CORRELATION OF HEAVY METALS CONTENT IN SHELL AND TISSUE OF SMOKED *Corbicula fluminea* (ETAK)

by

NURHAZIRAH BINTI ISHAK

A report submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Sustainable Science) with Honours

FACULTY OF EARTH SCIENCE

UNIVERSITI MALAYSIA KELANTAN

2018

DECLARATION

I declare that this thesis entitled "Correlation of Heavy Metals Content in Shell and Tissues of Smoked *Corbicula fluminea* (Etak)" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : ....
Name : Nurhazirah Binti Ishak
Date :

ACKNOWLEDGEMENT

It is a genuine pleasure to express my deep sense of thanks and highest gratitude to my philosopher and guide, Prof. Madya Dr. Aweng A/L Eh Rak, my supervisor, for his guidance, encouragement, ideas, supervision and patience throughout the study. His dedication and keen interest above all his overwhelming attitude to help his students had been solely and mainly responsible for completing my research. His timely advice, meticulous scrutiny, scholarly advice and scientific approach have helped me to a very great extent to accomplish this research.

Secondly, I would like to express my deepest appreciation to the post-doctorate fellow in this research, Dr. Faizuan bin Abdullah, to my seniors, Dee Koh Han and Nur Aini and also to all staff of Environmental Lab, Faculty of Earth Science especially to En. Mohamad Rohanif bin Mohamed Ali and Pn. Nur Izzati binti Salleh, who have assist and guide me throughout my research work. Their prompt inspirations, timely suggestions with kindness, enthusiasm and dynamism have enabled me to complete my research.

I would also like to express my special gratitude to my beloved parents, En. Ishak bin Nawi and Pn. Siti Zaliah binti Miskam and to my family who give me their support from the beginning until the end of this research work. Next, special thanks to Faculty of Earth Science, Universiti Malaysia Kelantan Jeli Campus which provided me a golden opportunity to learn and utilize all the instruments and facilities.

Last but not least, I owe a deep sense of gratitude to my lab mates, all of my lecturers and friends who guided me and accompanied me when I am doubt in conducting the laboratory work and thesis writing. I am extremely thankful for their kind help and cooperation throughout doing my research and study period and also for their keen interest on me at every stage of my research to assist and contribute their idea in completing my research.

Thank you.

ABSTRACT

Bivalve bio accumulate metals are useful as sentinel organisms for assessing the bioavailability of metal contaminants in aquatic ecosystems. Malaysian clams can be contaminated with toxic and heavy metals which are cumulative poison through long term ingestion. *Corbicula fluminea* which is one of the income sources that has become potentially important traditional food in Kelantan, was categorized as filter feeder organism which accumulate heavy metals from contaminated river or water stream and being absorbed from the shell into the soft tissue. Frequently, tissue metal concentrations are used by environmental monitoring studies to evaluate the potential exposure and the effects scenarios. However, bivalves may accumulate certain metals such as Cd, Cr, Cu, Fe, Pb and Zn to a significant extent in shells. As the water contamination increase at continuous rate, this clam was suspected contaminated. Lately, there are numerous reports in local newspapers that claim cause of health effects due to eating contaminated *C. fluminea*. Currently, there is no specific analysis showed the heavy metals content correlation between the shells and tissue from the contaminated *C. fluminea*. There may have a possibilities whether the heavy metals content from the tissue being effected from the shells, which is more exposed to the environmental problem and smoked method. Therefore, the purpose of this study is to analyse the heavy metals correlation between the shells and tissues content of smoked *C. fluminea* to achieve the permeable limits set by Malaysian Food Regulation 1985 (MFR) and FAO/WHO (1984). In this research, the contamination of *C. fluminea* was identified in term of heavy metal contamination across this smoked processing stage. The heavy metal analysis (Cd, Cr, Cu, Fe, Pb and Zn) were carried out using acid digestion and identified using Perkin Elmer PinAAcle 900F Atomic Absorption Spectrometer. Experimental results revealed that there is no correlation between the shells and the tissues in term of heavy metals contamination. The results showed all the heavy metals concentrations (µg/g) in the soft tissue of smoked *C. fluminea* (Cd: 1.16±0.09 µg/g, Cr: 3.42±0.32 µg/g, Cu: 34.93±7.02 µg/g, Fe: 211.23±24.20 µg/g, Pb: 2.34±0.24 µg/g and Zn: 110.62±11.96 µg/g) while for the shell (Cd: 0.48±0.16 µg/g, Cr: 6.08±0.70 µg/g, Cu: 0.56±0.07 µg/g, Fe: 25.86±2.64 µg/g, Pb: -60.72±5.29 µg/g and Zn: 9.53±0.91 µg/g). The range of heavy metals accumulated in soft tissues and shells were as followed: Soft tissues (Fe > Zn > Cu > Cr > Pb > Cd) ; Shells (Fe > Zn > Cr > Cu > Cd > Pb). The results obtained were compared with the permissible limits set by Malaysian Food Regulations 1985 (Cd: 1.0 µg/g, Cu: 30.0 µg/g, Pb: 2 µg/g and Zn: 100.0 µg/g) and FAO/WHO 1984 (Cr: 13 µg/g and Fe: 100.0 µg/g). The results of heavy metals indicated that the Cd, Cu, Pb, Fe and Zn are beyond the permissible limits set by Malaysia Food Regulation 1985 and FAO/WHO (1984). This study successfully determine the baseline concentration of the heavy metals content in shell and soft tissue of *C. fluminea* and their possible source from the environment.

ABSTRAK

Hidupan berkerang mengumpul logam dan ianya berguna sebagai organisma pengawal untuk menilai bioavailabiliti bahan pencemar logam dalam ekosistem akuatik. Hidupan berkerang Malaysia boleh tercemar dengan bahan toksik dan logam berat yang merupakan racun kumulatif melalui pengambilan jangka panjang. *Corbicula fluminea* merupakan salah satu sumber pendapatan yang menjadi makanan tradisional yang mendapat permintaan tinggi di Kelantan dikategorikan sebagai organisma penyerap penapis yang mengumpul logam berat dari sungai yang tercemar atau aliran air dan diserap dari cengkerang ke dalam tisu lembut. Kebiasaannya, kepekatan logam berat di tisu digunakan oleh kajian pemantauan alam sekitar untuk menilai potensi pendedahan dan senario kesan. Walau bagaimanapun, hidupan berkerang ini juga dipercayai boleh mengumpul logam tertentu dalam cengkerang. Oleh kerana peningkatan pencemaran air pada kadar yang berterusan, kerang ini disyaki tercemar. Akhir-akhir ini, terdapat banyak laporan di akhbar tempatan yang mengatakan penyebab kepada kemerosotan kesihatan akibat daripada memakan *C. fluminea* yang tercemar. Pada masa ini, tiada analisis khusus menunjukkan perhubungan kandungan logam berat di antara cengkerang dan tisu lembut dari *C. fluminea* yang tercemar. Berkemungkinan sama ada kandungan logam berat dari tisu dipengaruhi oleh kandungan logam berat di cengkerang yang lebih terdedah kepada masalah alam sekitar dan kaedah salai. Oleh itu, tujuan kajian ini adalah untuk menganalisis korelasi logam berat antara cengkerang dan tisu kandungan *C. Fluminea* yang telah disalai untuk mencapai had selamat yang ditetapkan oleh Peraturan Makanan Malaysia 1985 (MFR) dan FAO / WHO (1984) dan juga untuk menilai pengambilan dan pengedaran logam berat di *C. fluminea*. Analisis logam berat (Cd, Cr, Cu, Fe, Pb dan Zn) telah dijalankan menggunakan pencernaan asid dan dikenalpasti menggunakan Spektrometer Penyerapan Atom Perkin Elmer PinAAcle 900F Atom. Keputusan eksperimen menunjukkan bahawa tiada perhubungan di antara cengkerang dan tisu dalam konteks pencemaran logam berat. Hasil daripada kepekatan semua logam berat (µg / g) dalam tisu *C. fluminea* yang telah disalai menunjukkan (Cd: 1.16±0.09 µg/g, Cr: 3.42±0.32 µg/g, Cu: 34.93±7.02 µg/g, Fe: 211.23±24.20 µg/g, Pb: 2.34±0.24 µg/g dan Zn: 110.62±11.96 µg/g) manakala cengkerang pula adalah (Cd: 0.48±0.16 µg/g, Cr: 6.08±0.70 µg/g, Cu: 0.56±0.07 µg/g, Fe: 25.86±2.64 µg/g, Pb: -60.72±5.29 µg/g dan Zn: 9.53±0.91 µg/g). Keputusan yang diperolehi dibandingkan dengan had yang dibenarkan oleh Peraturan Makanan Malaysia 1985 (Cd: 1.0 µg / g, Cu: 30.0 µg / g, Pb: 2 µg / g dan Zn: 100.0 µg / g) dan FAO / WHO 1984 Cr: 13 µg / g dan Fe: 100.0 µg / g). Julat logam berat yang terkumpul dalam tisu lembut dan cengkerang adalah seperti berikut: Tisu lembut (Fe> Zn> Cu> Cr> Pb> Cd); Cengkerang (Fe> Zn> Cr> Cu> Cd> Pb). Hasil logam berat menunjukkan bahawa Cd, Cu, Pb, Fe dan Zn telah melebihi had yang telah ditetapkan oleh Peraturan Makanan Malaysia 1985 dan FAO / WHO (1984). Secara keseluruhan, kajian ini berjaya menentukan kepekatan kandungan logam berat dalam cengkerang dan tisu lembut *C. fluminea* dan kemungkinan sumbernya berpunca daripada alam sekitar.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AR	-	Aqua Regia
ISO	-	International Organization for Standardization
JECFA	-	Joint Food and Agriculture Organization / World Health Organization Expert Committee on Food Additives
AAS	-	Atomic absorption spectroscopy
BCF	-	Bioconcentration factors
LPO	-	Lipid peroxidation
SOD	-	Superoxide dismutase
GSTs	-	Glulathione stransferase
ICP	-	Inductively coupled plasma mass spectroscopy
MW	-	Microwave
OES	-	Optical emission spectrometer
GFAAS	-	Graphite furnace atomic absorption spectrophotometer
SRM	-	Switched reluctance motor
GPS	-	Global Positioning System
DWT	-	Constant dry weights
SPSS	-	Statistical Package for the Social Science

LIST OF SYMBOLS

$CaCO_3$	-	Calcium carbonate
HNO_3	-	Nitric acid
HCl	-	Hydrochloric acid
Pb	-	Lead
Zn	-	Zinc
Cu	-	Copper
Cd	-	Cadmium
Fe	-	Iron
Cr	-	Chromium
mg/kg	-	Miligram per kilogram
mg	-	Miligram
ppm	-	Part per million
µg/g	-	Microgram per gram
M	-	Molarity
%	-	Percentage
˚C	-	Degree celcius
g/l	-	Gram per litre
mL	-	Mililitre
mm	-	Milimetre
µm	-	Micrometer

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Corbicula fluminea (*C. fluminea*) as shown in Figure 1.1, which is also known as Etak in Malaysia, is a species of freshwater clam, an aquatic bivalve mollusc in the family Cyrenidae that usually live in most type of water body with fine sand and gravel. *C. fluminea* is an infaunal filter-feeding that feed primarily on phytoplankton (algae), which they filter from the sandy or muddy bottoms of streams, lakes or canals. It is not treated as aquaculture product like the other bivalves but they was collected from their natural habitat which is commonly harvested for human consumption. This bivalve commonly in the sandy-muddy bottoms of the interdal and sublittoral zones of the coastal environment (Argente & Estacion, 2014). The characteristics of the clams is it has solid shell, equivalve, inequilateral shell, which is triangularly elongated ovate in outlie with a smooth, highly glossy surface. It has a yellowish brown to black shell with concentric and evenly spaced ridges on the shell surface. They are usually less than 25 mm but can grow up to 50 to 65 mm in length. *C. fluminea* takes about 2-3 years to reach a marketable size and the weight are correlated with its length. The ages of the clams can be inferred from their lengths and rings. Based on Integrated Taxonomic Information System 2017

(Coordinators, 2017), the taxonomic hierarchy of *Corbicula fluminea* is illustrated in Table 1.1.

Figure 1.1 : *Corbicula fluminea* or Etak

Table 1.1 : Taxonomic hierarchy of *Corbicula fluminea*

(Sources : ITIS, 2017)

Kingdom	Animalia
Phylum	Mollusca
Class	Bivalvia
Subclass	Heterodonta
Order	Veneroida
Superfamily	Corbiculoidea
Family	Corbiculidae
Genus	Corbicula
Species	*Corbicula fluminea*

The life cycle of this Asian clam is variable which is commonly has an average ranging from 1 to 5 years. It is able to produce both eggs and sperm and capable of self-fertilizing or can be known as hermaphroditic species. The population of *C. fluminea* and its sex ratio was greatly influenced by the environment in which the animals lived. The reproduction happen when the shell size of *C. fluminea* reach around 10 mm which is between the age of 5 - 9 months (Basen, 2012). According to Pacific Northwest National Laboratory, one small of *C. fluminea* can produce to 400 larvae per day or up to 70,000 clams per year. As it was the hermaphrodite system, the sperm of *C. fluminea* is caught by another clam and broaded in the gills via water. The spawning process will occurred in water temperatures higher than 16℃ and for the clams released its larva, the water must be above 16℃. A single clam can release an average of 400 of juveniles a day and up to 70,000 per year. When the larvae of *C. fluminea* grow up, the juveniles are released and exposed to the natural river environment. Due to their small dimensions which is just around 250 µm, it can dispersed easily through water currents and anthropogenic activities such as attached to boats, carried in ballast water or used as bait.

Heavy metals could be classified as potentially dangerous and considered as one of the major anthropogenic contaminant in coastal, marine and freshwater environments worldwide (Ruilian *et al.*, 2008). Their toxicity, persistence and bioaccumulation characteristics make them known as a toxic or carcinogenic to humans and can pose a serious threat to human health, living organisms and natural ecosystems. Heavy metals such as cadmium, mercury and chromium can contribute to degradation of marine ecosystems by reducing species diversity and its abundance through accumulation of metals in living organisms and food chains (Conners *et al.*, 2009). These heavy metals can be introduced to coastal and marine environments through a variety sources including industrial, wastewaters and domestic effluents.

Trace metals in natural marine ecosystems are present in low concentration. Heavy metals contamination in coastal and marine environments is becoming an increasingly serious threat to both naturally stressed marine ecosystems and humans that rely on marine resources on food, industry and recreation. While for freshwater environments, the most common metal pollution in freshwater comes from mining companies which they usually use an acid mine drainage system to release heavy metals because metals are very soluble in an acid solution. After the drainage process, they disperse the acid solution in the groundwater, containing high levels of metals. Effluents arising from these human activities, infrastructure development and agricultural activities are the main contribution of the contamination in clam growing area nowadays (Ruilian *et al.*, 2008).

The ability of the clam to accumulate high concentration of heavy metals from its ecosystem due to its infaunal filter-feeding characterization make them as an ideal indicator organisms for this heavy metals content correlation study of its shell and tissue besides it has a sedentary life history and high numerical abundance. It also has a lengthy life span that permits sampling of more than 1 year throughout the monitoring period, so that the sample tissue is available for analysis and good adaptation to laboratory conditions.

Heavy metals have a great affinity for sulphur and attack sulphur bonds in enzymes of marine organisms and immobilizing the latter. Accumulation of these metals only begins after the organisms are faced with high concentrations in the surrounding medium (Al-Mohanna & Subrahmanyam, 2001). Bivalves are widely used as sentinel organisms for marine pollution detection due to their ability to accumulate metals several orders higher than what is present in the surrounding environment. The factors which influence metal concentration and accumulation are bioavailability of metals, season, size, sex, hydrodynamics of the environment, changes in tissue composition and reproductive cycle (Barlogie *et al.,* 2006). In addition, tissue metal concentration can vary with depth of water, salinity, temperature, pH and food availability. Basically, clams focussed on the use of total soft tissues of clams rather than the clams shell as a quantitative indicator to reflect the heavy metal contamination in the coastal area. However, not all the metals are effectively accumulated in the soft tissue of the clams depending on the capability of the clam species to accumulate and to regulate the metals.

If the data analysis of heavy metals content of *C. fluminea* between shells and tissue have not much difference and have a significant value, it means that each of them are correlate each other. The high sensitivity of the filter-feeder species like *C. fluminea* could be related with its trophic status, the individuals being exposed to both dissolved and particulate metallic pollution. The implementation of the standards level must be standardize to ensure the comprehensive is being done in the whole country. The Ministry of Domestic Trade, Co-operatives and Consumerism may issue a new law that the sale of *C. fluminea* should be without skin and the shell will be banned from being sold. Another outcome is by strictly monitoring and controlling each step of the smoked process at every sellers by Malaysia Health Department, there is less chance for hazards to occur. The identification and controlling process of major food risks such as microbiological, chemical and physical contaminants can being done.

The health impact towards consumers can be reduced as they will be more cautious to consume *C. fluminea* and be more educated towards the bad effects of contamination of heavy metals. Although the possibility of the sellers' economy level may be reduced but the contamination level towards human and the environment can be reduced respectively.

1.2 Problem Statement

C. fluminea clams in Malaysia have been known as popular seafood especially for Malaysian. This species have a potential to be exported to worldwide which is the largest importers of seafood. However, high concentration of heavy metals such as arsenic, chromium, copper, lead, magnesium, manganese, selenium, vanadium and zinc in *C. fluminea* can cause harmful to human body and can affected its quality. Its infaunal filter-feeding characterization which allowed the heavy metal in the river were absorbed in the tissue and organ of *C. fluminea* through the filter feeding ability make the clam has high potential to accumulate high concentration of heavy metals from its ecosystem. Even though the awareness among the communities and researchers had increased on this matter, there is no specific analysis showed the heavy metals content correlation between the shells and tissue from the contaminated *C. fluminea*. There may have a possibilities whether the heavy metals content from the tissue is being effected from the shells, which is more exposed to the environmental problem and smoked method. The ability of lime and calcium carbonate ($CaCO_3$) from the shell itself which is easily fragile and has high ability to be as absorbent of heavy metals for aquatic environment may possible effected its tissues by continually leaching the biocides contents (Srinivasan & Swain, 2007).

Since smoked *C.fluminea* has become special snack and is considered as an appetizer especially for the Kelantanese, therefore only a few people in this state are be aware and cautious in consume it. Smoked method process which is the process of flavouring, browning, cooking or preserving food by exposing it to smoke from burning or smouldering material, will exposed the clams to polycyclic aromatic hydrocarbons and may affect cancer to human health. The smoked processed clams might susceptible to the heavy metals contamination from the smoking process due to the presence ingredients and deposited of ash from charcoal combustion. Others than that, mostly the consumer will bite the clams' shell before eating to open up the tissue. It is believe that the easily fragile characterization from the shell may cause the heavy metals from the shells leaked out to the mouth and contaminated the consumers' body.

This analysis content correlation is important to ensure that community do not affect from contamination and to maintain the quality of the *C. fluminea*. The selling of smoked clam is also not covered and exposed to the contamination from the surrounding as most of the sellers sold their smoked etak at the nearby roadside and placed too exposed to the dust of the surroundings. Based on the statistics from Ministry of Health Malaysia, percentage rate for street food that safe to eat is only 41% and the others is categorize as highly risk to be eaten (Ministry of Health Malaysia, 2017). Others than that, the potential of *C. fluminea* itself as sentinels of fecal pollution in aquatic environment also can make it categorized as food in highly risk to be eaten because it may cause food infection diseases towards human such as acute diarrhoea and nausea. Besides, it is important to ensure that the concentration of heavy metal will not exceeding the maximum permissible limits which are stipulated under Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) and Malaysian Food

Regulations 1985 guidelines. The permissible concentration limit stated by Malaysia standards of Cd, Cu, Pb, Zn, Cr and Fe are 1.0 µg / g, 30.0 µg / g, 2 µg / g, 100.0 µg / g, 13 µg / g and 100.0 µg / g respectively (Wang *et al.*, 2015).

1.3 Significances of the Study

From the research, the data analysis process of heavy metals content in shells and tissue from *C. fluminea* is important to ensure that the quality is better and it below the standard limits stated by Malaysian. As this Asian clam has been vital food especially for the Kelantanese, the heavy metals content in it still cause a great concern towards the society. Therefore, through this research findings, the exact amount of heavy metals content in etak can be known, thus the safety of consumption can be taken and being more alert as precautious measure. The good quality product will reduce the risk towards human on toxicity effect of heavy metals contain in *C. fluminea* and to ensure that communities do not affect from any contamination. Hence, it will maintain the freshness and sustain good quality of *C. flumimea* because this is one of the food resources for local society especially in Kelantan.

The awareness among the communities also will be increase as they will be more educated and be more cautious towards their food consuming. This study also will get a specific analysis about the heavy metals content correlation between the shell and soft tissue from the contaminated *C. fluminea* as none of the previous study have done about this correlation study. There may have a possibilities whether the heavy metals content from the tissue is being effected from the shells and either is it can be proved that the easily fragile characterization from the shell may cause the heavy metals from the shells leaked out to the mouth and contaminated the consumers' body or not.

1.4 Objectives of the study

The objectives of the research are :

1. To determine heavy metals (Lead, Zinc, Iron, Copper, Cadmium and Chromium) in shell and tissue of smoked *Corbicula fluminea.*
2. To determine the correlation of heavy metals in shell and tissue of smoked *Corbicula fluminea.*

1.5 Scope of the study

This research had been conducted at Pasir Mas and Tumpat to investigate the heavy metals content of Pb, Zn, Cu, Cd, Fe and Cr from shells and tissue of *Corbicula fluminea* which involves sampling process of *C. fluminea* and chemical analysis process in the laboratory using aqua regia solution. Pasir Mas and Tumpat were being chosen as the site area as both districts were the main collection place to collect etak in Kelantan and main distributors place to distribute etak to other states for exporting activities. Most of the sellers in Kelantan have been focussed to these both districts to sell their clams there. This will be easier to the consumers to get their favourite clams in every districts they want.

For this research, the number of samples taken was 18 samples which three times of replicate sampling process had being done at each of the districts in each months. Six sellers have been chosen for the sampling process based on their continuous etak selling at that area which are Site 1, Site 2 and Site 3 from Pasir Mas while Site 4, Site 5 and Site 6 are sellers from Tumpat. The sampling and analysis of the heavy metals were carried out for three months duration (March – May) because based on the preliminary study, these three months were the peak reproduction months for etak (Achard *et al.*, 2004) and it can be seen that it was the most suitable time to exhibit the trend of heavy metal contents concentration and the exact heavy metals correlation between shell and soft tissues can be known. Pb, Zn, Cu, Cd, Fe and Cr are the six parameters that will be analysed in this research. This is because based on the previous study by Achard, Baudrimont, Boudou & Bourdineaud (2004), all of the six parameters have been proved have the highest heavy metals content in clams due to their significance value. Besides, all of the heavy metals that have been shortlisted for this research study had been identified as the causes which have high possibility that may effect to the human health.

CHAPTER 2

LITERATURE REVIEW

2.1 *Corbicula fluminea*

Bivalves can be defined as an aquatic life that can be categorized as molluscs' organism which their features are have a compressed body enclosed within a shell and without head such as oysters, mussels and scallops. Based on the National Oceanic and Atmospheric Administration, there are more than 15,000 species of bivalves exist and all of it are from variety of habitat including aquatic, marine and freshwater species. Bivalve is important in both aquatic and marine ecosystems by always filtering the water and serving as prey for the aquatic life (National Oceanic and Atmospheric Administration, 2017).

The shells of the bivalve is made up from calcium carbonate ($CaCO_3$**).** According to the Department of Fisheries Malaysia (2005), in 2005-2006, the Malaysia Fisheries Directory documented that there are approximately 27 species of bivalves in Malaysian coastal areas. Most of the clams are features of filter feeder organisms. They are a potential indicator of the bioavailability of toxic metal contamination in the estuary.

These animals ingest metal-enriched particles directly, thereby giving an indication of the bioaccumulation ability of metals.

Corbicula fluminea or can be known as etak is one of the freshwater clam that can be found living in the sandy bottom of streams. It was originated in the Eastern Asia and nowadays had been introduced to the worldwide state such as South America, North America and Europe (Zidkova *et al.*, 2014). This clam is self-fertilize and usually takes time for 4 years to reach maturity as it only grow 1 cm at each time it grow (Robutson *et al.*, 2001). This *C. fluminea* or Asian clam is really famous and well-known in Kelantan because the local communities there make them as snack food or appetizer and it is easy to get by the street or roadside.

For the habitat, *C. fluminea* can survive in almost any freshwater environment, including permanent brackish and estuarine waters. The clam live in various kind of substrates but preferable in clay, fine sand and coarse sand substrates. There are factors that may affect the population density and distribution of *C. fluminea* including temperature, salinity, hypoxia, pollution, bacteria, viral and parasitic infections, competition, predator and genetic changes (Robutson *et al.*, 2001). Besides, a high level of the contamination inhibit the growth and even increase the mortality of *C. fluminea* (Mouthon, 2001).

2.2 Heavy metals in Water

Marine sediments of the world are increasingly contaminated with heavy metals and other contaminants due to a history of industrial discharges and urban runoff. Marine sediments are an important environmental component when considering the fate and transport of metals within a watershed. The behaviour and distribution of metals in marine sediments is influenced by hydrodynamics, anthropogenic discharges, and biogeochemical processes (Zwolsman *et al.*, 2007). Marine bivalves (clams and mussels) have long been employed as pollution biomonitors in coastal environments. This is due to their intimate contact with the contaminated sediments and exceedingly high pumping activity and their responses are often proportional to ambient pollutant concentrations (Wang and Guo, 2001). Numerous factors need to be considered in marine environments when assessing bioaccumulation of metals in bivalve animals. Various conditions (organic content, pH and presence of sulphide) are considered potential metal sinks. An important sink of metals are hydrous iron oxides (a large part of oxidized sediments) acting by binding heavy metals to the surface of sediments (Luoma and Bryan, 2008). The strength of metal binding to the particulates contributes to the availability of the metal. Strongly bound metals are less available and weakly bounded metals are more available (Luoma, 2003). However, bound metals may be bioavailable depending on the feeding and biogeochemical characteristic of the organism. No universal way to assess the bioconcentration factors to benthic organisms exists for several reasons. The two most important factors include differences between organisms and inconsistent chemistry between metals and metal interactions. Redox cycles such as those caused by tidal action increased the metal availability to organisms by interfering with the metal species equilibrium (Simpson *et al.*, 2002)

Ahmed *et al.*, (2006) have done a further study about the heavy metal concentrations in water using *Leuciscus cephalus* from their muscles and gills as the bioindicator monitoring for the study. The finding results showed Cu and Zn correlate higher between the sediment and the tissue but the correlation showed negative between the heavy metals content in water and *L. cephalus* (muscle and gills) or the sediment itself. Lau *et al.*, (2008) supported that difference accumulation of heavy metals contamination in the water may due to the geographical structures not because of the water sediments. Other factors that may also effect the accumulation of heavy metals in the water is because of its pH, alkalinity, Total Dissolved Solids (TDS) and turbidity. The resulting effect of higher contamination of heavy metal mostly in the condition of water with higher pH and alkalinity.

The present study by El Sayed *et al.*, (2011) investigated the heavy metal residues that accumulated in the fish tissue (*Oreochromis niloticus*, *Clarias gariepinus* and *Bagrus bayad*). The study indicated that industrial waste and sewage waste are the main contributors to heavy metals in water others than agriculture drainage water. Cd, Cr, Pb, Al and Co were the heavy metals that higher accumulated in the water with beyond the permissible limit standards by WHO. Phytoplankton growth in water also played as important role in the heavy metals contamination process as it can absorb more residues in the water as the growth increases (Sareed *et. al.*, 2001).

2.3 Heavy Metals in Bivalves

Heavy metals pollution has been a worldwide issue for many years till nowadays and many environmental impacts have been effected by this problem. Most of the cases for this pollution is from anthropogenic inputs. Increased coastal population, rapid urbanization, oil and gas production, tourism development, heavy rainfall throughout the year and various economic activities have created numerous environmental and ecological problems in Malaysia's coastal areas including beach erosion, resource depletion and environmental degradation, and destruction of natural habitats (Yap *et al.*, 2003). From this problem, the bivalve molluscs such as oysters, clams, mussels and scallops acted as important role as pollution indicator of environmental pollution level due to their features of filter feeders. This type of natural bioindicator environmental monitoring should be used the indicators that really sensitive to the surrounding changes which can provide information based on their biological changes (National Oceanic and Atmospheric Administration, 2017).

Basically, the heavy metals content in clams are Cd, Cr, Cu, Fe, Pb and Zn (Fraysse *et al.,* 2002). These elements are widely distributed in coastal environments where bivalves usually grow. However the accumulation of toxic and heavy metals for clams depends on the proportion of these elements in a bio available form which is influences by pH, oxygen, salinity, temperature, and organic matter. Studied done by Mendez, Palacios, Acosta, Monsalvo-Spencer & Alvarez-Castaneda (2006) showed that the heavy metals concentration clams decrease from Fe, Zn, Cu, Mn, Cd, Ni, and Pb. The concentration is depends on the humidity at the site. As an example, concentration of Cd and Pb is high when dry season and the highest concentration of Ni was found in rainy season (Mendez *et al.*, 2006).

Ruilian *et al.*, (2008) had been studied the concentrations of Cd, Cu and Zn in a river water by using the Asiatic clam, *Corbicula fluminea* as a bioindicator potential and the significant correlation between water concentration and tissue accumulation was observed. In this 28 days exposure of the field research, the average of total aquatic concentration for Cu are 0.016 and 0.057 mg l^{-1} and its bioconcentration factors (BCF) are 22 571 and 17 720 respectively (Ruilian *et al.*, 2008). From the data, it can be showed that Cu has the highest degree of the tissue uptake and had a correlation coefficient of 0.639. For Cd, the average of its total aquatic concentration is intermediate which are 0.023 and 0.055 mg l^{-1} and its BCF 3,770 and 1,752 respectively with correlation coefficient of 0.758. The average of total aquatic concentration for Zn is the lowest which are 0.218, 0.433 and 0.835 mg l^{-1} with BCF 631, 358 and 511 respectively with its correlation coefficient of 0.478 (Ruilian *et al.*, 2008). After 11 days, Cd had reached a maximum rate of accumulation but not for Cu and Zn as their state condition was not observed and did not showed any relative increase until 28 days exposure period. The total uptake for this selected heavy metal that have been collected from this study can be as a prove for *C. fluminea* potential efficiency as a reliable indicator especially towards Cu (Ruilian *et al.*, 2008).

Heavy metals have a great affinity for sulphur bonds in enzymes of marine organisms that immobilizing the latter. Accumulation of these metals only begins after the organisms are faced with high concentration in the surrounding medium (Al-Mohanna *et al.*, 2001). Bivalves are widely used as sentinel organisms for marine pollution detection due to their ability to accumulate metals several orders higher than what is present in the surrounding environment. The factors which influence metal concentration and accumulation are bioavailability of metals, season, size, sex, hydrodynamics of the environment, changes in tissue composition and reproductive cycle (Kumari, Kaisary, & Rodrigues, 2006). In addition, tissue metal concentration can vary with depth of water, salinity, temperature, pH and food availability. Basically, clams focussed on the use of total soft tissues of clams rather than the clams' shells as a quantitative indicator to reflect the heavy metal contamination in natural habitat.

2.4 Heavy metals in Tissue

Based on the preliminary study by Bonnail *et al.*, (2016), the metal bioaccumulation intake of *G. paradoxa* that live in contaminated and non-contaminated sediment was totally different and have a huge range. On the basis of the calculated Biota-Sediment Accumulated Factor (BSAF) database, metal enrichment in the tissues of the clams was rank in the following order Hg>Mn>Fe (Bonnail *et al.*, 2016). From the finding results, Hg contamination levels were found to be higher in the clams than in the sediments. This results can be indicate that more concentration of Hg than the other heavy metals was accumulated by *G. paradoxa* due to higher of Hg concentration in the water content. Fe and Mn concentrations were generally lower in the clam tissues than in the sediments suggesting that the levels of contamination of these metals in the estuary do not exceed the clams' capacity to regulate them (Bonnail *et al.*, 2016). The differences of the bioavailability heavy metals intake by the clams are totally can be determine through the relationship between metal geochemistry and animal physiology (Wang *et al.*, 2015). The findings of the data supported that several variables control both the bioavailability and accumulation of heavy metals in individuals exposed to contamination. (Ansari *et al.*, 2004).

The similarities of the bioavailability heavy metals intake to the clams may the cause of the heavy metal concentrations in the clam tissues did not vary significantly with the sampling site (Ferreira *et al*., 2004). Based on the previous study, Szefer *et al*., (2002) stated that from the analysis that have been made in the previous research, there is totally no distinct relationship between heavy metals concentration in the clam tissue with the sediments sample. Other sources of heavy metals in bivalve tissues are derived from living or dead suspended particles and from dissolved metals in the water (Szefer *et al*., 2002). The relatively higher concentrations of Zn in the clam tissues compared to the concentrations in the sediments suggests a high rate of accumulation by the clams, a physiological mechanism induced by exposure or even a high relevance of the water as an additional source of contamination (Chan *et. al*., 2001).

Udechukwu and Ismail (2013) studied the Cu content in four different bivalve species (*Anadara granosa*, *Pholas orientalis*, *Fragnum unedo* and *Donax faba*) in Malaysia and reported that Cu concentrations were between 5.17 µg/g to 20.42 µg/g. Furthermore, Patrick *et al.* (2017) found that Cu concentration in the tissues of *C. fluminea* were significantly higher than in the sediments ($P<0.01$) even after they have been flushed with dechlorinated water. This is proof that flushing of water is ineffective in removing Cu from the bivalve. Moreover, Mouthon (2001) discovered that the Cu concentration in *C. fluminea* was significantly higher near a kaolin area. The Cu concentration in *C. fluminea* ranged from 5.7 µg/g to 133.17 µg/g (Lewbart *et al.*, 2010). The concentration of Cu that is permissible according to the Malaysian Food Regulation (1985) is 30.0 µg/g. Therefore, some Cu concentration in *C. fluminea* studies are higher than the acceptable values.

2.5 Heavy Metals in Shell

El Gamal *et al.*, (2011) examined the heavy metal contamination in the carpet shell clam, *Tapes deccussatus* from six sites in three main Egyptian natural fisheries (Ismailia, Alexandria and Damietta) over a six months period representing the winter and spring seasons (December 2010 – May 2011). The heavy metals that have been analysed were Pb, Cu, Cd and Zn. The clams were believed have been exposed to different levels of industrial wastes and municipal discharge in that area. From the results of the study, Zn was the only heavy metals that still under the permissible limit either during the winter season or spring season. Pb showed the highest contamination in Damietta but had the lowest concentration of Cu, Cd, and Zn. For Ismailia's data result, Pb contained high concentration for both of the seasons. From the data presented, this study can be concluded as most of the clam fisheries in this area were contaminated with heavy metals. Bivalves have great ability of bioaccumulation of contaminants from the surrounding environment. Therefore, the studied sites are suggested to be not suitable for use as grow out sites for clam aquaculture in Egypt (El Gamal *et al.*, 2011). Other than that, as a precautious measure to reduce the clam fisheries being contaminated, the discharged of wastes either municipal or other type of wates should be limited in the regulations for a better future.

The heavy metal concentration accumulated in shells in both marine and freshwater bivalve also had been widely studied (Freitas *et al.*, 2016) compared several heavy metal concentrations in *Corbicula* and *Elliptio hopetonensis*. It was found that heavy metal concentration in *Corbicula* is generally higher than *E. hopetonensis* except for Mn, which was to be significantly greater in *E. hopetonensis* (2259 μg/g to 7714 μg/g) than *Corbicula* (16.7 μg/g to 105 μg/g). The concentration of Mn in the shell of *C. fluminea* (29 μg/g to 500 μg/g) is significantly higher than in their tissue (2 μg/g to 17 μg/g) (Lewbart *et al.*, 2010). However, some of the Mn concentration in the soft tissue of *C. fluminea* is not safe to be consumed since the acceptable limit set by the FAO/WHO (1984) is 5.4 mg/day.

Wadige *et al.* (2014) used freshwater bivalve *Hyridella australis* as a bioindicator for the detection of Pb in sediment. It was found that Pb concentration of control bivalve (1.2 μg/g) increased to four fold (4.2 μg/g) after exposure to lead contaminated sediments (419 μg/g). In Vietnam, the Pb concentration of *Corbicula* sp. range from 0.37±0.27 mg/kg to 3.58±2.69 mg/kg from 2008 to 2012 and are significantly correlated with the sediments (Van Khanh *et al.*, 2013). Miedico *et al.* (2015) studied Pb concentration in 138 bivalve molluscs in the market and detected the Pb concentration ranged from 0.019 mg/kg to 2.520 mg/kg, where 2 samples were not compliant with the permissible limit. The concentration of Pb that are permissible by the Malaysian Food Regulation (1985) is 2.0 μg/g. Since Pb is more hazardous than other metals, the level of Pb in bivalve should be screened regularly for the safety of consumers.

2.6 Correlation of Heavy Metals in Shells and Tissues

From the studied done by Archard *et al.* (2004), three heavy metals which are Cr, Cd and Pb have been accumulated from *Tympanotonus fuscatus var radula* from Elechi Creek, Nigeria. The contaminants from the studied area were usually comes from effluents discharged from the industrial area and populated settlements. Cr is the highest concentrated of heavy metals contained after the water, sediments, soft tissues and shell were being processed and analysed. From the sizes of the shells, the contaminants and heavy metals that being absorbed by the species can be identified through its bioaccumulation factor (BCF) as the bigger the size of the samples, the more possibility of the periwinkles (soft tissue and shells) to accumulate the heavy metals content. Pb has the lowest concentrated of heavy metals contained in this study compared to Cd and Cr. According to Archard (2004), the result in terms of BCF is useful to compare the level uptake of heavy metals especially in soft tissues and shells as its observation reading has a high potential to concentrate the heavy metals content accumulation.

The studied done by Lau *et al.*, (2008) showed that human activities is the most contributed to the heavy metals accumulation in aquatic organisms. The study area which had been done in Sungai Sarawak Kanan was highly suspended with solid loads and most of them were trapped in the bottom sediment. *Brotia costula, Melanoides tuberculata* and *Clithon* sp. were the three chosen species of molluscs which two of them were purely from freshwater while the other one was from brackish water. From the results that have been obtained, *B. costula* sp. and *M. tuberculata* sp. contained higher efficiency of accumulation in shells compared to their tissue which are As, Cd, Pd and Zn while for *Clithon* sp., Cu and Zn contents were higher in tissue compared in shells. The feeding category of each species which acted as the specialized characteristics in mollusc was one of the differentiate causes of the heavy metals affinity accumulated. Acted as a deposit feeder in freshwater make *B. costula* sp. and *M. tuberculata* sp.tend to accumulate more metals. This was different with Clithon sp. as it will scraped the algae and diatoms by dwelling on rock surfaces for its food.

According to Gundacker (2010), *Scrobicularia plana* from Tamar has high possibility to be as an indicator towards environmental level problems. Cu, Fe, Mn and Zn are the heavy metals that have been measured and analysed on its mollusc and its soft tissues. Mn contributes to the highest heavy metals affinity contents in the shells as it caused by an increase metabolic rate leading to production and manufacture of gonad. While the affinity of heavy metals contents in the soft tissues showed that Cd, Cu, Cr, Pb, Zn and Ni were increased constantly based on its size. The possibility of big in size of samples will accumulate high metals contents due to the absorption rate as internal filter feeding towards the surroundings. The seasonal variation and the level of shore also contributed to this issues. From the results obtained, the total amount of metals contents in digestive gland of *S. plana* sp. is 75%, which are formed from Cu and Ag (30-40 %), Mn (51-80 %) and Fe (3-20%) (Gundacker, 2010). Some of the metals also adsorb onto the shells which the metal adsorption process are consists from additional 20% of the Mn, 50% of Cu, 60% of Fe and 70% of Zn.

2.7 Effect of Heavy Metals for Human

The environmental problems which involved the heavy metals contamination is one of the issue that we have familiar from previous. According to Sastre *et al.,* (2002), molluscs is the most constitute of major source of heavy metals in food after fish. The present study showed that the heavy metals contents that have been constituted in food at Cochin area, India were still under the limitations of Europian Union (EU) and United States Food and Drugs Administration (USFDA). Cd, Pb and Hg are the heavy metals that have been traced in the shell food, which contained 28%, 47% and 3% respectively.

In general, the fish and shell food were still under controlled and still safe for human consumption.

Based on the statistics from Department of Environmental Malaysia (2017), 80% of environment had been polluted by heavy metal which caused from anthropogenic activities by human. The needed of heavy metals in aquatic life is also important especially for their biological process and growth population (Achard, 2004). However, too much amount of heavy metals accumulated also may cause negative impacts especially to health effects. The essential amount that needed to be taken should not exceed the standard limit that have been regulated which is Malaysian Regulations Limit (1985).

Due to the increasing population in world and coupled with the rapid development of technology and industry, many heavy metals that have been discharged and may give bad impacts to the environment. (Department of Environmental Malaysia, 2017). Cd, Pb, Ni and Cr are some of heavy metals that can be categorized as non-essential because the amount of toxicity content in the metals are higher even at a very low concentration, thus they were not suitable for the essential use in metabolic activities. This also supported by Wang *et al.,* (2015) which stated that the contaminated bivalves is mostly being effected by the anthropogenic activities by humans that particulates more dangerous wastes to the surroundings and environment.

Lau *et. al.,* (2008) stated that food is the most exposure to heavy metals contamination. The hygiene preparation for the food should be aware to minimize the contaminants in the food, thus the health impacts from heavy metals contamination cases can be reduced. The metals that have been extensively studied and their effect are regularly reviewed by international bodies such as WHO are Cd, Pb, Hg and Ar (Foster *et al.*, 2017). Recent data indicate that adverse health effects of cadmium exposure may occur at lower exposure levels than previously anticipated, primarily in the form of kidney damage but possibly also bone effects and fractures.

CHAPTER 3

MATERIALS AND METHOD

This research study is mainly involved sampling process, analysis of heavy metal that include chemical analysis process, laboratory analysis process and data analysis process.

3.1 Study Area

Sampling process of *C. fluminea* were carried out in two districts in Kelantan, which are Pasir Mas and Tumpat. These two districts were being chosen as the site area because both districts were the main collection place to collect etak in Kelantan and main distributors place to distribute etak to other states for exporting activities. Pasir Mas districts (6.0424° N, 102.1428° E) which has a cover area of 614.15 km^2 with 185,741 people of the total population (Department of Statistics Malaysia, 2017), is a border of Malaysia with Thailand separated with Golok River as shown at Figure 3.2. This state also acts as the main gateway of the East Coast of Malaysia to Thailand.

While for Tumpat district (6.1991° N, 102.1694° E), it cover an area of 169.5km^2 with total population of 152,168 people (Department of Statistics Malaysia, 2017).

Tumpat is the northernmo location of Kelantan as it was bordering with Thailand across the Golok River. The speciality of Tumpat is its strategic end location make it as the end of the East Coast Line for train line operated by Keretapi Tanah Melayu and also other hub transportations in Kelantan.

Figure 3.1 : Location of Study Area

(Source from Google Maps)

3.2 Materials

Global Positioning System (GPS), plastic zip bag, digital callipers, balance callipers, blade, 35% of HNO_3 concentration, 65% of HCl concentration, 250 mL digestion flask, 50 mL standard flasks, deionized water, filter paper Whatsman No 41, .45 mm syringe filter, 150 ml fallon tube, Perkin Elmer PinAAcle 900F Atomic Absorption Spectrometer (AAS) based on the standard of Perkin Elmer Corporation 1996, Statistical Package for the Social Science application (SPSS) version 20.

3.3 Method

3.3.1 Sampling Process

Smoked *C. fluminea* samples for sampling process of this research study were collected from similar types at Pasir Mas and Tumpat to avoid environmental variations or sample bias. Details of the smoked etak including the weight of smoked etak analysed from each site, site descriptions such as location and name of sellers and coordinates of the sampling sites using Global Positioning System (GPS) were recorded. There times of sampling replication was done starting from March 2018 to May 2018. All the sampling points that were carried out from the districts aforementioned, which three site were located at Pasir Mas and three more sites were located at Tumpat, as shown in Table 3.1.

Table 3.1 : Coordinates of the sampling points

Pasir Mas	
Site 1	6.0495938° N, 102.1693547° E
Site 2	6.0497306° N, 102.1691667° E
Site 3	6.0497333° N, 102.1691667° E
Tumpat	
Site 4	6.1379039° N, 102.170987° E
Site 5	6.1467273° N, 102.218605° E
Site 6	6.1450348° N, 102.222301° E

The sampling process for the smoked etak was carried out in the smoking processing site as shown in Appendix A.1 and placed in a plastic bag. Six samples were collected aseptically for each month and was labelled with date and collecting site for each of the sample. After that, the samples were transported to the laboratory by kept it in the ice box with ice until further analysis.

3.3.2 Morphological Measurement Process

The objective of this process is to standardize the collected sample for *C. fluminea* species to avoid any rare species included and to minimize the bias or error that may happened. In addition, through this morphological measurement process, the common species for *C. fluminea* being used as experiment sample for this research can be identified. The features that need to be measured for morphology of *C. fluminea* were length, height, width and weight (mean ± standard error). For length, width and height data, the apparatus that being used was digital vernier calliper while for weight data, analytical balance was being used as shown in Appendix A.2. 100 samples of *C. fluminea* were taken randomly from all the samples collected for this process. As an average, the length, width, height and weight of *C. fluminea* were 16.25±0.74, 16.17±0.48, 21.98±0.68 and 5.11±0.27 respectively. The measurement process was being done before the samples being preserved in the freezer at about -20°C to maintain the morphology data of *C. fluminea*.

3.3.3 Sample Preparation Process

All the apparatus that being used in this research included plastics and glass material were rinsed and soaked in 10% of HNO_3 for an overnight. This acid washing is important to remove bound metals and essentials for some of highly sensitive experiments with metals (Lewenza, 2014). Then, the apparatus were rinsed with deionized water and dried them in oven commonly in 60˚C before used.

For the preparation of sample, the frozen smoked etak were opened and separated the soft tissues and the shells after being placed in room temperature to thaw them partially before opening for an hour. Next, the soft tissues and the shell samples of smoked etak were dried in an oven (as shown in Appendix A.3) at 65˚C, which was the most suitable temperature to remove all water from controlled sample until the weight does not vary more than 0.01% for 3 days until they reach constant dry weights (DWT) (Conners *et al.,* 2009). After obtained the constant weight, the samples were grinned by using blender until fine powders and kept in the desiccator for further analysis.

3.3.4 Chemical analysis process

For this research study, acid digestion is being used to analyse the heavy metal content in smoked *C. fluminea* based on the standard of Perkin Elmer Corporation 1996. Precisely, 5 g of dried and comminute soft tissues and shells sample of smoked *C. fluminea* were separately and accurately weight by using analytical balance. After that, the soft tissues and the shells sample were digested in 250 mL digestion flask by using aqua regia solution for acid digestion process.

i) Acid digestion for shell

8 mL of 35% hydrochloric acid (HCl) and 2 mL of 65% nitric acid (HNO_3) were being used for acid digestion process for shell sample. The samples were covered immediately with watch glass after all the samples being digested in the beaker. After the complete reaction of shell *C. fluminea* and the acid, the mixtures were heated at 60°C for 30 minutes by using hot plate (Appendix A.6). 4 mL of 35% HCl and 1 mL of 65% HNO_3 were added after the shell samples were allowed to cool. After that, the temperature of the hot plate were increased again slowly until 150°C for two hours until the colour of the solution become clear.

ii) Acid digestion for soft tissue

For soft tissue samples acid digestion process, 5 mL of 95% sulfuric acid (H_2SO_4) and 5 mL of 65% HNO_3 were being used. The samples were also covered immediately with watch glass same as the shell sample digestion process. After the complete reaction of soft tissue *C. fluminea* and the acid, the mixtures were heated at 60°C for 30 minutes by using hot plate. The samples were allowed to cool and then 20 mL of 65% HNO_3 were added. After that, the temperature were increased slowly until 150°C for two hours (repeated from shell sample digestion process) and after the solution were allowed to cool, 1 mL of 30% hydrogen peroxide (H_2O_2) was added until the colour become clear. The function of H_2O_2 is to stabilize the acidity content in the solution.

iii) Serial dilution method

The resultant clear solutions from shell and soft tissue sample were transferred into 50 mL standard flasks and made up to mark with deionized water. Filter paper Whatsman No 41 was being used to filter the previous solution for the preparation of stock solution. Then, 50 mL from the filter solution was transferred to the 150 mL Falcon tube by using 0.45 mm syringe filter. Original concentration solution was being made by transferred 15 mL from the stock solution to the Falcon Tube and 1.5 mL from the original solution were mixed with distilled water until made up to the 15 ml of the tube mark for dilution process. This dilution process was repeated until 10^{-4} solution (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). The last digested sample solution was determined its heavy metals concentrations content by using Atomic Absorption Spectrophotometer (AAS) and the average values of three determinations per sample were recorded.

3.3.5 Data Analysis

The stock solution of 1000 mg/L Cd, Cr, Cu, Fe, Pb and Zn based on the standard of Perkin Elmer Corporation were prepared. Each standard solution that reduced was top up by deionized water that contained the same volume percentage of acid as the digested samples Then, all of these elements were analyzed using Perkin Elmer PinAAcle 900F Atomic Absorption Spectrometer (AAS).

The correlation of heavy metals in shells and tissue of the sample were being analysed precisely by using Pearson's correlation in Statistical Package for the Social Science application (SPSS) version 20 as it was the most precise method for correlation analysis calculation (Conners *et al.,* 2009). In statistics, the Pearson's correlation coefficient (PCC) or the bivariate correlation is a measure of the linear correlation between two variables X and Y. It has a value between +1 and −1, where 1 is total positive linear correlation, 0 is no linear correlation and −1 is total negative linear correlation. The data were being analysed for each sampling from the average values of three determinations per shells and tissues sample that have been recorded from the AAS data. The content of Pb, Zn, Cu, Cd, Fe and Cr between the shells sample of smoked *C. fluminea* and tissue sample of smoked *C. fluminea* were the variables that being analysed in this correlation coefficient.

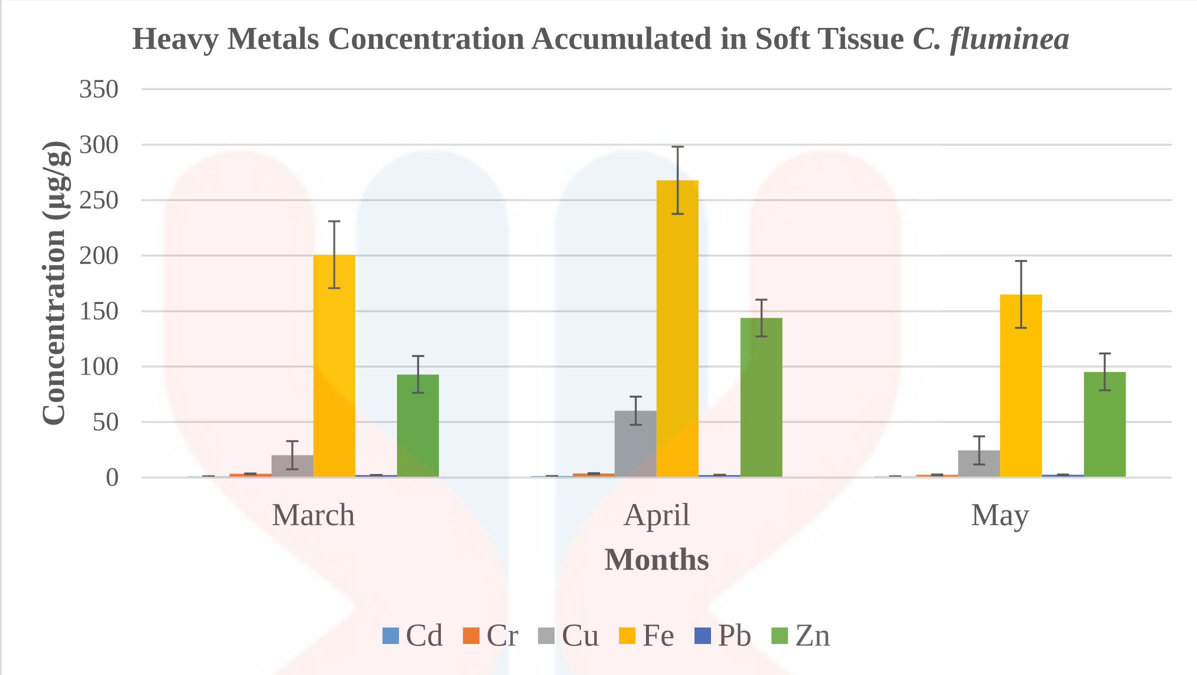
CHAPTER 4

RESULTS AND DISCUSSION

The research was done to study the correlation analysis of heavy metals in shells and tissues content of smoked *C. fluminea.* The correlation data of six heavy metals which were cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), zinc (Zn) and lead (Pb) were collected, analysed and summarized in this chapter.

4.1 Heavy Metals Content in Soft Tissue of *C. fluminea*

Concentration of heavy metals Cd, Cr, Cu, Fe, Pb and Zn in the soft tissue of smoked *C. fluminea* were analyzed for three times replication at Pasir Mas and Tumpat for three months (March-May). The trend of heavy metals concentration accumulated in soft tissues of *C. fluminea* for each month was illustrated in Figure 4.1.



	March	April	May
Cd	1.16	1.19	1.12
Cr	3.34	3.52	2.39
Cu	20.08	60.25	24.47
Fe	200.73	267.93	165.03
Pb	2.21	2.29	2.51
Zn	92.89	143.82	95.16

Figure 4.1 : Bar graph of heavy metal concentration accumulated in soft tissue *C. flumiea*

Figure 4.1 presented the comparison of all heavy metal concentration that have been analyzed (Cd, Cr, Cu, Fe, Pb and Zn) in the soft tissue of *C. fluminea* throughout three months. The range of heavy metals concentration accumulated was as followed : Fe : 165.03-267.93 µg/g, Zn : 92.89-143.82 µg/g, Cu : 20.08-60.25 µg/g, Cr : 2.39-3.52 µg/g, Pb : 2.21-2.51 µg/g and Cd : 1.12-1.19 µg/g.

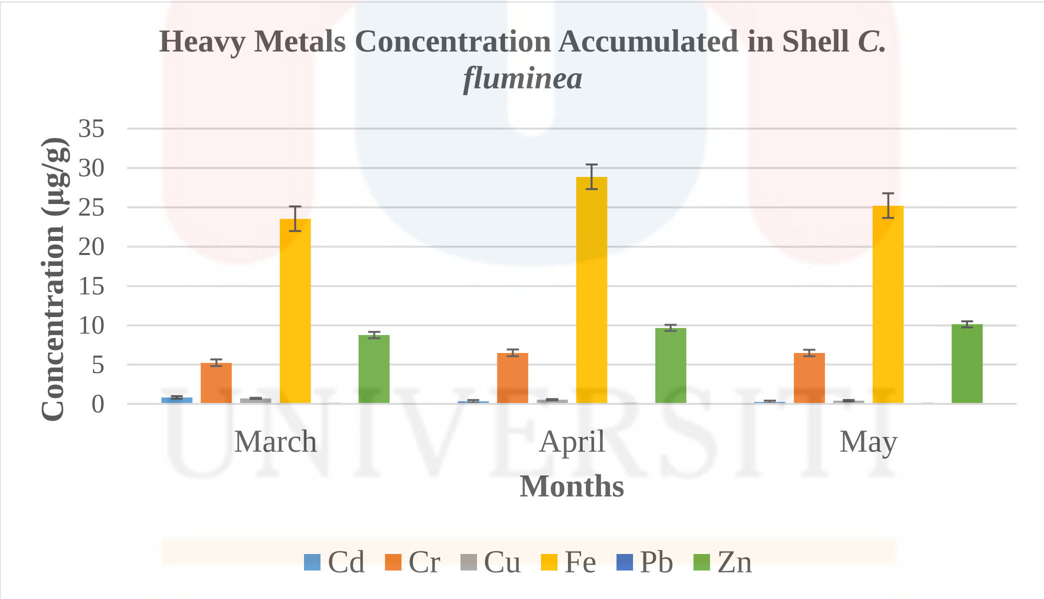
Based on the Figure 4.1, it clearly shown that Fe and Zn were the most abundant heavy metals accumulated in the soft tissues *C. fluminea* while Cd and Pb concentration levels were the lowest concentration accumulated. This indicates that the environment of *C. fluminea* is mostly surrounded with Fe and Zn as these two heavy metals were the major contributors in the environment especially for anthropogenic sources such as municipal waste disposal and discharge from industries (Shah *et al.*, 2016). As *C. fluminea* was a filter feeder organism, which strained all the food particles and suspended matter by water, the tendency of the soft tissue *C. fluminea* to absorb Fe and Zn from their habitat is highly possible. Other than that, Fe and Zn are the essential metals needed for the metabolic functions in the marine organisms (Hogstrand, 2012). These metals have high concentrations in the natural water especially in freshwater due to their accessibility to bind with water molecules. Thus, the bivalves tend to accumulate Fe and Zn in high concentration in their bodies.

Fe and Zn are the essential metals that play important role in human metabolism. However, the exceed intake of this metals causes adverse several effects. High levels of Zn in the body disrupt the homeostasis of other essential elements and suppress the absorption of Cu and Fe (Osredkar & Sustar, 2011). Besides, the excess of these metals can affect the brain system, disorder of respiratory system, gastrointestinal disease and prostate problem (Plum *et al.*, 2010).

After Fe and Zn, the concentration of heavy metals accumulated in soft tissue is Cu. The ranges for Cu concentration is 20.08-60.25 µg/g and mostly the sources of Cu concentration were comes from the pesticides from the agriculture activities and from the pathogens at fish culture ponds. This is supported by Mariam *et. al.*, (2016) indicates that Cu also one of the hazardous metals that needed in biological of aquatic life. For concentration of Cr (2.39-3.52 µg/g), it is lower than Cu but lower than Pb (2.21-2.51 µg/g) and Cd (1.12-1.19 µg/g) from the presented study. As a nutshell, the range of heavy metals accumulated in soft tissues of C. fluminea can be concluded as Fe > Zn > Cu > Cr > Pb > Cd.

4.2 Heavy Metals Content in Shell *C. flumimea*

The trend of heavy metals concentration (Cd, Cr, Cu, Fe, Pb and Zn) accumulated in shell of *C. fluminea* for each month (March – May) was illustrated in Figure 4.1.



	March	April	May
Cd	0.82	0.35	0.27
Cr	5.24	6.5	6.49
Cu	0.71	0.55	0.41
Fe	23.54	28.86	25.2
Pb	-55.13	-66.95	-60.09
Zn	8.77	9.67	10.13

Figure 4.2 : Bar graph of heavy metals concentration accumulated in shell *C. fluminea*

Figure 4.1 presented the comparison of all heavy metal concentration that have been analyzed (Cd, Cr, Cu, Fe, Pb and Zn) in the soft tissue of *C. fluminea* throughout three months. The range of heavy metals concentration accumulated was as followed: Fe : 23.54-28.86 µg/g, Zn : 8.77-10.13 µg/g, Cr : 5.24-6.5 µg/g, Cu : 0.41-0.71 µg/g, Cd : 0.27-0.82 µg/g and Pb : (-55.13) – (-66.95) µg/g.

Based on the Figure 4.1, it clearly shown that Fe, Zn and Cr were the most abundant heavy metals accumulated in the shell *C. fluminea* while Cd and Pb concentration levels were the lowest concentration accumulated. This indicates that *C. fluminea* accumulated both of the metals from their natural habitat as the concentration of Fe and Zn in shell is lower than in soft tissue. The smoking process for smoked *C. fluminea* may affect the content of the heavy metals accumulated as Fe and Zn were also has the highest concentration accumulated in shell but not as much as accumulated in soft tissues. Fe and Zn were acted as the major contributors in the environment especially for anthropogenic sources such as municipal waste disposal and discharge from industries (Shah *et al.*, 2016). As *C. fluminea* was a filter feeder organism, which strained all the food particles and suspended matter by water, the tendency of the soft tissue *C. fluminea* to absorb Fe and Zn from their habitat is highly possible.

After Fe and Zn, the order of concentration for heavy metals accumulated in shell were Cr (5.24-6.5 µg/g), Cu (0.41-0.71 µg/g), Cd (0.27-0.82 µg/g) and the lowest concentration is Pb with range from -55.13 to -66.95 µg/g. From Pb concentration in the presented study above, the negative concentrations that have been accumulated in the shells *C. fluminea* due to the existence of reaction with calcium carbonate ($CaCO_3$) that contained in the shell. In the shell of bivalve, mainly made of $CaCO_3$, calcite and aragonite are the prevalent forms but there are also highly unstable amorphous phases. The reaction of $CaCO_3$ with Pb is also indicates by Mendez (2006) which stated that Pb has an extreme toxicity content that the isotopes may react differently during chemical analysis. This post-transition metal type is a strong metallic that give positive reaction to the atmospheric nature (Mandez, 2006). The negative correlations of Pb accumulated in shell was considered as zero correlations in the Figure 4.2 and indicated as invalid content that did not accumulated in the shell. The amount of negative values in the correlations showed that there was no possible changes of the heavy metals content, either additional or reduction content, thus the heavy metals concentrations accumulated was considered as none (Peltier, 2016). As a nutshell, the range of heavy metals accumulated in shells of *C. fluminea* can be concluded as Fe > Zn > Cr > Cu > Cd > Pb.

4.3 Comparison of Heavy Metal Concentration Accumulated with Standard Regulation

The initial concentration of heavy metals in *C. fluminea* was determined to investigated the concentration of heavy metals in the clams before it undergo the acid digestion process and AAS analysis. Results of the average concentrations of heavy metals in clams and the permissible limit of Malaysian Food Regulation 1985 (MFR) and FAO/WHO (1984) were listed in the Table 4.1. The determination of initial concentration of heavy metals is important to determine the possible risk of *C. fluminea* consumption for human health and also for the exportation activities.

Table 4.1 : Concentration of heavy metals in tissues and shell of smoked *C. fluminea* (mean±SE) and the permissible limit of Malaysia Food Regulations (1985) and FAO/WHO (1984)

(Sources from Malaysian Food Regulations 2017)

	Cd (µg/g)	Cr (µg/g)	Cu (µg/g)	Pb (µg/g)	Zn (µg/g)	Fe (µg/g)
Concentration in Tissues	**1.16± 0.09**	3.42± 0.32	**34.93± 7.02**	**2.34± 0.24**	**110.62± 11.96**	**211.23± 24.20**
Concentration in Shells	0.48± 0.16	6.08± 0.70	0.56± 0.07	-60.72 ±5.29	9.53±0.91	25.86± 2.64
Permissible Limit :						
Malaysia Food Regulations (1985)	1.0	-	30.0	2.0	100	-
FAO/WHO (1984)	-	13.0	-	-	-	100.0

The concentration of heavy metals in *C. fluminea* was compared with permissible limit by Malaysia Food Regulations (1985) and FAO/WHO (1984). From the results, the average concentration of the heavy metals in the shells and tissues above such as Cd, Cu, Pb, Zn and Fe were already slightly exceed the permissible limit stated. Only concentration of Cr was still under the standard limit and considered as safe to be consumed. Previous studies done by Gatusso *et al.*, (2015) stated that the capacity of accumulating metals may vary among the aquatic organisms depending on their filtering activity and their positions in the water column. Thus, this study is very important to recognise which one of the heavy metals accumulated higher in *C. fluminea* in order to ensure it is safe to be consumed by human as well as the freshness of the etak needs to be maintained for the export purpose.

From the comparison data in Table 4.1, *C. fluminea* can be considered as highly risk to be consumed based on the variety of heavy metals concentration that has been accumulated in it. Most of the metals accumulated were highly contained in the soft tissue of *C. fluminea* compared to in shells mainly due to internal filter feeding characteristics which feed by straining suspended matter and food particles from water typically by passing the water over a specialized filtering structure. Age, growth rate, feeding habits of organisms, hardness and pH of water and availability of metal were suggested to be effective factors in the bioaccumulation of metals commonly for bivalves. (Demirak *et al.*, 2006). The possibility of the consumers to get osteomalacia (bone disease) from Cd, kidney malfunction from Fe (Wuana & Okiemen, 2011), liver disease from Cu, damage neurological system by Pb and brain system affection by Zn (Dubois *et al.*, 2007) is highly risk if too exceed the permissible limit.

4.4 Optimization of Correlation Analysis

In this research, data that being obtained from the Atomic Absorption Spectrophotometer (AAS) have been used for analysed the correlation coefficient between the heavy metals content in shells and the tissue of smoked *C. fluminea.* The type of correlation used in this analysis was Pearson's correlation as it was the most suitable correlation coefficient to find the linear relationship between two variables. Several parameters were varied in this analysis to assess their correlation content in shell and tissues such as Cd, Cr, Cu, Zn, Pb and Fe. The optimization analysis was done by having the results as showed in Appendix B.1.

4.4.1 Correlation of Cd Content

Table 4.2 showed that there is a strong relationship between Cd in shell and tissues content. This means that changes of Cd content in shell and tissues are strongly correlated with each other. As can referred to Table 4.2, the Pearson correlation is 0.972 and it is very close to 1. For this reason, this can be proved that there is a strong relationship between Cd in shell and tissues content.

The significance (2-Tailed) value for Cd content is equal to 0, which mean that there is a statistically significant correlations between two variables which is in tissues and in shells. That means, increases or decreases of Cd content in shells significantly relate to increases or decreases of Cd content in tissues.

Table 4.2 : Correlation of Cd content in shell and tissues of *C. fluminea*

		Cd in Tissues	Cd in Shells
Cd in Tissues	Pearson Correlation	1	0.972
	Sig. (2 tailed)		0
	N	16	16
Cd in Shells	Pearson Correlation	0.972	1
	Sig. (2 tailed)	0	
	N	16	16

4.2.2 Correlation of Cr Content

Table 4.3 showed that there is a very weak relationship between Cr in shell and tissues content. This means that changes of Cr content in shell and tissues are weakly correlated with each other. As can referred to Table 4.3, the Pearson correlation is 0.140 and it is very close to 0. This showed that Cr content in shell are not correlated with changes in the Cr content in tissues and vice versa. For this reason, this can be proved that there is a weak relationship between Cr in shell and tissues content.

The significance (2-Tailed) value for Cr content is equal to 0.604, which mean that there is no statistically significant correlations between two variables which are in tissues and in shells. That means, increases or decreases of Cr content in shells do not significantly relate to increases or decreases of Cr content in tissues.

Table 4.3 : Correlation of Cr content in shell and tissues of *C. fluminea*

		Cr in Tissues	Cr in Shells
Cr in Tissues	Pearson Correlation	1	0.140
	Sig. (2 tailed)		0.604
	N	16	16
Cr in Shells	Pearson Correlation	0.140	1
	Sig. (2 tailed)	0.604	
	N	16	16

4.2.3 Correlation of Cu Content

Table 4.4 showed that there is a very weak relationship between Cu in shell and tissues content. This means that changes of Cu content in shell and tissues are weakly correlated with each other. As can referred to Table 4.4, Pearson correlation is -0.217 and it is a negative correlation. For this reason, this can be proved that there is a weak relationship between Cu in shell and tissues content.

The significance (2-Tailed) value for Cu content is equal to 0.417, which mean that there is no statistically significant correlations between that two variables, in tissues and in shells. That means, increases or decreases of Cu content in shells do not significantly relate to increases or decreases of Cu content in tissues.

Table 4.4 : Correlation of Cu content in shell and tissues of *C. fluminea*

		Cu in Tissues	Cu in Shells
Cu in Tissues	Pearson Correlation	1	-0.217
	Sig. (2 tailed)		0.417
	N	16	16
Cu in Shells	Pearson Correlation	-0.217	1
	Sig. (2 tailed)	0.417	
	N	16	16

4.2.4 Correlation of Fe Content

Table 4.5 showed that there is a very weak relationship between Fe in shell and tissues content. This means that changes of Fe content in shell and tissues are weakly correlated with each other. As can referred to Table 4.5, Pearson correlation is 0.066 and it is very close to 0. This showed that Fe content in shell are not correlated with changes in the Fe content in tissues and vice versa. For this reason, this can be proved that there is a very weak relationship between Fe in shell and tissues content.

The significance (2-Tailed) value for Fe content is equal to 0.809, which mean that there is no statistically significant correlations between two variables which is in tissues and in shells. That means, increases or decreases of Fe content in shells do not significantly relate to increases or decreases of Fe content in tissues.

Table 4.5 : Correlation of Fe content in shell and tissues of *C. fluminea*

		Fe in Tissues	Fe in Shells
Fe in Tissues	Pearson Correlation	1	0.066
	Sig. (2 tailed)		0.809
	N	16	16
Fe in Shells	Pearson Correlation	0.066	1
	Sig. (2 tailed)	0.809	
	N	16	16

4.2.5 Correlation of Zn Content

Table 4.6 showed that there is a weak relationship between Zn in shell and tissues content. This means that changes of Zn content in shell and tissues are weakly correlated with each other. As can referred to Table 4.6, Pearson correlation is 0.201 and it is almost close to 0. This showed that Zn content in shell are weakly correlated with changes in the Zn content in tissues and vice versa. For this reason, this can be proved that there is a weak relationship between Zn in shell and tissues content.

The significance (2-Tailed) value for Zn content is equal to 0.455, which mean that there is no statistically significant correlations between two variables which are in tissues and in shells. That means, increases or decreases of Zn content in shells or tissues do not significantly relate to increases or decreases of Zn content in tissues.

Table 4.6 : Correlation of Zn content in shell and tissues of *C. fluminea*

		Zn in Tissues	Zn in Shells
Zn in Tissues	Pearson Correlation	1	0.201
	Sig. (2 tailed)		0.455
	N	16	16
Zn in Shells	Pearson Correlation	0.201	1
	Sig. (2 tailed)	0.455	
	N	16	16

4.2.6 Correlation of Pb Content

Table 4.7 showed that there is a moderate correlation relationship between Pb in shell and tissues content. This means that half or some changes of Pb content in shell and tissues are correlated with each other. As can referred to Table 4.7, Pearson correlation is 0.447 and it is in the intermediate range between 0 to 1. This showed that Pb content in shell are moderately correlated with changes in the Pb content in tissues and vice versa. For this reason, this can be proved that there is a moderate relationship between Zn in shell and tissues content.

The significance (2-Tailed) value for Pb content is equal to 0.082, which mean that there is no statistically significant correlations between two variables which are in tissues and in shells. That means, increases or decreases of Pb content in shells do not significantly relate to increases or decreases of Pb content in tissues.

Table 4.7 : Correlation of Pb content in shell and tissues of *C. fluminea*

		Pb in Tissues	Pb in Shells
Pb in Tissues	Pearson Correlation	1	0.447
	Sig. (2 tailed)		0.0882
	N	16	16
Pb in Shells	Pearson Correlation	0.447	1
	Sig. (2 tailed)	0.0882	
	N	16	16

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The results of the heavy metals concentration accumulated in *C. fluminea* showed the following order of abundance Fe> Zn> Cu> Cr > Pb > Cd in soft tissues while in shells, the order are as followed: Fe> Zn> Cr> Cu > Cd > Pb. The results of heavy metals indicated that the Cd, Cu, Pb, Zn and Fe are beyond the permissible limits set by Malaysia Food Regulation 1985 and FAO/WHO (1984). In this study shows that the shells of *C. fluminea* content of heavy metals in tissue is higher than the heavy metals content in the shells. Thus, the correlation of heavy metals content of smoked *C. fuminea* are mostly influenced from the soft tissue. According from this results, this etak were within the limit for human consumption. Mean metal concentration were found to be higher in the soft tissue than those in the shells. In this study also proved the limited ability of *C. fluminea* in accumulating various heavy metals from its habitat in different rates. Nevertheless, high concentration of metals in surrounding sediment might partially from anthropogenic activities which would reflect in metal levels in aquatic. From this study, the results showed the heavy metal content in *C. fluminea* are not accumulated from the shells. It is because all of the heavy metals correlation that have been analysed in shell is lower than in soft tissues.

The results revealed that the etak which have higher values of heavy metals content (Cd, Cu, Fe, Pb and Zn) than the food safety limits should be avoided in order to avoid any possible toxicological risks and heavy metal related diseases such as Parkinson' disease, Wilson's disease and Hallervorden-Spatz disease due to long term consumption. On the other hand, according to Malaysian Food Regulation (1985), about 30% and more than 50% sites are safe from Cd and Pb contamination, respectively and also the etak species from the other populations studied were safe for consumption.

There are a few recommendations for further study which are develop method to remove heavy metals in etak or bivalve to achieve the permissible limits set by Malaysian Food Regulations 1985 (MFR) and Commissions Regulation of EU (2006). More further study also should be carry out about others heavy metals that tend to have high possibilities accumulated in this *C. fluminea* such as mercury (Hg) which can cause food contamination. The details about the findings of the further study will help the sellers and the consumers know exactly the details of the contaminants content in smoked *C. fluminea* and be more aware when consume it. Others than that, the diversity of metal bioaccumulation patterns across both metals and bivalve species should be recognize and proper treatment in smoking process have to improve to reduce the heavy metal content in shell and soft tissue of *C. fluminea*. In summary, this research had successfully achieved its objectives study and will serve as a base for future studies and more further study need to be done in order to minimize the contaminants in the *C. fluminea*.

REFERENCES

Achard, M., Baudrimont, M., Boudou, A., & Bourdineaud, J. (2004). Induction of a multixenobiotic resistance protein (MXR) in the Asiatic clam Corbicula fluminea after heavy metals exposure. *Aquatic Toxicology, 67*(4), 347-357.

Al-Mohanna, S., & Subrahmanyam, M. (2001). Flux of heavy metal accumulation in various organs of the intertidal marine blue crab, Portunus pelagicus (L.) from the Kuwait coast after the Gulf War. *Environment International, 27*(4), 321-326.

Anacleto, P., Maulvault, A., Bandarra, N., Repolho, T., Nunes, M., Rosa, R., & Marques, A. (2014). *Effect of warming on protein, glycogen and fatty acid content of native and invasive clams, (64)*, 493-501.

Ansari, D., & Coch, D. (2006). Bridges over troubled waters: Education and cognitive neuroscience. *Trends in cognitive sciences, 10*(4), 146-151.

Antonkiewicz, J., Kołodziej, B., Bielińska, E. J., Witkowicz, R., & Tabor, S. (2018). Using Jerusalem Artichoke to Extract Heavy Metals from Municipal Sewage Sludge Amended Soil. *Polish Journal of Environmental Studies*, *27*(2), 513-527. doi:10.15244/pjoes/75200

Argente, F. A. T., & Estacion, J. S. (2014). Effect of different harvesting practices on the dynamics of Paphia textile (Gmelin 1792)(Bivalvia: Veneridae) populations at two sites in Zamboanga del Norte, Southern Philippines. *Environmental and Experimental Biology (12)*, 113-120.

Baby, J., Raj, J. S., Biby, E. T., Sankarganesh, P., Jeevitha, M., Ajisha, S., & Rajan, S. S. (2010). Toxic effect of heavy metals on aquatic environment. *International Journal of Biological and Chemical Sciences, 4*(4), 939-952.

Barlogie, B., Tricot, G., Anaissie, E., Shaughnessy, J., Rasmussen, E., van Rhee, Pineda-Roman, M. (2006). Thalidomide and hematopoietic-cell transplantation for multiple myeloma. *New England Journal of Medicine*, *354*(10), 1021-1030.

Basen, T., Gergs, R., Rothhaupt, K.-O., & Martin-Creuzburg, D. (2012). Phytoplankton food quality effects on gammarids: benthic–pelagic coupling mediated by an invasive freshwater clam. *Canadian journal of fisheries and aquatic sciences, 70*(2), 198-207.

Behjati, S., & Tarpey, P. S. (2013). What is next generation sequencing? *Archives of Disease in Childhood-Education and Practice, 98*(6), 236-238.

Bernardet, J.-F., & Nakagawa, Y. (2006). *An introduction to the family Flavobacteriaceae*: Springer.

Bharathi, K., Dwivedi, D., Agarwal, A. K., & Sharma, M. (2004). Diesel exhaust particulates characterization for heavy metals. *Indian Institute of Technology, 16*, 1-4.

Bhattacharya, P. T., Misra, S. R., & Hussain, M. (2016). Nutritional aspects of essential trace elements in oral health and disease: An extensive review. *Scientifica*, 12.

Bighiu, M. A., Eriksson-Wiklund, A.-K., & Eklund, B. (2017). Biofouling of leisure boats as a source of metal pollution. *Environmental Science and Pollution Research, 24*(1), 997-1006.

Bjerregaard, P., Andersen, C. B., & Andersen, O. (2015). Ecotoxicology of metals—sources, transport, and effects on the ecosystem *Handbook on the Toxicology of Metals (Fourth Edition)* (425-459): Elsevier.

Bonnail, E., Sarmiento, A., DelValls, T., Nieto, J., & Riba, I. (2016). Assessment of metal contamination, bioavailability, toxicity and bioaccumulation in extreme metallic environments (Iberian Pyrite Belt) using *Corbicula fluminea*. *Science of the Total Environment, 544*, 1031-1044.

Borgulat, J., Mętrak, M., Staszewski, T., Wiłkomirski, B., & Suska-Malawska, M. (2018). Heavy Metals Accumulation in Soil and Plants of Polish Peat Bogs. *Polish Journal of Environmental Studies, 27*(2), 362-370.

Broadley, M. R., White, P. J., Hammond, J. P., Zelko, I., & Lux, A. (2007). Zinc in plants. *New Phytologist, 173*(4), 677-702.

Budin, K., Praveena, S. M., Sakari, M., Hassan, S., & Ibrahim, E. I. (2014). Health risk assessment of heavy metals via consumption of bivalves species in Kota Kinabalu, Sabah, Malaysia. *From Sources to Solution* (585-590): Springer.

CABI. (2018). *Corbicula fluminea* (Asian Clam). Retrieved on July 10, 2018 from https://www.cabi.org/isc/datasheet/88200

Canfield, R. L., Jusko, T. A., & Kordas, K. (2005). Environmental lead exposure and children's cognitive function. *The Italian Journal of Pediatrics, 31*(6), 293-300.

Chen, M., & Ma, L. Q. (2001). Comparison of Three Aqua Regia Digestion Methods for Twenty Florida Soils Approved for publication as Florida Agricultural Experiment Station Journal Series no. R-06802. *Soil science society of America Journal, 65*(2), 491-499

Conners, D. E., Westerfield, S. M., Feyko, A., & Black, M. C. (2009). Lead accumulation in soft tissues and shells of Asiatic clams *(Corbicula fluminea)*. *Georgia Institute of Techology Library, 54*(9), 129-135.

Coordinators, N. R. (2017). Database resources of the national center for biotechnology information. *Nucleic acids research, 45*(Database issue), D12.

Da Silva, J. B. B., da Silva, M. A. M., Curtius, A. J., & Welz, B. (2009). Determination of Ag, Pb and Sn in aqua regia extracts from sediments by electrothermal atomic absorption spectrometry using Ru as a permanent modifier. *Journal of Analytical Atomic Spectrometry, 14*(11), 1737-1742.

DeForest, D. K., Brix, K. V., & Adams, W. J. (2007). Assessing metal bioaccumulation in aquatic environments: the inverse relationship between bioaccumulation factors, trophic transfer factors and exposure concentration. *Aquatic Toxicology, 84*(2), 236-246.

El-Gamal, M. (2011). The effect of depuration on heavy metals, petroleum hydrocarbons, and microbial contamination levels in Paphia undulata (Bivalvia: Veneridae). *Czech Journal of Animal Science, 56*(8), 345-354.

Food and Agriculture Organization. (2017). Globefish - Analysis and information on world fish trade. Retrieved on May 5, 2018 from http://www.fao.org/in-action/globefish/en/

Forde, B. M., & O'Toole, P. W. (2013). Next-generation sequencing technologies and their impact on microbial genomics. *Briefings in Functional Genomics, 12*(5), 440-453. doi:10.1093/bfgp/els062

Foster, A. M., Fuller, P., Benson, A., Constant, S., Raikow, D., Larson, J., *et al.* (2017). *Corbicula fluminea* (O. F. Müller, 1774): U.S. Geological Survey, Nonindigenous Aquatic Species Database, .

Frank, S. N., Singer, C., & Sures, B. (2008). Metallothionein (MT) response after chronic palladium exposure in the zebra mussel, *Dreissena polymorpha*. *Environmental Research, 108*(3), 309-314.

Fraysse, B., Baudin, J.-P., Garnier-Laplace, J., Adam, C., & Boudou, A. (2002). Effects of Cd and Zn waterborne exposure on the uptake and depuration of 57Co, 110mAg and 134Cs by the Asiatic clam (Corbicula fluminea) and the zebra mussel (Dreissena polymorpha)—whole organism study. *Environmental pollution, 118*(3), 297-306.

Freitas, P. S., Clarke, L. J., Kennedy, H., & Richardson, C. A. (2016). Manganese in the shell of the bivalve *Mytilus edulis*: Seawater Mn or physiological control? *Geochimica et Cosmochimica Acta, 194*, 266-278.

Gajardo, K., Rodiles, A., Kortner, T. M., Krogdahl, Å., Bakke, A. M., Merrifield, D. L., *et al.* (2016). A high-resolution map of the gut microbiota in Atlantic salmon (*Salmo salar*): A basis for comparative gut microbial research. *Scientific Reports, 6*, 30893.

Gakwisiri, C., Raut, N., Al-Saadi, A., Al-Aisri, S., & Al-Ajmi, A. (2012). A critical review of removal of zinc from wastewater. *Proceedings of the World Congress on Engineering, 1*, 627-630.

Ganguly, K., Levänen, B., Palmberg, L., Åkesson, A., & Lindén, A. (2018). Cadmium in tobacco smokers: a neglected link to lung disease? *European Respiratory Review, 27*(147).

Garcia-Aljaro, C., Momba, M., & Muniesa, M. (2017). *Pathogenic members of Escherichia coli & Shigella spp. Shigellosis*: Michigan State University, E. Lansing, MI, UNESCO.

García, R., & Báez, A. (2012). Atomic Absorption Spectrometry (AAS), Atomic Absorption Spectroscopy. Retrieved on February 24, 2018 from www.intechopen.com/books/atomic-absorptionspectroscopy/atomic-absorption-spectrometry-aas

Ferreira, K. N., Iverson, T. M., Maghlaoui, K., Barber, J., & Iwata, S. (2004). Architecture of the photosynthetic oxygen-evolving center. *Science, 303*(5665), 1831-1838.

Fu, F., & Wang, Q. (2011). Removal of heavy metal ions from wastewaters: a review. *Journal of environmental management, 92*(3), 407-418.

Graczyk, T., Conn, D., Marcogliese, D., Graczyk, H., & De Lafontaine, Y. (2003). Accumulation of human waterborne parasites by zebra mussels (Dreissena polymorpha) and Asian freshwater clams (Corbicula fluminea). *Parasitology Research*, *89*(2), 107-112.

Gundacker, C. (2010). Comparison of heavy metal bioaccumulation in freshwater molluscs of urban river habitats in Vienna. *Environmental pollution*, *110*(1), 61-71.

Hedouin, L., Metian, M., Teyssie, J.-L., Fowler, S., Fichez, R., & Warnau, M. (2006). Allometric relationships in the bioconcentration of heavy metals by the edible tropical clam Gafrarium tumidum. *Science of the Total Environment*, *366*(1), 154-163.

Jarup, L. (2003). Hazards of heavy metal contamination. *British medical bulletin*, *68*(1), 167-182.

Khan Academy. (2017). Polymerase chain reaction. Retrieved on August 18, 2017 from https://www.khanacademy.org/science/biology/biotech-dna-technology/dna-sequencing-pcr-electrophoresis/a/polymerase-chain-reaction-pcr

Kibret, M., & Abera, B. (2012). The sanitary conditions of food service establishments and food safety knowledge and practices of food handlers in bahir dar town. *Ethiopian journal of health sciences*, *22*(1), 27-35.

Kim, Y.-J., Yoshizawa, M., Takenaka, S., Murakami, S., & Aoki, K. (2002). Isolation and culture conditions of a *Klebsiella pneumoniae* strain that can utilize ammonium and nitrate ions simultaneously with controlled iron and molybdate ion concentrations. *Bioscience, Biotechnology, and Biochemistry*, *66*(5), 996-1001.

Kotteeswaran, M., Sundaresan, S., Preethy, R., & Senthilkumar, B. (2015). Analysis of some heavy metals in water, soil and fish from Pulliyankannu Lake at Ranipet, Vellore Dist., Tamilnadu, India. *International Journal of Pharmaceutical and Biological Archives*, *6*(2), 32-36.

Kraus, U., & Wiegand, J. (2006). Long-term effects of the Aznalcóllar mine spill—heavy metal content and mobility in soils and sediments of the Guadiamar river valley (SW Spain). *Science of the Total Environment*, *367*(2-3), 855-871.

Kucuksezgin, F., Kacar, A., Kucuksezgin, G., & Uluturhan, E. (2010). Monitoring metal contamination levels and fecal pollution in clam (*Tapes decussatus*) collected from Izmir Bay (Turkey). *Environmental Monitoring and Assessment*, *162*(1), 407-415.

Kucuksezgin, F., Pazi, I., Yucel-Gier, G., Akcali, B., & Galgani, F. (2013). Monitoring of heavy metal and organic compound levels along the Eastern Aegean coast with transplanted mussels. *Chemosphere*, *93*(8), 1511-1518.

Kumar, V., Sinha, A. K., Rodrigues, P. P., Mubiana, V. K., Blust, R., & De Boeck, G. (2015). Linking environmental heavy metal concentrations and salinity gradients with metal accumulation and their effects: a case study in 3 mussel species of Vitória estuary and Espírito Santo bay, Southeast Brazil. *Science of the Total Environment*, *523*, 1-15.

Kumari, L. K., Kaisary, S., & Rodrigues, V. (2006). Bio-accumulation of some trace metals in the short-neck clam Paphia malabarica from Mandovi estuary, Goa. *Environment International, 32*(2), 229-234.

La Guardia, M. J., Hale, R. C., Harvey, E., Mainor, T. M., & Ciparis, S. (2012). In situ accumulation of HBCD, PBDEs, and several alternative flame-retardants in the bivalve (Corbicula fluminea) and gastropod (Elimia proxima). *Environmental science & technology, 46*(11), 5798-5805.

Lau, S., Mohamed, M., Yen, A. T. C., & Su'Ut, S. (2008). Accumulation of heavy metals in freshwater molluscs. *Science of the Total Environment, 214*(1-3), 113-121.

Lewbart, G. A., Christian, L. S., Harms, C. A., & Van Wettere, A. J. (2010). A comparison of heavy metal concentrations and health assessment in Asian clams *Corbicula fluminea* from Florida and North Carolina. *Journal of Aquatic Animal Health, 22*(2), 73-77.

Li, X., Poon, C.-s., & Liu, P. S. (2001). Heavy metal contamination of urban soils and street dusts in Hong Kong. *Applied geochemistry, 16*(11-12), 1361-1368.

Lin, H.-T., Wong, S.-S., & Li, G.-C. (2004). Heavy metal content of rice and shellfish in Taiwan. *Journal of food and drug analysis, 12*(2), 167-174.

Martin, S., & Griswold, W. (2009). Human health effects of heavy metals. *Environmental Science and Technology briefs for citizens, 15*, 1-6.

Mazorra, M., Rubio, J., & Blasco, J. (2002). Acid and alkaline phosphatase activities in the clam Scrobicularia plana: kinetic characteristics and effects of heavy metals. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 131*(2), 241-249.

Mendez, L., Palacios, E., Acosta, B., Monsalvo-Spencer, P., & Alvarez-Castañeda, T. (2006). Heavy metals in the clam Megapitaria squalida collected from wild and phosphorite mine-impacted sites in Baja California, Mexico. *Biological trace element research, 110*(3), 275.

Mouthon, J. (2001). Life cycle and population dynamics of the Asian clam Corbicula fluminea (Bivalvia: Corbiculidae) in the Saone River at Lyon (France). *Hydrobiologia, 452*(1-3), 109-119.

Naser, H. A. (2013). Assessment and management of heavy metal pollution in the marine environment of the Arabian Gulf: a review. *Marine pollution bulletin, 72*(1), 6-13.

National Oceanic and Atmospheric Administration Coordinators, Forney, K. A., Muto, M. M., Baker, J., Barlow, J., Black, N., Calambokidis, J., Ellis, G. (2017).

Patrick, C. H., Waters, M. N., & Golladay, S. W. (2017). The distribution and ecological role of *Corbicula fluminea* (Müller, 1774) in a large and shallow reservoir. *BioInvasions Record, 6*(1), 39-48.

Rahman, M. S., Saha, N., Molla, A. H., & Al-Reza, S. M. (2014). Assessment of anthropogenic influence on heavy metals contamination in the aquatic ecosystem components: Water, sediment, and fish. *Soil and Sediment Contamination: An Internati*

Raj, S. P., & Ram, P. A. (2013). Determination and contamination assessment of Pb, Cd, and Hg in roadside dust along Kathmandu-Bhaktapur road section of Arniko Highway, Nepal. *Research Journal of the Chemical Sciences, 3*, 18-25.

Robertson, A. G., Kim, J., Al-Ahmadie, H., Bellmunt, J., Guo, G., Cherniack, A. D., . . . Akbani, R. (2017). Comprehensive molecular characterization of muscle-invasive bladder cancer. *Cell, 171*(3), 540-556. e525.

Ruilian, Y., Xing, Y., Yuanhui, Z., Gongren, H., & Xianglin, T. (2008). Heavy metal pollution in intertidal sediments from Quanzhou Bay, China. *Journal of Environmental Sciences, 20*(6), 664-669.

Sakan, S., Darvodevic, D., Devic, G., Relic, D., Andelkovic, I., & Duricic, J. (2011). A study of trace element contamination in river sediments in Serbia using microwave-assisted aqua regia digestion and multivariate statistical analysis. *Microchemical Journal, 99*(2), 492-502.

Sastre, J., Sahuquillo, A., Vidal, M., & Rauret, G. (2002). Determination of Cd, Cu, Pb and Zn in environmental samples: microwave-assisted total digestion versus aqua regia and nitric acid extraction. *Analytica Chimica Acta, 462*(1), 59-72.

Shau-Hwai, A. T., & Yasin, Z. (2003). Status of giant clams in Malaysia. *SPC Trochus Inform Bull, 10*, 9-10.

Siah, A., Pellerin, J., Amiard, J.-C., Pelletier, E., & Viglino, L. (2003). Delayed gametogenesis and progesterone levels in soft-shell clams (Mya arenaria) in relation to in situ contamination to organotins and heavy metals in the St. Lawrence River (Canada). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 135*(2), 145-156.

Srinivasan, M., & Swain, G. W. (2007). Managing the use of copper-based antifouling paints. *Environmental Management, 39*(3), 423-441.

Szefer, P., Frelek, K., Szefer, K., Lee, C.-B., Kim, B.-S., Warzocha, J., . . . Ciesielski, T. (2002). Distribution and relationships of trace metals in soft tissue, byssus and shells of Mytilus edulis trossulus from the southern Baltic. *Environmental pollution, 120*(2), 423-444.

Udechukwu, B. E., & Ismail, A. (2013). Copper and Zinc in commonly consumed bivalves from Pantai Remis Jeram, Selangor, Malaysia. *Journal of Tropical Marine Ecosystem, 3*(1)

Wang, H., Liu, Y., Zhao, J., Hu, S., Wang, Y., Liu, C., & Zhang, Y. (2015). A Simple Digestion Method with a Lefort Aqua Regia Solution for Diatom Extraction. *Journal of forensic sciences, 60*(1), 778-783.

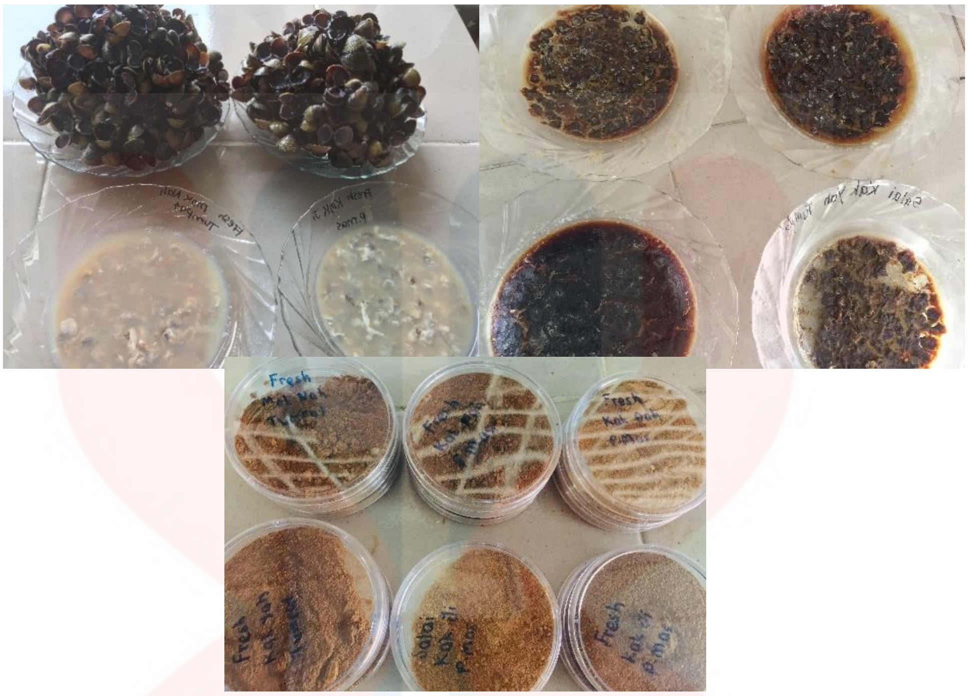
Yap, C., Ismail, A., & Tan, S. (2003). Can the byssus of green-lipped mussel Perna viridis (Linnaeus) from the west coast of Peninsular Malaysia be a biomonitoring organ for Cd, Pb and Zn? Field and laboratory studies. *Environment International, 29*(4), 521-528.

Zídková, J., Melčová, M., Bartošová, K., Šestáková, I., Zídek, V., Száková, J., . . . Tlustoš, P. (2014). Impact of cadmium on the level of hepatic metallothioneins, essential elements, and selected enzymes in the experimental rat model. *Czech Journal of Animal Science, 59*(12), 548-556.

APPENDIX A

Appendix A.1 : Sampling process of smoked *C. fluminea* : i) Smoking processing stage ii) One of the smoking processing site at Pasir Mas ; iii) *C. fluminea* before smoking stage

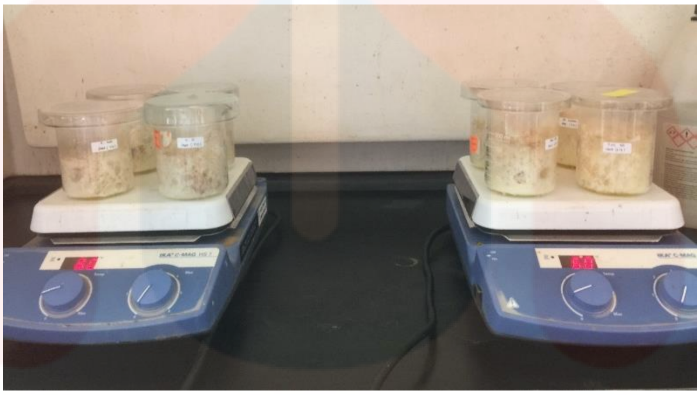
Appendix A.2 : Morphological Measurement Process : i) Measuring the width of *C. fluminea* by using digital vernier calliper : ii) Weighing *C. fluminea* using analytical balance



Appendix A.3 : Sample preparation process : i) Smoked *C. fluminea* after being thawed partially ; ii) Soft tissue of smoked C. fluminea after being dried ; iii) And after being grinded

Appendix A.4 : i) Shell of smoked *C. fluminea* after being dried ; ii) And after being grinded

Appendix A.5 : Preparation of acid digestion process



Appendix A.6 : Apparatus set up for acid digestion process

APPENDIX B

Appendix B.1 : Pearson's Correlation data of heavy metals concentration accumulated in shells and soft tissues of smoked *C. fluminea*

Correlations

		Tissue_Cd	Shell_Cd	Tissue_Cr	Shell_Cr	Tissue_Cu	Shell_Cu	Tissue_Fe	Shell_Fe	Tissue_Pb	Shell_Pb	Tissue_Zn	Shell_Zn
Tissue_Cd	Pearson Correlation	1	.972**	-.133	-.313	-.147	.477	-.055	-.196	.251	.330	-.008	.067
	Sig. (2-tailed)		.000	.622	.238	.587	.062	.841	.467	.349	.212	.976	.804
	N	16	16	16	16	16	16	16	16	16	16	16	16
Shell_Cd	Pearson Correlation	.972**	1	-.178	-.212	-.174	.536*	-.046	-.068	.103	.309	-.095	.014
	Sig. (2-tailed)	.000		.510	.431	.520	.033	.866	.802	.703	.244	.728	.958
	N	16	16	16	16	16	16	16	16	16	16	16	16
Tissue_Cr	Pearson Correlation	-.133	-.178	1	.140	-.167	.074	.138	-.122	.623**	.379	.264	-.337
	Sig. (2-tailed)	.622	.510		.604	.537	.785	.611	.652	.010	.148	.324	.202
	N	16	16	16	16	16	16	16	16	16	16	16	16
Shell_Cr	Pearson Correlation	-.313	-.212	.140	1	.049	-.322	-.059	.832**	-.220	-.326	-.201	-.010
	Sig. (2-tailed)	.238	.431	.604		.857	.223	.829	.000	.414	.218	.454	.969
	N	16	16	16	16	16	16	16	16	16	16	16	16
Tissue_Cu	Pearson Correlation	-.147	-.174	-.167	.049	1	-.217	.623**	.125	-.111	-.069	.082	-.213

	Sig. (2-tailed)	.587	.520	.537	.857		.419	.010	.644	.682	.801	.762	.428
	N	16	16	16	16	16	16	16	16	16	16	16	16
Shell_Cu	Pearson Correlation	.477	.536*	.074	-.322	-.217	1	-.024	-.055	-.043	.300	-.163	-.156
	Sig. (2-tailed)	.062	.033	.785	.223	.419		.931	.839	.875	.259	.545	.564
	N	16	16	16	16	16	16	16	16	16	16	16	16
Tissue_Fe	Pearson Correlation	-.055	-.046	.138	-.059	.623**	-.024	1	.066	.232	.175	.166	-.008
	Sig. (2-tailed)	.841	.866	.611	.829	.010	.931		.809	.387	.518	.538	.976
	N	16	16	16	16	16	16	16	16	16	16	16	16
Shell_Fe	Pearson Correlation	-.196	-.068	-.122	.832**	.125	-.055	.066	1	-.471	-.414	-.083	.162
	Sig. (2-tailed)	.467	.802	.652	.000	.644	.839	.809		.066	.111	.760	.548
	N	16	16	16	16	16	16	16	16	16	16	16	16
Tissue_Pb	Pearson Correlation	.251	.103	.623**	-.220	-.111	-.043	.232	-.471	1	.447	.216	.108
	Sig. (2-tailed)	.349	.703	.010	.414	.682	.875	.387	.066		.082	.423	.690
	N	16	16	16	16	16	16	16	16	16	16	16	16
Shell_Pb	Pearson Correlation	.330	.309	.379	-.326	-.069	.300	.175	-.414	.447	1	.067	-.506*
	Sig. (2-tailed)	.212	.244	.148	.218	.801	.259	.518	.111	.082		.807	.045
	N	16	16	16	16	16	16	16	16	16	16	16	16
Tissue_Zn	Pearson Correlation	-.008	-.095	.264	-.201	.082	-.163	.166	-.083	.216	.067	1	.201
	Sig. (2-tailed)	.976	.728	.324	.454	.762	.545	.538	.760	.423	.807		.455
	N	16	16	16	16	16	16	16	16	16	16	16	16

Shell_Zn	Pearson Correlation	.067	.014	-.337	-.010	-.213	-.156	-.008	.162	.108	-.506*	.201	1
	Sig. (2-tailed)	.804	.958	.202	.969	.428	.564	.976	.548	.690	.045	.455	
	N	16	16	16	16	16	16	16	16	16	16	16	16

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Appendix B.2 : Morphological Measurement of Data **of** Smoked *C. fluminea*

Sample	Length (mm)	Width (mm)	Height (mm)	Weight (g)
1	22.87	18.26	22.12	6.32
2	24.59	17.12	23.52	6.18
3	25.69	18.09	24.19	6.1
4	23.17	17.06	23	6.56
5	19.68	15.62	19.09	3.55
6	22.01	17.51	21.95	5.54
7	23.97	17	22.51	5.09
8	19.8	16.28	19.05	4.24
9	23.69	17.98	22.01	5.91
10	23.05	16.75	22.43	5.91
11	23.91	15.15	22.05	5.87
12	23.54	15.95	22.01	6.01
13	25.91	17.02	24.33	6.71
14	23.15	15.93	22.09	6.07
15	24.17	17.03	23.07	6.75
16	25.93	17.1	24.35	6.32
17	23.31	15.71	22.1	5.32
18	22.25	15.23	21.59	5.72
19	22.15	17.96	23.5	5.68
20	20.23	15.01	21.19	4.35
21	16.45	14.43	17.88	3.11
22	16.64	15.21	19.25	3.82
23	18.11	15.42	18.6	3.76
24	19.05	16.11	20.42	4.35
25	17.92	15.04	18.01	3.52

26	19.21	16.79	20.34	4.93
27	24.95	17.53	23.31	6.18
28	22.67	18.76	23.07	4.98
29	20.29	15.74	21.42	5
30	22.71	15.58	23.42	5.13
31	22.13	18.16	22.01	6.13
32	23.95	18.9	24.03	6.31
33	22.46	16.48	22.9	5.01
34	22.15	17.17	23.45	6.22
35	21.7	16.01	21.74	5.92
36	22.63	15.53	22.26	5.12
37	25.71	18.19	24.12	6.07
38	22.81	16.21	22.1	6.23
39	24.59	17.02	23.51	6.08
40	25.7	18.09	24.91	6.11
41	21.36	16.72	22.71	5.06
42	22.32	15.06	22.16	4.33
43	23.61	16.28	24.23	5.8
44	21.25	16.2	21.04	4.43
45	22.14	16.48	22.78	5.63
46	19.74	14.33	20.24	3.64
47	20.63	16.15	22.43	4.9
48	21.39	13.58	18.46	3.28
49	23.66	14.85	22.21	4.54
50	20.47	15.63	21.69	4.72
51	19.32	15.73	20.05	4.43
52	23.81	17.24	23.32	5.8
53	22.62	15.6	22.09	4.7

54	22.87	17.74	24.24	6.54
55	21.68	16.31	22.26	5.53
56	22.71	15.27	21.27	4.52
57	23.75	18.02	24.33	6.89
58	23.2	15.97	23.76	5.78
59	16.38	13.96	18.63	3.25
60	18.17	15.42	17.93	3.76
61	21.71	16.82	22.15	5.16
62	22.35	15.16	22.17	4.34
63	23.62	16.71	24.21	5.8
64	21.25	16.2	21.51	4.42
65	22.15	16.51	22.79	5.69
66	19.72	14.38	20.31	3.62
67	20.63	16.17	22.67	4.97
68	21.31	13.71	18.49	3.17
69	23.67	14.91	22.32	4.54
70	20.47	15.31	21.67	4.39
71	19.12	15.82	20.75	4.46
72	23.81	17.21	23.72	5.91
73	22.79	15.16	22.01	4.71
74	22.73	17.68	24.93	6.54
75	21.39	16.75	23.2	5.53
76	22.72	15.37	21.73	4.13
77	23.75	18.21	24.93	6.82
78	23.4	15.95	23.71	5.78
79	16.78	13.73	18.94	3.25
80	15.97	16.74	16.13	4.01
81	21.73	16.79	22.14	5.16

82	22.75	15.74	22.33	5.4
83	23.91	16.78	24.54	5.79
84	21.79	16.25	21.31	4.43
85	22.17	16.83	22.81	5.68
86	19.73	14.34	20.21	3.68
87	20.63	16.15	22.43	4.99
88	21.73	13.74	18.98	3.24
89	23.24	14.71	22.72	4.57
90	20.47	15.32	21.97	4.39
91	19.72	15.02	20.17	4.41
92	23.18	17.58	23.95	5.94
93	22.79	15.18	22.1	4.94
94	22.87	17.95	24.31	6.54
95	21.2	16.51	23.12	5.36
96	22.97	15.02	21.35	4.52
97	23.75	18.89	24.97	6.87
98	23.01	15.17	23.89	5.79
99	15.94	13.79	18.33	3.23
100	15.99	14.08	19.41	3.54
Mean :	16.278575	16.1701	21.9811	5.1142
Standard Deviation :	3.158473572	2.046280039	2.887662874	1.137569852
Standard Error :	0.7445	0.4823	0.6806	0.2681