Faculty of Resource Science and Technology

RAPID DETECTION OF *PSEUDO-NITZSCHIA* SPECIES USING WHOLE-CELL FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

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Rapid Detection of *Pseudo-nitzschia* Species Using Whole-cell Fluorescence *in situ* Hybridization (FISH)

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2011

Declaration

I hereby declare that this thesis is based on my original work except for quotations and citation, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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Table of Contents

			Page
	Acknowledgements		i
	Table of Contents		ii
	List of Abbreviations		iv
	List of Tables		v
	List of Figures		vii
	Abstract		ix
1.0	Introduction		1
2.0	Literature Review		
	2.1	Harmful Algal Blooms (HABs)	3
	2.2	History of Amnesic Shellfish Poisoning (ASP)	3
	2.3	Genus *Pseudo-nitzschia*	4
	2.4	Fluorescence *in situ* Hybridization (FISH)	7
	2.5	Ribosomal RNA Targeted Oligonucleotide Probe	7
3.0	Materials and Methods		
	3.1	Sample Collection	9
	3.2	Single Cell Isolation	10
	3.3	Culture Preparation	10
	3.4	Removal of Organic Acid Material with Acid Wash	11
	3.5	SEM Sample Preparation and Observation	12
	3.6	TEM Sample Preparation and Observation	12
	3.7	Genomic DNA Extraction	13
	3.8	LSU ribosomal DNA Amplification	14
	3.9	DNA Purification and Sequencing	15
	3.10	Phylogenetic Analysis	16
	3.11	*in silico* Oligonucleotide Probe Design	17
	3.12	Whole Cell FISH Protocol	21
	3.13	Fluorescent Intensity Comparison	21
	3.14	Application of Probes to Natural Spiked Samples	21
4.0	Results and Discussion		
	4.1	Sample Isolation and Culture Establishment	22
	4.2	Morphological Observation of *Pseudo-nitzschia* Cultures	22

4.2.1 *Pseudo-nitzschia brasiliana* 22
4.2.1 *Pseudo-nitzschia pungens* 23
4.3 Amplification of the LSU ribosomal DNA 27
4.4 Phylogenetic Inferences 28
4.5 *in silico* Oligonucleotide Probe Design 32
4.5.1 *Pseudo-nitzschia pungens* Probe 34
4.5.2 *Pseudo-nitzschia brasiliana* Probe 38
4.5.3 *Pseudo-nitzschia galaxiae* Probe 41
4.6 Whole Cell FISH 44
4.7 Probe Treatment 45
4.8 Fluorescent Intensity Comparison 52
4.9 Efficiency of Probes 54
5.0 Conclusion 59
6.0 References 60
Appendices
A Electropherogram 68
B Multiple Sequence Alignment Files 69
C Fluorescent Intensity Measurement 73
D Cell Enumeration 75

List of abbreviations

ASP	Amnesic Shellfish Poisoning
Bp	Basepair
BLAST	Basic Local Alignment Search Tool
CCD	Charge Coupled Device
CLSM	Confocal Laser Scanning Microscopy
CTAB	Cetyltrimetylammonium Bromide
CSP	Ciguatera Fish Poisoning
DA	Domaic Acid
DMF	Dimethylformamide
DSP	Diarrheic Shellfish Poisoning
EDTA	Ethylenediamine-Tetraacetic Acid
FITC	Fluorescein-5-isothiocyanate
FISH	Fluorescence *In* Situ Hybridization
FSW	Filtered Seawater
HMA	Heteroduplex Mobility Assay
ITS	Internal Transcribed Spacer
LSU rRNA	Large Subunit Ribosomal Ribonucleic Acid
MP	Maximum Parsimony
NJ	Neighbor-joining
NN	Nearest Neighbor
NSP	Neurotoxic Shellfish Poisoning
nt	Nucleotide
PAUP	Phylogenetic Analysis Using Parsimony
PCR	Polymerase Chain Reaction
Pm	Pulau Mamutik
Pn	*Pseudo-nitzschia*
PSP	Paralytic Shellfish Poisoning
RFLP	Restriction Fragment Length Polymorphism
Sb	Santubong
SEM	Scanning Electron Microscope
Sm	Semariang
SW II	Seawater II medium
TBR	Tree Bisectioning Reconstruction
TEM	Transmission Electron Microscope

List of Tables

		Page
Table 2.1	Occurrence of *Pseudo-nitzschia* species in Southeast Asia (Sidabutar et al. 2000; Lundholm et al. 2002a; Lundholm et al. 2002b; Priisholm et al. 2002; Larsen & Nguyen 2004; Bajarias et al. 2006; Suriyanti 2010; Lim 2011; Lim et al. submitted 2011).	6
Table 3.1	Ingredients of SWII medium (Iwasaki 1961).	11
Table 3.2	Reaction mixture of DNA amplification (Scholin et al. 1994b).	14
Table 3.3	Reaction parameters for LSU region amplification (Lenaers et al. 1989).	15
Table 3.4	LSU rRNA gene (D1-D3) sequences of *Pseudo-nitzschia* species used in this study with accession number, strain and references.	18
Table 4.1	Morphological characteristics of *Pseudo-nitzschia* species with shape of valve, transapical axis, apical axis, central interspace, row of poroids, poroids in 1 μm, fibulae in 10 μm and striae in 10 μm. The morphometric data was obtained from Lundholm et al. 2002a; Lundholm et al. 2002b; Bates 2009; Suriyanti 2010; Lim 2011.	33
Table 4.2	Oligonucleotide probes for *Pseudo-nitzschia* species.	32
Table 4.3	Proposed oligonucleotide probes of *Pseudo-nitzschia pungens* with nucleotide length, melting temperature (T_M), GC content (%), delta G (ΔG) and E value.	37
Table 4.4	Proposed oligonucleotide probes of *Pseudo-nitzschia brasiliana* with nucleotide length, melting temperature (T_M), GC content (%), delta G (ΔG) and E value.	40
Table 4.5	Proposed oligonucleotide probes of *Pseudo-nitzschia galaxiae* with nucleotide length, melting temperature (T_M), GC content (%), delta G (ΔG) and E value.	43
Table 4.6	Whole cell hybridization probe reactivity for *Pseudo-nitzschia* species and *Nitzschia* species. Each "+" indicates a strong probe reaction and "-" indicates no reaction was detected.	45
Table 4.7	Fluorescent intensity measurement between confocal laser scanning microscopy (CLSM) and epifluorescence micrographs.	53

Page

Table 4.8 A known number of cultured *Pseudo-nitzschia pungens* cells (500 μL) were added into filtered seawater (FSW) only and seawater (SW). Species-specific PuD1 probe was applied using centrifugation method and fluorescently labeled cells were counted in three replicate samples. 55

List of Figures

		Page
Figure 2.1	The structure of domaic acid.	4
Figure 3.1	Map showed the two sampling location, Santubong estuary and Semariang River in Kuching, Sarawak.	9
Figure 4.1	Micrographs of *Pseudo-nitzschia brasiliana* which isolated from Pulau Mamutik and Semariang. A-C: SEM micrographs, scale bar = 1µm. A: Acid cleaned valve showed fibulae and striae. B: central part of the valve showed absent of central interspace. C: broadly rounded apices of one valve's end. D-G: TEM micrographs. D: valve slightly tapering towards the end of the tip, scale bar = 0.2µm. E: central part of the valve, scale bar = 1µm. F-G: closed up of valve showed row of poroids in striae, scale bar = 0.2µm.	25
Figure 4.2	Micrographs of *Pseudo-nitzschia pungens* which isolated from Santubong and Semeriang. A: SEM. Acid-cleaned valve showed coarsely structure valve, fibulae and symmetrical margins, scale bar = 10µm. B, C, D: SEM and TEM. Central part of the cell showed absent of central interspace, scale bar = 1µm. E: TEM. Close up of valve showed two rows of poroids in each striae, scale bar = 0.1µm F: SEM. End of the cell of one valve from *Pseudo-nitzschia pungens* cell, the overall shape of pointed cell ends, scale bar = 1µm. G: TEM. Pointed end of the cell, scale bar = 2µm.	26
Figure 4.3	Gel images of PCR products loaded with 5µL per lane on 1% agarose gel with 1kb DNA ladder (Promega, USA). **1**.PnSb62; **2**. PnSb57; **3**. PnSm07; **4**. PnSb64; **5**. PnSb60; **6**. PnPm09; **7**. PnSb66; **8**. PnSb58; **9**. Negative control.	27
Figure 4.4	The maximum parsimony (MP) topology inferred from the 28s Large Subunit ribosomal RNA gene of *Pseudo-nitzschia* species with a tree length of 419 steps. Consistency Index (CI) = 0.7017, Homoplasy Index (HI) = 0.2983, CI excluding uninformative characters = 0.5915, HI excluding uninformative characters = 0.4085, Retention Index (RI) = 0.7811, Rescaled Consistency Index (RC) = 0.5481. Values in the internal nodes represent bootstrap supports of 1,000 replications. Outgroups are in red. Scale bar = 10 evolutionary steps.	30
Figure 4.5	Sequence logo of *Pseudo-nitzschia pungens* species-specific probes (A) Set 1, (B) Set 2 and (C) Set 3. High height position of alphabet (nucleotide) represented conserved region and low height position of nucleotide represent variables region with mismatches.	36

Figure 4.6	Sequence logo of *Pseudo-nitzschia brasiliana* signatures. (A) Set 1, (B) Set 2. High height position of alphabet (nucleotide) represented conserved region and low height position of nucleotide represent variables region with mismatches.	39
Figure 4.7	Sequence logo of *Pseudo-nitzschia galaxiae* species-specific probes (A) Set 1, (B) Set 2 and (C) Set 3. High height position of alphabet (nucleotide) represented conserved region and low height position of nucleotide represent variables region with mismatches.	42
Figure 4.8	*Pseudo-nitzschia pungens*. LM. (A) bright field, (B) cells hybridized with species specific probe under UV excitation.	44
Figure 4.9	Micrographs of cultured cells treated with PuD1 probe (*Pseudo-nitzschia pungens* specific probe) (A-D). Autofluorescence of cells showing chloroplast content (A´-D´) and bright field micrographs (A´´-D´´). Species tested are *Pseudo-nitzschia pungens* (A), *Pseudo-nitzschia brasiliana* (B), *Pseudo-nitzschia cuspidata* (C), *Nitzschia longissima* (D). Only *Pseudo-nitzschia pungens* showed green fluorescence.	46
Figure 4.10	Micrographs of cultured cells treated with UniC probe (A-D). Autofluorescence of cells showing chloroplast content (A´-D´) and bright field micrographs (A´´-D´´). Species tested are *Pseudo-nitzschia pungens* (A), *Pseudo-nitzschia brasiliana* (B), *Pseudo-nitzschia cuspidata* (C), *Nitzschia longissima* (D). All species showed green fluorescence.	47
Figure 4.11	Micrographs of cultured cells treated with UniR negative control (A-D). Autofluorescence of cells showing chloroplast content (A´-D´) and bright field micrographs (A´´-D´´). Species tested are *Pseudo-nitzschia pungens* (A), *Pseudo-nitzschia brasiliana* (B), *Pseudo-nitzschia cuspidata* (C), *Nitzschia longissima* (D). All species did not showed any green fluorescence.	48
Figure 4.12	Cell fluorescence of *Pseudo-nitzschia pungens.* Cells observed under a confocal laser scanning microscope (A) and epifluorescence microscope (B).	52
Figure 4.13	Bright field micrographs (A-D), cells treated with PuD1 probe micrograph (A´-C´) and autofluorescence of cells showing chloroplast content (A´´-C´´). A: seawater spiked sample. B: phytoplankton sample. C: filtered seawater spiked sample. D: lugol's solution samples. Only *Pseudo-nitzschia pungens* showed green fluorescence.	56

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ABSTRACT

The genus *Pseudo-nitzschia* comprised of thirty-four known species with half of them is reported to produce domaic acid (DA), a neurotoxin associated with Amnesic Shellfish Poisoning (ASP). Species identification of *Pseudo-nitzschia* mainly depends on detailed observations of the fine ultrastructure of frustules which cannot be easily distinguishable without electron microscope. In this study, a molecular approach for rapid detection of *Pseudo-nitzschia* spp. by using whole-cell fluorescence *in situ* hybridization (FISH) was developed. Cultured and natural samples of *Pseudo-nitzschia* used in this study were identified by transmission and scanning electron microscopy (TEM & SEM). Species specific oligonucleotide probes were designed i*n silico* to *P. pungens* (L-S-P.pu-0378-a-A-23), *P. brasiliana* (L-S-P.br-0129-a-A-23) and *P. galaxiae* (L-S-P.ga-0128-a-A-23) based on sequences obtained in this study and SILVA database. Synthesized *P. pungens* specie specific PuD1 probe was applied on both cultured and field samples with UniC and UniR probe used as positive and negative control. Efficiency of PuD1 probe in detection was consistently more than 93%. Probe PuD1 coupled with FISH method is equally efficiency in cell enumeration compared to traditional method with low discrepancy (<20%). This rapid detection method should be adopted in the HABs monitoring program for detection of harmful and potential harmful species of *Pseudo-nitzschia.*

Key words: *Pseudo-nitzschia*, fluorescence *in situ* hybridization (FISH), LSU rRNA oligonucleotide probe, cell enumeration, rapid detection

ABSTRAK

Genus Pseudo-nitzschia merangkumi tiga puluh empat spesies dengan separuh daripadanya dilaporkan menghasilkan asid domoik (DA), sejenis neurotoksik yang berkaitan dengan penyebab keracunan kerang-kerangan amnesik (ASP). Pengecaman Pseudo-nitzschia spesies bergantung kepada pencerapan terperinci ultrastruktur frustul halus yang tidak dapat dibezakan tanpa menggunakan mikroskop elektron. Dalam kajian ini, kaedah molekul untuk pengesanan Pseudo-nitzschia spesies dengan menggunakan seluruh sel hibridisasi in situ pendaran (FISH) dibangunkan. Pseudo-nitzschia kultur sampel dan sampel semulajadi dicam dengan menggunakan mikroskop transmisi dan imbasan elektron (TEM & SEM). Prob oligonukleotida spesies specifik terhadap P. pungens (L-S-P.pu-0378-a-A-23), P. brasiliana (L-S-P.br-0129-a-A-23) dan P. galaxiae (L-S-P.ga-0128-a-A-23) telah direka secara in silico berdasarkan jujukan gen yang diperolehi dalam penyelidikan ini dan pangkalan data SILVA. Prob spesies specifik P. pungens PuD1 yang disintesis dan diuji dengan sampel kultur dan semulajadi, dengan UniC dan UniR prob sebagai kawalan positif dan negatif. Kecekapan prob untuk pengesanan adalah sentiasa melebihi 93%. Kaedah FISH dengan prob PuD1 menunujukkan kecekapan penentuan kepadatan sel berbanding kaedah tradisi dengan perbezaan yang rendah (<20%). Kaedah pengesanan cekap ini harus digunapakai dalam program pemantauan HABs Pseudo-nitzschia spesies.

Kata kunci: Pseudo-nitzschia, hibridisasi in situ pendaran (FISH), LSU rRNA oligonukleotida prob, pengiraan cell, pengesanan cekap

1.0 Introduction

Diatoms form the main component in phytoplankton assemblage in coastal waters. Diatoms are receiving increasing attention from worldwide due to some of the diatom species produce toxins in seafood. One of the diatom species responsible for human intoxication is *Pseudo-nitzschia* spp., which produce the neurotoxin, domoic acid. *Pseudo-nitzschia* spp. are marine planktonic diatom. *Pseudo-nitzschia* spp. are pennate diatom that responsible for the occurrence of Amnesic Shellfish Poisoning (ASP). Harmful algal blooms that occur caused by *Pseudo-nitzschia* spp are increasing in density due to the changes in coastal nutrients (Anderson et al. 2002).

The genus of *Pseudo-nitzschia* can be identified to generic level by using light microscope based on their cell shape and characteristics on chain forming. However, that is impossible to determine *Pseudo-nitzschia* to species level without detail observation of cell ultrastructure under electron microscope (Hasle & Syvertsen 1997; Fryxell & Hasle 2003). Furthermore, sample preparation and species identification is a time consuming method and required taxonomical expertise in diatom.

Besides, traditional identification method is not suitable for rapid detection of their occurrence in marine habitat (Becerril 1998). With the recent advancement in technology developments, molecular approaches have been widely applied to replace traditional approaches in monitoring of HABs species. Production of DA by some *Pseudo-nitzschia* species are harmful to marine organisms and serious threat to public health, thus accurate and rapid and accurate identification toxic *Pseudo-nitzschia* species is essential (Greenfield et al. 2006). There are several kinds of molecular probe assays have been developed for detection of toxic *Pseudo-nitzschia* spp., i.e. Restriction Fragment Length Polymorphism (RFLP) (Adachi et al. 1994), FISH (Scholin et al. 1996a), Heteroduplex Mobility Assay (HMA) (Oldach et al. 2000), nucleic acid amplification methods that target specific

signature sequences (Bowers et al. 2000), sandwich hybridization (Scholin et al. 1996a & Tyrrell et al. 2001), probe arrays (Loy et al. 2002) and nanoparticle array technology (Galluzzi et al. 2006). FISH allows rapid separation of closely related or similar morphological species (Metfies et al. 2006).

The main objective of this study is to develop an optimal FISH protocol for rapid molecular detection of *Pseudo-nitzschia* species. The specific objectives in this study are as below:

1. To design *in silico* species-specific oligonucleotide probe based on signature sequences of LSU ribosomal RNA (rRNA) gene for a particular target species of *Pseudo-nitzschia*;
2. To optimize the hybridization conditions for FISH in detection of natural and cultured cells of *Pseudo-nitzschia* spp.;
3. To compare the efficiency of FISH method and conventional cell enumeration method of natural sample of *Pseudo-nitzschia* spp.

2.0 Literature review

2.1 Harmful Algal Blooms (HABs)

HABs are natural phenomena that happen in aquatic ecosystems. The surrounding physical, chemical, and biological environments contribute for the occurrence of HABs as well. Nowadays, the HABs are increasing in frequency, persistency, toxicity, and geographical extent (Landsberg 2002). It also caused a considerable economic impact on the surrounding coastal communities.

During HABs, algal toxins from toxic microalgae or phytoplankton can accumulate in the body of shellfish molluscs (filter feeder) from lower trophic level to higher trophic level in the food web. The HABs toxins cause intoxication to marine birds, marine mammals, and human when they consumed on the contaminated seafood (Backer & Mcgillicuddy 2006).

The first HABs and shellfish toxicity incident in Malaysia was reported in year 1976, due the bloom of toxic dinoflagellate *Pyrodinium bahamense* var. *compressum* occurred in the west coast of Sabah (Roy 1976).

2.2 History of Amnesic Shellfish Poisoning (ASP)

Amnesic Shellfish Poisoning (ASP) is type of seafood poisoning caused by contamination of domoic acid (DA) which is a naturally occurring marine toxin. The ASP was first reported at Prince Edward Island Canada in year 1987 (Bates et al. 1989), where the people consumed contaminated blue mussels. The ASP victims were reported had the gastrointestinal symptoms (such as vomiting, diarrhea, abdominal cramp and so on), and neurological symptoms (such as headache and short-term memory loss) (Perl et al. 1990). The causative organism of the event was identified later as diatom *Pseudo-nitzschia multiseries* (previously known as *Nitzschia pungens* f. *multiseries).*

DA acts as an excitatory neurotransmitter and is a strong glutamate receptor agonist. The domoic acid is a water-soluble tricarboxylic amino acid (Figure 2.1). It binds with high affinity to kainate and α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid subtypes of the glutamate receptor and lead to increasing of intracellular Ca^{2+} (Hampson & Manalo 1998). It is heat-stable and cannot be destroyed by cooking (Perl *et al*. 1990). In California, the mortality of marine organisms and seabird occur every year due to high level of domoic acid in anchovies (Scholin et al. 2000).

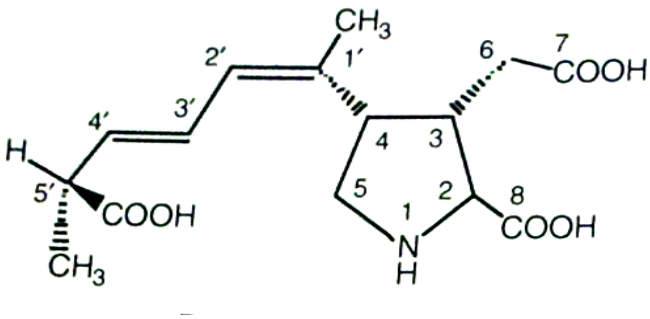

Figure 2.1: The structure of domaic acid.

2.3 Genus *Pseudo-nitzschia*

Genus *Pseudo-nitzschia* was originally belong to sub-sectioned to genus *Nitzschia* (Peragallo & Peragallo 1897-1908). It was later recognized as separate genus by Hasle (1994). The genus *Pseudo-nitzschia* consists of about thirty four known species throughout the world (Fryxell & Hasle 2003; Lundholm et al. 2003). Out of thirty four species, there is about fifteen species are potential toxin producer (domoic acid).

Fifteen potential toxic producers are *P. australis, P. calliantha, P. cuspidata, P. delicatissima, P. fraudulenta, P. galaxiae, P. heimii, P. multiseries, P. multistriata, P. pseudodelicatissima, P. pungens, P. subcurvata, P. subfraudulenta, P. seriata and P. turgidula* (Rhodes et al. 1998; Priisholm et al. 2002; Lundholm et al. 2002a; Orsini et al. 2002; Cerino et al. 2003; Lundholm et al. 2003; Lundholm et al. 2004). Among these

fifteen *Pseudo-nitzschia* species, *P. australis*, *P. multiseries* and *P. seriata* are highly toxic. They can produce DA level higher than 10pg/cell (Lundholm et al. 1994). In Malaysia, there are five *Pseudo-nitzschia* species were found, that are *P. pungens*, *P. dolorosa*, *P. brasiliana*, *P. cuspidata* and *P. micropora* (Suriyanti 2010; Lim 2011).

In Southeast Asia region, there is about seventeen *Pseudo-nitzschia* species had been detected. Among the seventeen species, five species was detected as toxin producer, which were *P. calliantha*, *P. delicatissima*, *P. fraudulenta*, *P. multistriata* and *P. pseudodelicatissima* (Table 2.1). Vietnam recorded most of the toxic *Pseudo-nitzschia* species that had been detected.

Table 2.1: Occurrence of *Pseudo-nitzschia* species in Southeast Asia (Sidabutar et al. 2000; Lundholm et al. 2002a; Lundholm et al. 2002b; Priisholm et al. 2002; Larsen & Nguyen 2004; Bajarias et al. 2006; Suriyanti 2010; Lim 2011; Lim et al. submitted 2011).

Pseudo-nitzschia species	Country				
	Malaysia	Thailand	Indonesia	Vietnam	Philippines
P. americana	-	-	-	+	-
P. brasiliana	+	+	+	+	+
P. caciantha	-	-	-	-	+
P. calliantha *	+	+	-	+	-
P. circumpora sp. nov.	+	-	-	-	-
P. cuspidata	+	-	-	+	-
P. delicatissima	+	+	-	+	-
P. dolorosa	+	-	-	-	-
P. fraudulenta *	-	-	-	+	-
P. cf. *granii*	-	-	-	+	-
P. heimii	-	+	-	-	-
p. inflatula	-	+	-	+	-
P. micropora	+	+	-	+	+
P. multistriata *	+	-	-	+	-
P. pseudodelicatissima *	-	-	-	-	+
P. pungens	+	-	+	+	+
P. cf. *sinica*	-	+	-	+	-
P.subpacifica	-	+	-	-	-

*toxic species

2.4 Fluorescence *in situ* Hybridization (FISH)

The FISH was developed in the 1960s and later was widely used in evaluation of phylogenetic identity, morphology, number and spatial arrangements of microorganisms in natural samples (Amann et al. 1995). Whole cell FISH also proven effective in detecting and identifying microbes, especially in the human disease study (Kim et al. 2005a). FISH involved the preparation probes, a short sequence of single-stranded DNA that complementary to the DNA sequences of the target organism. These probes were hybridized or binded to the complementary DNA. The location of sequence of DNA can be detected due to the present of fluorescent tags. Whole-cell FISH technique involved several main steps including fixation of the cells, probe hybridization, washing unbound probe, mounting and observation under fluorescence microscope.

2.5 Ribosomal RNA Targeted Oligonucleotide Probe

Ribosomal RNA (rRNA) was known as excellent targeted molecules due to their high natural concentration and high information content to provide signature nucleotide content for most phylogenetic taxa and most important was they presented in all organisms (Lipski et al. 2001). Genetic stability of ribosomal RNA makes it became common target molecule in designing oligonucleotide probes (Moter & Göbel 2000). Oligonucleotide probe targeted for rRNA was developed by Stahl *et al.* (1988) and nowadays became important tool for microorganisms' detection. Similar approach was applied in whole cell FISH to detect and enumerate natural sample of *Pseudo-nitzschia* species (Scholin et al. 1997).

A workable probe need to design and evaluate *in silico* to ensure the specificity, sensitivity and consistency was satisfied (Kumar et al. 2005). Probes should be specific to the targeted species only and not complementary to other sequences. Sensitivity was

required in probe design which self-complementarities. Probes that tend to hybrid to themselves rather than to their targeted sequences need to be avoided.

3.0 Materials and Methods

3.1 Sample Collection

Water samples were collected from Santubong and Semariang (Figure 3.1). Plankton samples were collected by using 20 μm mesh size plankton net. One liter of water samples were collected in a Van Dorn sampler.

Figure 3.1: Map showed the two sampling location, Santubong estuary and Semariang River in Kuching, Sarawak.

3.2 Single Cell Isolation

Pseudo-nitzschia cells were isolated by using the micropipette technique (Hoshaw & Rosowski 1973). A Pasteur pipette was drew into a fine capillary by flame of Bunsen burner. A few drops of medium were placed on the glass slide. Then, a natural field water sample was placed on the Petri dish. The *Pseudo-nitzschia* species was located with inverted microscope for isolation. The capillary action was practiced and the *Pseudo-nitzschia* species was transferred to a drop of sterile seawater on glass slide. The cell was washed with capillary pipette by transferring them one by one start from the first drop of the medium.

3.3 Culture Preparation

Cultures of *Pseudo-nitzschia* species used in FISH optimization were obtained from UNIMAS Harmful Algae Culture Collection (PnSb57, PnSb58, PnSb60, PnSb62, PnSb64, PnSb66, PnSm07 and PnSm09).

The clean test tubes were soaked in 10% HCl for at least one day. The culture test tubes were rinsed with tap water and followed with distilled water. The culture test tubes were filled with about 5 mL of distilled water. The cleaning of culture test tubes were carried out with double glove. The sterilization of culture test tubes were carried out by autoclaving at 121°C and about 20 min.

The cultures were maintained in enriched SWII medium (Iwasaki 1961). Filtered natural seawater was used as medium base. Salinity of filtered seawater was adjusted to 30PSU by adding 10 g of salt. The SWII medium was prepared by adding KNO_3, KH_2PO_4, Na_2-glycero.PO_4, Fe-EDTA, Tris-HCl, vitamin mix (mixture of B_{12} (cyanocobalamin), biotin, and Thiamine-HCl) and silicate into filtered seawater and make the final volume up to 1 L (Table 3.1).

Table 3.1: Ingredients of SWII medium (Iwasaki 1961).

	Stock concentration	Volume(mL)	Final concentration
KNO_3	7.2×10^{-1} mol/L	1.0	7.2×10^{-4} mol/L
KH_2PO_4	3.31×10^{-2} mol/L	1.0	3.31×10^{-5} mol/L
Na_2-glycero.PO_4	3.33×10^{-2} mol/L	1.0	3.33×10^{-5} mol/L
Fe-EDTA		1.0	1.19×10^{-6} mol/L
Tris-HCl (pH 7.8)		5.0	4.13×10^{-3} mol/L
Vitamin mix		1.0	
➢ Vitamin B_{12} (cyanocobalamin)			4.43×10^{-10} mol/L
➢ Biotin			4.1×10^{-9} mol/L
➢ Thiamine-HCl			3.0×10^{-7} mol/L
Silicate		0.5	

The pH of medium was adjusted to pH 7.8-7.9 by adding 10% HCl drop by drop. The medium was filtered by using hand pump. The medium was sent for autoclaving. The autoclaved medium was left at room temperature at least two days to allow the CO_2 gases diffuse into the medium.

Cultures transferring were carried out aseptically in a laminar flow hood. 70% EtOH was used for desk cleaning and a Bunsen burner was used to sterilize all the culture test tubes before culture transferring started. 25 mL of SWII medium was transferred into sterilized culture tubes. 15mL of culture was transferred into the culture test tube that filled with SWII medium. The cultures was kept at 25 °C of incubation temperature and 12:12 h light:dark photocycle. The old cultures was kept in 12:12 h light:dark photocycle culturing cabinet.

3.4 Removal of Organic Acid Material with Acid Wash

Samples were prepared at least one day before electronic microscope (EM) observation was done. 10 g of oxalic acid was weighted and added into 100 mL of distilled water and followed by stirring (preparation for five samples). 20 mL of water samples were transferred into a 50 mL centrifuge tube. 20 mL of 98% sulfuric acid (H_2SO_4) was added into each centrifuge tube that filled with different water sample and shook gently (carried

out in fume hood). A small amount of saturated $KMnO_4$ was added until the water samples turned into purple color. A small amount of 10% oxalic acid was added into the mixture samples until the mixture become clear. The mixture samples were centrifuged at 4000 rpm at 10°C for 10 min. The supernatant was discarded and added in distilled water until it reached 45 mL. The centrifugation steps and supernatant discarding steps were repeated at least five times in order to clean the cell.

3.5 SEM Sample Preparation and Observation

A small drop of the acid wash sample was pipette onto a polycarbonate membrane filter paper. The polycarbonate membrane filter paper was kept in the filter paper and kept in 60°C oven overnight for drying. The samples were mounted on an aluminium stub by using a double-sided carbon tape. Samples were placed into the chamber of the sputter coater for coating. A very thin film of gold or palladium was coated on each sample. The samples were put into JSM 6390LA SEM (JEOL, Japan) and were observed by using lens detector at 10KV in high vacuum condition. Micrographs were captured by using Gantan Digital Imaging Camera for each sample.

3.6 TEM Sample Preparation and Observation

A small drop of the acid wash sample was pipette and mounted on a formvar coated 300 mesh copper grid and the cell valves were observed under JSM-1230 TEM (JEOL, Japan). Species were identified after determined the transapical axis, apical axis, central interspace, rows of poroids, fibulae and striae (Hasle 1965; Hasle & Syvertesen 1997; Skov et al. 1999; Priisholm et al. 2002; Lundholm et al. 2003).