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THESIS ABSTRACT

**Ecobiological study on benthic diatom
producing amnesic shellfish toxins
in Asian waters**

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Introduction

Amnesic shellfish poisoning first occurred after the consumption of contaminated mussels in Prince Edward Island, Canada in 1987. The causative compound was traced to domoic acid (DA). And the toxic organism was later found and identified as a pennate diatom *Nitzschia pungense* forma *multiseries* (currently known as *Pseudo-nitzschia multiseries*). This diatom produces high levels of the neurotoxic amino acid, DA amounting >10 pg /cell in culture experiments. After the incident, screening of DA-producing *Pseudo-nitzschia* strains was done by many parts of the world, resulting in the discovery of more than ten toxin-producing species including *P. australis* and *P. seriata*. While plenty of researches have been performed in temperate waters, few have been done in tropical waters. During the screening of harmful algae in tropical waters, a curious diatom was isolated from a resting shrimp culture pond in Do Son, Vietnam and was found to produce significant level of DA. The species was identified as a new species, *Nitzschia navis-varingica*. This was the first confirmed report of a diatom not belonging to *Pseudo-nitzschia* that produces DA.

In this thesis, surveys were done to study the distribution of *N. navis-varingica* in Asian waters followed by experiments to ascertain the hypothesis that this diatom expands its distribution by the warm water current, to clarify the toxin production characteristics of the diatom in relation to the ecobiological role of the toxin production, and to verify the possibility of toxin accumulation in shellfish/snails around them.

Chapter 1 Distribution and toxin production characteristic of *N. navis-varingica*

After the first discovery of *N. navis-varingica* as a DA producer, screening of the *N. navis-varingica* was conducted outside Vietnam. DA analyses showed that *N. navis-varingica* distribute not only in Vietnam but also in Japan and the Philippines. Analyses further showed that *N. navis-varingica* also produces isodomoic acids A (IA) and B (IB) in addition to DA as the major toxin component. As *N. navis-varingica* has been found only in brackish water areas and was not from sea water nor fresh water areas, estuarine areas were selected for establishment of strains of *N. navis-varingica*. More detailed distribution of *N. navis-varingica* was screened in Japan and South East Asian countries such as the Philippines, Vietnam, Thailand, Indonesia and Malaysia. The locations of the screening are as follows. Japan; Estuarine area of Otsuchi Bay, Iwate Prefecture (September 2005) and estuarine areas of Okinawa Is (Naha, Suzaki, Mannza, Gesashi) and Ishigaki Is (Miyara, Nagura) (July 2005), Vietnam; Estuarine areas in Do Son, Dinh Vu and Ha Long Bay near Hai Phong (October, 2005). The Philippines; Iba Estuary in Zambales (March 2005, 2007), Estuarine areas of Bolinao and Alaminos in Pangasinan (December 2007, 2008), Bulacan and Cavite in Manila Bay (March 2005, December 2007), all are located in Luzon Is. Thailand; Estuarine areas of Chantaburi, Chonburi and Bangkok (including Mae-Klong River mouth, Samut Songkram Province and Choa Phuraya River mouth, Bangkok) (April 2007). Indonesia; Estuarine areas of Panyula, Bone in South Sulawesi (July 2008), Jakarta Bay (March 2009), Lampung Bay (March 2009) and Sangehe Is (June 2009). Malaysia; Estuarine areas of Kota Kinabalu, Sabah in Malaysian Borneo Is (May 2008).

Toxin production was surveyed for DA, IA and IB. *N. navis-varingica* producing DA and

IB were isolated from the estuarine areas surveyed in Japan (Otsuchi in Iwate Prefecture, Ishigaki and Okinawa Is in Okinawa Prefecture), Vietnam (Do Son, Dhin Vu and Ha Long), Thailand (Mae-Klong River mouth, Samut Songkram Province and Choa Phuraya River mouth, Bangkok), Indonesia (Panyula, Bone in South Sulawesi). Toxin production was analyzed by HPLC with fluorescence detection and confirmed by LC/MS/MS analyses using multiple reaction monitoring (MRM). *N. navis-varingica* producing IA-IB or IB or DA-IA-IB were isolated only from the estuarine areas in the Philippines (Iba in Zambales, Bulacan in Manila Bay, Bolinao and Alaminos in Pangasinan). As a result, toxin production types of *N. navis-varingica* were classified into four types. These are the types of DA-IB, IA-IB, DA-IA-IB and IB. Major type is DA-IB which is shown in the isolates of most areas (Japan, Philippines, Thailand, Vietnam, and Indonesia). Special toxin composition of DA-IA-IB, IA-IB and IB was seen only in above limited areas of the Philippines. Toxin production also showed that it is stable within each strain as shown by the same toxin production type by parent and sub-clones. Results also showed that ASP toxins are stable in cell free medium. Transformations were not observed even in the presence of bacteria.

Chapter 2 Dispersion of *N. navis-varingica* distribution

Studies showed wide distribution of *N. navis-varingica* in Asian waters. It's expansion mechanism was hypothesized to be transferred through the warm water current such as "Kuroshio." Surveys and corresponding experiments were performed to support the hypothesis. Culture experiments under various salinity conditions showed the highest growth rate and toxin production at salinity of 21–35. In addition, growth was also observed under wide range of salinities (7–35). Culture experiments under several temperature settings using isolates from both cold and tropical areas showed best growth at 25 °C and 30 °C and survival was recorded at 35°C. The toxin production rate (pg/cell/day) was observed at higher temperatures even in the isolate from cold area. Both salinity and temperature culture experiments showed survival of *N. navis-varingica* under varying environmental conditions brought about by the transfer through the warm water current.

Further surveys on *N. navis-varingica* distribution in key areas along the warm water current were done. Distribution and toxin production was confirmed in many areas along the warm water current (Shizuoka, Mie, Wakayama, Nagasaki, Kagoshima, Miyazaki, Fukuoka, Yamaguchi, all in Japan, Kota Bharu in Malaysia, Tainan and Bali in Taiwan and Laoag in the Philippines).

Genetic similarity of rDNA in the ITS1-5.8S-ITS2 regions was also analyzed using the strains isolated from these areas along the warm water current. Interestingly, the phylogenetic tree divided *N. navis-varingica* into two groups with difference in *p*-distance (*N. navis-varingica* in comparison to *Nitzschia* clade 2, 8.0-9.6%). However both clades were shown along the warm water current supporting the above distribution hypothesis. This represents the first report of two *N. navis-varingica* clades.

Major toxin composition type (DA-IB) was found along the water current and the special type (IA-IB) was found in newly isolated strains from the key areas were explainable by the transfer through the warm water current.

Chapter 3 Factors affecting the toxin production of *N. navis-varingica*

Several factors that affect toxin production were investigated. These include bacterial effect and short-term pH changes. The presence of bacteria in the cultures was found to change the toxin composition. Three parental strains prepared in axenic sub-cultures were composed of two DA-IB types and one IA-IB type. In the axenic cultures, IA-IB toxin type did not change toxin composition. However, in one of the two DA-IB types treatment, one did not change toxin composition whereas the other changed toxin type to IA-IB. Re-introduction of the converted IA-IB type into a cell free medium with bacteria of the parental strain caused the toxin composition to revert to DA-IB type. Short term pH changes which can be seen during tidal cycles tend to increase the production of DA while production of IB remains constant.

Chapter 4 ASP toxin contamination in shellfish by *N. navis-varingica*

ASP toxin contamination by *N. navis-varingica* was tested on shellfish and snails collected from the estuarine areas of Bolinao and Alaminos, Pangasinan in the Philippines where *N. navis-varingica* distributes. Meat of snails *Vittina* sp. and *Neritina* sp. were found to contain small amount of DA (1.0-4.2 µg/g, n=5) and shellfish *Polymesoda erosa* (mangrove clam) were also found to contain small amount of DA (0.20 and 0.24 µg/g, n=2). These contaminations were confirmed by LC/MS/MS analysis. This might be the first report of the ASP toxin accumulation in snail and shellfish from *N. navis-varingica*. Toxin levels are low enough against the international regulatory limit (20 µg/g), however continuous monitoring is needed as *Polymesoda erosa* (mangrove clam) is a common seafood in Southeast Asian countries.

Summary

It was shown that *N. navis-varingica* is widely spread in estuarine areas in Japan and Southeast Asia. The diatom produces not only DA but also IA and IB. Four types of toxin composition (DA-IB, IA-IB, DA-IA-IB, IB) were observed among strains. Type DA-IB was the major type in Asia whereas other types were observed mainly in the Philippines. When the water current is taken into account for the *N. navis-varingica* distribution, it was hypothesized that *N. navis-varingica* distribution happens via the warm water current. Further studies on the distribution of *N. navis-varingica* along the warm water current were done, resulting in the confirmation of their distribution. The diatom grew well at higher temperatures (25, 30°C) and survived even at the highest temperature (35°C). Toxin production was found higher when the culture grew at higher temperature. *N. navis-varingica* also grew/survived under wide range of salinities (7-35). Toxin production was maintained at these salinities. Genetic analysis for the rDNA ITS1-5.8S-ITS2 regions showed that the diatom is divided into two clades and both were distributed along the warm water current. All of these results support the expansion hypothesis of the *N. navis-varingica* distribution. Short term pH changes of brackish waters which can be seen during tidal cycles tend to increase the ratio of DA in DA-IB type strains. Analysis of shellfish from sites where *N. navis-varingica* was collected showed detectable amounts of ASP toxins, suggesting possible toxin accumulation.