

Morphological observation of two species of *Pseudo-nitzschia* (Bacillariophyceae)

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Abstract—About one third of the species in the genus *Pseudo-nitzschia* has been known to produce domoic acid, which is responsible for amnesic shellfish poisoning, resulting in human intoxication and the death of marine fauna. Precise identification to species level was done based on ultrastructure of frustules marking, which includes fibulae, striae, poroids and central interspace, as well as the cell dimensions. In this study, detail morphological observation of field samples and clonal cultures of *Pseudo-nitzschia* from estuarine waters in Kuching, Samariang Batu, Santubong and Muara Tebas was carried out using electron microscopy (SEM and TEM). Diatom cells were treated with acid to remove organic cellular contents before observation under SEM and TEM. Two morphotypes were observed from these three locations. One morphotype possesses a rectangular valve shape, the apical and transapical axis of 31.9–36.8 μm and 2.6–3.0 μm , respectively, with 21–25 fibulae and 21–24 striae in 10 μm . The other morphotype shows linear to lanceolate cell shape with the apical axis of 67.2–79.9 μm and the transapical axis of 3.0–3.5 μm . Numbers of fibulae and striae in 10 μm are 12–13 and 11–14, respectively. Central interspace is absent in both morphotypes. Based on the ultrastructure, the two morphotypes of *Pseudo-nitzschia* is designated as *P. brasiliana* and *P. pungens*, respectively.

Key words: *Pseudo-nitzschia*, *Pseudo-nitzschia brasiliana*, *Pseudo-nitzschia pungens*, amnesic shellfish poisoning, Malaysia

Introduction

Pseudo-nitzschia H. Peragallo is one of the most common diatom genera among marine phytoplankton and it is cosmopolitan as it can be found in polar, temperate, subtropical and tropical areas worldwide (Hasle 1972, Kaczmarek et al. 1986, Fryxell et al. 1991). Several *Pseudo-nitzschia* species had been reported to produce neurotoxin domoic acid (DA). Blooms of *Pseudo-nitzschia* in natural water may result in bioaccumulation of toxin in marine ecosystem, food web and thereby affecting marine mammals, seabirds and potentially humans (Bates et al. 1989, Work et al. 1993, Scholin et al. 2000).

Pseudo-nitzschia has received much more attention since the first occurrence of intoxication at Prince Edward Island, Canada in 1987 which caused illness of approximately 100 people while 4 patients died as a result of amnesic shellfish poisoning (ASP) after they consumed contaminated mussels (blue mussel, *Mytilus edulis*) (Perl et al. 1987). It was the first awareness of toxin producing diatoms (Bates et al. 1989).

Several species of *Pseudo-nitzschia* have been reported to produce neurotoxin domoic acid. These species include *P.*

australis, *P. calliantha*, *P. cuspidata*, *P. delicatissima*, *P. fraudulenta*, *P. galaxiae*, *P. heimii*, *P. multiseriata*, *P. multistriata*, *P. pungens*, *P. pseudodelicatissima*, *P. seriata* and *P. turgidula* (Perl et al. 1987, Hasle et al. 1996, Bargu et al. 2002, Lundholm et al. 2003, Bill et al. 2005). The focus of this study is to report the presence of *P. brasiliana* and *P. pungens* for the first time for Sarawak waters based on their morphological data.

Materials and Methods

Sampling and clonal cultures

Plankton samples were collected fortnightly from estuarine waters of Samariang Batu, Santubong and Muara Tebas, Sarawak from August to December 2008 using a 20 μm mesh plankton net (Fig. 1). The phytoplankton net samples were sent back to the laboratory and examined under Olympus IX51 inverted light microscope. Single chain of cells identified as *Pseudo-nitzschia* spp. was isolated with a micro-Pasteur pipette and transferred into 24-well microplates. The clonal cultures of *Pseudo-nitzschia* spp. were established in sterile SWII medium (Iwasaki 1961) with enrichment of silicate (pH adjusted to 7.8–7.9). Natural seawater

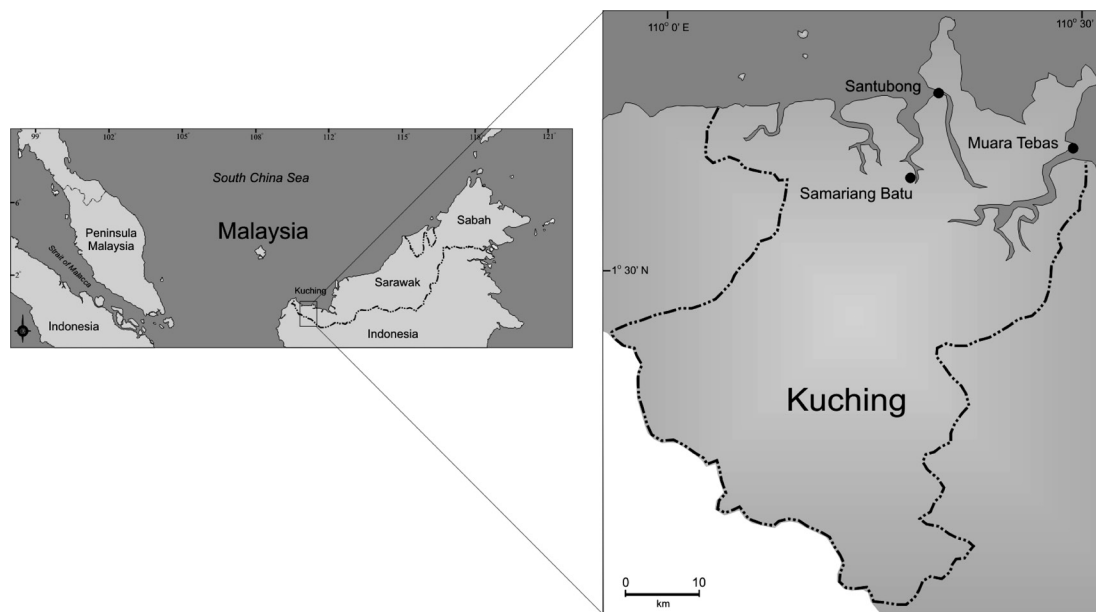


Fig. 1. Map showing the sampling locations in Kuching, Sarawak, Malaysia.

ter with 30 psu was used as medium base. Cultures were maintained at 25°C, light intensity of 200 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ with 12 : 12 hour light : dark photoperiod.

Light microscopy

Morphology of *Pseudo-nitzschia* from natural samples and clonal cultures was observed under light microscopy using phase contrast optic. A drop of each culture samples were transferred to a slide with cover slip and observed under a Nikon Eclipse 80 compound microscope with a Digital Sight DS-L1 camera. The percentage of cell ends overlap was recorded. The identification of *Pseudo-nitzschia* spp. using light microscopy was attempted on general features which include cell length and shape (Trainer and Suddleson 2005).

Electron microscopy

Clonal cultures of *Pseudo-nitzschia* spp. at stationary phase with cell density exceeding $10^4 \text{ cells L}^{-1}$ were harvested through centrifugation. Cells were treated with one drop of saturated KMnO_4 solution for 15 min, followed by addition of concentrated HCl (1 ml) for 30 min to remove all the organic matters (Sibel et al. 2002). Mixtures were then rinsed several times with distilled water to remove the remaining salt and acid. The acid cleaned materials were then examined under TEM JEM—1230 (JEOL DATUM LTD) at an accelerating voltage of 80 kV with beam current at 11.2 μA (57 %).

For SEM observation, a few drops of the acid-washed cells slurry were pipetted and filtered on track-Etch membrane filter paper (0.2 micron pores size) using vacuum-manifold, dried in oven, mounted onto aluminum stubs and sput-

ter coated with gold palladium by auto fine coater. Specimens were examined under a JSM—6390LA (JEOL DATUM LTD) scanning electron microscope with an accelerating voltage of 10 kV.

Morphometric characteristics

The cells identified as *Pseudo-nitzschia* were examined for several characteristics under the electron microscope including the width and length of the valve, numbers of fibulae and striae in 10 micron, number of poroids in 1 micron and number of row of poroids. About 30 valves from each specimen/strain were randomly selected and morphometric measurements were performed.

Results

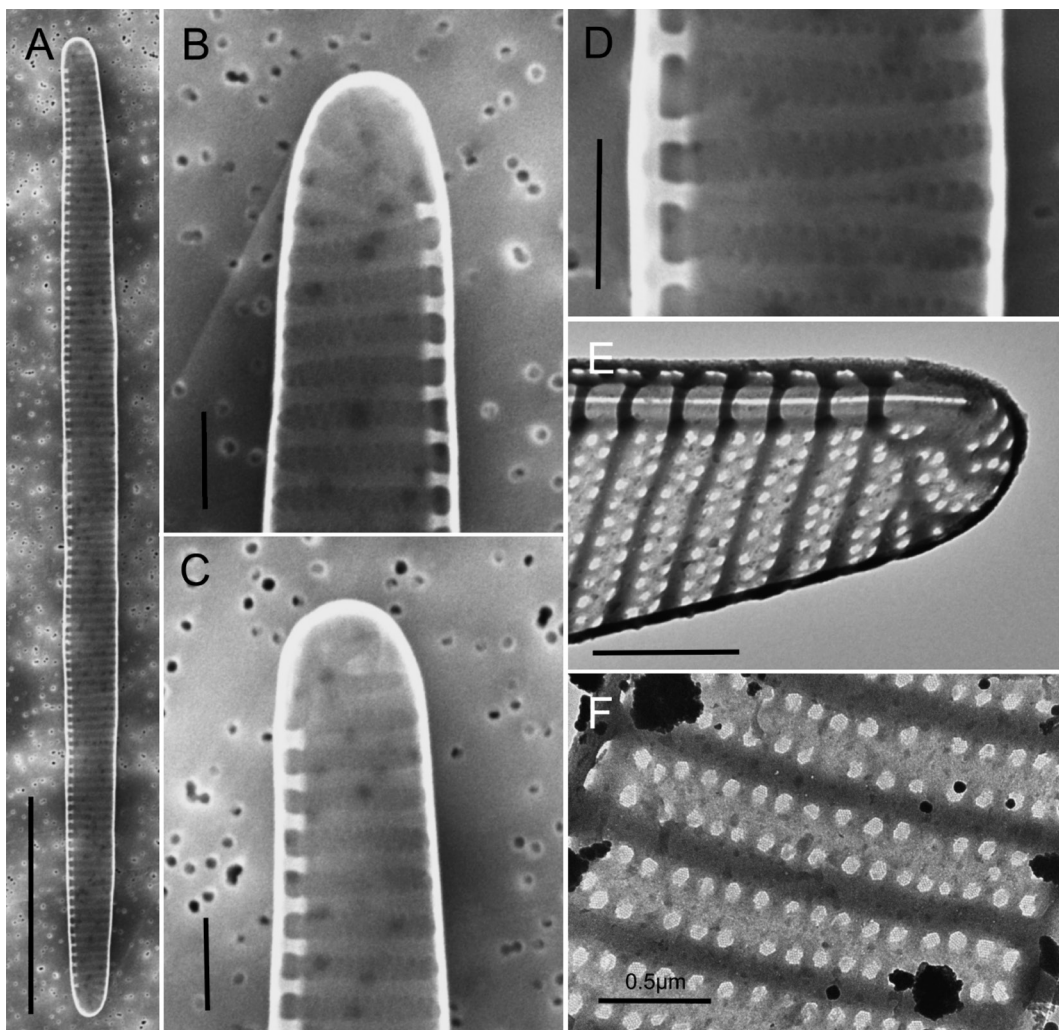
Two species of *Pseudo-nitzschia*, *P. brasiliiana* and *P. pungens* were found and documented for the first time in Malaysia based on detailed morphological observation (Table 1). Here we describe the detailed morphological features of the two species based on natural samples and clonal cultures of *P. brasiliiana* (PnSm20) and *P. pungens* (PnMt45).

Pseudo-nitzschia brasiliiana (Lundholm) Hasle et Fryxell 2002

The appearances of the cells are symmetric in valve view with slight tapering towards the broadly rounded tips (Fig. 2A–C). The cells are interconnected with 1/10 of cell overlap into a motile chain. The apical axis is 31.9–36.8 μm and the transapical axis is 2.6–3.0 μm . *P. brasiliiana* have equal number of fibulae and striae in 10 micron, i.e., 21–25

Table 1. Morphometric data recorded under TEM and SEM for two *Pseudo-nitzschia* species observed in Sarawak waters compared with literature data. Numbers in square brackets indicate number of cells used for measurements.

Species	Valves		Fibula		Striae (in 10 μm)	Poroid		Cell overlap
	Length (μm)	Width (μm)	Number in 10 μm	Central interspace		Number in 1 μm	Rows in 1 stria	
<i>P. brasiliiana</i>								
PnSm20	32–37 [30]	2.6–3.0 [30]	21–25 [4]	absent	21–24 [6]	9–10 [5]	2 [3]	1/10
Brazil ^a	30–49	2.0–3.4	20–26	absent	20–26	7–10	2 (3)	
Spanish ^b	34–39	2.3–3.3	22–27	absent	23–28	7–10	2	
<i>P. pungens</i>								
PnMt45	67–80 [20]	3.0–3.5 [20]	12–13 [4]	absent	11–14 [5]	3–4 [3]	2 (3) [4]	1/4
Field samples	88–104 [16]	2.7–3.0 [16]	12–13 [8]	absent	10–11 [8]	2–4 [5]	2 [5]	1/4
USA ^c	96–160	2.0–4.0	10–16	absent	10–16	4–5	2 (3)	
Ireland ^d	61–156	2.2–5.4	10–16	absent	10–16	3–4	2	
Spanish ^b	70–156	2.2–4.8	9–13	absent	9–13	2–3	2	

References: ^a Villac et al. (2005), ^b Quijano-Scheggia et al. (2008), ^c Sterh et al. (2002), ^d Cusack et al. (2004)**Fig. 2.** *Pseudo-nitzschia brasiliiana*, PnSm20, isolated from Kuching. (A–D) Scanning electron micrographs. (A) Acid cleaned valve showing fibulae and striae, scale bar, 10 μm . (B–C) Two end of one valve, broadly rounded apices, scale bar, 1 μm . (D) Central part of the valve showing absent of central interspace, scale bar, 1 μm . (E–F) Transmission electron micrographs. (E) Valve slightly tapering towards the tip, scale bar, 1 μm . (F) Close-up of valve showing row of poroids in striae, scale bar, 0.5 μm .

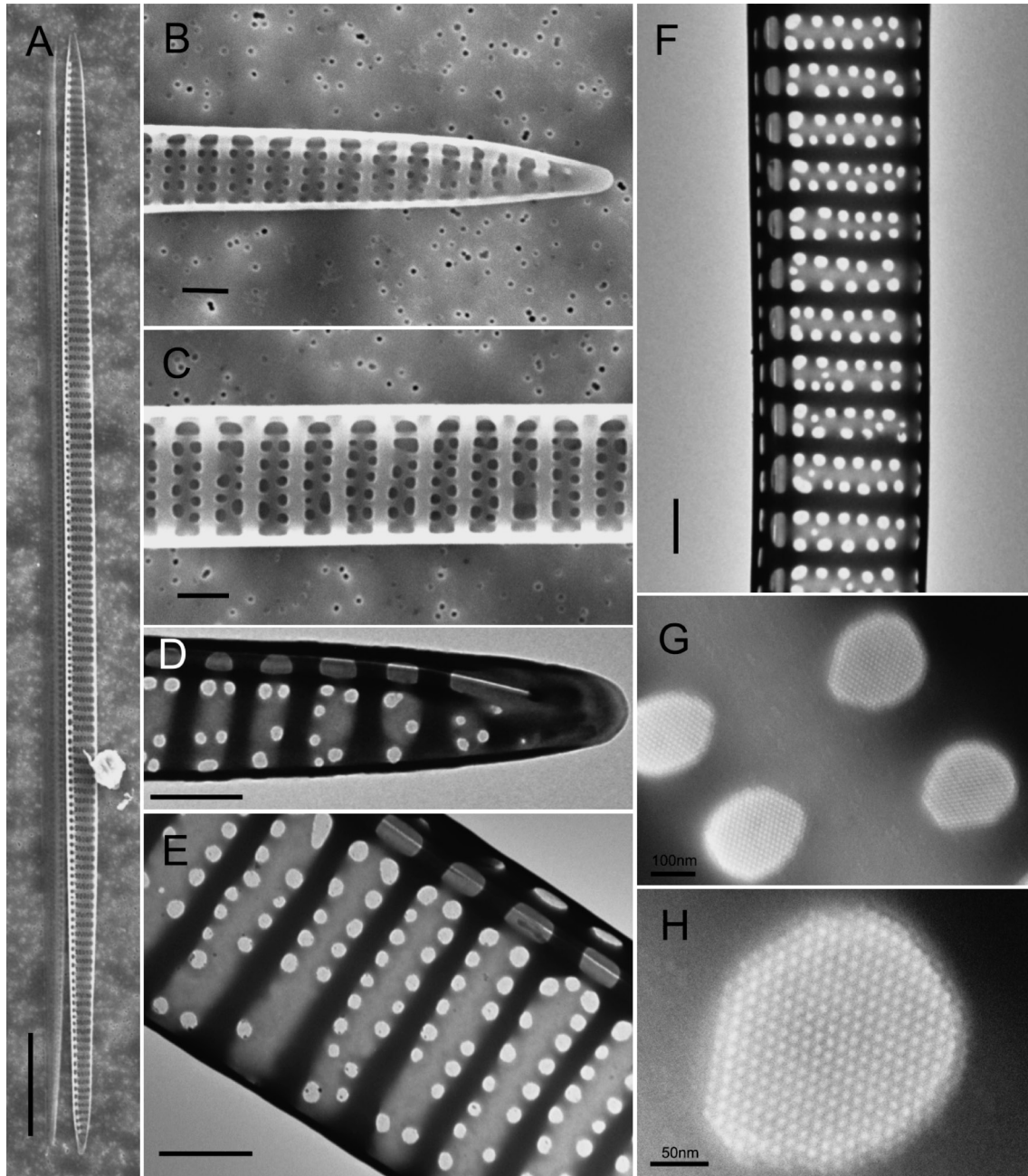


Fig. 3. *Pseudo-nitzschia pungens*, strain PnMt45 and cells from field samples. (A–C) SEM. (A) Acid cleaned frustules showing coarsely structured valve, scale bar, 10 μm . (B–C) End and centre part of valve, scale bar, 1 μm . (D–H) TEM. (D) Pointed cell end, scale bar, 1 μm . (E–F) Central part of the valve showing absent of central interspace, scale bar, 1 μm . (G–H) Close-up of valve showing two rows of poroids in each striae.

and 21–24 respectively. Striae consist of two rows of poroids with 9–10 poroid in one micron (Fig. 2F). Central interspace is not present (Fig. 2D). *P. brasiliensis* belongs in the *delicatisima*-group with valve width less than 3 μm .

Pseudo-nitzschia pungens (*Grunow ex P.T.Cleve*) Hasle 1993

The cells of *P. pungens* are linear to lanceolates, symmetric along the apical axis and pointed ends in girdle and valve view. The apical and transapical axis for the valve is 67.2–79.7 μm and 3.0–3.5 μm , respectively. In this species,

central interspace is absent (Fig. 3A–C). The numbers of striae and fibulae in 10 micron are 13–14 and 12–13, respectively. Consistently, two rows of round poroids occur in each stria and poroids are spaced at 3–4 per one micron (Fig. 3D–F). The hymen is round, simple without any perforation (Fig. 3G–H). The overlap of cell ends is approximately 1/4 of the cell length. *P. pungens* belongs in the *seriata*-group with valve width more than 3 μm .

Discussion

Pseudo-nitzschia brasiliiana and *P. pungens* are reported for the first time in Sarawak coastal waters. The species have previously been reported in other parts of the world (Hasle et al. 1996, Sterh et al. 2002, Cusack et al. 2004, Villac et al. 2005, Quijano-Scheggia et al. 2008, Turrell et al. 2008). In addition, this study contributes to the regional check-list of harmful or potentially harmful algae species in Malaysian waters.

The two species showed some similarity in morphology. Both species showed absence of large central interspace, simple hymen without perforation and two rows of poroids positioned close to the interstriae in striae. However, several other features such as number of fibulae and striae in 10 micron, the width, length and shape of valves differed and could be used for differentiating the two species from each other.

P. brasiliiana is very similar to *P. americana* in terms of the cell features. According to Larsen and Nguyen (2004), *P. americana* does not form colonies, while *P. brasiliiana* makes colonies in the form of chain cell. *P. brasiliiana* has approximately same number of interstriae and fibulae in 10 μm and the interstriae are regularly spaced, while *P. americana* has more interstriae than fibulae in 10 μm (26–31 and 18–24) (Lundholm et al. 2002). *P. brasiliiana* can be distinguished from other species in *delicatissima*-group, which lacks central interspace, by the two rows of poroids and the equal number of fibulae and striae. *P. cf. granii*, *P. micropora* and *P. multistriata* have similar size in terms of valve length and valve width, but they have very characteristic cell shapes, fibulae and interstriae (Quijano-Scheggia et al. 2008, Larsen and Nguyen 2004).

P. pungens differs evidently from *P. australis* and *P. multiseriis* in terms of valve width and poroids and rows of poroids, even though they are quite similar to each other in a few features. According to Cusack et al. (2004), *P. australis* has wider transapical axis (5.3–8.0 μm) and about 4–5 poroids in 1 μm , whereas *P. pungens* is narrower (3.0–3.5 μm) and has 3–4 poroids in 1 μm . These two features distinguish *P. pungens* from *P. australis*, as the other features of *P. australis* more or less resemble *P. pungens* which has 15–19 striae and fibulae in 10 μm , two rows of poroids, no large central interspace and 1/4 cell overlap (Turrell et al. 2008). *P. pungens* is very similar to *P. multiseriis*, but both can be distinguished by the number of poroids in 1 μm and rows of poroids. *P. multiseriis* has 5–6 poroids in 1 μm and 4–5 rows of poroids in each striae, while *P. pungens* has 3–4 poroids in 1 μm and 2 rows of poroids in each striae (Hasle et al. 1996). *P. pungens* can be distinguished from other species in *seriata*-group, which do not possess large central interspace, by 3–4 poroids in 1 μm , 2 rows of poroids and narrower valve width. Even though *P. australis*, *P. multiseriis* and *P.*

seriata might have the characteristic of equal number of striae and fibulae, they are wider in valve width and different in terms of poroids and rows of poroids (Cusack et al. 2004, Kaczmarek et al. 2005).

Although most species of marine diatoms are harmless, a few produce neurotoxin domoic acid and causes illness and even death to marine mammals, marine birds, and sometimes even humans (Scholin et al. 2000). *Pseudo-nitzschia* comprises both toxic and non-toxic species (Bates 2000). *P. brasiliiana* is non-toxic, while *P. pungens* produced low level of domoic acid. The strains found in Kuching were negative in production of domoic acid based on High Performance Liquid Chromatography (HPLC) analyses (Lim et al., 2010). However, we cannot conclude that they are non-toxic, as different culture conditions may change the toxin production mechanisms (Smith et al. 1990, Bates et al. 1991). Further toxin analysis on clonal cultures of these species at different growth conditions is underway.

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References

- Bates, S. S., Bird, C. J., De Freitas, A. S. W., Foxall, R., Gilgan, M. W., Hanic, L. A. Johnson, G. E., McCulloch, A. W., Odense, P., Pocklington, R. G., Quilliam, M. A., Sim, P. G., Smith, J. C., Subba Rao, D. V., Todd, E. C. D., Walter, J. A. and Wright, J. L. C. 1989. Pennate diatom, *Nitzschia pungens*, as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edward Island, Canada. *Can. J. Fish. Aqua. Sci.* 46: 1203–1215.
- Bates, S. S. 2000. Domoic acid producing diatoms: another genus added. *J. Phycol.* 36: 978–985.
- Bates, S. S., deFreitas, A. S. W., Milley, J. E., Pocklington, R., Quilliam, M. A., Smith, J. C. and Worms, J. 1991. Controls on domoic acid production by the diatom *Nitzschia pungens* f. *multiseriis* in culture: nutrients and irradiance. *Can. J. Fish. Aqua. Sci.* 48: 1136–1144.
- Bargu, S., Koray, T. and Lundholm, N. 2002. First report of *Pseudo-nitzschia calliantha* Lundholm, Moestrup & Hasle 2003, a new potentially toxic species from Turkish Coasts. *J. Fish. Aqua. Sci.* 19: 479–483.
- Bill, B., Lundholm, N., Connel, L., Baugh, K. A. and Trainer, V. L. 2005. Domoic acid in *Pseudo-nitzschia cuspidata* from Washington State coastal waters. Abstract from the 3rd Symposium on Harmful Algae in the US. Monterey, CA. Oct, 2–7, 2005, pp. 77.
- Cusack, C., Raine, R. and Patching, J. W. 2004. Occurrence of species from the genus *Pseudo-nitzschia* Peragallo in Irish waters. *Biology and Environment: Proceedings of the Royal Irish*

- Academy 104B(1): 55–74.
- Fryxell, G. A., Garza, S. A. and Roelke, D. L. 1991. Auxospore formation in an antarctic clone of *Nitzschia subcurvata* Hasle. *Diatom Res.* 6: 235–245.
- Hasle, G. R. 1972. The distribution of *Nitzschia seriata* Cleve and allied species. *Nova Hedwigia, beiheft.* 39: 111–119.
- Hasle, G. R., Lange, C. B. and Syvertsen, E. E. 1996. A review of *Pseudo-nitzschia*, with special reference to the Skagerrak, North Atlantic, and adjacent waters. *Helgol. Meeresf.* 50: 131–175.
- Iwasaki, H. 1961. The life-cycle of *Porphyra tenera* in vitro. *Biol. Bull.* 121: 173–187.
- Kaczmarek, I., Fryxell, G. A. and Watkins, T. P. 1986. Effect of two Gulf Stream warm-core rings on distributional patterns of the diatom genus *Nitzschia*. *Deep-Sea Res.* 33: 1843–1868.
- Larsen, J. and Nguyen, N. L. 2004. Potentially toxic microalgae of Vietnamese waters. *Opera Botanica* 140: 23–31, 43–45.
- Lim, H. C., Su, S. N. P., Mohamed-Ali, H., Kotaki, Y., Leaw, C. P. and Lim, P. T. 2010. Toxicity of diatom *Pseudo-nitzschia* (Bacillariophyceae) analyzed using high performance liquid chromatography (HPLC). *Journal of Science and Technology in the Tropics* 6: S116–S119.
- Lundholm, N., Hasle, G. R., Fryxell, G. A. and Hargraves, P. E. 2002. Morphology, phylogeny and taxonomy of species within the *Pseudo-nitzschia americana*—complex (Bacillariophyceae), with description of two new species. *Phycologia* 41: 480–497.
- Lundholm, N., Moestrup, O., Hasle, G. R. and Hoef-Emden, K. 2003. A study of the *Pseudo-nitzschia pseudodelicatissima/ cuspidata* complex (Bacillariophyceae): What is *P. pseudodelicatissima*? *J. Phycol.* 39: 797–813.
- Perl, T., Bedard, L., Remis, R., Kosatsky, T., Hoey, J. and Masse, R. 1987. Intoxication following mussel ingestion in Montreal. *Canada District Weekly Rep.* 13–49: 114–225.
- Quijano-Scheggia, S., Garces, E., Sampedro, N., van Lenning, K., Flo, E., Andree, K., Fortuna, J.-M. and Camp, J. 2008. Identification and characterization of the dominant *Pseudo-nitzschia* species (Bacillariophyceae) along the NE Spanish coast (Catalonia, NW Mediterranean). *Sci. Mar.* 72: 343–359.
- Scholin, C. A., Gulland, F., Doucette, G. J., Benson, S., Busman, M., Chavez, F. P., Cordaro, J., Delong, R., De Vogelaere, A. D., Harvey, J., Haulena, M., Lefebvre, K., Lipscomb, T., Loscutoff, S., Lowenstine, L. J., Marin III, R., Miller, P. E., McLellan, W. A., Moeller, P. D. R., Powell, C. L., Rowles, T., Silvagni, P., Silver, M., Spraker, T., Trainer, V. and Van Dolah, F. M. 2000. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature* 403: 80–84.
- Sibel, B., Koray, T. and Lundholm, N. 2002. First report of *Pseudo-nitzschia calliantha* Lundholm, Moestrup & Hasle 2003, a new potentially toxic species from Turkish coasts. *E. U. J. Fish. Aqua. Sci.* 19: 479–483.
- Smith, J. C., Cormier, R., Worms, J., Bird, J. C., Quilliam, M. A., Pocklington, R., Angus, R. and Hanic, L. 1990. Toxic blooms of the domoic acid containing diatom *Nitzschia pungens* in the Cardian River, Prince Edward Island. *In Toxic marine phytoplankton.* Graneli, E., Sundstrom, B., Edler, L. and Anderson, D. M. (eds), pp. 227–232, New York, Elsevier.
- Sterh, C. M., Connell, L., Baugh, K. A., Bill, B. D., Adams, N. G. and Trainer, V. L. 2002. Morphological, toxicological, and genetic differences among *Pseudo-nitzschia* (Bacillariophyceae) species in inland embayments and outer coastal waters of Washington State, USA. *J. Phycol.* 38: 55–65.
- Trainer, V. L. and Suddleson, M. 2005. Monitoring approaches for early warning of domoic acid events in Washington State. *Oceanography* 18: 228–237.
- Turrell, E., Bresnan, E., Collins, C., Brown, L., Graham, J. and Grieve, M. 2008. Detection of *Pseudo-nitzschia* (Bacillariophyceae) species and amnesic shellfish toxins in Scottish coastal waters using oligonucleotide probes and the Jellet Rapid Test (TM). *Harmful Algae.* 7: 443–458.
- Villac, M. C., Melo, S., Menezes, M. and Tenenbaum, D. R. 2005. *Pseudo-nitzschia brasiliiana* (Bacillariophyceae), an opportunistic diatom on the coast of the state of Rio de Janeiro, Brazil. *Atlantica, Rio Grande* 27: 139–145.
- Work, T. M., Beale, A. M., Fritz, L., Quilliam, M. A., Silver, M., Buck, K. and Wright, J. L. C. 1993. Domoic acid intoxication of brown pelicans and cormorants in Santa Cruz, California. *In Toxic phytoplankton blooms in the sea.* Smyada, T. J. and Shimizu, Y. (eds), pp. 643–649, Elsevier, Amsterdam.