight increase was observed when the cell density of *A. minutum* started to decline (Fig. 2). The cellular toxins of the 800 ppm CO_2 group were higher than that of the other two groups. Additionally, intracellular toxin quantity per milliliter of algal culture increased first and then decreased slowly in the three experimental groups (Fig. S3-a). The composition of intracellular GTXs at different concentrations of CO_2 was also calculated. The proportion of GTX2&3 gradually decreased, whereas the percentage of GTX1&4 had an apparent rise during the experiment. At a CO_2 concentration of 1200 ppm, GTX2 decreased from 55% to 13%, whereas GTX1 increased from 15% to 55%. Likewise, the toxin proportion remained relatively stable at 800 ppm before the decline phase, after which GTX2 decreased by 30% with an increase in GTX4. In contrast, only slight variations in composition were observed at 400 ppm.

3.3. Extracellular toxin concentration and composition

As shown in Fig. 3, the concentration of extracellular toxins in the culture medium of *A. minutum* was relatively low during the first 9 days. After the exponential growth phase, however, the extracellular toxin

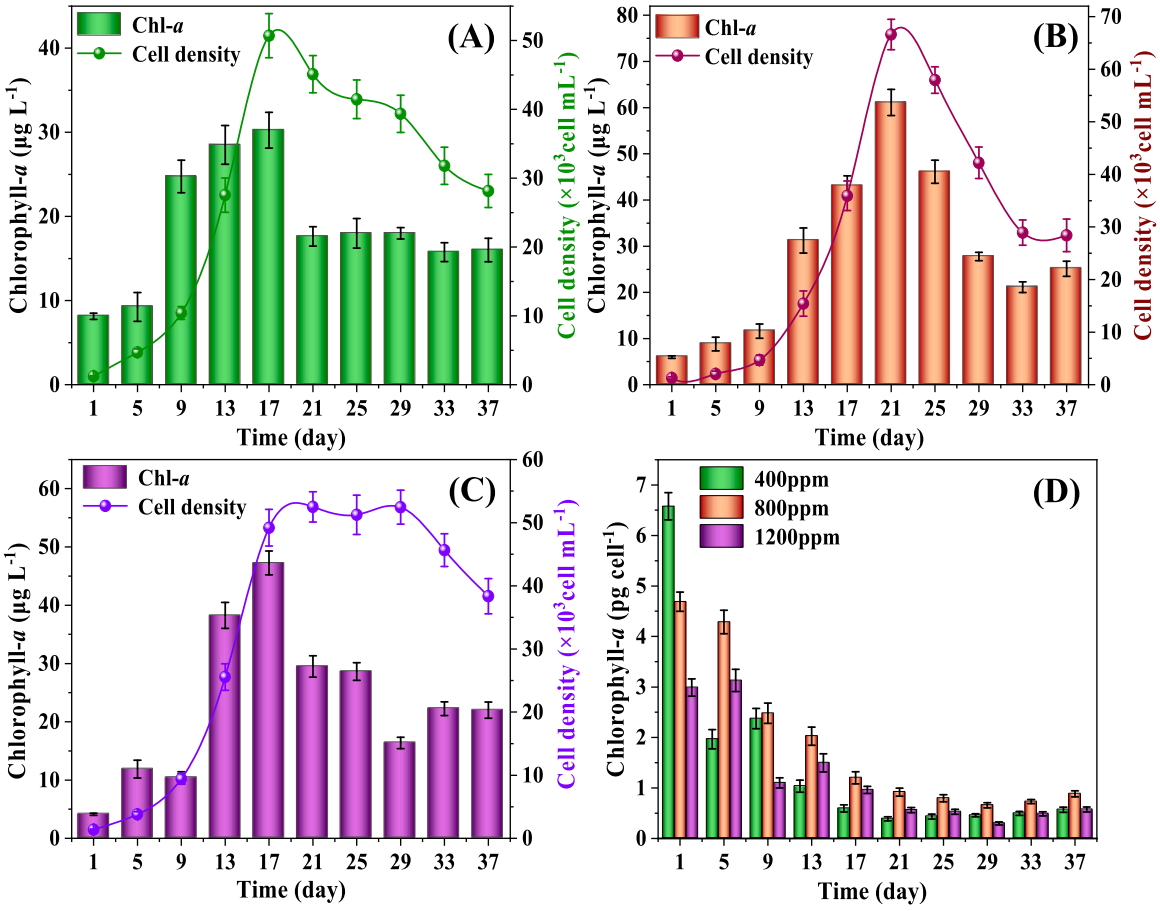

Fig. 1. Effect of different CO_2 levels on the cell density and chlorophyll-*a* content of *A. minutum*. **A**-400 ppm, **B**-800 ppm, **C**-1200 ppm, **D**-the chlorophyll-*a* content in single cell. The error bars represent the mean standard deviation of triplicate measurements.

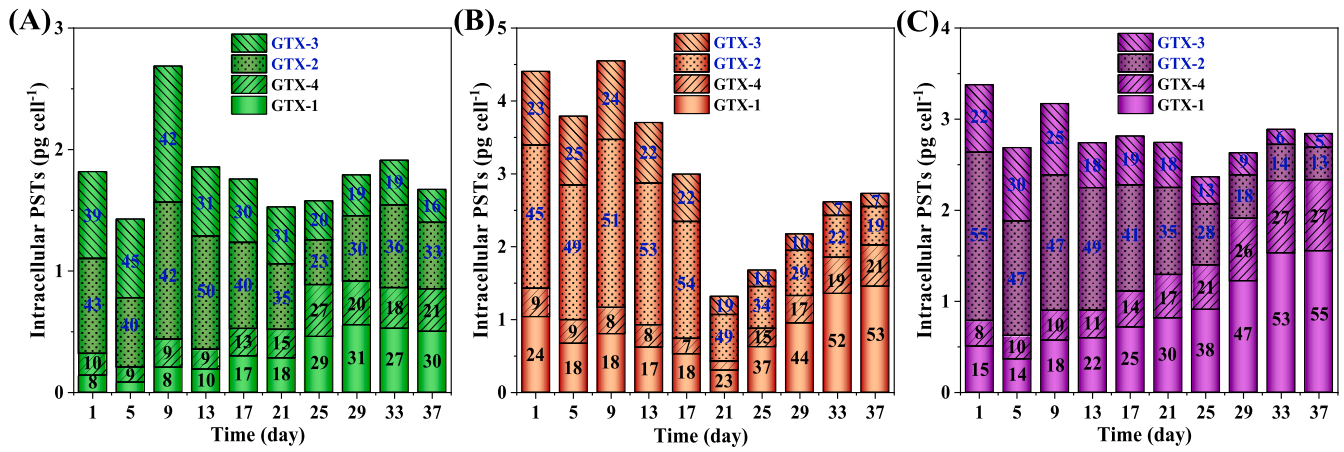

Fig. 2. The intracellular paralytic shellfish toxins (PSTs) content of *A. minutum* at different CO_2 levels. **A**-400 ppm, **B**-800 ppm, **C**-1200 ppm. The number on the cylinder represents molar fractions of gonyautoxin (GTX)1, 2, 3 and 4.

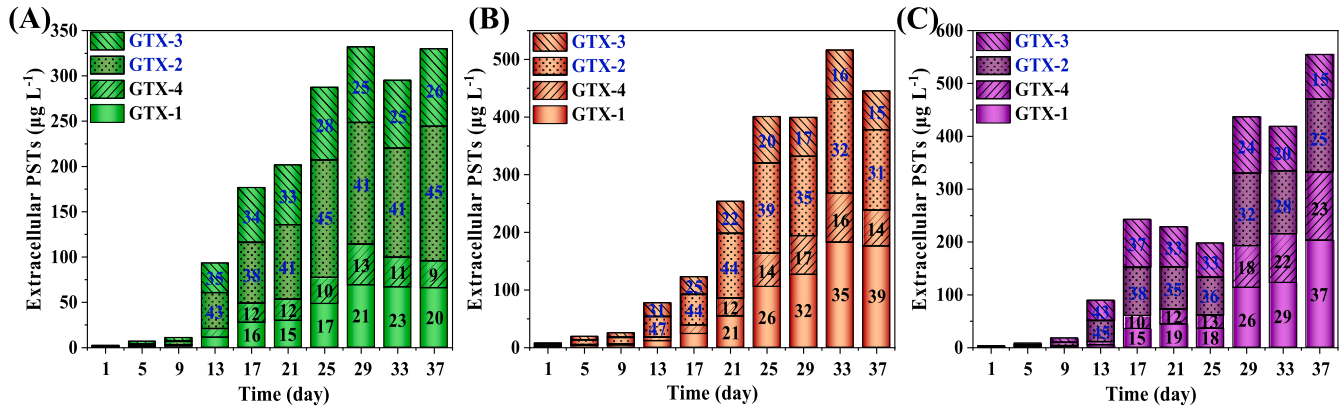

Fig. 3. Concentrations of extracellular paralytic shellfish toxins (PSTs) in the culture media of *A. minutum* at different CO_2 levels. **A**-400 ppm, **B**-800 ppm, **C**-1200 ppm. The number on the cylinder represents the molar fractions of gonyautoxin (GTX)1, 2, 3 and 4.

concentration increased remarkably. The groups cultured under 800 and 1200 ppm CO_2 exhibited higher levels of extracellular toxins than the 400 ppm group. As measured at the cell level (Fig. S3-b), the accumulated amount of extracellular toxins had a slight fluctuation before the late exponential phase but then increased remarkably after day 21. The composition of extracellular PSTs at different growth phases was further analyzed and there was a clear transformation among the four analogs of GTXs under different CO_2 conditions. Similar to the intracellular toxins, the proportion of GTX2&3 decreased gradually, whereas GTX1&4 had a remarkable increase during the entire culture cycle. As the CO_2 concentrations increased, GTX2&3 decreased, and GTX1&4 became the dominant toxin congeners.

3.4. Total yield of paralytic shellfish toxins and the ratio of extra-/intracellular toxins

To evaluate the effects of CO_2 on PST production by *A. minutum*, the total toxin yield was calculated using the data of both intracellular and extracellular toxins. As shown in Fig. 4-A, extracellular toxins represent a large proportion of toxins produced by *A. minutum*. Total toxins had two distinct tendencies during the whole culture period. Before the late exponential phase (≤ day 17), the total toxin trend was largely consistent with that of intracellular toxins. Afterward, its variation pattern was similar to that of the extracellular toxins. The enrichment of CO_2 has an apparent promoting effect on toxin synthesis, particularly at the late decline phase. Total toxins reached 664 μg L^{-1} (1200 ppm, day 37) and 592 μg L^{-1} (800 ppm, day 33) respectively, which constituted a 1.7 and 1.5-fold change compared with the 400 ppm group (Fig. S3-c).

The extracellular toxin to intracellular toxin molar ratio, hereinafter referred to as the C_e/C_i ratio, was calculated and illustrated in Fig. 4-B. Our findings indicated that C_e/C_i was lower at the first day, ranging from 0.5 to 1.2 under the three culture conditions. After the exponential growth phase, there was an obvious rise in the C_e/C_i ratio, which reached its peak at the decline phase (≥ day 25) in a range of 5.1–7.0. In this case, the C_e/C_i ratio was greater than 1, indicating that the toxins released by *A. minutum* had exceeded the level of intracellular toxins. At higher CO_2 levels, there was an apparent decrease in C_e/C_i ratio. Upon analyzing the ratios of four individual GTX components (Fig. S4), our findings indicated that the GTX2 and GTX3 components had an apparent increase in three experimental groups and achieved their highest levels (10.1 for GTX2 and 15.8 for GTX3) at the late decline phase. In contrast, the ratios for GTX1 and GTX4 were below 5 throughout the growth cycle.

To better estimate the potential toxicity of *A. minutum*, the total toxicity of each analog must first be determined. In this way, the toxicity of a mixture of PSTs can be expressed in terms of "saxitoxin equivalents (STX eq)". Total cellular toxicity was calculated using conversion factors based on the specific toxicity of each derivative reported by Oshima (1995a) and illustrated in Fig. 4-C. The 800 and 1200 ppm CO_2 groups had a remarkable influence on the cellular toxicity of *A. minutum*, which was significantly higher than that of the 400 ppm group ($p \leq 0.01$). The results of the normal distribution test, homogeneity test of variance, and Kruskal-Wallis test are summarized in Table S5.

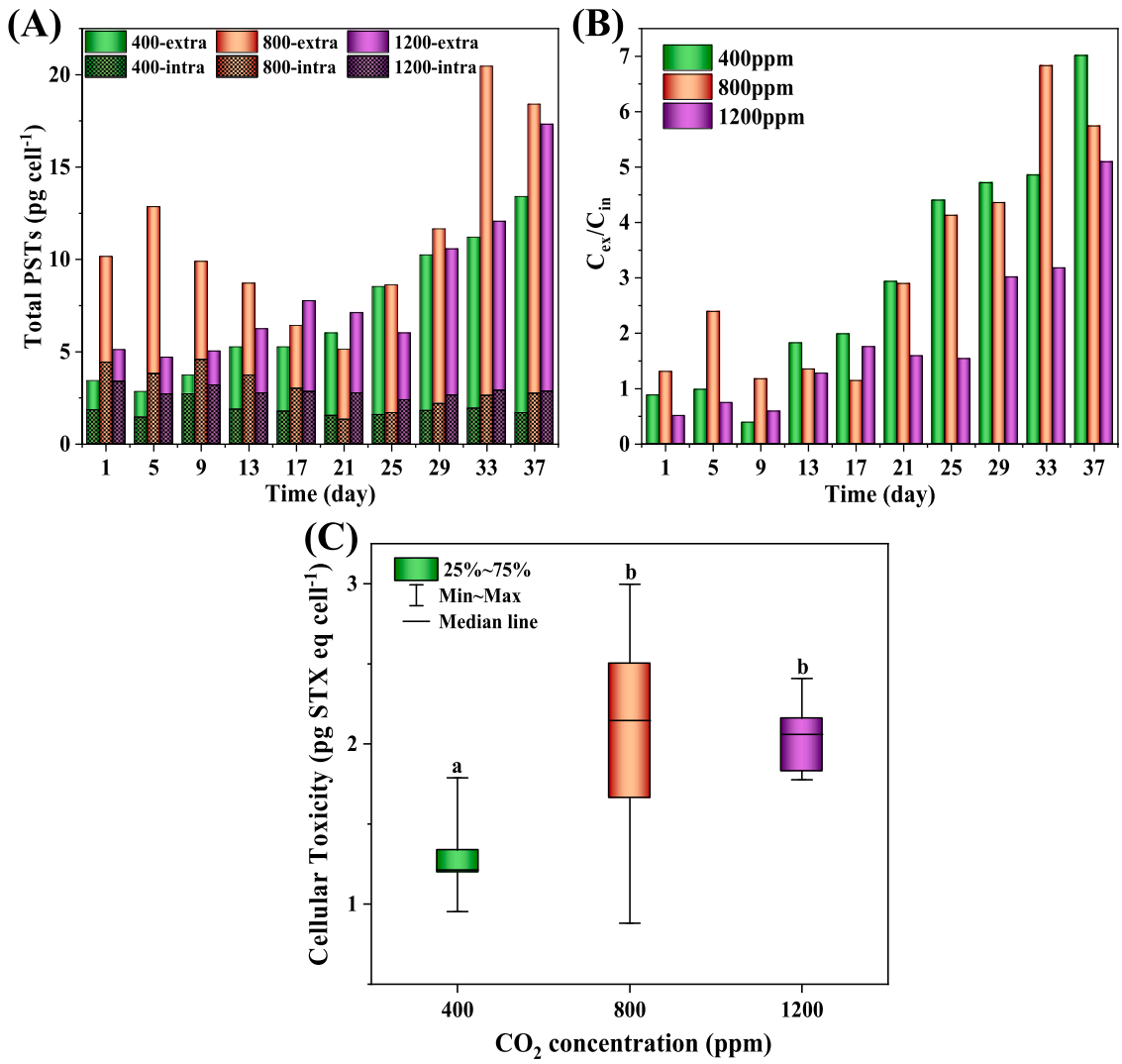

Fig. 4. The total yields of paralytic shellfish toxins (**A**), molar ratio of extracellular to intracellular toxins (**B**), and cellular toxicity (**C**) of *A. minutum* to different levels of CO_2. Statistical analysis of the data was performed using SPSS 25.0 (IBM Corp., USA) with non-parametric independent sample test (Kruskar -Wallis) followed by Bonferroni statistical tests. The line in the middle bar represents the median and different letters represent significant difference ($p \leq 0.01$).

3.5. Amino acid content and enzyme activity

Next, we analyzed the levels of crucial amino acids related to PST biosynthesis, including arginine (Arg), citrulline (Cit), and methionine (Met) (Fig. S5). In the 800 ppm group, the content of Arg and Met in *A. minutum* cells increased significantly in response to elevated CO_2 levels during the first 10 days and then decreased. The cellular content of Arg, the core amino acid for PST biosynthesis, reached levels as high as 341 pmol on the 5th day in the 800 ppm group. Among the three experimental groups, the lowest amino acid levels were observed in the 1200 ppm group.

The enzyme activities of ASS, AST and CPS were determined, and the results were shown in Fig. S6. The activities of the three enzymes at different CO_2 levels decreased significantly at the late exponential phase (days 10–15) and varied slightly after day 21. Particularly, toxin yields began to increase after day 17 and lagged behind those of enzymes and amino acids. In the 800 ppm group, the maximum levels for the activities of three enzymes were achieved on the day 10, and the enzymes of the 1200 ppm group had a much lower activity. The variations in the levels of crucial amino acids and enzymes involved in PST biosynthesis in *A. minutum* are summarized and illustrated in Fig. S7. Additionally, multiple regression analysis (MRA) was performed to develop a regression model between intracellular PSTs and different factors including three key amino acids and culture time. The PST concentration was set as the dependent variable for MRA. Some variables were removed according to the determinant coefficients of each regression model, which filtered out the causative variables in a stepwise manner. The regression equations are shown in Table S6. The results indicated that PST content was positively correlated with the interaction between Arg and Met at 400 ppm group. At high levels of 800 ppm and 1200 ppm, there was a positive relationship with the interaction between Arg and the culture time.

4. Discussion

In our study, the 800 ppm CO_2 group exhibited the highest *A. minutum* cell density, suggesting that high CO_2 concentrations promoted *A. minutum* growth. Furthermore, the levels of intracellular toxins of *A. minutum* cultured at 800 ppm and 1200 ppm were significantly higher than those at 400 ppm, particularly after the exponential phase. These results indicate that elevated CO_2 levels could promote toxin production by *A. minutum*. Regarding the proportions of toxin components, a gradual increase in GTX1&4 proportion was accompanied by a decrease in GTX2&3, and toxin composition varied primarily as a function of the different growth periods. Collectively, our findings indicated that elevated CO_2 levels remarkably changed both the content

and composition of toxins in the cells of *A. minutum*.

The rising CO_2 in seawater was advantageous to the growth of *A. minutum*, *A. catenella* and *A. tamarense* (Flores-Moya et al., 2012; Guan et al., 2018; Tatters et al., 2013). In fact, a previous study demonstrated that the photosynthetic properties of *A. tamarense* (i.e., maximal PSII quantum yield) responded positively to increasing CO_2 levels (Pang et al., 2017). In terms of toxin content and composition, different species within the genus *Alexandrium* might respond differently to rising CO_2 concentrations. Tatters et al. (2013) found that the production of PSTs would increase at a high partial pressure of CO_2, suggesting that acidification may increase the toxicity of harmful algal blooms. However, Kremp et al. (2012) found no significant differences in the content of intracellular toxins in *A. ostenfeldii* cultured at high CO_2 levels, but there were clear differences in toxin composition, particularly STX.

The regulation and efficiency of CO_2-concentrating mechanisms vary considerably in a species- and phylogenetic group-dependent manner (Ratti et al., 2007; Trimborn et al., 2008). With the rise of CO_2 concentration, these carbon-concentrating mechanisms might become active in dinoflagellates to adapt to environmental changes that could affect amino acid synthesis and enzyme activity related to toxin synthesis (Pang et al., 2017). As a precursor of PSTs, Arg is one of the most important amino acids for toxin biosynthesis, and Arg content was much higher than that of the other two amino acids examined herein. In fact, the variations in the amino acid compositions were more accentuated than the variations in the toxins themselves. Specifically, the 800 ppm CO_2 group had the most obvious impacts on the synthesis of key amino acids. Arg and Met levels increased rapidly in the first few days of the experiment. Afterward, the concentration of these amino acids decreased, which was likely due to the synthesis of other amino acids or toxins via the urea and citric acid cycles. The saxitoxin molecule is synthesized from three Arg molecules, one Met molecule, and one acetate molecule, and this process is catalyzed by a set of core enzymes. Enzymes from secondary metabolic pathways are responsible for assembling other analogs of PSTs with more complex structures. In this study, the content of CPS and ASS increased significantly in the 800 ppm group. We speculate that elevated CO_2 levels could stimulate the activity of CPS and ASS and promote the synthesis of Arg and Met, thereby indirectly increasing the toxin yield.

Due to the limitations in toxin analytical methods, most previous studies have not accounted for the levels of extracellular toxins produced by dinoflagellates. However, extracellular PSTs could represent a high proportion of the total toxins synthesized by toxic algae such as the cyanobacterium *Aphanizomenon issatschenkoii* (Dias et al., 2002). The extracellular STX levels in the culture media of *Alexandrium* spp. isolates from Sequim Bay ranged from 12 to 31 μg STX eq L^{-1}, as determined by the receptor binding assay (Lefebvre et al., 2008). The study also demonstrated that field-collected seawater contained STX levels of up to 0.8 μg STX eq L^{-1}, thus confirming that extracellular PSTs associated with toxic blooms of *Alexandrium* spp. were present in the marine environment. Our findings indicated that extracellular toxins constituted a large proportion of total PSTs, particularly at the late stage of growth. Elevated CO_2 levels can significantly promote PST yields, resulting in an apparent increase in extracellular toxin levels.

The variations in the patterns of extracellular toxin composition were similar to those of intracellular toxins, with a gradual decline in GTX2&3 and a remarkable increase in GTX1&4 throughout the culture cycle. Specifically, elevated CO_2 levels can accelerate the transition from GTX2&3 to GTX1&4. In turn, this transition might result from enzymatic oxidation or bacterial effects at the N1 position of the tetrahydropurine ring (Raposo et al., 2020). Oshima (1995b) reported the occurrence of an oxidase activity in *A. tamarense*, which could catalyze the transformation of GTX2&3 into GTX1&4. Two PST-transforming bacteria isolated from a toxic dinoflagellate species *A. minutum* and a non-toxic species *Scrippsiella trochoidea* were able to hydroxylate GTX2&3 to produce GTX1&4 (Smith et al., 2002). However, it is still unclear whether the transformation of GTX analogs results from the direct impacts of CO_2 on *A. minutum* or is mediated by associated bacteria. Importantly, we found that the overall toxicity of *A. minutum* increased significantly when exposed to rising CO_2 concentrations, which was attributed to potential changes in the synthesis, transformation, and release of PSTs. Our study established a methodological framework to comprehensively predict the impacts of rising CO_2 levels on the toxicity of toxic algae, which accounts for the combined effects on cell growth, toxin synthesis, transformation, and release processes.

5. Conclusion

In the study, we examined the growth and toxin production responses of *A. minutum* under three different concentrations of CO_2. High levels of CO_2 (800 and 1200 ppm) significantly promoted the growth of *A. minutum* compared to the group representing the current CO_2 level (400 ppm). High CO_2 levels also increased the PST yield of *A. minutum*, as demonstrated by the induction of core enzyme activity (i.e., carbamoyl phosphate synthetase and argininosuccinate synthetase) and the cellular content of key amino acids (arginine and methionine) for PST synthesis. Our experiments also indicated that high levels of CO_2 could promote the transformation from GTX2&3 to GTX1&4. All in all, these processes collectively resulted in an apparent increase in *A. minutum* toxicity, suggesting that rising CO_2 levels might exacerbate the severity of toxic *A. minutum* blooms in the future. The results of this study demonstrated the importance of extracellular toxins and supported our idea that both intracellular and extracellular toxins should be considered to assess the responses of toxic *Alexandrium* to ocean acidification.

CRediT authorship contribution statement

Ziru Lian: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Data curation, Supervision and Resources. **Fang Li**: Methodology, Investigation, Performing the experiments, Data collection and Analysis, Writing – original draft. **Xiuping He**: Performing the experiments, Scientific concept, Technical facilities, Proof reading. **Junhui Chen**: Scientific concept, Experimental guiding, Validation, Co-supervision. **Ren-Cheng Yu:** Data curation, Validation, Revising and proof reading of the entire manuscript, Co-supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.128627.

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