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Toxicity of diatom *Pseudo-nitzschia* (Bacillariophyceae) analyzed using high performance liquid chromatography (HPLC)

Hong-Chang Lim[1], Suriyanti Nyun-Pau Su[1], Hartina Mohamed-Ali[1], Yuichi Kotaki[2], Chui-Pin Leaw[3] and Po-Teen Lim[1]*

[1] Department of Aquatic Resource Science, Faculty of Resource Science and Technology, University Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia
(* ptlim@frst.unimas.my)
[2] Laboratory of Biological Chemistry, School of Fisheries Science, Kitasato University, Sanriku, Ofurato Iwate, 022-0101 Japan
[3]Institute of Biodiversity and Environmental Conservation, Universiti Malaysian Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia

Abstract Amnesic shellfish poisoning (ASP) is a type of shellfish poisoning due to the consumption of shellfish mollusks contaminated with domoic acid (DA). The toxin was first reported in the chain-forming pennate diatom, *Pseudo-nitzschia* and subsequently in other diatom species. In this study, clonal cultures of *Pseudo-nitzschia* were established from plankton samples collected from Sarawak and Sabah coastal waters. Clonal cultures were maintained in SWII medium with the addition of silicate at 25°C, 30 PSU and under 12:12 light-dark photoperiod. Fifteen milliliters of late exponential phase cultures were collected for toxin analysis and subsamples were taken for cell count. Cellular toxin was extracted by boiling in medium at 100°C for 5 minutes. The extracts were filtered to remove cell debris before being analyzed with HPLC using standard domoic acid, isodomoic A and B as reference toxins. All the 32 strains of *Pseudo-nitzschia* sp. analyzed in this study showed the absence of peaks corresponding to the three ASP toxins. This implies that non-toxic strains of *Pseudo-nitzschia* sp. are common in Malaysian waters. Further study will be carried out to include more strains along the coastal waters of Borneo as well as selected sites with shellfish farming activities in Peninsula Malaysia.

Keywords domoic acid – amnesic shellfish poisoning (ASP) – *Pseudo-nitzschia* sp. – HPLC

INTRODUCTION

Amnesic shellfish poisoning (ASP) was first reported in Prince Edward Island, Canada in 1987 with density of the pennate diatom *Pseudo-nitzschia multiseries* (previously described as *Nitzschia pungens*) reaching up to 15×10^6 cells/L [1]. The shellfish poisoning caused three deaths and 105 cases of acute human intoxication after the consumption of contaminated blue mussels (*Mytilus edulis*) containing 900 µg/kg domoic acid [2]. A toxic bloom of *Pseudo-nitzschia pseudodelicatissima* was later reported at mussel cultivation areas in 1989 [3, 4].

In subsequent years, bloom event of *Pseudo-nitzschia australis* (7×10^5 cells/L) in Monterey Bay, California in 1991 was reported with mortality of 100 brown pelicans and cormorants [5]. In the same year, dozens of cases of human illness were reported along the Pacific coasts of Washington and Oregon, where razor clams, Dungeness crabs, blue mussels and oyster were found to contain up to 154 µg/g of domoic acid [6]. The victims exhibited gastrointestinal disorders after digestion of any contaminated shellfish. The symptoms include nausea, vomiting abdominal cramps, headache, diarrhea, and memory loss [6]. The loss of memory in patients intoxicated with ASP appeared to be similar to patients with Alzheimer's disease [7].

Domoic acid (DA) is the compound responsible for ASP (Fig. 1). It is a water soluble tricarboxylic amino acid with a molecular weight of 311.14, ($C_{15}H_{21}NO_6$). DA acts as an analogue of the neurotransmitter glutamate and is a potent glutamate receptor agonist [8] that has high binding affinity towards both kainate and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid) [9].

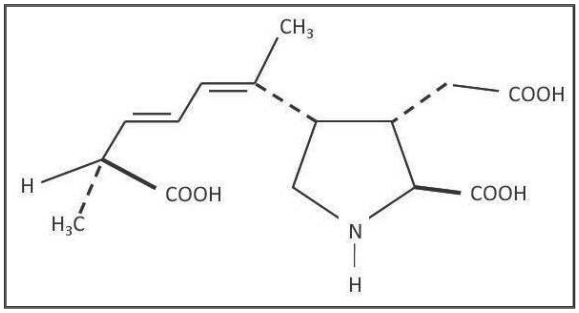


Figure 1. Structural formula of domoic acid (DA).

Currently, shellfish poisoning and plankton monitoring by the related authority in Malaysia is only focused mainly on the west coast of Sabah and selected locations in Peninsula Malaysia, and only paralytic shellfish poisoning and its related causative organisms are monitored. Little is known about the status of ASP in Malaysia though *Pseudo-nitzschia* has been commonly found in coastal waters. In this study, clonal cultures of *Pseudo-nitzschia* sp. were established from five locations along the coast of Sabah and Sarawak, Malaysia. Toxin analysis was carried out using high performance liquid chromatography (HPLC) with fluorescence detection method on all the strains in the collection.

MATERIALS AND METHODS

Phytoplankton samples

Plankton samples were collected from four sampling locations in Sarawak *viz*. Santubong, Muara Tebas, Semariang Batu, and Bintulu and one sampling location in Sabah i.e. Kota Kinabalu (Fig. 2). They were collected using a 20 µm vertical plankton net.

Pseudo-nitzschia cultures

Each single chain cells of *Pseudo-nitzschia* sp. found in the plankton net sieved samples was isolated using micropipette under an Olympus inverted light microscope (Olympus IX51). The single chain cells were inoculated into Nunc's multiwell plates with filtered seawater. SWII (Si) medium at 30 PSU was added to the wells after any observation of cell division. The culture strains established were then grown in the SWII (Si) medium (Iwasaki, 1979) in 250 mL Erlenmeyer flasks with cool fluorescence illumination at 25°C on a 12:12 hr light-dark photoperiod.

Toxin analysis

The 150 mL clonal cultures of *Pseudo-nitzschia* sp. were harvested during late exponential phase and boiled in 15 mL screwed centrifuge tubes for 5 minutes. Another 15 mL of cultures were kept in 15 mL screwed centrifuge tubes and cell densities were determined through microscopy count.

The HPLC analysis was performed by pre-column derivatization with 9-fluorenylmethylchlorofromate (FMOC) followed by reversed-phase HPLC with fluorescence detection. Subsample (1 mL) of each strain was filtered through a 0.22 µm disposable filter. Samples were treated using 50 µL borate buffer, 10 µL DHKA internal standard solution followed by 250 µL FMOC-CL reagent solution. The mixture was then extracted with 500 µL ethyl acetate. The aqueous bottom layer was transferred to a vial for HPLC analysis [10].

RESULTS AND DISCUSSION

Since the first incidence of ASP in Canada, DA-producing *Pseudo-nitzschia* species have been increasingly reported from other parts of the world. To date, 10 species of *Pseudo-nitzschia* are known to produce DA [11, 12]. They are *P. australis,*

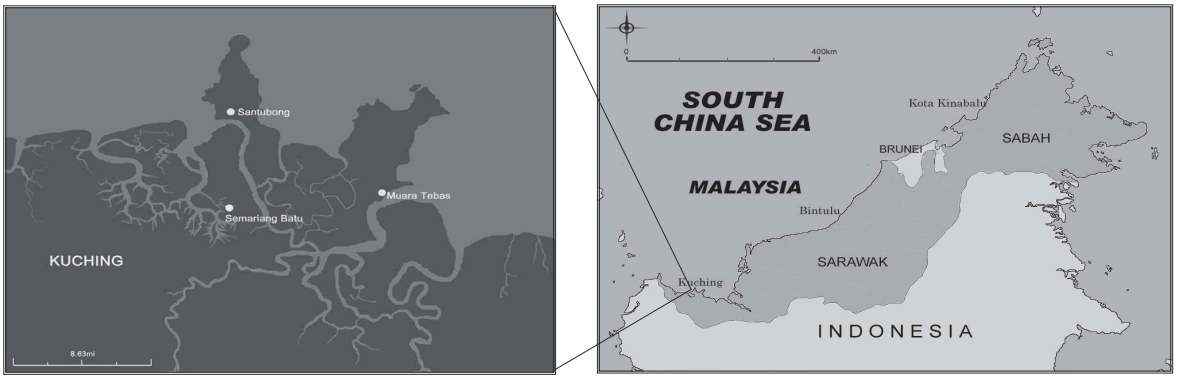
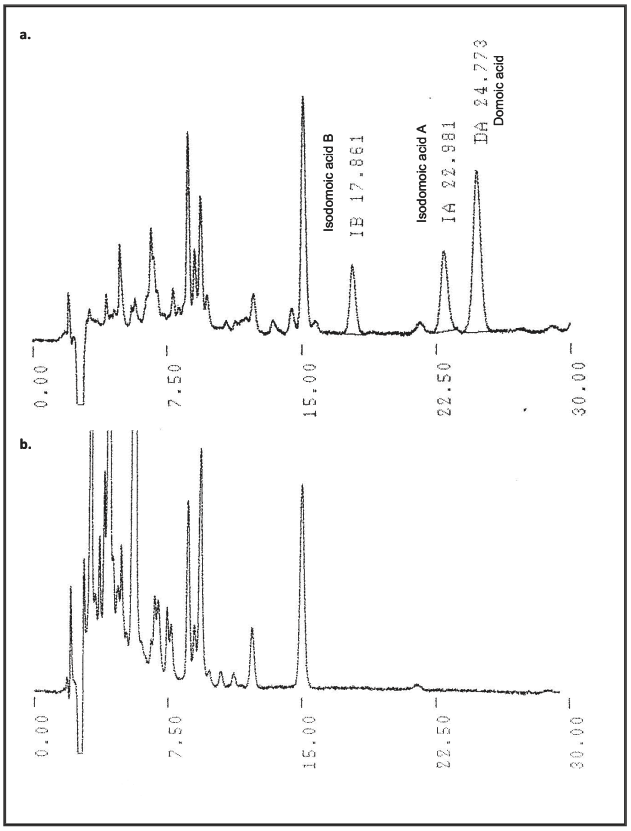


Figure 2. Sampling locations on the west coasts of Sabah and Sarawak.

P. calliantha, P. cuspidate, P. delicatissima, P. fraudulenta, P. galaxiae, P. multiseries, P. multistriata, P. pungens, P. seriata and *P. turgidula* [11, 12]. Almost all toxigenic species are primarily coastal species, although some may be found up to 150 km offshore. Most of the DA-producing *Pseudo-nitzschia* species are cosmopolitan except *P. seriata*, which is a potentially toxic species that has caused toxic blooms restricted to cold water of the North Atlantic Ocean [13]. This implies that *Pseudo-nitzschia* diatoms involved in ASP are widely distributed in the world. However, there has been no documentation on ASP neither has any survey been conducted in Malaysian coastal waters.

In total, 37 strains of *Pseudo-nitzschia* sp. were cultured in June, July and August 2008. All of the cultures were extracted and analyzed for DA toxin. There were five strains from Santubong, eight from Semariang Batu, 16 from Muara Tebas, five from Bintulu and three from Kota Kinabalu. Among the strains, none of the extracts showed significant DA-like peak identical to the retention time of standard DA (Fig. 3).



Figur 3. HPLC chromatograms of domoic acid analysis. (a) Domoic acid (DA) standard, and (b) *Pseudo-nitzschia* with no significant DA-like peak.

In this study, some cultures with small cell dimension could grow up to 568,000 cells mL^{-1} while the density of large cells was only about 3000 cells mL^{-1}. Low cell density in some of these cultures used for toxin extraction might contribute to undetectable DA in the samples. Cell density of *Pseudo-nitzschia* sp. will be increased to 100,000 cells mL^{-1} in future toxin analysis.

According to Kotaki *et al*. 14] *P. multiseries* produced DA during late stationary phase. The production of domoic acid increased remarkably when it reached the late stationary phase. Similar characteristic was also reported in *P. autralis* [6]. Several studies have shown that DA production by *Pseudo-nitzchia* sp. can be very high when either phosphate or silicate level is low while nitrogen level is high 15]. Depletion of silicate or phosphate content in water enhances and triggers the production of DA in *P. multiseries* [16] and *P. pungens* [17]. Cell division is reduced during limiting amounts of phosphorus, causing deficiency of phospholipids and reduced cell integrity by slowing the membrane forming processes. Silicate limitation also inhibits *Pseudo-nitzschia* from the normal progression of the cell cycle. These two limitations enhance DA production [18].

According to Lundholm *et al*. [19], *P. seriata* and *P. multiseries* produce a small amount of DA during the exponential phase, and toxin production is enhanced during stationary phase. In contrast, *P. australis* begins to produce DA during the exponential phase and remains either stable or declines during the stationary phase under similar culture conditions [6]. The differential production of DA by the same diatom species under the same culture condition may be related to their genetic makeup.

CONCLUSION

Our preliminary study indicated that non-toxic *Pseudo-nitzschia* strains/species are more common in Borneo waters. However, further study is needed with more samples and cultures from more locations.

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