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Domoic acid production by *Pseudo-nitzschia calliantha* Lundholm, Moestrup et Hasle (bacillariophyta) isolated from the Black Sea

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Abstract

A species of *Pseudo-nitzschia* isolated from Sevastopol Bay, Black Sea, was examined for its toxicity. The species was identified as *P. calliantha* Lundholm, Moestrup et Hasle based on SEM and TEM examination. Domoic acid (DA) was detected in batch culture throughout the growth cycle of *P. calliantha*. The production of DA by this diatom species was confirmed by fluorenylmethoxycarbonyl (FMOC) derivatization and HPLC-fluorescence method. The cellular DA level was higher in the early exponential phase, with the maximum value of 0.95 pg DA cell^{-1}. In the stationary phase, the cellular DA levels declined. This is the first record of a DA producing diatom isolated from the Black Sea.
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Keywords: Domoic acid; Harmful algae; Diatom; *Pseudo-nitzschia calliantha*; Black Sea

1. Introduction

Production of domoic acid (DA), the toxin responsible for amnesic shellfish poisoning (ASP), has been reported for several species of the diatom genus *Pseudo-nitzschia* H. Peragallo. Marine invertebrates, birds and mammals can accumulate considerable amounts of this toxin. Filter feeding bivalves consuming toxin producing *Pseudo-nitzschia* species can accumulate DA to high concentrations and human consumption of these contaminated bivalves can result in ASP. This event was first recognized in Prince Edward Island, Canada, in 1987 with three deaths and over 100 people becoming ill after consuming blue mussel *Mytilus edulis* (Bates et al., 1989). In 1991, the California coast *P. australis* bloom contaminated anchovies with DA, resulting in the deaths or neurological symptoms of more than 100 brown pelican and other marine birds after eating the anchovies (Garrison et al., 1992). Marine mammals deaths were recorded first time due to the DA poisoning along the California coast (Lefebvre et al., 1999; Scholin et al., 2000). Shellfishery sectors are highly susceptible to impacts of DA toxicity. The high levels of DA in shellfish have caused the closure of harvesting areas in Oregon and Washington State (Horner and Poster, 1993), in the Gulf of St. Lawrence (Bates et al., 2002), and in UK waters (Bogan et al., 2007; Campbell et al., 2001; Gallacher et al., 2001).

Pseudo-nitzschia species have been commonly observed in the Black Sea (Morozova-Vodyanytskaya, 1954; Proschkina-Lavrenko, 1955; Belogorskaya and Kondratjeva, 1965; Bodeanu, 1987/1988; Rat'kova, 1989; Ryabushko, 1991, 2003a,b; Mikaelyan et al., 1992; Davidovich and Bates, 1998; Bologa et al., 1999; Ryabushko et al., 2000; Moncheva et al., 2001; Vershinin and Kamnev, 2001; Uysal, 2002; Turkoglu and Koray, 2002; Eker-Develi and Kideys, 2003; Vershinin et al., 2004, 2005). *Pseudo-nitzshia pseudodelicatissima, P. pungens, P. delicatissima*, *P. seriata* and *P. calliantha* were reported in Turkish waters of the Black Sea (Turkoglu and Koray, 2002; Uysal, 2002; Bargu et al., 2002; Eker-Develi and Kideys, 2003). High abundance of *P. seriata* and *P. delicatissima* were documented along the Bulgarian coasts (Moncheva et al., 2001). Ryabushko (2003a,b) recorded five *Pseudo-nitzschia* species in the Black Sea and in the Azov Sea; *P. delicatissima*, *P. fraudulenta*, *P. pseudodelicatissima*, *P.*

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pungens and *P. seriata*. Vershinin and Kamnev (2001) cited the blooming of *P. pseudodelicatissima* and *P. seriata* (over 10^6 cells L^{-1}) during the winter–spring period in the Black Sea.

Pseudo-nitszchia calliantha was recorded for the first time in the Turkish waters of the Black Sea by Bargu et al. (2002). The culture isolated from the Karadag, southern coast of the Crimea, was ascribed to *P. pseudodelicatissima* by Davidovich and Bates (1998) but after re-examination of the culture the species was identified as *P. calliantha* (Lundholm et al., 2003).

Toxic and non-toxic clones of *Pseudo-nitzschia calliantha* from different geographical areas have been reported. Domoic acid production of *P. calliantha* was reported in the Bay of Fundy, Canada, (Martin et al., 1990) and in Danish waters (Lundholm et al., 1997 cited in Lundholm et al., 2003), the species was ascribed to *P. pseudodelicatissima.* The isolates of *P. calliantha* from Vietnam waters did not show any presence of domoic acid (Lundholm et al., 2003).

The aims of the present study were to isolate *P. calliantha* from the Black Sea and to test it for the ability to produce DA in the laboratory culture.

2. Material and methods

2.1. Culture

An unialgal culture of *P. calliantha* was isolated in October 2005 from Sevastopol Bay, Black Sea. A stock culture was kept in F/2 medium in 1 L flask at 19 ± 2 °C in ~20 ppt salinity water, under an irradiance of 65 μEinsteins $m^{-2} s^{-1}$ (12 h light: 12 h dark cycle). Irradiance was measured with the LI-COR Spherical quantum sensor (model L1-193SA, USA).

2.2. Microscopy

For studies of the morphology of *P. calliantha*, the organic material of a subsample of culture was removed by oxidation (Lundholm et al., 2002). A mid-exponential phase culture was fixed in 2% glutaraldehyde. The organic material was removed by adding 0.4 ml 30% H_2SO_4 and 2 ml saturated $KMnO_4$ to a 2 ml sample. After 24 h, the sample was cleared by addition of 2 ml saturated aqueous solution of oxalic acid. The sample was then centrifuged at 1500 rpm for 10 min and then rinsed with double distilled water. This last step was repeated several times until the removal of oxalic acid. For SEM examination, samples were filtered on 0.45 μm pore size, 47 mm diameter cellulose acetate membrane filters, and dried in oven at 35 °C for 24 h. SEM pictures were taken in TUBITAK-Marmara Research Center, Kocaeli, Turkey. For TEM, drops of cleaned material were placed on formvar-coated copper grids, dried and studied in a JEOL, JEM-101L electron microscope in Mersin University, Mersin, Turkey.

2.3. Toxin analysis

Subsamples were taken from the stock culture every 2–3 days until the mid-stationary phase. Aliquots around 15–70 ml (containing total around 2×10^6 cells) taken from the culture were filtered through GF/F (25 mm diameter) filters and kept at −20 °C until HPLC analysis. The sample was extracted in 10% methanol by sonicating for 2 min at 100 W. Finally, the extract was centrifuged at 4000 rpm for 10 min and filtered through a 0.2 μm disposable acrodisc (25 mm surfactant-free cellulose acetate membrane, Nalgene, Hereford, UK) to remove cell debris, and frozen at −20 °C before analysis.

DA was analyzed using the fluorenylmethoxycarbonyl (FMOC) derivatization and HPLC-fluorescence method (Pocklington et al., 1990), with the following modifications. The chromatographic system consisted of an Agilent 1100 HPLC equipped with a fluorometric detector (264 nm excitation; 313 nm emission). Separations were performed on a Vydac RP 18 column (250 mm × 4.6 mm i.d.). The mobile phase consisted of acetonitrile and 0.1% trifluoroacetic acid (TFA) and pumped at 0.2 ml/min. Gradient elution was programmed linearly from 30% to 40% acetonitrile over 10 min and

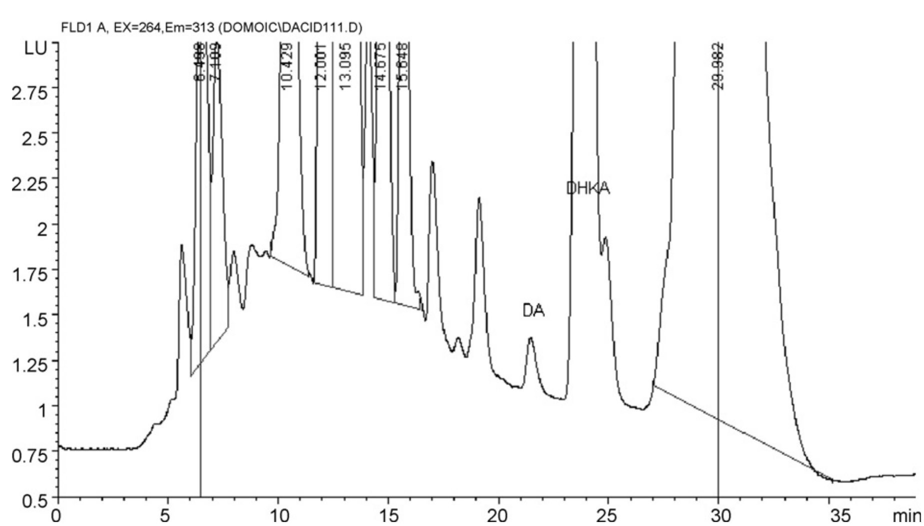

Fig. 1. HPLC-fluorescence chromatogram of domoic acid (DA). Domoic acid has a retention time around 21 min.

maintained for 10 min. Elution was then followed by an increase to 100% acetonitrile over 2 min which was maintained for 3 min before programming back to initial conditions over 2 min. Initial conditions were maintained for a further 12 min, resulting in a total cycle time of 39 min. The column temperature was 55 °C. Injections were done by manual injector (Rheodine) equipped with 20 µl loop. DACS 1 (Marine Analytical Chemistry Standards Program of the NRC, Halifax, NS) was used for instrument calibration solution and dihydrokainic acid (Sigma) was used as an internal standard. DA per cell was calculated by dividing the DA concentration of the aliquots by the cell number. Representative HPLC chromatogramme of DA was given in Fig. 1. Domoic acid has a retention time around 21 min (Fig. 1).

3. Results

3.1. Morphology

Ultrastructural examination by SEM and TEM revealed the cultured isolate from the Sevastopol Bay, Black Sea, as *Pseudo-nitzschia calliantha*. The appearances of the cells are linear shape in valve view and overlapping in colonies. The tapering part of the valve toward the tips is very short (Fig. 2A–C), and the eccentric raphe is divided in the middle by a central nodule (Fig. 2D). The apical axis ranged from 47 to 115 µm (commonly 60–90 µm), while the transapical axis of valves is between 1.8 and 3.6 µm (commonly 2.2–3 µm). Fibulae are regularly spaced, with 16–17 in 10 µm (Fig. 2D; Fig. 3A and B). The central part of the valve has central nodule. Interstriae number 34–37 in 10 µm (Fig. 2D). Striae are composed of a single row of round to square poroids, with 5–6 poroids in 1 µm (Fig. 3A and B). Each poroid is divided into 3–8 sectors (Fig. 3A and B). Valvo-copula are 2–3 poroids wide and 3–4 poroids high (Fig. 3C).

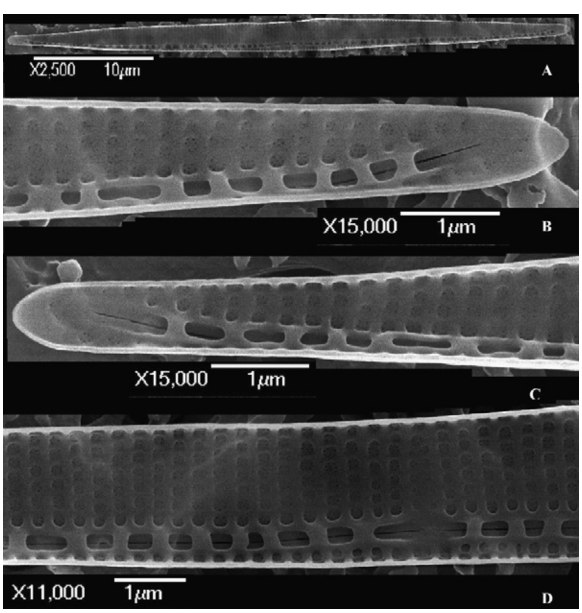

Fig. 2. Scanning electron micrographs of *Pseudo-nitzschia calliantha* isolated from Sevastopol Bay, Black Sea. (A) Whole valve. (B–C) Tips of valves. (D) Central part of valve with central nodule.

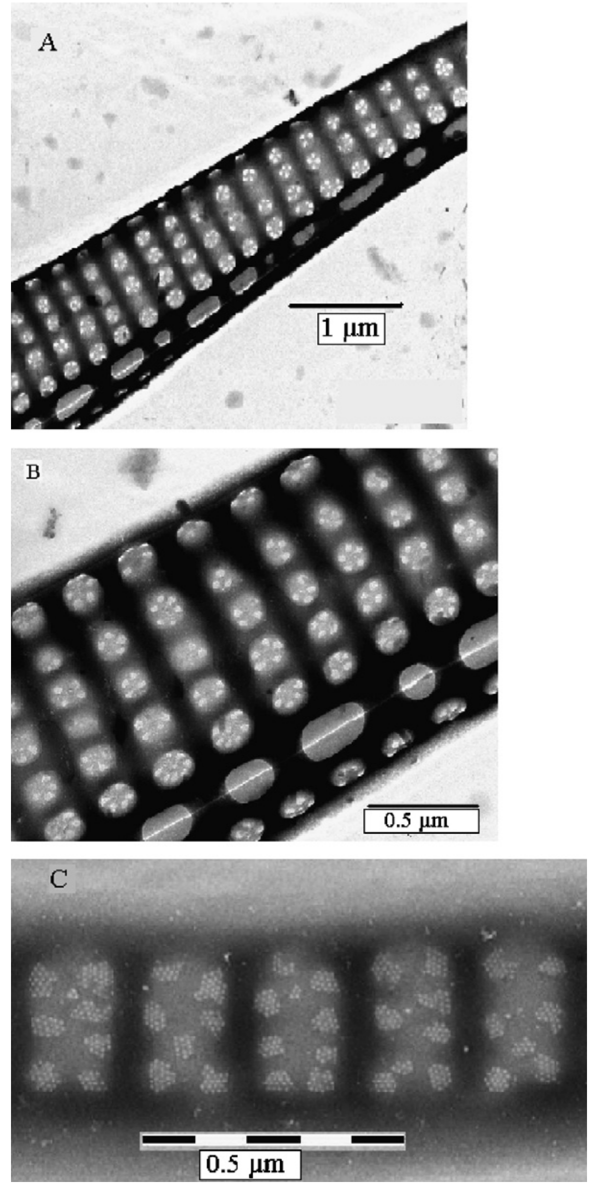

Fig. 3. Transmission electron micrographs of *Pseudo-nitzschia calliantha* isolated from Sevastopol Bay, Black Sea. (A–B) parts of the valves showing the poroid structures. (C) Cingular band.

3.2. DA content

Culture growth was followed for 27 days and it remained in exponential growth phase until day 21 (Fig. 4). The highest cell concentration was recorded as 245,000 cell ml^{-1} in stationary phase. Domoic acid levels were highly variable over time within the cells. The maximum cellular DA values were observed during early exponential phase with the average value of 0.7 ± 0.85 at day 3, and 0.95 pg DA cell^{-1} at day 5. During the mid-exponential phase DA was not detectable, and during late-exponential and stationary phases, cell DA levels ranged from 0.47 pg cell^{-1} at day 17 to 0.11 pg cell^{-1} at day 21 (Fig. 4).

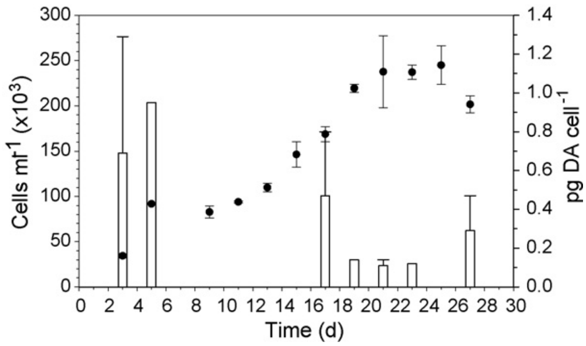

Fig. 4. *Pseudo-nitzschia calliantha* cell growth (dots) and domoic acid (DA) concentrations (bars) as pg DA cell^{-1} in the culture, grown at an irradiance of ~65 μEinsteins m^{-2} s^{-1} (12:12 h L:D cycle) at 19 ± 2 °C ($n = 2$, ± S.E.).

4. Discussion

The genus *Pseudo-nitzschia* is a regular component of the marine phytoplankton in the Black Sea waters, with the cell concentrations reaching more than 1–2 × 10^6 cells L^{-1} in spring in Sevastopol Bay (Ryabushko et al., 2000; Ryabushko, 2003a,b). A red-tide event was observed in summer 1989 near the Bulgarian coast due to the bloom of the dinoflagellate *Noctiluca scintillans* together with the diatom *Pseudo-nitschia* (Ryabushko, 1991, 2003a,b). *Pseudo-nitzschia pseudodelicatissima* has been found in the planktonic community during the mussel harvesting period in Caucasian coasts of the Black Sea, and DA assays in mussel samples have given a negative result (Vershinin et al., 2005). This study presents the first identification of *P. calliantha* from Sevastopol Bay, Black Sea, as a DA producer. Domoic acid production has been documented previously in *P. calliantha* in the Bay of Fundy, Canada, (Martin et al., 1990) and in Danish waters (Lundholm et al., 1997, cited in Lundholm et al., 2003). In both studies, the species was ascribed to *P. pseudodelicatissima*, but after re-examination, it has been confirmed to belong to *P. calliantha* (Lundholm et al., 2003). Other cultures from Vietnam waters of the same species appear to be non-toxic (Lundholm et al., 2003). We found the toxin level in the cell between non-detectable and 1.3 pg DA cell^{-1} using HPLC–FMOC method. These values are comparable with values measured by Martin et al. (1990) who measured cellular DA levels ranging from 0.007 to 0.098 pg DA cell^{-1} in the Bay of Fundy, Canada. In laboratory batch cultures, high DA production was generally observed in the late exponential or during stationary phase (Bates et al., 1991; Pan et al., 1996a,b; Cusack et al., 2002; Fehling et al., 2004). In our *P. calliantha* culture, the high DA level was measured in early exponential phase. A similar pattern was reported by Pan et al. (2001) for *P. pseudodelicatissima* from Gulf of Mexico.

Detailed examination of the species revealed that the morphology of the species corresponded to published *Pseudo-nitzschia calliantha* descriptions in Lundholm et al. (2003). However, there are some variations. The cell width of *P. calliantha* from Sevastopol Bay is 1.8–3.6 μm whereas cells described in Lundholm et al. (2003) are 1.4–1.8 μm wide. Instead of 7–10 poroid sectors (Lundholm et al., 2003), our culture has 3–8 poroid sectors. These differences may result from environmental variabilities that affect the growth rates of the individuals.

Since our study indicates that *Pseudo-nitzschia calliantha* from the Black Sea is a DA producer, a detailed phytoplankton monitoring program to identify *Pseudo-nitzschia* species and their ability to produce DA needs to be carried out in the Black Sea.

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