# CLEARANCE RATE OF TWO COMMERCIALLY IMPORTANT BIVALVES FEEDING ON *PSEUDO-NITZSCHIA* SPECIES (BACILLARIOPHYCEAE)

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This project is submitted in partial fulfillment of the requirement for the degree for Bachelor of Science with Honors
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## List of Abbreviations

| | |
|---|---|
| Paralytic Shellfish Poisoning | PSP |
| Neurotoxic Shellfish Poisoning | NSP |
| Diarrhetic Shellfish Poisoning | DSP |
| Amnesic Shellfish Poisoning | ASP |
| Domoic Acid | DA |
| South East Asia | SEA |
| Harmful Algal Blooms | HAB |
| Clearance rate | CR |
| Ingestion rate | IR |

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# CLEARANCE RATE OF TWO COMMERCIALLY IMPORTANT BIVALVES FEEDING ON *PSEUDO-NITZSCHIA* SPECIES (BACILLARIOPHYCEAE)

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## Abstract

The chain-forming pennate diatom, *Pseudo-nitzschia* is a cosmopolitan diatom. Contamination of domoic acid (DA), derived from several toxic species of *Pseudo-nitzschia* in filtered feeding bivalves, has subsequently caused seafood poisoning which is commonly known as amnesic shellfish poisoning (ASP). In this study, a feeding experiment was carried out to determine the clearance rates (CR) of two commercial bivalves, blood cockles, *Anadara granosa* and benthic clams, *Polymesoda expansa* feeding on *Pseudo-nitzschia* species. Mass clonal culture of *Pseudo-nitzschia* sp. strain PnMt43 from Muara Tebas was used as prey in this experiment. The bivalves were fed with different initial cell density ranging from 100 to 5000 cell $mL^{-1}$. Subsamples were taken half-hourly for a period of six hours for cell counts. Our results showed two distinct clearance responses between the two bivalves ($P = 0.0020$). *P. expansa* had the higher CR with 7.316 cell $gram^{-1}$ $mL^{-1}$ day than *A.granosa* with 0.389 cell $gram^{-1}$ $mL^{-1}$. The half-saturation concentrations (*K*) for *A. granosa* and *P. expansa* were 955 and 437 cell $mL^{-1}$, respectively. The higher clearance rate of *P. expansa* indicates the tendency to accumulate higher level of toxin during a *Pseudo-nitzschia* bloom. However, *P. expansa* with higher CR has lower K due to the difference in consumption pattern. Simulation of toxin kinetic of the two bivalves was not able to be conducted due to the absence of toxic *Pseudo-nitzschia* strains. Further studies on feeding responses and toxin accumulation-depuration rates are important to understand the mechanism of toxin kinetic of DA in these two commercially important bivalves.

*Keywords:* Clearance rate; *Pseudo-nitzschia; Anadara granosa; Polymesoda expansa*

## Abstrak

*Pseudo-nitzschia adalah diatom pembentuk rantaian yang mempunyai taburan kosmopolitan. Pencemaran asid domoik (DA) terbitannya daripada beberapa spesies Pseudo-nitzschia oleh dwicengkerang penurasan semasa pemakanan akan menyebabkan keracunan makanan laut yang dikenali sebagai Keracunan Kerang-kerangan Amnesia. Dalam kajian ini, satu eksperimen penyuapan telah dijalankan untuk menentukan kadar pembersihan spesies Pseudo-nitzschia oleh dua jenis dwicengkerang komersial, kerang, Anadara granosa dan lokan, Polymesoda expansa. Pengkulturan klon Pseudo-nitzschia sp. PnMt43 dari Muara Tebas telah digunakan sebagai makanan dwicengkerang dalam eksperimen ini. Dwicengkerang telah diberi kepadatan sel pemulaan dengan julat kepadatannya dari 100 hingga 5000 sel $mL^{-1}$. Subsampel telah diambil setiap setengah jam selama enam jam untuk pengiraan sel. Keputusan kita menunjukkan perbezaan kadar pembersihan yang ketara diantara dua dwicengkerang tersebut ($P = 0.0020$). P. expansa mempunyai kadar pembersihan yang lebih tinggi, 7.316 sel $gram^{-1}$ $mL^{-1}$ hari berbanding dengan A. granosa iaitu 0.389 sel $gram^{-1}$ $mL^{-1}$ hari. Kepadatan penepuan separuh (K) untuk A. granosa dan P. expansa adalah masing-masing 955 sel $mL^{-1}$ dan 437 sel $mL^{-1}$. Kadar pembersihan yang lebih tinggi menunjukkan P. expansa mempunyai kecerunan untuk mengumpul lebih toksin semasa ledakan Pseudo-nitzschia berlaku. Simulasi toksin kinetik ke atas kedua-dua dwicengkerang tidak dapat dijalankan disebabkan oleh ketiadaan Pseudo-nitzschia toksik. Kajian lanjutan mengenai tindakbalas penyuapan dan kadar pengumpulan-depurasi toksin adalah penting untuk memahami mekanisma kinetik toksin DA dalam dua jenis dwicengkerang ini yang bernilai komersial.*

*Kata kunci: Kadar pembersihan; Pseudo-nitzschia; Anadara granosa; Polymesoda expansa*

## 1.0 INTRODUCTION

Shellfish poisoning was caused by the consuming of contaminated molluscan shellfish through bioaccumulation. The filter feeding shellfish sieve the water column and feed on phytoplankton including the toxic species. These shellfish consume compounds that are seemingly harmless to them, but are highly toxic to the organisms that are on top of the food chain.

Paralytic Shellfish Poisoning (PSP), Neurotoxic Shellfish Poisoning (NSP), Diarrhetic Shellfish Poisoning (DSP) and Amnesic Shellfish Poisoning (ASP) are four major types of shellfish poisoning found throughout the world (Hallegraefff, 1993). Among the four types of shellfish poisoning, ASP is the only shellfish intoxication caused by a diatom from the *Pseudo-nitzschia* genus.

The first reported occurrence of ASP was documented in Prince Edward Island, Canada in 1987. Studies by Wright *et al.* (1989) and Perl *et al.* (1990) identified the toxin compound as Domoic Acid (DA) derived from the diatom *Pseudo-nitzschia multiseries* (previously reported as *Nitzschia pungens f. multiseries*) (Subba-Rao *et al*., 1988; Bates SS *et al*., 1989). The symptoms were observed in 15 minutes to 28 hours after consuming the contaminated shellfish. The common symptoms observed in ASP victims were nausea, vomiting, abdominal cramps, diarrhea and headache followed by memory loss. Among the other hospitalized patients, symptoms such as confusion, disorientation, coma, hiccups and emotional liability were also observed (Teitelbaum *et al*., 1990).

The cosmopolitan distribution of the causative organism of DA is a concern in food safety as it has potential to widespread illness through commercial bivalves. The safety limit of DA is 2 mg per 100 g of tissue (20 ppm) was established on animal studies (International

Oceanographic Commission, 1995). However, another study suggested that the tolerable regulatory level should be 20 ppm for razor clams and 32 ppm for Dungeness crabs (Marien, 1996). These regulatory limits have resulted in periodic closures of fisheries in a few areas (Wekell *et al*., 1994).

No human outbreaks of ASP have occurred since the 1987 incident in Prince Edward Island Canada. However, DA has been identified as the causative agent in the mass mortality of pelicans and cormorants in 1991 (Fritz *et al*., 1992; Work *et al*., 1993) and the extensive die-off of California sea lions in 1998 in Monterey Bay, California (Gulland, 1999; Scholin *et al*., 2000). The geographical distribution of confirmed ASP events was not only limited to the eastern Canada. DA has also been reported in North America, Europe and Japan (Loscutoff, 1992; Zaman *et al*., 1997). Although there were no reports concerning ASP in the South East Asia (SEA) region, there is a trend of increasing toxic blooms (Belltran *et al*., 1997).

Currently there is still lack of ASP studies and monitoring in Malaysia. Shellfish toxicity monitoring for PSP is the only program available but confined to the west coast of Sabah. The ASP toxicity monitoring is crucial as none of its precaution measurement is available at the moment. Vietnam, a neighboring country of Malaysia had reported massive bloom of *Pseudo-nitzschia* in January 2003 and 2004 around Haiphong area (Nguyen *et al*., 2005). Since we share the same South China Sea, it is possible that *Pseudo-nitzschia* blooms may also occur in our waters.

According to Shumway (1990) and Lassus (1999), their studies shows that oyster take up lesser toxin compared to mussels when both are exposed simultaneously to a toxic algal bloom and there are different uptake rate of DA among shellfish species. However, these studies were conducted on *Pseudo-nitzschia* and mollusk from the temperate water which has different

physiological characteristics compared to the species of tropical origin. Thus it is important to know the feeding responses of *Pseudo-nitzschia* in commercially important shellfish in our waters.

The objectives of this study are to determine the clearance rate of commercially important bivalves when fed with *Pseudo-nitzschia*, to compare the feeding responses between *A. granosa* and *P. expansa* as well as to assess the potential DA accumulation in selected bivalves.

## 2.0 LITERATURE REVIEW

This section will include harmful algal blooms in Malaysia, amnesic shellfish poisoning, domoic acid, *Pseudo-nitzschia*, clearance rate, *Anadara granosa* and *Polymesoda expansa*.

### 2.1 Harmful Algal Blooms in Malaysia

Malaysia is a country affected by Harmful Algal Blooms (HAB). PSP cases have been documented in Sabah, East Malaysia. In 1991, PSP was documented in West Malaysia when three people were ill after consuming mussels from the Straits of Malacca. The event has resulted in the establishment of shellfish toxicity monitoring (Usup *et al*., 2002). Unfortunately, ten years later six teenagers were hospitalized including one death after consuming clams harvested from a coastal lagoon in Tumpat, Kelantan. High density of *Alexandrium minutum,* was found in the inner part of the lagoon after the incident (Lim *et al*., 2004).

### 2.2 Amnesic Shellfish Poisoning

Japanese had been using the extract of red algae *Chondria armata* which contain DA as an antihelminthic agent (Takemoto & Daigo, 1960). In another study DA was also used as an insecticidal compound (Maeda *et al.*, 1984). However no negative effect was reported.

DA intoxication in autumn 1987 has raised the concern of the presence of this neurotoxin in the natural environment when it caused ASP in Prince Edward Island, Eastern Canada (Bates *et al*., 1989). The case causes 3 deaths and 105 cases of acute poisoning after consuming contaminated blue mussels (*Mytilus edulis*) from the area of Cardigan Bay (Addison & Stewart, 1989). Several events of ASP reported in the subsequent years are shown in Table 2.1.

Table 2.1: Historical events of Amnesic Shellfish Poisoning occurrence since 1987

| Year | Location/ Country | Vector | Consequences | References |
|---|---|---|---|---|
| 1987 | Cardigan River region of eastern Prince Edward Island, Canada | Blue mussels (*Mytilus edulis L.)* | 153 cases of acute intoxication and 3 deaths. | Addison & Stewart, 1989 |
| 1991 | California | anchovies | pelicans and cormorants poisoned | Fritz *et al*., 1992; Work *et al*., 1993 |
| 1994 | Oregon and Washington | razor clams and crabs | 25 illnesses | Washington Department of Health, 1994 |
| 1998 | Monterey Bay, California | Anchovies and sardines | 70 female sea lions (*Zalophus californianus)* died | Gulland, 1999; Scholin *et al*., 2000 |
| 2004 | Portuguese coast | *Eledone cirrhosa* and *E. moschata* (Cephalopoda, Octopoda) | | Pedro *et al*., 2004 |
| 2005 | Ireland | mussels (*M. edulis*), oysters (*C. gigas*), king scallops (*P. maximus*) and razor clams (*E. siliqua*), | | Kevin *et al*., 2005 |

Symptoms such as vomiting, abdominal cramps, and diarrhea were observed within one day after consuming the affected seafood. On the second day, patients show symptoms such as headache and short-term memory loss. Some symptoms were only observed in certain patients for example vertigo, disorientation and confusion (Nijjar & Nijjar, 2000). It should be cautioned that the effect of the toxin is cumulative and permanent. A person might not show any symptoms under low level of intoxication.

## 2.3 Domoic acid and *Pseudo-nitzschia*

Domoic Acid is a naturally occurring marine toxin. The chemical structure of DA is shown in Figure 2.1. It is a water soluble tricarboxylic amino acid that acts as an analog of the neurotransmitter glutamate and is a potential glutamate receptor agonist (Van Dolah, 2000). It is related both structurally and functionally to the excitatory neurotoxin kainic acid isolated from the red macroalga *Digenea simplex* (Murakami *et al*., 1953). It is heat-stable and cannot be destroyed by cooking (Perl *et al*., *1990*). It binds at the same receptor site in central nervous system (Baden *et al*., 1995). DA as the major component have nine derivatives namely 5'-epi-Domoic acid, Isodomoic acid A, Isodomoic acid B, Isodomoic acid C, Isodomoic acid D, Isodomoic acid E, Isodomoic acid F, Isodomoic acid G and Isodomoic acid H. Isomers such as Isodomoic acid D to F are weaker than DA although they are structurally similar (Hampson *et al*., 1992). Structures are shown in Figure 2.2.

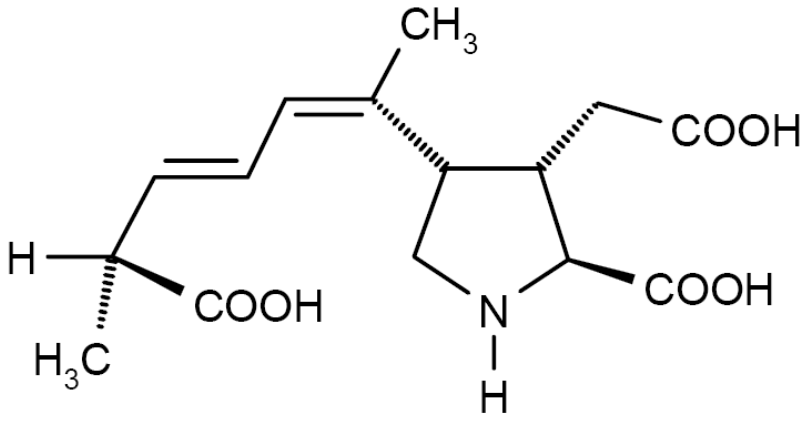


**Figure 2.1**: Chemical structure of Domoic Acid (adopted from Quilliam, 2003).

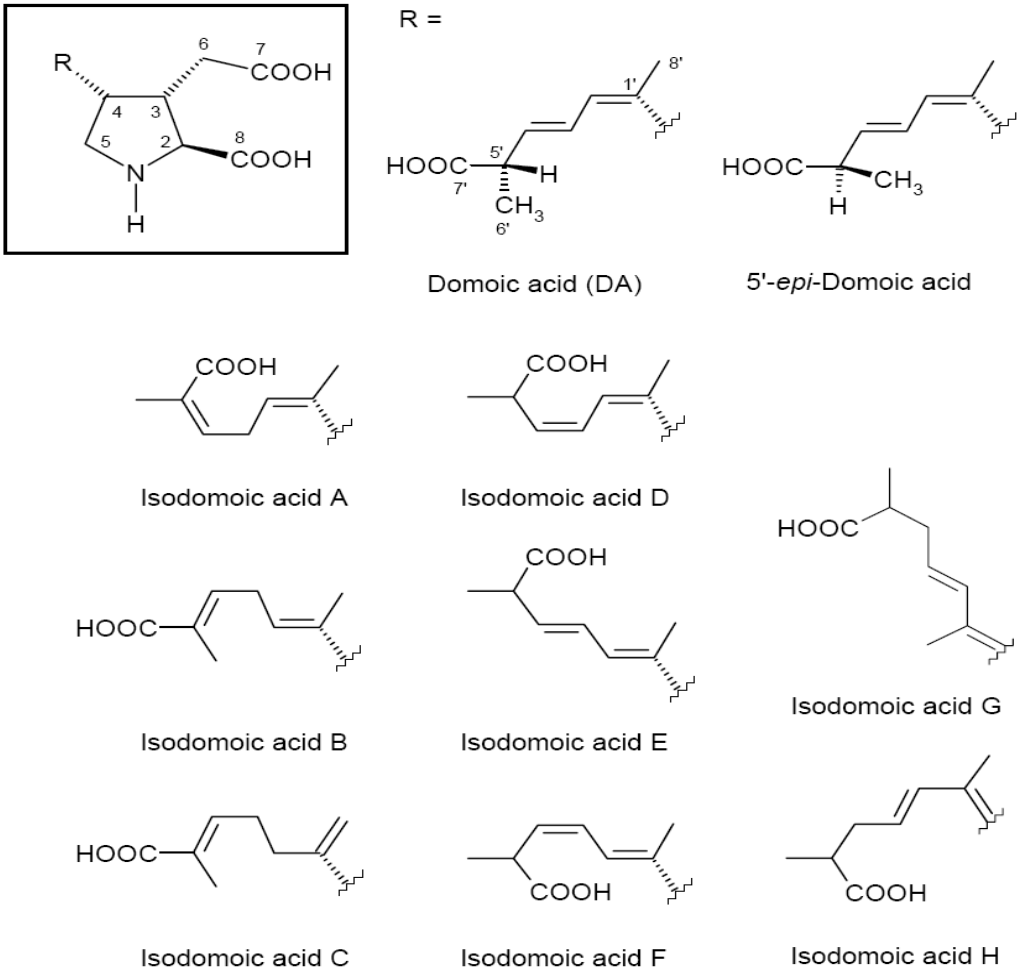


**Figure 2.2**: Domoic Acid and its derivatives (adopted from Quilliam, 2003).

This toxin is produced by several genera of diatom species such as *Pseudo-nitzschia*, *Nitzchia* and also red alga *Chondria armata.* List of diatoms know to be associated with ASP are shown in Table 2.2.

Table 2.2: Domoic Acid Producing Diatoms (*Pseudo-nitzschia*).

| **Diatom** | **DA level**[a] | **Country**[b] |
|---|---|---|
| ***Pseudo-nitzschia*** | | |
| *multiseries* | High | Canada |
| *australis* | High | USA |
| *seriata* | High | Denmark |
| *delicatissima* | Low | New Zealand |
| *pseudodelicatissima* | Low | Canada |
| *pungens* | Low | New Zealand |
| *turgidula* | Low | New Zealand |
| *fraudulenta* | Low | New Zealand |
| *multistriata* | Low | Italy |
| *cuspidata* | Low | Japan |
| *subpacifica* | Low | Japan |
| *subfraudulenta* | Low | Japan |
| *heimii* | Low | Japan |
| *Amphora coffeaeformis* | Low | Canada |
| *Nitzschia navis-varingica* | Medium | Vietnam |

High: > 10 pg cell$^{-1}$, Medium 1-10 pg cell$^{-1}$, Low: < 1 pg cell$^{-1}$

[a] Maximum DA content of the diatom culture

[b] First incident

Source: Kotaki (2008)

## 2.4 Clearance rate

Clearance rate (CR) is expressed as volume of water cleared of suspended particles per unit of time. The CR (L $h^{-1}$) was calculated using the following equation (Coughlan, 1969; Vanessa and Maria, 2006):

$$\mathrm{CR} = \frac{V}{nt}\left(ln\frac{C_1}{C_2}\right)$$

Where V is the volume of seawater in the experiment tank (liters),

n is the average weight of one individual,

t is the time interval (minutes),

$C_1$ and $C_2$ is the cell density at the beginning and end of the experiment

According to Bontes *et al*. (2007), there are three basic types of functional responses in bivalves when fed with the prey. Figure 2.3 shows the three basic types of functional responses for clearance rate in bivalves .The Type I show a linear increase of the ingestion rate (IR) with the food concentration up to a concentration where the maximum amount of food that can be ingested by an organism is reached. Type II decreases progressively towards zero with increasing food concentrations. Type III have IR accelerates more than linearly at low food densities, which for example may be the case with reward-dependent feeding behavior.

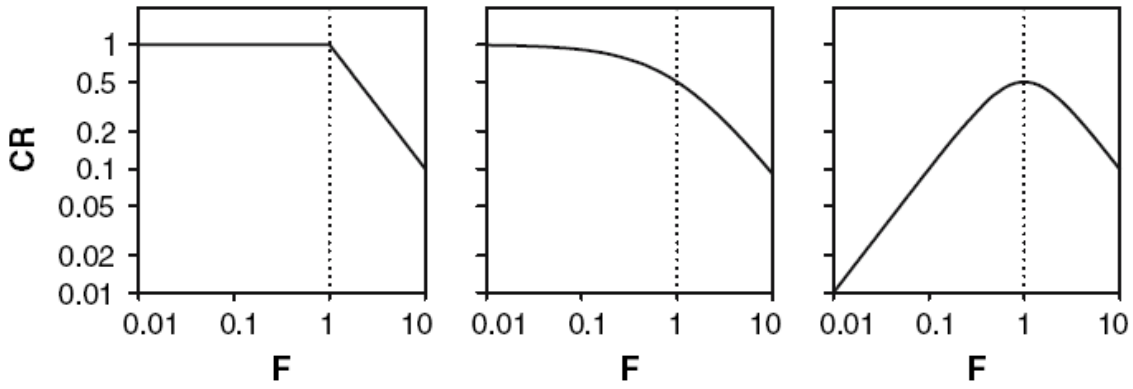


**Figure 2.3:** Three basic types of functional responses for clearance rate (CR), respectively, as a function of food concentration (F) (solid lines). (Bontes *et al.*, 2007)

## 2.5 *Anadara granosa* and *Polymesoda expansa*

According to Morris & Purchon (1981), there are 185 species from 44 families of bivalve molluscs in Malaysia. *A. granosa* and *A. antiquata* are two commercially important cockle species. In 2002, cockle production was 78,706.64 metric tons covering 6891.17 ha $m^{-2}$ involving 297 personnel. In 2003, cockle production was 71,067.29 metric tons covering 7447.06 ha $m^{-2}$ involving 311 personnel. In 2004, cockle production was 64,564.75 metric tons covering 6662.7 ha $m^{-2}$ involving 276 personnel (Othman, 2006).

Aquaculture from marine sector of year 2004 contributed about 133 to 146 thousand metric tons annually. This represented about 8 to 33% of total fish production in the country. There are six major sectors which contributed to the production. The most and traditional contributor is from cockle cultivation. Cockle contributes the most or about 40% harvest from aquaculture sector. Since the past 3 years the production from cockle annually was in the range of 70,000 metric tons. The value from sale of cockle in 2004 was about RM54 millions.

At the moment *A. granosa* is the most important aquaculture bivalve species in Malaysia (Ong, 1981, 1983; Ong & Liong, 1988). This has been possible due to the abundant supply of natural seeds and extensive areas of mudflats on the sheltered west coast of Peninsular Malaysia which is suitable for cockle cultivation. The prices of bivalves are relatively low for example; cockle is about RM 1 to RM 10 per kilogram.

*Polymesoda expansa* also known locally as 'lokan', is one of the suspension filter feeder species distributed in the mangrove swamp. This species was one of the largest mangrove bivalves that can attain diameter up to 8 cm during adult stage (Ng & Sivasothi, 1999). Once harvested, they were subsequently handled and processed without any additives or chemical preservatives and distributed on the same day to the market or freezing as only mean of preservation (Bilung *et al.*, 2005).

## 3.0 MATERIALS AND METHODS

### 3.1 Mass culture of *Pseudo-nitzschia*

A clonal culture of *Pseudo-nitzschia* sp. (PnMt43) was used in this study. The isolate was isolated from Muara Tebas (1°38'N 110°28'E) and maintained in 0.22 µm filtered sterile natural seawater containing SWII medium. SWII medium was prepared according to table 3.1. Natural seawater from Santubong with salinity of 30 psu was used as medium base. The culture was kept in a light and temperature controlled incubator at 25$^{o}$C under a 12:12h light: dark photoperiod.

**Table 3.1**: Chemical components and concentrations used in SW II medium (modified from Iwasaki, 1961)

| **Ingredient** | **Stock conc.** | **Volume** | **Final conc.** |
|---|---|---|---|
| $KNO_3$ | $7.2 \times 10^{-1}$ mol/ L | 1.0 mL | $7.2 \times 10^{-4}$ mol/ L |
| $KH_2PO_4$ | $3.31 \times 10^{-2}$ mol/ L | 1.0 mL | $3.31 \times 10^{-5}$ mol/ L |
| $Na_2$-glycero•$PO_4$ | $3.33 \times 10^{-2}$ mol/ L | 1.0 mL | $3.33 \times 10^{-5}$ mol/ L |
| Vitamin mixture: | | | |
| (a) Vitamin $B_{12}$ (Cyanocobalamin)<br>(b) Biotin<br>(c) Thiamine-HCL | | | a) $4.43\times 10^{-10}$ mol/ L<br>b) $4.43\times 10^{-10}$ mol/ L<br>c) $3 \times 10^{-7}$ mol/ L |
| Tris-HCL (pH 7.8) | 4.13 m | 1.0 mL | $4.13 \times 10^{-3}$ mol/ L |
| Fe-EDTA | $1.19 \times 10^{-3}$ mol/ L | 1.0 mL | $1.19 \times 10^{-6}$ mol/ L |
| Silicate | | 1.0 mL | $1\times 10^{-4}$ mol/ L |

Make final volume up to 1.0 L with distilled water. Adjust pH to 7.8 – 7.9 using 10% HCl and autoclaved.

Mass culture of *Pseudo-nitzschia* sp. was carried out in 5 L flasks containing 2 L of SWII medium. The late exponential phase cultures (approximately 12-day old culture with a density of ca. 182,233 cell $mL^{-1}$) were used for the feeding experiments as described below.

The *Pseudo-nitzschia* cells were harvested by gently sieving through a 20 µm nylon mesh sieve and resuspended in 100 mL of filtered seawater. Duplicate subsamples of 0.5 mL were taken for cell counting. Cell count was carried out in a Sedgwick-Rafter slide under a light compound microscope with a magnification of 40×.

### 3.2 Preparation of bivalves

Two edible bivalves that are available locally, *Polymesoda expansa* (lokan) and *Anadara granosa* (blood cockle) were used in the experiments. The bivalves were brought back to the laboratory and their epibionts were cleaned off with a brush under running tap water to remove micro-organisms adhered to the shells. The shell width of the bivalves was measured using a caliper. *P. expansa* (5.103 cm to 5.968 cm width) from Asajaya and *A. granosa* (1.658 cm to 2.376 cm width) from Kampung Buntal were used. The *P. expansa* bought from the seafood stall in Kota Samarahan comes from Asajaya. The salinity of seawater from their habitat was determined using a hand refractometer and filtered seawater was adjusted to the salinity by diluting with distilled water ($dH_2O$). Eighty individuals of *P. expansa* were placed evenly in 8 tanks. Each tank is filled with 2.5 liters of filtered seawater and equipped with aeration system. The *P. expansa* were left to starve in the filtered seawater for 24 to 48 hours.

### 3.3 Bivalves feeding Experiment

The bivalves feeding experiments were carried out in a static system where they were exposed to a range of four *Pseudo-nitzschia* cell concentrations (100, 500, 1000, and 5000 cells $mL^{-1}$). Individuals with shell length of 5.10 – 5.97 cm ($n$ = 80) for *P. expansa* and 1.66 – 2.38 cm ($n$ = 320) for *A. granosa* were cleaned and placed randomly in a tray containing 2.5 L of filtered seawater for the experiment. The experiments were carried out in duplicate trays for each of the *Pseudo-nitzschia* cell concentrations.

The experiment started off ($T_{0min}$) when the *Pseudo-nitzschia* cells were added into each tray. During the experiment, gentle aeration was applied in the trays to keep the cells in suspension, but not disturbing the bivalves. Water samples (6 mL) were taken from all trays at an interval of 30 minutes for a total of 6 hours. The samples were then preserved in acid Lugol's solution and cell density was determined microscopically.

### 3.4 Cell counting

Cell count was carried out in a Sedgwick-Rafter slide under a light compound microscope with a magnification of 40×. Cell counts were performed in triplicates and then recorded into a table attached in Appendix 1.

### 3.5 Determination of clearance rate

Clearance rate of the bivalves were calculated as follows (Coughlan, 1969; Vanessa & Maria, 2006):

$$\mathrm{CR} = \frac{V}{nt}\left(ln\frac{C_1}{C_2}\right)$$

where V is the volume of seawater in the experiment tank (2.5L), *n* is the dry weight of a single bivalve (gram), t is the duration of the experiment (in minutes), $C_1$ is the concentration of *Pseudo-nitzschia* in the experiment tank during that time, $C_2$ is the concentration of *Pseudo-nitzschia* in the experiment tank 30 minutes after $C_1$.

## 4.0 RESULTS

The *Pseudo-nitzschia sp*. strain PnMt43 was successfully cultured in the lab's light and temperature controlled incubator. Live chain-forming cells were taken under light microscope as in Figure 4.1a. Detail outer morphology of cells in chain was shown in Scanning Electron Microscope in Figure 4.1b. The cell used in the experiment is about 87.7 µm in length based on the measurement of 20 replicates (n=20) (Figure 4.2).

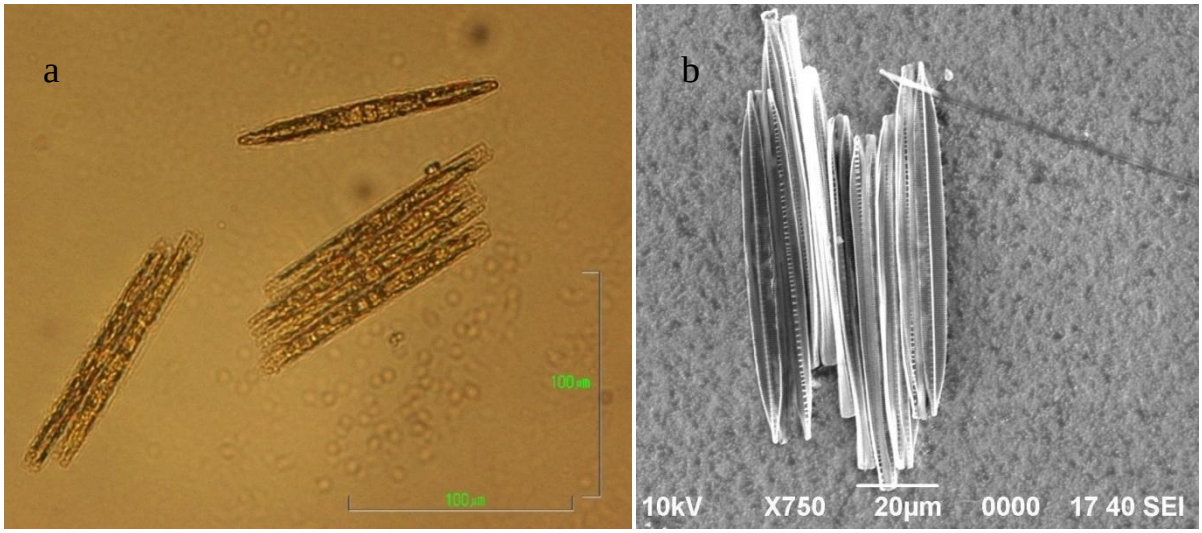


**Figure 4.1:** *Pseudo-nitzschia* PnMt43 under light and electron microscope. **a.** light microscope showing colony of *Pseudo-nitzschia* (Scale=100 µm). **b.** Scanning Electron Microscope (Scale= 20 µm)

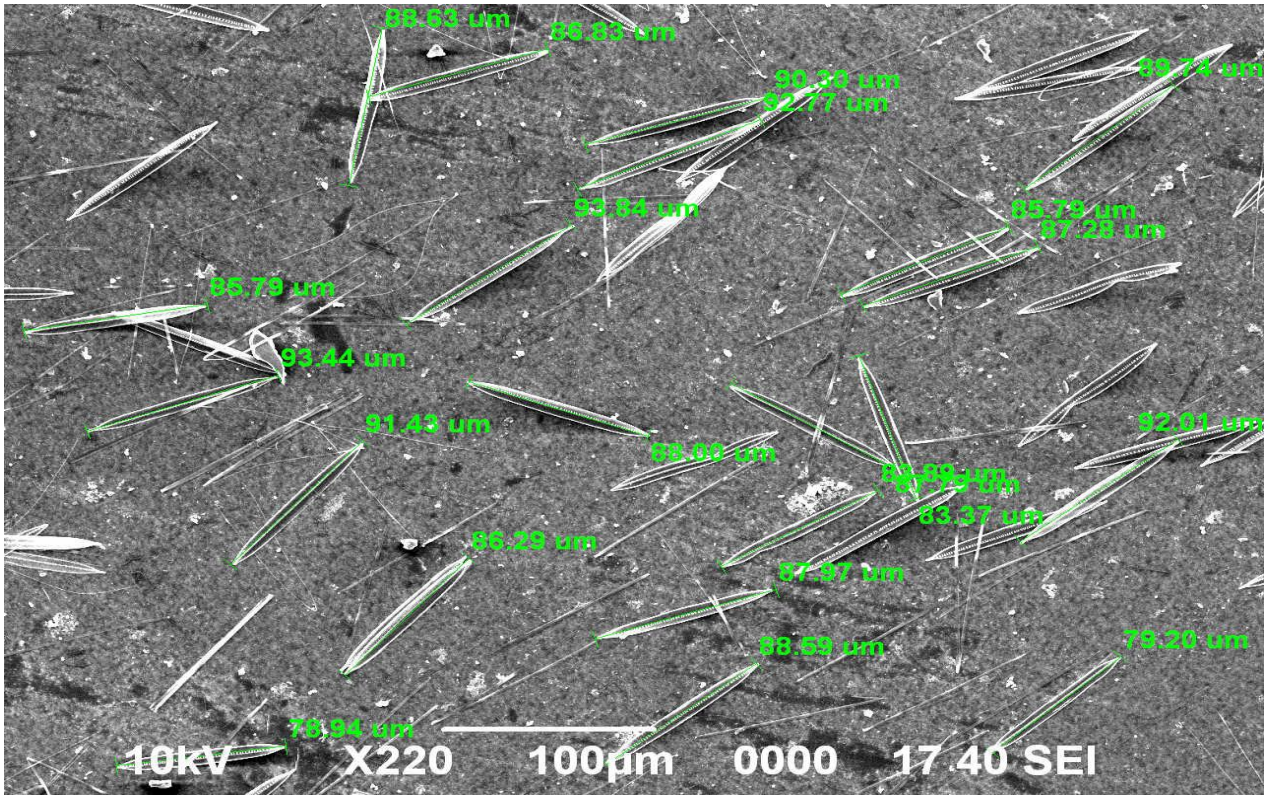


**Figure 4.2:** Measurement of 20 replicates of *Pseudo-nitzschia* under Scanning Electron Microscope. (Scale= 100 µm)