



# Novel Non-paralytic Shellfish Toxin and Non-spirolide Toxicity to Finfish, Brine Shrimp, and Rotifer Observed in a Culture of the Dinoflagellate *Alexandrium insuetum* Isolated From the Coastal Water of China

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The genus *Alexandrium* is one of the major harmful algal blooms (HABs)-forming dinoflagellate group and at least half of ~40 described species have been reported to produce paralytic shellfish toxins (PSTs). The potentially harmful species *Alexandrium insuetum* has been reported from many countries of Asia and Europe, and to have paralytic shellfish poisoning toxicity, but no mortality of marine animals was observed during its bloom. Therefore, it is ecologically important to characterize the possible toxicity and toxins of this organism. In this study, based on the establishment of two clonal cultures through cyst germination collected from the Yellow Sea, we identified *A. insuetum* from China as the first record *via* light microscopy (LM) and scanning electron microscopy (SEM) observations and phylogenetic analyses. The cultures of *A. insuetum* were further observed to be toxic to finfish and zooplankton and deleterious to rotifer eggs *via* laboratory bioassays. The exposure bioassays using rotifer (*Brachionus plicatilis*), brine shrimp (*Artemia salina*), and larval finfish (*Oryzias melastigma*) demonstrated that *A. insuetum* caused significant lethal effects on finfish and zooplankton species. Rotifer bioassays using cell-free culture medium, heat-treated cultures, and water, methanol, and trichloromethane extracts of algal cells revealed that *A. insuetum* produced heat-labile, water-soluble toxin(s) that could be excreted from *A. insuetum* cells and steadily accumulated in the medium during the growth phases. Hatching success of rotifer eggs was also found to be seriously affected by the exposure to *A. insuetum*. Importantly, ultra-high performance liquid chromatography-tandem mass spectrometry [UPLC (or LC)-MS-MS] analyses suggest the above-described toxicity of *A. insuetum* was caused by neither PSTs nor spiroimines (13-desmethyl spirolide C and gymnodimine). Collectively, our findings demonstrated the novel toxicity to finfish and zooplankton in *A. insuetum*, which is ecologically important in not only possibly contributing to population dynamics and

even the formation of HABs of the species, but also affecting the on-the-spot survival and the reproduction potency of marine animals. The present work is believed to set a cornerstone for the monitoring and risk assessment of the species along the coastal waters of China and for understanding the general ecology of *A. insuetum*.

**Keywords:** harmful algal blooms (HABs), *Alexandrium insuetum*, morphology, phylogeny, novel toxicity, fish kill

## INTRODUCTION

Dinoflagellates are not only important primary producers and grazers of aquatic ecosystems, but also responsible for the most harmful algal blooms (HABs) (Hallegraeff et al., 2021). The dinoflagellate genus *Alexandrium* Halim, one of the most important genera responsible for causing HABs, has negative impacts on marine ecosystems, economy, and human health worldwide (Anderson, 1998; Anderson et al., 2012). Many *Alexandrium* species have been proved to produce toxins, e.g., paralytic shellfish toxins (PSTs, saxitoxin and its derivatives), spiroidines (spiroclides and gymnodimines), goniotoxins, and lytic compounds or allelochemicals (Cembella et al., 2000; Ichimi et al., 2002; Nascimento et al., 2005; Hsia et al., 2006; Tang et al., 2007; Espina et al., 2016; Blossom et al., 2019; Mertens et al., 2020; Shang et al., 2021). Furthermore, at least more than a half of *Alexandrium* species have been shown having a resting cyst stage in their life histories, which can be associated with seeding recurrent HABs and geographic expansion of populations (Anderson et al., 2005, 2012; Bravo et al., 2006; Nagai et al., 2009; Gu et al., 2013; Tang et al., 2016, 2021). The range, frequency, and intensity of HAB events caused by *Alexandrium* species have been increasing worldwide during the past decades (Hosoi-Tanabe and Sako, 2006; Anderson et al., 2012; Zou et al., 2014; Yu and Liu, 2016).

In the coastal waters of China, about 11 *Alexandrium* species have been documented, including *Alexandrium affine*, *Alexandrium andersonii*, *Alexandrium catenella*, *Alexandrium fraterculum*, *Alexandrium leei*, *Alexandrium minutum*, *Alexandrium ostenfeldii*, *Alexandrium pacificum*, *Alexandrium pseudogonyaulax*, *Alexandrium tamiyavanichii*, and *Alexandrium tamutum* (Gu, 2011; Gu et al., 2013; Huang et al., 2021; Tang et al., 2021; Xu et al., 2021). In Chinese coastal waters, large-scale HABs caused by species of this genus have been reported frequently since 2000. For instance, recurrent blooms of toxic *Alexandrium* spp. have been detected from 2001 to 2006 in East China Sea (Zhou and Zhu, 2006; Yu and Liu, 2016). Many blooms that were attributed to *A. tamarensis* (but possibly either *A. catenella* or *A. pacificum*; see John et al., 2014; Fraga et al., 2015), a PSTs-producing species, have been also observed in the coastal waters of Dalian, Yantai, Zhoushan, and Xiamen, China, causing massive wild and caged fish kills (Zhou and Zhu, 2006; Zou et al., 2014; Yu and Liu, 2016). It is essential to identify *Alexandrium* species accurately, as this genus includes many hard-to-differentiate and toxic species that cause diverse impacts in aquaculture, human health, and marine ecosystems (Bravo et al., 2006).

*Alexandrium insuetum* was first described by Balech (1985) from Korea, and it has been then reported from the coastal areas of Korea (Shin et al., 2014), Japan (Yuki and Yoshimatsu, 1990; Kim et al., 2005), Greece (Nikolaidis et al., 2005; Spatharis et al., 2007), France (Guillou et al., 2002), and Tunisia (Daly Yahia-Kefi et al., 2001). Both toxic or nontoxic strains of *A. insuetum* have been reported (Adachi et al., 1994; Lilly et al., 2005) and paralytic shellfish poisoning (PSP) toxicity was also detected in a strain isolated from Uchinoumi, Japan, using high performance liquid chromatography (HPLC)-fluorometric method (Sako et al., 2004), whereas many other strains have not been subjected to toxins analysis (Guillou et al., 2002; Kim et al., 2002; Spatharis et al., 2007; Shin et al., 2014). In April 2003, an *A. insuetum* bloom (max.  $2.54 \times 10^6$  cells·L<sup>-1</sup>) was reported for the first time in Amvrakikos Bay, Greece, which caused brownish water discoloration but without death of marine organisms being observed, and blooms of this species reoccurred and lasted for 3 weeks in the following year (Nikolaidis et al., 2005). During the winter of 2004–2005, co-occurrence of *A. insuetum* (max.  $1.4 \times 10^5$  cells·L<sup>-1</sup>) with *Pseudo-nitzschia calliantha* (Bacillariophyceae) was observed and developed into dense blooms in the Aegean Sea, Greece (Spatharis et al., 2007), but no death of marine animals were reported. *Alexandrium insuetum* has been generally considered to be a potentially harmful species (Sako et al., 2004; Spatharis et al., 2007; Shin et al., 2014), but the characterization of its toxicity and toxins has never been well-accomplished.

Vegetative cells of *A. insuetum* are solitary unless two daughter cells in division, oval in shape, and also characterized by highly reticulated cell surface and presence of a ventral pore located on the first apical (1') plate (Balech, 1985; Yuki and Yoshimatsu, 1990; Shin et al., 2014). *Alexandrium insuetum* is morphologically similar to *A. minutum* and *A. tamutum* (Lilly et al., 2005) in all having a small cell size and a posterior sulcal (sp) plate wider than its length (Balech, 1989; Montresor et al., 2004; Shin et al., 2014). Moreover, *A. insuetum* is phylogenetically close to *A. minutum* in terms of forming the *minutum/insuetum* group (Lilly et al., 2005; John et al., 2014; Shin et al., 2014). Compared with most species of the *A. minutum/insuetum* group, however, *A. insuetum* has a feature that the pore plate (Po) and the 1' plate are completely disconnected (Lilly et al., 2005; Shin et al., 2014). It has been speculated that there have been only a few records of this species in the literature due to the remarkable morphological and phylogenetic similarities between *A. insuetum* and *A. minutum* (Lilly et al., 2005; Spatharis et al., 2007; Shin et al., 2014). Thus, more detailed observations on the isolates of this species from different geographic regions are needed for accurate identification of the species group in the future.

In this study, we established two clonal cultures of *A. insuetum* via germinating resting cysts collected from the coast of Lianyungang, China, and examined their morphology via light microscopy (LM) and scanning electron microscopy (SEM) and phylogeny based on the large subunit (LSU) rDNA sequences. More importantly, we characterized their toxicity to marine animals (rotifer, brine shrimp, and finfish) via laboratory bioassays and ultra-high performance liquid chromatography-tandem mass spectrometry [UPLC (or LC)-MS-MS] analyses for their toxin profiles. Our results are believed to set a cornerstone for the monitoring and risk assessment of the species along the coastal waters of China and important knowledge for the general ecology of *A. insuetum*.

## MATERIALS AND METHODS

### Sediment Sample Collection and Clonal Culture Establishment

Surface sediment samples (0–2 cm) were collected from the coast of the Yellow Sea, China (34.78° N, 119.45° E; water depth: 10 m), on March 22, 2016, using a grab corer, and stored in the dark at 4°C until further treatment. Cyst assemblage was concentrated using a protocol of sodium polytungstate (SPT) gradient centrifugation (Bolch, 1997). The single cysts were washed several times using sterile seawater, then inoculated into the individual wells of 24-well tissue culture plates (Corning, USA) filled with 2 mL *f*/2-Si medium (Guillard, 1975) and a final concentration of 2% antibiotic solution (a mixture of 10,000 IU Penicillin and 10,000  $\mu\text{g}\cdot\text{mL}^{-1}$  streptomycin; Solarbio, China). Two clonal cultures of *A. insuetum* (strains AILYG12 and AILYG23) were established by transferring vegetative cells into fresh *f*/2-Si medium using micropipettes after cysts germination. All cultures were maintained in the incubator at 21°C, light intensity of  $\sim 100 \mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ , and a 12:12 h light:dark cycle.

### Light Microscopy and Scanning Electron Microscopy

Cysts and vegetative cells were observed and photographed using an inverted microscope (IX73, Olympus, Japan) and a light microscope (BX53, Olympus, Japan) equipped with a digital camera (DP80, Olympus, Japan). Vegetative cells were stained with SYBR Green (Solarbio, Beijing, China), viewed and photographed for chlorophyll-induced red autofluorescence and SYBR Green-induced green fluorescence of nucleus. Cells sizes for 50 live cells at the mid-exponential growth phase were measured at  $\times 200$  magnification using a DP80 digital camera (Olympus, Tokyo, Japan). For plate pattern identification, live cells were stained with calcofluor white and examined using the epifluorescence microscope (BX53, Olympus, Japan) with a UV filter set (Fritz and Triemer, 1985).

For SEM observation, vegetative cells at mid-exponential growth stage were fixed with glutaraldehyde (2.5% final concentration) for 40–50 min, then gently filtered onto an 11- $\mu\text{m}$  pore size Millipore nylon membrane, dehydrated in an acetone series (10%, 30%, 50%, 70%, 90%, and three times in 100%,

15 min for each step), critical point-dried with liquid CO<sub>2</sub> (EM CPD300, Leica, Austria), sputter-coated with gold (EM ACE200, Leica, Austria), and observed using a S-3400 N SEM (Hitachi, Japan; Hu et al., 2020).

### DNA Extraction, PCR Amplification, and Sequencing

Total DNA was extracted from the cells using a plant DNA extraction kit (Tiangen, China) following the protocol of the manufacturer. About 1,400 bp of LSU rDNA sequence was amplified using the primer set, D1R (F: 5'-ACCGCTGAATTTAAGCATA-3') (Scholin et al., 1994) and 28-1483R (R: 5'-GCTACTACCACCAAGATCTGC-3') (Daugbjerg et al., 2000), and an  $\sim 1,800$  bp of the small subunit (SSU) rDNA was amplified using primers EukA (forward, 5'-ACCTGGTTGATCCTGCCAGT-30) and EukB (reverse, 5'-TCACCTACGGAAACCTTGT-30) (Medlin et al., 1988). The PCR reactions were conducted in a total volume of 20  $\mu\text{L}$ , containing 7  $\mu\text{L}$  ddH<sub>2</sub>O, 10  $\mu\text{L}$  High Fidelity (HiFi) PCR SuperMix (Transgen, China), 1  $\mu\text{L}$  of each PCR primer, and 1  $\mu\text{L}$  DNA template. The PCR protocol was as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min, and extension for 10 min at 72°C. PCR-amplified products were confirmed by 1.0% agarose gel electrophoresis. The amplicons were purified with agarose gel DNA fragment recovery kit (GENEray, China) and ligated into the pMD-19T vector (TaKaRa, Japan), and then transformed into *Escherichia coli* DH5 $\alpha$  (Biomed, China), the positive cloning were sequenced (TSINGKE, China). New sequences were deposited in GenBank with accession numbers MZ489186 and MZ489188 (LSU rDNA), and MZ489187 (SSU rDNA).

### Phylogenetic Analysis

Large subunit rDNA gene sequences of *A. insuetum* and other closely related *Alexandrium* species were used for phylogenetic analysis. A sequence of *Lingulodinium polyedra* (accession No. EF613357) was used as an out-group. Sequence alignments were performed using the MAFFT v7.475 with the default settings (Kato et al., 2019) (<http://mafft.cbrc.jp/alignment/server/>) and then modified with BioEdit v7.2.5 (Hall, 1999). The final alignment of LSU rDNA sequences included 1,725 characters, including gaps introduced from alignment. The general-time reversible model with estimated proportion of invariant sites and gamma-distributed rate variation among sites (GTR+I+G) substitution model was selected as the best-fit model using jModelTest 2.1.4 (Darrriba et al., 2012) based on the Akaike information criterion (Akaike, 1974). Phylogenetic trees were constructed using Bayesian inference (BI) and maximum likelihood (ML) analyses. The BI analysis was performed with MrBayes 3.2.6 (Ronquist et al., 2012) using the best-fitting substitution model (GTR+ I + G). Four independent Markov chain Monte Carlo simulations were run simultaneously for 10 million generations and trees were sampled every 1,000 generations. The initial 25% of trees were discarded as burn-in, and convergence was judged based on the average SD of split frequencies (all < 0.01). The remaining trees were used to



generate a consensus tree and calculate posterior probabilities for all branches using a majority-rule consensus approach. ML analysis was conducted with RaxML v7.2.6 (Stamatakis, 2006) using the model GTR + I + G, and node support was assessed with 1,000 bootstrap replicates. FigTree v1.4.4 was used to visualize consensus tree.

## Exposure Experiments of Marine Animals (Rotifer, Brine Shrimp, and Finfish)

### Experiment 1: Bioassays of Rotifer and Brine Shrimp With the Live Cell Culture and the Cell Lysate

In order to test the toxicity of *A. insuetum* culture, exposure experiments of marine zooplankton (rotifer, *Brachionus plicatilis*; and brine shrimp, *Artemia salina*) were conducted using the live cell culture and cell lysates of *A. insuetum*. The resting eggs of rotifer *B. plicatilis* (L-type) and brine shrimp *Ar. salina* were bought from Ningbo Futian Biotechnology Co., Ltd (China) and Boaifeng Biological Products Co., Ltd (China), respectively, and 2-day-old neonates were used in this study. Cell lysates were prepared by sonicating the live cell culture on ice at 250 W for 60 × 10 s with a high energy JY92-II sonicator (Scientz Biotechnology Co., Ltd., China), microscopic examination confirmed no intact cells present. Test animals were exposed to a series of densities of live cell cultures and cell lysates (7,000, 4,000, 2,000, and 1,000 cells·mL<sup>-1</sup>, respectively), which were both obtained from diluting the initial culture (7,000 cells·mL<sup>-1</sup>, stationary phase) using f/2-Si medium. The bioassays were conducted in 24-well culture plates, with one test animal and 2 mL prediluted culture added to each well ( $n = 24$ ). The f/2-Si medium and *Isochrysis galbana* (strain T-ISO, nontoxic prey) were used as negative controls. Test animals were observed every 12 h within a period of 120 h, and the death of the animal was recorded when a lack of movement. Dissolved oxygen (DO) levels of culture medium were measured at the beginning of the experiment and immediately after animal death, or at the end of the experiment.

### Experiment 2: Fish Bioassays in the Live Cell Culture and Cell-Free Culture Medium

For fish bioassay, experiments were performed in six-well tissue culture plates (Labselect, China) with transwell polycarbonate membrane inserts (8.0 μm pore size) that divided each well into two portions using larval medaka *Oryzias melastigma* to measure the toxicity of live culture and cell-free culture medium to finfish. The larval fish was purchased from Qingdao Feiruite Biotechnology Co., Ltd., China. A volume of 6 mL culture of *A. insuetum* (4,000, 6,000, and 8,000 cells·mL<sup>-1</sup>, respectively) was added into the insert and the membrane with pore size of 8.0 μm would allow the cell-free culture medium to seep out the insert, but prevent *A. insuetum* cells (>10 μm) moving out the insert, meanwhile, we examined that no cells passed through the membrane during the experiment under a light microscope (IX73, Olympus, Japan). Three- to five-day-old juveniles (3–4 mm in length) were placed into the inserts (live cell culture) and the outer compartments (cell-free culture medium) when the culture mediums of two compartments maintained osmotic balance (about 1:1 in volume for inside and outside; one fish for

each compartment, 10 replicates for each treatment). The f/2-Si medium and *I. galbana* (T-ISO) were used as negative controls. The state of the finfish was monitored every 3 h within the first 24 h and then observed every 12 h for a period of 96 h. Death time was recorded once the heart stopped beating. The DO levels were measured before the addition of fish and the time of fish death.

### Experiment 3: Bioassays of Rotifer With Cell-Free Culture Mediums at Different Growth Phases

The toxicity of cell culture at the initial, exponential, and stationary growth phases were examined. Algal cultures collected at day 3, 6, and 9 were used in this experiment (2,840, 4,410, and 7,625 cells·mL<sup>-1</sup>, respectively). The cell-free culture medium was prepared by gravity filtration of algal culture through a 5-μm pore size nylon membrane (Millipore, USA) and the filtrate was further filtered through a syringe filter of 0.22-μm pore size (Jinteng Experiment Equipment Co., Ltd., China), no intact cells were observed in the cell-free culture medium with a light microscope (IX73, Olympus, Japan). Exposure bioassays of rotifer were conducted as described above in experiment 1 ( $n = 24$ ).

### Experiment 4: Characterizing the Chemical Nature of *A. insuetum* Toxin(s)

To further determine the chemical nature of toxin(s) produced by *A. insuetum*, toxin(s) were extracted using three solvents, i.e., sterile filtered seawater enriched with f/2-Si medium, methanol, and trichloromethane. Cells at exponential phases were harvested by centrifuging and removal of supernatant, cell pellets were suspended in 600 μL solvents and extracted by reciprocal shaking at 6 m s<sup>-1</sup> with lysing matrix E (MP Biomedicals, USA) for 60 s (30 s for each time) in a MP FastPrep tissue homogenizer (MP Biomedicals, USA). The extracts were then centrifuged at 12,000 rpm for 5 min, and the resultant supernatants (600 μL) were used as the toxin extracts. For water and methanol extracts, the supernatants were filled with fresh f/2-Si culture medium to volume of 60 mL (1% final concentration); for trichloromethane extracts, the supernatants were evaporated and the residue redissolved in 300 μL dimethylsulfoxide (DMSO) for further exposure bioassays (60 mL final volume, 0.5% of DMSO). Therefore, 240,000, 420,000, 600,000, 3,000,000, and 6,000,000 cells were used in each treatment, corresponding to the equivalent cell concentration of 4,000, 7,000, 10,000, 50,000, and 100,000 cells·mL<sup>-1</sup>, respectively. Exposure bioassays of rotifer were also conducted as described in experiment 1 to examine the toxicity of these extracts ( $n = 12$ ), mortalities were calculated every 12 h for a period of 60 h. Fresh f/2-Si culture medium, with and without addition of 1% methanol or 0.5% DMSO, nontoxic to rotifer, were used as negative controls.

To test the heat stability of toxin(s) from *A. insuetum*, exposure experiments of rotifer were also conducted using both heat-killed cell cultures and heated water extracts. The culture mediums were prepared by heating the cell cultures (10,000 cells·mL<sup>-1</sup>) or water extracts (equ. 10,000 cells·mL<sup>-1</sup>) in a 100°C water bath for 30 min, and then cooling to room temperature before testing. The same cell cultures and water extracts of *A. insuetum* without heating were used as positive controls, f/2-Si

medium and *I. galbana* (T-ISO) culture were used as negative controls. The bioassays were conducted in 24-well culture plates (one rotifer for each well,  $n = 24$ ) for a period of 96 h, and the state of rotifer was monitored every 12 h. DO levels were also detected during the bioassays.

## Influence of *A. insuetum* on the Hatching of Rotifer Eggs

Eggs produced by rotifers were found not being able to hatch success when exposed to the culture of *A. insuetum* in the exposure experiment 1 and 2 above, thus, exposure bioassays of rotifer eggs to cell-free culture mediums and whole-cell cultures at different growth stages were conducted to examine the influence of toxic *A. insuetum* on the hatching success of healthy eggs. The rotifer eggs were purchased from Ningbo Futian Biotechnology Co., Ltd, China. The hatching experiments were performed in 24-well culture plates, with one resting egg in each well ( $n = 24$ ). Each well-contained 2 mL of test culture medium, which included live cell culture or cell-free culture medium of *A. insuetum* at different growth phases (3, 6, and 9 d after inoculation, respectively). Cell-free culture mediums were acquired followed procedure in experiment 3 above. The cell culture and cell-free culture medium of *I. galbana* (T-ISO), and f/2-Si medium were set as the controls. The hatching of neonates was conducted at 21°C with an irradiance of 100  $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$  and a12:12 h light: dark photoperiod. The resting eggs were checked every 12 h, hatching rates were calculated for the 72 h-incubation experiments.

## Analyses of PSTs and Spirolides

For the analysis of PSTs, aliquots of 200 mL culture at the mid-exponential, late exponential, and stationary growth phases were collected by centrifugation at 5,000 rpm for 5 min. Cell pellets were suspended in 10 mL acetic acid (0.1 M), extracted with a high energy JY92-II DN ultrasonic probe (Ningbo Scientz Biotechnology Co., Ltd., China) on ice bath, then centrifuged at 12,000 rpm at 4 °C for 15 min. The extracts were filtered through a syringe filter of 0.22  $\mu\text{m}$  pore size and stored at  $-20^\circ\text{C}$  until analysis. PSP toxins were analyzed by UPLC-MS-MS method using the Agilent 1290 Infinity II UPLC coupled to an Agilent 6470 triple quadrupole mass spectrometry system. The detailed procedures of the chromatographic separation and mass detection for PSTs were shown in **Supplementary Material 1**.

For the analysis of toxin 13-desmethyl spiroside C (SPX1), the major component of spiroside in *A. ostensfeldii* and *A. peruvianum* (Sleno et al., 2004; Borkman et al., 2012), and eight common lipophilic algal toxins (LMATs), including gymnodimine (GYM), okadaic acid (OA), dinophysistoxin (DTX1, DTX2), azaspiracids (AZA1, AZA2, and AZA3), and pectenotoxin (PTX2) were also detected in *A. insuetum*. The toxin extraction and determination procedure followed that in Gao et al. (2016) and Wang et al. (2021), aliquots of 200 mL culture were collected by centrifugation, followed by homogenization with 6 mL methanol for 5 min (400 W, 2 s/5 s work-rest cycles) using an ultrasonic treatment (Ningbo Scientz Biotechnology Co., Ltd., China), and then ultrasonic-assisted extraction in an ice-cold bath (60% power for 30 min). The extracts were centrifuged at 12,000 rpm for 5 min. Then, the

supernatant was transferred to a clean tube and methanol was added to 10 mL and filtered through a 0.22  $\mu\text{m}$  syringe filter. Three replicates were performed in this study. The toxin extracts were stored at  $-20^\circ\text{C}$  and further analyzed by online solid phase extraction (SPE) coupled with LC-MS/MS method (Wang et al., 2021). The detailed information was shown in **Supplementary Material 1**.

## Statistics

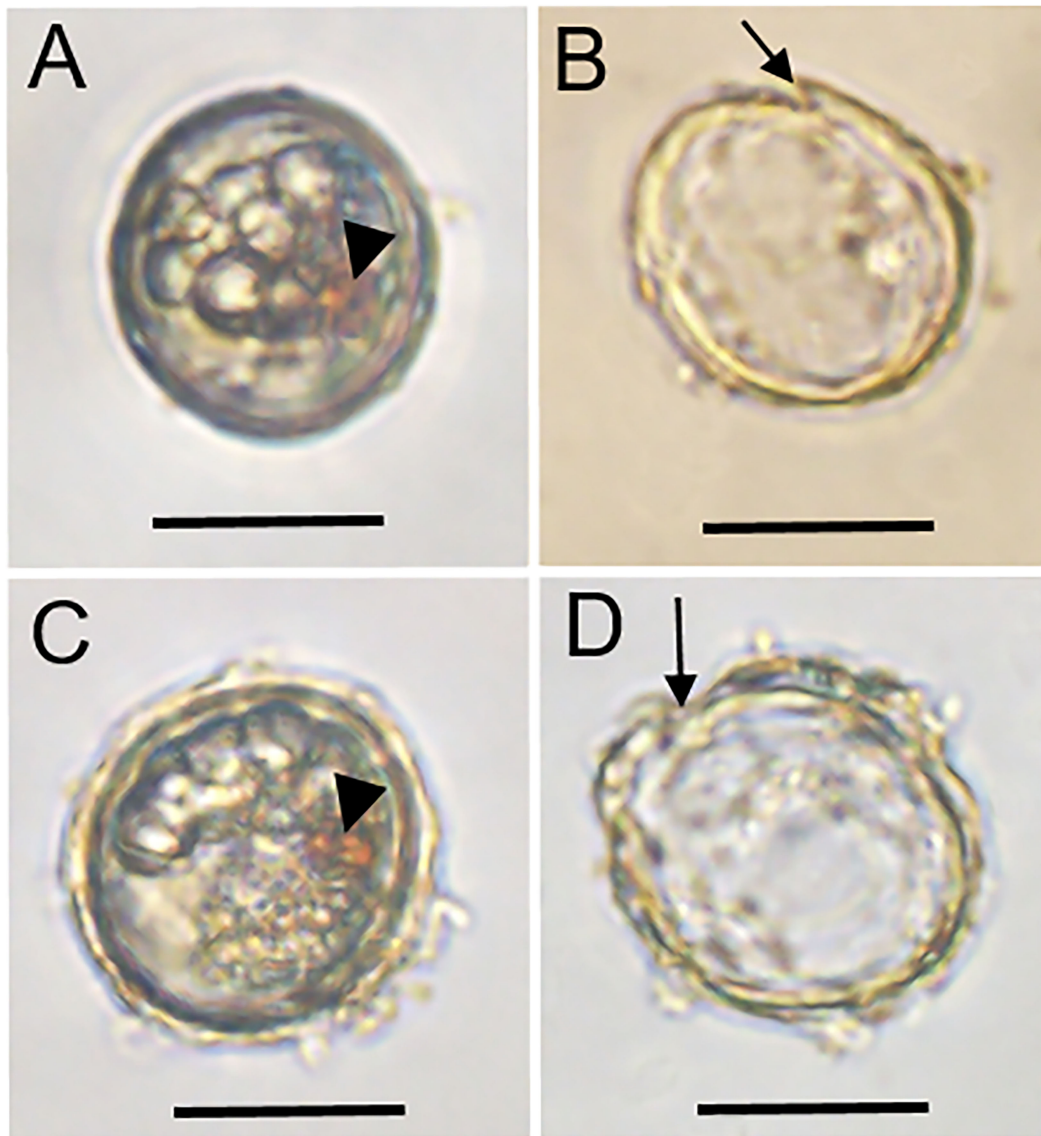
Differences among treatments were generally analyzed using *t*-test or One-way ANOVA followed by least significant difference (LSD) *post-hoc* test in the toxicity experiments. The regression analysis of fish bioassays was performed by linear regression using IBM Statistical Product and Service Solutions (SPSS) Statistics 22. Significance level was set at  $p < 0.05$  in all cases unless otherwise indicated.

## RESULTS

### Morphological and Molecular Confirmation of *A. insuetum*

Two cysts of *A. insuetum* were both spherical with a diameter of 28.6 and 29.9  $\mu\text{m}$ , respectively, had globular and granular content and a prominent orange-red accumulation body (**Figures 1A,C**). Cyst wall was slightly thick and smooth relatively (**Figures 1A,C**). The two resting cysts were germinated after 14 (**Figures 1A,B**) and 5 (**Figures 1C,D**) days' incubation, and the archeopyle was chasmic (**Figures 1B,D**). Vegetative cell of *A. insuetum* was very small in size, 11–25  $\mu\text{m}$  long ( $15.93 \pm 2.44 \mu\text{m}$ ,  $n = 50$ ) and 10–17  $\mu\text{m}$  wide ( $13.39 \pm 1.70 \mu\text{m}$ ,  $n = 50$ ). Cells were always solitary, had a slightly conical epitheca and round hypotheca (**Figures 2, 3**). The nucleus was located in the hypocone of cell (**Figure 2B**), and numerous rod-like chloroplasts were located almost throughout the cell (**Figure 2E**). The cell surface was very rough and highly reticulated, both on the epitheca and the hypotheca (**Figure 3**). The plate formula is: Po, 4', 6'', 6C, 5''', 2'''' (**Figures 2, 3**). The first apical plate (1') was rhomboidal in outline with a complete disconnection to the apical pore plate (Po) (**Figure 3**). The Po was drop-shaped, with the apical pore comma-shaped on the center, and small pores on the plate margin (**Figures 3C,G-I**). The sulcal plate (sp) was wider than its length with the irregular pentagon, attachment pore was absent on the Sp plate (**Figure 3A**).

One SSU rDNA and two partial LSU rDNA sequences of *A. insuetum* were obtained from our clonal cultures established from resting cyst germination. We compared the SSU rDNA sequence of the Chinese isolate (strain AILYG12; 1,800 bp, GenBank accession No. MZ489187) with that of other strains of *A. insuetum* in the National Center for Biotechnology Information (NCBI) database and found it was 99.8% (1,797 bp/1,800 bp) and 99.8% (1,754 bp/1,757 bp) identical to the Japanese strains AI104 and CCMP2082 (GenBank accession Nos. AB088298, JF521630). We also compared our 1,487 bp LSU rDNA sequence (strain AILYG12; accession No. MZ489186) of *A. insuetum* with other strains and found that it was 99.7% (1,483/1,487 bp), 99.4% (712/716 bp), and 99.6% (673/676 bp), 99.6% (1,186/1,191 bp), 97.9% (702/717 bp) identical to the Japanese strains CCMP2082, AI104, and



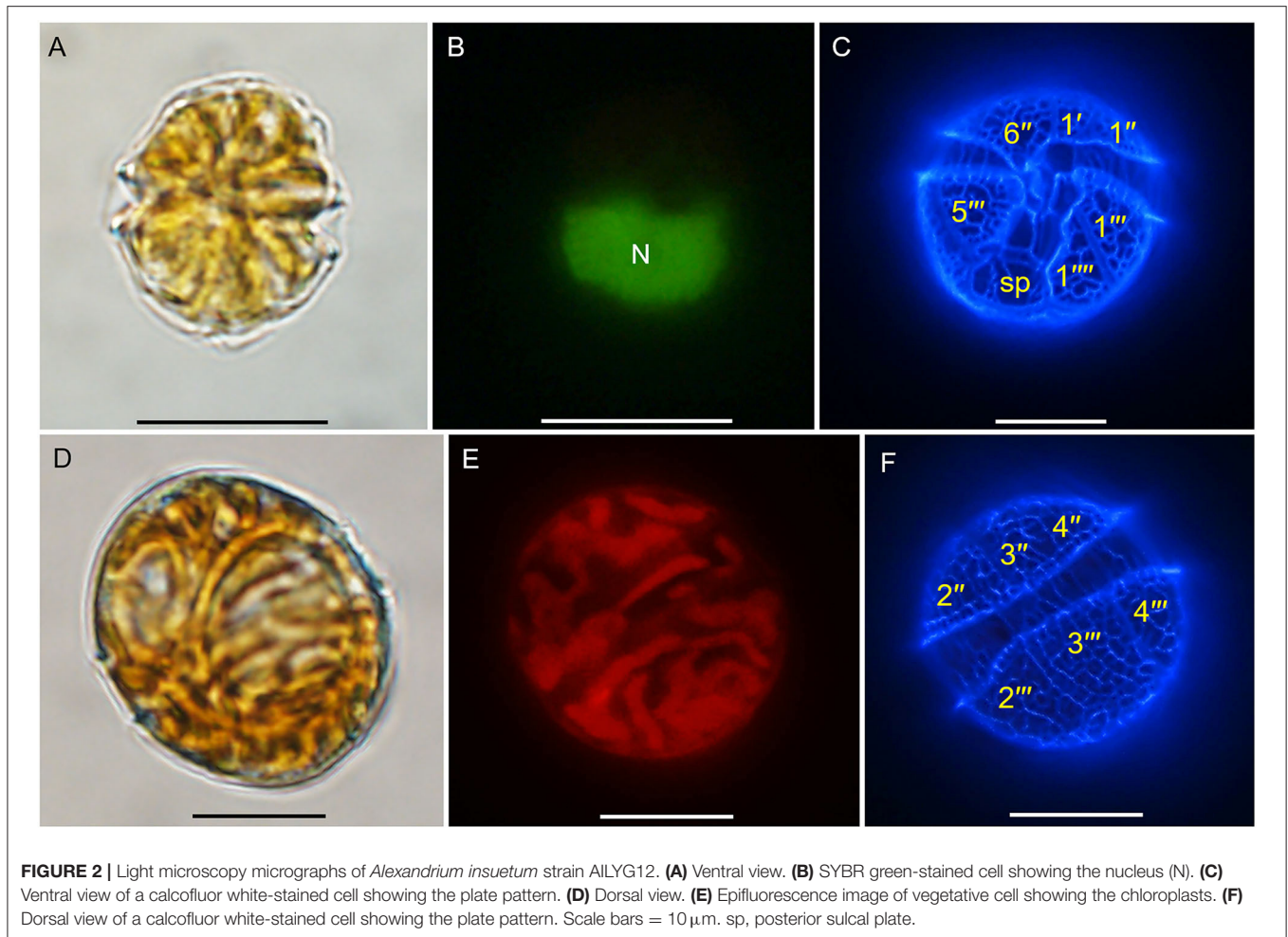
**FIGURE 1** | Light microscopy micrographs of two living (**A,C**) and the corresponding empty (**B,D**) cysts of *Alexandrium insuetum*. Arrowheads indicate the red accumulation body, the archeopyle (arrow) appeared after cyst germination. Scale bars = 20  $\mu\text{m}$ .

D-155-B-1 (GenBank accession Nos. JF521630, AB088249, AY962834), Korean strain LMBE-C7 (GenBank accession No. KF752546), French strain X6 (GenBank accession No. AF318233), respectively. Phylogenetic analyses using BI and ML generated similar phylogenetic trees (**Figure 4**). All sequences of *A. insuetum* separated from the other *Alexandrium* species and grouped together with a higher support (0.74 PP). The well-resolved *A. insuetum* clade included strains from China, Japan, Korea, and France (**Figure 4**). Our isolates and the isolates from Japan and Korea formed a coherent clade with maximal support (1.00 PP), and a sister group with the French isolates (0.74 PP; **Figure 4**).

### Toxicity of *A. insuetum* to Rotifer, Brine Shrimp, and Finfish

Both the whole cell culture and cell lysate of *A. insuetum* exhibited strong toxicity to rotifer and brine shrimp (**Figure 5**). In the exposure bioassays of rotifer, the rotifers *B. plicatilis* exposed to the live cell culture showed significantly higher mortality compared with those exposed to the sonicated cell lysate at the same cell concentration, with 38–75% mortality observed in the live cell cultures at the cell densities of 1,000–7,000 cells·mL<sup>-1</sup> within 120 h, but only 21–46% rotifer kills observed in the treatment of cell lysates with equivalent cell densities ( $p < 0.05$ , paired *t*-test; **Figure 5A**). Relatively low

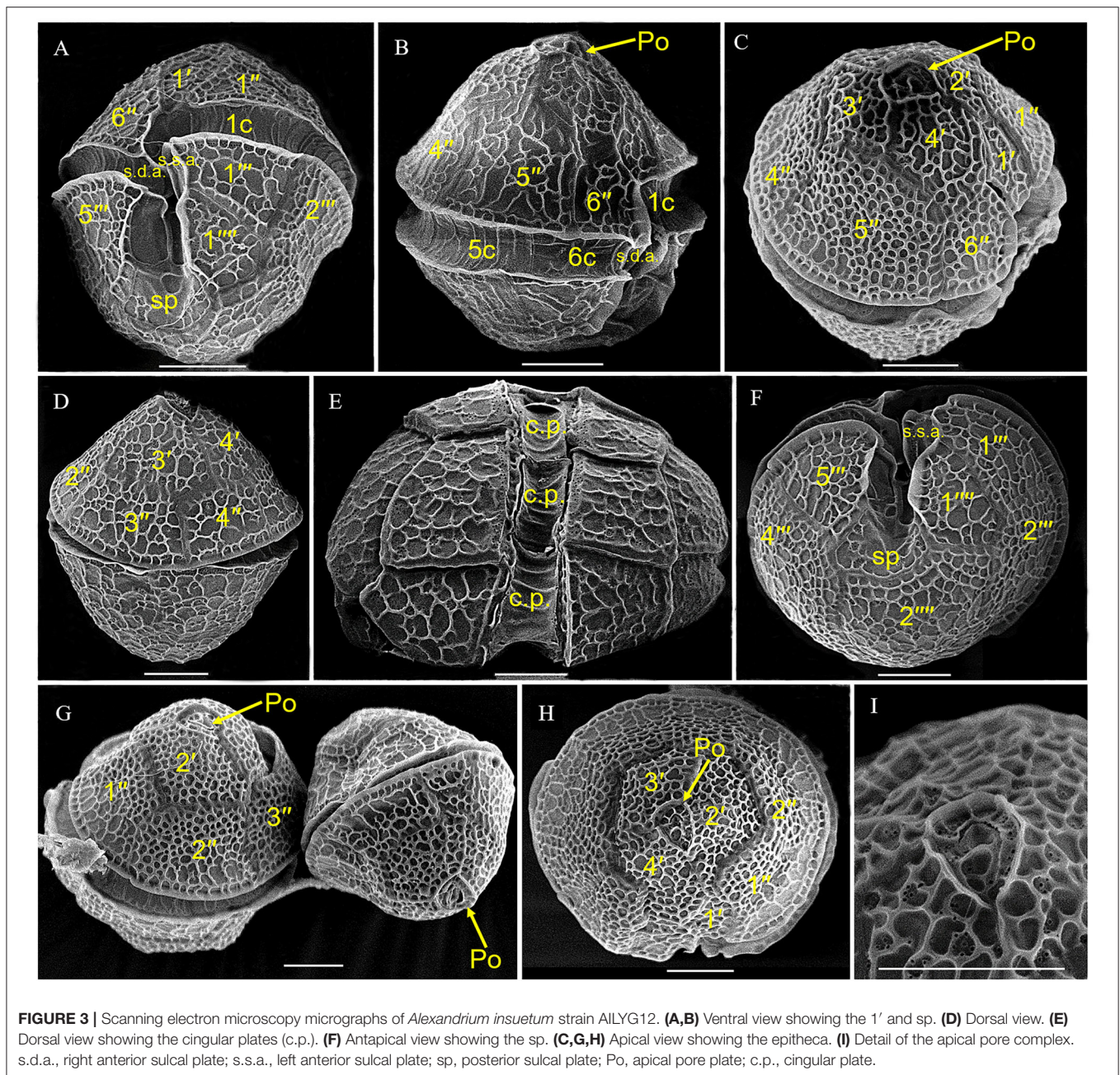




mortalities (4–21%) of rotifer exposed to the whole cell cultures (cell densities: 1,000, 2,000, 4,000, 7,000 cells·mL<sup>-1</sup>) were observed in the first 60 h, then it reached to 38–75% at the end of the experiments (120 h; **Figure 5A**). Mortalities of rotifer exposed to the cell lysates were mainly observed in the first 60 h (55–100%), but death of rotifer was not observed in the sonicated cultures of 1,000 and 7,000 cells·mL<sup>-1</sup> after 60 h (**Figure 5A**). The DO levels were 80–90% of the saturated DO concentrations at 21°C and no rotifer died in the negative controls within 120 h. The surviving rotifers exposed to culture mediums could feed on *A. insuetum* and were able to produce eggs during the bioassays, but no successful hatch was observed (**Supplementary Figure 1**). For brine shrimp bioassays, the mortalities were significantly higher than that of rotifer exposed to the same culture medium with same cell densities at the 120 h exposure ( $p < 0.05$ , paired *t*-test; **Figure 5B**). Mortalities of brine shrimps at 120 h exposure were 83–96 and 79–83% for the treatments of live cells and cell lysates, respectively, and mortality of brine shrimp exposed to cell culture was slightly higher than that in the cell lysate with the same cell density ( $p = 0.06$ , paired *t*-test; **Figure 5B**). A 17% mortality of brine shrimp was observed in the f/2-Si medium while no death was present in the

algal culture of *I. galbana*, and the DO levels were above 7.30 mg·L<sup>-1</sup> in the course of bioassays, indicating the brine shrimps in the medium control died as a result of starvation (**Figure 5B**).

In the fish bioassays using culture plates with inserts, fish mortality was observed only in the side with live cells (inside the inserts) within 24 h, with 10, 50, and 90% fish mortality in the treatments with the initial cell densities of 4,000, 6,000, and 8,000 cells·mL<sup>-1</sup>, respectively (**Table 1**). Finfish mortality (*M*) was positively correlated with the cell density (*D*) of *A. insuetum* within 96 h [Regression equation:  $M = 0.022D - 79.481$ ,  $R^2 = 0.981$  ( $n = 17$ ,  $p < 0.01$ )], but the time of fish death (*T*) was negatively correlated with the density (*D*) of algal culture within 24 h [Regression equation:  $T = -0.001D + 18.158$ ,  $R^2 = 0.921$  ( $n = 15$ ,  $p < 0.01$ )]. However, all fish survived in the bioassays using cell-free culture medium within 48 h, only 10% mortality was observed at 96 h exposure with a cell density of 6,000 cells·mL<sup>-1</sup>, and no fish died in the negative controls. It was possible that the toxin content of cell-free culture medium with all cell concentration we used was not lethal to the larval fish. The DO levels measured immediately after fish death



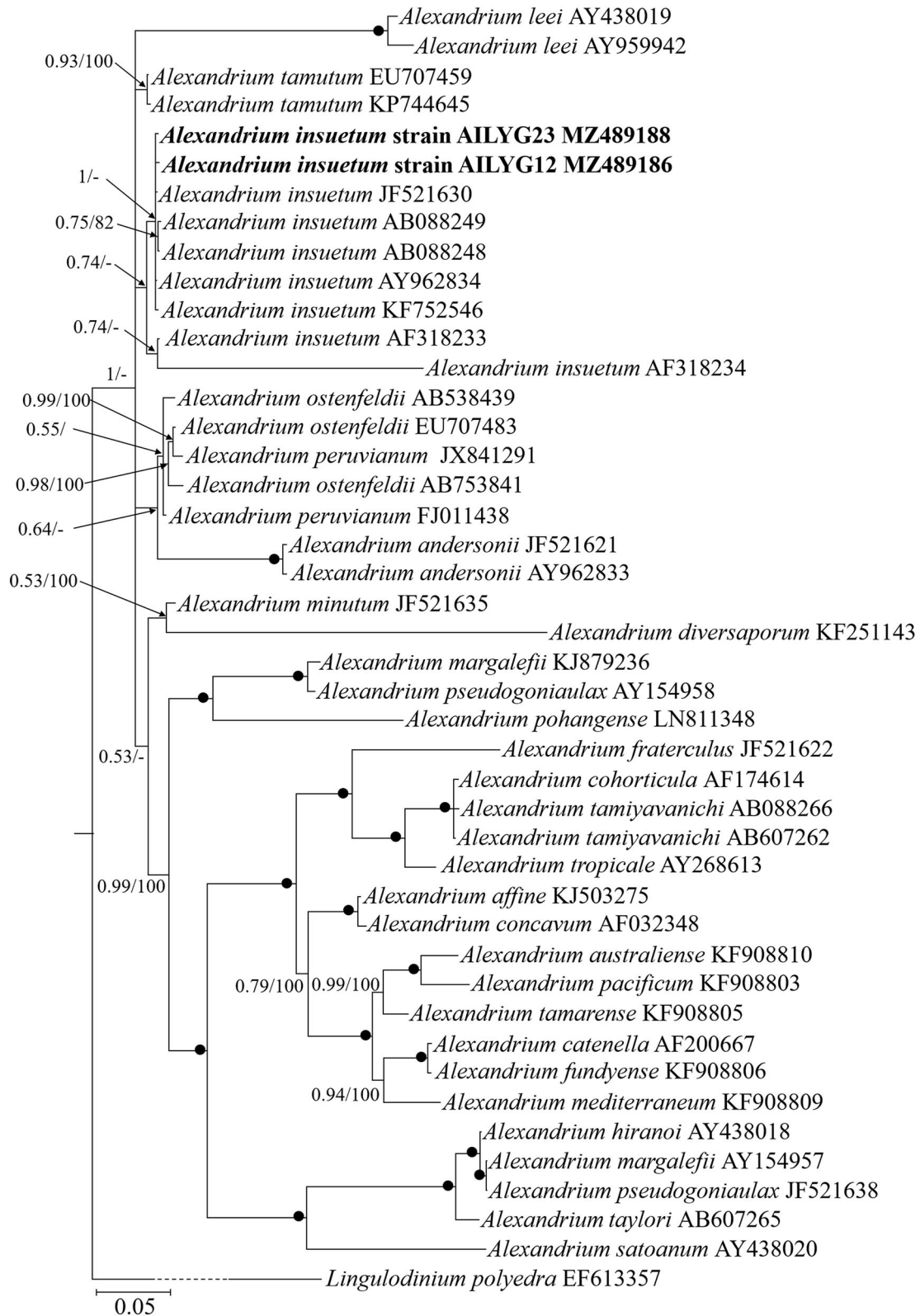
were in the range of 7.29–7.44 mg L<sup>-1</sup>. The lethal effect of *A. insuetum* algal culture on finfish larvae was observed with an inverted epifluorescence microscope with a filter for blue excitation (450–480 nm), cells of *A. insuetum* (red in micrographs) were observed in the intestine and near the gills (Figure 6).

### Toxicity of Cell-Free Culture Mediums of the Cultures at Different Growth Phases

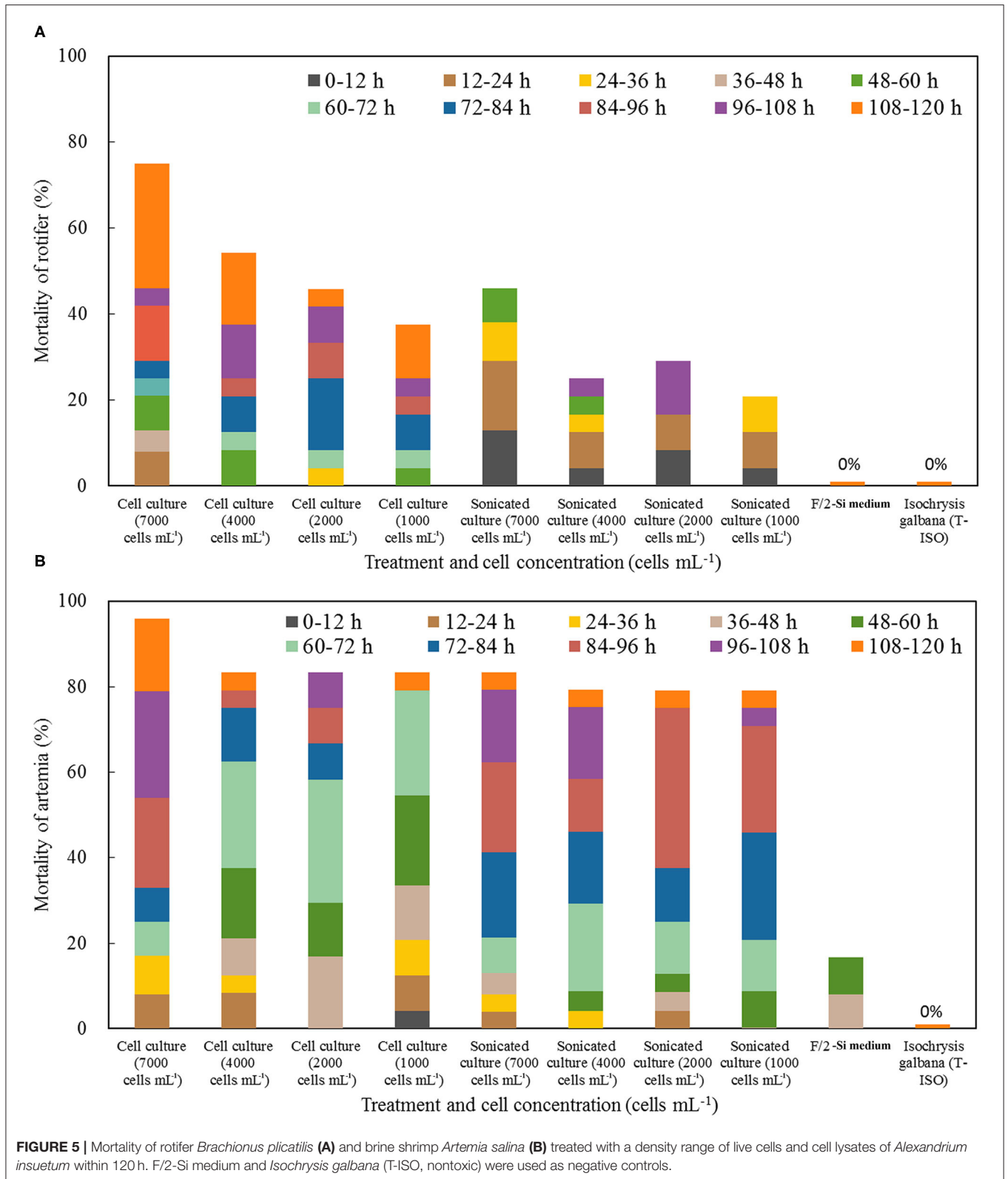
The results of exposure experiments on two zooplankton species showed the toxicity was increased in the cell-free culture medium

with the incubation time (Figure 7). The cell-free culture media of day 3, 6, and 9 caused 29, 41, and 54% mortalities of rotifer within 120 h, respectively, contrasting to 0 and 8% mortalities in the negative controls of *I. galbana* culture and f/2-Si medium. Higher mortality of brine shrimp was also observed at the 120 h exposure to cell-free culture medium as compared to the mortality of rotifer exposed to the culture medium at the same cell densities, with 54% rotifer mortality and 88% brine shrimp mortality observed at 120 h exposure to the cell-free culture medium collected on the day 9, but the difference was that death





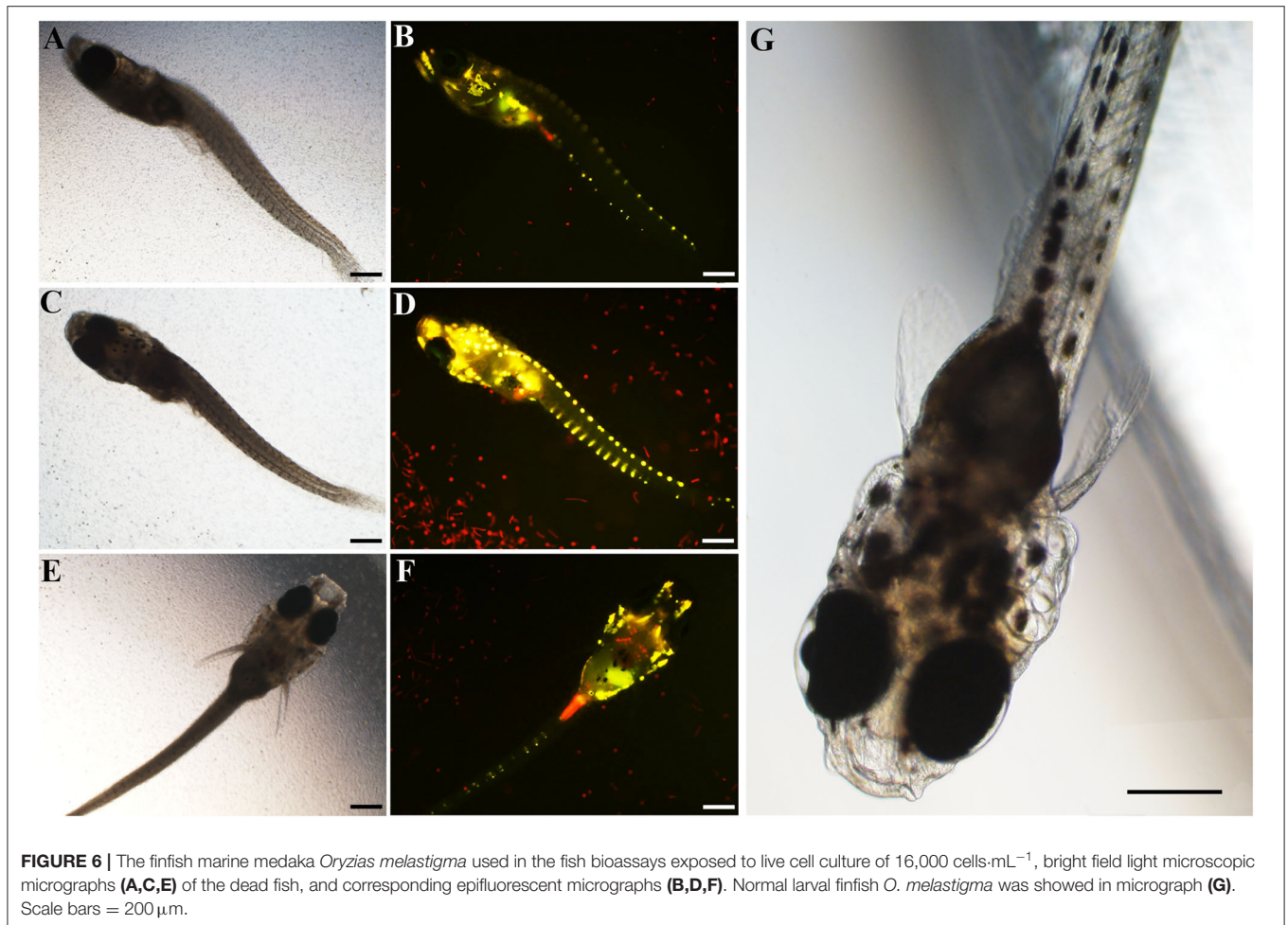
**FIGURE 4 |** Molecular phylogeny of Chinese strain of *Alexandrium insuetum* inferred from partial large subunit rDNA sequences based on Bayesian inference (BI) analysis with *Lingulodinium polyedra* (EF613357) as the outgroup. New sequences of *A. insuetum* are indicated in bold. Numbers on branches are statistical support values (left, Bayesian posterior probabilities; right, maximum likelihood (ML) bootstrap support values). Bootstrap values >50% and posterior probabilities (pp) above 0.5 are shown. Black circles (●) indicate maximal support (pp = 1.00 in BI and bootstrap support = 100% in ML, respectively). All branches are drawn to scale.



**TABLE 1** | Fish bioassays using partitioned culture plates showing the toxicity of live cell culture and cell-free culture medium within 96 h.

Treatment and the initial cell concentration		No. of fish dead within 24 h	No. of fish dead within 48 h	No. of fish dead within 96 h	Average death time (h) within 24 h
<i>A. insuetum</i> (4,000 cells·mL <sup>-1</sup> )	Live cell culture	1	1	2	15 ± 0
	Cell-free culture medium	0	0	0	–
<i>A. insuetum</i> (6,000 cells·mL <sup>-1</sup> )	Live cell culture	5	5	5	12.4 ± 1.36
	Cell-free culture medium	0	0	1	–
<i>A. insuetum</i> (8,000 cells·mL <sup>-1</sup> )	Live cell culture	9	10	10	11.55 ± 2.61
	Cell-free culture medium	0	0	0	–
Controls	f/2-Si medium	0	0	0	–
	<i>Isochrysis galbana</i> (T-ISO)	0	0	0	–

f/2-Si medium and non-toxic *Isochrysis galbana* culture (T-ISO) were used as negative controls. The dissolved oxygen levels were in the range of 7.29–7.44 mg·L<sup>-1</sup> when the larval fish died.



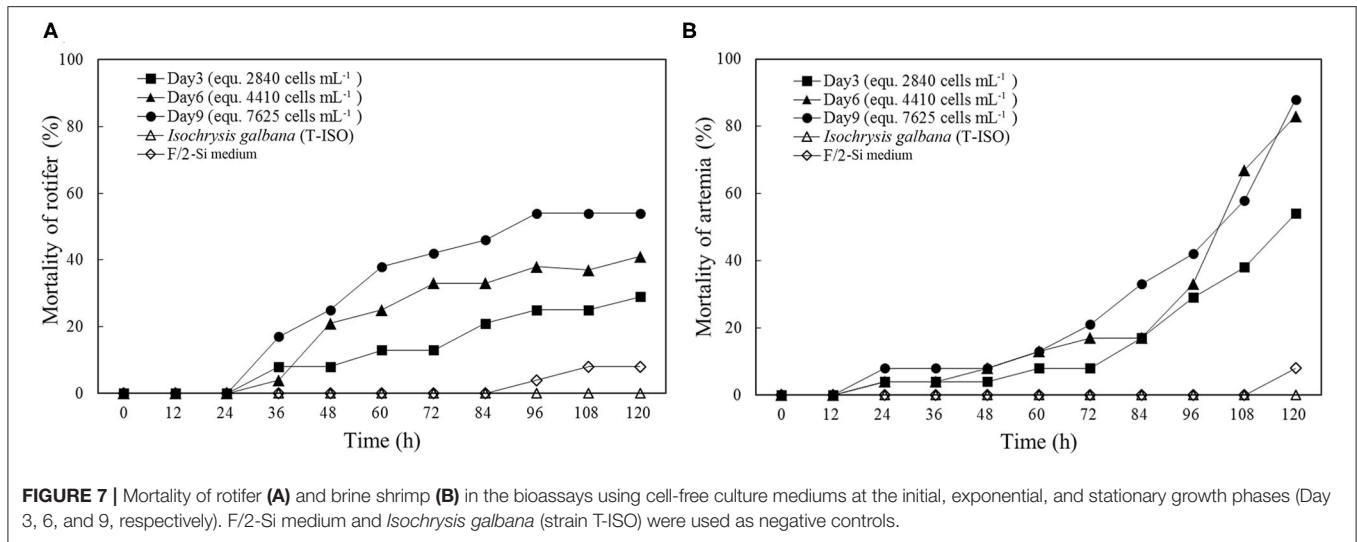
of rotifer mainly observed within the first 96 h, however, most brine shrimp kills were occurred after the initial 72 h.

### Characterization of the Chemical Nature of Toxin(s)

The rotifer bioassays using the crude toxin extracts that were extracted using solvents with different polarities demonstrated that the rotifer mortalities caused by water extract were

significantly higher than those caused by methanol extract at the equivalent cell densities ( $p < 0.05$ , paired  $t$ -test; **Figure 8A**). The methanol extract caused higher mortalities of rotifer than did by the trichloromethane extract at the equivalent cell densities ( $p < 0.05$ , paired  $t$ -test; **Figure 8A**). Lethal effect of the trichloromethane extract was observed only if the equivalent cell densities were above 10,000 cells·mL<sup>-1</sup> ( $p < 0.05$ , One-way ANOVA; **Figure 8A**).





The heat stability of the toxins of *A. insuetum* was tested via exposing rotifers to the whole live culture and the water extract of algal cells with or without heat pretreatment. The rotifers exposed to the whole culture and the water extract without heat treatment exhibited significantly higher mortalities compared with that exposed to the whole culture and the water extract with heat treatment ( $p < 0.05$ , One-way ANOVA; **Figure 8B**). In addition, there was no significant difference in rotifer mortality among the heat-treated whole culture, heat-treated water extract, and the negative controls (i.e. fresh culture medium;  $p < 0.05$ , One-way ANOVA; **Figure 8B**).

### Effect of *A. insuetum* on Hatching of the Rotifer Eggs

Since in the experiments of exposing rotifer to the whole live culture, cell lysate, and cell-free culture medium, rotifers that survived were observed being capable of producing eggs (**Supplementary Figure 1**), hatching experiments of healthy rotifer eggs were performed using the whole live culture and cell-free culture medium. Hatching success of healthy eggs was observed to be significantly influenced by the live cell cultures and cell-free culture media of *A. insuetum* (**Figure 9**). The hatching rate of eggs was significantly reduced in the cell-free culture medium on day 9 compared with that in the controls ( $p < 0.05$ , One-way ANOVA; **Figure 9**), where 70.8 and 91.7% of eggs successfully hatched in the cell-free culture medium and the fresh culture medium (control), respectively (**Figure 9**). The hatching rates of eggs that were exposed to the whole live culture on day 3 and day 6 were 75 and 70.83%, respectively, significantly lower than that in the fresh culture medium control ( $p < 0.05$ , One-way ANOVA; **Figure 9**).

### Analyses of PSTs and Spirolide

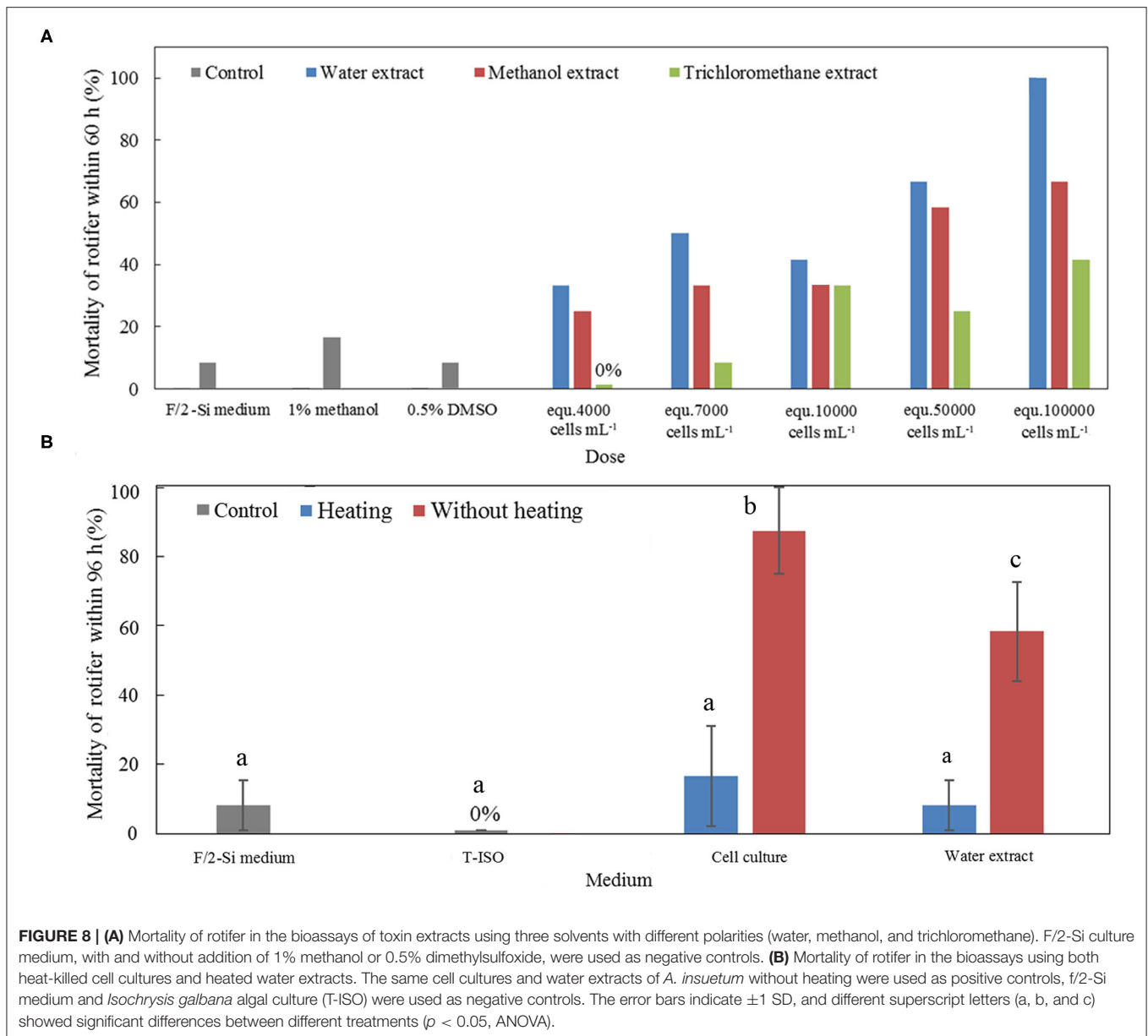
PSP toxins and nine lipophilic marine algal toxins (SPX1, GYM, OA, DTX1, DTX2, AZA1, AZA2, AZA3, and PTX2) were detected in *A. insuetum* strain AILYG12 (**Supplementary Figures 2, 3**). The 13 derivatives of PSTs

(C1, C2, GTX1, GTX4, GTX2, GTX3, GTX5, dcGTX2, dcGTX3, STX, dcSTX, NEO, and dcNEO) were not detected or under the detection limit in *A. insuetum* (**Supplementary Figure 2**) with UPLC-MS-MS analysis. The online SPE coupled with LC-MS/MS analysis showed no indicative peaks of any lipophilic marine algal toxins (**Supplementary Figure 3**), including 13-desmethyl spirolide C (SPX1), which had been detected from some strains of *A. ostenfeldii* (Cembella et al., 2000; MacKinnon et al., 2006; Paredes-Banda et al., 2018) and *A. peruvianum* (Borkman et al., 2012; Tomas et al., 2012).

## DISCUSSIONS

### Morphological and Molecular Characterization

To monitor the dynamics of the toxic *Alexandrium* blooms and the spread of the toxic population, it is extremely important to identify the species/strains accurately (Bravo et al., 2006; Anderson et al., 2012). While the morphology of our isolate is almost identical to the original species descriptions of *A. insuetum* (Balech, 1985), the cell size of our isolates was smaller than that of the isolates from Japan, Korea, and France (Yuki and Yoshimatsu, 1990; Spatharis et al., 2007; Shin et al., 2014). *A. insuetum* is characteristic in small cell size, highly reticulated thecal plates, a disconnection between Po and 1' plate, and the absence of the attachment pore on the Sp plate, which would allow for *A. insuetum* to be differentiated from other *Alexandrium* species (Lilly et al., 2005; Shin et al., 2014; Kim et al., 2017). Cyst morphology of *A. insuetum* was first described by Shin et al. (2014), which is a spherical, 20–25  $\mu\text{m}$  in diameter, and of granular content and three or four red accumulation bodies. This description somewhat differs from our observations in cyst size and general appearance as described above. The significant difference in cyst morphology of *A. insuetum* between the Chinese and Korean strains suggests that it could be extremely difficult to accurately identify the cysts of *Alexandrium* species based on their morphological features only.

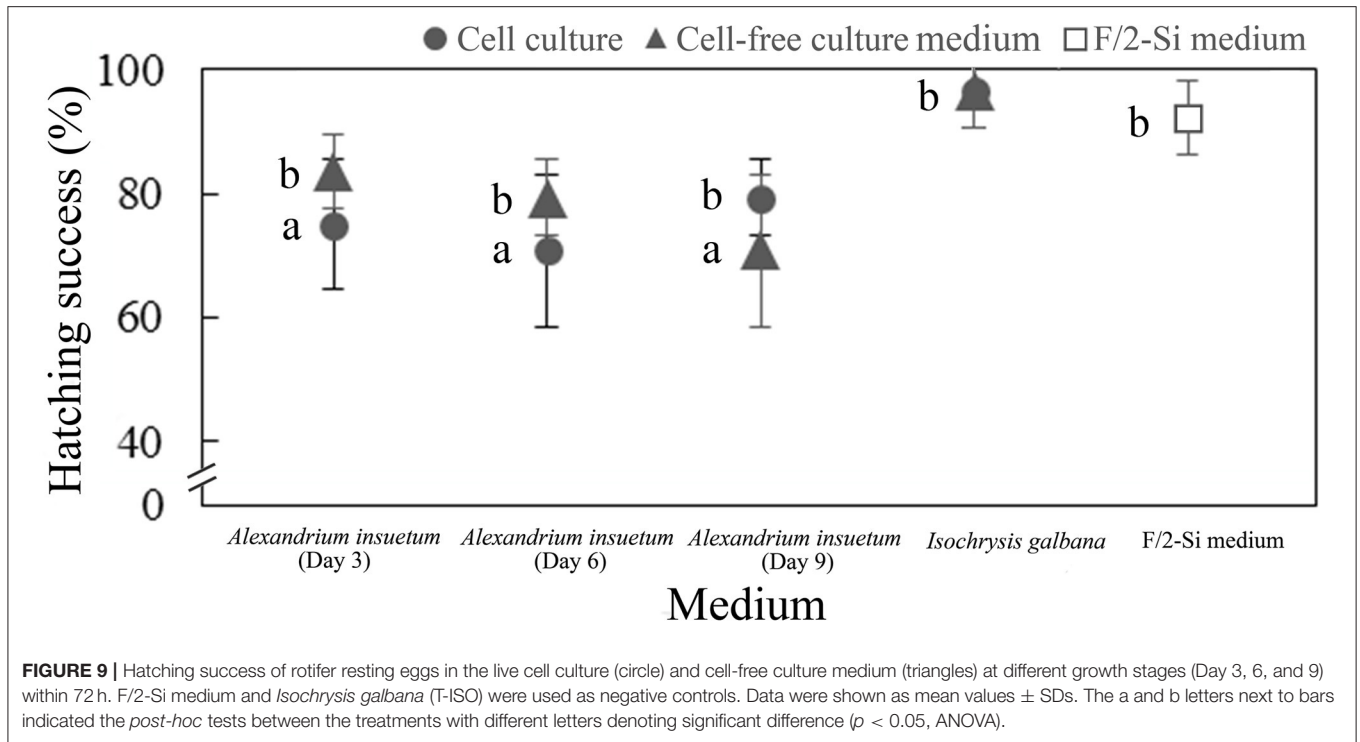


In the phylogenetic tree, while all strains of *A. insuetum* formed a clade with high support, the clade, however, divides into a sub-clade of Asian strains and a sub-clade of French strains, which is in accordance with the results conducted by Lilly et al. (2005) and Shin et al. (2014). The clustering and separation of Asian and French strains agree with their geographic distance, indicating that there may be intraspecific variations in LSU rDNA sequences and even other traits among different geographic populations of *A. insuetum*.

## Toxicity of *A. insuetum* and Its Ecological Implication

Our results clearly showed that *A. insuetum* exhibited significant toxicity to rotifer, brine shrimp, and finfish. Mortality of rotifer

exposed to live cell culture was significantly higher than that exposed to cell lysate and cell-free culture medium, and the death of rotifer was mainly occurred in the last 60 h when exposed to the whole-cell culture, whereas most of the mortalities happened in the first 60 h of bioassays using the cell lysate and cell-free culture medium. Similarly, the whole live culture showed significantly higher toxicity to fish than did the cell-free culture medium and the epifluorescence LM micrographs of dead fish showed the presence of algal cells in the gut, suggesting that the toxins of *A. insuetum* could be accumulated along with the test animals ingesting algal cells during the bioassays, which explains the higher mortality of test animals caused by the whole live culture than that by the cell-free culture medium and cell lysate. Moreover, a number of algal cells appeared near



the finfish gills (see **Figure 6F**), which could be another reason for the fish death. *A. insuetum* with the same or equivalent cell density showed variable lethal effects among the different test animals, which exhibited different sensitivity to the toxins among various groups of animals and, in this work, an order of sensitivity (e.g., brine shrimp, rotifer, and the larval fish). Considering that the test animals differ in body size, body structure, feeding mechanism, and physiological character, we are not able to provide a convincing explanation for their differences in sensitivity, but we believe that the complete identification of toxin(s) will facilitate the intoxication mechanisms in these different animals. In addition, the higher mortality of test animals (rotifer and brine shrimp) caused by the “older” cell-free culture medium indicates that toxins of *A. insuetum* could be continuously released into and accumulated in the culture medium and the toxins may be stable at least for days under room temperatures.

Some *Alexandrium* species may expand their populations to form HABs by suppressing micro-zooplankton (Wang et al., 2003; Lin et al., 2015). For instance, the marine copepod *Acartia tonsa* displayed reproductive impairment when feeding on *A. catenella* alone (Abdulhussain et al., 2021). Our results also revealed that hatching success of rotifer eggs was reduced by *A. insuetum*. More importantly, the eggs produced by the rotifers that had survived the exposure to either the whole live culture or cell-free culture medium of *A. insuetum* exhibited no hatching success at all, suggesting that the sublethal toxicity of *A. insuetum* to the rotifers led to the production of unhealthy eggs. Therefore, the ecological consequences of toxin production in *A. insuetum* could be

viewed from two aspects: while the *A. insuetum*-caused mortality in marine animals will reduce the grazing pressure and thus enhance the growth of *A. insuetum*, on the other hand, the toxicity could affect marine animals not only in on-the-spot survival but also in the reproduction potency as reflected in the no or lowered hatching success of rotifer eggs, which will subsequently influence the population recruitment of the animals.

### Characterization of Toxin(s) Produced by *A. insuetum*

Our work clearly demonstrated that the live cell culture, cell-free culture medium, and crude toxin extracts exhibited lethal effects on rotifers. Taken these results together, the toxins produced by *A. insuetum* were both intra- and extracellular, that is, the toxins were not only present in algal cells but also continuously released into the surrounding medium. Furthermore, the mortality of rotifer exposed to the extracts using water, methanol, and trichloromethane indicates that the toxin(s) is (are) of high polarity (more easily dissolve in water and methanol). We also found that the toxins were not heat stable.

Within the genus *Alexandrium*, PSP toxins have been considered as the most important toxins because they are produced by a number of well-known species and have potent toxicity to human health (Anderson et al., 2012), e.g., *A. catenella*, *A. tamarense*, *A. pacificum*, and *A. minutum* are responsible for the majority of PSP events worldwide (Anderson et al., 2012; Zou et al., 2014; Gu et al., 2021). Spirolides, a group of macrocyclic imines, have been isolated from some strains of *A. ostensfeldii* (Hu et al., 1995; Cembella et al., 2000; Cembella,



2001; MacKinnon et al., 2006; Paredes-Banda et al., 2018) and *A. peruvianum* (Borkman et al., 2012; Tomas et al., 2012). In addition, *A. hiranoi* and *A. monilatum* have been reported to produce goniodomins (Espina et al., 2016). Our previous studies demonstrated that *A. leei* also produces potent allelochemicals (affect phytoplankton) and ichthyotoxins that are hydrophilic, heat-stable, non-PSTs, and non-spiroimidines (Tang et al., 2007; Shang et al., 2021). In this study, analyses by UPLC-MS/MS and SPE-LC-MS/MS demonstrated that the toxins produced by *A. insuetum* were also neither PSTs nor spiroimidines (13-desmethyl spiroide C and gymnodimine), but are heat unstable, suggesting that the toxins of *A. leei* and *A. insuetum* are different. However, PSP toxin has once been detected from a strain of *A. insuetum* isolated from Uchinoumi, Japan (Sako et al., 2004), but without the quantity of specific toxins, while non-toxic strains have also been reported for the isolates from Ofunato Bay and Shodoshima, Japan (Adachi et al., 1994; Lilly et al., 2005). These findings, thus, indicate that toxicity and toxin profiles may vary among different strains and geographic populations of *A. insuetum*.

## CONCLUSIONS

This work represents the first characterization of the morphology, molecular phylogeny, and toxicology of *A. insuetum* from Chinese coastal water. The Chinese isolate exhibited significant lethal effects on rotifer, brine shrimp, and finfish, and also impacted the hatching success of rotifer eggs. Moreover, chemical characterization and toxin analyses demonstrated that the toxin(s) of *A. insuetum* is (are) hydrophilic, heat-labile, non-PSTs, and non-spiroimidines. Therefore, *A. insuetum* may produce novel toxins that are different from those well-known, PSTs-producing *Alexandrium* species, and the toxicity to marine animals may not only contribute to the survival and HABs formation of *A. insuetum*, but also negatively impact the on-spot survival and the population recruitment of marine animals (rotifers in particular, as evidenced in the current work). Therefore, further investigations for the toxin(s) identification are highly desired.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

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## AUTHOR CONTRIBUTIONS

XS, ZH, and YT contributed to the conception and design of the study. XZ isolated the algal cultures of *Alexandrium insuetum*. ZH and XZ performed the LM and SEM microscopic images. XZ conducted the PCR reactions. ZH analyzed the rDNA sequence and constructed the phylogenetic tree. JC and SH performed the PSP and the lipophilic marine algal toxins analysis. LS, YD, and ZC helped with the design of laboratory bioassays. XS conducted the toxicity bioassays, extracted the lipophilic marine algal toxins, performed the statistical analyses, and wrote the primary draft manuscript. XZ, SH, LS, ZC, JC, and ZH revised the draft manuscript. YT perceived the project, designed the experiments, and revised the draft manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.735752/full#supplementary-material>

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