

OCCURRENCE OF *PSEUDO-NITZSCHIA* SPECIES (BACILLARIOPHYCEAE) AT KUCHING ESTUARIES FROM YEAR 2007 TO 2010

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ABSTRACT

Amnesic shellfish poisoning (ASP) is a type of seafood poisoning due to human consumption on shellfish mollusks contaminated with algal toxin, domoic acid (DA) derived from the diatom *Pseudo-nitzschia*. Bloom dynamics of this diatom in tropical waters were poorly understood due to lack of long term study on the organism. Occurrence of *Pseudo-nitzschia* species was investigated at Santubong and Samariang estuarine waters, Kuching, Sarawak from 2007 to 2010. The estuaries were characterized by shallow water with semi-diurnal tidal cycle. Cell abundance was determined by microscopic enumeration. Temperature, pH, salinity and macronutrients at the sampling sites were determined at each sampling occasions. Highest cell density of *Pseudo-nitzschia* spp. was recorded 8.0×10^3 cells L⁻¹ in Santubong in April 2009 and *P. brasiliiana* cells were found highest in October 2007 (2×10^4 cells L⁻¹). Increase in cell abundance was coincided with high salinity and low precipitation rate. Water temperature and pH showed insignificant influence on the abundance of *Pseudo-nitzschia* spp. The result obtained in this study provided fundamental understanding on bloom dynamic of potential harmful *Pseudo-nitzschia* species in the tropical estuarine waters.

Keywords: *Pseudo-nitzschia*, domoic acid, cell abundance, morphology, Kuching.

INTRODUCTION

Pseudo-nitzschia is one of the main genera that cause Amnesic Shellfish Poisoning (ASP) by production of neurotoxin domoic acid (DA). Almost 40 species of *Pseudo-nitzschia* have been documented worldwide, with some species only restricted to specific region while the others are cosmopolitan (Hasle, 2002; Lelong et al., 2012). In Southeast Asia, a total of 22 species of *Pseudo-nitzschia* has been recorded thus far, with eight species were known to be potentially toxic (Lundholm, 2011). Our recent study also showed high species richness of *Pseudo-nitzschia* in Malaysia coastal water than previously thought (Lim et al., 2012a; Teng et al., accepted). Bioac-

cumulation of DA through the food web had been caused the death of marine mammals and birds (Scholin et al., 2000). Although no human ASP case has been reported in Southeast Asian region, contaminations of domoic acid in some bivalve species was confirmed at least in two neighbouring countries (Ha et al., 2006; Ha et al., 2009; Takata et al., 2009; Ha et al., 2012).

Nutrient loading in coastal ecosystem has been recognized as one of the major driving factor of harmful algal blooms (Glibert et al., 2001), including bloom of diatom, *Pseudo-nitzschia*. In the coast of US, blooms of *Pseudo-nitzschia* spp. occurred in response to nutrient enriched waters from spring runoff (Heisler et al., 2008), excessive nutrient from riverine input (Parsons et



Figure 1. Map showing the two sampling sites, Santubong estuary and Samariang River in Kuching, Sarawak.

al., 2002) or nutrient flux from coastal upwelling events (Trainer et al., 1998; Anderson et al., 2006). Alternation of macronutrient composition to lower Si:N and Si:P in coastal waters due to various anthropogenic sources (Anderson et al., 2002), shown to associated with the increases in *Pseudo-nitzschia* blooms and DA production (Pan et al., 1996a; Anderson et al., 2006). Since the first ASP in late 1980s, more than ten events related to DA contamination have been reported but occurrences confined only to temperate regions (Bates et al., 1989; Martin et al., 1990; Fritz et al., 1992; Scholin et al., 2000; Lefebvre et al., 2002; Marchetti et al., 2004).

To gain insight in the bloom dynamics of *Pseudo-nitzschia* species, long term studies have been carried out in wide geographical areas covered various types of coastal habitats. However, the occurrence and bloom dynamics of *Pseudo-nitzschia* in the tropical waters remained unknown. In this study, seasonal occurrence of *Pseudo-nitzschia* species in Samariang and Santubong estuaries, Kuching, Sarawak was investigated. The environmental factors at estuarine waters and its nutritional conditions were investigated to relate with the abundance of *Pseudo-nitzschia*. Sampling was carried out

fortnightly and water physico-chemical parameters were measured *in-situ*. Field and culture samples of *Pseudo-nitzschia* species were used for fine morphological observation by using scanning and transmission electron microscopy.

MATERIAL AND METHODS

Study areas and sampling

Fortnightly samplings were carried out in Santubong estuary (1°42'59.82"N, 110°19'40.84"E) and Samariang estuary (1°35'51.82"N, 110°18'58.19"E) (Fig. 1) from July 2007 to March 2010. The samples were collected at the jetty of Fisheries Department (Santubong and Samariang Branch). Sampling site in Santubong located at area adjacent to mangrove forest near to the river mouth. Several shrimp farms are operating at the area. Sampling station of Samariang is located upstream of Samariang estuary. The area is within the vicinity of village of Samariang and is prone to domestic waste (solid or liquid) from the settlement along the river with poor sanitary.

Surface water samples were collected by using a Van-Dorn water sampler and a 20 µm mesh size plankton net at less than 10 m depth. Water samples in duplicates were stored in bottles with one litre each and brought back to the laboratory for further analyses. Water physical parameters, i.e.: pH, temperature and salinity were measured *in-situ* using HANNA electronic pH meter (Hanna Instrument, USA) and AGATO hand refractometer (Agato, Japan).

Sample preservation and macronutrient analysis

Filtered water samples for nutrient analysis were kept frozen at -20°C until further analysis. Nitrate-nitrogen (NO₃-N), reactive phosphorus (PO₄³⁻-P) and silicate (SiO₂) in water samples were analyzed using HACH Odyssey 2500 UV spectrophotometer (HACH Company, Colorado, USA) according to manufacturer's instructions. NO₃-N concentrations were determined at wavelength of 507 nm according to the Cadmium reduction method. Reactive phosphorus was analyzed using PhosVer3 (Ascorbic Acid) Method

Heteropoly blue method was used to determine SiO₂ concentration in the sample.

Cell enumeration

One litre of plankton sample was concentrated to 50 mL through filtration and preserved with acidic Lugol's solution. Samples were stored in dark at ambient temperature. Preserved plankton sample was observed using a Sedgwick-Rafter counting chamber under a Leica compound light microscope (Leica, Germany) at a magnification of 100×. All of the cell counting was carried out in triplicate. *Pseudo-nitzschia* cells found in the samples were counted using tally counter and manually recorded. Species enumeration and identification through light microscopy was conducted based on common features of *Pseudo-nitzschia* spp. that is visible under light microscopy. The morphological characteristics includes cells in chain-form, cell overlapped, flattened and elongated, and presence of chloroplasts in each polar according to description by Hasle et al. (1996).

Data analysis

Cell density and physico-chemical readings were input into the statistic program Graphpad Prism ver. 5.0. General *t* statistical analysis was performed to investigate the significance of each variable by using Prism. Statistic ordination method of principle component analysis (PCA) (Jolliffe, 2002) was applied to examine pattern and distribution of *Pseudo-nitzschia* composition. The data were transformed to log (*x* + 1) to improve normality distribution. Monthly average precipitation rate of Kuching were obtained from the Meteorology Department of Malaysia. Precipitation was measured in millimeters (mm).

RESULTS

Pseudo-nitzschia abundance

Cell density of *Pseudo-nitzschia* at Santubong estuary was examined from a total of 98 samples since July 2007 until March 2010. Generally, *Pseudo-nitzschia* cells were observed between April and September each year and most abundant

in April–May. From July onwards, cells of *Pseudo-nitzschia* were present in low abundance. Cells were absent during the December–January intervals of sampling period (Fig. 2).

Lower cell density was observed in 2007–2008 compared to those in recent samples where *Pseudo-nitzschia* cells were encountered at almost 50% out of total samples starting April 2009 until March 2010. In 2007, cells were present at a very low density from July until year end. Only a single occurrence of *Pseudo-nitzschia* appeared in 2008 between May and June. A peak with cell density at approximately 8,000 cells L⁻¹ was observed in April 2009, which was also the highest density recorded in Santubong thus far (Fig. 2).

Occurrence of *Pseudo-nitzschia* was less frequent in Samariang for the duration from 2007

to 2010. Only a single bloom of *Pseudo-nitzschia* at 2.9×10⁴ cells L⁻¹ were observed in October 2007 and once in May for year 2008. Similar to Santubong, cells present at low density (± 300 cells L⁻¹) in between end of April to September 2009. Occurrence of cells was most likely from April to May in each year. The average cell density in the estuary remained low to absent throughout the study period (Fig. 3).

Water physical data in the two estuaries

Salinity in Santubong (Fig. 4a) ranged between 12–33 PSU whereby in Samariang the range was in 10–30 PSU (Fig. 4b). Lower salinity in Samariang was mainly due to its location, at the upstream of Samariang River. Lowest salinity at both locations was observed during the months

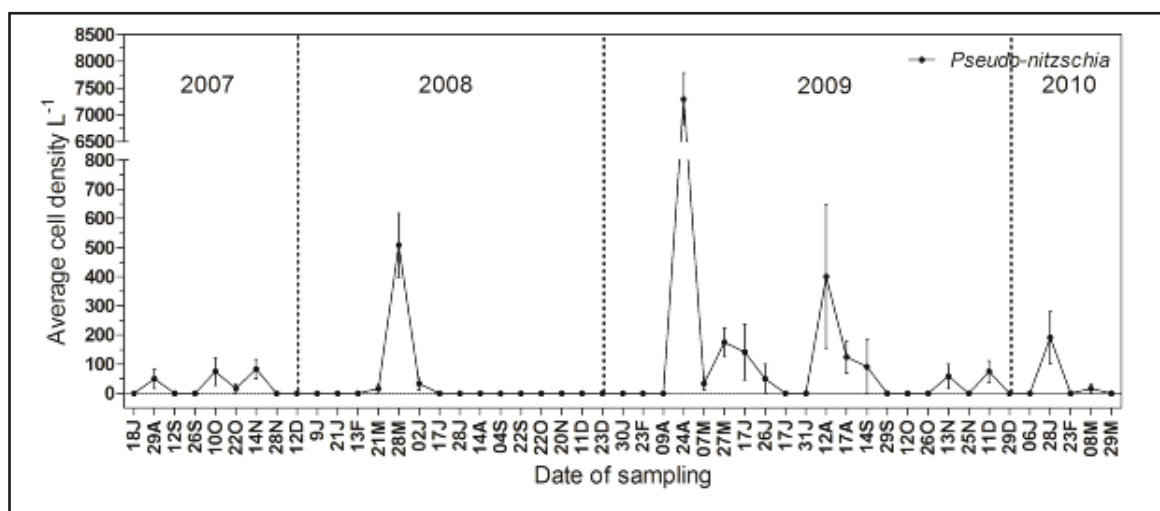


Figure 2. *Pseudo-nitzschia* spp. abundance (mean ± SD) at Santubong estuary from 18 July 2007 to 29 March 2010.

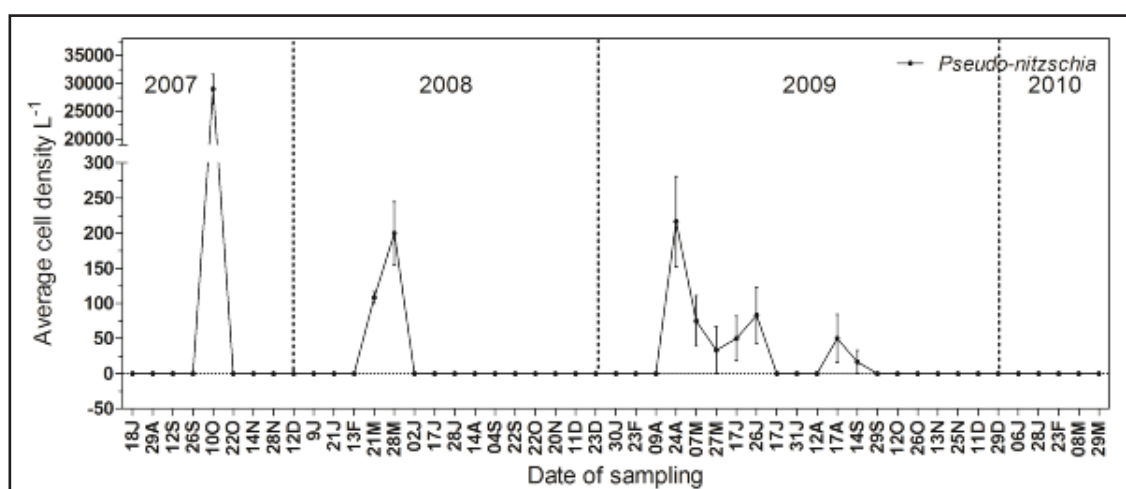


Figure 3. *Pseudo-nitzschia* spp. abundance (mean ± SD) at the Samariang estuary from 18 July 2007 to 29 March 2010.

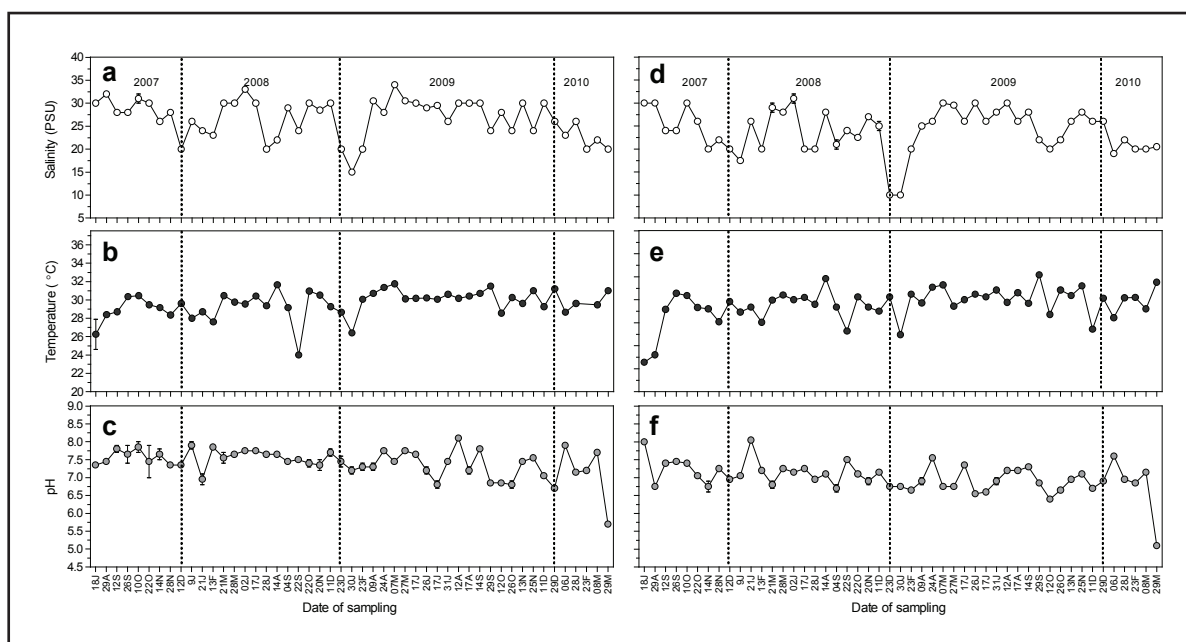


Figure 4. In situ physical data of salinity (a, b), temperature (c, d) and pH (e, f) at Santubong and Samariang estuary from July 2007 until March 2010.

with high precipitation (e.g. December 2008–January 2009).

Water temperature of Santubong (Fig. 4c) and Samariang (Fig. 4d) ranged from 22.5 to 33.0°C. Minor fluctuation in temperature is typical for tropical water. Similar to salinity, water temperature was slightly lower when samples were collected during the day with high precipitation. There was no much difference in water temperature at the two locations. No significant relationship between the water temperature and *Pseudo-nitzschia* cell densities ($p > 0.05$) in this study. The lowest pH recorded in Santubong was 5.7 (Fig. 4e) and 5.1 in Samariang (Fig. 4f). The highest pH value for both estuaries was 8.1. Elevation of pH in estuaries was attributed to the flood tide over the semi-diurnal tidal cycle in the areas.

Macronutrients

Nitrate-nitrogen concentrations at both locations varied during the study period. The nitrate-nitrogen concentration of Santubong varied between 0–0.48 mg L⁻¹ (Fig. 5a) and from 0–0.45 mg L⁻¹ in Samariang (Fig. 5b). Both locations recorded low average nitrate concentration (≤ 0.1 mg L⁻¹) from July 2007 to September 2008. Elevation of nitrate nitrogen concentration was observed for three subsequent months. Similar

elevation of nitrate nitrogen was observed in 2009, but happened several months. Nitrate nitrogen levels were higher for both locations compared to the previously years. However, increased cell density in Santubong (May 2008, April–September 2009) and Samariang (October 2007, May 2008, April–September 2009) did not show significant relationship with the nitrate-nitrogen concentration ($p > 0.05$).

Phosphate concentration in Samariang was lower than Santubong. Low concentrations of phosphate were observed when dissolved nitrate-nitrogen was high and vice versa. When phosphate was available, both locations gave a range of reading from below detectable level to 1.76 mg L⁻¹ (Fig. 5c) and 1.86 mg L⁻¹ at each estuary respectively (Fig. 5d). The highest phosphate concentration in Santubong during 2007 was 1.37 mg L⁻¹ but phosphate concentrations at Samariang were at trace level during the period. In May–September 2008, elevated phosphate was recorded in Santubong and Samariang. In Santubong, phosphate increased from 0 to 0.83 mg L⁻¹ and gradually decreased to 0.28 mg L⁻¹ and 0.11 mg L⁻¹ in the next samplings (Fig. 5c). Final increase to the highest concentration of 0.93 mg L⁻¹ was recorded before the concentration decrease to trace level in the remaining months of 2008 (Fig. 5c).

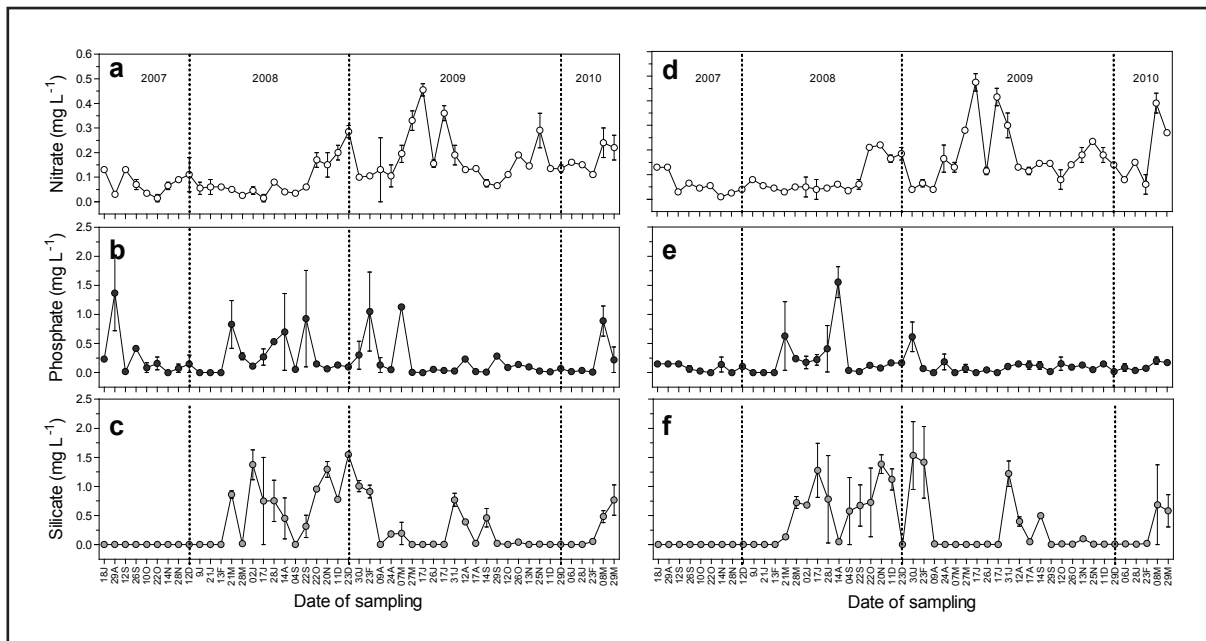


Figure 5. Macronutrients (nitrate-nitrogen, phosphate, silicate) reading at Santubong and Samariang estuary from July 2007 until March 2010.

In Samariang, sharp increase of phosphate concentrations to 0.63 mg L^{-1} was also observed in May 2008 (Fig. 5d). Elevation in phosphate concentration from trace level to relatively high concentrations had been recorded several times from both locations in 2009–2010. Lowered phosphate in the water may have related to nutrient uptake by higher cells abundance in the recent years (2009–2010). Changes in phosphate availability did not portray close relationship with the cell densities in most sampling ($p > 0.05$).

Silicate concentrations in Santubong and Samariang estuaries during 2008 until early 2009 recorded a high variability. Silicate content in Santubong and Samariang ranged from below detectable level to 1.547 mg L^{-1} (Fig. 5e) and 1.532 mg L^{-1} (Fig. 5f), respectively. High dissolved silicate available in May 2008 might have stimulated the growth of *Pseudo-nitzschia* in the two estuaries whereby high cell densities were always followed by decline in silicate. Silicate was enriched again particularly during end and early of year (Fig. 5e, f). Since April 2009, frequent record of *Pseudo-nitzschia* occurrence coincided with decline in silicate concentrations and sometimes to below detection limit, with exception to several occasions when silicate slightly increased for a short period.

Monthly rainfall

Average monthly precipitation rate is shown in Fig. 6. Data showed precipitation occurred all year round confined to the tropical monsoon. There are two main monsoon seasons, namely, north-east monsoon (November–March), south-west monsoon (May–September) and two short periods of inter-monsoon seasons. Kuching coastal region is more exposed to the north-east monsoon season whereby precipitation rates are more pronounced in January (Fig. 6). High precipitation rate above 400 mm and more than 20 days per month were recorded during early and end of year. Minimum rainfall occurred in the month of June or July in each year. Annual precipitation read at approximately 2,000 to 2,500 mm.

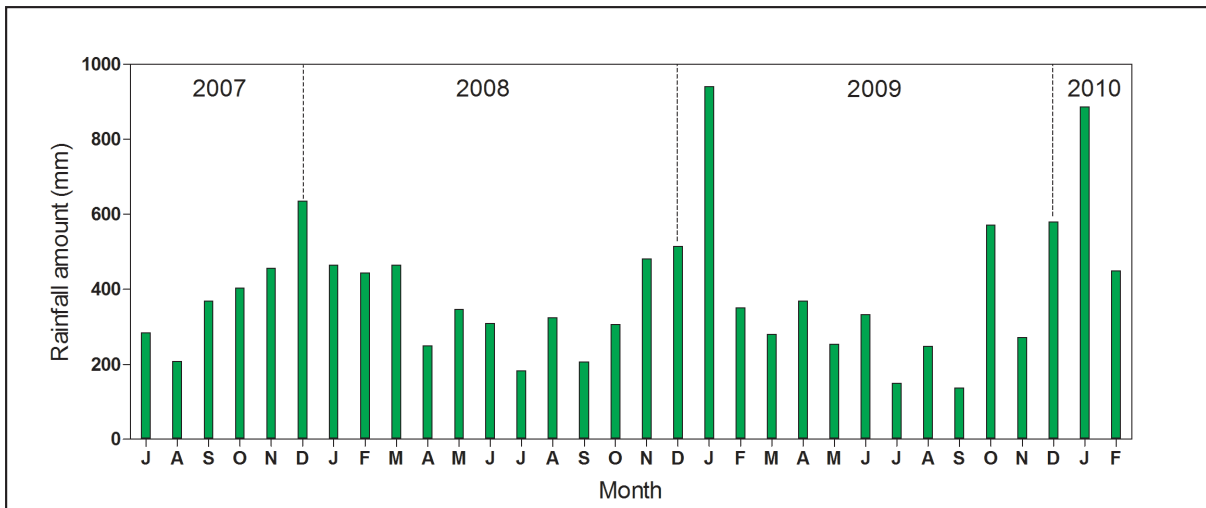


Figure 6. Monthly precipitation rate of Kuching (Source: Department of Meteorology, Malaysia).

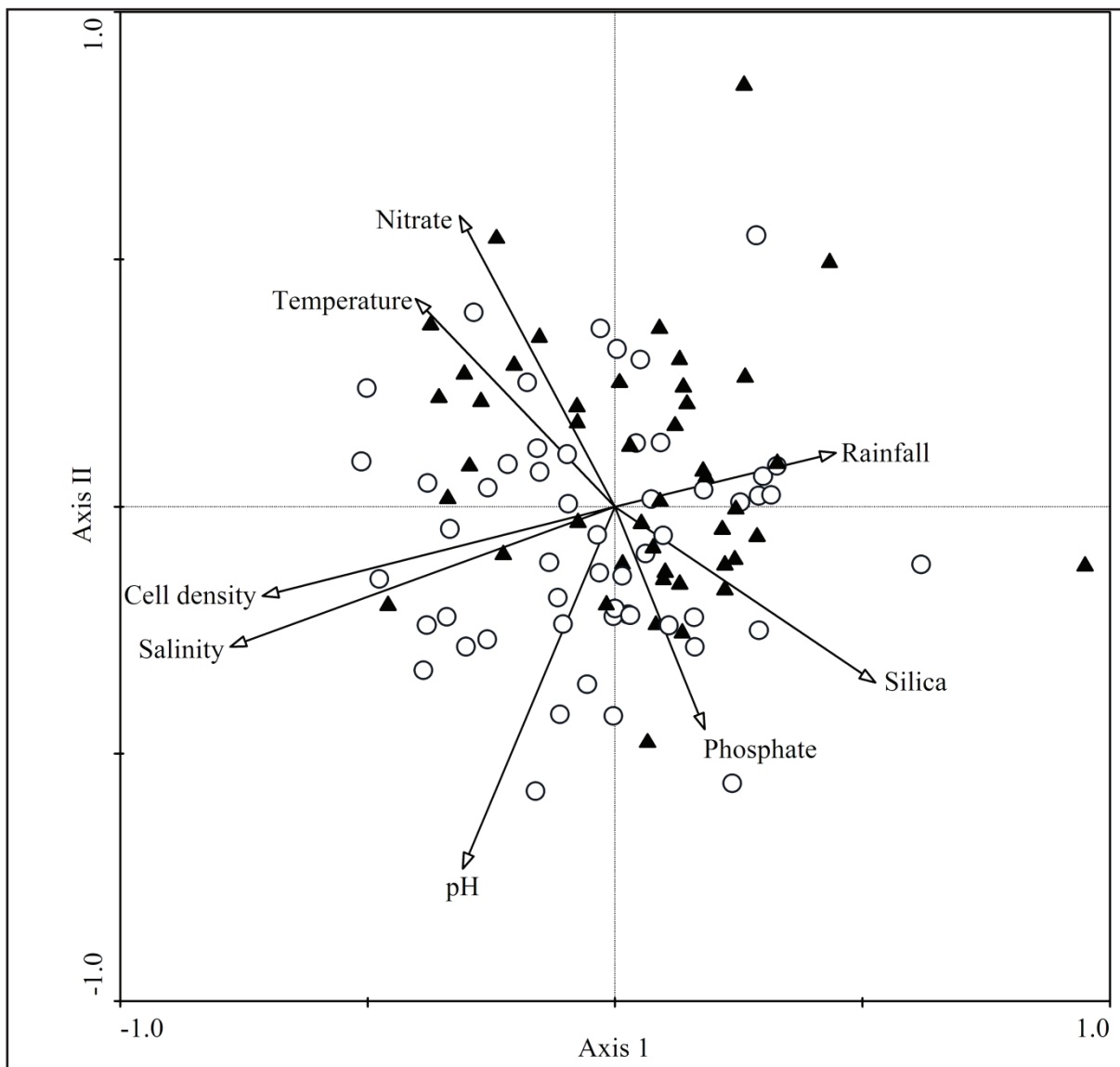


Figure 7. Biplot of Principle Component Analysis showing cell density structure and relationships with environmental variables. Solid circles represent *Pseudo-nitzschia* cell densities of Santubong and Samariang (missing values for silica variables were calculated by regression with salinity).

Principle Component Analysis (PCA)

PCA was used to investigate *Pseudo-nitzschia* cell density in relation to the environmental variables. Analyses were performed on the data set of 196 samples from both locations for the period of 33 months. The environmental variables examined were salinity, temperature, pH, nitrate, phosphate, silica and precipitation. Data were centred and standardized because variables were in different unit. Axes 1 and 2 accounted for 43.6% variance out of 69.8% total variance of species data (Fig. 7). The Eigenvalues of axes were: 0.247, 0.189, 0.143 and 0.118, respectively. The circles represent the *Pseudo-nitzschia* spp. cell densities at Santubong and Samariang (Fig. 7). The positions of the circular plots represent the cell abundance association to the environmental factors represented by arrows. The narrowest angle between particular parameter arrows with the cell density arrow reflects the most significant parameters affecting the cell density.

Based on the biplot, salinity and pH were the most influential factors in regulating the cell density of *Pseudo-nitzschia*, whereas cell abundance was weakly affected by temperature, nitrate and phosphate. Silica and rainfall was negatively related to the cell densities. Plots close to the centre in the ordination diagram denoted that cell density was poorly related to environmental factor and suggests that it could be driven by other factors not considered in the analysis.

DISCUSSION

Increased occurrence of phytoplankton blooms in recent years has been attributed to coastal eutrophication and global climate change, in addition to better awareness and documentation of bloom events (Hallegraeff, 2003), with no exception in occurrence of *Pseudo-nitzschia* and ASP events. ASP not only poses impacts and threat to aquatic mammals and birds, but also to human consumers via bioaccumulation. In the past decades, many efforts have taken through field investigation and laboratory experiments in various environmental parameters. Among others is to determine factor inducing bloom and toxin production of *Pseudo-nitzschia*, especially in temperate and subtropical regions. In this paper,

one of the few studies on occurrence of *Pseudo-nitzschia* in the tropical waters is reported.

In the present study, *Pseudo-nitzschia* colonies were identified to generic level based on stepped cells in chain for enumeration under light microscopy. Species identification is challenging as closely related species is differentiated based on minute structures of frustules and required the aids of electron microscope. However, some *Pseudo-nitzschia* species can be distinguished based on the sparse features of size and cell shape. In some studies earlier, *Pseudo-nitzschia* were identified to genus and counted as a whole, while some studies grouped different morphotypes based on their size ranges (e.g. Dortch et al., 1997; Trainer et al., 1998; Kaczmarek et al., 2007; Almandoz et al., 2008; Klein et al., 2010), but only in a few studies, cell was identified and enumerated to species level (Cusack et al., 2004; Marchetti et al., 2004).

Generally, *Pseudo-nitzschia* cells in Santubong were greater in length and formed longer chain compared to samples from Samariang. Electron microscopy observations of the isolates from both estuaries confirmed with at least two species of *Pseudo-nitzschia*. *Pseudo-nitzschia brasiliensis* and *P. pungens* were predominantly found in the study areas. Studies in Lim et al. (2012b) and Teng et al. (accepted) also shown the wide distributions of this two species.

Both Santubong and Samariang estuaries shared the same range of salinity. The salinity ranged from 15–34 PSU in Santubong and 10–30 PSU in Samariang, respectively. However, salinity at Samariang was lower compared to Santubong at most of the time during the sampling period. In Santubong, *Pseudo-nitzschia* was observed at 6% of samples taken at salinity <25 PSU and 55% of samples taken at >25 PSU. Higher cell abundance of *Pseudo-nitzschia* spp. was apparently present at salinity of >25 PSU at Santubong estuary (>250 cells L⁻¹). In Samariang, none of the cell was present in samples taken <25 PSU and cells were only encountered in 37% of samples taken at salinity >25 PSU. Absence of *Pseudo-nitzschia* in most of the Samariang samples may due to low preference of *Pseudo-nitzschia* in low salinity environment. Peak concentrations of *Pseudo-nitzschia* were observed at a close range of salinity (28–30 PSU) in both estuaries. According to Thessen et al.

(2005), species of *Pseudo-nitzschia* demonstrated euryhaline characteristics, growing over a wide range of salinities with selective salinity preference among species isolated from different geographical area (e.g. lagoon, pool rock). *Pseudo-nitzschia subcurvata* and *P. turgiduloides*, species that restricted to cold Antarctic waters were found in high abundance in salinity regimes of 33–34 PSU (Almandoz et al., 2008). In this study, *P. pungens*, which is known to be cosmopolitan, was observed in samples with salinity more than 20 PSU.

Another environment variable that affects the species composition is water temperature (Almandoz et al., 2008). Seasonal blooms have been observed in many *Pseudo-nitzschia* species in the temperate regions where day lengths were variable with season (Fehling et al., 2005). *Pseudo-nitzschia* spp. cell density in this study however did not correlate well with temperature. This is not surprising as water temperature remained relatively constant throughout the year in tropical water. In temperate species, densities of *P. americana* and *P. australis* were positively correlated with temperature (Klein et al., 2010) in culture experiments. The cold water species *P. seriata* was at greatest abundance in spring whereas *P. multiseriata* and *P. delicatissima* favoured the autumn seasons (Hasle et al., 1996). High abundance of cosmopolitan *P. pungens* had been recorded during summer (Hasle et al., 1996; Rhodes et al., 1996). Seasonal shifts of *Pseudo-nitzschia* in temperate waters suggested that most species are less tolerable to high variation in temperature (Fehling et al., 2005). *Pseudo-nitzschia seriata* was observed to grow underside of sea ice of the Arctic Ocean, as well as in the water column when they were released as the ice melts (Bates et al., 2004). However when it is in culture condition, *Pseudo-nitzschia* can tolerate a tremendously high range of temperature (5–30°C) (Thessen et al., 2005).

In general, pH of Santubong ranged from 6.7 to 8.1 and Samariang was between 6.4 and 8.1. pH values at both estuaries were slightly distinct throughout the study probably due to different distance from the river mouth. Fluctuations of pH at Santubong and Samariang estuaries were mainly due to intrusion of seawater during intertidal changes. High concentration of *Pseudo-nitzschia* in Santubong (8.0×10^3 cells mL⁻¹)

and Samariang (2.0×10^4 cells mL⁻¹) was not affected by pH. Lundholm et al. (2004) reported that marine and smaller *Pseudo-nitzschia* species could tolerate higher upper limit of pH for growth. In this study, smaller *Pseudo-nitzschia* cells were encountered in samples with pH value ranging from 6.5–7.5 in Samariang. On the other hand, larger *Pseudo-nitzschia* cells in Santubong were found in pH ranging from 7.0–8.0. Alkalinity unlikely be the limiting factor in abundance since the two forms of *Pseudo-nitzschia* spp. can hypothetically tolerate pH ≥ 9.0 based on model described by Lundholm et al. (2004). However this study showed that *Pseudo-nitzschia* cells were absent in high pH value (≥ 7.0). The reason might lie in other factors such as higher precipitation rate especially the northeast monsoon season, depletion and competition of essential nutrients for growths or other environmental conditions that may stunt their bloom in the area. In nutrient-enriched condition, pH was the limiting factor in algal growth (Lundholm et al., 2004). Absence of cells during low tide, hence the lower pH may again support the original idea that they were marine origin and their existence in the estuarine waters was driven by flooding. Most *Pseudo-nitzschia* species can grow in pH up to 8.7, for instance *P. delicatissima* can tolerate higher pH of 9.8 (Lundholm et al., 2004).

Cell density of *Pseudo-nitzschia* spp. at both estuaries was affected by monthly precipitation rates. This close relationship was also supported by the PCA analysis whereby rainfall was inversely related to the cell densities (Fig. 7). High precipitation of more than 400 mm of more than 20 days per month decreased the density of *Pseudo-nitzschia* at Santubong and Samariang. Low cell density of *Pseudo-nitzschia* spp. during precipitation may probably due to high freshwater dilution rate and decreased light penetration as a consequence of increase in total suspended solid, thus increased turbidity and reduced water transparency. Increased concentration of *Pseudo-nitzschia* cells was observed after a decrease in monthly rainfall, and the abundance was sustained in dry season of May–September. This is coincided with other studies conducted in Thailand and Philippines, where *Pseudo-nitzschia* cells appeared during December–May, with low precipitation (Udomratana et al., 2008; Yap-Dejeto

et al., 2008). Calm water condition coupled with high dissolution rate of *Pseudo-nitzschia* frustules (Dickman and Glenwright, 1997) may have contributed to the amount of dissolved silicate essential for growth. Enhanced river flow especially after precipitation may be the reason for high value of macronutrient recorded at a particular time. However, short resident time prevents the proliferation of diatom such as *Pseudo-nitzschia* spp. in turbulence water. The first bloom of *P. multiseriata* was resulted from high precipitation after a prolonged dry period (Bates et al., 2004). Previous study done by Spatharis et al. (2007) also demonstrated that the density of *P. calliantha* was extremely high after a raining period. Nutrient loading into the water column from land runoffs may explain the enhanced cell concentrations after precipitation (Dortch et al., 1997; Scholin et al., 2000). Change of nutritional condition due to land runoffs helps regulate eutrophication (Glibert et al., 2001). Relatively lower cell density in this study compared to other studies in the temperate region (e.g., Dortch et al., 1997; Klein et al., 2010), was probably related to higher precipitation and dilution rate in tropical country. Therefore, salinity and pH became the limiting factor in such nutrient rich condition.

Light availability is considered one of the main aspects, which determined different responses in phytoplankton confined to high latitudes (Brand and Guillard, 1981). The content of algal pigments, growth rates and dark respiration rate in phytoplankton were strongly influenced by the light regimes (Gilstad and Sakshaug, 1990). In tropical regions, heavy cloud cover during monsoonal seasons can be a proxy to photoperiod and may limit the light availability. Cloudy conditions during heavy raining period may responsible for the absence of *Pseudo-nitzschia* cell densities as a result from light limitation. A minimum irradiance of $\geq 100 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ was required for optimal growth and DA productivity (Bates, 1998). Sexual reproduction in *P. multiseriata* has been reported to be affected by photoperiod as well (Hiltz et al., 2000). Healthy *Pseudo-nitzschia* cells were also reported in low irradiance to aphotic zone (Trainer et al., 1998). *Pseudo-nitzschia delicatissima* and *P. seriata* demonstrated higher division rate and

toxin productivity in long photoperiod and reduced almost twofold in shorter photoperiod (Fehling et al., 2005). Adenosine triphosphate (ATP) reserves build up upon photosynthetic energy in longer light exposure and are important for DA production (Bates et al., 1989; Pan et al., 1998). Decrease in light phase duration also restricted the cells' ability to divide rapidly due to prolongation of the G2 phase in cell cycle (Fehling et al., 2005). Therefore, the timing of sampling (before, during or after raining) is one important factor that affects cell counts in the sample collected.

The average nitrate-nitrogen concentrations at Santubong and Samariang estuaries were in the same range. The nitrate-nitrogen in Santubong was relatively low compared to other dissolved nutrients. The highest nitrate-nitrogen recorded in Santubong was 0.45 mg L^{-1} in June 2009. However, no cell of *Pseudo-nitzschia* was observed at the time. In Samariang, the average nitrate-nitrogen concentration was slightly higher than phosphate but much lower compared to silicate. Absent of *Pseudo-nitzschia* cells at highest concentration of nitrate at 0.51 mg L^{-1} was recorded in Samariang. This may indicate that nitrate-nitrogen may not be the main factor that triggers the growth of *Pseudo-nitzschia* spp. in this study. According to Klein et al. (2010), only *P. delicatissima* and *P. fraudulenta* abundance is related to high concentration of dissolved nitrate while *P. americana*, *P. australis*, and *P. pungens* were not affected by the amount of nitrate in natural environment. Nonetheless, previous studies had proven that nitrate is one of the requirements for DA production in *Pseudo-nitzschia* spp. (Bates et al., 1991).

Pseudo-nitzschia is capable of utilizing both organic and inorganic form of nitrogen sources (Howard et al., 2007). Ammonia-nitrogen is one of nitrogen sources commonly found in the natural water column. However, no significant difference was observed in *P. australis* cultures growth (ca. 0.9 day^{-1}) in either nitrate or ammonium used as the sole nitrogen source (Howard et al., 2007). Furthermore, the function of ammonium in sustaining phytoplankton blooms is still not clear (Maguer et al., 2004). In growth experiment conducted by Hillebrand and Sommer (1996), *P. multiseriata* exhibited higher preference in nitrate uptake when both ammonium and nitrate were

present. Excessive supplement of ammonium promotes toxic condition and inhibits algae growth (Hillebrand and Sommer, 1996).

Despite the absence of correlation between *Pseudo-nitzschia* cell densities with nitrogenous concentration in both estuaries, loadings of urea from the adjacent human habitation into the estuaries should be given more attention. Abundance of *P. multistriata* in natural environment was regulated by the concentration of urea (Klein et al., 2010). Howard et al. (2007) showed that DA production rate was higher in urea-grown *P. australis* in comparison with other nitrogen sources. This implied that intensified agricultural runoffs and sewage discharge from the nearby area might increase the chances of toxic bloom of *Pseudo-nitzschia* spp. in Santubong and Samariang. In addition, nitrogen source uptakes in phytoplankton in combination with a particular amount of phosphate were to be considered as factor affecting cell densities and growth rates (Howard et al., 2007; Lim et al., 2010).

The orthophosphate analysed from the samples of both estuaries gave the value ranging 0–2.00 mg L⁻¹. Sewage and semi-composed organic matter runoffs from the mangrove area might contribute to phosphorus loading into Santubong and Samariang estuaries. Previous study showed that growth rate of *P. pungens* was at optimum level when exposed to low-phosphorus concentrations (Hu et al., 2008). However, nutrients preferences in *Pseudo-nitzschia* spp. were species-specific (Fehling et al., 2004a). Phosphate limitation was proven to induce DA production in *P. multiseries* (Pan et al., 1996b; Bates, 1998) and *P. seriata* (Fehling et al., 2004b). In this study, abundance of *Pseudo-nitzschia* was coincided with orthophosphate concentration below 0.5 mg L⁻¹ and this was supported by the PCA biplot where orthophosphate showed weak relationship to *Pseudo-nitzschia* cell densities.

Nitrogen Phosphorus (NP) ratios fluctuate in wide ranges in estuarine regions (Dortch, 1997; Tanaka and Choo, 2000). In this study, NP ratios (data not shown) were observed varied from 5:1 to 500:1 at Santubong and 7:1 to 133:1 in Samariang. High NP at both estuaries (>100) may explain the low cell densities of *Pseudo-nitzschia*. According to Brzezinski (1985), optimum growth for diatom

was obtained at Redfield-Brzezinski nutrient ratios, Si:N:P=15:16:1.

Increase in *P. australis* abundance in Monterey Bay during an ASP event was suspected as a response to elevated dissolved silicate concentrations from the coastal runoffs (Scholin et al. 2000). Similarly in France, *P. australis* and *P. americana* increased in abundance when silicates concentration were high (Klein et al. 2010). Contrarily, high silicate content did not accommodate the growths of other *Pseudo-nitzschia* spp. such as *P. delicatissima* and *P. fraudulenta* (Klein et al., 2010). In this study, highest silicate concentration (1.589 mg L⁻¹) in the waters was coincided with low *Pseudo-nitzschia* cell density and vice versa. Siliceous diatoms like *Pseudo-nitzschia* take up silicate for their formation of cell walls. Therefore, low silicate content in the water might have correlated with growth periods of *Pseudo-nitzschia* and other occurring diatoms. Dissolution of emptied frustules of siliceous diatom such as *Pseudo-nitzschia* particularly (Dickman and Glenwright, 1997), in the water column may explain the sustainability of *Pseudo-nitzschia* during low nutrient conditions during dry seasons (Fehling et al., 2005). Increment of silicate concentration only achieved after the density of diatom reduced.

Santubong and Samariang estuaries had maximum tidal range at approximately 5 meters and both were less likely to be stratified due to daily mixing of water during semi diurnal tide cycle. Twenty-five genera of diatom can be found in Santubong and Samariang (Hilaluddin, 2009; Kamarudin, 2009). However, *Pseudo-nitzschia* spp. contributed in less than 5% of total diatom density at the two locations (Hilaluddin, 2009). Phytoplankton assemblages were dominated by diatom in Santubong with the highest record of diatom to dinoflagellate ratios (DD) in August (117:1) and lowest DD ratio observed in December–January interval (Kamarudin, 2009). In Samariang, lower diatom densities compared to dinoflagellates were reported in July, September and December 2008 (Kamarudin, 2009). Similar to *Pseudo-nitzschia* cell densities in Santubong, abundance of *Pseudo-nitzschia* did not coincide with the mass development of other diatom or dinoflagellates. We hypothesize that the condition is due to lack of competitive advantages

in *Pseudo-nitzschia* in its ecological adaptation (especially nutrient uptake, salinity tolerance) over other species in the phytoplankton assemblages.

In this study, the smaller size *Pseudo-nitzschia* was always predominant compared to the bigger counterpart. Two or more *Pseudo-nitzschia* spp. might co-exist in the phytoplankton assemblage. However, abundance of some species such as *P. multiseriata* decreased with the occurrence of other *Pseudo-nitzschia* (Hasle et al., 1996).

In this study, *Pseudo-nitzschia* tended to be found after a period of high precipitation followed by an extended dry season. It is noteworthy that the occurrence of *Pseudo-nitzschia* at the Santubong had been observed frequently and was more intense in number of cells in the recent years. Similarly, *Pseudo-nitzschia* species was reported at inshore or offshore, but peak abundance of *Pseudo-nitzschia* tends to appear in continental shelf ($\geq 10^6$ cells L⁻¹) compare to estuaries (Dortch et al., 1997; Thessen et al., 2005).

Pseudo-nitzschia was only encountered in 53 out of total 196 replicates (27%) obtained from the two estuaries. Two *Pseudo-nitzschia* spp. were identified at Santubong and the small size *Pseudo-nitzschia* was the most commonly found in the samples. *Pseudo-nitzschia* cell densities at Samariang recorded a bloom at 2.9×10^4 cells L⁻¹ in October 2007 and only a predominant small-sized *Pseudo-nitzschia* was observed during the peak concentration and throughout study at the estuary. The density in Samariang during the bloom in October 2007 (2.9×10^4 cells L⁻¹) was significantly higher than in Santubong during the highest density (8.0×10^3 cells L⁻¹) record. *Pseudo-nitzschia* cells were most likely to appear in April to September. Simultaneous emergence of *Pseudo-nitzschia* was recorded at both estuaries in May each year. A study on *Pseudo-nitzschia* temporal distribution by Santosa (2004) in Gaya Bay, Sabah showed that *Pseudo-nitzschia* cells were present during the whole year and most abundant between October-December and were lowest in March until May, which contradicted to finding in this study. The reason may lie behind the sampling location whereby the abundance and species diversity of *Pseudo-nitzschia* were

reported to be higher in the shelf compare to the estuaries (Dortch et al., 1997).

Above all, not every phytoplankton yields positive reactions towards ambient dissolved nutrients and the same species may response differently to the environmental variables (Klein et al., 2010). Environmental variables had weak relationship with the density of *P. lineola* (Almandoz et al., 2008) and *P. pungens* (Klein et al., 2010). Low cell density in certain depth may have related to *Pseudo-nitzschia* capability to sink from low-nutrient water into the more nutritious bottom layer (Trainer et al., 1998). The biomass may also be affected by the grazing activity by other organism such as the metazoans (Buck et al., 1992) and meso-zooplankton (Lefebvre et al., 2002). The grazing is also selective towards *Pseudo-nitzschia* species with small cells (Fehling et al., 2005).

CONCLUSION

Pseudo-nitzschia cell commonly be found in low precipitation months from April to September. Highest densities were observed especially in May each year. Apart from the precipitation rate, the cell density has apparently affected by the water salinity, pH and the availability of dissolved silicate. The two *Pseudo-nitzschia* species found in Kuching estuaries are non-toxic and cell densities in both estuaries were relatively low compared to other studies. The frequency of occurrence derived from this study revealed low probability of ASP in Malaysian waters particularly at the estuarine waters.

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