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SCREENING OF TOXIC MARINE *NITZSCHIA* SPECIES (BACILLARIOPHYCEAE) IN MALAYSIA

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ABSTRACT

Amnesic Shellfish Poisoning (ASP) is a type of intoxication caused by the neurotoxin domoic acid (DA). The diatom genus Nitzschia is capable of producing this toxin. Screening for the presence of toxic Nitzschia spp. was carried out at various estuaries in Malaysia. Nitzschia-like cells were isolated and established into clonal cultures. Late stationary phase of cultures were harvested and tested for toxin production using HPLC. Toxin production and compound was verified by LC-MS. From the analyses, at least three cultures were detected with DA, while the rest of the cultures did not show detectable amounts of DA. The localities of the toxic species are Johor and Sabah. Here we conclude that toxic Nitzschia species are present in Malaysian water.

Keywords: diatom, estuary, intoxication, safety, toxic

INTRODUCTION

Domoic acid (DA) is a neurotoxin produced by the diatom *Pseudo-nitzschia* sp. that cause Amnesic Shellfish Poisoning (ASP) in Prince Edward Island, Canada in 1987 (Bates *et al*. 1989). The toxin accumulates in shellfish among others where signs of intoxication include memory loss in human (Perl *et al*. 1990) and even fatality in mammals (Scholin *et al*. 2000) and birds (Fritz *et al.* 1992). Recent studies had discovered DA producing capability in the genus *Nitzschia* (Kotaki *et. al*. 2000; Smida *et al*. 2014).

Eventhough HABs in Malaysia have always been attributed to dinoflagellates (Roy, 1977; Ting and Wong, 1989; Usup *et al.,* 1989; Usup *et al.,* 2002), the nation is susceptible to ASP outbreak as toxic *Nitzschia* sp. is widely distributed in Asia (Kotaki *et al*., 2004). Thus, we took the effort to screen for toxic *Nitzschia* species focusing on the marine water of Malaysia.

MATERIALS AND METHODS

Sample collections and culture establishments

Phytoplankton samples were collected at various estuaries in Malaysia (Figure 1) using a 20 µm mesh size plankton net between March 2012 and October 2014. Live samples were brought back to the laboratory for further processing. *Nitzschia*-like cells were isolated using a finely-tipped micropipette into 96-well plates. Healthily divided cells were transferred into culture tubes containing silica-enriched SWII media (Iwasaki, 1961) and maintained at 26 °C in 14:10 light:dark cycles.

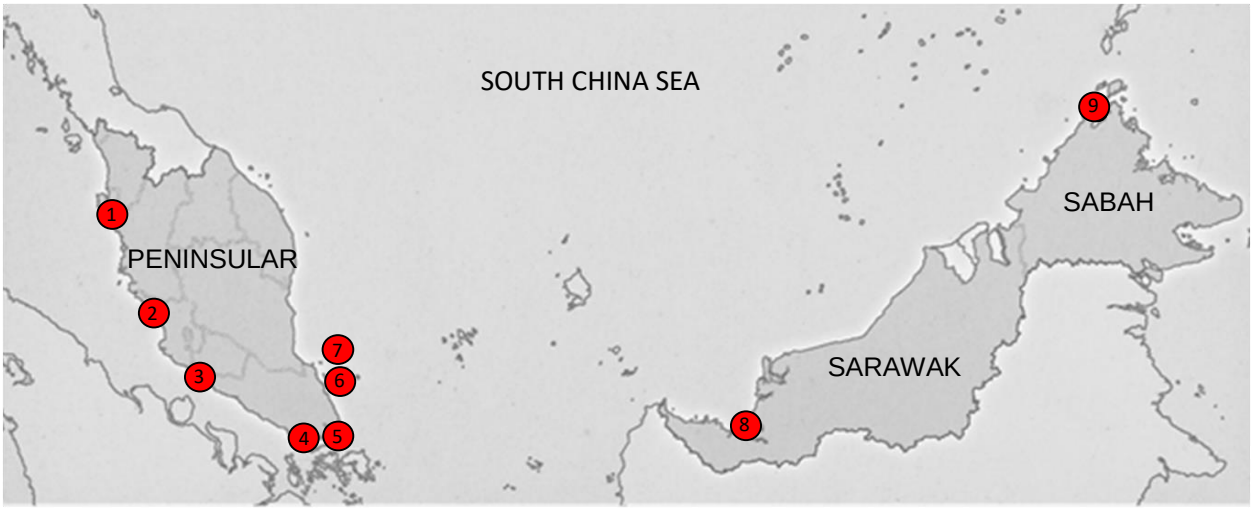

Figure 1. Map of Malaysia. Phytoplankton collections at different estuaries (●).

Screening for ASP toxin

One liter of *Nitzschia* cultures were harvested after 30-days by manual filtration over 20 µm sieves. Harvested cells were transferred into 1.5 mL microcentrifuge tubes. Domoic acid extractions and HPLC-UV (Shimadzu Corporation, Japan) conditions were carried out according to the Manuals for Determination of Marine Biotoxins Protocol for ASP toxins. Extracts with detected toxin production were verified using LC-MS micrOTOF-Q (Bruker, Germany).

RESULTS AND DISCUSSION

Cultures and screening

Out of 106 total isolates from all locations, only 12 were successfully established into cultures (Table 1). Other isolates were either did not survive in isolation wells or were other diatoms instead of *Nitzschia* species. Kudat has the most culture strains followed by Kuala Selangor. All other locations were represented by at least 1 culture strain, except for Santubong, Sarawak where none of the isolates survived. Seven cultures are marine origin while 6 were isolated from mangrove areas. Three from 12 culture strains analyzed using HPLC analysis had a peak corresponding to domoic acid (data not shown).

Table 1. Localities and habitats of culture strains tested for toxin production.

No	Locality	Culture	Habitat	Toxin test
1	Teluk Kumbar, Penang	TK47	Marine	-
2	Kuala Selangor, Selangor	KS58	Mangrove	-
	Kuala Selangor, Selangor	KS55	Mangrove	-
3	Port Dickson, Negeri Sembilan	PD1	Marine	-
4	Sungai Pendas, Johor	P22C7	Mangrove	+
		P22G9	Mangrove	+
5	Teluk Sengat, Johor	NTS105	Mangrove	-
6	Pulau Sibu, Johor	PS8	Marine	-
7	Pulau Tioman, Pahang	TMN26	Marine	-
8	Santubong, Sarawak	n.d.	Mangrove	-
9	Kudat, Sabah	KD89	Marine	-
	Kudat, Sabah	KD92	Marine	-
	The Tip of Borneo, Sabah	TOB54	Marine	-

nd: no data; (+) DA detected; (-) DA undetected.

Little study has been done on this genus as the taxonomy of this diatom is very complex particularly for those who have the limited access to electron microscopy and literatures. There are more than 500 described species of *Nitzschia* and some of them are still being reclassified and verified (Guiry and Guiry, 2014). So far, there are two toxic species in the genus, namely *N. navis-varingica* Lundholm et Moestrup and *N. bizertensis* Smida, Lundholm, Salda, Hadj Mabrouk. Toxic *Nitzschia* species is widely distributed in temperate to tropical water including Vietnam (Kotaki *et al*. 2000; Lundholm and Moestrup, 2000), Japan (Kotaki *et al*., 2004), the Philippines (Kotaki *et al*., 2005; Bajarias *et al.,* 2006), Thailand (Romero *et al.,* 2008), Indonesia (Romero *et al*., 2011) and Tunisia (Smida *et al*., 2014). Previous study done in southern parts of Asia which included several sites in Malaysia reported that toxic *Nitzschia* species was rare due to unfavourable environmental conditions (Thoha *et al*., 2012). This study focuses on estuarine areas as toxic *Nitzschia* species were reported from brackish to marine areas (Kotaki *et al*., 2000; Lundholm and Moestrup, 2000; Kotaki *et al*., 2004; Kotaki *et al*., 2005; Bajarias *et al.* 2006; Romero *et al.,* 2008; Romero *et al*., 2011; Smida *et al*., 2014). More species are expected if extensive sampling is carried out at locations we did not cover.

Toxin profiles

In LC-MS analyses of all toxic strains, a peak at the same retention time as DACS-1D standard was observed (Fig. 2a and b). The MS of the substance showed an ion peak at m/z 312 which is the $[M + H]^+$ of DA (Fig. 2b and c). The highest relative toxin intensity is from P22C7 culture which is 1.041×10^5 compared to the intensity of the standard which is 9.871×10^5. The concentration of the standard is 87.7 µg/mL. These results verified that *Nitzschia* culture strains P22C7, P22G9 and KD89 produce domoic acid.

The extracts used for toxin analyses were obtained from cells in 30-days old non-axenic cultures eventhough the average growth cycle for *Nitzschia* spp. is 7 days (Kotaki *et al*., 2000; Smida *et al*., 2014). High levels of DA in the samples could be mainly due to stationery growth phase of cultures where DA production was the highest (Kotaki *et al*., 2000; Trainer *et al*., 2012). The extracts also had been left at room temperature for days before LC-MS analyses. It was found that the DA concentration was not affected, meanwhile a very low amount of DA was observed in standard kept in 4°C. Therefore, temperature could be one of the factors that affect DA concentration. In addition, toxin production had been associated with bacteria where non-axenic cultures produce more domoic acid than axenic cultures (Bates *et al*., 1995), though presence of bacteria did not necessarily contribute to the toxin production (Douglas and Bates, 1992). Maximum cellular DA contain of toxic *Nitzschia* spp. ranges from 2×10^{-4} pg cells^{-1} to 15.3 pg cells^{-1} (Kotaki *et al*., 2004; Smida *et al*., 2014). Toxin composition and concentration in toxic *Nitzschia* sp. differ geographically (Bajarias *et al*., 2006). Physiological studies on the toxic strains are necessary to obtain more information.

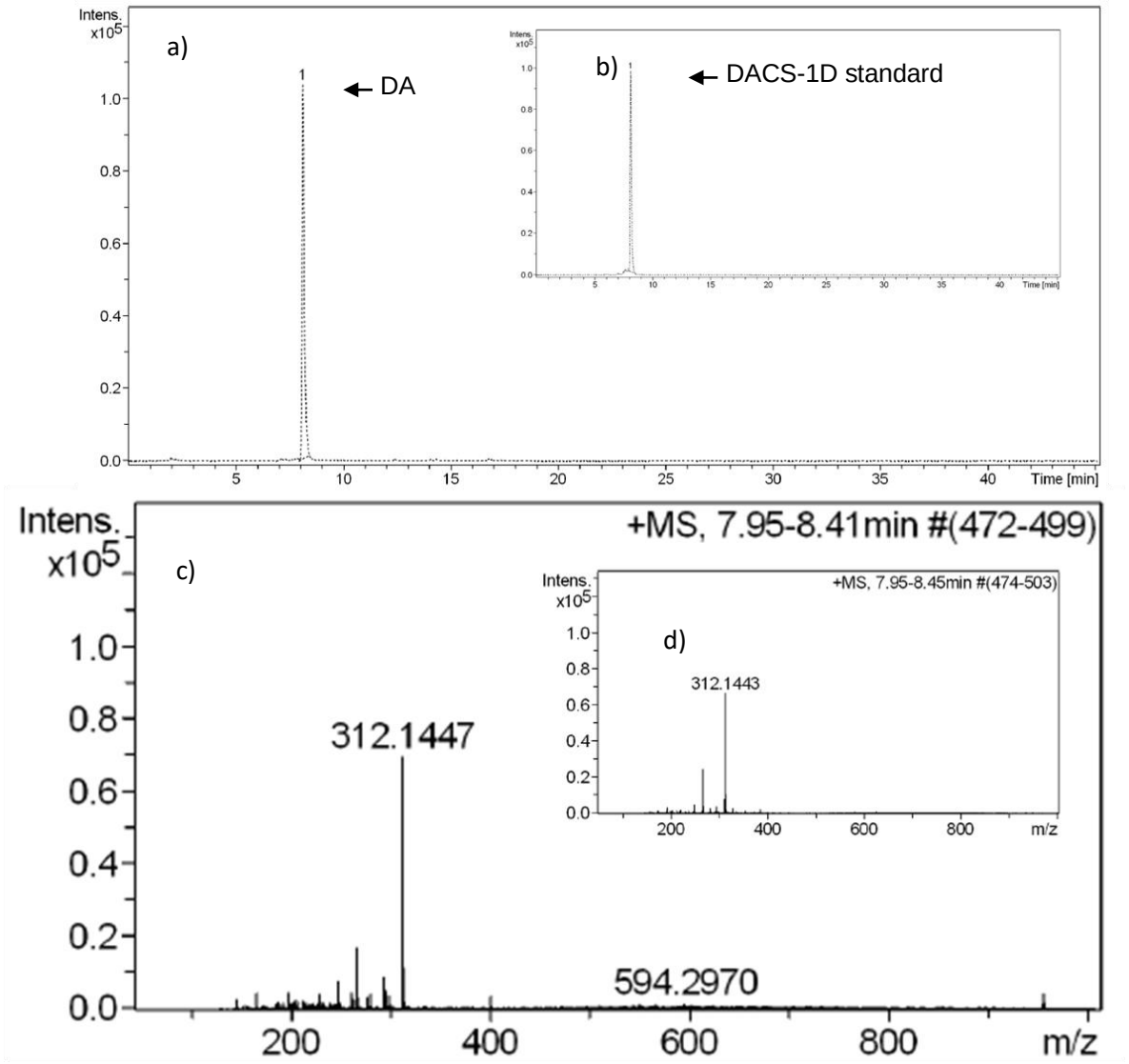

Figure 2. Results of liquid chromatography-mass spectrometry (LC-MS) of P22C7 culture extract against a domoic acid standard of 87 µg/mL (insets). a) Mass chromatogram of DA substance in P22C7 culture extract; b) Mass chromatogram of DACS-1D standard; c) Mass spectrum of DA substance in culture extract scanned at retention time of 8.13 min; d) Mass spectrum of DA substance in DACS-1D standard scanned at retention time of 8.10 min.

CONCLUSIONS AND SUGGESTION

From this study, we conclude that toxic *Nitzschia* species are present in Malaysian water. Precautious measure should be implemented to ensure seafood safety, avoid economic losses and to protect estuarine mammals.

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