Faculty of Resource Science and Technology

APPLICATION OF SOIL FLOCCULATION IN REMOVAL OF TOXIC *ALEXANDRIUM MINUTUM* AND *PROTOCERATIUM RETICULATUM* (DINOPHYCEAE)

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Application of Soil Flocculation in Removal of Toxic *Alexandrium minutum* and *Protoceratium reticulatum* (Dinophyceae)

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This project is submitted in partial fulfillment of the requirement for the degree for Bachelor of Science with Honours (Aquatic Resource Science and Management)

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Declaration

I hereby declare that the thesis is based on my original work except for quotation and citation, which have been duly acknowledged. I also declare that no portion of this dissertation has been submitted for any other degree of Unimas or any other university or other institutions.

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List of Abbreviations

c	Cingulum
CPD	Critical point drying
HABs	Harmful Algal Blooms
LM	Light microscopy
Po	Apical pore plate
PSP	Paralytic shellfish poisoning
SEM	Scanning electron microscopy
S.a.	Anterior sulcal plate
S.d.a.	Right anterior lateral plate
S.d.p.	Right posterior lateral plate
S.p.	Posterior sulcal plate
S.s.a.	Left anterior lateral plate
S.s.p.	Left posterior lateral plate
STX	Saxitoxin
YTX	Yessotoxin
vp	Ventral pore
1′	First apical plate
2′	Second apical plate
3′	Third apical plate
1′′	First precingular plate
2′′	Second precingular plate
3′′	Third precingular plate
4′′	Forth precingular plate
5’’	Fifth precingular plate
6′′	Sixth precingular plate
1′′′	First postcingular plate
2′′′	Second postcingular plate
3′′′	Third postcingular plate
4′′′	Forth postcingular plate
5′′′	Fifth postcingular plate
6′′′	Sixth postcingular plate
1′′′′	First antapical plate
1p	Posterior intercalary plate

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ABSTRACT

Aquacultures industries in Malaysia suffered great losses in recent year due to the occurrence of harmful algal blooms (HABs), partly due to absent of mitigation strategies in dealing with these events. Clay flocculation has been known to effective in HABs mitigation and widely applied in East Asian countries. However, efficiency of this mitigation method differed due to the properties of soil material. The objective of this study was to assess the efficiency of two local soils in removal of several known harmful dinoflagellates, i.e. *Alexandrium minutum* and *Protoceratium reticulatum*. Both soil samples used in this study composed mainly clay (45-68%), followed by sand (15-22%) and silt (17-33%). Removal efficiency increased significantly ($p < 0.05$) with higher clay concentration, but differed in the two species and soils tested. Soil sample B showed high efficiency in removal of *A. minutum* cells with total removal (Bmax ~ 100%) achieved at concentration as low as 0.2 g. For *P. reticulatum*, maximum removal of cells by both soils was achieved within 2 h. This study clearly showed the potential of local soils in mitigation of HABs. Field trial of clay dispersal during the blooms event is essential to verify the outcome of this study.

Key words: Harmful algal blooms (HABs), *Alexandrium minutum*, *Protoceratium reticulatum*, Clay flocculation,

ABSTRAK

Industri akuakultur di Malaysia telah mengalami kerugian yang besar pada tahun kebelakangan ini disebabkan oleh ledakan alga merbahaya (HABs) yang meningkat dan ketiadaan strategi mitigasi untuk menangani masalah ini. Pemflokulatan tanah telah digunakan dalam menangani masalah algal berbahaya dengan berkesan dan secara luas di negara-negara Asia Timur. Namun, kecekapan kaedah mitigasi adalah berbeza kerana cirri-ciri tanah. Tujuan kajian ini ialah untuk menganalisa kecekapan bagi kedua-dua tanah tempatan dalam pengawalan dinoflagellata berbahaya iaitu Alexandrium minutum dan Protoceratium reticulatum. Kedua-dua tanah yang digunakan dalam kajian ini mengandungi tanah liat (45-68%), diikuti oleh pasir (15-22%) dan lumpur (17-33%). Kecekapan dalam mengawal sel telah meningkat ($p < 0.05$) dengan menggunakan kepekatan tanah yang tinggi tetapi berbeza dengan kedua-dua spesies dan tanah yang diuji. Tanah B telah menunjukkan kecekapan yang tinggi dalam pengawalan sel A. minutum dengan jumlah pengawalan (Bmax ~ 100%) yang dicapai pada berat yang serendah 0.2 g. Untuk sel P. reticulatum, pengawalan maksimum sel oleh kedua-dua tanah ini adalah dalam waktu 2 jam. Penyelidikan ini telah jelas menunjukkan potensi bagi tanah tempatan untuk megawal algal berbahaya. Penyebaran tanah liat melalui percubaan lapangan semasa ledakan algal berbahaya adalah penting untuk mengesahkan hasil kajian ini.

Kata kunci: Ledakan algal merbahaya (HABs), Alexandrium minutum, Protoceratium reticulatum, Pemflokulatan tanah

1.0 Introduction

Microalgae play an important role in the aquatic food webs. Their organic material production support zooplankton and other small aquatic organisms. Filter-feeding organisms are also depend on phytoplankton as food sources (Paz et al., 2008). Virtually algal blooms are harmless; it benefits aquaculture and wild fisheries (Paz et al., 2008). High density of algae leads to the death of aquatic organisms due to hypoxia conditions (Paz et al., 2008). This causes major environmental problems and economic losses (Paz et al., 2008). Toxins produced by certain microalgae have an adverse effect to human health and shellfish industries (e.g. Hallegraff, 1993; Paz et al., 2008; Wiese et al., 2010). These toxic algae blooms are known as Harmful Algal Blooms (HABs).

According to Hallegraff (1993), the strategies or methods have risen to control HAB's directly due to its wide distribution and impacts. Different controlling methodologies have been proposed, tested and applied to disrupt or kill the HAB cells during blooms. The controlling agents covered the wide ranges of mechanisms, these included chemical, physical, biological or mechanical (Anderson et al., 2001). Perhaps the most promising controlling strategy is flocculation and sedimentation with clay (Beaulieu et al., 2005). This approach minimizes costs and environmental impacts, and maximizing its capability in removing cells from water column (Anderson, 1997; Boesch et al., 1997; Anderson et al., 2001; Sengco, 2001). Over the past 25 years, clays were used to remove harmful algae by different countries (Shirota, 1989; Yu et al., 1994; Bae et al., 1998). Some countries such as Australia (Atkins et al., 2001), Japan (Shirota, 1989), and South Korea (Choi et al., 1998) have applied clay dispersal to control HABs in the field.

In this study, two clonal cultures of harmful dinoflagellates, *Alexandrium minutum* and *Protoceratium reticulatum* were used in clay flocculation experiments. Both

dinoflagellates are toxin producers of saxitoxin (STX) and yessotoxin (YTX) respectively. Saxitoxin is a complex guanidine-based alkaloid with more than 30 identified analogues in nature (Llewellyn, 2006). STX disrupts the flow of ions through voltage gate sodium channels (Catterall, 1992; Cestele and Catterall, 2000). Currently, it is listed as a chemical weapon in Schedule 1 of the Chemical Weapons Convention due to its acute toxicity (Llewellyn, 2006). The consumption of seafood containing STX by human can lead to paralytic shellfish poisoning (PSP) cases. The PSP events were considered problem that happened throughout the world (Anderson et al., 1994; Hallegraff et al., 1995). There is no known antidote for PSP toxins to date (Anderson et al., 2001).

YTX is a chiral molecule with high polarity (Caron et al., 2010). To date, nearly 100 analogs of YTX have been recognized (Ciminiello et al., 1998, 2000, 2001; Daiguji et al., 1998; Satake et al., 1997, 1999; Miles et al., 2004, 2005a, b, 2006; Paz et al., 2006). It is produced by the dinoflagellates *Protoceratium reticulatum*, *Gonyaulax spinifera* and *Lingulodinium polyedrum* (Paz et al., 2008). It is one of the major concerns in shellfish aquaculture (Guerrini et al., 2007) and widely distributed throughout the world (Satake et al., 1997, 1999; Ciminiello et al., 2003; Paz et al., 2007).

To date, study on clay flocculation on HABs in the country has little attention paid to it. As such, the ability of local soil to remove harmful algal cells is not known. Thus, the main aim of the present study is to investigate a variety of locally available soils on their removal ability against harmful dinoflgellates, *Alexandrium minutum* and *Protoceratium reticulatum* found in the Malaysian waters. The study was performed under controlled laboratory conditions with two clonal cultures of *A. minutum* and *P. reticulatum* used. The specific objectives are as below:

1. to establish clonal cultures of dinoflagellate species from Kuching waters;

2. to investigate the morphology of cultures by using light and scanning electron microscopy (LM & SEM);
3. to determine the properties of local soils;
4. to compare the efficiency of local soils in removal of *A. minutum* and *P. reticulatum*.

2.0 Literature review

2.1 Saxitoxins (STXs) and the toxin-producing dinoflagellates

In Malaysia waters, eight marine dinoflagellates species were known to STX producer. They were *Alexandrium affine*, *Alexandrium leei*, *Alexandrium minutum*, *Alexandrium tamarense*, *Alexandrium tamiyavanichii*, *Pyrodinium bahamense var compressum*, (Usup et al., 2002), *Alexandrium peruvianum* and *Alexandrium taylori* (Lim et al., 2005). Victim who consumed the seafood that contaminated with STX may had minor symptoms within 30 min such as diarrhea, vomiting, tingling sensation of the tips and face, salivation, dizziness, headache, intense thirst and perspiration and stomach cramp (Llewellyn, 2006). A high dose STX if consumed can result in death within hours due to muscular paralysis and difficult in respiratory followed by complete respiratory arrest (Caron et al., 2010).

2.1.1 *Alexandrium minutum*

Alexandrium minutum is a small dinoflagellate found for the first time in the red tide at *Alexandrium* harbor (Halim, 1960). It is widely distributed in global (Hallegraeff et al., 1988; Maguer et al., 2000; Yoshida et al., 2000; Daly Yahia Kefi et al., 2001a, b; Usup et al., 2002). The *Alexandrium minutum* was originally found in Tumpat, Peninsular Malaysia in September 2001 (Lim et al., 2004).

2.2 Yessotoxins (YTXs) and the toxin-producing dinoflagellates

YTX was originally isolated from the digestive gland of the scallops (*Patinopecten yessoensis*) which collected in Mutsu Bay, Japan in 1986 (Murata et al., 1987). Biological targets of YTX were the immune system, lysosomes and thymus (Franchini et al., 2004; Malagoli et al., 2006) and other reports were indicated cardiotoxic effects (Terao et al., 1990; Ogino et al., 1997; Aune et al., 2002). YTX was excluded from the DSP group recently because it does not lead to diarrhea (Tubaro et al., 2003; Ogino et al., 1997) and does not inhibit protein phosphatases (Ogino et al., 1997).

2.2.1 *Protoceratium reticulatum*

Protoceratium reticulatum is alike a polyhedron with heavy theca which made up of several plates (Paz et al., 2008). The theca has prominent reticulation with pores (Paz et al., 2008). The *Protoceratium reticulatum* has been found in very different locations so it is reasonable to believe that it is able to grow within a wide range of temperature conditions, salinities, light, pH and nutrient conditions (Rodríguez et al., 2007). For instance, *P. reticulatum* from different places in Japan (Satake et al., 1999; Eiki et al., 2005; Suzuki et al., 2007), in Spain (Paz et al., 2004; Paz et al., 2007), in the Adriatic Sea in Italy (Ciminiello et al., 2003), in Norway (Samdal et al., 2004) and in Nova Scotia, Canada (Stobo et al., 2003).

2.3 Mitigation approaches in HABs

For the chemical control, copper sulfate was adopted to mitigate the Florida harmful algal cells in 1957 (Rounsefell and Evans, 1958). But, it was only effective in small area and was too slow to be applying on a major outbreak of algal blooms (Anderson et al., 2001).

According to Rounsefell and Evans (1958), the copper dusting was too expensive for used in major outbreak of red tides. Besides that, the use of copper may bring the other negative impact to the ecological life such as lethal to the aquatic plants and animals (Anderson et al., 2001). On the other hand, McCoy and Martin (1977) suggested aponin used as a mitigate agent for toxic *Gymnodinium breve* blooms. The aponin caused breakage of *G. breve* within 4-10 days. However, the usages of aponin can leads to many objections (Steidinger, 1983). In general, the chemical control undesired to be used as it may cause mortality to other organisms in environment (Anderson, 2009).

For the physical control, it includes the skimming of surface water and ultrasonic destruction of HAB cells. Shirota (1989) describes that a study in collecting HAB planktons by using pressure flotation separation equipment that conducted in Japan. The HAB algae are theoretically scavenged by fine bubbles released into the water (Shirota, 1989). The cells were removed by a skimmer when they float to the sea surface (Shirota, 1989). However, a prototype of this equipment was tested in 1973 but the results were not encouraging (Shirota, 1989). Next, the usage of ultrasound to destruct of HAB cell was abandoned (Shirota, 1989) because it is ineffective at low cell concentrations and water depth level is less than 50 cm (Shirota, 1989).

Clay is an example of mechanical control used effectively in HABs. They are abundant natural materials and available with low cost (Sengco, 2009). Many laboratory was applied and with some field demonstrations in several countries (Yu et al., 1994; Sengco et al., 2001; Pierce et al., 2004; Beaulieu et al., 2005). The environmental impacts of clay flocculation are generally positive although there have negative effects (Pan et al., 2011). Clay particles flocculate with algal cells caused rapid sedimentation of algal cells (Beaulieu et al., 2005). Clay flocs entrain algal cells and the loss of algal cell motility due to physical-chemical interaction with clay particles (Beaulieu et al., 2005).

For biological control, the environmental-friendly feature helps them to be applied in mitigation of HABs (Jeong et al., 2000). Bacteria are ubiquitous and abundant in marine environment (Yu et al., 2005). Natural assemblage of bacteria had used in controlling of HABs (Suttle, 1994). For example, the bacteria of *Cytophaga* sp. were found in a *Chattonella* bloom in the Harima Sea (Furuki and Kobayashi, 1991). Besides that, parasite has potential to become biological control agent for HABs due to it has highly virulent nature (Kim, 1998). There are a variety of different parasite species which can infect marine organisms (Anderson et al., 2001). Some of them are dinoflagellates such as *Amoebophrya ceratii* which is known as intracellular parasite of free living dinoflagellates (Taylor, 1968; Nishitani et al., 1984). In addition, viruses are very abundant in coastal seawater and essential to the dynamics of marine ecosystems (Fuhrman and Suttle, 1993). They could infect algae in red or brown tide bloom. For example, *Emiliania huxleyi* blooms were collapsed due to the breakout of viruses (Bratbak et al., 1993).

In addition, some workers have proposed use *Nannochloris* sp. to control the *Gymnodinium breve* blooms in Florida (Taft and Martin, 1986). This strategy may be exceedingly difficult to evaluate due to the lack of experimental data. Moreover, a zooplankton that can consider as a biological control agent due to the zooplankton graze them as food (Anderson et al., 2001). According to Shirota (1989), he indicates that the Japanese use zooplankton like *Acartia clausi* in controlling red tide blooms. But, it is unrealistic if respect to cost, space, and facilities (Anderson et al., 2001). Bivalves is also can be used as biological mitigating agent in controlling the biomass of phytoplankton in water column (Officer et al., 1982; Alpine and Cloern, 1992). Polyculture of bivalves in shallow shrimp ponds can control the blooms of algae and prevent the shrimp mortalities cases occur in areas of extensive shrimp culture such as the Bohai region in China (Anderson et al., 2001).

3.0 Materials and methods

3.1 Plankton Samples

Plankton samples were collected by using 20 μm mesh size plankton net. The plankton samples were collected fortnightly from August to December 2010 during high tide in Santubong and Samariang estuaries (Figure 3.1). The live plankton samples were used for cell isolation to establish clonal cultures.

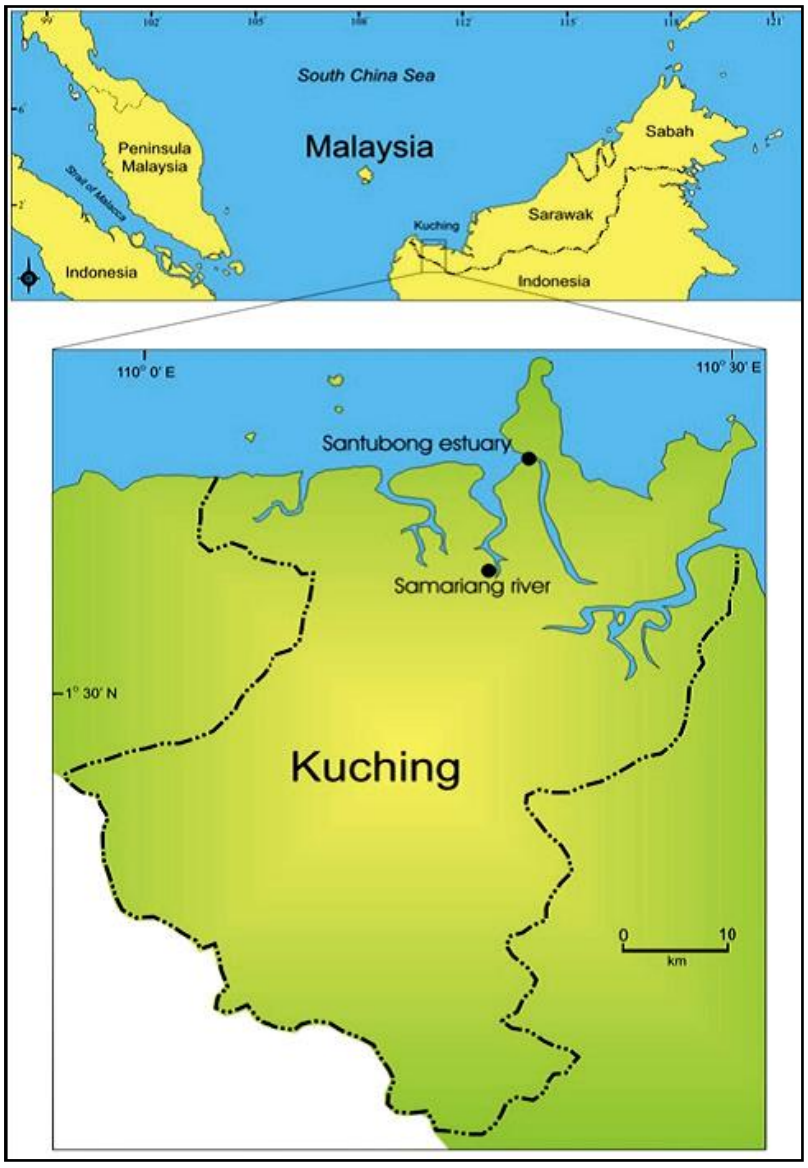

Figure 3.1: Map showing sampling sites at Santubong and Samariang estuaries (black fill circles) (Hilaluddin et al., 2010).

3.2 Establishment of clonal culture

Live specimens of dinoflagellates were examined by an Olympus IX71 microscope (Tokyo, Japan) with 40× magnifications. Cell isolation was carried out by using the micropipetting technique (Hoshaw and Rosowski, 1973). The Pasteur pipette was used to isolate individual cells. The cells then were placed in a 96 well tissue culture plate containing filter-sterile seawater. Cell divisions were examined daily until cell density achieved roughly 100 cells and cells were transferred into test tubes. The clonal cultures established were maintained in SW II (Iwasaki, 1961) or ES-DK (Kokinos and Anderson, 1995) medium at 25^{o}C under a light intensity of 70 μmol photons m^{-2} s^{-1} on a 12:12 hr light: dark photocycle.

3.3 Species identification

Identification of dinoflagellate species were carried out by using light microscopy (LM) and scanning electron microscopy (SEM). For LM, live cells were observed using an Olympus IX51 compound microscope (Tokyo, Japan) under 400× magnifications. The chloroplast arrangement of cells was observed by using epi-fluorescence microscopy with UV filter (Tokyo, Japan).

For SEM, cells were fixed with 2 % glutaraldehyde for one hour and 1% osmium tetroxide (OsO_4) for one hour at room temperature. The cells then were dehydrated in a graded series of ethyl alcohol concentration and substituted with the intermedium, amyl acetate. The cells were underwent critical point drying (CPD), followed by coating with gold-palladium by using the JEOL sputter coater (JFC-1600, Japan) before observing under JEOL SEM (JSM-6390LA, Japan).

3.4 Soil particle size analysis (Buchnan, 1984)

Two soil samples were collected in UNIMAS. The soil samples were ground by hand with a mortar and pestle (Figure 3.2). Soil powders were preheated in the oven at 60 ^{o}C before conducting soil particle size analysis. There have three steps were found in the soil particle size analysis: pretreatment, initial splitting of silt-clay fraction and pipette method (Figure 3.2). During pretreatment, 40 g of dried sediment were weighted. Then the sediment was transferred to a beaker. A total of 100 mL 6% hydrogen peroxide was added to the sediment sample and sealed with parafilm left it overnight (Figure 3.2). The content was filtered through a filter paper (Whatman 50). The sediment sample was rinsed thoroughly with distilled water. Then, the filtered sediment sample was subsequently back-washed with roughly 200 to 300 mL of distilled water into a beaker contained 10 mL of sodium hexametaphosphate solution (6.2 g/L) (Figure 3.2). The sample was stirred for 15 min, sealed with parafilm and left overnight (Figure 3.2).

To initial splitting of silt-clay fraction, the sediment was stirred again for 15 min. The sediment was then transferred into a 63 μm sieve which placed in a big pail (Figure 3.2). The distilled water was added about 300-400 mL which is sufficient to flood the sieve surface. However, the volume of the water in the pail should not be more than 1 L. The sediment in the sieve was agitated and puddled in the pail of water until most all of the fraction had passed through the sieve.

The weight of the Petri dish was measured before transferring the sediment sample. The soil fraction that left on the sieve was transferred into the Petri dish and rinsed with distilled water until no soil fraction was left on the sieve (Figure 3.2). The Petri dish was dried in oven at 80 ^{o}C. To obtain the sand weight, the weight of the sample and Petri dish was subtracted with the pre-weight of the Petri dish.

For the pipette method, it was took place at 20 ^{o}C. The materials in the pail were rinsed into the liter cylinder with distilled water until 1 L (Figure 3.2). The cylinder was covered with lid, then shaked and turned to suspend the sediment evenly throughout the water column (Figure 3.2). To get the first pipette sample, the cylinder placed upright and immediately withdrew a 20 mL pipette sample from a depth of 20 cm. For the second pipette sample, a few seconds before the expiry of 7′ 44′′ according to the Time Table of Pipette Withdrawal (Appendix C), the pipette tip was lowered to a depth of 10 cm to obtain 20 mL of sample. Then the third pipette sample was obtained after a time interval of 2 h 3 min. All the samples of the first, second and third pipette must be transferred into separate crystallizing dish where the pre-weight of the dish was obtained. The samples were dried in an oven at 100 ^{o}C and weighted.

Different particle size of soil components were calculated based on the calculation as below:

(Sample + Petri dish) – pre-weighted Petri dish = Pipette sample's weight

Silt (62-15.6 µm) = (1^{st} pipette's weight × 50) – (2^{nd} pipette's weight × 50)

Silt (15.6-3.9 µm) = (2^{nd} pipette's weight × 50) – (3^{rd} pipette's weight × 50)

Clay (< 3.9 µm) = 3^{rd} pipette's weight × 50

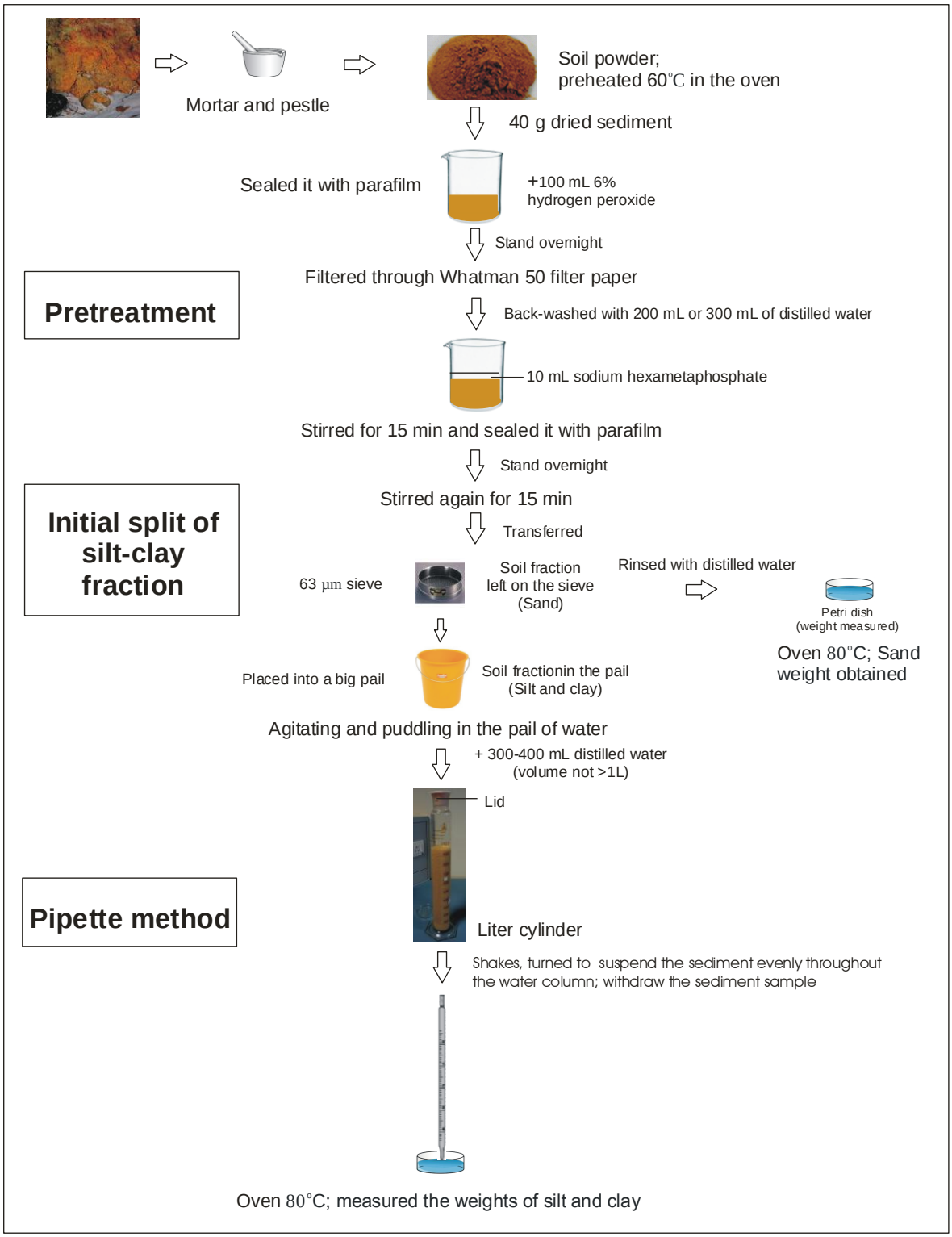

Figure 3.2 The flow chart procedure of the soil particle size analysis experiment.

3.5 Soil flocculation experiment on *Alexandrium minutum* and *Protoceratium reticulatum*

Clonal cultures of *A. minutum* and *P. reticulatum* were maintained in 1 L conical flask with SWII at salinity of 15 PSU and ES-DK medium at salinity of 30 PSU. The cultures were grown in the light-temperature controlled growth incubator at 25 ^{o}C under 70 μmol photons m^{-2} s^{-1} on a 12:12 hr light: dark photocycle. Cell growths were monitored by microscopic cell counts. Experiments were performed with cultures in early to mid-exponential growth only. The initial concentration of cells was subsampled into microcentrifuge tubes and preserved with a few drops of Lugol's solution (Figure 3.3). The fixed samples were transferred to a 1-mL Sedgewick-Rafter counting chamber for cell counting under light microscope at 40× (Figure 3.3). Cultures with cell concentrations of 30,000 to 50,000 cells/mL for *A. minutum* and 20,000 to 30,000 cells/mL for *P. reticulatum* were used for the experiments.

In the clay experiments, a range of soil loadings were applied (0.2, 0.4, 0.8, 1.0, 1.2, 1.8, and 2.0 g). All treatments were run in triplicates in a test tube format. A total of 25 mL of culture cells were used in each treatment. Immediately after soil addition, the suspensions were mixed briefly by hand until the soil was thoroughly dispersed. The soil-cell resuspension then was allowed to flocculate under quiescent condition (Figure 3.4). The flocculation experiments encompassed three time series (30 min, 2 h and 12 h) (Figure 3.3). Triplicate subsamples of 1 mL culture medium were collected and transferred to the 1.5 mL microcentrifuge tubes (Figure 3.3). The cells were fixed with a few drops of Lugol's solution and counted under light microscope (Figure 3.3). The soil flocculation experiments were repeated with other soil samples.