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Acute toxicity of the cosmopolitan bloom-forming dinoflagellate *Akashiwo sanguinea* to finfish, shellfish, and zooplankton

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ABSTRACT: The unarmored dinoflagellate *Akashiwo sanguinea* is a well known cosmopolitan harmful alga; however, the toxic nature of this alga has yet to be examined using multiple clonal isolates. Here we examined the toxicity of 3 clonal cultures of *A. sanguinea*, including JX13 and JX14, isolated from Daya Bay, South China Sea, and AS2, isolated from Chesapeake Bay, USA, to multiple aquatic animals including species of finfish, shellfish, shrimp, and zooplankton. The whole-cell cultures of *A. sanguinea* exhibited acute lethal effects on the shrimp *Litopenaeus vannamei*, the bivalve *Meretrix meretrix,* and 2 species of fish (*Mugil cephalus* and Mugilidae sp.) with 72 h mortalities ranging from 20 to 100%. The sonicated and filtrated cultures were lethal to brine shrimp *Artemia salina*, while the filtrates of whole-cell cultures were not, suggesting that the toxins are intracellular. Boiling and freezing led to significant reductions in toxicity. *A. sanguinea* toxicity differed among the Chinese strains, and the hemolytic activity of 1 Chinese strain was 3-fold greater than that of the US strain. Cultures in exponential phase displayed stronger toxicity, and the greatest toxicity of *A. sanguinea* was observed at 20°C and a salinity of 35, conditions optimal for growth of the alga. Toxicity was enhanced by increased nutrient supply, suggesting that this species could both directly (via increased growth) and indirectly (e.g. via enhanced toxin production) become more toxic in response to eutrophication. Collectively, our findings suggest that the ability to produce and release toxin(s) may promote *A. sanguinea* blooms by suppressing predators and competitors.

KEY WORDS: *Akashiwo sanguinea* · Toxicity · Hemolytic activity · Algal toxin · Aquatic animal · Harmful algal bloom

INTRODUCTION

The unarmored (or naked) dinoflagellate *Akashiwo sanguinea* is a common phytoplankton species in estuarine and coastal waters worldwide. It is a signature bloom species in the Peruvian upwelling system (Smayda & Trainer 2010) and has caused recurrent harmful algal blooms (HABs) in Korean coastal waters for more than 30 yr (Park et al. 2013). In the last few decades, blooms of *A. sanguinea* have been increasing in geographical distribution and frequency. To date, blooms of *A. sanguinea* have been reported in North America (Horner et al. 1997, Robichaux et al. 1998, Kudela et al. 2008, Jessup et al. 2009, Du et al. 2011, Badylak et al. 2014), Brazil (Domingos & Menezes 1998), Peru (Kahru et al. 2004), the Black

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Sea (Gómez & Boicenco 2004), Ireland (O'Boyle & McDermott 2013), Korea (Lee et al. 2005, Park et al. 2013), Japan (Matsubara et al. 2007), and China (Wu et al. 2001, Hodgkiss & Lu 2004, Su 2009, Yang et al. 2012). Recently it has been established that this alga is capable of producing resting cysts that may facilitate its rapid global expansion (Tang & Gobler 2015).

Field studies have shown that blooms of *A. sanguinea* have been associated with mortalities of and harm to shellfish, finfish (Rensel & Prentice 1980, Harper & Guillen 1989, Wu et al. 2001, Kahru et al. 2004), and marine birds (Jessup et al. 2009, Du et al. 2011). For example, more than 13 million fish and blue crabs were killed during a bloom of *A. sanguinea* in Galveston, Texas, USA (Harper & Guillen 1989). A laboratory study showed that a strain of *A. sanguinea* isolated from False Bay, South Africa, caused severe mortality in abalone larvae (Botes et al. 2003). In other cases, *A. sanguinea* has been considered non-toxic, with mortalities of fish and invertebrates observed during blooms attributed to oxygen depletion resulting from bloom respiration, death, and/or decay (Horner et al. 1997, Wu et al. 2001, Kahru et al. 2004). Kim et al. (1999) found that *A. sanguinea* can produce low concentrations of reactive oxygen species that may be a source of toxicity. Recently, the stranding of marine birds during *A. sanguinea* blooms was found to be related to surfactants released by algal cells, which led to saponification of the oil in bird feathers and resulted in hypothermia and death (Jessup et al. 2009, Du et al. 2011). Considering the serious ecological damage and economic loss associated with these blooms, a clearer understanding of *A. sanguinea* toxicology is warranted, particularly since the definitive mechanism by which this species may be harmful is currently unknown (Landsberg 2002).

The first report of *A. sanguinea* bloom in China was from Sishili Bay, Yellow Sea, in 1998, which resulted in a significant loss of aquacultured shellfish (Wu et al. 2001). More recently, blooms of *A. sanguinea* have been observed in coastal waters of Zhejiang, Fujian, and Guangdong provinces occupying large areas (up to 100 km^2), with cell densities exceeding 1500 cells ml^{-1} (Cai et al. 2006, Yang et al. 2012). While blooms of *A. sanguinea* have become annual events in China (Wu et al. 2001, Cai et al. 2006, Yang et al. 2012), their possible effects on marine organisms and ecosystems have not been closely investigated. In this study, we established 2 clonal cultures of *A. sanguinea* from Daya Bay, South China Sea. We performed toxicity experiments with these isolates as well as an isolate from Chesapeake Bay, USA. Our results demonstrate that *A. sanguinea* cultures are toxic to multiple marine animals. We further investigated biotic and abiotic factors regulating the toxicity of these isolates as well as the genetic relatedness of global isolates of this species.

MATERIALS AND METHODS

Cultures, culturing conditions, and target animals

Two clonal cultures of *Akashiwo sanguinea*, JX13 and JX14, were established by pipetting single cells under an inverted microscope from samples collected from coastal water of Daya Bay, South China Sea (22.686° N, 114.557° E) on 21 April 2011. Identification of the 2 isolates was based on morphological observations and sequencing of the large subunit (LSU) rDNA (see below). Morphological characteristics were observed and photographed under a light microscope (Olympus CX41). A culture of *A. sanguinea*, strain AS2, was isolated from Chesapeake Bay (Virginia, USA) (Tang et al. 2010).

Cultures were grown in sterile f/2 medium with a salinity of 33 made with 0.2 µm filtered and autoclaved seawater at 23°C in an incubator with a 12:12 h light:dark cycle illuminated by a bank of fluorescent lights providing a light intensity of ~100 µmol m^{-2} s^{-1}. All cultures used for experiments were in mid-exponential growth phase unless otherwise noted.

Seven indigenous aquatic animals were used as targets for toxicity assays in this study including 3 finfish (*Mugil cephalus* and Mugilidae sp. from Longsheng Aquaculture Center, Zhuhai; *Chrysiptera parasema* from Huadiwan Market, Guangzhou), the bivalve *Meretrix meretrix* (from Shipai Market, Guangzhou), the whiteleg shrimp *Litopenaeus vannamei* (from Shipai Market, Guangzhou) and the brine shrimp *Artemia salina* previously hatched in the laboratory. The ages and sizes of all target animals are listed in Table 1. All test animals were maintained in filter-sterilized seawater collected from Daya Bay at room temperature (23 ± 1°C) for 24 to 48 h prior to use in experiments.

Morphological observation and phylogenetic analysis of *A. sanguinea*

Identification of 2 isolates of *A. sanguinea*, JX13 and JX14, was based on morphological observation and 28S rDNA sequences. Morphological character-

Table 1. Marine animal bioassays examining the toxicity of *Akashiwo sanguinea* strain JX14. All assays were repeated at least 3 times; data are means ± SD. p-values were determined with a *G*-test; na: not applicable

Culture	Density (cells ml^{-1})	Target animal	Age	Length (mm)	Weight (g)	Mortality in 72 h (%)	Time to death (h)	p
Whole cell	3000	*Litopenaeus vannamei*	3 mo	–	2.3 ± 0.5	100	11.1 ± 5.1	<0.01
Whole cell	3000	*Meretrix meretrix*	1 yr	–	7.4 ± 5.1	50	59.8 ± 12.2	<0.05
Whole cell	3000	Mugilidae sp.	3 mo	94.1 ± 3.8	3.6 ± 0.3	0	na	na
Whole cell	8000	Mugilidae sp.	1 mo	35.7 ± 0.8	0.1 ± 0.0	40	50.5 ± 21.5	<0.05
Whole cell	8000	*Mugil cephalus*	1–2 d	<1.5	–	22	45.5 ± 24.5	<0.05
Whole cell	8000	*Artemia salina*	2 d	0.9 ± 0.2	–	0	na	na
Sonicated	8000	*Litopenaeus vannamei*	3 mo	–	2.3 ± 0.5	100	<12.0	<0.01
Sonicated	8000	*Chrysiptera parasema*	3 mo	36.5 ± 3.5	1.0 ± 0.2	100	<23.3	<0.01
Sonicated	8000	*Artemia salina*	2 d	0.8 ± 0.2	–	91	42.0 ± 30.0	<0.01

istics were observed and photographed under a light microscope (Olympus CX41). For molecular identification, *A. sanguinea* cultures (100 ml) were concentrated at 1400 × *g* for 10 min, after which the pellet was frozen at –80°C and then thawed in a 80°C water bath for 5 min. DNA was then extracted with a UNIQ-10 plant genomic DNA prep kit following the manufacturer's instructions. A fragment of the 28S rRNA gene was amplified using the primers GymnLsuL (5′-AGG ATT CCC THA GTA ATG GCG AAT G-3′) (designed for the present study), and 28-1483R (Daugbjerg et al. 2000). PCR was conducted in 25 µl solution containing 1 U of *Taq* DNA polymerase, 2.5 µl 10× buffer (20 mmol dm^{-3} $MgCl_2$) (Takara), 1 µl dNTP (2.5 mmol dm^{-3}), 2.5 µl of each primer (10 pmol dm^{-3}), 1.5 µl DMSO, and 1 µl of genomic DNA as a template (~1.0 µg). The thermocyclic conditions for PCR included an initial cycle of denaturation at 94°C for 1 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min via Biometra UNO II. The PCR products were assessed with 2% agarose gel electrophoresis visualized by ultraviolet illumination (Bio-Rad Universal Hood II), purified with gel extraction and used as templates for DNA sequencing with the aforementioned PCR primers on a ABI3730 Genetic Analyzer using a BigDye Terminator 1 Cycle sequencing kit (Applied Biosystems) at Beijing Genomic Institute. Phylogenetic analysis was based on the partial 28S rRNA gene sequence.

Sequencing chromatograms were manually evaluated and assembled in BioEdit7.2.5 (Hall 1999). Homologous sequences were retrieved from GenBank. The collected rDNA sequences were aligned using Clustal 2.1 (Larkin et al. 2007) and trimmed to 1292 bp. Bayesian analysis was performed in MrBayes 3.2 (Ronquist et al. 2012) for 3000000 generations by running 4 independent chains with the GTR substitution model, sampled every 100 generations, with the consensus tree drawn using the last 22500 samples after the first 7500 samples (25%) were discarded as burn-in, using *Cochlodinium* as the outgroup. A neighbor-joining (NJ) tree was also constructed with the Kimura 2-parameter model by using MEGA6.06 (Tamura et al. 2013) with *Cochlodinium* as the outgroup, and 1000 bootstrap replicates for each sequence were made to estimate the confidence of the branches in the phylogenetic tree.

Experiment 1: toxic effects of *A. sanguinea* on multiple aquatic animals

To examine the toxic effects of *A. sanguinea*, the above-mentioned 7 marine animals were exposed to *A. sanguinea* JX14 at different cell densities (triplicates for each) for 72 h under the conditions used for maintaining cultures. Fresh sterile f/2 medium was used as a control. Dead animals were removed immediately during the experiments. Daily measurements of dissolved oxygen (DO% of the saturated concentration) and pH of treatments and controls were carried out daily or immediately after animals died.

Whole-cell experiments

Mugil cephalus, Mugilidae sp. (1 mo old), Mugilidae sp. (3 mo old), *Meretrix meretrix*, *L. vannamei* (3 mo old), or *Artemia salina* were co-cultured with *Akashiwo sanguinea* at initial cell densities of 3000 or 8000 cells ml^{-1}, respectively (Table 1). Experiments for *M. cephalus* and *A. salina* were established in 6-well culture plates (Nunc) with each well con-

taining 5 ml culture of JX14 (or culture medium as control) and 3 fish (or 15 brine shrimp). Experiments for *M. meretrix* and Mugilidae sp. (1 mo old) were performed in 50 ml glass beakers containing 40 ml JX14 culture (or medium) and 1 clam or fish in each beaker. Experiments for *L. vannamei* were set up in 3 l beakers containing 2 l of JX14 culture and 3 shrimp in each beaker. Mugilidae sp. (3 mo old) was examined in 5 l beakers containing 3.5 l of JX14 culture (or medium) and 3 fish in each beaker. Parallel control vessels for all experiments were established with animals placed in culture medium only. All control and treatment vessels were established in triplicate.

Sonicated-cell experiments

A. sanguinea JX14 cultures in mid-exponential growth phase were sonicated with an ultrasonic processor (JYD-900L) for 30 min on ice and examined under a microscope to ensure all cells were broken. The test animals *A. salina*, *L. vannamei*, and *C. parasema* were then transferred into the sonicated cultures of *A. sanguinea* with initial cell densities of about 8000 cells ml^{-1}, respectively. *A. salina* was tested in 6-well culture plates (Nunc), with each well containing 5 ml sonicated culture and 45 brine shrimp, while *L. vannamei* and *C. parasema* were tested in 50 ml beakers with 40 ml sonicated culture and 1 shrimp or fish in each beaker. Parallel control vessels for all experiments were established with animals placed in culture medium only. All control and treatment vessels were established in triplicate.

Experiment 2: variations in *A. sanguinea* toxicity among strains and growth phases

To determine whether the toxic effects of *A. sanguinea* are unique to strain JX14, the toxicity of 3 strains of *A. sanguinea* (JX13, JX14, and AS2) were compared. Cultures were diluted using the culture media to concentrations of 500, 1000, 2000, 4000, and 8000 cells ml^{-1}. Cultures were then sonicated with the ultrasonic processor for 30 min on ice to assure cells were lysed. Experiments were performed in 6-well culture plates, with each well containing 5 ml of sonicated cultures and 15 brine shrimp (triplicates for each treatment) for 72 h under the same condition used for maintaining cultures. Sterile f/2 medium instead of *A. sanguinea* culture was used as the control.

To further explore the toxic mechanisms of *A. sanguinea* and the variation in toxicity among different strains, we performed a hemolytic assay with cultured rabbit blood cells to compare the toxicity of *A. sanguinea* JX14 to AS2. One liter culture of JX14 or AS2 at exponential growth phase (initial cell density: 6480 and 7140 cells ml^{-1}, respectively) were used for the extraction of hemolytic substances. Procedures for hemolytic substance extraction and the erythrocyte hemolysis test followed the methods of Peng et al. (2005). A calibration curve based on digitonin concentrations and the corresponding hemolytic activities was used as a reference for *A. sanguinea*, and the hemolytic activity was expressed as digitonin equivalents.

The effects of algal cultures within different growth stages on toxicity were assessed with *A. sanguinea* JX14 cultures in different growth phases (lag, exponential, stationary, and decline phase, 1, 4, 7, and 11 d after inoculation, respectively) and brine shrimp *A. salina*. Experiments were conducted with the same procedures as described above.

Experiment 3: toxicity of different fractions of *A. sanguinea* cultures

To better understand the nature of *A. sanguinea* toxicity, experiments were performed with different fractions of *A. sanguinea* cultures. Cultures of *A. sanguinea* JX14 were first diluted with the corresponding culture filtrates to create a concentration gradient of cell densities: 500, 1000, 2000, 4000, and 8000 cells ml^{-1}. Next, 3 treatments were applied to each concentration in triplicate. For the first set, cultures were sonicated as described above and filtered using 0.22 or 0.45 µm membranes, respectively. For the second and third sets, cultures were either frozen at −80°C for 2.5 h and then sonicated, or boiled for 15 min and cooled to the room temperature for the experiments.

Since brine shrimp were highly sensitive to *A. sanguinea* during the initial experiments and have been commonly used in toxicological experiments of HAB species (Marcoval et al. 2013), they were used as a model organism in this study. Experiments were performed in 6-well culture plates with each well containing 5 ml of *A. sanguinea* culture or culture fractions prepared above and 15 brine shrimp (triplicates for each treatment) for 72 h under the same condition used for maintaining cultures. Sterile f/2 medium and unfiltered sonicated cultures were used as negative and positive controls, respectively.

Experiment 4: environmental factors affecting toxicity of *A. sanguinea* cultures

To elucidate the effects of environmental factors on the toxicity of *A. sanguinea*, different temperatures (15, 20, 25°C) and different salinities (15, 25, 35) were set up for laboratory cultures. Before conducting the experiments, *A. sanguinea* JX14 was acclimated to the corresponding temperatures or salinities for at least 3 transfers. Cultures in exponential growth phase were diluted in a concentration gradient of 500, 1000, 2000, 4000, and 8000 cells ml^{-1} using sterile medium and then sonicated with an ultrasonic processor. The toxicity of sonicated cultures was evaluated with brine shrimp as described above.

Effects of nitrogen concentrations on the toxicity of *A. sanguinea* was evaluated with *A. sanguinea* culture grown under low (0.059 mg N l^{-1}), moderate (0.559 mg N l^{-1}), and high (12.4 mg N l^{-1}) nitrate levels. Other nutrient components followed f/2 medium concentrations. Before the experiments, *A. sanguinea* JX14 was acclimated to different nitrogen concentrations for at least 3 transfers. Cultures in mid-exponential growth phase were diluted in a concentration gradient of 500, 1000, 2000, 4000, and 8000 cells ml^{-1} using sterile medium. The cultures were then sonicated and the toxicity was tested with brine shrimp following the same procedure as above.

Effects of the presence of other phytoplankton on the algal toxicity were also assessed. *A. sanguinea* JX14 (initial cell density: 5320 cells ml^{-1}) was co-cultured with the diatom *Skeletonema costatum* (initial cell densities: 3.3×10^2, 3.3×10^3, 3.3×10^4, and 3.3×10^5 cells ml^{-1}) for 24 h, and the mixtures were then sonicated. The toxicity tests were performed using brine shrimp following the same procedure as described above.

Statistics

Mortalities of target animals were calculated with Eq. (1):

$$M = (N_{control} - N_{survival})/N_{control} \quad (1)$$

where M is the mortality, $N_{control}$ is the number of live individuals in controls, and $N_{survival}$ is the number of live individuals in treatments.

For marine animal bioassays, a goodness of fit test (*G*-test) was used to statistically assess the effect of treatments on the survival of test animals, as this is a robust test for analysis of differences in percent survivorship among treatments (Sokal & Rohlf 1995).

In dilution experiments, the mortalities of target animals followed a sigmoidal pattern when plotted against log-transformed *A. sanguinea* cell densities. Estimates of LC_{50}, i.e. the cell density of *A. sanguinea* yielding a 50% mortality in target animals, were determined by fitting the data points to Eq. (2) using the non-linear fit procedure of Origin 7.5 for Windows (modified from Tillmann et al. 2008):

$$M = 1 - \frac{1}{1 + (x / LC_{50})^h} \quad (2)$$

where M is the mortality of *A. salina*, x is the log-transformed cell concentration of *A. sanguinea*, and LC_{50} and h are fit parameters. Results are expressed as LC_{50} (cells ml^{-1}) including 95% confidence intervals. The significance of differences among treatments (including controls) was tested with 2-way ANOVA and Tukey's HSD post hoc tests.

RESULTS

Confirmation of identification of *Akashiwo sanguinea* based on phylogenetic analyses

The Bayesian tree (Fig. 1A) and NJ tree (Fig. 1B) demonstrated that all isolates of *A. sanguinea* were clustered into a monophyletic clade with 3 geographically defined subclades: East Asia (including the 2 isolates JX13 and JX14 from Daya Bay, South China Sea, GenBank accession numbers KF793277 and KF793278, respectively), USA (including isolate AS2, GenBank accession number KJ655530, and an entity from Bermuda, DQ779988), and Singapore, respectively. While the genetic distance deduced from the K2P model within the 3 subclades ranged from 0.000 to 0.001, the genetic distances among the 3 groups ranged from 0.017 to 0.029, i.e. the latter is much larger than the former, which confirmed the existence of 3 geographically distinct subclades.

Toxic effects of *A. sanguinea* on multiple aquatic animals

A. sanguinea cultures caused extensive mortalities in 6 target aquatic animals including 3 finfishes, i.e. *Mugil cephalus*, Mugilidae sp., and *Chrysiptera parasema*, the shellfish *Meretrix meretrix*, the shrimp *Litopenaeus vannamei*, and the brine shrimp *Artemia salina* (Table 1). In whole-cell experiments, *L. vannamei* was the most sensitive animal, experiencing 100% mortality in 24 h and a

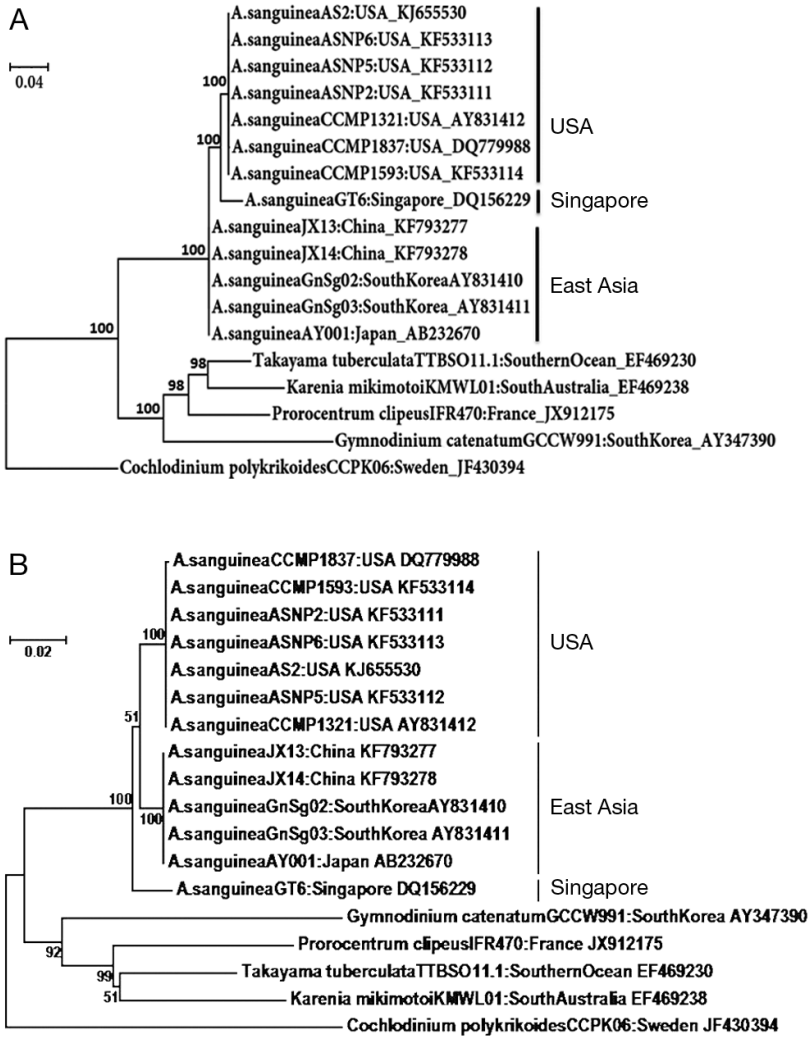

Fig. 1. Phylogenetic trees based on partial 28S ribosomal RNA sequences for (A) Bayesian inference and (B) neighbor-joining (NJ), with *Cochlodinium polykrikoides* as an outgroup. Sample ID number, country of origin, and GenBank accession number are listed after taxon names. Support values are shown for Bayesian posterior probabilities and NJ bootstrap percentages, respectively. Three subclades of *Akashiwo sanguinea* are indicated, and the branch lengths are drawn to scale, with the scale bars proportional to genetic distances. Note that sequences of the *A. sanguinea* isolates JX13, JX14, and AS2 were obtained in the present study, while the remaining sequences were retrieved from GenBank

mean time to death of 11 h. The 72 h mortalities of *M. meretrix*, Mugilidae sp. (1 mo old), and *M. cephalus* were 50, 40, and 22%, respectively. In contrast, Mugilidae sp. (3 mo old) and *A. salina* survived 72 h exposure to *A. sanguinea* cells. Lysed *A. sanguinea* cells were significantly more toxic than whole cells, with 100% mortality observed in *L. vannamei* and *C. parasema* after 12 and 23 h, and 91% mortality of *A. salina* in 72 h. During the experiments, levels of pH and DO saturation were 7.47–8.03 and 70–80%, respectively, indicating that the mortality of animals was not caused by stress of DO or pH. All test animals in control treatments displayed 100% survival throughout the 72 h experiments.

Variation in *A. sanguinea* toxicity among strains and growth phases

A. sanguinea JX14 at 8000 cells ml^{-1} yielded 91% mortality in brine shrimp *A. salina* after 72 h, a percentage significantly higher than that observed for strain JX13 (51%; $p < 0.01$; Fig. 2A). Further, the LC_{50} for JX14 (1895 cells ml^{-1}) was much lower than that for JX13 (7369 cells ml^{-1}), demonstrating a higher potency of JX14 (Fig. 2B; $R^2 = 0.9741$, n = 45, $p = 0.00306$). Brine shrimp displayed 100% survival in negative controls.

Hemolytic assays demonstrated that both JX14 and AS2 yielded lysis of rabbit erythrocytes (Fig. 3). The toxic effects were positively correlated with the cell density of *A. sanguinea* (Fig. 3A), with the toxicity of JX14 (9.85×10^{-5} hemolytic units [HU] $cell^{-1}$) being almost 3 times that of AS2 (3.72×10^{-5} HU $cell^{-1}$; Fig. 3B).

Comparisons among cultures of *A. sanguinea* at different growth phases with similar cell densities showed that cultures of JX14 at exponential stages caused significantly higher mortalities (>50% after 24 h) in *A. salina* than did cultures at lag and declining stages (0 and 30%; $p < 0.05$; Fig. 4A). Mortalities of *A. salina* increased with time, and final mortalities among all cultures were not significantly different at the end of the experiments ($p > 0.05$). Non-linear fitting of LC_{50} using 72 h mortalities of *A. salina* showed that toxicities of *A. sanguinea* JX14 at exponential and lag growth phases displaying LC_{50} of 2097 and 2145 cells ml^{-1} were significantly higher than those observed in declining and stationary phases (LC_{50} of 5238 and 5806 cells ml^{-1}, respectively; $p < 0.01$, Fig. 4B). *A. salina* exhibited 100% survival in negative controls.

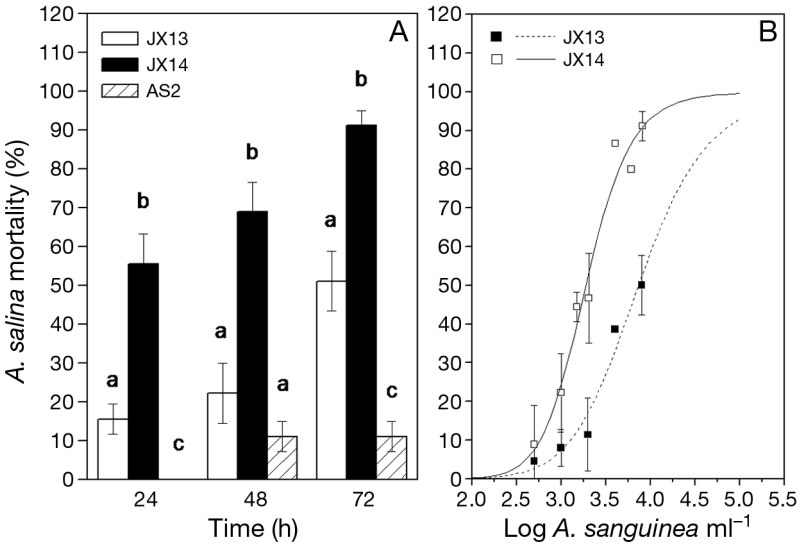

Fig. 2. (A) Toxicity of *Akashiwo sanguinea* strains JX13, JX14, and AS2 to *Artemia salina* at a density of 8000 *A. sanguinea* cells ml^{-1}. (B) Dose-dependent toxicity of JX13 and JX14. Values of 72 h LC_{50} for JX13 and JX14 were 7369 and 1895 cells ml^{-1}, respectively. Results are triplicate mean ± 1 SD. Significant differences ($p < 0.05$) in *A. salina* mortality at each timepoint are denoted with different letters

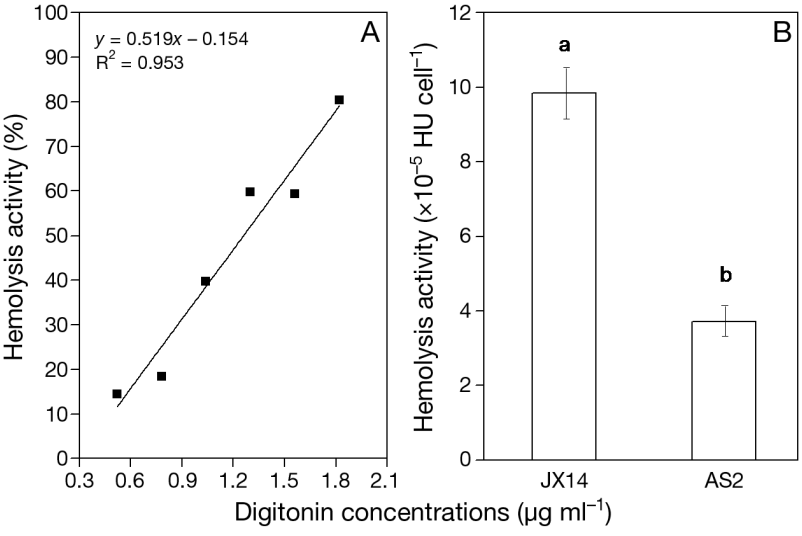

Fig. 3. Hemolytic assay with rabbit blood cells to compare the toxicity of *Akashiwo sanguinea* strains JX14 and AS2. (A) Standard hemolytic curve based on digitonin concentration was used as a reference, and the hemolytic activity of the culture extract was estimated as digitonin equivalent. (B) Hemolytic activity of 1 l of JX14 or AS2 culture during exponential growth phase (initial density: 6482 and 7135 cells ml^{-1}, respectively) used for the extraction of hemolytic substances. Results are triplicate mean ± 1 SD. Significant difference ($p < 0.05$) in hemolysis activity is denoted with different letters. HU: hemolytic units

Toxicity of different fractions of *A. sanguinea* cultures

Filtrate of sonicated *A. sanguinea* JX14 culture was toxic to *A. salina*, causing 51 to 69% mortality after 72 h, although mortality rates were significantly lower than that caused by sonicated cultures without filtration (about 90%; $p < 0.05$; Fig. 5A). A 72 h exposure to boiled *A. sanguinea* yielded low mortality rates that were not significantly different from the negative controls ($p > 0.05$; Fig. 5B). In contrast, frozen cultures of *A. sanguinea* were lethal to *A. salina* (20–27% mortality rates after 48 and 72 h), but with a lower mortality rate than live cultures (about 90%; $p < 0.05$; Fig. 5B). Brine shrimp displayed 100% survival in negative controls.

Environmental factors affecting toxicity of *A. sanguinea* cultures

Temperature altered the toxicity of *A. sanguinea* to *A. salina*, with the highest toxicity (91% mortality) observed at 20°C (vs. mortalities of 56 and 37% at 15 and 25°C, respectively; $p < 0.05$, ANOVA; Fig. 6A), the temperature where the highest growth rate was observed (0.46 d^{-1}). Salinity also altered the toxicity of *A. sanguinea* to *A. salina* (Fig. 6B), with the highest mortality (96%) observed at the highest salinity applied (35), where the highest growth rate of *A. sanguinea* was observed. Regarding nitrate levels, *A. sanguinea* JX14 grew rapidly at high (12.4 mg N l^{-1}) and moderate (0.6 mg N l^{-1}) NO_3^- levels but very slowly at low NO_3^- (0.06 mg N l^{-1}) level. Mortalities of *A. salina* in cultures with moderate and high NO_3^- levels were significantly higher than that in low N treatments at 72 h ($p < 0.05$; Fig. 7). After 24 h co-culturing with *Skeletonema costatum*, the toxicity of *A. sanguinea* to *A. salina* decreased significantly in a dose-dependent manner, with higher cell densities of *S. costatum* causing lower mortalities and longer time to death of *A. salina* ($p < 0.05$; Fig. 8). Brine shrimp displayed 100% survival in negative controls.

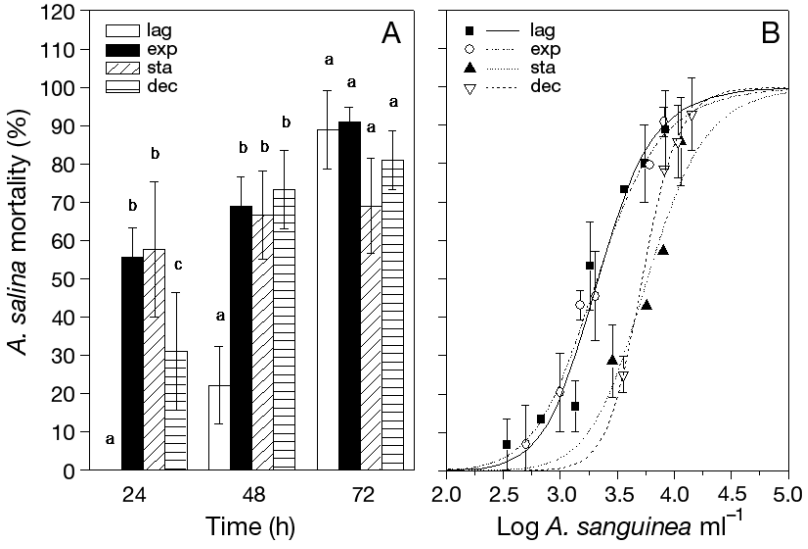

Fig. 4. Effects of growth stage on the toxicity of *Akashiwo sanguinea* strain JX14. (A) Comparison of lag, exponential (exp), stationary (sta), and decline (dec) phase at a density of 8000 JX14 cells ml^{-1}. (B) Dose-dependent toxicity of *A. sanguinea* in different growth phases. Values of 72 h LC_{50} for lag, exponential, stationary, and decline phase were 2145, 2097, 5806, and 5238 cells ml^{-1}, respectively. Results are triplicate mean ± 1 SD. Significant differences ($p < 0.05$) in *Artemia salina* mortality at each timepoint are denoted with different letters

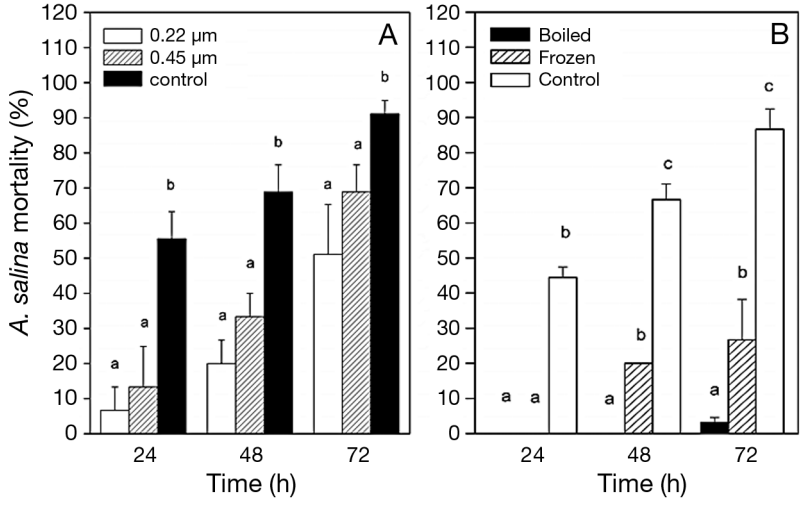

Fig. 5. Bioassays using *Artemia salina* exposed to different fractions of *Akashiwo sanguinea* JX14 culture. (A) Culture of 8000 JX14 cells ml^{-1} was sonicated and filtered (pore size: 0.22 or 0.45 µm) or unfiltered as a control, and (B) sonicated cultures (8000 *A. sanguinea* cells ml^{-1}) were boiled or frozen or left unamended as a control in the experiments. Results are triplicate mean ± 1 SD. Significant differences ($p < 0.05$) in *A. salina* mortality at each timepoint are denoted with different letters

DISCUSSION

Dynamics of *Akashiwo sanguinea* toxicity

A. sanguinea has long been associated with mortality of finfish, shellfish, and other marine animals and is generally considered a harmful alga, and mechanisms controlling deleterious effects observed during blooms are unknown (Landsberg 2002). In this study, clonal cultures of *A. sanguinea* JX14 isolated from the South China Sea exhibited significant lethal effects on multiple aquatic animals including finfish (*Chrysiptera parasema*, Mugilidae sp., *Mugil cephalus*), bivalve *Meretrix meretrix*, shrimp *Litopenaeus vannamei*, and brine shrimp *Artemia salina*. DO was not likely responsible for the animal kills, because mortalities of fish, shellfish, and shrimp were also observed even when DO concentrations were elevated within whole-cell experiments, and the DO measurements at the end of experiments were always above 6 mg l^{-1}, a level known to be supportive of marine life (Fox & Taylor 1955, Mitchell & Geddes 1977, Vaquer-Sunyer & Duarte 2008).

Our study showed that cultures of *A. sanguinea* displayed acute toxicity on multiple test animals (Table 1). For example, the shrimp *L. vannamei* experienced 100% by both whole-cell as well as sonicated cultures within 12 h, and the finfish *C. parasema* also experienced 100% mortality by sonicated culture within 24 h. All test animals in control treatments displayed 100% survival throughout the 72 h experiments, which indicated that sterile f/2 medium does not cause mortality of the test animals under the experimental conditions. Bacterial infection was unlikely the dominant factor responsible for the mass mortality in a short time period. In addition, Expt 1 showed that sonicated *A. sanguinea* culture was lethal to brine shrimp (91% mortality), which was in sharp contrast to the whole-cell culture (0% mortality), suggesting that intracellular substances are the primary factor causing the mass mortality of *A. salina*. Although toxic effects of *A. sanguinea* on test animals may be a reasonable explanation for this result, indirect consequences of increased organic load stimulating (or altering) the bacterial community activity could not be excluded.

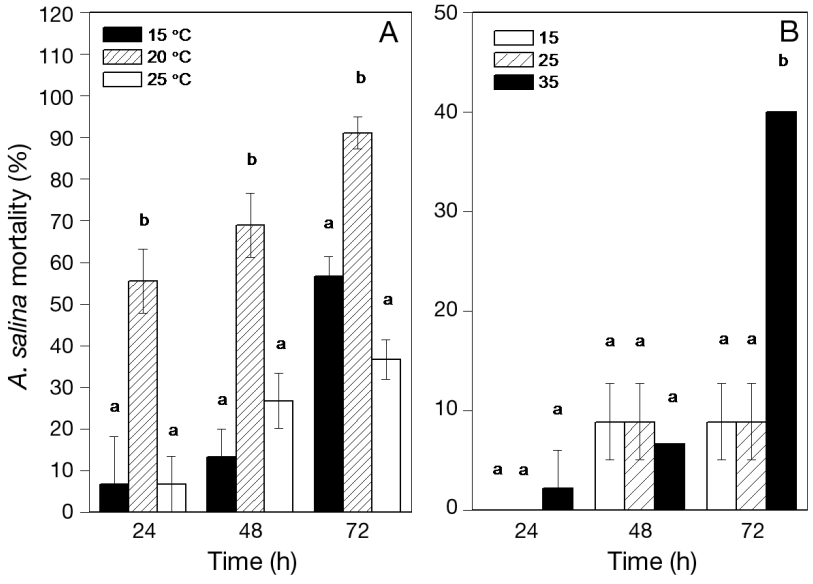

Fig. 6. Effects of temperature and salinity on the toxicity of *Akashiwo sanguinea* JX14 to *Artemia salina*. Densities of *A. sanguinea* JX14 were (A) 8000 cells ml^{-1} for the temperature experiment and (B) 2000 cells ml^{-1} for the salinity experiment. Results are triplicate mean ± 1 SD. Significant differences ($p < 0.05$) in *A. salina* mortality at each timepoint are denoted with different letters

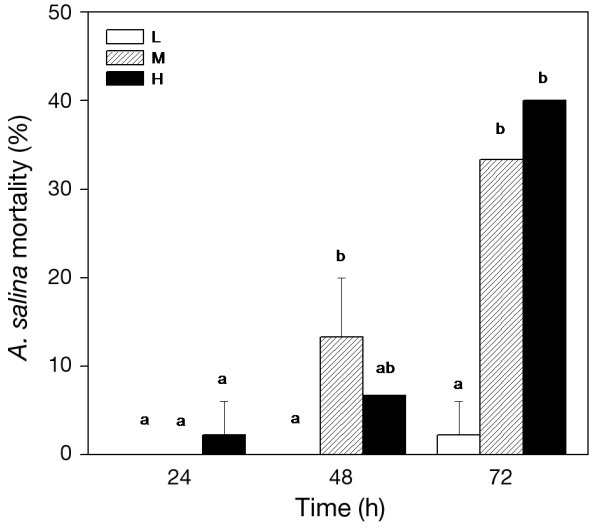

Fig. 7. Effects of nitrogen supply on the toxicity of *Akashiwo sanguinea* JX14 to *Artemia salina*. *A. sanguinea* JX14 was grown under low (L: 0.059 mg N l^{-1}), moderate (M: 0.559 mg N l^{-1}), and high (H: 12.4 mg N l^{-1}) nitrate levels. Toxicity of sonicated JX14 culture was tested with *A. salina*. Cell density of *A. sanguinea* was 2000 cells ml^{-1}. Results are triplicate mean ± 1 SD. Significant differences ($p < 0.05$) in *A. salina* mortality at each timepoint are denoted with different letters

In Expt 2 using sonicated cultures of 3 strains of *A. sanguinea* (JX13, JX14, and AS2), *A. salina* mortality exhibited big differences among different treatments: the highest mortality (90%) was observed in the JX14 treatment, followed by the JX13 treatment (50%), which was in contrast with the AS2 treatment (<10%; Fig. 2A). This experiment demonstrated that treatments using different strains of *A. sanguinea* at similar cell density caused very different mortalities in test animals. Similar experimental results were also found in Expt 3 using different fractions of *A. sanguinea* cultures (Fig. 5B). We believe that microbial effects could not explain the large differences among *A. sanguinea* strains with the equivalent organic loading, and these experiments provided comparable examples supporting that *A. sanguinea* toxin(s) are responsible for mass mortality of brine shrimp during the experiments. Furthermore, the hemolytic assays showed that both JX14 and AS2 yielded lysis of rabbit erythrocytes, which provided direct evidence demonstrating that cells of *A. sanguinea* are capable of producing hemolytic toxin(s). Hemolytic activity of species of *A. sanguinea* on different categories of marine animals requires further investigation. Therefore, the results presented in this study provide confirmative evidence that this species is toxic and thus can cause large kills of animals as previously described during *A. sanguinea* blooms reported in the literature (e.g. Harper & Guillen 1989, Wu et al. 2001, Kahru et al. 2004).

A. sanguinea was more toxic during the exponential phase than during the stationary or decline phases, which suggests that cells at actively growing stages produce more toxins. Similarly, the dinoflagellate *Cochlodinium polykrikoides* and the raphidophyte *Heterosigma akashiwo* have also been shown to be most toxic during active growth (Tang & Gobler 2009).

There were remarkable differences in the toxicity of *A. sanguinea* among strains. The strains of *A. sanguinea* from the South China Sea, i.e. JX13 and JX14, were significantly different in their toxicity to *A. salina*, with LC_{50} being 7369 and 1895 cells ml^{-1}, respectively, while they displayed significantly higher toxicity than did the strain AS2 isolated from Chesapeake Bay, USA. Furthermore, the hemolytic toxicity of JX14 (9.85×10^{-5} HU cell^{-1}) was almost 3 times that of AS2 (3.72×10^{-5} HU cell^{-1}). A strain of *A. sanguinea* isolated from False Bay, South Africa, caused severe mortality in abalone larvae but was

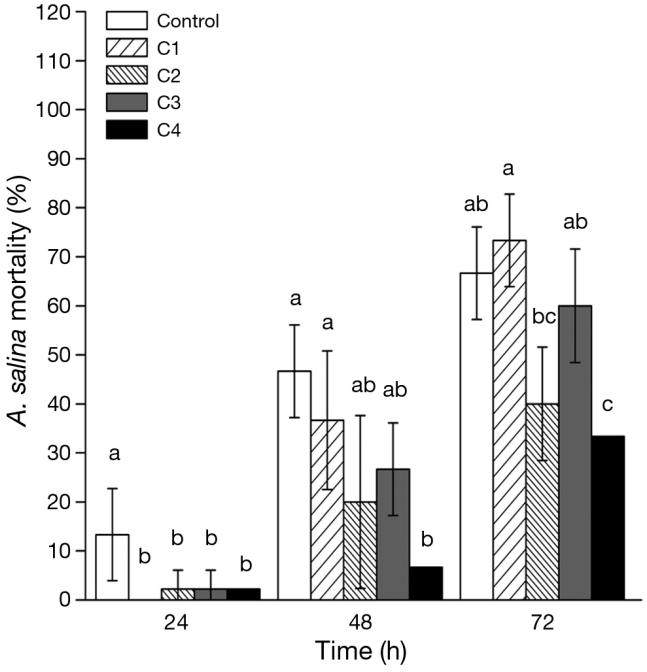

Fig. 8. Effects of co-occurring phytoplankton *Skeletonema costatum* on the toxicity of *Akashiwo sanguinea* JX14 to *Artemia salina*. *A. sanguinea* JX14 (initial cell density 5320 cells ml^{-1}) was co-cultured with the diatom *S. costatum* (initial cell densities C1–C4 were 3.3×10^2, 3.3×10^3, 3.3×10^4, and 3.3×10^5 cells ml^{-1}) for 24 h, and then the mixtures were sonicated and toxicity was tested using brine shrimp. Results are triplicate mean ± 1 SD. Significant differences ($p < 0.05$) in *A. salina* mortality at each timepoint are denoted with different letters

not lethal to *Artemia* larvae (Botes et al. 2003). Therefore, strain-specific differences may be one of the key factors that partly accounts for the differences in the toxic effects of *A. sanguinea* to marine animals. Phylogenetic analyses indicated that *A. sanguinea* isolates are divided into 3 phylogroups: Singapore, East Asian Pacific, and USA–Atlantic, with minor intra-group genetic differences (0–0.001) and larger inter-group genetic differences (0.017–0.029). These genetic differences generally parallel their toxicity, with the East Asian Pacific strain being highly toxic and Atlantic strains being less so, and may further account for prior reports of toxic and non-toxic populations of *A. sanguinea* (Landsberg 2002). Still, even within the same phylogroup, there can be varying toxicity, as exemplified by the 2 strains isolated from Daya Bay (JX13 and JX14).

Effects of environmental factors on *A. sanguinea* toxicity

A. sanguinea is considered a eurythermal and euryhaline organism, able to grow at temperatures from 10 to 30°C and salinities from 10 to 40, exhibiting a maximum growth rate of 1.13 divisions d^{-1} at a combination of 25°C and salinity of 20 (Matsubara et al. 2007). Field studies have shown that *A. sanguinea* blooms in waters with wide ranges of temperature (11–28.5°C) and salinity (26–33.6; Table 2). For example, Park et al. (2013) investigated the outbreaks of blooms dominated by dinoflagellates in the coastal waters of Korea from 1981 to 2009 and found that *A. sanguinea* red tides occurred primarily from May to October but also during February, April, November, and December. In the coastal waters of China, blooms of *A. sanguinea* appeared in the cold season (February–March) as well as warm seasons (May–June, August–September; Wu et al. 2001, Yang et al. 2012), affirming its ability to form blooms across a broad temperature and salinity window. During this study, the optimal temperature and salinity for the growth of strain JX14 were 20°C and 35, respectively, which provided further evidence that strains from the same regions can have different physiological characteristics, including toxicity. Further, we found that the toxicity of *A. sanguinea* peaked under the same temperature and salinity

Table 2. Review of maximum abundance (MA) of *Akashiwo sanguinea* and *in situ* environmental conditions, including water temperature (T), salinity (S), dissolved inorganic nitrogen (DIN), dissolved inorganic phosphorus (DIP), and N:P ratio during blooms. np: data not provided

Location	MA (cells ml^{-1})	T (°C)	S	DIN (µM)	DIP (µM)	N:P ratio	Reference
Yantai, China	1560	25–26	30.6–31.5	2.57–6.43	0.23–0.73	5.64	Wu et al. (2001)
Xiamen, China	117	23–27	26–30	32.6–302.9	0.81–6.84	2.87–27.33	Yang et al. (2012)
Korea	3000	12.0–28.5	21.6–33.6	1.7–36.0	0.1–2.9	0.9–8.6	Park et al. (2013)
California, USA	9880	15.1–15.6	33.5–33.6	0.05–0.35	0.41–3.56	0.01–0.85	Kudela et al. (2008)
Oregon, USA	348	11–12	32–33	0.89–1.55	0.33–0.58	0.70–2.29	Du et al. (2011)
Co. Cork, Ireland	16 900	np	27.2–31.1	~50	~0.8	np	O'Boyle & McDermott (2014)

conditions that were optimal for growth. This suggests that fast-growing cells are more toxic. This positive feedback may favor the reproduction of *A. sanguinea* populations and thus facilitate the formation and development of blooms that may cause serious harm to marine animals (Sunda et al. 2006). Since a temperature around 20°C is common in the spring (from March to May) and autumn (from October to December) in the South China Sea, potential ecological risks of *A. sanguinea* blooms may be higher in these seasons.

The increase of HAB events has frequently been attributed to excessive nitrogen and phosphorus supplies in worldwide coastal waters (Anderson et al. 2002, Heisler et al. 2008). However, due to the differences among HAB species and their different responses to multiple ambient nutrient forms, the role of nutrients during blooms can be difficult to estimate (Kudela et al. 2010). As one of the most common HAB species in Korean waters, *A. sanguinea* accounted for 11% of the total dinoflagellate red tides from 1981 to 2009 (Park et al. 2013). Long-term data of nutrients showed that the decrease in dissolved inorganic nitrogen (DIN) was partially responsible for the decrease in *A. sanguinea* red tides after 2000 (Park et al. 2013). However, based on field studies during *A. sanguinea* blooms from different geographic areas (Table 2), this species is able to bloom under both high (in the case of Xiamen, China) and low (in the case of California and Oregon, USA) DIN and dissolved inorganic phosphorus (DIP) levels (Kudela et al. 2008, Du et al. 2011, Yang et al. 2012). Despite the complicated correlation between nutrient levels and *A. sanguinea* blooms, there was a similarity in the low N:P ratios among these bloom events, suggesting that nitrogen supplies may control the growth of these blooms (Table 2). In our study, we found that an elevated nitrogen supply enhanced the toxicity of *A. sanguinea* to brine shrimp. Thus the role of nutrients may be dual for *A. sanguinea* blooms: stimulating the proliferation of populations and strengthening their chemical defense against potential grazers.

In recent years, anthropogenic nutrient loading has increased significantly in many estuarine and coastal areas, with the concentration and percentage of nitrogen as dissolved organic nitrogen (DON), including urea, increasing substantially (Anderson et al. 2002, Glibert et al. 2006). The potential connection between organic nutrients and HABs has raised concerns in the scientific community (Anderson et al. 2008, Heisler et al. 2008, Hu et al. 2014). Laboratory studies have indicated that *A. sanguinea* was able to utilize urea, ammonium, and nitrate, showing a preference for urea (Kudela et al. 2008). Reifel et al. (2013) indicated that effluent discharge and urban runoff can stimulate blooms of *A. sanguinea*. Our unpublished data showed that urea significantly enhanced the toxicity of *A. sanguinea*. As a similar case, domoic acid production by *Pseudo-nitzschia australis* was enhanced when urea was supplied as the sole N source (Cochlan et al. 2008). *A. sanguinea* is a mixotrophic dinoflagellate with a preference for organic nitrogen such as urea (Kudela et al. 2008). Based on our present findings, this species may respond to anthropogenic urea loading directly (via increased growth) and indirectly (e.g. via enhanced toxin production). Our field data showed that the average concentration of urea was 2.0 µg N l^{-1} in aquaculture areas of Daya Bay, which represented about 20% of DON (Xu et al. 2012). Therefore, the increasing anthropogenic nitrogen loading as urea could favor the growth of this species, and thus act as an important regulating factor for the occurrence of *A. sanguinea* blooms.

Phytoplankton densities may play an important role in regulating chemical defenses of harmful algae (Prince et al. 2008, Tang & Gobler 2009). In the present study, we found that the presence of *Skeletonema costatum* significantly mitigated the toxicity of *A. sanguinea* (Fig. 8). Because cell density of *S. costatum* was set up before the 24 h co-culture procedure, the exact cell number in each treatment may have changed prior to the assay. Our result showed that the lowest mortality of brine shrimp occurred in the treatment with the highest *S. costatum* cell concentration. Similarly, as *C. polykrikoides* blooms develop, other dinoflagellates or cryptophytes such as *P. minimum* and *Rhodomonas salina* may co-dominate phytoplankton communities (Gobler et al. 2007) and reduce the toxicity of *C. polykrikoides* cells (Tang & Gobler 2009). Tang & Gobler (2009) indicated that the ecological impacts of *C. polykrikoides* likely depend not only on the absolute cell densities achieved by blooms but also on the relative dominance of *C. polykrikoides* among the total plankton community. The dilution effect caused by increasing the adsorption surface area of algal cells may partly explain the observations in the laboratory and field. It has been previously reported that *S. costatum* reduced the allelopathic growth-inhibiting effects of bloom exudates of *Karenia brevis* (Prince et al. 2008). Thus, this cosmopolitan diatom species may partly mitigate the toxicity of *A. sanguinea*. To date, the effects of the toxin(s) in *A. sanguinea* on the growth of co-occurring phytoplankton are unknown.

The potential resistance of other phytoplankton species to the toxicity of *A. sanguinea* requires further investigation.

Possible toxic mechanisms of *A. sanguinea* and its ecological implications

In this study, we found that the sonicated *A. sanguinea* culture displayed higher toxicity to the target species (brine shrimp, 91% mortality) than did whole-cell culture, which suggested that toxin(s) are produced intracellularly and may be released under certain circumstances, such as when cells are grazed or lysed. Therefore, the toxicity of *A. sanguinea* may serve as a chemical defensive mechanism targeting predators exerting grazing pressure on this alga. Further, *A. sanguinea* toxin(s) may also act as allelochemicals, providing an advantage in outcompeting other phytoplankton (Granéli & Salomon 2010, Tang & Gobler 2010, Xu et al. 2015). If this was the case, the ability to produce broad toxin(s) that target both predators and competitors would be a highly effective competition strategy, and may play an important role in the formation and sustaining of *A. sanguinea* blooms.

Our culture fraction experiments showed that the toxic chemical(s) were partly stable after freezing (70.6% decrease in the mortality of brine shrimp), but instable after boiling (almost 100% loss in its toxicity to brine shrimp). The filtrate of sonicated *A. sanguinea* culture displayed lower toxicity than the unfiltered control. It was likely that some toxin(s) were retained and/or adsorbed on the filter used (pore size: 0.22 and 0.45 µm). Tillmann et al. (2008) also found that allelopathic effects of culture filtrate (<10 and <0.2 µm) were significantly lower than those of whole-cell treatments of *Alexandrium catenella* and *A. taylori*. Given that the exact composition of toxin(s) in *A. sanguinea*, and the ability of such toxin(s) to accumulate and even transfer in marine food webs are unknown, further research on these topics is needed.

Our laboratory study provided evidence to support the supposition that *A. sanguinea* produces toxic chemical(s) responsible for prior observations of mass mortality of marine animals during blooms of this species (Harper & Guillen 1989, Wu et al. 2001, Kahru et al. 2004). However, our findings also showed that the toxin(s) were mainly stored intracellularly, suggesting that toxic effects may occur during bloom demise or when blooms experience rapid cell turnover. Regardless, the ability of *A. sanguinea* to cause rapid death in multiple species of organisms that may also have grazing control on this species provides a mechanism by which blooms may form (grazer-deterrence) and may partly explain the common occurrence of these blooms.

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