Coastal Marine Science 35(1): 34–38, 2012

Screening of diatoms that produce ASP toxins in Southernmost Asian waters

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Received 14 December 2009; accepted 15 September 2011

Abstract — In order to explore the detailed distribution of *N. navis-varingica* that produces ASP toxins in Asian waters, a screening was made in four areas of Indonesia and a part of Malaysia. Diatom samples were collected by a plankton net. *Nitzschia*-like diatoms were isolated from crude pre-cultures and cultured in f/2 medium followed by HPLC-fluorescence analysis. Unlike other Asian countries, *Nitzschia navis-varingica*-like diatoms were rare in Indonesian and Malaysian samples, which is partly attributable to the extreme pH and salinity at some of the localities. Fifteen, six and eleven isolates were obtained from South Sulawesi, Lampung and Sangihe Island in Indonesia, respectively, none from Jakarta Bay, Indonesia and eight strains from Kota Kinabalu, Malaysia. All of the fifteen strains from South Sulawesi (Panyula) produced toxins. They produced on average 2.7 pg toxin cell^{-1} with the toxin profile of DA : IB (98 : 2) similar to results on strains from Thailand and northern Japan. This toxin production was confirmed by LC-MS/MS analysis. These toxin-producing strains were identified as *N. navis-varingica*. Eleven sub-strains of a single parental strain showed the same toxin profile (on average 1.1 pg toxin cell^{-1}, DA : IB=94 : 6). The remaining *Nitzschia* strains did not produce ASP toxins. Twenty isolates of *Pseudo-nitzschia* from Lampung Bay were not found to produce ASP toxins.

Key words: domoic acid, isodomoic acid, *Nitzschia navis-varingica, Pseudo-nitzschia,* Indonesia

Introduction

Domoic acid (DA) was first recognized as the toxin responsible for the amnesic shellfish poisoning (ASP) in Canada (Wright et al. 1989). The causative organism was traced to a pennate diatom *Pseudo-nitzschia multiseries* (Bates et al. 1989). After this, the search for DA-producing *Pseudo-nitzschia* species has resulted in finding 12 of the more than 30 *Pseudo-nitzschia* species to be DA-producers, although the toxin level varies considerably according to the species (Bates 2000, Kotaki 2008, Trainer et al. 2008).

During a search for DA-producing diatoms in tropical waters, *Nitzschia navis-varingica* from Vietnam was found to produce DA at levels similar to *Pseudo-nitzschia* (Kotaki et al. 2000). *Nitzschia navis-varingica* was also isolated from brackish waters in the Philippines, Thailand and Japan as well as in Vietnam (Kotaki et al. 2004) and was found to produce not only DA but also isodomoic acids A (IA) and B (IB) as major toxin components (Kotaki et al. 2005) (Fig. 1). The most frequent toxin type among *N. navis-varingica* strains are either mainly production of DA, with traces of IB (expressed as DA-traceIB type; IB ratio is less than 10%), or production of DA with substantial amount of IB (expressed as DA-IB type; IB ratio is 20 to 80%). There is a tendency for higher IB-ratios in more southern areas. One exception is the isolates from Thailand in which all strains have the DA type of toxin composition, similar to northern Japan (Romero et al. 2008). Strains from three areas of Luzon Island (Bulakan and Iba Estuary, Bacoor Estuary, Alaminos Estuary) have special toxin composition types: IA-IB, DA-IA-IB and only IB, respectively (Kotaki et al. 2008, Kotaki 2008). An investigation of strains from southernmost Asian areas like Indonesia and Malaysia has not been performed before. Similarly, *Pseudo-nitzschia* strains from Indonesia have never been examined for toxin production. We report here the first findings of *N. navis-varingica* from Indonesia and Malaysia that produce ASP toxin with a toxin composition of the DA type, similar to the strains from northern Japan and Thailand (Kotaki et al. 2008, Kotaki 2008, Romero et al. 2008). We also report the preliminary screening results of *Pseudo-nitzschia* strains from Indonesia.

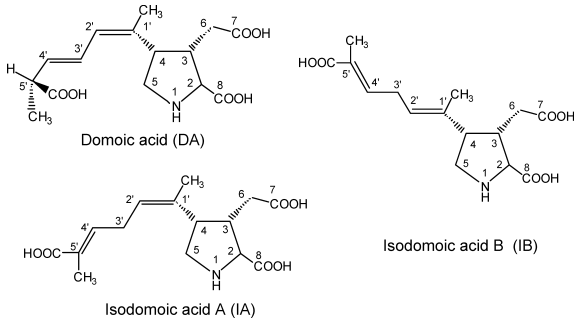

Fig. 1. Structure of domoic acid and isodomoic acids A and B.

Fig. 2. Sampling areas in Indonesia and Kota Kinabalu, Malaysia.

Materials and Methods

Collection of diatoms

Plankton samples were collected using a scoop plankton net (20 μm) and pre-cultured before isolation of single cells by the capillary washing method. Several brackish water sites were sampled in each of the following localities: Panyula in South Sulawesi (July 2008), Jakarta Bay (March 2009), Lampung Bay (March 2009) and Sangihe Island (May 2009), all in Indonesia and Kota Kinabalu, Malaysia (May 2008) (Fig. 2, Table 1). *Pseudo-nitzschia* samples were collected from Lampung Bay, Indonesia (Jun and August 2007). Water parameter like salinity, pH and temperature were measured at the collection sites wherever possible (Table 1).

Screening of DA-producing diatoms

Established strains of *Nitzschia*-like pennate diatoms were analyzed for the major ASP toxins of DA, IA and IB during a batch culture experiment. The strains were cultured in f/2 medium with a salinity of 27 psu. The experimental conditions was 25°C under an irradiance level of 70 mmol photons m^{-2} sec^{-1} with light : dark cycle of 16 : 8. Three mL of whole culture was harvested into test tubes at the stationary growth phase of the 3-week culture period. Cell concentration was determined at the time of sampling for toxin analysis. Each sample was extracted by boiling for 8 min. The extract was centrifuged and the supernatant was analyzed for ASP toxins (DA, IA and IB) by HPLC-fluorescence analysis with pre-column derivatization using 9-fluorenylmethylchloroformate (FMOC-Cl) according to slightly modified method of Pocklington et al. (1990) in which a Develosil ODS column (4.6×250 mm, Nomura, Seto Aichi, Japan) and a mobile phase of 40% acetonitrile in 20 mM phosphate buffer (pH 2.5) were used (Kotaki et al. 2004).

One representative strain positive for ASP toxin production was cultured in a one liter scale experimental flask, and during toxin sampling the cellular fraction on the filter was extracted by sonication and analyzed by LC-MS/MS using multiple reaction monitoring (MRM, *m*/*z* 312-266, 312-248 and 312-161) (Takata et al. 2009).

The *Pseudo-nitzschia* strains were also cultured and analyzed in the same way as the *Nitzschia* strains.

Species identification of the diatoms

Three representative strains positive for ASP toxin production were selected and examined using transmission electron microscopy (TEM) (see Lundholm and Moestrup 2000), and identified according to Lundholm and Moestrup (2000) and Kotaki et al. (2004) using light microscopy (LM) and TEM.

Results and Discussion

Isolation of diatoms

Fifteen strains of *Nitzschia*-like diatoms from Panyula, Bone, South Slawesi, six from Lampung Bay and eleven from Sangihe Island, all Indonesia and eight strains from Kota Kinabalu, Malaysia were obtained (Table 1). No isolates were obtained from Jakarta Bay (Fig. 2). Twenty strains of *Pseudo-nitzschia* were obtained from Lampung Bay, Indonesia.

DA analysis in the isolates

All of the 15 strains from Panyula, South Sulawesi produced ASP toxin of either of the two different toxin composition types: only DA (four strains) and DA-trace IB (eleven strains). Average toxin content of DA and IB of all the strains were 2.38 and 0.06 pg $cell^{-1}$, respectively (ratio of DA : IB was 98 : 2) (Fig. 3). Cell extract from one representative strain (IBNB 08-2) was analyzed by LC-MS/MS (MRM), which confirmed the presence of DA and IB.

This toxin composition type found was the same as in the strains from northern Japan and Thailand, and different from the strains from Okinawa, southernmost Japan, Vietnam and the Philippines (Kotaki 2008, Romero et al. 2008). This result shows that the toxin composition is not related to latitude as previously hypothesized (Kotaki 2008).

Eleven sub-strains were established from the same parental strain, IBNB 08-2, and the toxin composition of all of them showed the same composition as in their parental

Table 1. Water parameters of each sampling site

Sampling area	Code name	pH	Salinity	Temperature	Sampling date
Indonesia					
Jakarta Bay	IJAKA09	7.4	5	31	2009/3/14
	IJAKB09	8.4	—	32	2009/3/14
	IJAKC09	7.9	24	33	2009/3/14
	IJAKD09	7.4	3	34	2009/3/16
	IJAKE09	7.5	2	31	2009/3/16
	IJAKF09	7.5	6	29	2009/3/16
	IJAKG09	7.4	5	32	2009/3/16
Jakarta Bay	IJAK2A09	7.2	25	30	2009/4/23
	IJAK2B09	7.2	20	30	2009/4/23
	IJAK2C09	7.5	1	30	2009/4/23
	IJAK2D09	7.6	1	30	2009/4/23
	IJAK2E09	7.6	1	30	2009/4/23
	IJAK2F09	7.6	4	30	2009/4/23
Lampung Bay	ILAMPA	7.9	32	30	2009/3/24
	ILAMPB	8.3	32	30	2009/3/24
	ILAMPC	—	—	—	2009/3/24
	ILAMPD	8.4	32	30	2009/3/26
	ILAMPE	—	—	—	2009/3/26
	ILAMPF	8.5	32	30	2009/3/26
	ILAMPG	—	—	—	2009/3/26
	ILAMPH	—	—	—	2009/3/26
	ILAMPI	—	—	—	2009/3/26
Sangihe Island	ISANGIHEA	6.2	10	29	2009/5/18
	ISANGIHEB	6.2	10	29	2009/5/18
	ISANGIHEC	6.2	10	29	2009/5/18
	ISANGIHED	6.2	10	29	2009/5/18
	ISANGIHEE	6.2	10	29	2009/5/18
	ISANGIHEF	6.2	10	29	2009/5/18
	ISANGIHEG	6.4	30	31	2009/5/20
	ISANGIHEH	6.4	30	31	2009/5/20
	ISANGIHEI	6.4	30	31	2009/5/20
	ISANGIHEJ	6.4	30	31	2009/5/20
Malaysia					
Kota Kinabalu	MLIK 08	-	25	31	2008/5/22
	MINA 08	—	22	32	2008/5/22
	MKIN 08	—	30	32	2008/5/22
	MLKK 08	—	31	33	2008/5/22
	MPUT 08	—	32	31	2008/5/22

(—) not measured

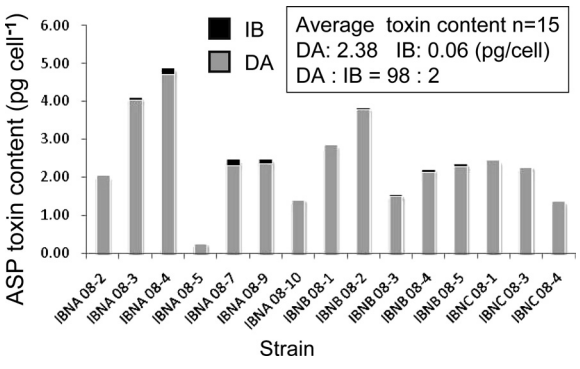

Fig. 3. Toxin composition of *N. navis-varingica* isolates from Panyula, Bone, South Sulawesi, Indonesia.

strain (toxin composition type DA-trace IB). Average toxin cellular contents of DA and IB were 1.02 and 0.07 pg cell^{-1}, respectively, with an average toxin composition ratio of DA : IB being 94 : 6. This shows that toxin composition of a strain is stable. All the other 25 strains from Lampung Bay, Sangihe Island and Kota Kinabalu did not show ASP toxin production. All the 20 isolates of *Pseudo-nitzschia* gave negative results when tested for DA, IA and IB production.

Species identification of the Nitzschia

The morphological characteristics of the three *Nitzschia* strains (IBNA08-3, IBNB08-2, IBNC08-3) which showed positive ASP toxin production agreed with the description of

N. navis-varingica by Lundholm and Moestrup (2000). In LM, the cells were yellow-brown and possessed two chloroplasts at each end of the cell; the cells were lanceolate in valve view, 38–110 μm long and 9–11 μm wide; in girdle view, they were rectangular and slightly indented at the middle. Most cells formed ribbon-shaped colonies whilst growing. In TEM, the characteristic silica ridges were seen in the wall of the raphe canal and on the mantle. The girdle bands were ornamented by silica warts and the valvovopula had 2–3 rows of poroids. On the valve, the density of interstriae were 26–28 in 10 μm and the poroids in the uniseriate striae had a density of 3–5 poroids in 1 μm. The raphe was interrupted in the middle by a central interspace and the fibulae that did not show a central larger interspace had a density of 8–11 in 10 μm. The density of the poroids on the girdle bands was 32–40 in 10 μm.

The *Pseudo-nitzschia* strains which did not produce ASP toxins were not identified.

Water parameters at the collection sites

Water temperature, salinity and pH were measured if possible. Unfortunately, they were not measured at Panyula where the strains positive for ASP toxin production were obtained (Table 1). *N. navis-varingica* strains were not obtained from any of the sampling sites in Jakarta Bay, Indonesia and were rare at the other sampling localities in Indonesia and Malaysia. This is unlike several other countries in Asia such as Vietnam, Philippines, Thailand and Japan where *N. navis-varingica* is a very common species (Kotaki 2008, Kotaki et al. 2008). These differences may partly be explained by the very low salinity in some of the sites in Jakarta Bay (1–6 psu), or the very low pH in Sangihe Island (pH 6.2 or 6.4). These water parameters showed that the sampling sites are not similar to the brackish water areas where *N. navis-varingica* usually exists (Kotaki et al. 2004, Kotaki 2008). We have no other possible explanation for the low frequency of *N. navis-varingica.* Water parameters like especially salinity and pH should be measured continuously in order to determine whether these factors limit the distribution of *N. navis-varingica* in brackish water areas. Further studies with more intense sampling and ecophysiological laboratory studies are therefore needed, especially in Indonesian waters.

Acknowledgement

This work was supported in part by a multilateral cooperative research project (coastal oceanography) of the Japan Society for the Promotion of Science (JSPS).

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