Faculty of Resource Science and Technology

Rapid detection of toxic dinoflagellate, *Alexandrium minutum* (Dinophyceae) using whole-cell fluorescence *in situ* hybridization (FISH)

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This project is submitted in partial fulfillment of the requirement for the degree of
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Resource Biotechnology Programme
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Universiti Malaysia Sarawak
2012

Declaration

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

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List of Abbreviations

HABs	Harmful algal blooms
PSP	Paralytic shellfish poisoning
STX	Saxitoxin
FISH	Fluruoscence *in situ* hybridization
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
rRNA	ribosomal Ribonucleic acid
rDNA	ribosomal deoxyribonucleic acid
SHA	sandwich hybridization assay
CCD	Charge coupled device
HPLC	High performance liquid chromatography
mRNA	messenger Ribonucleic acid
CY5	Cyanine Dyes (Indodicarbocyanine)
HCI	Hydrochloric acid/ Hydrogen chloride
UV	Ultraviolent
TIF	Tagged image file
LSU	large subunit
SSU	small subunit
MP	maximum parsimony
ML	maximum likelihood
BI	Bayesian inference
TBR	tree bisection reconnection
CI	consistency index
RI	retention index
RC	rescaled consistency index
HI	homoplasmy index
AIC	Akaike Information Criterion
PDT	PROBE Design Tool
PMT	PROBE Match tool
BLAST	Basic local alignment search tool
Blastn	nucleotide blast
FITC	Fluorescein-5-isothiocyanate
NCBI	National Center for Biotechnology Information
APC	apical pore complex
RDP	ribosomal database project
PBS	phosphate buffer saline
SEM	Scanning Electron Microscope

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ABSTRACT

Harmful algal blooms (HABs) are phenomena known as sudden increase in microalgal population that cause not only human seafood poisoning but also impact to the marine ecosystem. The dinoflagellates particularly species of *Alexandrium* have been known as producers of paralytic shellfish poisoning (PSP) toxins, a type of sodium blocking neurotoxins collectively called saxitoxin (STX). Species identification in the genus was commonly done under conventional light microscope. However variation in morphological characteristics used in delineating species is often hard to detect which requires taxonomic expertise. Hence, this study adopts a molecular detection approach to rapidly detect the species of *Alexandrium* by using whole-cell fluorescence *in situ* hybridization (FISH). Ribosomal RNA-targeted oligonucleotide DNA probe targeting the toxic *Alexandrium minutum* were designed *in silico*. Specificity and accessibility of designed probes were further verified *in silico* comparing parameters that influenced the hybridzation kinetics. An *A. minutum* species-specific probe region was successfully identified, and designated as L-S-Amin-569-A-18. The probe was synthesized and tested on clonal cultures of *A. minutum*. Samples were fixed and then underwent whole-cell FISH protocol prior to observation under an epi-fluorescence microscope. Optimization on FISH procedure was conducted to determine the optimum hybridization conditions. The result showed that the DNA probe had high specificity towards *A. minutum* with no cross-reactivity towards other *Alexandrium* (*A. tamiyavanichii*, *A. tamutum* and *A. affine*). The FISH protocol had been proven as rapid detection tool for *A. minutum* in regional of Malaysia. Hence, this approach is proposed to be adopted in the national harmful algal monitoring.

Key words: Paralytic shellfish poisoning (PSP); *Alexadrium minutum;* whole-cell fluruoscence *in situ* hybridization (FISH); *in silico* rRNA-targeted oligonucleotide DNA probe

ABSTRAK

HABs merupakan fenomena peningkatan microalga populasi secara menjujuk dan kesannya bukan sahaja terhadap keracunan makanan laut manusia tetapi juga terhadap ekosistem marin. Kumpulan dinoflagella terutamanya spesies-spesies Alexandrium telah dikesan sebagai pengeluar toksin yang menyebabkan keracunan kerang-kerangan melumpuhkan (PSP). Ia merupakan sejenis penghalang natrium yang dikenali sebagai saxitoxin (STX). Pengenalpastian spesies umumnya melalui pemerhatian dengan mikroskop cahaya, namun demikian perbezaan dari segi morfologi dalam pengenalpastian spesies amat sukar dan memerlukan kepakaran taksonomi. Justeru itu, pendekatan molekul dengan menggunakan hybridisasi pendaran in situ (FISH) seluruh sel telah diaplikasikan dalam kajian ini untuk tujuan pengesanan spesies beracun Alexandrium secara pantas dan berkesan. Hybridisasi pendaran in situ penjujuk DNA prob oligonukleotida rRNA terhadap A. minutum telah ditentukan secara in silico. Prob spesifik telah diuji dengan kultur. Prob spesifik untuk A.minutum bernama L-S-Amin-569-A-18 telah dikenal pasti. Sampel diawet dan protokol hydridisasi pendaran in situ (FISH) seluruh sel dijalankan sebelum permerhatian di bawah mikroskop pendaran sipi. Pengoptimunan prosedur hydridisasi pendaran in situ (FISH) dijalankan. Keputusan kajian ini menunjukkan Prob DNA tersebut mempunyai spesifikasi tinggi terhadap A. minutum dan tidak mempunyai aktiviti silang terhadap Alexandrium spesies yang lain (A. tamiyavanichii, A. tamutum dan A. affine). Justeru, protokol FISH dibuktikan boleh digunakan sebagai kaedah pengesanan secara pantas untuk A. minutum di serantau Malaysia. Oleh itu, pendekatan ini dicadangkan boleh digunapakai dalam pemantauan kebangsaan untuk alga yang berbahaya.

Kata kunci: keracunan kerang-kerangan melumpuhkan (PSP); Alexadrium minutum; hydridisasi pendaran in situ (FISH); in silico oligonukleotida rRNA sasaran DNA prob

1.0 INTRODUCTION

Detection and enumeration of harmful algal species is pivotal in preventing human intoxication and impacts on ecological and economic aspects (Kao, 1993). Dinoflagellates comprise toxic and nontoxic species which are commonly found in marine ecosystem. Certain toxic dinoflagellate species of the genus *Alexandrium* are lethal to human. Paralytic shellfish poisoning (PSP) is caused by a group of neurotoxins which collectively known as saxitoxin (STX) that blocked sodium channels in mammalian nerve cells and thus preventing signal propagation along the neuron (Schantz *et al*., 1966; Kao, 1993).

Worldwide distribution of this genus has increased the severity of PSP that threaten human health and fisheries industries. Thus far, PSP causative organisms in Malaysia were mainly found as marine dinofalgellates. They are *Pyrodinium bahamense, Alexandrium tamiyavanichii* and *Alexandrium minutum*. In Malaysian waters, *A. minutum* and *A. tamiyavanichii* were responsible for PSP events in the Peninsular Malaysia (Usup et al. 2002; Lim *et al*., 2004, 2007; Leaw *et al*., 2005).

Since the toxins are tasteless, odorless, and cannot be differentiated using normal screening, it required rapid and suitable molecular method to identify the presence of toxic dinoflagellates. Therefore, a variety of detection techniques using molecular approaches such as fluorescence *in situ* hybridization (FISH) (e.g. Simon *et al*., 2000; Groben & Medlin, 2005), DNA microarrays, real-time PCR (Galluzzi *et al*., 2004) and sandwich hybridization assays (SHAs) (Scholin *et al*., 1996) have been employed for rapid detection of harmful algal species (Diercks *et al*., 2008). Moreover, rapid detection of toxic dinoflagellates, identification of the species, and characterization of their abundance and distribution in algal blooms would aid in the protection of sensitive aquaculture areas and also prevent human intoxication.

Monitoring of toxic algae involved accurate morphological identification and enumeration of species by using standard microscopic procedures. *Alexandrium minutum* was difficult to differentiate among species as it was only characterized by minute morphological details in the theca plates. Hence, FISH with chemiluminescent detection was a more suitable tool for rapid and reliable detection of harmful algae (Anderson *et al*., 2005; Groben & Medlin, 2005; Hosoi-Tanabe & Sako, 2005). Moreover, this method required considerable short time compare to other methods.

In this study, species-specific oligonucleotide probe was designed *in silico* in the large subunit ribosomal RNA using the ARB package. The designed probes were subsequently evaluated based on factors that influenced hybridization kinetics and efficiency. The optimum probe was then tested and applied on cultured *Alexandrium* species. Targeted cell were detected with the assistance of light and epi-fluorescence microscope equipped with a charge coupled device (CCD) camera. Probe optimization such as probe stability, probe intensity and cross reactivity of the probe was carried out in order to validate the efficiency of probe.

The main objective of this study was to apply a molecular method of whole-cell FISH for rapid detection of toxic *A. minutum* either in cultured cells or natural seawater samples by using rRNA-targeted oligonucleotide DNA probe. The specific objectives were as below:

1. To develop *in silico* species-specific rRNA-targeted oligonucleotide DNA probe targeting toxic *A. minutum*;
2. To determine the best *A. minutum* species-specific probe using *in silico* approach;
3. To optimize the hybridization conditions for whole-cell FISH;
4. To apply whole-cell FISH on the target species, *A. minutum*.

2.0 LITERATURE REVIEW

2.1 Harmful algal blooms (HABs)

A bloom begins when a small population of toxic dinoflagellates cells in lag phase or in resting cysts resides in the bottom sediment. The origin of marine algal toxins were unicellular algae with favorable conditions of environmental and thus proliferated or aggregated to form dense conditions of cells or "bloom" (Dolah, 2000). At high level of bloom, the water may assume a fluorescent reddish color referred to as red tide (RaLonde, 1996).

In recent decades, the frequency and geographic extent of HABs have increased worldwide. This magnitude of HABs is estimated to be on the increase due to variety of mechanisms like shifts in microalgae assemblages towards more flagellates and some others (Shears & Ross, in press). Global distribution and increased in of HABs cases from 1970s to 1990s has been determined (Figure 2.1). Toxin-producing HABs species are particularly dangerous to sea water creatures as well as towards human. There were expansion of PSP in Southeast Asia and South America.

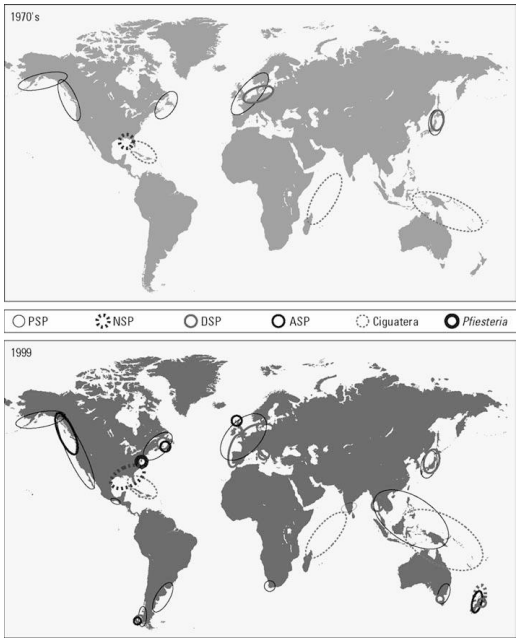

Figure 2.1 Global distribution and increasing of HABs cases from 1970s to 1990s. (Source: Sellner *et al*., 2003)

Harmful algae not only can be found in a single class or in few genera, but among six taxonomic groups (diatom, dinoflagellates, haptophytes, raphidophytes, cyanophytes and pelagophytes) (Zingone & Enevoldsen, 2000).

Consumption of seafood contaminated with algal toxins result in the following poisoning syndromes. These primary grouping of HABs toxins include paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), amnestic shellfish poisoning (ASP), diarrhetic shellfish poisoning (DSP), azaspiracid shellfish poisoning (AZP), ciguatera fish poisoning (CFP) and cyanobacteria toxin poisoning (CTP) (Sellner *et al*., 2003).

Human illness was mainly caused by ingesting seafood (primary shellfish) contaminated with natural toxins produced by HAB organisms. There were variety complications due to consumption of these HABs toxin. These include respiratory and digestive problems, memory loss, seizures, lesions, skin irritation, fatalities in fish, bird and mammal (include human) (Sellner *et al*., 2003). Furthermore, there were some being acutely lethal due to such kinds of HABs poisoning.

2.2 Paralytic shellfish poisoning (PSP)

PSP produced by dinoflagellates group such as *Alexandrium*, *Pyrodinium* and also *Gymmodinium*. Toxin algae of the genus *Alexandrium* are the dominant sources of PSP toxins in contaminated bivalves (Li & Wang, 2001). Approximately 2,000 cases of human poisoning through fish and shellfish consumption had been reported each year and achieved as high as 15% mortality rate among the PSP cases (Hallegraeff, 2003).

PSP is a biological intoxication resulting from ingestion of marine mollusks containing potent neurotoxins derived from dinoflagellates. This is caused by a group of toxin named saxitoxin (STX) (Figure 2.2). The name saxitoxin is derived from the butter clam, *Saxidomus giganteus* where saxitoxin was originally extracted and identified

(RaLonde, 1996). PSP happened in people who had ate bivalve shellfish (clams, mussels, scallops and etc) and subsequently contaminated with one or more group of structurally related congeners of saxitoxin (Dolah, 2000). High-performance liquid chromatography (HPLC) had been used to make analysis of shellfish toxins that provides excellent peak resolution and high sensitivity (Oshima *et al*., 1989; Ledoux *et al*., 1993).

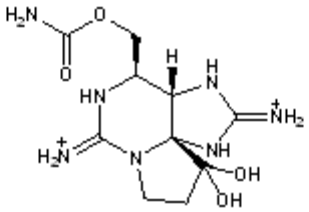

Figure 2.2: Structure of saxitoxin (Oshima, 1995)

Generally, STX are soluble in water and can withstand high temperature. Even with high pressure cooked addition with high temperature of 250°F for 15 min, PSP remains toxic (RaLonde, 1996). It is stable in acidic conditions but degraded in alkaline conditions. In addition, acid hydrolysis increases six-fold in toxicity of STX when SO_3^- group separate from position 21on STX molecule (RaLonde, 1996).

One of the first PSP recorded cases was in 1973 in Pacific Northwest of USA (Nishitani & Chew, 1988). Although the main cases are in America or Europe, there were also some cases occurred in Malaysia, Philippines, Indonesia, Venezuela, Guatemala, China and South Africa as well (Backer *et al*., 2003). There were outbreak of PSP in Brunei began in 1976, central Philippines in 1983, Northern Phillippines in 1987 and also Malaysia in 1988 (Dolah, 2000).

PSP symptoms are neurological and their onset is rapid. Neuronal and muscular voltage-gated sodium channels of the excitable membrane were blocked and thus prevented the propagation of action potential in nerve axons and skeletal muscle fibers

(Kao, 1993). Initially paresthesia, numbness around lips and mouth can be observed within 30 minutes and would gradually spread to the face and neck. Victims also experienced nausea and vomiting. Onset of PSP symptoms usually occur within 30 min to 3h. In severe cases, respiratory seizure occurred less than 24 hours after consumption of toxic shellfish (Daranas *et al*., 2001). Human are most sensitive with that the fatal oral doses of saxitoxin range from one to four mg (Levin, 1992).

In Malaysia, PSP toxin producers were found, for example *Pyrodinium bahamense* var. *compressum* at Sabah in 1976, *A. tamiyavanichii* at Sebatu Melaka in 1991 and *A. minutum* at Tumpat Kelantan in 2001 (Usup *et al*., 2002). The first PSP incident occurred in the east coast of Peninsular Malaysia involving six persons who were poisoned with one fatality after consumption of the contaminated benthic clam *Polymesoda sp*. collected from a coastal lagoon in Tumpat, Kelantan in September 2001 (Usup *et al*., 2002). Before that, at the early 1991, PSP occurred for the first time outside Sabah. There were three person poisoned after consuming mussel collected from mussel farm in Sebatu in the Straits of Malacca which after that had been subjected to be toxins due to discovery of *A. tamiyavanichii* (Usup *et al*., 2002).

2.3 The genus *Alexandrium* (Halim)

The genus *Alexandrium* was first described by Halim (1960) with *A. minutum* as the predominant one. The species are planktonic-sized marine thecate and photosynthetic dinoflagellates. There are 33 species *Alexandrium* currently described (Leaw *et al*., 2009). Some species have the ability to cause PSP while some are not responsible to PSP occurrences. One third of these species had been reported to cause PSP due to STX and its analogues that contaminate edible shellfish mollusk (Anderson *et al*., 1990). *Alexandrium*

species that cause PSP include *Alexandrium acatenella, A. catenella, A. fraterculus, A. fundyense, A. minutum, A. ostenfeldii, A. tamarense* and *A. tamiyavanichii* (Balech, 1995).

Identification of the genus was based on cell shape and Kofoidian theca plate tabulation. Generic characteristics can be primarily based on details theca plate pattern, with the Kofoidian formula apical pore (Po), fourth apical (4´), sixth precingular (6˝), fifth postcingular (5´˝), second antapical (2˝´), sixth cingular (6c) and 9-10sulcals (Hansen *et al*., 2003; Leaw *et al*., 2005).

While as features used for species separation primarily minute details of plate pattern like shape and size of 1´, 6˝, anterior sulcal plate (s.a.) and posterior sulcal plate (s.p.), details of Po plate and also presence or absence of ventral pore that located at the margin between 1´ and 4´ plates (Hansen *et al*., 2003; Leaw *et al*., 2005).

Theca plate tabulation of *Alexandrium* species shows ventral, dorsal, apical and antapical view (Figure 2.3). Due to these minute morphological differences between the species, morphological of species can only be observed under high magnifying fluorescence microscope. It is difficult to identify *Alexandrium* spp. alone because they vary widely due to various factors and with present of some microalgae (Hosoi-Tanabe and Sako, 2005). This obstacle had rinsed up the application of probe design as well as whole-cell FISH technique.

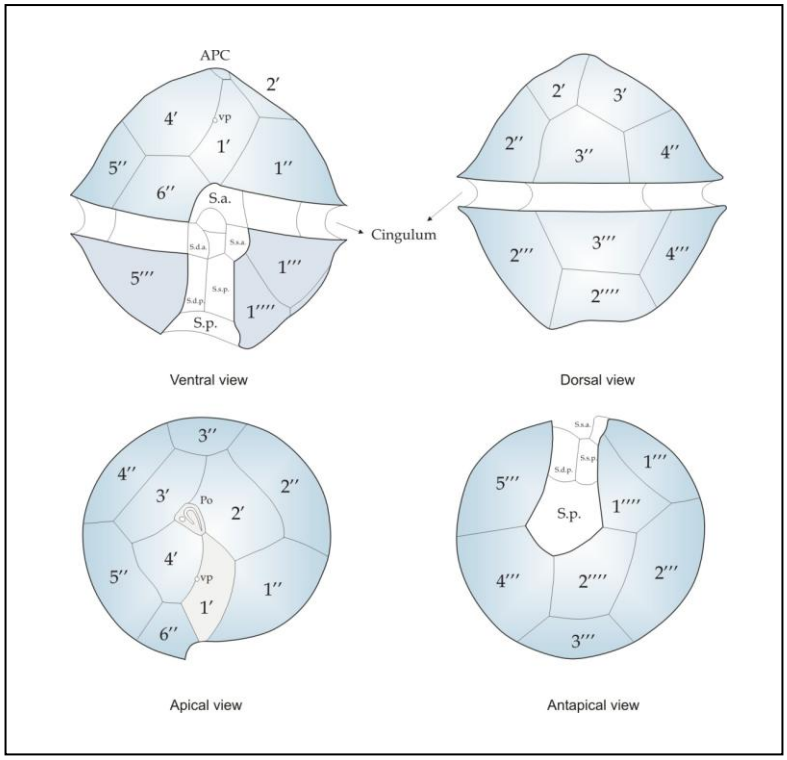

Figure 2.3: Thecal plate tabulation of *Alexandrium* based on Kofoidian system (Leaw, 2005).

2.3.1 *Alexandrium minutum*

In Malaysia, blooms of the species *A. tamiyavanichii* and *A. minutum* had resulted in PSP incidence. As described by Halim (1960) and Balech (1995), *A. minutum* is a toxic single-armoured dinoflagellate and also the smallest cell of *Alexandrium* with the short posterior sulcal plate. Cells are spherical in shape and small-sized, 15 to 29 µm in diameter. The cell is green-brown in colour with a theca (clear protective covering) (Bravo *et al*., 2006). There are small details that can differentiate *A. minutum* with the other *Alexandrium* species. This species has wide posterior sulcal plate and narrow but long 6´´ plate. The s.a. is longer than width with a straight anterior margin (Lim *et al.*, 2003).

A. minutum belongs to most potent PSP-toxin producers from many studies in worldwide (Honsell *et al.* 1996, Chang *et al*. 1997). *A. minutum* can be found whole

around and its huge geographic range since with the increasing bloom frequency (Leaw *et al*., 2005; Lim *et al*., 2004, 2006; Lily *et al*., 2005).

2.4 Oligonucleotide ribosomal RNA

Oligonucleotides are ideal for *in situ* hybridization due to its small sizes and thus allow easier penetration into the cells or tissue of interest. In addition, they are single stranded synthetically designed and hence exclude the chance of renaturation (Tang *et al*., 2005) Molecular biological techniques have greatly enhanced our ability to understand phylogenetic relationships among organisms and to develop means to detect species. Various numbers of genetic markers used and oligonucleotide ribosomal RNA (rRNA) genes have historically figured most prominently in studies of harmful algae. Large abundance of ribosomal RNA molecules made them excellent markers (Woese, 1987) because conserved and variable region of molecule advanced the development of oligonucleotide in identification of phytoplankton.

2.5 Whole-cell fluorescence *in situ* hybridization (FISH)

First review for the use of molecular probes as tools to aid the identification of harmful algal species was presented by Anderson (1995) FISH uses a fluorescently labeled probe that specially designed to recognize a specific sequence of a particular organism. The probe is hybridized inside the intact cells and thus the cells that associate with specific sequences and intact fluorescent labeled probe were consequently detected using epi-fluorescence microscopy (Hosoi-Tanabe and Sako, 2005). Rapid detection of different algal group can be performed under epi-fluorescence microscopy.

Whole cell FISH use rRNA-targeted oligonucleotide probes. It is different with common FISH method used since it binds to whole part of the cells and only the desired

part of cells will emit light (Lipski *et al*., 2001). Whole cell FISH is a method of localizing and detecting specific mRNA sequences in morphologically preserved cells prepared by hybridizing the complimentary strands of nucleotide probe to the sequences of interest. This hybridization with rRNA-targeted probe had been a suitable tool for assistant of phylogenetic supportive and also applied on environmental study (Amann *et al*. 1995).

There had been various studies being conducted on HABs species using FISH and also this method had been showed to be a powerful tool to detect species specific samples from *Pseudo-nitzschia* (Miller & Scholin, 1998; 2000) and moreover also *Alexandrium* species (Kim *et al*., 2004; 2005; John *et al*., 2003; Sako *et al*., 2004). FISH has been applied to discriminate toxigenic dinoflagellates *A. tamarense* and *A. ostenfeldii* from Scottish coastal (John *et al*., 2003). Besides that, *A. tamiyavanichii* can be rapidly detected using FISH method using DNA probe (Kim *et al*., 2004; 2005)

FISH technique start with fixed sample to preserve overall cell morphology, reduce autofluorescence and permeabilize the cell wall to exchange probes and hybridization solutions (Metfiers, 2006). Whole cell FISH protocol involve the following steps that are fixation of cells, probe hybridization, washing off unbound probe and observation under modified light and epi-fluorescence microscope (Figure 2.4).

The beneficial use of fluorescence labeling (FISH) of marine microalgae for a rapid detection has been reported in many studies (Scholin *et al*., 1994; 1996; Knauber *et al.*, 1996; Simon et al., 1997; John *et al*., 2003, 2005; Groben *et al*., 2004; Groben and Medlin, 2005). Single probe for some species had been developed and tested specificity by means of FISH (Simon *et al*., 1997; Tobe *et al*., 2006) such as application of whole cell FISH.

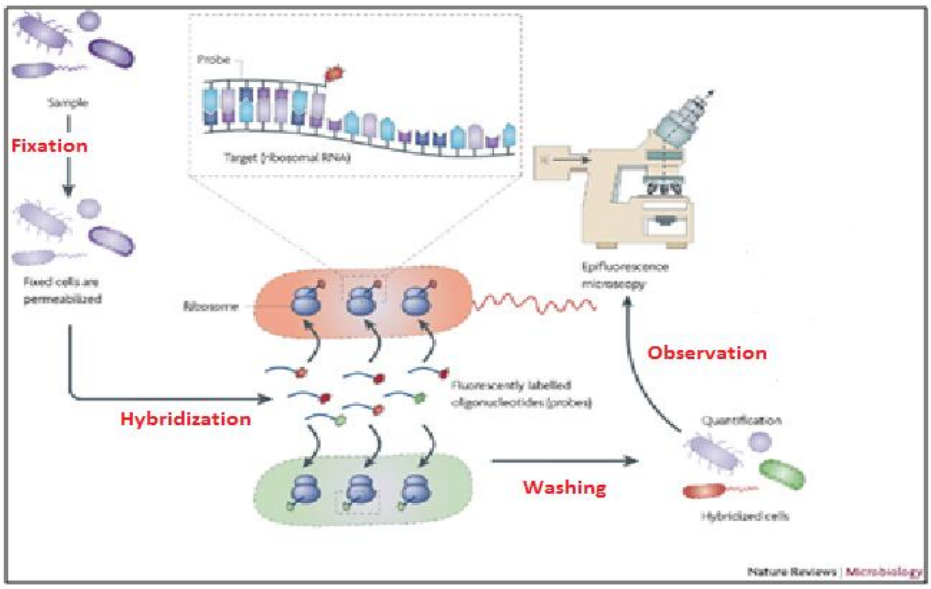

Figure 2.4: Schematic diagram of FISH method (Source: Amann & Fuchs, 2008).

2.6 Epi-fluorescence Microscopy

In dinoflagellate taxonomy, epi-fluorescence microscopy had been widely used for species identification (Usup *et al*., 2002; Lim *et al*., 2003) and also for enumeration and determination of suitable probes which associate with FISH (Kim *et al*., 2004; 2005; John *et al*., 2003; Sako *et al*., 2004). In addition application FISH allows the rapid detection of different algal groups using epi-fluorescence microscopy and even can be utilized for separation of closely related and morphologically similar species (Tobe *et al*., 2006).

Epi-fluorescence microscopy utilizes two types of light sources as to provide adequate fluorescence for imaging. There are high-pressure mercury vapors (lamp that produce light in ultraviolent to short blue wavelength) and xenon (lamp that produce light across the visible spectrum or ultraviolent light).

The range of fluorescence microscopic is limited by spectral sensitivity of human eye that is restricted to range between 400-700 nm. As a result, far-red fluorochromes such as CY5 can only be recorded by using camera or other detectors. For digital imaging, charge coupled device (CCD) cameras are most frequently as compare to the other camera

such as tube-type cameras. Multiple-beam microscope has the ability readily capture images with an array detector, such as CCD camera system.

Epi-fluorescence microscope enables the observation of visible fluorescence opaque as well as transparent samples. The wavelength of light reaching the sample as apply excitation theory and then the light viewed through eyepieces (fluorescence) is regulated via different filter sets which are built up of excitation filter, a dichroic mirror and an emission filter (Dykstra & Reuss, 2003).

3.0 MATERIALS AND METHODS

3.1 Sample collection and cell isolation

Plankton samples were collected from Samariang River and Santubong estuary, Kuching, Sarawak (Figure 3.1) by using a 20 µm-mesh plankton net. Sampling was carried out fortnightly from September 2011 until April 2012. Live samples were brought back to the laboratory for cell isolation.

Figure 3.1: Map showing Santubong and Samariang sampling site

Cells of interest were isolated using micropipetting technique (Hoshaw & Rosowski, 1973). Targeted dinoflagellate cells were located using inverted microscope with magnification up to 200×. Few drops of filter-sterile seawater were placed on a glass slide. Cell was picked up and rinsed several times in the seawater droplets before transferring into a tissue culture plate. Cells were kept in light-temperature controlled growth chamber at 25^{o}C under 12:12 h light: dark cycle, a photon flux of 140 µmol photon