***Centrodinium punctatum* (Dinophyceae) produces significant levels of saxitoxin and related analogs**

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Abstract :

Centrodinium punctatum is a fusiform dinoflagellate with a global marine distribution. Due to a close phylogenetic relationship of one *C. punctatum* strain to *Alexandrium* species, toxin production of this *C. punctatum* strain was assessed using liquid chromatography coupled to tandem mass spectrometry. The paralytic shellfish toxin (PST) profile of *C. punctatum* was dominated by six analogs, i.e. STX (30%), GTX-1 (20%) and neoSTX (24%), followed by GTX-2 (9%), GTX-4 (9%) and GTX-3 (8%); deoxy-STX was also putatively identified while no gymnodimines, spirolides or goniodomins were detected. This is the first record of *C. punctatum* producing saxitoxins. The estimated cellular toxicity was rather elevated, between 91 and 212 pg cell⁻¹ (or 259 and 601 fmol cell⁻¹). When considering the toxicity equivalent factors, results suggest that this species can produce high cellular toxicity compared to other STX-producing dinoflagellates. Morphological details of the sulcal area and the hypotheca of *Centrodinium punctatum* were re-examined by scanning electron microscopy (SEM); this revealed that in the sulcal area, the left posterior sulcal plate (Ssp) is larger and longer than the left posterior sulcal plate and extended into the hypotheca. Based on the morphological observation, a revised interpretation of the sulcus and hypotheca is proposed.

Keywords : Dinoflagellate, *Alexandrium*, PST, STX, neoSTX, Gonyautoxin

1. Introduction

The structure of saxitoxin has been clarified in 1975 and > 50 analogs have now been identified in several marine dinoflagellates and some freshwater cyanobacteria (Wiese et al., 2010), and paralytic shellfish poisoning (PSP) has been reported for more than 80 years (e.g. Sommer et al. 1937; extensively reviewed in Anderson et al., 2012; Yñiguez et al., 2020).— Especially in the 1980s and 1990s, several PSP outbreaks in many coastal areas have caused human intoxications and death, serious economic losses in fisheries industries and negative impacts on marine ecosystems (e.g. Chang et al. 1987; Hallegraeff, 1993; Azanza and Taylor, 2001; Anderson et al. 2012). Due to such outbreaks, regular monitoring of dinoflagellates in the water column and of toxins in shellfish is typically carried out in many coastal areas of the world (e.g. EFSA, 2009; Nagai et al., 2019).

To date, ~~16 a dozen~~ species ~~belonging to of~~ three genera (~~14~~ *Alexandrium* species, *Gymnodinium catenatum* and *Pyrodinium bahamense*) have been reported as the main toxic dinoflagellates producing STX and analogs and are hence responsible for PSP (Harada et al., 1982; Oshima 1987; Anderson et al., 1994; Taylor et al., 2003; Moestrup et al., 2009; Tomas et al., 2012; John et al., 2014; Murray et al., 2015; Lassus et al., 2016; Band-Schmidt et al., 2019; [Mertens et al., 2020](#)). Of these, *Alexandrium* species are most significant because they are frequently abundant and globally spread STX producers in subarctic, temperate and tropical areas (Anderson et al., 1994; Taylor et al., 2003; Hallegraeff, 2003). According to Anderson et al. (2012), among the more than 30 species in the genus *Alexandrium*, at least half are known to be toxic or to have otherwise harmful effects, and in particular, the *Alexandrium tamarense* complex (~~A. tamarense and related species~~), and *A. minutum* are currently considered to be important toxic species in many coastal areas.

The taxonomic identification of *Alexandrium* species is based on a unique combination of morphological characters. Such characters include the cell size and shape, the ~~shape form~~ of the first apical and sixth precingular plate, the thecal ornamentation, the presence or absence of sulcal lists, the shape of the apical pore, the size and shape of the sulcal plates, the presence or absence of a ventral pore, and the connection between the pore plate and the 1' plate (Balech, 1995). More recent studies combine this with additional molecular sequence data for species identification (Lilly et al., 2005; Orr

et al., 2011; Gu et al., 2013; Shin et al., 2014). ~~In~~ Li et al. (2019), ~~we~~ found that the genus *Alexandrium* is not monophyletic, due to the nesting of the fusiform dinoflagellate *Centrodinium punctatum* within *Alexandrium* species, despite morphological differences between both genera. *Centrodinium* species have rarely been reported, and their taxonomic and phylogenetic positions, distribution and eco-physiological characteristics are unstudied.

Li et al. (2019) postulated that *C. punctatum* does not produce STXs, based on lacking *sxtA* gene, and thus no analysis of STXs was carried out. However, such causal relationship between *sxtA* gene and STX production requires further investigation, as some exceptions are known (Stüken et al., 2011; Orr et al., 2013). *C. punctatum* displayed the closest genetic resemblance to *A. affine* (Li et al., 2019). According to Orr et al. (2011), *A. affine* is a non-STX producing species at the base of a clade containing most of the PST producing *Alexandrium* species. *A. affine* lacks the saxitoxin genes (Stüken et al., 2011). However, Nguyen-Ngoc (2004) reported that a strain of *A. affine* that can produce low levels of STXs. This indicates that although *C. punctatum* lacks the *sxtA* gene, it might still produce STXs, leading us to re-investigate STX production also in *C. punctatum*.

~~In this study we focused principally on~~ The present study aimed to detection the PSTs of *C. punctatum* that has been maintained under the best culture conditions as obtained in preliminary experiments-optimal culture conditions. Based on the results obtained by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), the present study ~~we~~ reports presence of STXs in a strain of *C. punctatum* and describes *C. punctatum* as a PST producer. In addition, as the molecular phylogeny and morphological relationships between *Centrodinium* and *Alexandrium* species has attracted increasing attention (e.g. Gómez and Artigas, 2019; Mertens et al., 2020), the plate configuration is reinvestigated to correct the sulcal area of *C. punctatum*.

~~because Gómez and Artigas (2019) suggested inaccuracies in the hypothetical tabulation of *C. punctatum* described by Li et al., (2019), the plate configuration in the sulcal area of *C. punctatum* is reinvestigated.~~

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2. Materials and methods

2.1. Culture maintenance

111 The geographic origin of the strain (LIMS-PS-2525) of *C. punctatum* was the East China Sea
 112 (31°59'53"N, 127°41'59"E). Cultures were established from the strain maintained in the Library of
 113 Marine Samples, Korea Institute of Ocean Science and Technology, Republic of Korea. The strain was
 114 maintained in 2L culture bottles (SPL, Pocheon, Korea) containing f/2 culture medium (Marine Water
 115 Enrichment Solution, Sigma Aldrich, St. Louis, MO, USA), prepared with sterile seawater (filtered
 116 through 47 mm membrane filter and autoclaved). Cultures were maintained at 20 °C and ca 100 μmol
 117 photons m⁻² s⁻¹ ~~with~~ cool-white illumination under a 24L:0D photo-cycle on a roller apparatus
 118 (Wheaton, Millville, NJ, USA), as ~~a preliminary comparison~~^{the culture test} indicated that cell growth
 119 of *C. punctatum* under a 12L:12D photo-period is lower than that under a 24L:0D photo-cycle does not
 120 ~~stimulate the growth of this strain of *C. punctatum*~~ (Fig. S1), and that *C. punctatum* could not grow
 121 without a roller apparatus (Fig. S2). The samples for toxin analysis were harvested after 30 days of
 122 incubation. Cells incubated under a 12L:12D photo-period might be sufficient for toxin analysis, but
 123 need to be tested (Fig. S1).

124 2.2. Morphological study of *Centrodinium punctatum*

125 To study the morphological features of *C. punctatum*, including the sulcus, cells were isolated from
 126 culture flasks and were photographed using an AxioCam MRc digital camera on an upright microscope
 127 (Axio Imager 2; Carl Zeiss, Jena, Germany).

128 For scanning electron microscopy (SEM) of the established cultures, 2 mL of the cultures were
 129 fixed with 2 % acidic Lugol's solution (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at room
 130 temperature. Subsequently, the fixed cells were passed through a 2.0 μm pore size membrane filters (22
 131 mm diameter). The filters were rinsed with deionized water and dehydrated in a graded ethanol series
 132 (10–99.9% in eight steps) for 15 min at each step. The filters were then critical point dried using a
 133 critical point drying (SPI-Dry Regular Critical Point Dryer, SPI Supplies, West Chester, PA, USA) from
 134 liquid CO₂. Finally, the filters were mounted on stubs, coated with platinum and examined at a voltage
 135 of 5 kV under a JEOL JSM 7600F field emission scanning electron microscope (JEOL Ltd, Tokyo,
 136 Japan).

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2.3. Toxin analysis

For toxin analysis, 2L of bulk cultures were filtered using a nylon net with a mesh size of 20 µm. Cells were transferred into a 1.5 ml tube and centrifuged at 3000 × g at 4 °C for 10 min. The supernatant was gently removed and the cell pellets were freeze-dried at -70 °C (FDU-7012, Operon, Korea). Freeze-drying has been used for over a decade for the stabilization of shellfish tissues contaminated with lipophilic algal toxins (McCarron et al., 2007). C-toxins have also been shown to be stable in freeze-dried tissues of shellfish tissues (Band-Schmidt, 2005). More recently, freeze-drying has been systematically compared to wet-frozen storage and has been shown to be a better stabilization technique for saxitoxins, including C-toxins, in biological tissues (Turner et al., 2013). The same technique has also frequently been used for sample pretreatment concerning dinoflagellate cells, including saxitoxin producers (e.g. Halme et al., 2012).

Duplicate samples (~~LIMS-P2-2525-C1 and LIMS-P2-2525-C2~~) containing 412,500 and 420,000 cells, respectively, were used for PST~~toxin~~ analysis (Table 1). For the analysis of PSTs, the freeze-dried samples were extracted with acetic acid 1% (Turner et al., 2019), in an ultrasonic bath (15 min, 25 kHz). After centrifugation (10,000 g at 4 °C for 5 min), the supernatant was purified by Solid Phase Extraction (SPE) with an ENVI-carb cartridge (250 mg, Supelco), based on the method of Boundy et al. (2015). The analysis of PSTs was performed by HILIC-MS/MS (hydrophilic interaction liquid chromatography).

Liquid chromatography was conducted according to Turner et al. (2019), with a minor modification: the use of a Poroshell 120 HILIC-Z analytical column (150 x 2.1 mm, 2.7 µm, Agilent) coupled to a Nexera Ultra-Fast Liquid Chromatography system (Shimadzu, Tokyo, Japan~~France~~). The linear gradient elution program of the binary mobile phase (solvent A: water/formic acid/ammonium hydroxide (500:0.075:0.3, v/v/v); solvent B: acetonitrile/water/formic acid (900:100:0.1 v/v/v)) was as follows: 0 min, 15% A; 8.25 min, 25% A, 9 min, 50% A; 11.25 min, 50% A, 12 min, 15% A, maintained for 7 min. The flow rate was 0.5 mL min⁻¹, the column temperature 30 °C while samples were kept at 4 °C and the injection volume was 5 µL.

The MS/MS analysis was conducted with a hybrid triple quadrupole/linear ion-trap mass spectrometer (QTRAP 5500, ABSciex, Redwood city, CA, USA). The electrospray switched between negative and positive ionizations and the detection was performed by MRM (Multiple Reaction monitoring) with two transitions per toxin (Supplementary table S1). Samples were quantified with matrix-matched calibration curves prepared by spiking an acetic acid extract of a non-PST producing *A. minutum* strain before SPE clean up. Limits of detection and quantification were provided in table S1 and ranged from 1.3 to 46 fg cell⁻¹ depending on the PST analog.

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Certified calibration solutions for the PSP toxins were acquired from the National Research Council Canada (NRCC, Halifax, NS, Canada) and included C toxins (C1 and C2), gonyautoxin (GTX-1 to -6, and decarbamoyl dcGTX-2 and dcGTX-3) and saxitoxins (STX, neoSTX (NEO) and decarbamoyl dcSTX and dcNEO). In addition, analysis of spirolides (17 analogs), gymnodimines (7 analogs) and gonidomin A and Bs was performed by LC-MS/MS following extraction with 500 µL of methanol of a third independent replicate replicate LIMS P2-2325-C3 (80,000 cells) according to previously published protocols (Mertens et al., 2017; Krock et al., 2018; Qiu et al., 2018; Zurhelle et al., 2018; Supplementary table S2). Limits of detection and quantification for the two available standards (13-desmethyl-spirolide C and GYM A obtained from NRCC, Halifax, NS, Canada) were 8.8 fg cell⁻¹, as given in supplementary table S2.

3. Results and discussion

3.1. Morphology of *Centrodinium punctatum*

Morphological features of *C. punctatum* were previously documented (Li et al., 2019). The thecal surface of cell was strongly areolated, which was clearly visible with a light microscope (Fig. 1A–C), and the nucleus was elongated and located on the right-lateral side of the cell (Fig. 1C). The epitheca was prolonged in an apical horn (Fig. 1A, D), and the hypotheca also extended into an antapical horn, which was longer than the apical horn (Fig. 1A, D). The cingulum descended by about one cingulum width (Fig. 1D). The cells displayed a plate formula of Po, 3', 1a, 6'', 6C, 8S, 5''', 2'''' (Fig. 1D–I). ~~In~~-Li

et al. (2019), ~~we~~ described that the cells had an additional posterior intercalary plate (1p) that contacted plates 1^{'''} and 2^{'''}, and that the right (Sdp) and left posterior sulcal (Ssp) plates had a similar length, based on an internal view of sulcal plates (Li et al., 2019). However, new SEM observations revealed that the left posterior sulcal plate (Ssp) extended into the hypotheca (Fig. 1G, I) and was larger and longer than the Sdp. This agrees with Gómez and Artigas (2019), who pointed out this inaccuracy in the hypothecal tabulation induced by mislabeling of the Ssp of *C. punctatum*. However, the drawing of ~~the~~ hypotheca provided by Gómez and Artigas (2019) is here considered inaccurate, presumably because of the lack of detailed SEM observations; in particular, Gómez and Artigas (2019) did not correctly describe the shape and location of the posterior sulcal plate (Sp). Therefore, ~~we re-document~~ the sulcal plates and hypothecal plate configuration of *C. punctatum* are reinvestigated in this study. ~~There~~ are eight sulcal plates in *C. punctatum*, consisting of the anterior sulcal plate (Sa), the right (Sda) and left (Ssa) anterior sulcal plates, the anterior median sulcal plate (Sma), the posterior median sulcal plate (Smp), right (Ssp) and left (Sdp) posterior sulcal plates, and the posterior sulcal plate (Sp) (Fig. 2A). ~~Tand~~ the hypotheca is composed of five postcingular plates and two antapical plates, and the Sp is polygonal, larger than plates 1^{'''} and 2^{'''} and contacted ~~with~~ plates 4^{'''}, 5^{'''}, 1^{'''}, 2^{'''} and Ssp (Fig. 2B).

3.2. Toxin profile and toxicity of *Centrodinium punctatum*

Several PST analogs were detected in the *C. punctatum* strain (Table 1 and Fig. 3). The total PST concentrations for ~~the duplicate two individual~~ samples (LIMS-PS-2525-C1 and C2) were 91 and 212 pg cell⁻¹ (or 259 and 601 fmol cell⁻¹), corresponding respectively to 99 and 230 pg STXeq cell⁻¹ when toxicity equivalent factors based on Botana et al. (2017) were considered. It is not clear why the two replicates, established from separate cultures maintained under the same conditions, showed such a large difference in PST concentrations. Despite of the difference in total PSTs, the proportions of each analog for the two samples were in excellent agreement, showing that STX, GTX-1 and neoSTX were the principle analogs (>70% of the profile) followed by GTX-4, -2 and -3 (Fig. 3 and Fig. S3). In addition, deoxydecarbamoyle STX (do-STX) was putatively detected at trace level; as no standard was available for confirmation, identification was based on both MRM transitions and the relative retention

time compared to STX (Boundy et al., 2015). Neither C-toxins nor decarbamoyl analogs (except a trace of doSTX) were detected in the strain of *C. punctatum*. Similarly, no spirolide, gymnodimine and gonidomin could be detected.

Despite of morphological differences between *C. punctatum* and *Alexandrium* species, *C. punctatum* is phylogenetically closely related to *Alexandrium* species, and in particular to *A. affine* (see Figs 9 and 10 in Li et al., 2019). Some studies found that strains of *A. affine* do not produce PSTs (Band-Schmidt et al., 2003; Wang et al., 2006; Orr et al., 2011), whereas Nguyen-Ngoc (2004) documented a strain of *A. affine* that produces six PSTs consisting of neoSTX, STX, GTX-1, GTX-2, GTX-3 and GTX-4. Toxin profiles of *A. affine* in Nguyen-Ngoc (2004) are similar to the profile found in this study of *C. punctatum*. This indicates that the close phylogenetic relationship between *A. affine* and *C. punctatum* could potentially also be reflected in a similarity between both toxin profiles. However, doubts about detection limits and analogue identity due to the fluorescence detection method used by Nguyen-Ngoc (2004) suggest that *A. affine*'s toxin profile should be re-investigated.

Although PST profiles and concentrations are affected by variable factors, the PST profiles of *Alexandrium* species such as *A. acatenella*, *A. affine*, *A. andersonii*, *A. australiense*, *A. catenella*, *A. cohorticula*, *A. fundyense*, *A. leei*, *A. minutum*, *A. ostenfeldii*, *A. pacificum*, *A. peruvianum*, *A. tamiyavanichii*, and *A. taylorii* usually include STXs, GTXs, and C-toxins (Anderson et al., 1994; Yoshida et al., 2001; Hansen et al., 2003; Orr et al., 2011; John et al., 2014; Murray et al., 2015; Lassus et al., 2016; but see Tillmann et al., 2020 for *A. taylorii*), and some possess high concentrations of STX and neoSTX (Anderson et al., 1994; John et al., 2014). Importantly, in several toxin profiles, the C-toxins are consistently detected in many toxic strains of *Alexandrium* species (e.g., Yoshida et al., 2001; Lim et al., 2006; Wang et al., 2006; Orr et al., 2011; Laabir et al., 2013; John et al., 2014; Band-Schmidt et al., 2019), however this was not the case for *C. punctatum*. Interestingly, Pacific strains of *A. minutum* do not appear to produce C-toxins, while strains from Denmark and France do produce C-toxins (Lassus et al., 2016; Lewis et al., 2018). Such differences clearly indicate the need to evaluate more strains of *C. punctatum* from different geographical regions for their toxin production. Additionally, according to the results on toxin contents between life stages of *A. fundyense* by Persson et al. (2012), gonyautoxins

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such as GTX-1 and -2 are present or absent in different life stages, whereas the presence of C-toxins such as C2 is conserved. Such studies indicate that the PST-profile can be life stage dependent; so far no other life stage is known for *C. punctatum*, and thus this variability cannot be assessed here. Among other PST-producing dinoflagellates, *G. catenatum* can also produce the C toxins, however this species has been reported to produce benzoyl analogs (GC toxins) that have not been detected in toxic *Alexandrium* species and not screened for in the present study (Band-Schmidt et al., 2019), similar to M-toxins, another PST variant recently also discovered in cyanobacteria (D'Agostino et al., 2019).

The toxin profile of the strain of *C. punctatum* is highly unusual, with high proportions of STX and neoSTX (that accounted for ca. 48% of the total toxins), which have not commonly been reported in strains of toxic *Alexandrium* species. The gonyautoxins in this strain of *C. punctatum* accounted for the rest of the total toxin concentrations. According to Botana et al. (2017), in the mouse bioassays, STX and neoSTX are the most toxic and followed by gonyautoxins, indicating that *C. punctatum* can produce a high level of toxicity compared to *Alexandrium* species. In addition, the maximal cellular PST quota (230 pg STXeq cell⁻¹ or 601 fmol cell⁻¹) of *C. punctatum* is rather high. Indeed, from many strains of worldwide distributed PST-producing dinoflagellates such as *G. catenatum* and *P. bahamense*, the highest reported cellular toxicity was 450 fmol cell⁻¹ (Laabir et al., 2013 and references therein). However, in fact, it is quite difficult to compare the cellular toxicity between *C. punctatum* and other related species since cellular toxicity of *Alexandrium* species PST-producing dinoflagellates varies considerably interwithin and intraspecifically between species, and between geographically distinct populations (e.g., Anderson et al., 1990, 2012; Cembella, 1998; Orlova et al., 2007; Persson et al., 2012; Van de Waal et al., 2013).

In this study, the strain of *C. punctatum* was established from a single cell isolated in the East China Sea (Li et al., 2019). The East China Sea is connected with the East Sea (Sea of Japan) of Korea (Sea of Japan) through the Korea Strait, and opens to the north into the Yellow Sea. Along the continental slope of the East China Sea, the Taiwan Warm Current and the Tsushima Current that branch from the Kuroshio Current, enter into the Korean and Japanese coastal areas (Lie and Cho, 1994; Hu and Yang, 2001). These currents have been suggested to transport harmful species such as

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Margalefidinium polykrikoides and *Prorocentrum obtusidens* into Korean and Japanese coastal areas (Lee, 2006; Shin et al., 2019), a mechanism which may also apply to intrusions of *C. punctatum*. Even though blooms or harmful effects caused by *C. punctatum* have not yet been recorded in Korean and Japanese coastal areas, *C. punctatum* may have been overlooked and monitoring of this species now appears necessary to protect both the public health and the shellfish industry. In addition, further research is needed to clarify changes in toxicity of *C. punctatum* with differences in environmental factors such as temperature, salinity and nutrient or bacterial interactions and to clarify the relationship between *Centrodinium* and *Alexandrium* species. Furthermore, since *C. punctatum* and related species are widely distributed, the toxicity of other strains should be investigated in future studies as well.

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300 **References**

301

302 Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., Lee, C., 1990. Dynamics and physiology of
303 saxitoxin production by the dinoflagellate *Alexandrium* spp. Mar. Biol. 104, 511–524.

304

305 Anderson, D.M., Kulis, D.M., Doucette, G.J., Gallagher, J.C., Balech, E., 1994. Biogeography of toxic
306 dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. Mar. Biol.
307 120, 467–478.

308

309 Anderson, D.M., Alpermann, T.J., Cembella, A.D., Collos, Y., Masseret, E., Montresor, M., 2012. The
310 globally distributed genus *Alexandrium*: Multifaceted roles in marine ecosystems and impacts on human
311 health. Harmful Algae 14, 10–35.

312

313 Azanza, R.V., Taylor, F.J., 2001. Are *Pyrodinium* blooms in the Southeast Asian region recurring and
314 spreading? A review at the end of the millennium. Ambio 39, 356–364.

315

316 Balech, E., 1995. The genus *Alexandrium* Halim (Dinoflagellata). Sherkin Island Marine Station,
317 Sherkin Island Co., Cork, Ireland.

318

319 Band-Schmidt, C.J., Lilly, E.L., Anderson, D.M., 2003. Identification of *Alexandrium affine* and *A.*
320 *margalefii* (Dinophyceae) using DNA sequencing and LSU rDNA-based RFLP–PCR assays.
321 Phycologia 42, 261–268.

322

323 Band-Schmidt, C.J. 2005. Paralytic shellfish toxin profile in strains of the dinoflagellate *Gymnodinium*
324 *catenatum* Graham and the scallop *Argopecten ventricosus* G.B. Sowerby II from Bahía Concepción,
325 Gulf of California, Mexico. Harmful Algae 4, 21–31.

326

327

328 Band-Schmidt, C.J., Durán-Riveroll, L.M., Bustillos-Guzmán, J.J., Leyva-Valencia, I., López-Cortés,
329 D.J., Núñez-Vázquez, E.J., Hernández-Sandoval, F.E., Ramírez-Rodríguez, D.V., 2019. Paralytic toxin
330 producing dinoflagellates in Latin America: Ecology and physiology. Front. Mar. Sci. 6, 42. doi:
331 10.3389/fmars.2019.00042

332 Botana, L.M., Hess, P., Munday, R., Nathalie, A., DeGrasse, S.L., Feeley, M., Suzuki, T., van den Berg,
333 M., Fattori, V., Garrido Gamarro, E., Tritscher, A., Nakagawa, R., Karunasagar, I., 2017. Derivation of
334 toxicity equivalency factors for marine biotoxins associated with Bivalve Molluscs. Trends Food Sci.
335 Techn. 59, 15-24. doi:<https://doi.org/10.1016/j.tifs.2016.09.015>
336
337 Boundy, M.J., Selwood, A.I., Harwood, D.T., McNabb, P.S., Turner, A.D., 2015. Development of a
338 sensitive and selective liquid chromatography–mass spectrometry method for high throughput analysis
339 of paralytic shellfish toxins using graphitised carbon solid phase extraction. J. Chromatogr. A 1387, 1–
340 12. doi:10.1016/j.chroma.2015.01.086
341
342 Cembella, A.D., 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine
343 microalgae. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of
344 Harmful Algal Blooms. Springer Verlag, Heidelberg, pp. 381–403
345
346 Chang, D.S., Shin, I.S., Pyeun, J.H., Park, Y.H., 1987. A study on paralytic shellfish poison of sea
347 mussel *Mytilus edulis*. Bull. Kor. Soc. Fish. Tech. 20, 293–299.
348
349 EFSA, 2009. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from
350 the European Commission on Marine Biotoxins in Shellfish – Saxitoxin Group. The EFSA Journal
351 1019, 1–76.
352
353 Gómez, F., Artigas, L.F., 2019. Redefinition of the dinoflagellate genus *Alexandrium* based on
354 *Centrodinium*: Reinstatement of *Gessnerium* and *Protogonyaulax*, and *Episemicolon* gen. nov.
355 (Gonyaulacales, Dinophyceae). Hindawi J. Mar. Biol., 1284104.
356
357 Gu, H., Zeng, N., Liu, T., Yang, W., Müller, A., Krock, B., 2013. Morphology, toxicity, and phylogeny
358 of *Alexandrium* (Dinophyceae) species along the coast of China. Harmful Algae 27, 68–81.
359
360 Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase.
361 Phycologia 32, 79–99.
362
363
364 Hallegraeff, G.M., 2003. Harmful Algal Blooms: a global overview. In: Hallegraeff, G.M., Anderson,
365 D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. Monographs on oceanographic
366 methodology 11. UNESCO Publishing, Paris, pp. 25–49.
367

368

369 Halme, M., Rapinaja, M.-L., Karjalainen, M., Vanninen, P., 2012. Verification and quantification of

370 saxitoxin from algal samples using fast and validated hydrophilic interaction liquid chromatography–

371 tandem mass spectrometry method. *Journal of chromatography. B, Analytical technologies in the*

372 *biomedical and life sciences.* 880, 50-57.

373

374 Hansen, G., Daughjerg, N., Franco, J.M., 2003. Morphology, toxin composition and LSU rDNA

375 phylogeny of *Alexandrium minutum* (Dinophyceae) from Denmark, with some morphological

376 observations on other European strains. *Harmful Algae* 2, 317–335.

377

378 Harada, T., Oshima, Y., Kamiya, H., Yasumoto, T., 1982. Confirmation of paralytic shellfish toxins in

379 the dinoflagellate *Pyrodinium bahamense* var. *compressa* and bivalves in Palau. *Bull. Japan Soc. Sci.*

380 *Fish* 48, 821–825.

381

382 Hu, D.X., Yang, Z.S., 2001. The key process of marine fluxes in East China Sea. Ocean Press, Beijing.

383

384 John, U., Litaker, R.W., Montresor, M., Murray, S., Brosnahan, M.L., Anderson, D.M., 2014. Formal

385 revision of the *Alexandrium tamarense* species complex (Dinophyceae) taxonomy: The introduction of

386 five species with emphasis on molecular–based (rDNA) classification. *Protist* 165, 779–804.

387

388 Laabir, M., Collos, Y., Masseret, E., Grzebyk, D., Abadie, E., Savar, V., Sibat, M., Amzil, Z., 2013.

389 Influence of environmental factors on the paralytic shellfish toxin content and profile of *Alexandrium*

390 *catenella* (Dinophyceae) isolated from the Mediterranean Sea. *Mar. Drugs* 11, 1583-1601.

391

392

393 ▲

394 Lassus P., Chomérat, N., Hess, P., Nézan, E., 2016. Toxic and Harmful Microalgae of the World Ocean

395 / Micro-algues toxiques et nuisibles de l’océan mondial. Denmark, International Society for the Study

396 of Harmful Algae / Intergovernmental Oceanographic Commission of UNESCO. IOC Manuals and

397 Guides, 68 [Bilingual English/French].

398

399 Lewis, A.M., Coates, L.N. ~~Coates, Turner, A.D.~~ ~~Turner, Percy, L.~~ ~~Percy,~~ and Lewis, J. ~~Lewis.~~ 2018. A

Formatted: Font

review of the global distribution of *Alexandrium minutum* (Dinophyceae) and comments on ecology and associated paralytic shellfish toxin profiles, with a focus on Northern Europe. J. Phycol. 54, 581-598.

Lee, Y.S., 2006. Factors affecting outbreaks of high-density *Cochlodinium polykrikoides* red tides in the coastal seawaters around Yeosu and Tongyeong, Korea. Mar. Pollut. Bull. 52,1249–1259.

Lilly, E.L., Halanych, K.M., Anderson, D.M., 2005. Phylogeny, biogeography, and species boundaries within the *Alexandrium minutum* group. Harmful Algae 4, 1004–1020.

Li, Z., Mertens, K.N., Nézan, E., Chomérat, N., Bilien, G., Iwataki, M., Shin, H.H., 2019. Discovery of a new clade nested within the genus *Alexandrium* (Dinophyceae): Morpho-molecular characterization of *Centrodinium punctatum* (Cleve) F.J.R. Taylor. Protist 170(2), 168–186.

Lie, H.J., Cho, C.H., 1994. On the origin of the Tsushima Warm Current. *J. Geophys. Res.* 99, 25081–25091

Lim, P.T., Leaw, C.P., Usup, G., Kobiyama, A., Koike, K., Ogata, T., 2006. Effects of light and temperature on growth, nitrate uptake, and toxin production of two tropical dinoflagellates: *Alexandrium tamiyavanichii* and *Alexandrium minutum* (Dinophyceae). J. Phycol. 42, 786–799.

McCarron, P., Emteborg, H., Hess, P., 2007. Freeze-drying for the stabilisation of shellfish toxins in mussel tissue (*Mytilus edulis*) reference materials. Anal. Bioanal. Chem. 387, 2475-2486.

Mertens, K.N., Adachi, M., Anderson, D.M., Band-Schmidt, C.J., Bravo, I., Brosnahan, M.L., Bolch, C.J.S., Calado, A.J., Carbonell-Moore, M.C., Chomérat, N., Elbrächter, M., Figuera, R.I., Fraga, S., Gárate-Lizárraga, I., Gracés, E., Gu, H., Hallegraeff, G., Hess, P., Hoppenarath, M., Horiguchi, T., Iwataki, M., John, U., Kremp, A., Larsen, J., Leaw, C.P., Li, Z., Lim, P.T., Litaker, W., MacKenzie, L., Masseret, E., Matsuoka, K., Moestrup, Ø., Montresor, M., Nagai, S., Négzan, E., Nishimura, T., Okolodkov, Y.B., Orlova, T.Y., Reñe, A., Sampedro, N., Satta, C.T., Shin, H.H., Siano, R., Smith, K.F., Steidinger, K., Takano, Y., Tillmann, U., Wolny, J., Yamaguchi, A., Murray S., 2020. Morphological and phylogenetic data do not support the split of *Alexandrium* into for genera. Harmful Algae 98.

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434 [101902.](#)

435
436
437 Moestrup, Ø., Akselmann-Cardella, R., Churro, C., Fraga, S., Hoppenrath, M., Iwataki, M., Larsen, J.,
438 Lundholm, N., Zingone, A. (Eds.), 2009 onwards. IOC-UNESCO Taxonomic Reference List of
439 Harmful Micro Algae. Accessed at <http://www.marinespecies.org/hab> on 2020-02-20.
440 <https://doi:10.14284/362>

441
442 Murray, S.A., Diwan, R., Orr, R.J., Kohli, G.S., John, U., 2015. Gene duplication, loss and selection in
443 the evolution of saxitoxin biosynthesis in alveolates. *Mol. Phylogenet. Evol.* 92, 165–180.

444
445 Nagai, S., Chen, H., Kawakami, Y., Yamamoto, K., Sildever, S., Kanno, N., Oikawa, H., Yasuike, M.,
446 Nakamura, Y., Hongo, Y., Fujiwara, A., Kobayashi, T., Gojobori, T., 2019. Monitoring of the toxic
447 dinoflagellate *Alexandrium catenella* in Osaka Bay, Japan using a massively parallel sequencing
448 (MPS)-based technique. *Harmful Algae* 89, 101660.

449
450 Nguyen-Ngoc, L., 2004. An autecological study of the potentially toxic dinoflagellate *Alexandrium*
451 *affine* isolated from Vietnamese waters. *Harmful Algae* 3, 117–129.

452
453 Orr, R.J.S., Stüken, A., Rundberget, T., Eikrem, W., Jakobsen, K.S., 2011. Improved phylogenetic
454 resolution of toxic and nontoxic *Alexandrium* strains using a concatenated rDNA approach. *Harmful*
455 *Algae* 10, 676–688.

456
457 Orr, R.J.S., Stüken, A., Murray, S., Jakobsen, K.S., 2013. Evolutionary acquisition and loss of saxitoxin
458 biosynthesis in dinoflagellates: lessons from the second “core” gene – *stxG*. *Appl. Environ. Microbiol.*
459 79, 2128–2136.

460
461 Orlova, T.Y., Selina, M.S., Lilly, E.L., Kulis, D.M., Anderson, D.M., 2007. Morphogenetic and toxin
462 composition variability of *Alexandrium tamarense* (Dinophyceae) from the east coast of Russia.
463 *Phycologia* 46, 534–548.

464
465 Oshima, Y., Hasegawa, M., Yasumoto, T., Hallegraeff, G., Blackburn, S., 1987. Dinoflagellate
466 *Gymnodinium catenatum* as the source of paralytic shellfish toxins in Tasmanian shellfish. *Toxicon* 25,
467 1105–1111.

468
469 Persson, A., Smith, B.C., Alix, J.H., Senft-Batoh, C., Wikfors, G.H., 2012. Toxin content differs

470 between life stages of *Alexandrium fundyense* (Dinophyceae). Harmful Algae 19, 101–107.
 471
 472 Shin, H.H., Baek, S.H., Li, Z., Han, M.S., Oh, S.J., Youn, S.H., Kim, Y.S., Kim, D., Lim, W.A., 2014.
 473 Resting cysts, and effects of temperature and salinity on the growth of vegetative cells of the potentially
 474 harmful species *Alexandrium insuetum* Balech (Dinophyceae). Harmful Algae 39, 175–184.
 475
 476 Shin, H.H., Li, Z., Seo, M.H., Soh, M.Y., Lim, W.A., Park, J.W., 2019. Harmful dinoflagellate
 477 *Prorocentrum donghaiense* Lu is widely distributed along the East China Sea and Korean coastal area.
 478 Ocean Sci. J. 54, 685–691.
 479
 480 Sommer, H., Whedon, W.F., Kofoid, C.A., Stohler, R., 1937. Relation of paralytic shellfish poison to
 481 certain plankton organisms of the genus *Gonyaulax*. Arch. Pathol. 24, 537–559.
 482
 483 Stüken, A., Orr, R.J.S., Kellman, R., Murray, S.A., Neilan, B.A., Jakobsen, K.S., 2011. Discovery of
 484 nuclear-encoded genes for the neurotoxin saxitoxin in dinoflagellates. PLoS ONE 6–(5), 20096.
 485 <https://doi.org/10.1371/journal.pone.0020096>
 486
 487 Taylor, F.J.R., Fukuyo, Y., Larsen, J., 2003. Taxonomy of Harmful Dinoflagellates. In: Hallegraeff,
 488 G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. Monographs
 489 on oceanographic methodology 11. UNESCO Publishing, Paris, pp. 389–432.
 490
 491 Tillmann, U., Krock, B., Wietkamp, S., Beran, A., 2020. A Mediterranean *Alexandrium taylorii*
 492 (Dinophyceae) Strain Produces Goniodomin A and Lytic Compounds but Not Paralytic Shellfish
 493 Toxins. Toxins 12, 564. <https://doi:10.3390/toxins12090564>
 494
 495
 496 Turner, A., Lewis, A., Hatfield, R., Higman, W., Burrell, S., 2013. A feasibility study into the
 497 production of a freeze-dried oyster reference material for paralytic shellfish poisoning toxins. Anal.
 498 Bioanal. Chem. 405, 8621–8632.
 499
 500 Turner, A.D., Dhanji-Rapkova, M., Fong, S.Y.T., Hungerford, J., McNabb, P.S., Boundy, M.J.,
 501 Harwood, D.T., 2019. Ultrahigh-Performance Hydrophilic Interaction Liquid Chromatography with
 502 Tandem Mass Spectrometry Method for the Determination of Paralytic Shellfish Toxins and
 503 Tetrodotoxin in Mussels, Oysters, Clams, Cockles, and Scallops: Collaborative Study. J AOAC Int.
 504 <https://doi:10.5740/jaoacint.19-0240>
 505

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506 Van de Waal, D.B., Tillmann, U., Zhu, M., Koch, B., Rost, B., John, U., 2013. Nutrient pulse induces
 507 dynamic changes in cellular C:N:P, amino acids, and paralytic shellfish poisoning toxins in
 508 *Alexandrium tamarense*. Mar. Ecol. Prog. Ser. 493, 57–69.
 509
 510 Wang, D.Z., Zhang, S.G., Gu, H.F., Chan, L.L., Hong, H.S., 2006. Paralytic shellfish toxin profiles,
 511 and toxin variability of the genus *Alexandrium* (Dinophyceae) isolated from the Southeast China Sea.
 512 Toxicon 48, 138–151.
 513
 514 Wiese, M., D’Agostino, P.M., Mihali, T.K., Moffitt, M.C., Neilan, B.A., 2010. Neurotoxic alkaloids:
 515 Saxitoxin and its analogs. Mar. Drugs. 8, 2185–2211.
 516
 517 Yñiguez, A.T., Lim, P.T., Leaw, C.P., Jipanin, S.J., Iwataki, M., Benico, G., Azanza, R.V., 2020. Over
 518 30 years of HABs in the Philippines and Malaysia: What have we learned? Harmful Algae, 101776.
 519 <https://doi.org/10.1016/j.hal.2020.101776>
 520
 521 Yoshida, T., Sako, Y., Uchida, A., 2001. Geographic differences in paralytic shellfish poisoning toxin
 522 profiles among Japanese populations of *Alexandrium tamarense* and *A. catenella* (Dinophyceae).
 523 Phycol. Res. 49, 13–21.

Figure legends

Fig. 1. Light micrographs and scanning electron micrographs of *Centrodinium punctatum*. (A) Ventral view showing the sulcus and cingulum. (B) Dorsal view showing the cingulum. (C) Mid focus on right lateral view showing the nucleus (n). (D) Ventral view showing the left posterior sulcal plate (Ssp). (E) Dorsal-left lateral view. (F) Right lateral view. (G-H) Details of the sulcal area. (I) Antapical view showing Ssp and the posterior sulcal plate (Sp). Scale bars: A-F = 10 μm ; G-I = 3 μm .

Fig. 2. Diagrammatic line drawings of the sulcal area (A) and the hypotheca (B) of *Centrodinium punctatum*, based on scanning electron microscope observation.

Fig. 3. PST profiles (average molar concentration) of *Centrodinium punctatum* strain LIMS-PS-2525.

Fig. 1.

Fig. 2.

Fig. 3.

Table 1. Toxin content (pg cell⁻¹) and toxicity (pg STX-eq. cell⁻¹, obtained by weighting by the Toxicity Equivalent Factors, TEFs, defined by Botana et al. 2017) of *Centrodinium punctatum*.

	GTX-1	GTX-2	GTX-3	GTX-4	NEO	STX	Total (pg cell ⁻¹)
LIMS-PS-2525-C1	21	8,7	7,2	9,7	20	24	91
LIMS-PS-2525-C2	50	21	18	23	45	53	212
	GTX-1	GTX-2	GTX-3	GTX-4	NEO	STX	Total (pg STX-eq. cell ⁻¹)
LIMS-PS-2525-C1	21	3,5	4,3	6,8	39	24	99
LIMS-PS-2525-C2	50	8,5	11	16	91	53	230

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