Biochemical characteristics support the recently described species *Karlodinium zhouanum* (Gymnodiniales, Dinophyceae)

Xiaojuan Zhu,1,2* Chengxu Zhou,1,2†* Ran Meng,1,2* Shuang Li,1 Keyi Fang,3 Zhaohue Luo,4 Jilin Xu,1 Shan He,2 Qijun Luo1 and Xiaojun Yan1,2,*

1Key Laboratory of Marine Biotechnology of Zhejiang Province, Ningbo University, Ningbo, China, 2Li Dak Sum Yip Yio Chin Kenneth Li Marine Biopharmaceutical Research Center, Ningbo University, Ningbo, China, 3Ningbo Entry-Exit Inspection and Quarantine Bureau Technology Center, Ningbo, China, 4Third Institute of Oceanography, State Oceanic Administration, Xiamen, China and 5Zhejiang Ocean University, 316022, Zhoushan, China

SUMMARY

The small athecate dinoflagellate *Karlodinium zhouanum* is a species recently described in the coastal waters of China. *K. zhouanum* is morphologically similar to *Karlodinium veneficum*, a typical ichthyotoxic blooming karlotoxin-producing species, and it is impossible to distinguish between these two species based on light microscopy. In this study, strains of *K. zhouanum* isolated from the East China Sea were studied. By analyzing toxins, toxicity, lipid characteristics and typical molecular and physiological traits of this species, *K. zhouanum* was shown to be nontoxic to brine shrimp and widely spread over the coastal waters of China. No karlotoxin-like toxin was detected by liquid chromatography-mass spectrometry (LC-MS). Instead of gymnodinosterol, the critical sterol in toxic *K. veneficum*, 27(nor)-24S-4α-Methyl-5α-ergosta-8(14)-en-3β-ol (NEE) was dominant in *K. zhouanum*, while gymnodinosterol was absent. These sterol characteristics may provide not only support for the species separation between toxic and nontoxic species of *Karlodinium* but also environmental survey tools to differentiate the contribution of nontoxic *Karlodinium* strains, which has been unclear until now.

Key words: DNA size, fatty acid, *Karlodinium zhouanum*, sterol, toxicity.

INTRODUCTION

The dinoflagellate *Karlodinium zhouanum* (Gymnodiniales) is a recently described species in the coastal areas of China (Luo et al. 2018). This species is similar to *K. veneficum*, a toxic species that has caused massive aquaculture losses (Place et al. 2012). Morphologically, the cell size of *K. zhouanum* ranges from 9.9–13.3 μm long and 7.4–10.1 μm wide, which is within the same size range as *K. veneficum*. These two species have similar traits, such as a sulcal intrusion and short straight apical groove, when observed via scanning electron microscopy (SEM). *K. zhouanum* is a fucoxanthin producer, like *K. veneficum* and other species in the *Karlodinium* genus, which are typical species used to study tertiary endosymbiosis of dinoflagellates with fucoxanthin-containing haptophytes (Yoon et al. 2002; Danne et al. 2012). It is impossible to separate the two species solely by light microscopy. The obvious morphological differences between these two species are that *K. zhouanum* lacks ventral pores in SEM. Some other species in the genus *Karlodinium*, such as *K. ballantinum* Salas and *K. antarcticum* Salas (de Salas et al. 2010), also lack ventral pores. However, these cells differ in size, nucleus location, or apical groove length (Luo et al. 2018).

Most *Karlodinium* species are regarded as toxic, even though nontoxic species have been reported (Ballantine 1956; Vaqué et al. 2006; Adolf et al. 2007; Place et al. 2012). Because blooms of toxic *K. veneficum* associated with marine aquatic faunal mortalities have been discovered in America, Europe, Asia, Australia, and other regions (Place et al. 2012), it is critical to differentiate between toxic and nontoxic species. Historically, *Karlodinium* species with no toxins have been reported. *K. vitiligo* (Ballantine 1956), originally isolated in the Plymouth area, is nontoxic and harmless to shellfish (Place et al. 2012). Nontoxic *K. veneficum*, strain MD5, isolated from the Choptank River, Maryland, presented no typical UV absorption of karlotoxin and no negative effects on predators (Adolf et al. 2007). Strain CSIC1, isolated from Alfacs Bay, Spain, was found to reduce egg viability of the copepod *Acartia margaleti* but was not lethal to the copepods, and no toxin was identified in this strain via LC–MS (Vaqué et al. 2006). Nontoxic *Karlodinium* species have not been studied to the same extent as toxic *Karlodinium* species. This discrepancy probably exists because more attention was paid to the toxic species that lead to harmful consequences. However, nontoxic species play roles as primary producers from an ecological point of view, and these cultures are also potential resources for good feed in aquaculture applications. As *K. zhouanum* is a recently established species that is closely

---

*To whom correspondence should be addressed.
Email: zouchengxu@nbu.edu.cn; yanxiaojun@nbu.edu.cn
†These authors contributed equally in this study.
Communicating editor: Kazuhiro Kogame
Received 16 November 2018; accepted 28 April 2019.

© 2019 The Authors. Phycological Research published by John Wiley & Sons Australia, Ltd on behalf of Japanese Society of Phycology

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
related to the toxic blooming species with a worldwide distribution (K. veneficum), examining the eco-physiological and biochemical characteristics of K. zhouanum is necessary.

Karlodinium zhouanum has been reported from the South China Sea and Yellow Sea of China (Luo et al. 2018). The ITS sequence of K. zhouanum was identical to that of the Korean strain KAMS0708, which was found to be a sister clade of K. veneficum with maximal support in molecular phylogenetic analysis (Luo et al. 2018). In this study, we report the unique eco-physiological and biochemical characteristics of K. zhouanum strains newly isolated from the East China Sea (ECS) area. Six strains of K. zhouanum were comparatively studied to profile the characteristics of this species. Taxonomic analysis was performed based on cell morphology and molecular analysis. Toxicity was determined based on the brine shrimp mortality test, and potential toxins were investigated via LC-MS. The growth rates, nuclear DNA size, and fatty acid and sterol composition of the six strains were studied.

MATERIALS AND METHODS

Cell isolation and culture maintenance

Six strains of K. zhouanum were established from field samples at the sites of a coastal survey cruise on June 16–17, 2016. The cruise was carried out in the outer bay area of Xiangshan Bay in the ECS (28.9833°N latitude and 121.9068°E longitude) (Table S1). Aliquots of field water samples from each location were grown in wells of 24-well plates enriched with f/2-Si medium. After Karlodinium-like cells became dominant in the wells, single cells were isolated and transferred into 96-well plates, also enriched with f/2-Si medium, with a micropipette under a light microscope. The plates were incubated at 20–22°C under 50 μmol photon m⁻² s⁻¹ illumination with a light:dark cycle of 14:10 h. After further purification, six non-axenic monoalgal strains were established, namely GM15, −16, −17, −20, −21, −22, and were applied in this study.

Morphology and growth rates

The morphology of the ECS strains was examined and photographed using SEM according to the method of Luo et al. (2018). Briefly, 600 μl of cultures in the exponential phase were fixed with 200 μl of 4% OsO4 (v/v, in filtered seawater) for 1 h. The fixed cells were attached to poly-L-lysine (molecular weight 70 000–150 000) coated coverslips by placing the samples on coverslips for 30 min. The cells were then washed subsequently in filtered seawater, solution of distilled water and filtered seawater (1:1, v/v), and Milli-Q water (Millipore, Bedford, MA, USA) for 10 min each time. To dehydrate the cells, the samples were placed in ethanol solutions containing of 10, 30, 50, 70, 90% ethanol for 10 min at each concentration, followed by three 10 min dehydration steps in 100% ethanol. The samples were critical point dried with a K850 critical point dryer (Quorum/Emitech, West Sussex, UK), sputter-coated with gold, and examined with a Zeiss Sigma FE SEM (Carl Zeiss Inc., Oberkochen, Germany).

To study the growth of the K. zhouanum strains, triplicates of the exponential phase precultures were cultivated in f/2-Si medium (25 psu) with an initial cell density of 3000 cells·ml⁻¹ in 250 ml polystyrene culture flasks with a final water volume of 150 ml. The cultures were placed in an incubator at 20°C under 40 μmol·m⁻²·s⁻¹ illumination with a 12:12 h light:dark cycle. The cell number was determined using a hemocytometer (Sigma-Aldrich, St. Louis, MO, USA) every 2 days. The growth rate was calculated as the slope of the linear phase using In-transformed data.

Cell size was measured by examining and photographing cells using a Nikon microscope (ECLIPSE Ti-U) equipped with a Nikon image analysis system. A drop of each culture in the exponential growth phase was mounted on the microscope. The average cell size (width and length) was obtained by randomly measuring 50 cells.

Nucleotide sequences and cellular DNA content analysis

Genomic DNA extraction according to the manufacturer’s instructions was performed with approximately 10 ml of the culture in the logarithmic growth phase using the EZUP Column Bacterial Genomic DNA Extraction Kit (Sangon Biotech, Shanghai, China). The primers for amplification were P2: 5'-GTAGTGTAACCTGCAAGGATCA-3' and P4: 5'-CGACAKATGC TTAARTTCAGCGG-3'. The sequences covered the whole ITS1-5.8S-ITS2 region and partial SSU and LSU regions. Additionally, the primers Dino1662F(5'CCGATTGAGTGTCCGCGTGAATAA-3') and LSU R2(5' ATTCGGACGTGATGTTGTT AC-3') were used to amplify the LSU sequence. The thermal cycling procedure was 2 min at 94°C, followed by 30 cycles for 30 s at 94°C, 30 s at 55°C, 30 s at 72°C, and a final extension for 2 min at 72°C with a Mastercycler (Eppendorf, Hamburg, Germany) following the manufacturer’s protocol (TakaRa). The PCR products were collected using a DNA gel extraction kit (OMEGA), cloned into the pMD18-T vector (TakaRa) and sequenced.

Phylogenetic relationships based on the LSU sequence were inferred using the neighbor-joining (NJ) method. The pairwise nucleotide differences of ITS sequences among the different strains were calculated with MEGA version 7.0. Standard deviations were calculated by 10 000 bootstrap replicates.

For DNA content analysis, 100 ml of each culture in the exponential growth phase (6000–8000 cells·ml⁻¹) was synchronized by placing the culture in darkness for 48 h. Twenty microliters of the culture was fixed with 1% paraformaldehyde for 10 min and washed in PBS (pH 7. Sigma-Aldrich) by centrifugation at 1200 g for 10 min. The pellet was resuspended in 0.6 ml PBS containing 1.25 ng Hoechst 33342 and 100 μg RNaseA. A total of 0.5 ml of the suspension was analyzed by a Beckman Gallios Flow Cytometer (Beckman Counter, Inc., Brea, CA, USA) with a laser emitting at 405 nm. Samples were run at medium speed, and data were acquired in linear and log modes until approximately 10 000 events had been recorded. Human hepatocyte L-02 cells from the China Center for Type Culture Collection (Wuhan, China) were used as internal standards. These cells were freshly prepared in Dulbecco's Modified Eagle’s Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) (Gibco BRL, Grand Island,
NY, USA) maintained in a humidified incubator at 37°C with 5% CO₂ and 95% air. The fluorescence emission of dsHoechst 33 342 was detected at 450 nm. Cell ploidy, peak numbers, coefficients of variation (CVs) and the peak ratios for DNA fluorescence distributions in a population were computed with a ModFit LT (Verity software House). Hepatocyte L-02 cells in the G0 phase were selected as the standard for calculating the sample DNA content according to the following equation:

$$\text{sample DNA content} = \frac{a}{b} \times c,$$

where a is the sample fluorescence signal value, b is the standard fluorescence signal value, and c is the standard DNA content.

Aliquots of Hoechst 33342-dyed cells were mounted on a Zeiss laser scanning confocal microscope (LSM880; Carl Zeiss, Germany) to observe and photograph cell nuclei.

Toxicity and toxin detection

The toxic strain of K. veneficum (GM2, in which toxin had been demonstrated by Cai et al. (2016)) was used as a positive control in this study. Algal cultures grown to exponential phases in sterile natural seawater (25 psu) enriched with f/2-Si medium were used for the toxicity and karlotoxin detection analyses.

Toxicity was determined based on the mortality of brine shrimp. Briefly, active nauplii of brine shrimp freshly hatched in 24 h were added to 24-well plates, with nine nauplii per well, in up to 1 ml of total water volume. An additional 1 ml of algal culture in the exponential phase was added to each well. The final density of each algal strain was approximately 1.5 x 10⁵ cells/ml. Nauplii grown in fresh culture medium without any food were used as the starving control. All treatments were replicated six times. The plates were placed under conditions described above. Dead nauplii were counted at 12, 24, 48, and 72 h after the initiation of incubation. The percentage of dead nauplii out of the nine initial nauplii was documented per well.

For karlotoxin analysis, the methods of Bachvaroff et al. (2008) and Krock et al. (2017) were adopted with modifications. Briefly, 600 ml of each algal culture was used for toxin extraction. UV absorption of the extracts was measured using a Thermo Evolution 201 spectrophotometer and was further analyzed on a UPLC-Q-TOF-MS (Waters, Milford, MA, USA). Two formerly purified karlotoxin solutions from GM2, 4, 5-dihydro-dechloro-KmTx 2 and 4, 5-dihydro-KmTx 2 (Cai et al. 2016), were run in precursor ion experiments with typical KmTx fragments to determine the possible presence of KmTx-related compounds.

Fatty acids and sterols

Cultures of K. zhouanum strains were grown in f/2-Si medium to a final volume of 4000 ml in 5000 ml glass flasks in an air-conditioned culture room at 20 ± 2°C under 40 μmol·m⁻²·s⁻¹ illumination with a 12:12 h light:dark cycle. Cultures in the exponential phase were harvested by centrifugation at 12 000 g at 4°C. The algal pellets were freeze-dried and stored at −20°C until analysis.

Analysis of fatty acids, sterols, sterol silyl ethers were performed according to previously published methods (Xu et al. 2008) with some modifications. Briefly, the total lipids from 200 mg of each frozen dried microalgal sample were extracted using the modified Bligh-Dyer method (Bligh & Dyer 1959). Following saponification in MeOH-H₂O (4:1, v/v) with 5–6% KOH under an N₂ atmosphere at 60°C for 2 h and acidification with hydrochloric acid (1:1) to adjust the pH to less than 1, a mixture of fatty acids and sterols was obtained via three successive extractions with hexane-chloroform (4:1, v/v). Subsequently, fatty acid methyl esters were formed via esterification by heating the mixture with 14% BF₃-CH₃OH at 60°C for 1 h, and the trimethylsilyl ethers of sterols were formed by treatment with bis(trimethylsilyl) trifluoroacetamide (BSTFA) for 0.5 h at 80°C. The sample was then dried with N₂, dissolved in hexane and analyzed using a gas chromatography-triple quadrupole mass spectrometer (Agilent 7890B-7000C, Palo Alto, CA) with an HP-5 MS column (0.25 μm, 30 m x 0.25 mm, Agilent, USA). The fatty acid structures were identified by NIST and WILEY library entries. In addition, the sterol structures were identified by comparing the mass spectra according to the typical mass spectra patterns of sterol trimethylsilyl (TMS) derivatives (Miao et al. 2008), NIST and WILEY library entries, and previously published GC/MS data on sterols (Barrett et al. 2010; Leblond and Chapman, 2010b; Leblond and Volkman et al. 1998; Volkman 2003). Peak areas were used to determine the relative fatty acid and sterol proportions.

RESULTS

Morphology and growth rates

The cell size of the ECS strains of K. zhouanum ranged from 10.12–11.31 μm in length and 6.61–7.45 μm in width. The

| Strain | Length(L) (μm) | Width(W) (μm) | L/W ratio | Strain | DNA content (pg/cell)(CV) | Strain | Growth rate (/d) |
|--------|----------------|--------------|-----------|--------|--------------------------|--------|-----------------|
| GM20   | 11.31 ± 0.98   | 7.45 ± 0.88  | 1.53 ± 0.20| GM22   | 8.81(2.27)               | GM15   | 0.29            |
| GM22   | 10.17 ± 0.73   | 7.63 ± 0.94  | 1.47 ± 0.15| GM17   | 7.21(2.78)               | GM22   | 0.26            |
| GM17   | 10.60 ± 1.07   | 7.10 ± 1.02  | 1.51 ± 0.20| GM20   | 6.91(2.90)               | GM20   | 0.25            |
| GM21   | 10.54 ± 1.17   | 6.97 ± 0.73  | 1.52 ± 0.15| GM15   | 6.21(3.92)               | GM21   | 0.25            |
| GM15   | 10.37 ± 1.14   | 6.78 ± 0.98  | 1.55 ± 0.21| GM21   | 5.61(3.57)               | GM17   | 0.24            |
| GM16   | 10.12 ± 1.10   | 6.61 ± 0.80  | 1.55 ± 0.22| GM16   | 5.51(3.64)               | GM16   | 0.23            |
Sterol of nontoxic Karlodinium zhouanum

Nucleotide sequences and nuclear DNA content analysis

The ITS sequences of the six strains were aligned with those of strain TIO398 from Daya Bay, South China Sea, and the Korean strain KAMS0708 (Fig. S2). The 5.8S regions of the strains were identical, except for strain GM17, which had one base variation. Among the strains, base variations mainly occurred in the ITS1 region (five positions) and ITS2 region (three positions) (Fig. S2). Phylogenetic analysis using MEGA7.0 based on the nuclear-encoded LSU sequences showed that all ECS strains of K. zhouanum were clustered with strain TIO398 of the same species from Daya Bay, South China Sea, forming a sister clade with the species in the genus Karlodinium (Fig. 2).

The DNA content of the six ECS strains were evaluated with human hepatocyte L-02 cells as a reference standard using a Beckman Gallios Flow Cytometer based on the linear and log modes (Fig. S3). As shown in Table 1, the DNA content ranged from 5.51 to 8.81 pg./cell. The coefficients of variation (CVs) were less than 4, indicating the method's accuracy and the instrument's stability.

Toxicity and toxin detection

No toxic activity on brine shrimp was detected for either ECS K. zhouanum strains in the 72 h exposures (Fig. 3). While the K. veneficum strain GM2 of the same density had significant lethal effects on brine shrimp and increasing mortality was also found in the pure culture medium controls as a result of starvation, no dead brine shrimp were found in cultures of K. zhouanum. In fact, brine shrimp were found feeding on K. zhouanum, completely eliminating the algae from the wells by the end of the detection period.

HPLC analysis of the karlotoxin reference standard, 4, 5-dihydro-dechloro-KmTx 2 and 4, 5-dihydro-KmTx 2, purified from K. veneficum (strain GM2, Cai et al. 2016) showed two major peaks with typical karlotoxin UV characteristics at 225 nm and 235 nm (Fig. 4a). However, no such peaks were present in extracts of the ECS K. zhouanum strains (Fig. 4b, strain GM17 as representative). In mass spectrum analysis, while the karlotoxin reference standard showed typical mass peaks of 1313.8630 in positive ion mode and 1311.8474 and 1345.8052 in negative ion mode (Fig. 5a), no characteristic karlotoxin-like mass peaks appeared at the appropriate retention times for the elution of K. zhouanum strains, either in the positive or the negative mode (Fig. 5b, results of GM17 as representative). According to the results from the toxicity and toxin analysis, K. zhouanum is a nontoxic species.

Fatty acid and sterol content

Regarding fatty acid composition (Table 2), a total of 15 fatty acids were detected in the studied species. C14:0 (18.26–23.95%), C16:0 (15.15–20.54%), C16:1 (7.35–12.27%), C18:1 (8.68–12.79%) and C22:6 (18.90–22.62%) were the predominant fatty acids. Saturated fatty acids accounted for 39.88–47.21% of the total fatty acid content, with the major saturated fatty acids comprising myristic acid (C14:0) and palmitic acid (C16:0), followed by...
stearic acid (C18:0). Monounsaturated fatty acids composed
18.06 – 22.78% of the total fatty acid content, with only three
fatty acids (C16:1(n-7), C18:1(n-9) and C18:1(n-7)) present
ubiquitously. Polyunsaturated fatty acids accounted for vari-
ous percentages of the total fatty acid content (33.07% –
41.01%), and docosahexaenoic acid (DHA C22:6) was the
predominant polyunsaturated fatty acid found in all strains
(18.90 – 22.62%), followed by C20:5, C18:3 and C18:2.

In total, seven sterols, which mainly contained 27 to 30 car-
bons, were detected in K. zhouanum (Table 3). Regarding the
sterol composition, 27(nor)-24S-4α-Methyl-5α-ergosta-8(14)-
en-3β-ol (NEE, Table 3) was the most dominant sterol structure
found in all six strains of the nontoxic species K. zhouanum
and accounted for 54% to 84% of the total sterol content. Ste-
rol 27(nor)-24S-4α-Methyl-5α-ergosta-8(14), 22-dien-3β-ol
(NED, Table 3) accounted for 4% to 10% of the total sterol
content. Some common sterols, such as stigmasterol [C29:2
(Δ5,24Ethyl)], beta-sitosterol [C29:2(Δ5,24Ethyl)] and C29:2
(Δ5,24(28)/24Ethyl), were also observed in considerable
amounts, accounting for 4% to 15% of the total sterol
content.

**DISCUSSION**

Morphological differences between K. zhouanum and closely
related species have been extensively compared when the
new species was first described (Luo et al. 2018). Based on
the results of morphological and molecular sequence ana-
lyses, the existence of K. zhouanum in the ECS area was dem-
strated in the present study. From results of this study and
the study of Luo et al. (2018), the distribution of this species
extends from the south to the north of the coastal area of
China.
Small athecate dinoflagellates are normally found in estuaries or coastal areas worldwide (Ballantine 1956; Hallegraeff 1992; de Salas et al. 2003, 2005; Dai et al. 2014; Luo et al. 2018). Because of the multiple toxins, such as karlotoxins, gymnodimines and brevetoxins, produced by species in the family Kareniaceae, blooms of toxic species have caused massive fishery losses (Ballantine 1956; Baden 1989; Kempton et al. 2002; Adolf et al. 2006; Marrouchi et al. 2012). However, it is difficult to identify or differentiate dinoflagellate species in the family Kareniaceae, which contains three genera, Karlodinium, Karenia, and Takayama, because the cells are mostly small and identical when observed using light microscopy (Bergholtz et al. 2010). Physiological and biochemical traits are useful taxonomical characteristics in differentiating species. What is more is that, recognizing and discerning Kareniaceae cells are important for evaluating their significance in ecosystems. Even though nontoxic Karlodinium species have been frequently reported and described (Ballantine 1956; Vaqué et al. 2006; Adolf et al. 2007; Place et al. 2012), there are few extensive studies on these species. In the present study of K. zhouanum, no karlotoxin was detected via LC–MS, and no toxicity was indicated against brine shrimp. In fact, K. zhouanum supported the growth of brine shrimp. In the sampling areas of this study, both toxic K. venezicum and nontoxic K. zhouanum were detected (Huang et al. 2019). Questions about how these two closely related species interact with each other, the ecological significance of population dynamics in the field, and whether the potentially nontoxic K. zhouanum supports the growth of the omnivorous mixotrophic K. venezicum should be considered for future studies in the laboratory and in the field.

It is generally believed that the main fatty acids of dinoflagellates are C16:0, C18:4(n-3), C18:5(n-3), C20:3(n-3) and C22:6(n-3). Interestingly, C18:5(n-3) was once considered a useful signature compound of photosynthetic dinoflagellates (Joseph 1975) but is now known to be present in other algal classes (Volkman et al. 1989; Dunstan et al. 1992) and to be absent in strain GM20 (this study) and Amphidinium carterae Hulburt CCMP1687 (Leblond & Chapman 2010a). In addition, the content of C14:0 in Dinophyceae has been reported as less than 10% but was greater than 18% (18.26–23.95%) in the present study. In addition, C18:2, C18:3 and C20:5 were detected in all strains, with a smaller content (1.13–5.70%) than that of C14:0.

For mixotrophic toxic K. venezicum, karlotoxins confer advantages. Firstly, karlotoxins can be excreted out of the cells to paralyze prey (Sheng et al. 2010). Secondly, karlotoxins target unique sterols in the cell membrane of prey, generating pores that result in cellular malfunction, such as osmotic loss and cell lysis (Deeds 2003; Deeds & Place 2006). Thirdly, unique sterols of toxic K. venezicum help the species itself to avoid the adverse effects of karlotoxins (Deeds & Place 2006). However, the sterol compositions in the new species of nontoxic K. zhouanum exhibit very distinctive characteristics such as the absence of 24S-4α-Methyl-5α-ergosta-8(14),22-dien-3β-ol (ED) and the existence of a new dominant sterol, 27(nor)-24S-4α-Methyl-5α-ergosta-8(14),en-3β-ol (NEE), as well as 27(nor)-24S-4α-Methyl-5α-ergosta-8(14),22-dien-3β-ol (NED). Based on recent studies, ED and NED are considered unusual sterols in Karlodinium and Karenia and possess uncommon Δ8(14) nuclear unsaturations, as indicated during a survey of the sterol compositions of related dinoflagellate species (Leblond & Chapman 2010b). ED and NED were further named...
Fig. 5. Liquid chromatography traces for purified toxin extracts in *Karlodinium veneficum* strain GM2 (a) and in *K. zhouanum* (b), strain GM17 as representative) performed on a UPLC-Q-TOF-MS (Waters, USA). Purified karlotoxin solutions from GM2, 4, 5-dihydro-dechloro-KmTx 2 and 4, 5-dihydro-KmTx 2 (Cai et al. 2016), were run in precursor ion experiments with typical karlotoxin mass peaks to determine the possible presence of karlotoxin-like compounds in the sample extracts. No typical mass peak appeared in extracts of *K. zhouanum*.

Table 2. Fatty acid composition of strains of *Karlodinium zhouanum* isolated from the East China Sea

|       | GM15 | GM16 | GM17 | GM20 | GM21 | GM22 |
|-------|------|------|------|------|------|------|
| C14:0 | 18.85| 21.30| 18.86| 23.95| 20.22| 18.26|
| C16:0 | 16.13| 20.20| 17.14| 15.15| 17.88| 20.54|
| C18:0 | 4.90 | 5.71 | 5.49 | 6.57 | 6.49 | 5.78 |
| Saturates sum | 39.88 | 47.21 | 41.49 | 45.67 | 44.59 | 44.59 |
| C16:1 n7 | 10.43 | 7.35 | 10.93 | 12.27 | 11.98 | 7.54 |
| C18:1 n9 | 5.53 | 6.46 | 7.11 | 4.93 | 7.00 | 9.27 |
| C18:1 n7 | 3.15 | 4.25 | 4.74 | 2.62 | 3.35 | 3.52 |
| Monounsaturates sum | 19.11 | 18.06 | 22.78 | 19.82 | 22.33 | 20.33 |
| C16:3 n3 | 2.00 | 1.91 | 0.00 | 2.28 | 1.64 | 0.00 |
| C16:2 n6 | 0.00 | 0.96 | 0.00 | 0.00 | 0.00 | 0.00 |
| C18:5 n3 | 2.05 | 0.46 | 2.23 | 0.00 | 2.62 | 3.55 |
| C18:4 n3 | 3.63 | 3.06 | 3.15 | 2.26 | 1.93 | 0.00 |
| C18:3 n3 | 3.40 | 2.63 | 1.13 | 5.70 | 1.79 | 5.47 |
| C18:2 n6 | 3.57 | 3.16 | 3.61 | 3.52 | 4.69 | 4.84 |
| C20:5 n3 | 3.55 | 1.22 | 1.14 | 1.63 | 1.51 | 2.13 |
| C20:4 n3 | 0.19 | 0.00 | 2.02 | 0.00 | 0.00 | 0.00 |
| C22:6 n3 | 22.62 | 21.33 | 22.45 | 19.12 | 18.90 | 19.09 |
| Polynsaturates sum | 41.01 | 34.73 | 35.74 | 34.50 | 33.07 | 35.08 |
gymnodinosterol and brevesterol, respectively, by Giner et al. (2010) to underline their importance as potential chemotaxonomic biomarkers, as supported by Mooney et al. (2010). Interestingly, Deeds and Place (2006) suggested that these two sterols, especially gymnodinosterol, can provide self-protection from the karlotoxins released from toxic *K. venenatum*. Therefore, the absence of gymnodinosterol and the occurrence of 27(nor)-24S-4α-ethyl-â-ergosta-8(14), en-3β-ol (NEE) in nontoxic *K. zhouanum*, particularly since toxic *K. venenatum* contains gymnodinosterol and brevesterol, deserves more attention in the future. Further studies should investigate the chemical information of these species, as the occurrence of ED (gymnodinosterol) in toxic *Karlodinium* species and NEE in nontoxic *Karlodinium* species not only provide support for the separation between toxic and nontoxic species of genus *Karlodinium* but also provide environmental survey tools to differentiate the contribution of nontoxic strains, which has been unclear until now.

**ACKNOWLEDGMENTS**

This work was supported financially by the National Key Research and Development Program of China (2018YFD0900702); the Earmarked Fund for Modern Agro-industry Technology Research System, China (CARS-49); the Li Dak Sum Yip Yio Chin Kenneth Li Marine Biopharmaceutical Development Fund; the National 111 Project of China; and partially supported by the Laboratory of Marine Ecosystem and Biogeochemistry, Second Institute of Oceanography, China (LEMB201705); the K.C. Wang Magna Fund in Ningbo University; and high throughput screening of marine toxins in marine products based on LC-HRMS (2017K070).

**REFERENCES**

Adolf, J., Bachvaroff, T., Krupatkina, D. et al. 2006. Species specificity and potential roles of *Karlodinium micrum* toxin. *S Afr. J. Mar. Sci.* 28: 415–9.

Adolf, J., Krupatkina, D., Bachvaroff, T. and Place, A. 2007. Karlotoxin mediates grazing by *Oxyrrhis marina* on strains of *Karlodinium venenatum*. * Harmful Algae* 6: 400–12.

Bachvaroff, T., Adolf, J., Squier, A., Harvey, H. and Place, A. 2008. Characterization and quantification of karlotoxins by liquid chromatography-mass spectrometry, *Harmful Algae* 7: 473–84.

Baden, D. G. 1989. Brevetoxins: unique polyether dinoflagellate toxins. *FASEB J.* 3: 1807–17.

Ballantine, D. 1956. Two new marine species of Gymnodinium isolated from the Plymouth area. *J. Mar. Biol. Assoc. U. K.* 35: 467–74.

Barrett, S., Volkman, J., Dunstan, G. and Jeannie-Marie, L. 2010. Sterols of 14 species of marine diatoms (Bacillariophyta). *J. Phycol.* 31: 360–9.

Bergholtz, T., Daugbjerg, N. and Moestrup, O. 2010. On the identity of Karlodinium venenatum and description of Karlodinium armiger sp. nov. (Dinophyceae), based on light and electron microscopy, nuclear-encoded LSU rDNA, and pigment composition. *J. Phycol.* 42: 170–93.

Bligh, E. and Dyer, W. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37: 911–7.

Cai, P., He, S., Zhou, C. et al. 2016. Two new karlotoxins found in Karlodinium venenatum (strain GM2) from the East China Sea. *Harmful Algae* 58: 66–73.

Dai, X., Lu, D., Guan, W. et al. 2014. Newly recorded Karlodinium venenatum dinoflagellate blooms in stratified water of the East China Sea. *Deep-Sea Res. II Top. Stud. Oceanogr.* 101: 237–43.

Danne, J., Gornik, S. and Waller, R. 2012. An assessment of vertical inheritance versus endosymbiotic transfer of nucleus-encoded genes for mitochondrial proteins following tertiary endosymbiosis in *Karlodinium micrum*. *Protist* 163: 76–90.

Deeds, J. 2003. Toxins and toxicity from the cosmopolitan, bloom-forming dinoflagellate *Karlodinium micrum*. Ph.D. thesis, University of Maryland, Centre of Marine Biotechnology, College Park, MD.

Deeds, J. and Place, A. 2006. Sterol-specific membrane interactions with the toxins from *Karlodinium micrum* (Dinophyceae) — a strategy for self-protection? *Afr. J. Mar. Sci.* 28: 421–5.

de Salas, M., Bolch, C., Botes, L., Nash, G., Wright, S. and Hallgraeff, G. 2003. Takayama gen. nov. (Gymnodiniales, Dinophyceae), a new genus of unarmored dinoflagellates with sigmoid apical grooves, including the description of two new species. *J. Phycol.* 39: 1233–46.

de Salas, M., Laza-Martinez, A. and Hallgraeff, G. 2010. Novel unarmored dinoflagellates from the toxigenic family Kareniaceae (Gymnodiniales): five new species of *Karlodinium* and one new *Takayama* from the Australian sector of the southern ocean. *J. Phycol.* 44: 241–57.

de Salas, M., Rhodes, L., Mackenzie, L. and Adamson, J. 2005. Gymnodinoid genus Karenia and Takayama (Dinophyceae) in New Zealand coastal waters. *New Zeal. J. Mar. Freshw. Res.* 39: 135–9.

Dunstan, G., Volkman, J., Jeffrey, S. and Barrett, S. 1992. Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae lipid classes and fatty acids. *J. Exp. Mar. Biol. Ecol.* 161: 115–34.

Giner, J., Faraldos, J. and Boyer, G. 2010. Novel sterols of the toxic dinoflagellate *Karenia brevis* (Dinophyceae): a defensive function for unusual marine sterols? *J. Phycol.* 38: 315–9.

Hallgraeff, G. 1992. Harmful algal blooms in the Australian region. *Mar. Pollut. Bull.* 25: 186–90.

Huang, H., Shao, Q., Zhu, X. et al. 2019. Distribution of Karlodinium venenatum in the coastal region of Xiangshan Bay in the East China Sea, as detected by a real-time quantitative PCR assay of ribosomal ITS sequence. *Harmful Algae* 81: 65–76.
Joseph, J. 1975. Identification of 3, 6, 9, 12, 15-octadecapentaenoic acid in laboratory-cultured photosynthetic dinoflagellates. *Lipids* **10**: 395–403.

Kempton, J., Lewitus, A., Deeds, J., Law, M. and Place, A. 2002. Toxicity of *Karenia mikimotoi* (Dinophyceae) associated with a fish kill in a south Carolina brackish retention pond. *Harmful Algae* **1**: 233–41.

Krock, B., Busch, J., Tillmann, U. et al. 2017. LC-MS/MS detection of karotoxins reveals new variants in strains of the marine dinoflagellate *Karlodinium veneficum* from the Ebro Delta (NW Mediterranean). *Mar. Drugs* **15**: 391.

Leblond, J. and Chapman, P. 2010a. Lipid class distribution of highly unsaturated long chain fatty acids in marine dinoflagellates. *J. Phycol.* **36**: 1103–8.

Leblond, J. and Chapman, P. 2010b. A survey of the sterol composition of the marine dinoflagellates *Karenia brevis*, *Karenia mikimotoi*, and *Karlodinium mikromicrum*; and *Karlodinium veneficum* distribution of sterols within other members of the class Dinophyceae. *J. Phycol.* **38**: 670–82.

Luo, Z., Wang, L., Chan, L., Lu, S. and Gu, H. 2018. *Karlodinium zhouanum*, a new dinoflagellate species from China, and molecular phylogeny of *Karenia digitata* and *Karenia longicanalis* (Gymnodiniales, Dinophyceae). *Phycologia* **57**: 401–12.

Marrouchi, R., Benoit, E., Kharrat, R. and Molgó, J. 2012. Gymnodimines: a family of phyctotoxins contaminating shellfish. In Benoit, E., Goudey-Perrêtre, F., Marchot, P. and Servent, D. (Eds). *Toxins and Signalling*. ISSN: 1760-6004. SFET Publications, Châtenay-Malabry, France, pp. 79–83.

Miao, M., Ji-Lin, X. U., Yan, X. J. and Hou, Y. D. 2008. Study of the regular pattern of Mass spectrometry of TMS derivatives of sterols. *J. Instrum. Anal.* **27**: 59–62.

Mooney, B., Nichols, P., De Salas, M. and Hallegreff, G. 2010. Lipid, fatty acid, and sterol composition of eight species of Kareniaceae (Dinophyta): Chemotaxonomy and putative lipid phycotoxins. *J. Phycol.* **46**: 101–11.

Place, A., Bowers, H., Bachvaroff, T., Adolf, J., Deeds, J. and Sheng, J. 2012. *Karlodinium veneficum* —The little dinoflagellate with a big bite. *Harmful Algae* **14**: 179–95.

Sheng, J., Malkiel, E., Katz, J., Adolf, J. and Place, A. 2010. A dinoflagellate exploits toxins to immobilize prey prior to ingestion. *Proc. Natl. Acad. Sci. U. S. A.* **107**: 2082–7.

Vaqué, D., Estrada, M., Calbet, A., Felipe, J. and Sala, M. 2006. Effects of the toxic dinoflagellate *Karlodinium* sp. (cultured at different N/P ratios) on micro and mesozooplankton. *Sci. Mar.* **70**: 59–65.

Volkman, J. 2003. Sterols in microorganisms. *Appl. Microbiol. Biotechnol.* **60**: 495–506.

Volkman, J., Barret, S., Blackburn, S., Mansour, M., Sikes, E. and Gelin, F. 1998. Microalgal biomarkers: a review of recent research developments. *Org. Geochem.* **29**: 1163–79.

Volkman, J., Jeffrey, S., Nichols, P., Rogers, G. and Garland, C. 1989. Fatty acid and lipid composition of 10 species of microalgae used in mariculture. *J. Exp. Mar. Biol. Ecol.* **128**: 219–40.

Xu, Z., Yan, X., Pei, L., Luo, Q. and Xu, J. 2008. Changes in fatty acids and sterols during batch growth of *Pavlova viridis* in photobioreactor. *J. Appl. Phycol.* **20**: 237–43.

Yoon, H., Hackett, J. and Bhattacharya, D. 2002. A single origin of the peridinin- and fucoxanthin-containing plastids in dinoflagellates through tertiary endosymbiosis. *Proc. Natl. Acad. Sci. U. S. A.* **99**: 11724–9.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Growth of ECS *Karlodinium zhouanum* strains (GM15, −16, −17, −20, −21, −22) in 1/2-Si medium (salinity 25 psu) under 20 °C at 40 μmol·m⁻²·s⁻¹ illumination (light: dark = 12:12 h).

**Fig. S2.** Alignment of ITS sequences of strains of *Karlodinium zhouanum*. GM15, −16, 17, −20, −21, −22 from the ECS area, strain TIO398 from Daya Bay, South China Sea, and strain KAMS0708 from Korean coastal water.

**Fig. S3.** Flow cytometry histograms of the cellular DNA content of the ECS strains of *Karlodinium zhouanum* compared with human hepatocyte L-02 cells.

**Table S1.** Strain information of the *Karlodinium* species used in the study. NBAlgae: The Microalgae Collection Center of Ningbo University.