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Consistency between the ichthyotoxicity and allelopathy among strains and ribotypes of *Margalefidinium polykrikoides* suggests that its toxins are allelochemicals

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Harmful algal blooms (HABs) of the ichthytoxic dinoflagellate *Margalefidinium polykrikoides* have caused mass mortality of marine life around the world. While its toxic effects can impact fish, bivalves, coral, zooplankton, and even other phytoplankton, the toxin(s) and allelochemical(s) eliciting these impacts have yet to be definitely identified, leaving open the question as to whether its toxicity and allelopathic effects are caused by the same chemical agents. In this study, we investigated the ability of 10 strains of *M. polykrikoides* with different geographic origins and ribotypes to cause mortality in two strains of the dinoflagellate, *Akashiwo sanguinea* (allelopathy), and the sheepshead minnow, *Cyprinodon variegatus* (toxicity). Results showed that the potency of allelopathy against both strains of *A. sanguinea* and toxicity to the fish were significantly correlated across strains of *M. polykrikoides* ($p < 0.001$ for all). These results strongly support the notion that the major allelochemicals and toxins of *M. polykrikoides* are identical chemicals, an ecological strategy that may be more energetically efficient than the separate synthesis of toxins and allelochemicals as has been reported in other HABs. Our results also highlight the vital significance of the definitive identification of allelochemicals and toxins of *M. polykrikoides* and of the quantitative characterization of these compounds in the field where HABs of *M. polykrikoides* occur during blooms.

KEYWORDS

***Margalefidinium polykrikoides*, ichthyotoxicity, allelopathy, strain variation, toxins, allelochemicals**

Introduction

Margalefidinium (= *Cochlodinium*) *polykrikoides* is a harmful algal bloom (HAB) species that has been responsible for mass mortalities of aquatic organisms worldwide (Dorantes-Aranda et al., 2009b; Jiang et al., 2009; Jose Dorantes-Aranda et al., 2010; Kudela and Gobler, 2012; Cui et al., 2020; Basti et al., 2021). As an ichthyotoxic and HAB-causing species, *M. polykrikoides* not only has caused fish kills across North America and Asia, but also has been known to cause rapid mortality in bivalves, zooplankton, and corals (Kim et al., 1999; Dorantes-Aranda et al., 2009b; Jiang et al., 2009; Tang and Gobler, 2009b; Tang and Gobler, 2009a; Bauman et al., 2010; Tang and Gobler, 2010; Griffith et al., 2019). Beyond being toxic to marine animals, *M. polykrikoides* is also strongly allelopathic to other phytoplankton (Kim et al., 1999; Dorantes-Aranda et al., 2009b; Tang and Gobler, 2009b; Tang and Gobler, 2009a; Tang and Gobler, 2010). Still, the traits and interactions of toxicity and allelopathy and how they may facilitate *M. polykrikoides* blooms remain unclear.

Allelochemicals are chemical agents secreted by photosynthetic organisms that affect (mainly negatively) the growth, health, behavior, or population biology of organisms, mainly plants (Whittaker and Feeny, 1971). Smayda (1997) hypothesized that harmful algae form blooms by four major pathways, with two of them pertinent to allelochemically enhanced interspecific competition. Oppositely, the target species of toxicity are animals (Rice, 1979; Smayda, 1997; Legrand et al., 2003; Granéli and Hansen, 2006).

Many harmful algae can be both allelopathic to other algae and toxic to marine animals. In several cases, allelochemicals and toxins seem to be different compounds as have been demonstrated for the karlotoxin-producer *Karlodinium veneficum* (Yang et al., 2019), the paralytic shellfish toxin-producer *Alexandrium* spp. (Tillmann and John, 2002; Tillmann et al., 2007; Tillmann et al., 2008), and the brevetoxin producer *Karenia brevis* (Kubanek et al., 2005). For some algae, however, their toxicity to animals and allelopathic effects on phytoplankton appear to be caused by common mechanisms, for instance, for *Prymnesium parvum* (Singh et al., 2001; Granéli and Hansen, 2006).

The identity and mechanism of toxins and allelochemicals of *M. polykrikoides* have been the subject of debate. The toxins of *M. polykrikoides* cause damage to different cell types, including hemolysis in fish erythrocytes (Kim et al., 1999; Dorantes-Aranda et al., 2009a; Kim and Oda, 2010). The conceivable ichthyotoxic substances produced by *M. polykrikoides* have been hypothesized to include reactive oxygen species (ROS) (Tang and Gobler, 2009a), sterols, fatty acids (Giner et al., 2016), and mucopolysaccharides (Kim et al., 2002; Kim and Oda, 2010). The short-term nature of *M. polykrikoides* toxicity (minutes in the absence of live cells) and the ability of anti-oxidation compounds to mitigate its toxicity have suggested that ROS are a likely source of this HABs toxicity (Tang and Gobler, 2009b; Jiang et al., 2009). Further studies have also suggested that the existence of a synergistic action of ROS and polyunsaturated fatty acids, docosahexaenoic and eicosapentanoic, that are produced by *M. polykrikoides* may contribute to lipid peroxidation (Dorantes-Aranda et al., 2009a; Dorantes-Aranda et al., 2009b), which is associated with an increase in the solute permeability in the membrane cells, causing swelling and lysis of the vacuoles of the membrane liposomes (Girotti, 1990) and severe damage in fish gill liposomes (Kim et al., 1999). In addition, polysaccharides can exert a damaging effect on branchial cells (Dorantes-Aranda et al., 2009a; Dorantes-Aranda et al., 2009b; Kim and Oda, 2010), which may also contribute to the ichthyotoxic effects of *M. polykrikoides*. As for the allelochemicals of *M. polykrikoides*, they have been reported to affect the growth and survival of many planktonic species (Gobler et al., 2008; Mulholland et al., 2009; Tang and Gobler, 2009b; Tang and Gobler, 2009a; Richlen et al., 2010; Koch et al., 2014; Pérez-Morales et al., 2017; Hattenrath-Lehmann et al., 2019). However, the exact allelopathic mechanisms remain controversial and largely unknown. Prior investigations of *M. polykrikoides* (Kim et al., 1999; Tang and Gobler, 2010) and other allelopathy-causing species (Oda et al., 1992; Marshall et al., 2005; van Rijssel et al., 2008) have suggested that various compounds including ROS, PUFA (polyunsaturated fatty acid), and unidentified toxic metabolites may act as allelochemicals.

The goal of this study was to compare the toxic effects of *M. polykrikoides* on fish to its allelopathic effects on other phytoplankton in terms of characterizing the chemical nature of the toxin(s) and allelochemical(s) produced by the species. Given that prior research has established significant variation in the strength of allelopathy and toxicity among clonal isolates of *M. polykrikoides* (Tang and Gobler, 2010; Wang et al., 2020), we compared the ability of 10 clones isolated from the Atlantic and Pacific Oceans and representing two major ribotypes. Results demonstrated a high degree of similarity between allelochemical potency and ichthyotoxicity across the clones studied.

Materials and methods

Cultures and culturing conditions

Ten strains of *M. polykrikoides* were isolated from coastal areas of the United States, Mexico, and Japan (Table 1). The identity of all strains was confirmed with large subunit (LSU) rDNA sequencing. Two strains of *Akashiwo sanguinea* (ASNP6 and AS2) isolated from the Northport Bay, New York, USA in 19 August 2011 (Tang and Gobler, 2015) were used as the target species of allelopathic tests. All cultures were maintained in exponential phase growth in sterile GSe medium with a salinity of 31–32 made with an autoclave and 0.2 μm filtered seawater,

TABLE 1 Strains of *Margalefidinium polykrikoides* used in this study.

Strain name	Isolated area	Ribotype	Isolated time
CP1	Flanders Bay, NY	American/Malaysian	31 August 2006
CPCB10	Cotuit Bay, MA, USA, MA	American/Malaysian	September 2001
CPPV1	Bahía de La Paz, Mexico	American/Malaysian	Unknown
CPSB-1B	Shinnecock Bay, NY	American/Malaysian	5 August 2010
CPSB-1G	Shinnecock Bay, NY	American/Malaysian	5 August 2010
CPSB-2A	Shinnecock Bay, NY	American/Malaysian	5 August 2010
CPINS129	Japan	East Asian	Unknown
CPNB-3	Noyak Bay, NY	American/Malaysian	16 August 2011
CPNB-6	Noyak Bay, NY	American/Malaysian	16 August 2011
CPGSB-1	Great South Bay, NY	American/Malaysian	17 August 2011

and maintained at 21°C in an incubator with a 12-h light:12-h dark cycle providing ~100 µmol quanta m^{-2} s^{-1}. Cultures were grown with a mixture of penicillin and streptomycin (2% v/v dilution of stock with 200 U·ml^{-1} penicillin and 0.2 mg·ml^{-1} streptomycin) to discourage the growth of bacteria.

Testing the allelopathy of *M. polykrikoides*

Cultures of CPSB-1B, CPSB-1G, CP1, CPNB-3, CPNB-6, CPSB-2A, CPGSB-1, and ASNP-6 were maintained in exponential growth phase in 500-ml conical flasks in GSe medium with 2% antibiotics mixture. For experiments, 10 ml of *M. polykrikoides* and 1 ml of ASNP-6 (both at ~10^3 cells ml^{-1}) were added to one well of a six-well culture plate. One milliliter of ASNP-6 was added into 10 ml of GSe medium as control. Each treatment and control were established in triplicate. After 48 h, all samples (11 ml) were preserved with 2% Lugol's solution (final) and quantified under a light microscope. Cultures of CPSB-2A, CP1, CPSB-1B, and CPSB-1G were also co-cultured with *A. sanguinea* (ASNP-6) for 72 h to test the allelopathic effects using the same methods described above for the 48-h assay, the only difference being that six replicates were set in each group of the latter experiment ($n = 6$).

Other strains of *M. polykrikoides*, CPINS129, CPCB10, CPPV1, and CP1, were tested with *A. sanguinea* strain AS2 as target species. All strains of *M. polykrikoides* were diluted to different cell densities (650–4,000 cells ml^{-1} for CP1, 2,300–4,300 cells ml^{-1} for CPINS129, 500–1,700 cells ml^{-1} for CPCB10, and 5,300–9,900 cells ml^{-1} for CPPV1) and then co-cultured with AS2 in a six-well culture plate with 9 ml of *M. polykrikoides* and 1 ml of *A. sanguinea* culture in one well, respectively. One milliliter of AS2 with the same cell density as treatments was added to 9 ml of GSe medium as control. All the treatments and controls were in triplicate. Samples were preserved after 24 h with Lugol's solution (2% final) for subsequent cell density quantification.

To quantify and compare the allelopathic effects of *M. polykrikoides* among strains with different cell densities, a parameter, "Relative Mortality compared with control of *A. sanguinea*", was defined as: Relative Mortality (AS) = [(Mean cell density of mono-cultured *A. sanguinea* – mean cell density of *A. sanguinea* co-cultured with *M. polykrikoides*)/mean cell density of mono-cultured *A. sanguinea*] × 100%, but is called "Mortality (AS)" below for simplicity.

Testing the toxicity of *M. polykrikoides*

We used 14-day-old (0.4–0.5 cm in length) *Cyprinodon variegatus* (sheepshead minnows) for toxicity experiments. All 10 strains of *M. polykrikoides* (CP1, CPINS129, CPCB10, CPPV1, CPSB-1B, CPSB-1G, CPSB-2A, CPNB-3, CPNB-6, and CPGSB-1) were maintained in exponential growth phase and diluted to several cell densities (350–4,700 cells ml^{-1} for CP1, 1,200–4,800 cells ml^{-1} for CPINS129, 300–700 cells ml^{-1} for CPCB10, 1,500–3,000 cells ml^{-1} for CPPV1, 1,200–4,000 cells ml^{-1} for CPSB-1B, 1,800 cells ml^{-1} for CPSB-1G, 2,700–5,500 cells ml^{-1} for CPSB-2A, 450–1,700 cells ml^{-1} for CPNB-3, 1,400 cells ml^{-1} for CPNB-6, and 1,400 cells ml^{-1} for CPGSB-1) before the experiment. Fish bioassays were performed in six-well plates with 10 ml of culture and one fish in each well, and one fish was added to 10 ml of GSe medium as control. All the treatments and controls were in replicates of six ($n = 6$) and were maintained at room temperature without aeration. The survival of fishes was recorded at 24 h. Some treatments (CP1, CPSB-1B, CPSB-1G, CPSB-2A, CPNB-3, CPNB-6, and CPGSB-1) lasted for 6 days to compare the mean death time of fishes in different cultures. A probit regression analysis of cell densities of CP1, CPINS129, and CPSB-2A, and mortality of sheepshead minnows was used to determine the median lethal dose (LD_{50})

and compare the toxicity of different strains of *M. polykrikoides* when the cell densities were unequal across experiments.

Statistics

One-way ANOVAs and multiple comparison tests were used to compare the cell densities of *M. polykrikoides*, mortalities of *A. sanguinea* compared with control, and death time of sheepshead minnows among treatments using SPSS. For the toxicity experiments, G-tests were performed to assess the significance of toxic effects (Woolf, 1957). Spearman correlation coefficients were calculated to examine the correlation between the rank of allelopathy and toxicity of different *M. polykrikoides*. In all cases, the significance level was set at $p < 0.05$.

Results

Comparing allelopathic intensity of different strains of *M. polykrikoides*

During 48-h experiments with *A. sanguinea* strain ASNP-6, cell densities of seven strains of *M. polykrikoides* were similar with the exception of CPSB-1B (H), which was higher than CPSB-1G (H) (Figure 1, Table S1-2, $p = 0.001$), but this made no difference in Mortality (AS) (Table S1-1, $p = 0.55$), indicating that CPSB-1G was allelopathically stronger than CPSB-1B. CPSB-1B was more allelopathic than CPNB-6 given that CPSB-1B (L) had higher Mortality (AS) (Figure 1, Table S1-1, $p = 0.001$) but had equal cell density (Table S1-2, $p = 0.08$). CPNB-6 had a lower cell density than CP1 (Figure 1, Table S1-2, $p = 0.01$) but induced equal Mortality (AS) as CP1 (Figure 1, Table S1-1, $p = 0.68$), indicating that CPNB-6 was more allelopathic than CP1. As for CPNB-6 and CPGSB-1, the two strains exhibited no difference in cell density (Table S1-2, $p = 0.69$) but the former strain was more allelopathic (Figure 1, Table S1-1, $p = 0.01$). Similarly, CP1 and CPSB-2A exhibited no difference in cell density (Figure 1, Table S1-2, $p = 0.66$), but the former strain induced higher Mortality (AS) (Figure 1, Table S1-1, $p = 0.01$). Moreover, CPNB-3 (H), CPSB-2A, and CPGSB-1 led to roughly equal inhibition to ASNP-6 (Table S1, $p > 0.05$ for each pairwise comparison of the three strains) but decreased in order in cell density (Figure 1, Table S1, $p < 0.05$ in each pair of the three strains), indicating that the allelopathic strength was CPGSB-1 > CPSB-2A > CPNB-3. Although we cannot discern the difference in allelopathic strength of CP1 and CPGSB-1, the two strains were weaker than CPNB-6 but stronger than CPSB-2A, meaning the overall rank order of strength was CPSB-1G > CPSB-1B > CPNB-6 > CP1 ≈ CPGSB-1 > CPSB-2A > CPNB-3.

In the experiment of ASNP-6 co-cultured with CPSB-2A, CP1, CPSB-1G, and CPSB-1B for 72 h, CPSB-2A had the highest cell density and the lowest Mortality (AS) (Figure 2, $p < 0.05$, one-way ANOVA) and thus was the least allelopathic strain. CP1 had a higher cell density than CPSB-1G (Figure 2, $p = 0.001$) but showed no difference in inducing Mortality (AS) (Figure 2, $p = 0.13$). Additionally, CP1 showed no difference in cell density with CPSB-1B (Figure 2, $p = 0.09$) but induced lower Mortality (AS) (Figure 2, $p = 0.001$). Thus, CP1 ranked after CPSB-1B and CPSB-1G in allelopathic intensity. Concluding from the above results, the allelopathic intensity in decreasing order was CPSB-1G > CPSB-1B > CPNB-6 > CP1 ≈ CPGSB-1 > CPSB-2A > CPNB-3.

In the allelopathic experiment using AS2 as the target species, four *M. polykrikoides* clones, namely, CP1, CPINS129,

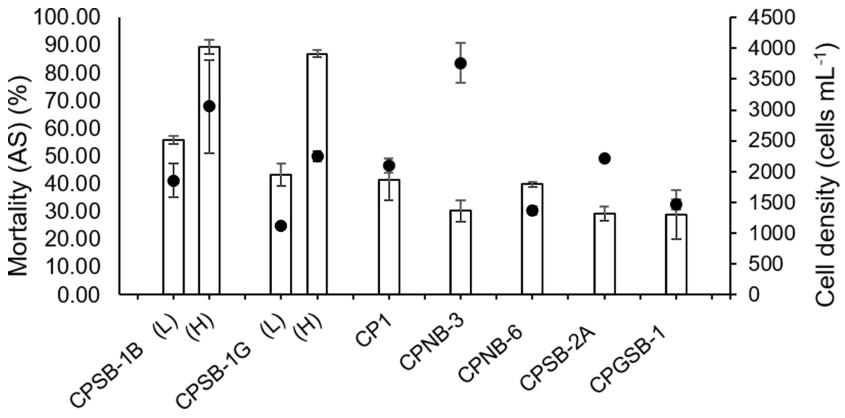

FIGURE 1
Allelopathic effects of *M. polykrikoides* on ASNP-6 in 48 h expressed as Mortality compared with control of *A. sanguinea* [Mortality (AS)]. The dots represent the cell density of different strains of *M. polykrikoides*. Each data point is the mean of triplicates ($n = 3$). Error bars indicate ±1 SD of $n = 3$.

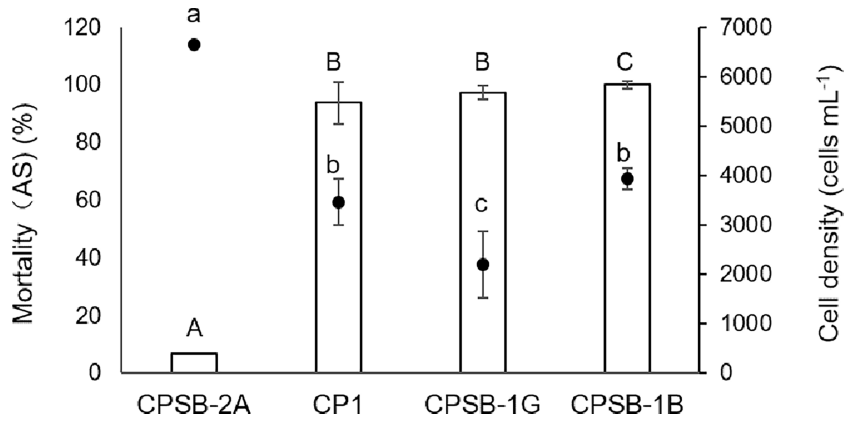

FIGURE 2
Allelopathic effects of *M. polykrikoides* on ASNP-6 in 72 h expressed as Mortality compared with control of *A. sanguinea* [Mortality (AS)]. The dots represent the cell density of different strains of *M. polykrikoides*. Each data point is the mean of sextuplicate (n = 6). Error bars indicate ±1 SD of n = 6. Different letters in uppercase indicate significant differences ($p < 0.05$) among mortalities, and different letters in lowercase indicate significant differences ($p < 0.05$) among cell densities.

CPCB10, and CPPV1, were tested (Figure 3). As shown in Mortality (AS), CPCB10 at 500 cells ml^{-1} was more potent than CP1 at 650 cells ml^{-1} (Figure 3, Table S2-1, p = 0.001) despite similar cell densities (Table S2-2, p = 0.05). Compared with CPINS129 at a cell density of 2,300 cells ml^{-1}, CP1 of 1,900 cells ml^{-1} had a lower cell density (Figure 3, Table S2-2, p = 0.001) but had a higher Mortality (AS) (Figure 3, Table S2-1, p = 0.00). In addition, CCPV1 of 8,300 cells ml^{-1} and CPINS129 of 4,300 cells ml^{-1} led to similar Mortality (AS) (Figure 3, Table S2-1, p = 0.09); however, the former had a higher cell density (Figure 3, Table S2-2, p = 0.00). As a result, the allelopathic intensity in decreasing order of the four strains was CPCB10 > CP1 > CPINS129 > CPPV1.

Comparing ichthyotoxicity among *M. polykrikoides* strains

Ten strains of *M. polykrikoides* at different cell densities were co-cultured with 14-day-old sheepshead minnows to test their toxicity (Figure 4). Four groups of *M. polykrikoides* (CPCB10 in 700 cells ml^{-1}, CPSB-1B in 1,200 cells ml^{-1}, CPNB-6 in 1,400 cells ml^{-1}, and CPSB-1G in 1,800 cells ml^{-1}) all caused 100% mortality of sheepshead minnows within 24 h (Figure 4, Table S3, $p < 0.05$ for pairwise comparisons), and the mean death time of sheepshead minnows was 1.9 h, 4.5 h, 5.4 h, and 13.1 h, respectively, suggesting that their toxic intensity decreased in the order CPCB10 > CPSB-1B > CPNB-6 > CPSB-1G.

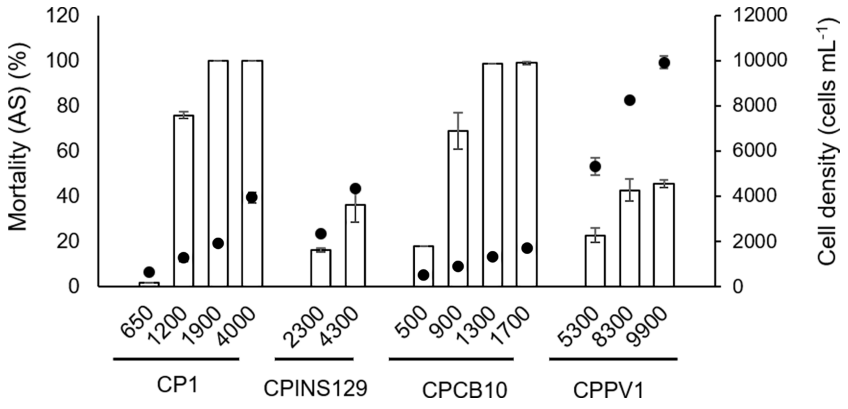

FIGURE 3
Allelopathic effects of *M. polykrikoides* on AS2 in 24 h expressed as Mortality compared with control of *A. sanguinea* [Mortality (AS)]. The dots represent the cell density of different strains of *M. polykrikoides*. Each data point is the mean of triplicates (n = 3). Error bars indicate ±1 SD of n = 3.

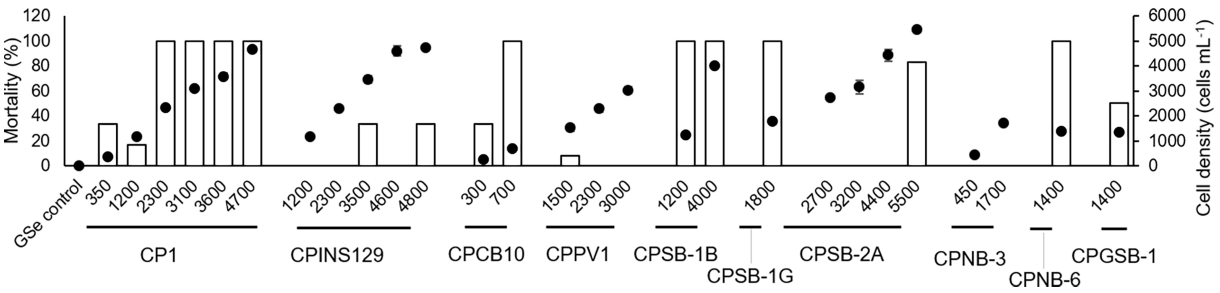

FIGURE 4
Toxic effects of *M. polykrikoides* on sheepshead minnows *Cyprinodon variegatus* in 24 h expressed as mortality (%) of sheepshead minnows (bars). The corresponding cell density of different strains of *M. polykrikoides* was also shown as dots for comparison. Error bars indicate ±1 SD of n = 6.

According to the results of regression analysis, CP1 caused 80% mortality of sheepshead minnows at a cell density of 1,874 cells ml^{-1} (Figure S1), which was lower than 100% mortality of sheepshead minnows co-cultured with 1,800 cells ml^{-1} of CPSB-1G (Figure 4). CP1 caused 60% mortality of sheepshead minnows at a cell density of 1,400 cells ml^{-1} (Figure S1), which was higher than the mortality of sheepshead minnows caused by CPGSB-1 (50%) at the same cell density (Figure 4). The LD_{50} dose of CPINS129 and CPSB-2A was 6,611 cells ml^{-1} and 5,170 cells ml^{-1}, respectively (Figure S1), indicating that CPSB-2A was more ichthyotoxic than CPINS129. Moreover, CPPV1 and CPNB-3 caused the lowest mortality of sheepshead minnows among the 10 strains (Figure 4). Conclusively, the decreasing order of toxic intensity of the 10 strains was CPCB10 > CPSB-1B > CPNB-6 > CPSB-1G > CP1> CPGSB-1 > CPSB-2A> CPINS129 > CPPV1 ≈ CPNB-3.

The death time of sheepshead minnows was also recorded in some groups (Figure 5). In groups of CPSB-2A (5,500 cells ml^{-1}), CP1 (3,100 cells ml^{-1}), CPSB-1G (1,800 cells ml^{-1}), CPNB-6 (1,400 cells ml^{-1}), and CPSB-1B (1,200 cells ml^{-1}), the death time of sheepshead minnows was less than 20 h and did not differ among the strains (Figure 5, Table S4-2, $p > 0.05$ for pairwise comparisons), and cell densities of these groups decreased in order [Figure 5, Table S4-1, $p < 0.05$ for pairwise comparisons excluding groups CPNB-6 in 1,400 cells ml^{-1} vs. CPSB-1B in 1,200 cells ml^{-1} ($p = 0.13$)], indicating that the order of ichthyotoxicity of the five strains was CPSB-1B, CPNB-6 > CPSB-1G > CP1 > CPSB-2A. The death time of sheepshead minnows in groups of CPSB-2A and CPGSB-1 showed no significant difference (Figure 5, Table S4-2, $p = 0.23$), but CPGSB-1 had a lower cell density (Figure 5, Table S4-1, $p = 0.001$), indicating that the toxicity of CPGSB-1 was greater than CPSB-2A. The death time of sheepshead minnows in groups of CPNB-3 was significantly longer than in other groups as most fishes survived the duration of the experiment (Table S4-2, $p = 0.001$), indicating that the toxicity of CPNB-3 was the weakest among the seven strains.

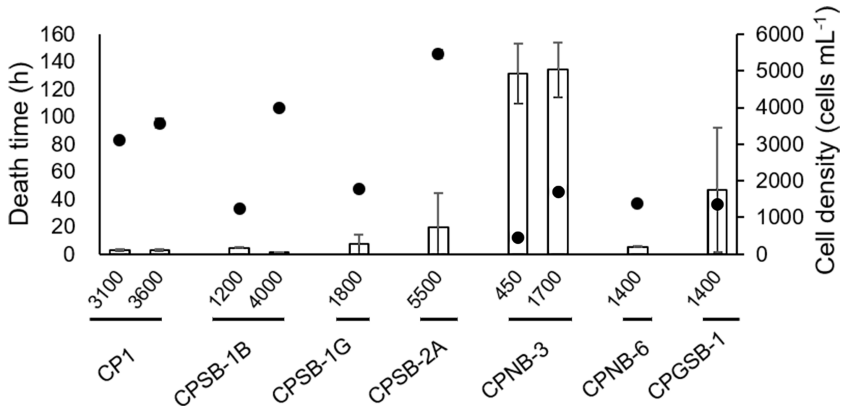

FIGURE 5
Toxic effects of *M. polykrikoides* on sheepshead minnows *Cyprinodon variegatus* expressed as death time. The dots represent the cell density of different strains of *M. polykrikoides*. Error bars indicate ±1 SD of n = 6.

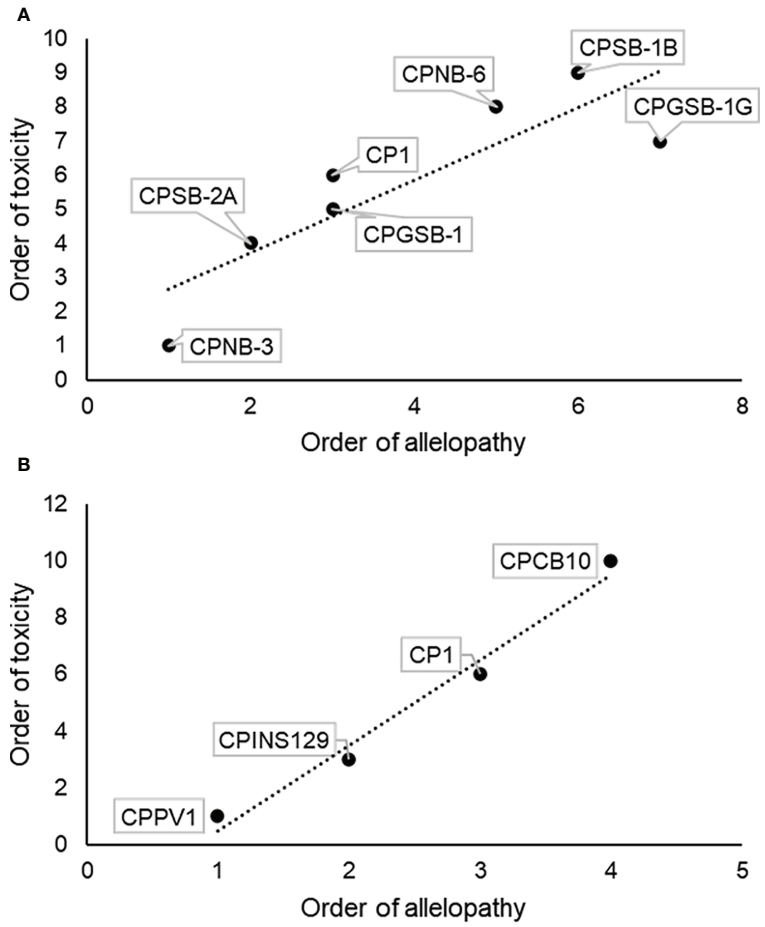

FIGURE 6
Correlation analysis of allelopathic intensity and toxic intensity of different strains of *M. polykrikoides*. **(A)** Order of allelopathic effects of *M. polykrikoides* on ASNP-6 and toxic effects of *M. polykrikoides* on sheepshead minnows. **(B)** Order of allelopathic effects of *M. polykrikoides* on AS2 and toxic effects of *M. polykrikoides* on sheepshead minnows.

Correlation of allelopathy and toxicity of *M. polykrikoides*

The allelopathic and toxic strengths of each strain were ranked according to the orders of allelopathy, which were CPSB-1G > CPSB-1B > CPNB-6 > CP1 ≈ CPGSB-1 > CPSB-2A > CPNB-3, and CPCB10 > CP1 > CPINS129 > CPPV1, and the order of toxicity, which was CPCB10 > CPSB-1B > CPNB-6 > CPSB-1G > CP1> CPGSB-1 > CPSB-2A > CPINS129 > CPPV1 ≈ CPNB-3 (Table S5), upon which the allelopathy and ichthyotoxicity of the strains were compared and found to be linearly correlated (Figure 6). Spearman's rank correlation analysis showed that the allelopathic effects and ichthyotoxicity were significantly positively correlated (Spearman's correlation coefficient = 0.88, $p = 0.01$, $n = 7$ for toxicity to sheepshead minnows and allelopathy to ASNP-6; Spearman's correlation coefficient = 1.0, $p < 0.0001$, $n = 4$ for toxicity to sheepshead minnows and allelopathy to AS2; Figure 6), illustrating consistency between the allelopathy and ichthyotoxicity of the *M. polykrikoides* strains.

Discussion

The consistency between allelopathy and toxicity among strains of *M. polykrikoides*

Margalefidinium polykrikoides strains exhibit variations in both toxicity and allelopathy (Tang and Gobler, 2010; Wang et al., 2020). By phylogenetic analysis of LSU rDNA of *M. polykrikoides* strains, at least four ribotypes have been identified globally (Iwataki et al., 2008; Reñé et al., 2013). Prior research has shown that different ribotypes of *M. polykrikoides* differ in toxicity (Wang et al., 2020), making this trait useful for providing perspective to investigate the relationships and chemical nature of allelopathy and toxicity of *M. polykrikoides*.

Here, 10 strains were studied that varied in different geographic origins, ribotypes, and isolation seasons (Table 1) but were cultured under uniform conditions and maintained in exponential growth. While differences in growth rates and maximum cell concentrations sometimes caused differences in experimental cell densities used, a gradient in cell densities was typically made for comparable treatment densities across strains. Fortunately, we obtained enough data to compare the allelopathy and toxicity between two strains, which yielded similar results and a similar order of strain potency.

Even though the intensity of allelopathy and toxicity of *M. polykrikoides* varied in different strains, especially strains of different ribotypes (Wang et al., 2020), the potencies in toxicity and allelopathy for the different strains of *M. polykrikoides* were consistent, as strains displaying potent toxicity also exhibited strong allelopathy. This consistency provides potent evidence for the hypothesis that chemical agents that are responsible for inhibitory and lethal effects on phytoplankton and marine animals, i.e., allelochemicals and toxins, were the same compounds.

Possible ecological implications of the consistency between allelopathy and toxicity

Plant secondary metabolism is a term for pathways and small-molecule products of metabolism (i.e., secondary metabolites) that are non-essential for the survival of the organism (Kossel, 1891). However, a wide variety and high diversity of secondary metabolites produced by plants are an important part of plant defense system against pathogenic attacks and environmental stresses, including toxins and allelochemicals (Yang et al., 2018). Producing secondary metabolites is thought to have a minor energetic cost (Waterman, 1992). Many harmful algae (e.g., *Alexandrium* spp., *K. brevis*, and *K. veneficum*) produce toxins and allelochemicals that are different compounds (Tillmann and John, 2002; Kubanek et al., 2005; Tillmann et al., 2007; Tillmann et al., 2008; Yang et al., 2019). In contrast, the findings of this study suggest that *M. polykrikoides* produces a singular class of compounds that inhibit competitors and potential predators (e.g., zooplankton and planktivorous fish), representing a potential energetic cost-saving for this HAB-causing species.

Expectations in identifying toxins and allelochemicals of *M. polykrikoides*

While prior studies have identified several kinds of compounds that may be the toxins made by *M. polykrikoides*, more evidence is needed to verify the actual toxicity of these substances. It has been suggested that the toxins of *M. polykrikoides* may be ROS (Kim et al., 1999; Tang and Gobler, 2009a). ROS-scavenging enzymes (peroxidase and catalase) have been shown to mitigate the toxicity and allelopathy of *M. polykrikoides* and multiple attributes of the toxicity are consistent with ROS being the toxic principle. For example, the rapidly diminished toxicity (in minutes) observed in *M. polykrikoides* cells that were freshly killed (Tang and Gobler, 2009a) was consistent with the short half-life of ROS compounds. Furthermore, *M. polykrikoides* exhibited the highest toxicity during the exponential growth phase of cultures, which aligns with reports of ROS production by actively growing, rather than stationary phase, cells of the species (Tang and Gobler, 2009a). While one study found that the O_2^- and H_2O_2 in a toxic strain of *M. polykrikoides* were at trace levels (Kim and Oda, 2010), other ROS compounds were not measured in that study. Mucopolysaccharides produced by *M. polykrikoides* may be attributed to the smothering of fishes (Kim and Oda, 2010), but no study has affirmed this finding and there were no visual signs of polysaccharides on fish during our study. Giner et al. (2016) extracted lipids of *M. polykrikoides* cells and analyzed the compositions of fatty acid and sterol in crude lipids, which consisted of a high proportion of PUFAs (47% of total fatty acids), dinosterol (40% of total sterols), and dihydrodinosterol (32% of total sterols). The identified fatty acids and sterols may contribute to long-term deleterious effects on invertebrates but were unlikely to be effective substances responsible for the acute toxicity to fish (Giner et al., 2016). In addition, according to the definition of allelopathy, allelochemicals *sensu stricto* refer to the substances that are excreted from the producing cells. Thus, the crude lipids extracted with organic solvents from cells may include many more substances than extracellular secretions. While some fatty acids with hemolytic property have also been identified in *M. polykrikoides* (Dorantes-Aranda et al., 2009a), bioassays of these substances have not been implemented and thus their toxic effects remain unknown. In addition, it has been proved that direct physical contact between test animals and algal cells is not necessary for *M. polykrikoides* to cause mortality, which means the toxins of *M. polykrikoides* could be easily released to the extracellular milieu (Tang and Gobler, 2009a).

Our finding strongly suggests that the allelochemicals and toxins of *M. polykrikoides* are the same chemical agents, which could be a cost-saving or energy-saving and thus ecologically advantageous strategy, especially so if the toxin(s) and allelochemical(s) are synthesized *via* a simple pathway. In this regard, the multiple chemical agents proposed to be responsible for the toxicity and allelopathy of the species as reviewed above (e.g., ROS, mucopolysaccharides, fatty acids, and sterols) are certainly not to be all true. It is, therefore, important to fully identify the toxins and allelochemicals of *M. polykrikoides* for the sake of both understanding the bloom ecology and mitigating the harmful effects of the species in the field.

Conclusion

We confirmed that the ichthyotoxicity and allelopathy of *M. polykrikoides* are strain specific and vary with different geographic origins and ribotypes. We further found that the order of ichthyotoxicity and allelopathy from strong to weak of

the 10 strains of *M. polykrikoides* was positively correlated. These results strongly suggest that major allelochemicals and toxins of *M. polykrikoides* are identical chemicals, which could be an energy-saving and thus ecologically advantageous strategy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

HY analyzed the data, searched the literature, and wrote the manuscript. CG supervised the research, edited the manuscript, and acquired the funding. YT designed and performed the experiments, edited the manuscript, and acquired the funding. All authors read and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.941205/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Regression curves about cell density of *M. polykrikoides* strains (CP1, CPINS129 and CPSB-2A) and mortality of sheepshead minnows by probit regression analysis.

SUPPLEMENTARY TABLE 1
Multiple comparison test of allelopathic effects of *M. polykrikoides* on ASNP-6 in 48 h.

SUPPLEMENTARY TABLE 2
Multiple comparison test of allelopathic effects of *M. polykrikoides* on AS2 in 24 h.

SUPPLEMENTARY TABLE 3
Multiple comparison test of cell densities of *M. polykrikoides* in toxic experiment co-cultured with sheepshead minnows in 24 h.

SUPPLEMENTARY TABLE 4
Multiple comparison test of 6-day toxic effects of *M. polykrikoides* on sheepshead minnows.

SUPPLEMENTARY TABLE 5
Ranks of allelopathic and toxic intensities of different *M. polykrikoides* strains.

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