DIATOM AS FORENSIC INDICATOR: IN VIVO STUDIES OF INGESTED DIATOMS FOR INTERPRETING DIATOM TEST RESULTS

by

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ABSTRAK

Diatom seringkali dikaitkan dengan diagnosis kes lemas dalam bidang diatomologi forensik. Penemuan diatom di dalam tubuh individu yang mati bukan disebabkan lemas telah mencabar ujian diatom sebagai indikator lemas ante-mortem. Kehadiran diatom dalam sumber makanan dikatakan punca kepada kebarangkalian diatom boleh memasuki badan melalui laluan gastrointestinal. Dalam kajian ini tikus Sprague – Dawley (SD) telah digunakan sebagai model haiwan bagi memahami nasib diatom yang ditelan sekaligus menilai kesahihan ujian diatom. Sejumlah 60 tikus SD dibahagi sama rata kepada dua kumpulan, control dan eksperimen. Kumpulan ini dibahagikan lagi kepada enam set lima ekor tikus. Kumpulan eksperimen diberi makan cecair diatom dos bermeter secara intragastrik tiga hari dalam seminggu. Setiap dua bulan, satu set dari kumpulan kontrol dan eksperimen akan dikorbankan dan organ dalaman diambil. Organ yang diambil akan dihadamkan menggunakan asid nitrik pekat dan setelah beberapa kali disentrifugasi, sedimen yang terhasil akan diperiksa secara mikroskopik. Jumlah diatom sempurna dan pecahan diatom yang dijumpai dikira dan direkodkan. Diatom dijumpai di dalam organ hati, jantung, otak, buah pinggang dan paru-paru hanya dari kumpulan

eksperimen dan jumlah diatom yang dijumpai adalah sangat kecil iaitu maksima adalah 14 diatom yang dijumpai dalam organ hati set ke enam kumpulan eksperimen. Penemuan ini membuktikan bahawa diatom yang dimakan boleh masuk ke dalam peredaran sistemik melalui dinding gastrointestinal. Penemuan daripada kajian ini memerlukan anjakan paradigma dalam penggunaan diatom sebagai indikator penentuan lemas ante-mortem apabila terjumpanya frustul di dalam organ individu yang ditemui mati di dalam air.

DIATOM AS FORENSIC INDICATOR: IN VIVO STUDIES OF INGESTED DIATOMS FOR INTERPRETING DIATOM TEST RESULTS

ABSTRACT

Diatoms have constantly been associated with the diagnosis of drowning in the field of forensic diatomology. The finding of diatoms in the bodies of non-drowned deceased has challenged the reliability of the diatom test as indicative of ante-mortem drowning. The availability of diatoms in the foodstuffs has been attributed to the possibility of diatoms to entering the systemic circulation via the gastrointestinal tract. Sprague – Dawley (SD) rats were used as animal models to understand the fate of ingested diatoms inside the human body while evaluating the reliability of the diatom test. A total of 60 SD-rats were equally divided into two groups, control and experimental, and each group was again divided into six batches of five rats in a batch. Three times a week, the experimental group was intragastrically administered with metered-doses diatom frustules from a stock solution. Every two months one batch from control and one batch from experimental groups were sacrificed and the internal organs were harvested. The harvested organs were digested with concentrated nitric acid and after repeated centrifugation, the resulting sediment was microscopically examined. The number of diatoms, intact and fragments, recovered from the digested organs was counted and recorded. Diatoms were recovered from the liver, heart, brain, kidneys and lungs from the animals of the experimental group alone although the number was small, the maximum being 14 from the liver from animals of the sixth batch. The recovery of

diatoms from the internal organs of the experimental animals proved that the ingested diatoms can enter into the systemic circulation via gastrointestinal lining. The above finding of this research necessitates a paradigm shift in treating diatoms as indicators of ante-mortem drowning when frustules are recovered from the organs of dead bodies found in water.

CHAPTER I

INTRODUCTION

1.1 INTRODUCTION

Drowning can be defined as death due to full or partial submersion in a fluid. While the precise mechanism of drowning is a complex event modified by the medium involved and other factors, in most cases, inhalation of fluid resulting in respiratory tract obstruction is the essential cause of death (Moar, 1983; Krstic *et al.*, 2002).

Once decomposition sets in, death by drowning is difficult to determine and is often diagnosed by eliminating all other potential causes of death. The presence of aquatic diatoms in a cadaver has long been held by some to be a clear indicator of death by drowning. Research into the presence of diatoms in bone marrow, lungs, liver, spleen, kidney, and brain tissue has led to the development of the diatom test, a direct screening test for drowning, whereby the presence of diatoms can be verified and analyzed both quantitatively and qualitatively (Rohn & Frade, 2006).

According to many authors (Hendey, 1980; Auer & Mottonen, 1988; Pollanen, 1998b; Yange *et al.*, 1999; Piette & De Letter, 2006; Kumar *et al.*, 2011), the diatom examination is a reliable and practical method that is used to identify

whether a deceased drowned before his/her death or was thrown into water after his/her death. Specifically when a corpse is heavily decomposed or skeletonized, diatom test forms the only method (Yange *et al.*, 1999).

The diatom test is based on the inhalation of the diatoms suspended in the fluid medium in the process of drowning. Diatoms can enter the ruptured pulmonary alveolar capillaries and reach the organs in the greater circulation (Lunetta *et al.*, 1998; Sitthiwong *et al.*, 2011). There has been indication of diatoms entry via pulmonary lining through passive absorption. Due to low salinity of the water, precipitous absorption into the circulation takes place (Spitz & Fischer, 1980).

However, questions on the validity of diatom test have been raised by some groups of researchers (Peabody, 1980; Foged, 1983; Yen and Jayaprakash, 2007; Giancamillo *et al.*, 2010; Ago *et al.*, 2011). Controversies raised regarding the reliability of diatom method in determining death by drowning lead to the beginning of scientific debate on validity of the diatom test (Peabody, 1980; Krstic *et al.*, 2002). The principal grounds for the criticism on diatom test lies in the fact that diatoms have also been found in some non-drowning bodies (Foged, 1983; Calder, 1984; Pachar and Cameron, 1993; Pollanen, 1998b; Seo *et al.*, 2013) while they were found absent in bodies with known history of death by drowning (Timperman, 1972; Azparren *et al.*, 1998).

The presence of diatoms in living persons has been attributed to sources such as air (Spitz & Schneider, 1964; Dayan *et al.*, 1978), food (Pachar & Cameron, 1993; Krstic *et al.*, 2002; Yen & Jayaprakash, 2007) and also other daily products

(Peabody, 1980). Although there have been indications on the possible entry of diatom frustules into the human body via food and drinks (Peabody, 1980; Krstic *et al*., 2002; Yen & Jayaprakash, 2007), how the frustules can penetrate through the gastrointestinal wall and reach the bloodstream as well as the duration of their persistence in the organs of the body are still mystery (Yen & Jayaprakash, 2007).

The research reported in this thesis was designed to study the fate of metered doses of diatom periodically fed to Sprague Dawley (SD) rats (*Rattus norvegicus*) that were reared for a period of one year. Over the above period, batches of experimental and control rats were sacrificed once in two months. The visceral organs were preserved and acid digested for extracting diatom frustules in the organs. Thus the study focused on the presence of the ingested diatom frustules in the organs to draw the inference that the fed diatoms cross the gastrointestinal lining and enter the systemic circulation and thereafter reach the internal organs of the body.

1.2 STATEMENT OF PROBLEM

Diatom test had once served as a “gold standard” for the diagnosis of drowning. At one point of time, the association between diatoms and drowning was considered so strong, to the extent that the recovery of only one diatom frustule from the bone marrow was considered a positive indication of death by drowning. However, further research by various independent researchers over a period of time revealed contradicting findings. These findings initiated a so called “war on diatoms”.

Criticisms on the reliability of diatom test for drowning had been attributed to a number of reasons, the following being more important. First, diatoms have been found in the internal organs of non-drowned deceased. Second, the absence of diatoms in internal organs of drowned deceased. Third, dissimilarity of diatom species found in internal organs of drowned subjects compared to the diatom species in the drowning medium and finally the reports on the prevalence of diatoms in food, water, air and the environment.

The findings of diatoms in the internal organs of non-drowned deceased and the absence of diatoms in organs of known drowned deceased confused the interpretation of death by drowning especially when the body is decomposed wherein the physical signs and symptoms of drowning are lost and no longer visible. These findings led to questioning the validity and reliability of diatoms as indicator of drowning. A recent report on the prevalence of diatoms in foodstuffs has also added merit to the above criticisms.

Since the past six decades the controversies regarding the use of diatom test for diagnosis of drowning are being debated among the medico-legal forensic practitioners all over the world. A series of questions had been raised by the scholars with regard to the above matter. The issues include the following:

a) Why diatoms were absent in the bodies of known drowned and dead individuals?
b) Why diatoms were also found in the bodies of non-drowned victims?
c) Why is that, the species of diatoms found in the drowned deceased were not similar to the species of diatoms found in the drowning medium?
d) Is there any possibility that diatoms from the air, water, food and other materials in day-to-day use can enter the human body even when drowning does not occur?
e) How long would diatoms persist once they enter the organs in human body?
f) Can diatoms that are ingested along with the foodstuffs enter the systemic circulation through the gastro intestinal lining and reach the visceral organs?

The last of the issues listed above was chosen as the problem to be tested during this study.

1.3 RESEARCH OBJECTIVES

1.3.1 General Objectives:

To explore the fate of ingested diatoms for interpreting the cause of death by drowning.

1.3.2 Specific Objectives:

1. To determine whether the ingested diatoms enter into the systemic circulation.
2. To study the pathway and accumulation of ingested diatoms in the various organs.
3. To evaluate the reliability of diatom test as forensic indicator of drowning based on the above two objectives.

1.3.3 Scope of the study

This study used SD rats as representative of human to obtain evidence whether the diatoms that are orally ingested over a period of one year can penetrate the wall of the gastrointestinal tract and enter into blood circulation and finally reach the major organs including the bone marrow. The diatoms that were used for the preparation of stock solution were dead diatoms frustules since only dead frustules are detected during diatom test. This study focused on the quantity and morphological similarity of the ingested diatom frustules vis-à-vis the diatom frustules that were periodically recovered from the internal organs of experimental and control rats that were sacrificed.

CHAPTER II

LITERATURE REVIEW

2.1 DIATOMS

2.1.1 Biology of Diatoms

Diatoms are eukaryoticunicellular microorganisms,belonging to major aquatic vegetation known as algae. Algae can be divided into nine phyla namely, *Cyanophyta, Chlorophyta, Crysophyta, Euglenophyta, Crytophyta, Pyrophyta, Rhodophyta, Phaeophyta*and*Chloromonadophyta.Crysophyta* can be divided into four classes;*Xanthophyceae, Chrysophyceae, Haptophyceae*and *Bacillariophyceae.* Diatoms, belongs to the class of *Bacillariophyceae* (Shamsudin, 1991). Diatoms exist in different shapes and their sizes vary from 1 to 500 µm (Singh *et al.*, 2006). They have inorganic cell walls known as frustules which aremade up of silica oxide (SiO_2). The frustules consist of two parts known as valves (Round *et al.*, 1990).

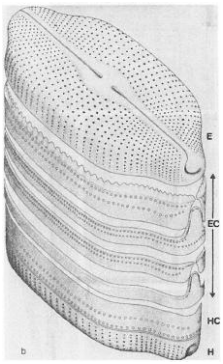

Figure 2.1 Composition of diatom frustules (Source: Round et.al., 1990)

Diatoms are pigmented and photosynthetic and are a part of the planktonic communities (Round *et al.*, 1990). Planktons are known as the major primary producers which form the food for many aquatic organisms that are filter feeder. Shamsudin (1991) quoted that Alan and Nelson in 1910 succeeded in reproducing diatom species in a continuous culture and used them in the breeding of echinoderms, molluscs and copepods which are known filter-feeders. There are over 100 000 recognized diatom species, with many more being constantly identified (Pollanen, 1998a; Punia, 2011). Generally, diatoms are divided into two orders based on shape; the pennate diatoms which are bilaterally symmetric and the centric diatoms which are radially symmetric. Round et. al. (1990) further classified diatoms into three classes; centric diatom, pennate diatoms without a raphe and pennate diatoms with a raphe. Diatoms are also classified as pinnate, centric and chains diatom (Vargas *et al.*, 2007) however this classification is not commonly used.

2.1.2 Reproduction

Reproduction in diatoms can be divided into two categories namely sexual reproduction and vegetative multiplication (asexual reproduction).Most diatom species reproduce asexually by means of dividing the valves to create more diatoms. The separated valves will serve as moulds for the production of yet another new set of valves. In this type of reproduction process, the sizes of the new generations will become smaller and smaller as compared to the parent generations. This is due to the newly synthesize valves being formed within confines of the parent frustules. It has been cautioned that the valve morphology may appear different during the division process and thus confuse taxonomic identification (Round *et al.*, 1990).

When the cells have become smaller following a phase of vegetative multiplication, some diatom species will revert to sexual reproduction by means of auxospore formation to restore balance of diatom sizes in the abundance (Pouličkova, 2008). In some species the auxospore formation are known as vegetative enlargement (Round *et al.*, 1990).

2.1.3 Prevalence of diatoms

Diatoms have been found prevalent in the environment. In the water, diatoms can be found roaming around at the pelagic level especially those of motile species while some can be found laying on the benthic level making up the water bed (Yallop & Anesio, 2010). Some species are suitable to live in fresh water environment and some are adapted to suit the salinity of the saltwater (Shamsudin, 1991) and some species are able to live in the brackish water of the river mouth. Diatoms have also been found in soil (Peabody, 1977; Kakizaki *et al.*, 2011), sediment and surfaces of rocks (Shamsudin, 1991). Diatoms in the air have been attributed to findings of diatoms in the filtrate of air filter proving their prevalence in the air (Spitz & Schneider, 1964). Certain foodstuffs such as vegetables, fruits and seafood have been shown to contain diatoms. The availability of diatoms in vegetables and fruits has been attributed to the soil-species diatoms, while non-vegetarian foodstuffs such as filter feeders had been attributed to the water-species diatoms (Yen & Jayaprakash, 2007). It has been estimated that in 50 g of prawn and *kerang,* there are a total of 8,360 and 29,054 diatoms respectively (Yen & Jayaprakash, 2007).

Apart from their natural abundance, diatoms are also commercially available as "Diatomaceous Earth" (DE). DE is fossilized diatoms harvested off the water bed and has been popularly used as filtration material in water filtration systems (Modell *et al.*, 1999) and as abrasive in metal industries (Dolley, 2000). Food grade DE has also been traditionally prescribed to naturally kill bed bugs and control pest infestation without harming the humans as DE is non-chemical. DE mechanically acts by grazing in between the scale layers of the bugs and removing the scales that form the protective layer which eventually causing the bugs to die off dehydration (Quarles, 1992). Authors have indicated that diatoms used in such items as abrasives may also gain entry into the human body via inhalation (Peabody, 1977).

2.1.4 Earlier studies in diatomology

Diatomology can be defined as a study of diatoms. Studies in the field of diatoms revolve around biology of diatoms, environmental studies and also drowning associated diatoms (Round *et al.*, 1990). Diatomologists study the biology of diatoms focusing particularly on species identification, nutrition, reproduction and ecological aspects of diatoms. Environmentalists use diatoms as indicators of water pollution. The higher the amount of diatoms found in a water body, the higher the pollution level in that particular water. This is because water polluted with certain trace elements, favours the growth of diatoms. Environmentalists also associated the diatoms with what is called "red-tide phenomena" in which the sea water appears red as certain diatom species bloom during a particular season. This area where the water appears red should be avoided from physical contact as the species that bloom causing the red-tide (*Pseudo-Nitschia sp.*) are said to be poisonous (Bates, 2004).

Studies on diatoms in the medico-legal field associate diatoms with drowning. The study on drowning associated diatoms acquired relevance while investigating decomposed suspected drowned individuals where the typical pathological signs and symptoms of drowning have been lost. These studies mostly report finding of diatoms in drowned (Auer & Mottonen, 1988; Pollanen, 1996; Pollanen, 1997; Pollanen, 1998b) and non-drowned cases (Foged, 1983; Calder, 1984) either as case studies or the hypothetical studies (Lunetta *et al.*, 1998; Giancamillo *et al.*, 2010), the advancement in extraction methodology (Ming *et al.*, 2007; Fucci, 2012; Hu *et al.*, 2013) and the issues on the reliability of diatom test for drowning (Spitz & Schneider, 1964; Peabody, 1980; Piette & De Letter, 2006; Giancamillo *et al.*, 2010). Issues in reliability of diatom test invariably included the possibility of diatoms entering via gastrointestinal tract as unsolved question (Spitz & Schneider, 1964; Foged, 1983; Yen & Jayaprakash, 2007; Giancamillo *et al.*, 2010). However, reports on specific experimental findings on the possibility of diatoms entering through the gastrointestinal tract do not appear in the literature.

2.2 DROWNING

Drowning is considered a rapid anoxial process due to obstruction of the airway with inhaled fluid, associated with acute electrolyte changes. Physical signs commonly evident in fresh dead bodies where death is due to ante-mortem freshwater drowning include cadaveric spasm due to instantaneous rigor mortis, the formation of mushroom-like stable white foam emerging from the nose, mouth and tracheobronchial tree, “washerwoman skin” and “kissing lungs” or hyperinflated lungs (Knight & Saukko, 2004; Farrugia & Ludes, 2011).

Champignon de mousse or the mushroom-like foam exuding from the nose of freshwater drowning victim is produced by the mixture of air, mucus and water in the presence of respiratory movement (Spitz & Fischer, 1980). The presence of foam is indicative that the victim was alive at the time of submersion. During the process of drowning, a large amount of water was inhaled. Since the water is hypotonic relative to blood, water move from the alveoli into the blood circulation through diffusion and osmosis. During this phase that particles like diatom passing through the alveolar-capillary interface before reaching internal organs (Farrugia & Ludes, 2011).

2.3 DIATOMS AS INDICATOR OF DROWNING

2.3.1 History

The use of diatoms for the diagnosis of drowning has been reported as early as the work of Guy (1861) who has observed penetration of water that carried with it mud and fragments of aquatic plants into trachea and bronchial tubes up to their most minute ramifications in a drowned subject. In 1896, Hofmann discovered diatoms in fluid from a dissected lung section. However, it was not until Ravenstorf in 1904, who credited Hofmann for the discovery, which began the association of diatoms and drowning when he attempted to correlate the diatoms found in the lungs with the diagnosis of drowning (Pollanen, 1998b).

2.3.2 Principle

Fundamental principle of the diatom testing in drowning cases investigation is based on postulation that diatoms are present in the water medium where the drowning took place. The inhalation of water into the lungs in the event of drowning leads to penetration of diatoms into alveolar system and the systemic blood stream Blood circulation being still active, the diatoms entering into the bloodstream are deposited in the internal organs such as brain, kidney as well as in the bone marrow of large bones (Krstic *et al.*, 2002; Lunetta & Modell, 2005). Bone marrow being encapsulated within the long bones and thus secured from contamination, many authors have suggested its use for diagnosing the presence of diatoms in decomposed bodies (Timperman, 1972; Hendey, 1980; Pollanen, 1998b). The finding of diatoms in the bone marrow (Pollanen, 1997; Gruspier & Pollanen, 2000) and other internal organs (Sidari *et al.*, 1999; Krstic *et al.*, 2002) indicates that the decedent was

breathing at the time of submersion into the water and died, at least in part, due to drowning (Pollanen, 1998a). In case the victim was already dead and the dead body was submerged or thrown into the water, the transport of diatom frustules to various organs is prevented due to lack of circulation (Krstic *et al.*, 2002). Testing for diatoms in the water in which the person drowned is useful, if it is found that the types of diatom evidence in the internal organs are similar to those in the water medium (Pollanen, 1998a).

2.3.3 Drowning associated diatoms

A positive diagnosis of death by drowning can be very difficult particularly when the body is in an advanced state of decomposition wherein the basic physical findings of drowning are rendered unrecognizable (Peabody, 1980; Knight & Saukko, 2004). When a person drowns in fresh water, diatoms are taken not only into the lungs but are also dispersed among other internal organs (Knight & Saukko, 2004). In the absence of other evidence, diatoms detected in the body tissues are the most reliable indicator of freshwater drowning (Ludes *et al.*, 1994). Even when there are only skeletal remains, diatoms can be detected in the bone marrow of drowned victims (Ludes *et al.*, 1999). In cases of suspected drowning, 20 diatoms from a 10 g of lung sample, or five complete diatoms from other organs, including bone marrow, are normally required for a positive diagnosis (Ludes *et al.*, 1999). In general, drowning associated diatoms are diatoms in the range 10 to 40 µm in sizes and are seldom longer than 50 µm. The diatoms typically recovered from the bone marrow extract are small pennate diatoms, having size of less than 30 µm (Pollanen, 1998b).

2.4 CRITICISMS ON THE RELIABILITY OF DIATOM TEST

The principal criticism on diatom test is the fact that diatoms have also been found in some non-drowned corpses (Piette & De Letter, 2006; Ming *et al.*, 2007; Lucci *et al.*, 2008) and the low positive rates of diatoms test among truly drowned bodies (Pollanen, 1996). Diatoms can be absent when macroscopical diagnosis of drowning is obvious such as when the victim died following a very short agony or the drowning occurred in water deprived of diatoms (Geertinger & Voigt, 1970; Krstic *et al.*, 2002; Piette & De Letter, 2006) or when diatoms density varied due to climatic influences (Piette & De Letter, 2006; Pogule *et al.*, 2007). On the other hand, diatoms have also been recovered from people who died due to causes other than drowning (Krstic *et al.*, 2002; Piette & De Letter, 2006) and there are cases where diatoms found in the drowned victims did not match the diatoms species found in the water where the victims were found (Giancamillo *et al.*, 2010; Ago *et al.*, 2011).

The presence of diatoms in non-drowned decedents and the dissimilarity between diatoms in the surrounding medium to those found in the victim's body indicate the possibility of diatoms being already prevalent in the victim's body prior to death. There has been suggestion in the literature postulating the possible sources of diatom in consumable goods of day-to-day use (Peabody, 1977). Certain diatom species are air borne (Krstic *et al.*, 2002), thus representing a continual source of diatom cells that might be inhaled (Dayan *et al.*, 1978). Food and water are suggested to be one of the major sources of diatoms in daily consumption (Pachar & Cameron, 1993; Krstic *et al.*, 2002; Yen & Jayaprakash, 2007; Giancamillo *et al.*,

2010). Meanwhile raw fruits and vegetables are suggested to be the sources of soil diatoms.

The proposition of diatoms being already prevalent in the human body is possibly based on the ingestion of foodstuffs containing diatoms (Yen & Jayaprakash, 2007). Pertinently, Giancamillo et. al. (2010) found a marine diatom while decomposing piglets in fresh water and observed that the indication by Yen & Jayaprakash (2007) deserved consideration. Consumption of diatom-rich foodstuffs indirectly means ingestion of the diatoms too. That these diatoms reach the stomach is beyond doubt. There is no experimental proof on the possibility of the consumed diatoms entering the circulation. In case such ingested diatoms enter the systemic circulation, they will also finally reside at internal organs of the body for unknown periods and would form the source of diatoms inside the human body during life i.e. prior to death by drowning. The probability that diatoms may reach the blood circulation during the process of absorption of digested food via gastrointestinal lining remains to be tested on empirical basis.

2.5 ANIMAL EXPERIMENTS

2.5.1 Hypothetical studies regarding drowning

Animals such as rats (Lunetta *et al.*, 1998; Xu *et al.*, 2011), rabbits (Ming *et al.*, 2007; Hu *et al.*, 2013) and pigs (Giancamillo *et al.*, 2010) had been extensively used by various researchers to study drowning and drowning-associated studies. Rats have been used in hypothetical studies of drowning to simulate human drowning.

2.5.2 Sprague – Dawley rats

The most commonly used rat species in experimental design studies are Winstar and SD rats. Even though both SD and Winstar rats did not show much significant differences, experience with handling both species proved that SD rats are less aggressive in behavior thus increased their popularity as rats of choice for hypothetical animal studies. The femur of the rat is fairly large as compared to mice and can be easily cut to obtain the marrow.

2.6 EXTRACTION OF DIATOM FRUSTULES

Various methods have been suggested for extracting diatom frustules from the water samples as well as organ samples. These include the use of enzymes, chemical solubilizers, mechanical action related centirifugation processes and strong acids for digesting the other organic material so that the insoluble diatom frustules can be extracted.

2.6.1 Enzymatic digestion method

Enzymatic digestion method was also being used as one of the method for diatom extraction (Kobayashi *et al.*, 1993; Azparren *et al.*, 1998; Sitthiwong *et al.*, 2011). It is actually adapted from DNA extraction techniques in which Proteinase K and sodium dodecyl sulphate (SDS), traditionally used to digest proteins and cell plasma membranes in DNA extraction, have proven to be effective in isolating diatoms (Kobayashi *et al.*, 1993).

2.6.2 Soluene-350 method

Soluene-350 is a solubilizer which has gained some popularity to be used in diatoms extraction. This method has been employed for the destruction of tissue sample (Fukui *et al.*, 1980). In 1999, this method was again used for the extraction of diatoms from fresh water and seawater samples (Sidari *et al.*, 1999).

2.6.3 Other method

Colloidal silica gradient centrifugation method has been reported to successfully separated diatoms from tissue samples (Terazawa & Takatori, 1980). Ming et. al. (2007) invented a can that can be used to destruct organic materials for diatoms isolation. Hu et. al. (2013) employed microwave digestion combined with membrane filtering method in extracting diatom from water and tissue.

2.6.4 Acid digestion method

One of the most commonly known methods is the acid digestion method. This method has been employed by researchers all over the world (Spitz & Schneider, 1964; Timperman, 1972; Pollanen, 1998b; Yen & Jayaprakash, 2007). The most common acids used in acid digestion methods are nitric acid, sulfuric acid and hydrochloric acid. However, this method was criticized to be hazardous, pollutive, disorganized and dangerous (Matsumoto & Fukui, 1993). It was shown to destroy zooplankton (Kobayashi *et al.*, 1993). Methods involving the use of concentrated oxidizing acids usually lead to partial or complete dissolution of the frustules (Round *et al.*, 1990).

In this study, acid digestion using nitric acid was chosen for the extraction of diatoms from internal organs of the study animals. The nitric acid was chosen because it is cheap, easy to obtain and effective in extracting diatom from organ samples.

CHAPTER III

MATERIALS & METHODS

3.1 OVERVIEW

This research aims to study the fate of ingested diatoms inside the body. Rats were chosen to simulate the human subjects in view of the similarity in the digestive system. The methodology was divided into three parts. The first part was the preliminary part followed by the animal study and finally the laboratory analysis. The preliminary part involved the preparation of diatom stock solution which is the most crucial part as it served as the feed for the next part which is the animal study. During the animal study, the rats reared for one year were periodically administered with metered doses of the diatom from the stock solution following a pre-determined schedule and the animals were sacrificed to harvest the organs. In the last part, the harvested organs were acid digested to extract diatoms following standard analytical procedure. The laboratory analysis was basically divided into two parts; the chemical analysis that digested the organs and microscopic analysis to detect and count diatoms. The chemical analysis was conducted to extract any possible diatoms that might be lodged in the harvested internal organs of the experimental animals. The extracts from the acid digestion were then subjected to microscopic analysis to visually determine the presence or absence of diatoms or their fragments in the extracted materials. The findings were then used to determine the fate of ingested diatoms and to evaluate the reliability of diatoms as indicator of drowning. The details of each part are described in the following sections.

3.2 PREPARATION OF DIATOM STOCK SOLUTION

3.2.1 Harvesting diatoms from suitable sources

Preliminary study was conducted to determine the suitable sources of diatoms. Diatoms have been reported to be abundant in water; marine or freshwater, pumice (Shamsudin, 1991) as well as shellfish (Yen & Jayaprakash, 2007). Water samples were collected in a 50 ml beaker (Pyrex, USA) from a few fish ponds, pools and drains within the campus of USM Kubang Kerian. Each of the water samples was then examined under the microscope (Zeiss Axiostar Plus) to determine the availability of the diatoms.

"Kerang" or cockles (*Anadara granosa*) (Brown, 1985) were bought at the local wet market, boiled until they were cooked, de-shelled and acid digested (procedure is described in 3.4.1 Extraction of diatoms frustules by acid digestion method) to harvest the diatoms. The sediment from the acid extract was examined under the microscope to determine the availability of the diatoms. From the preliminary study conducted, it was found that cockles or "kerang" proved to be a more suitable source to harvest diatoms since the number of diatoms available was in abundance as compared to those in the water samples. Thus, the cockles were chosen as the source for diatoms and diatoms were harvested from cockles by means of acid digestion method and the sediment was suspended in diatom-free distilled water in a test tube. The suspension was microscopically examined and the presence of diatoms in the suspension was confirmed.

3.2.2 Segregating diatoms into required size by filtering

During drowning, the entry of diatoms into the systemic circulation is a passive process and it has been indicated that diatoms that enter the pulmonary linings are often in the size range of about 30 µm (Pollanen, 1998b). Hence, the size of diatoms frustules required for this study was decided to be in the size range of 5 to 50 µm irrespective of the species as well as the shape. The higher limit of size range of 50 µm was a constraint on account of the pore size of the filter that was available viz. 53 µm.

In order to segregate diatoms frustules into desired size range i.e., a fine polyester cloth filter, PES 110/35 PW made in Germany with the pore size of 53µm was used as the filter. A 10 cm x 10 cm portion of the cloth was cut and the piece of cut portion was placed over a 100 ml beaker, secured with a rubber band around the mouth of the beaker to form a sieve. As the filter pore size was very fine, it was hard for even plain water to pass through the filter, therefore a 5 ml syringe was used to deliver the water-diatom suspension as a jet through the improvised sieve until the suspension was exhausted. The thrust from the syringe aided in effective passage of the suspension through the fine filter ensuring that desired diatoms were collected in the filtrate and other debris as well as diatoms of sizes larger than 53 µm were trapped on the filter. The filtrate was transferred into a 10 ml plastic test tube with a cap and a sample of the stock was microscopically examined using Olympus BX41.CVXS Image Analysis System, to confirm the presence of diatoms and for determining the size range of the diatoms in the filtrate. The diatom frustules collected in the filtrate were determined to be in the size range of 5 µm to 50 µm which included the desired size range and was acceptable to be used in this study. In

the stock solution, the proportion of diatoms in the size range 40 to 50 µm was about 20%, the remaining being in the size range of 5 to 39µm.

3.2.3 Preparing the metered-dose stock of diatoms in distilled water

Capped test tube containing diatom laden distilled water was vortexed for ensuring uniform distribution of diatom. A total of 10 µm of this stock solution was pipette out using Eppendorf 10 – 100 µm pipette and transferred to a chamber of a haemocytometer covered with glass slip. The number of diatoms in the content was counted using haemocytometer under the microscope. This process was repeated three times. The haemocytometer was thoroughly cleaned and dried in between repeating the counting and a new cover slip was used for each repeat to prevent cross contamination.

Another slide was prepared by air-drying 10 µm stock solution on a cover slip that was flipped over mounted on a glass slide and the edges sealed with DPX. The process of counting the number of diatoms present in 10 µm stock solution was again conducted by manually counting each and every diatoms present in the slide. This process was also repeated three times. The numbers of diatoms in 10 µm stock solution counted using haemocytometer and without haemocytometer in the second method were compared and the consistency of counts between the two was considered as the concentration of diatoms in distilled water. The concentration of the diatom stock solution was determined to be about 220 diatoms in 10 µm or 22,000 diatoms in 1 ml.

Metered dose of diatom laden distilled water was prepared by adding 40 µm of diatom stock solution to distilled water to make up 1 ml to obtain a concentration of 4 diatoms per 1 µm. The concentration of the diatom laden distilled water stock was calculated in similar manner as the determination of diatom stock solution concentration by comparing counting using haemocytometer and counting without using haemocytometer as described above.

3.3 ANIMAL EXPERIMENTS

3.3.1 Ethical approval

Prior to the commencement of the animal experiments, the proposal of this research was presented before the Animal Ethics Committee, Universiti Sains Malaysia on the 12th January 2011. After successful defense session, the animal ethics approval was obtained in USM/Animal Ethics Approval/2011/(64)(265) dated 31.01.2011.

3.3.2 Design of animal experiments

Young adult (aged about 3 months) SD rats, both males (n=15) and females (n=45) (this was a constrain due to availability) formed the experimental animals; half as experimental group (n=30) and the rest (n=30) as control group. The animals were duly tagged for their identity.