UNITED KINGDOM · CHINA · MALAYSIA

School of Environmental and Geographical Sciences, Faculty of Science

Functional Ecology of Freshwater Mussels in Peninsular Malaysia

by

Farah Najwa Mahadzir

(Bsc. Biotechnology)

Thesis submitted to the University of Nottingham for the degree of Master of Research

October 2017

Contents Pages

List of Figures Pages

List of Tables Pages

Acknowledgements

First and foremost, I would like to express my sincere appreciation to my Principal Supervisor and Co-supervisors, Dr. Alexandra Zieritz, Dr Tuong Thuy Vu and Dr. John-James Wilson for their constant guidance and encouragement, without which this work would not have been possible. For the unwavering support and patience, I am truly grateful. Thank you as well to all the lecturers in the School of Environmental and Geographical Sciences and Faculty of Science Staff for their help and support.

I would also like to thank my family; especially my parents for the endless support and encouragement and to Nadia for accompanying me all the weekends that I needed to work in the lab. A special thanks goes to Siti Nur Khatijah bte Che Samsuddin, for the endless hours on the road, and every step of the way through the fieldwork, and thank you as well to my colleague Nabilah Fatin for the support throughout our MRes journey. To Azmaiha, Adeela, Muaz and Fatin, I'm grateful for your help with the fieldwork and to Chan Wei Ning, thank you with your help during laboratory work.

My gratitude also goes to Dr. Subha Bhassu from the University of Malaya for taking me under her wings and providing me with complete laboratory to work in and guidance when things started to look uncertain and to VC Lim who had always been an awesome mentor to look up to and a great motivator to learn from.

Thank you to Mr. Mahza for allowing me to set up my project in his orchard in Kampung Hayat, Bentong and many other locals who helped us throughout the course of my fieldwork. Last but not least, thank you all my friends and colleagues at the University of Nottingham Malaysia Campus for their companionship and moral support which made my studies more enjoyable.

Abstract

In Southeast Asia, freshwater mussel diversity, distribution and conservation status is very poorly understood, with limited data and studies available on its functional ecology as compared to freshwater mussels in temperate regions. The present thesis aims to investigate the functional ecology of freshwater mussels in Peninsular Malaysia. Firstly, the effects of mussels on the water column were investigated in a filtration experiment combining mesocosm in natural habitat and laboratory experiment involving river mussel community and lake mussel community. Second, the effects of mussel presence on benthic macroinvertebrates were investigated through biodiversity survey in 28 sites with and without mussel presence across Peninsular Malaysia.

While in situ clearance shown no significant differences between mussel treatments and controls, ex situ clearance experiment suggest that both river and lake mussel communities affect the ecosystem through different ways. River mussel have significant significantly reduces Soluble Reactive Phosphorus (SRP) ($P=0.048$), indicating removal of nutrient from the water column. Inversely, lake mussel community significantly increase Total Phosphorus (TP) ($P<0.001$) concentration, indicating biodeposition taking place instead. This difference was further supported the observation that River mussels significantly decreasing the concentration of chlorophyll a ($P=0.007$) whereas lake mussel significantly increases the concentration of chlorophyll a ($P<0.001$). The results suggest that tropical freshwater mussels from different freshwater ecosystems may perform different ecological functions.

Biodiversity survey indicates that freshwater mussels in Peninsular Malaysia have significant effect on higher species richness in tropical streams ($P=0.047$) similar to freshwater mussels in temperate regions, although invertebrate taxa with clear preference for mussel presence differed the two systems. The order Hemiptera had shown significant preference for mussel sites ($P=0.008$), and orders Plecoptera and Decapoda had shown positive correlation to mussel presence although not to a statistically significant level. The utilisation of DNA barcoding for macroinvertebrate identification also indicates several species sampled have yet to be recorded into the Genebank library. Further studies into the functional roles of freshwater mussels in tropical ecosystem coupled with advanced methods such as DNA barcoding should be carried out in the future to develop a better management and conservation strategy for Unionidae in Peninsular Malaysia.

Chapter 1- General introduction to freshwater bivalves

1.1 Taxonomic placement and patterns of diversity

Freshwater mussels (order Unionida), also known as freshwater mussels or pearly mussels, belong to the phylum Mollusca and class Bivalvia. Unionida spans about 800 species and represents the only bivalve order that is restricted to freshwater habitats (Bogan, 2008). Within the Unionida, the family Unionidae is one of the most widely-distributed groups of freshwater bivalves, spanning across the globe from North America all the way to the African continent, most parts of Europe, Southeast Asia and Eastern Asia (Bogan, 2008) (Lopes-Lima *et al*. 2014) (Fig. 1). According to Zieritz *et al.* (2016), all Malaysian Unionida species fall into the Unionidae family.

Figure 1: The global distribution of Unionidae. Adapted from Bogan (2008).

Graf & Cummings (2007) stated that North America exhibits the highest Unionida species richness in the world, with 300 recognized native species out of the 800 species recognized globally. However in Southeast Asia freshwater mussel diversity, distribution and conservation status is very poorly understood, with limited data and studies available on the subject matter (Lopes-Lima *et al* 2014). Once the unionids in the Indotropical region are re-examined using more modern methods, Graf & Cummings (2007) predicted species and level of diversity will be found to be greater than what is currently understood.

The first comprehensive assessment of the diversity and distribution of freshwater mussels in Peninsular Malaysia done by Zieritz *et al*. (2016) shows that the distributions of the native species are very restricted, in some cases to single river basins, while the non-native *Sinanodonta woodiana* is widely distributed all over the peninsula. Nine native species of freshwater mussels were found during the survey, with two species not having been recorded from Peninsular Malaysia in the past while three other species reported in historical records were not found in the assessment (Zieritz *et al*. 2016).

1.2 Morphology and lifecycle

The typical body plan of a freshwater mussel consists of a visceral mass and foot, which are surrounded by a pair of valves made of calcium carbonate, and the lobes of the mantle that secrete them (Cummings & Graf, 2015). Besides providing protection, the shell also allows the attachment of prominent muscle systems, with an elastic dorsal ligament articulating the two calcified valves. On either side of the mouth, the visceral mass bore the labial palps, with a pair of comb-like structures that are called ctenidia, extending into the posterior mantle cavity that serve as gills. In the first ten years, the shells of most temperate Unionida species grow about 5mm per year, and once they approach asymptotic length, shell length becomes a less reliable indicator for age of the mussels (Aldridge, 1999).

The life history of Unionida is unique, as it involves an obligatory parasitic stage on fish or other vertebrate hosts for dispersal purposes (Bogan, 1993) (Helfrich *et al.* 2005). Reproduction of mussels occurs when male mussels release sperm into the water column, which later will be siphoned into the female mussel to fertilize the eggs. The reproduction process is thought to be triggered by an increase in water temperature and day length. The reproductive success of a mussel is affected by its maximum size and longevity, whereas age of maturity and growth rate affects its lifetime productivity (Aldridge, 1999).

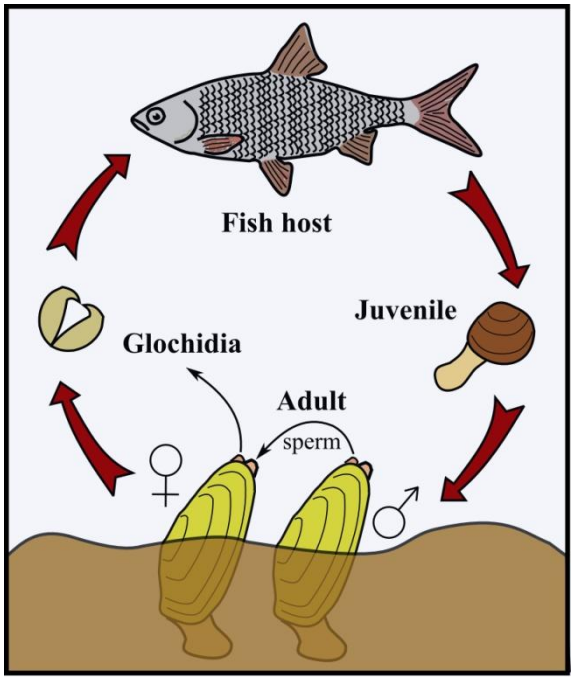

Figure 2: The lifecycle of Unionida freshwater mussels (Zieritz, 2010) .

Mussel larvae are developing and retained in the mother's marsupial demibranchs for about one to ten months (Cummings & Graf, 2015). Once mature enough, the fully developed larvae (termed glochidium in the most unionoid families) will be expelled from its parent (Fig.2). The glochidium will then attach itself to the gills or fins of a living host fish, forming a parasitic relationship, undergoes metamorphosis and drops from the host to begin their life as juveniles (Bogan, 1993). If the condition is suitable for living, the juveniles will be able to live on and finally growing into an adult mussel (Helfrich *et al*. 2005).

1.3 Life-habit and feeding

Most Unionida species are classified as infaunal organism and usually burrow themselves into the benthic substrate (Bogan, 2008). Unionida tend to be half-buried in the sediment, anchored by their extended foot with their siphons extended into the water column (Vaughn *et al.* 2008) (Fig. 2). Though predominantly sessile, freshwater mussels are more mobile and are able to burrow through sediments using their foot compared to typical marine mussels, which attach themselves to rocks and other underwater structures with threads called byssus (Bogan, 2008). The variety among freshwater mussel species in sizes, shell morphologies, and behaviours often cause different species to reside at different angles and depths above and below the sediment-water

interface (Vaughn *et al.* 2008). In North America, Unionida further tend to occur as diverse, multispecies assemblages as compared to aggregations of one to a few species like marine bivalves and zebra mussels (Vaughn, 1997) (Fig. 3).

Figure 3: *Pseudodon vondembuschianus* mussels. (Photograph by author taken on 2016.10.17).

Unionida are suspension feeders or also known as filter-feeders, a process that is facilitated by a series of cilia on the inner surface of the mantle, demibranchs and visceral mass (Vaughn *et al*. 2008). In smaller bivalves such as sphaeriids as well as juvenile unionoids, the ctenidia are often not effective enough for suspension feeding, hence the need to resort to deposit feeding instead (Cummings & Graf, 2015). *Corbicula* on the other hand, is able to perform both filter-feeding and deposit-feeding, with significant impacts on the benthic community as a whole (Hakenkamp & Palmer, 1999).

There are three main processes in the filter-feeding mechanisms of bivalves: (1) filtration, (2) ingestion and (3) assimilation (Saraiva *et al*. 2011). In the first step, the filtration and extraction of seston from the water column is controlled by the activity of cilia generating the water flow and the retainment of the particles in the gills of the mussels. Water currents were generated inside and outside the shell by the synchronous movement of the cilia, bringing in a continuous supply of freshwater with

food and O_2 inside the shell (Vaughn *et al*. 2008). Initially, water filtration by mussels were thought to occur only through the mussel's inhalant and exhalant siphons, however Nichols *et al.* (2005) found that the opening of shell also enables water to enter the shell from the anterior. Once the particles are retained in the gills, particles are selected for ingestion whilst being transported to the mouth by labial palps. Waste products are removed as water exits the shell, while cirri sweep potential food items within the water toward the mouth (Vaughn *et al*. 2008). The particle handling time limits the amount of particle that can be processed, and rejected particles are returned into the water column in the form of pseudofaeces (Saraiva *et al*., 2011). In the final (3) assimilation step, ingested particles are absorbed and converted into the organism's reserves.

In the broadest outline, Unionids capture particulate nutrients in the form of seston and phytoplankton (both living and non-living), and later convert these filtered nutrients into bivalve tissues, particulate biodeposits and dissolved nutrients (Strayer, 2014). Hence, freshwater mussels provide nutrients from the water column to other organisms as well as to the rest of the mussel community in 3 ways: i) through excretion of NH3 and P, ii) through biodeposition activity and iii) through the release of stored nutrients as dead mussels decay (Vaughn *et al.* 2008).

It is assumed that the absorbed food and the reserves need to have similar chemical composition, and that any difference between that of the bivalve reserves and ingested food determines the biodeposition of faeces. Although freshwater mussels were mainly suspension-feeders and feeds on freely floating organic particulate in the water column, there had been evidence of benthic and planktonic food supply accession, indicating their adaptability in obtaining food resources (Nichols *et al.* 2005). Through the filter-feeding and biodeposition activity of bivalves, the rates of downstream loss of nutrients and particulates are being reduced (Vaughn & Hakenkamp, 2001; Howard & Cuffey, 2006).

1.4 Freshwater mussels as ecosystem engineers

Compared to other freshwater macroinvertebrates, mussels generally are long-lived and often dominate in term of benthic biomass; therefore their activities such as filter-feeding and excretion can have strong effects on the environment (Strayer *et al.* 1994 ; Gutierrez *et al.* 2003 ; Haag & Williams, 2014). Freshwater mussels have been shown to influence the distribution and abundance of co-occurring macroinvertebrates, and have been recognised as ecosystem engineers, i.e. "organisms that modulate the

availability of resources to other species, either directly or indirectly, by causing physical state changes in biotic or abiotic materials" (Jones *et al* . 1994).

The presence of freshwater mussel community in a freshwater habitat provides valuable ecosystem services such as energy transfer by depositing organic matter used by other organisms (Howard & Cuffey, 2006), nutrient cycling by controlling the nutrient concentration in water column (Soto & Mena, 1999), and improving the availability and quality of habitat by providing shelter for smaller invertebrates in the interstitial spaces of their shells (Spooner & Vaughn, 2006). As filter-feeders, they remove suspended materials from the water column, improving the water clarity for macrophytes growth and preventing eutrophication or algal bloom incidents (Atkinson *et al.* 2013; Zhu *et al.* 2006).

Apart from affecting nutrient availabilities across multiple trophic levels through filtration and biodeposition, freshwater mussels also affect their ecosystem physically by (1) providing substrata for attachment, (2) providing shelter for macroinvertebrates from predation and other physical and physiological stress, and (3) controlling the transport of particles and solutes in the benthic ecosystem via bioturbation (Gutierrez *et al.* 2003; Vaughn & Spooner, 2006).

1.5 Threats and conservation efforts

Although having a widespread global distribution, Unionida are also one of the most imperilled animals in fresh waters around the world (Bogan, 1993; Strayer, 2004). In North America,around 30 taxa have become extinct while the remaining 65% of species are considered endangered, threatened or vulnerable in the IUCN Red List in the last 100 years (Haag & Williams, 2014). In Europe, Lopes-Lima *et al*. (2017) comprehensively reviewed the collective understanding of the ecology, distribution, and the conservation status of 16 native species of European freshwater mussel in order to suggest a logical path for future management actions. The decline in freshwater mussel populations across the world is caused by a variety of factors that are usually interrelated (Bogan, 1993; Watters, 1999). Some major factors of the rapid decline are the modification and destruction of habitat (Bogan, 2008) and the degradation of water quality and water pollution (Downing *et al*. 2010).

The destruction of habitat is thereby often the consequence of the construction of dams and canals as well as the changes in the deposition of fine particle such as sand silt (Bogan, 2008). Due to their nearly sessile life habit, freshwater mussels are particularly prone to environmental changes and require stable substrata. As filter-feeders, freshwater mussels further require high water quality and are not adapted to living in soft, silty substrate and incapable of withstanding heavy loads of silt (Bogan, 2008). Being covered with a layer of such substrate will simply suffocate the bivalves as they are unable to escape (Bogan, 1993).

The second major threat to freshwater mussels is failed recruitment due to a lack of appropriate host fish species (Downing *et al.* 2010). Since freshwater mussels depend on host fish species for reproduction, any threats towards the local host fish population will indirectly impact the local freshwater mussel population as well. Habitat destruction may thereby affect freshwater mussels directly by rendering their environment unsuitable as well as indirectly by affecting their host fish populations, which will indirectly have an impact on the reproduction of native freshwater mussel species.

In the worst case scenario, an additional threat can be posed by introduction of non-native species, which may outcompete native freshwater mussels that are already under stress by habitat alteration and loss of host fish (Nichols *et al*. 2005; Parker *et al*. 1998). The reduction of both food and habitat by invasive species with broader trophic niches poses a threat towards the population of native unionid mussels (Atkinson *et al.* 2011). Freshwater mussels are also threatened by direct human exploitation for centuries; for example, these animals had been gathered for their pearls and mother-of-pearl shells for pearl-button industry (Bogan, 1993; Strayer *et al*. 2004).

Besides commercial activities, freshwater mussels are also harvested as a source of food (Bogan, 2008; Strayer *et al*. 2004; Zieritz *et al*. 2016). The loss of freshwater mussel populations and whole species can be detrimental to their ecosystems, which in turn will affects human's livelihood, since mussels fulfil several of important ecosystem functions (Borthagaray & Carranza, 2007; Vaughn & Hakenkamp, 2001).

The main key to successful biomonitoring, management and conservation of habitat is the ability to perform frequent assessments of biodiversity using a method that has a low impact and is affordable in a long term (Lim *et al*. 2016). One example of this method is a molecular technique called DNA barcoding. The concept of DNA barcoding

is the comparison between a query sequence and a DNA barcode reference library comprised of known species (Hajibabaei *et al.* 2011). DNA metabarcoding - a term coined by Taberlet *et al*. (2012) - refers to the high-throughput identification of multispecies (or higher-level taxon) using degraded DNA extracted from an environmental sample (eDNA). According to Hajibabaeil *et*. *al*. (2011) DNA metabarcoding also includes the species identification of entire organisms isolated prior to analysis from bulk samples. DNA metabarcoding is the most widely used method of molecular identification , and has been shown to improve the capacity of bioassessments by reducing the cost and time required for taxonomic identification (Carew *et al.* 2013).

1.6 Aim of this thesis

An understanding of the functional role of freshwater mussels in an ecosystem provide valuable insights into water quality and changes in the availability of benthic resource (Howard & Cuffey, 2006). However, studies on the functional ecology of freshwater mussels have so far been carried out mostly in Europe and North America regions, and to a far lesser extent in the African and Southeast Asian region (Lopes-Lima *et al*. 2014). As a result, almost everything that is known in regard of the functional ecology of freshwater mussels is based on mussels in temperate systems, and very little are known about Unionida mussels in tropical regions.

My aim for this thesis is to investigate the functional role of freshwater mussels in the tropical streams across Peninsular Malaysia. Considering that there is a big difference in term of seasonal variation between tropical regions and temperate regions (Boulton *et al.* 2008), it will be interesting to see whether the functional role of freshwater mussels in tropical regions is similar to their temperate counterparts. If there is a large difference between the findings, a further study should be conducted on the possibility of climate playing an important role in affecting the functional ecology of freshwater mussels in Peninsular Malaysia.

Chapter 2: The effects of filter-feeding of freshwater mussels towards the water column

2.1 Introduction

Although freshwater mussels are not as extensively-studied as marine species, it is widely known that their suspension-feeding have significant impacts on the ecosystem, as shown by *Diplodon chilensis* (Soto & Mena, 1999), *Margaritifera falcata* (Howard & Cuffey, 2006), and *Anodonta anatina* (Dionisio Pires *et al*. 2007). The filter-feeding of freshwater mussels has been associated with increased water clarity, which in turn affects the growth of macrophytes (Chowdhury *et al*. 2016). Through filter-feeding and biodeposition of pseudofaeces (material rejected prior to the gut passage) and faeces (material rejected prior to absorption is termed faeces), mussels remove nutrients and energy sources such as phytoplankton and zooplankton from the water column to the sediment (Vaughn & Hakenkamp, 2001) (Saraiva *et al.* 2011). This transfer increases the abundance of accessible nutrients for benthic community and stimulate production across different trophic levels (Howard & Cuffey, 2006; Spooner & Vaughn, 2006).

As small suspended particles otherwise would not settle from the water under natural conditions in flowing water bodies, the presence of freshwater mussel in riverbeds increases food availability and structural resources of the benthic community, thereby increasing benthic productivity. Despite their ability to filter out nutrients and other materials from the water column, mussels do not really digest everything that is ingested. Most of the filtered particles are <20 µm in size, survive the gut passage alive and undamaged, and is deposited at the benthic surface (Vaughn *et al*. 2008; Saraiva *et al.* 2011).

Under a condition where the biomass of bivalves is declining and the population release more nutrients than they absorb, these bivalves serve as nutrient sources (Vaughn & Hakenkamp, 2001). Additionally, stored nutrients are released as dead mussels decay. This excretion activity increases the water column N:P which alleviates strict N-limitation in streams, leading to a subsequent change in algae community (Atkinson *et al*. 2013). In three different rivers in south central of United States (Kiamichi, Little and Mountain Fork), sites without mussels were found to be N-limited, whereas

sites with high mussel densities were co-limited by N and P, alleviating the strict N-limitation (Atkinson *et al*. 2013).

The filtration rate of a mussel is defined as the volume of water that passes through the mussel's gills per unit time, and can be approximated as clearance rates (McIvor, 2004; Riisgård, 2001). The clearance rate at which mussels filter materials from the water column have often been measured from the clearance of chlorophyll a from the water column (Vaughn *et al.* 2004). The filtration rate of an individual mussel depends on various abiotic and biotic factors, such as animal size, species, water temperature and population density (Vaughn *et al.* 2008).

Filtration rates for Unionida estimated in laboratory (in situ) experiments to date lie around 0.2-1.5 L/h, with a strong positive correlation between filtration rate and mussel size. McIvor (2004) recorded that the filtration rate for a 61-mm-long mussel to be between ~0.5 to 1 L/h. Cyr *et al.* (2017) founded that a native species of New Zealand, Echyridella *menziesii* (Gray 1943) have a filtration rate of 0.02-1.3 L/h per mussel. *Parreysia caerulea* (Lea, 1831) and *Lamellidens marginalis* (Lamarck, 1819) from Dhanmondi Lake, Bangladesh, on average filtered about 0.2 L/h, with filtration rate as high as 0.9 L/h in largest mussels (Chowdhury *et al*. 2016). However, the actual rates of filtration in nature are difficult to measure, whilst values for freshwater mussel filtration rates assessed in the laboratory are often considered underestimations due to unnatural experimental conditions (Vaughn & Hakenkamp, 2001).

Studies assessing the effects of freshwater mussel filtration on their ecosystem in situ are scarce, but some examples exist. In Southern Chile, *Diplodon chilensis* (Gray, 1828) has shown the ability to mitigate the effects hypereutrophic situation from salmon farming to an oligotrophic-mesotrophic one (Soto & Mena, 1999). The presence of *D. chilensis* in salmon tanks significantly reduced the concentration of chlorophyll a (from ~300 µg l^{-1} to 3 µg l^{-1}). The concentration of Total Phosphorus (TP) was also significantly different in salmon tanks with mussels and control tanks, respectively (336µg l^{-}1 vs. 23µg l^{-1}).

In the heavily polluted Dhanmondi Lake in Dhaka, Bangladesh, presence of freshwater mussels co-occurred with a heavily reduced biomass of phytoplankton and improved water clarity (Chowdhury *et al*. 2016). These effects were observed despite the abundance of N and P availability for algal growth derived from anthropogenic activities around the lake.

Large amount (3-10 times the dissolved nutrients excreted) of captured material are deposited onto the sediments as faeces and pseudofaeces (Nalepa *et. al* 1991). However, biodeposition are not spread evenly over the entire area of a lake or river, but probably are more or less tightly focused in and around mussel beds. In general, biodeposition rates of freshwater mussels appear to be highly variable, depending on environmental conditions and characteristics of the mussel assemblage (Vaughn *et al*. 2004; Hakenkamp & Palmer, 1999). For example, *D. chilensis* in salmon farms was estimated to produce a daily total of 329 kg of biodeposits (Soto & Mena, 1999), whilst *Margaritifera falcata* in the South Fork Eel river deposited only a fraction of this, with an average of about 300 mg per day in October (Howard & Cuffey, 2006).

Considering the generally high temperature and food availability in tropical systems, biodeposition rates in tropical freshwater mussel species would be expected to be on the higher end of this range, though no data are available to date. Similar to effects of filter-feeding, the effects of mussel deposition are significant for the ecosystem even at low deposition rates. For example, the amount of fine benthic matter was significantly greater in mussel areas than non-mussel areas for the *M. falcata* – South Fork Eel river system, with organic matter making up 25% of the biodeposited material (Howard & Cuffey, 2006). Similarly, the mean weight of deposited organic material was significantly higher in mussels treatments compared to controls in mesocosm experiments conducted by (Vaughn *et al*. 2004).

Mussel communities in different habitats affect their ecosystems in different ways, especially between regions with very distinct climate and hydrological factors (Vaughn *et al.* 2004). In our case for example, the mussel communities found here in Peninsular Malaysia faces warmer waters and more frequent flooding events as compared to their temperate counterparts of which most of the knowledge about the functional ecology of freshwater mussels were based on. However a complete set of data for the functional ecology of freshwater mussels in the tropics in general are very scarce and inadequate to make a proper comparison (Zieritz *et al.* 2016).

The present study aims at assessing (1) in situ clearance rates and (2) ex situ clearance + deposition rates of nutrients and bioseston (approximated by pigment concentrations) by freshwater mussel communities in a tropical lake and stream, respectively. This is achieved through replicated field and laboratory filtration experiments on mussel community of an artificial lake and a natural stream in Peninsular

Malaysia. My study is one of the few experiments that studies of the functional ecology of freshwater mussels that combines field experiment with a laboratory experiment, and one of the first to do so in a tropical region.

2.2 Materials and Methods

The experiment was conducted in two parts; I) field (mesocosm) experiment and II) laboratory experiment. In part I, the experiment was carried out in the field site and in part II the experiment was carried out in the laboratory using mussels taken from the field sites. The sites involved were Tasik Semenyih, Selangor (GPS: 2.9470278, 101.859778) and Sungai Muar, Negeri Sembilan (GPS: 2.766111, 102.39592).

Table 1 : Information on the study sites, Muar River in Negeri Sembilan and Semenyih Lake in Selangor

Parameters/Site	Muar River	Semenyih Lake
Chlorophyll a (mg L^{-1})	2 ± 0.801	16 ± 9.891
Total Phosphorus(mg L^{-1})	46 ± 7.18	36 ± 2.183
Ammonia (mg L^{-1})	23 ± 33.261	57 ± 28.791
Nitrate (mg L^{-1})	2 ± 0.72	n.d
Species of mussel found	***Contradens contradens*** **(Lea, 1838)** ***Pseudodon vondembuschianus*** **(Lea, 1840)**	***Sinanodonta woodiana*** **(Lea, 1834)**
Density of Mussel/m^2	7±4.375	4±2.746

Tasik Semenyih is a man-made lake located close to a residential area and used for fishing and other recreational activities. The lake is eutrophic and exhibits moderate to high chlorophyll a concentration, and fairly high concentrations of Total Phosphorus (TP) (Table 1).The freshwater mussel species found here is *Sinanodonta woodiana* (Chinese pond mussel) (Fig.4) which is an invasive species native to China found widely in ponds and rivers across Peninsular Malaysia (Zieritz *et al*. 2016).

The study site at the Muar River flows through a mosaic of oil palm plantation and secondary forest, several km from the nearest human settlement. Based on its low chlorophyll a concentrations, the river can be categorised as oligo-mesotrophic with high TP content (Table 1). The species of mussels found here are *Contradens contradens* and *Pseudodon vondembuschianus*, both of which are native species of Peninsular Malaysia and usually found in medium-sized streams with a mixture of muddy and rocky substrate (Zieritz *et al*. 2016) (Fig.4).

Figure 4: Species of freshwater mussels found in Muar River (a-*Pseudodon vondembuchianus*, b-*Contradens contradens*) and Semenyih Lake site, (c-*Sinanodonta woodiana*).

Field Experiment (clearance rate)

Field experiments were carried out in April and May 2016. At each of the two study sites, twelve pairs of black bins (diameter = 43cm, height = 51cm) with the bottoms cut off were placed at randomly-selected spots across a 100 metres stretch of river/lake where mussels were found, at a maximum of 3 metres distance from the shore. All mussels were removed from one bin of each pair, which served as a control and the bin without mussels removed will serve as the mussel treatment. Subsequently, all bins were lifted to let water flow until the disturbed water was replaced by new water after which the bins were planted back exactly in the same spot.

Water samples (1L) were taken from each of the bins close to the surface at the beginning of the experiment and again after 3 hours. This corresponds to water before mussel filter-feeding activity and after filter-feeding activity takes place respectively. After the filtration period, all the mussels from the mussel treatment bins were collected and measured to ±0.1 cm accuracy using a sliding calliper.

Lab experiment (clearance + deposition rate)

The laboratory filtration experiments were carried out in the University of Nottingham Malaysia Campus in July and August 2016 for the samples from Muar and Tasik Semenyih site, respectively. In the morning of the day of the experiment at 10 am, approximately 80L of river/lake water, and a total of 20 (10 *C. contradens* and 10 *P. vondembuschianus*) and 15 (for *S. woodiana*) mussels, respectively, were taken from each of the two sites and transported to the laboratory in darkened and cooled containers. In the laboratory, 18 and 23 jars for Muar and Semenyih experiment, respectively, were set up with 3.5L of lake water and air stone connected to an air pump

in each. A 0.5L water sample was taken from each jar for analysis before individual mussels were put inside all but three of the jars, which served as controls. At the end of the experiment, 3 hours after the mussels had been placed in the jars, mussels were removed from the jars, jars closed and repeatedly inverted for homogenisation and a 1L water sample collected. The shell length of each mussel was then measured to ±0.1 cm accuracy using a sliding calliper.

Experimental Design

The in situ experiment was carried out in a Matched-Pair Randomization design, in which paired treatment and control bins were placed in completely randomized positions along the stretch of the river / lake. For ex situ experiment, the experiment was carried out in a Completely Randomized Design, in which all the mussel tanks and the control tanks were numbered in a randomized way.

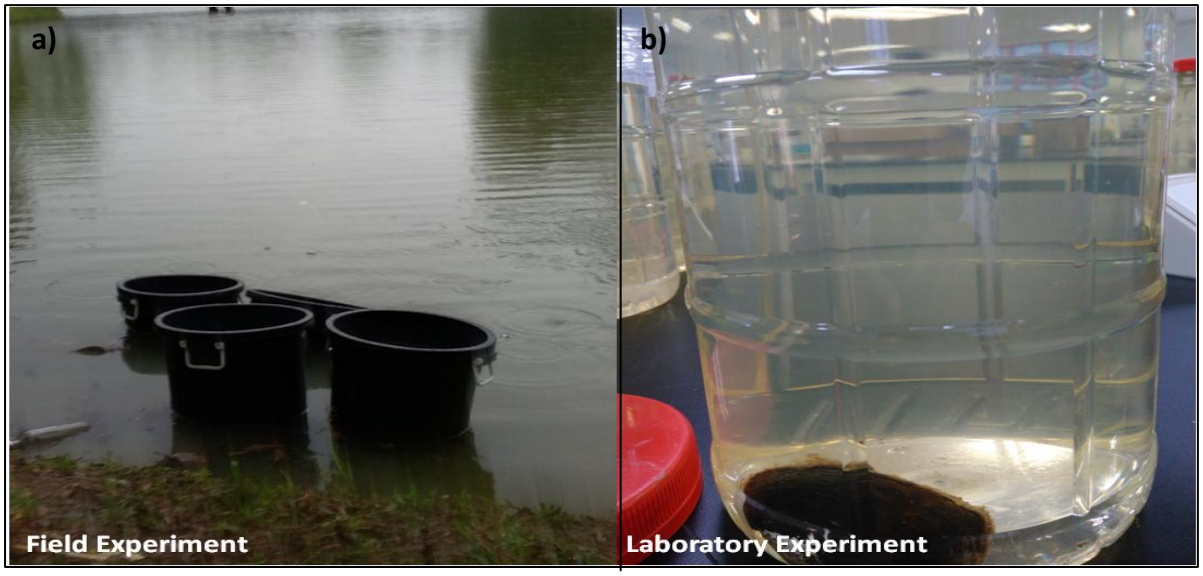

Figure 5: a) The setup for in-situ (Field) clearance rate experiment. 12 pairs of bins with the bottom cut off were placed along in mussel natural habitat. All mussels were removed from one bin for each pair as a control, while no disturbance was made in the bins with mussels. b) The setup for ex-situ (Laboratory) clearance+deposition rate experiment. Jars filled were with 3 litres of water taken from the mussels' original habitat with one mussel placed in each jar. Several jars with only water without mussels were used as controls.

Water chemistry

Water samples were held in darkness and cool condition, and processed as soon as possible and always on the day of the experiment. A known volume of each water sample (0.2-0.4 L) was filtered using two sets of Whatman® GF/C Glass microfiber filters using a glass filtration apparatus and vacuum pump. One batch of pre-weighed (to ± 0.001 mg accuracy) filter papers was designated for Total Suspended Solids (TSS)

and Organic Matter (OM) content analysis and another batch of filter papers were for pigment analysis of the samples. The water samples were then subjected to water chemistry analysis, with unfiltered water being tested for the TP content in each treatment, whereas the filtered water was tested for Nitrate (NO3) content, Soluble Reactive Phosphorus (SRP) content and Ammonium (NH4) content. These parameters were assessed using standard photometric/colorimetric methods (Mackereth *et al*. 1978).

For TSS and OM analysis, the batch of pre-weighed filter papers was dried overnight in an oven at 104^{o}C before being weighed on an electronic scale to ± 0.001 mg accuracy. This weight was recorded as the dry weight (DW) of the filter papers. Next, the filter papers were burned inside a furnace at 550^{o}C for 3.5 hours to burn off all the suspended organic matter that was filtered from the water samples. After the papers had cooled off, the weight of each filters were taken once again, this time recorded as the burned weight (BW) of the filter paper. These weighs were used to calculate TTS and OM of the water samples.

Total suspended solids (TTS; mg/L)	$= \frac{[\mathrm{DW(mg)} - \mathrm{filter(mg)}]}{\mathrm{Volume\ of\ filtered\ water\ (L)}}$
Organic matter content (OM; mg/L)	$= \frac{[\mathrm{DW(mg)} - \mathrm{filter(mg)}] - [\mathrm{BW(mg)} - \mathrm{filter(mg)}]}{\mathrm{Volume\ of\ filtered\ water\ (L)}}$

For pigment analysis, an extraction solution was made up from acetone: methanol: distilled water at 80: 15: 5 ratio. Each filter was cut into thin strips and put into an individual glass bottle. Extraction solution was added into the bottles until the papers filters were completely submerged, before being left overnight in the fridge. After the incubation period, the extraction solution was transferred from each of the bottle into Falcon tubes and made up to 10 ml, followed by centrifugation at 7000 rpm for 10 minutes. The extraction solvent was used as a ‘blank’ to calibrate the spectrophotometer before the samples were measured at seven different wavelengths (750nm, 665nm, 645nm, 630nm, 480nm, 430nm, 410nm). The cuvettes were rinsed with acetone in between each reading.

The readings obtained from the samples were analysed and used to calculate the concentration of Chlorophyll a (Chl a), Chlorophyll b (Chla b), Chlorophyll c (Chl c) and Carotenoid. The formula used to calculate the concentrations of the chlorophylls are:

Chlorophyll a (µg L^{-1})	=[11.85*(A_{665}-A_{750}) – 1.54*(A_{645}-A_{750}) – 0.08*(A_{630}-A_{750})] x E/V
Chlorophyll b(µg L^{-1})	=[21.03*(A_{645}-A_{750}) – 5.43*(A_{665}-A_{750}) – 2.66*(A_{630}-A_{750})] x E/V
Chlorophyll c (µg L^{-1})	=[24.52*(A_{630}-A_{750}) - 1.67*(A_{665}-A_{750}) – 7.60* (A_{645}-a_{750})] X E/V
Total chlorophyll (µg L^{-1})	=Sum (Chl a + Chl b + Chl c)
Carotenoid (µg L^{-1})	= 10*A_{480}* (E) / (V x D)

E=volume of extraction solvent (ml) V=Total volume of water filtered (L). D= Path length of spectrophotometer cell.

Statistical Analysis

The change in the concentration of each dependent variables (∆NO3, ∆NH4, ∆SRP, ∆TP, Δ Total chlorophyll, Δ Chlorophyll a, Δ Chlorophyll b, Δ Chlorophyll c, etc.) for each replicate bin (for in situ experiment) and jars (for ex situ experiment) in the course of the 3-hour experiment was calculated by subtracting concentrations at the start of the experiment from the concentration at the end of the experiment. Data were tested for normality, and non-normally distributed data were transformed with Boxcox transformation prior to further analysis. The obtained changes in nutrient and pigment concentration were later subjected to statistical analysis testing.

To determine whether the presence of mussels altered the nutrient concentration of water column, a Paired T-test was performed in order to test for significant differences between paired control and mussel treatments of the in situ field (mesocosm) experiment. I also ran ANCOVA correlation test between mussel treatments and the changes in nutrients with the density of mussels as co-variate to see whether the number of mussels per litre have a significant effects on the changes in nutrient concentration.

Similarly, to determine whether mussel treatments have significant effects on the changes in nutrient concentration in water column during ex situ experiment, Welch Two-Sample T-test were performed to test for differences between mussel and control treatments. Besides testing for significant differences, a regression analysis test was also carried out to find out the correlation between the mussel shell lengths (cm) and the changes on the nutrient concentration in the water column. All statistical analyses were conducted in R software with significant value at $P < 0.05$.

2.3 Results

In situ clearance (field experiment)

Paired t-test had shown no significant differences (P > 0.05) between mussel treatments and controls on the changes in nutrient concentration (Fig. 6) and pigment concentrations (Fig. 7) during in-situ experiment (field experiment) in both Muar River and Semenyih Lake. Differences between mussel treatments and controls were notable with respect to the change in NH_4 (Fig. 6a) in both sites and Chlorophyll a concentration in Semenyih Lake (Fig. 7b), but not to the extent of being statistically significant. In addition, at Semenyih Lake, changes in TP concentration were in higher magnitude (~199 mg/L) as compared to the TP changes in Muar River (~43 mg/L).

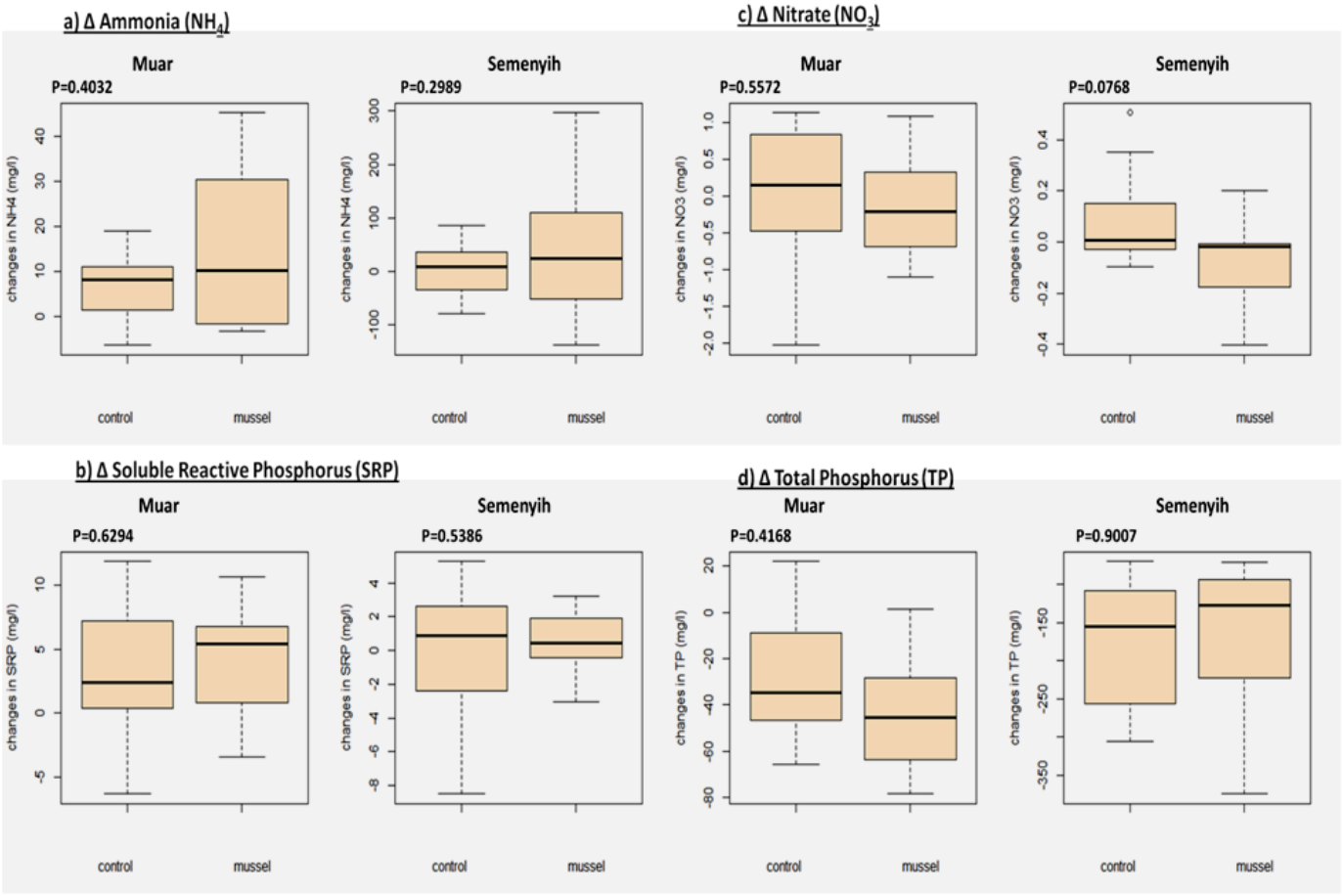

Figure 6: Box plots of the changes in concentration of nutrients after 3 hours of field experiment in sealed water containers without (control) and with freshwater mussel (mussel) for Muar River and Semenyih Lake and P-values of Paired t-test (n=12) (*p<0.05, **p<0.01, and ***p<0.001).

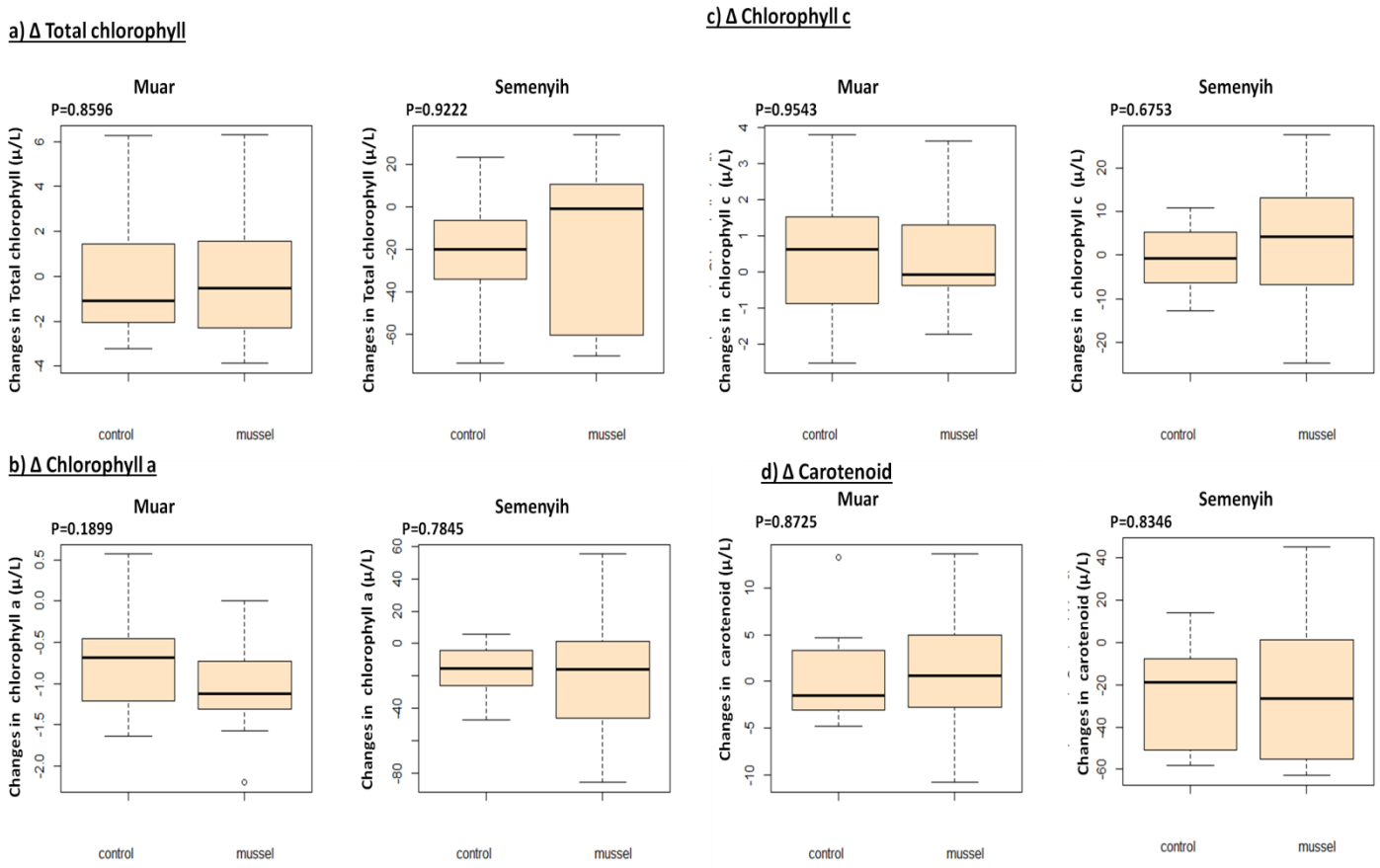

Figure 7: Box plots of the changes in concentration of pigments after 3 hours of field experiment in sealed water containers without (control) and with freshwater mussel (mussel) for Muar River and Semenyih Lake and P-values of Paired t-test (n=12) (*p<0.05, **p<0.01, and ***p<0.001).

.ANCOVA analysis with mussel density as co-variate in Muar River also had shown no significant differences in the nutrient concentration changes. However, there are several significant correlations ($P < 0.05$) between the number of mussels per litre with the changes in pigment concentration (Fig. 8). Increasing mussel density per litre was shown to be significantly correlated with the ΔTotal Chlorophyll (P-value=0.0226) (Fig.8a), ΔChlorophyll a (P-value= 0.0107) (Fig. 8b), ΔChlorophyll c (P-value=0.0474) (Fig.8c) and Δ Carotenoids (P-value=0.0069) (Fig. 8d). This significant correlation indicates that increasing number of mussel per litre leads to a decrease in the concentration of pigment within the water column, which may explain the clearer water I observed in mussel bins towards the end of the experiment.

Similar to Muar River, no significant correlations observed from ANCOVA analysis between mussel density co-variate and the changes in nutrient concentrations in Semenyih Lake. For changes in pigment concentration, significant correlations were recorded between the mussel density co-variate with ΔChlorophyll a (P-value= 0.021) (Fig.10a) and Δ Carotenoids (P-value=0.011) (Fig. 10b). At both sites, weak correlations between initial mussel density before removal and changes in pigment

concentrations were also observed in control bins (Figures 9 & 10). However, the correlation was generally stronger and more extreme in mussel bins compared to control bins.

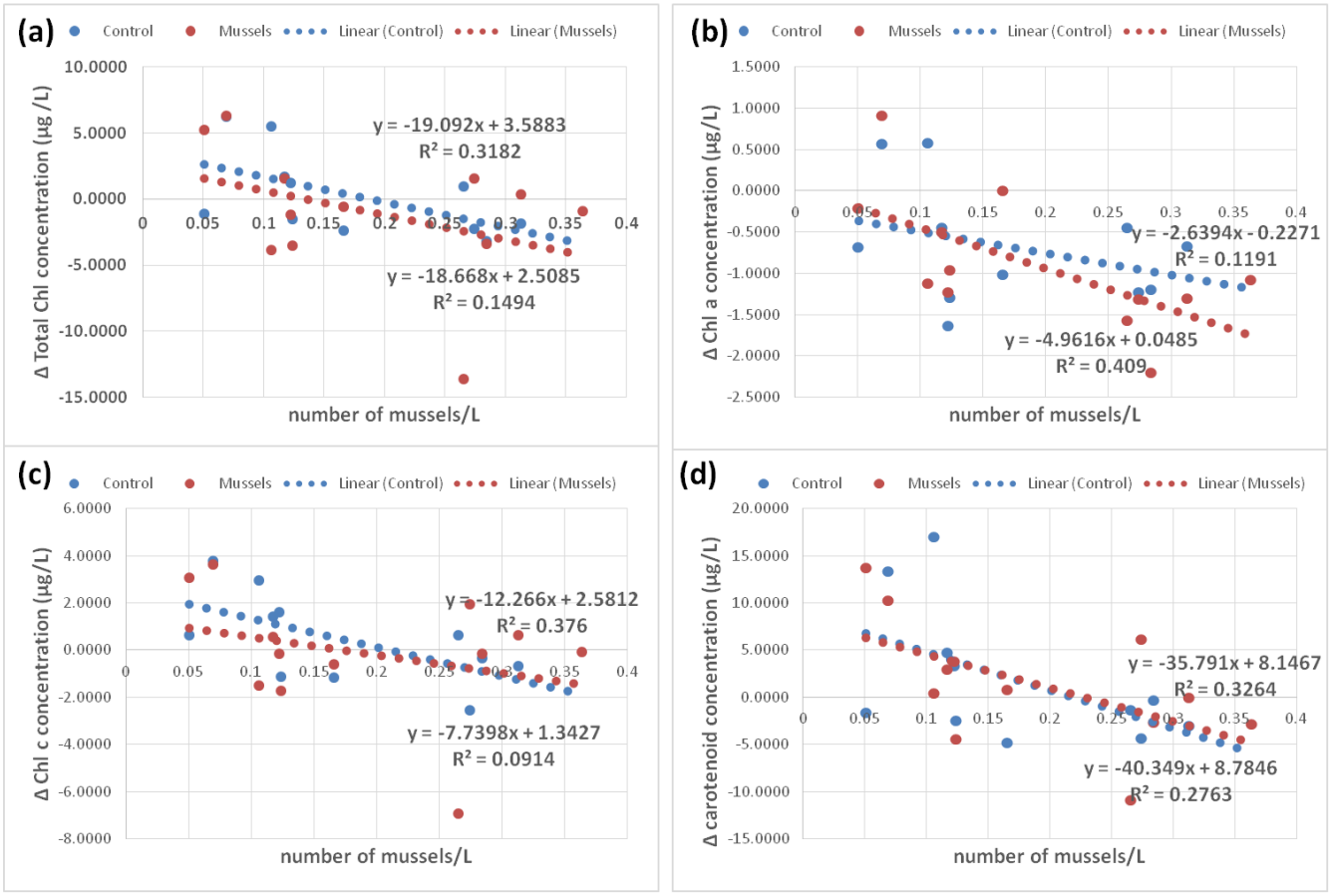

Figure 8: Linear Regression test indicates significant correlation between number of mussels per litre and ΔTotal Chlorophyll, ΔChlorophyll a, ΔChlorophyll c, and ΔCarotenoid observed in field experiment at the Muar River. P-values for mussel treatments (n=12)., a) Total chlorophyll (adj. r^2= 0.187, p= 0.0226), b) Chl a (adj. r^2 = 0.2373, p=0.0107), c) Chl c (adj. r^2 = 0.1351, mussel p=0.0474), d) Carotenoid (adj. r^2 = 0.2664, mussel p= 0.00685).

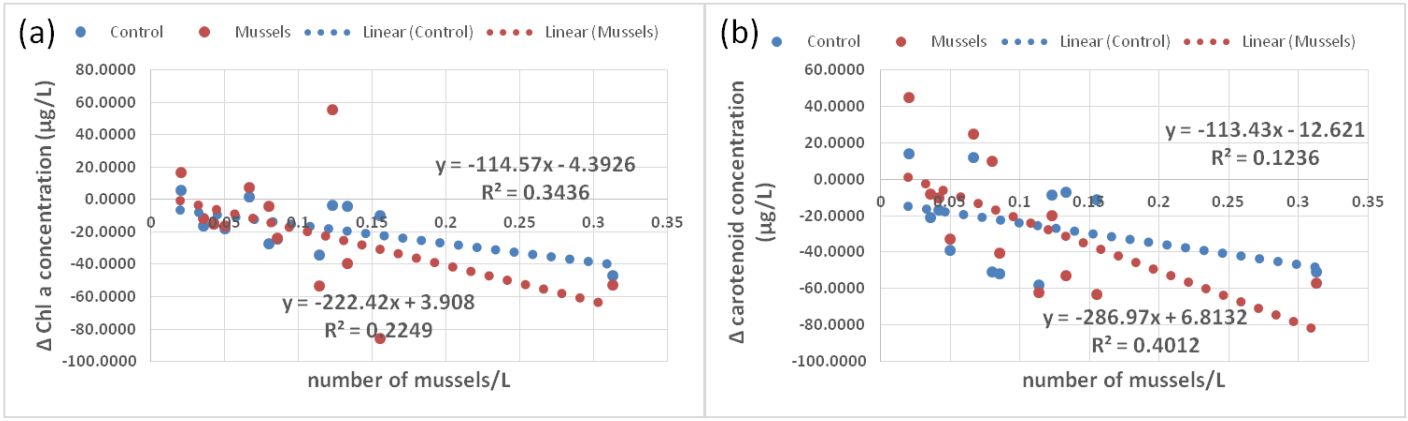

Figure 9 : Linear regression test indicates significant correlation between the ΔChlorophyll a and ΔCarotenoid with the number of mussels per litre for field experiment in Semenyih Lake. P-values for mussel treatments (n=12), a) Chl a (adj. r^2 = 0.184, p=0.02096), b) Carotenoid (adj. r^2 = 0.2252, p=0.0111).

Ex-situ clearance (laboratory experiment)

Whilst in-situ experiments focused exclusively on effects to the water column, in ex-situ (laboratory) experiments, I investigated the effects of mussels on nutrient-and-pigment concentrations in the water column and deposited material combined (i.e. combined clearance + deposition rate). In the Muar River system, mussel presence significantly affected ΔSRP (P-value = 0.0478) (Fig.11b) and ΔChl a (P-value = 0.007) (Fig. 12b), both of which showed a larger decrease in mussel jars compared to the control jars. In the Semenyih Lake system, a significant effect of mussel presence on nutrients was observed with respect to ΔNH_4 (P-value<0.001) (Fig. 11a) and ΔTP (P-value <0.001) (Fig. 11d). On its effect on pigment concentration, mussel treatments had shown significant differences with controls in ΔTotal Chl (P-value<0.001) (Fig. 12a), ΔChl a (P-value<0.001) (Fig. 12b) and Δcarotenoids (P-value = 0.0103) (Fig. 12d). Mussel presence in the lake system results in a strong increment of NH_4 (Fig. 11a), but the NH_4 increment is not statistically significant in the Muar River system.

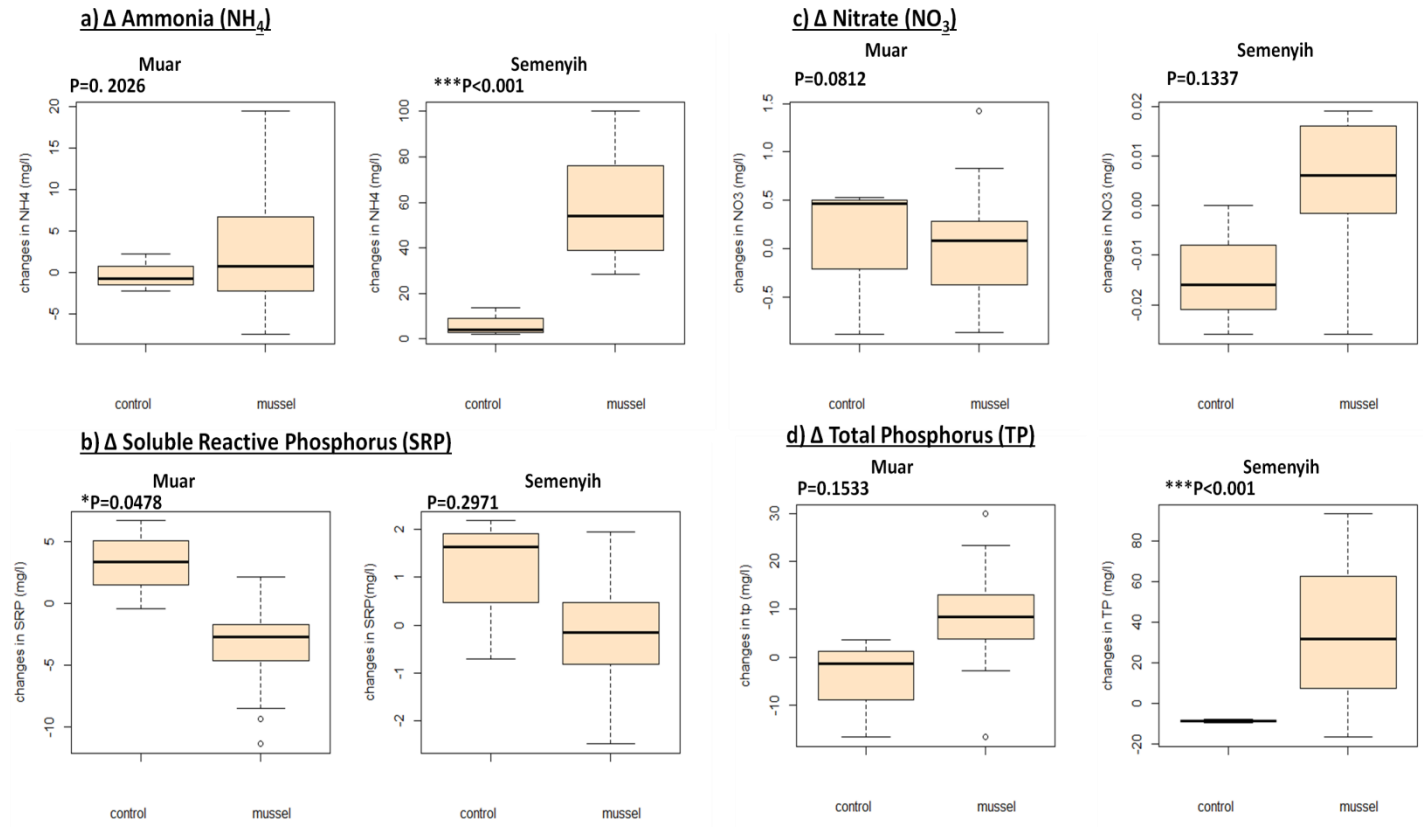

Figure 10 Box plots of the changes in concentration of nutrients after 3h in 3L of water without (control) and with one freshwater mussel (mussel) for Muar River (n=33) and Semenyih Lake (n=23), and P-values of Welch Two Sample t-test, (*p<0.05, **p<0.01, and ***p<0.001).

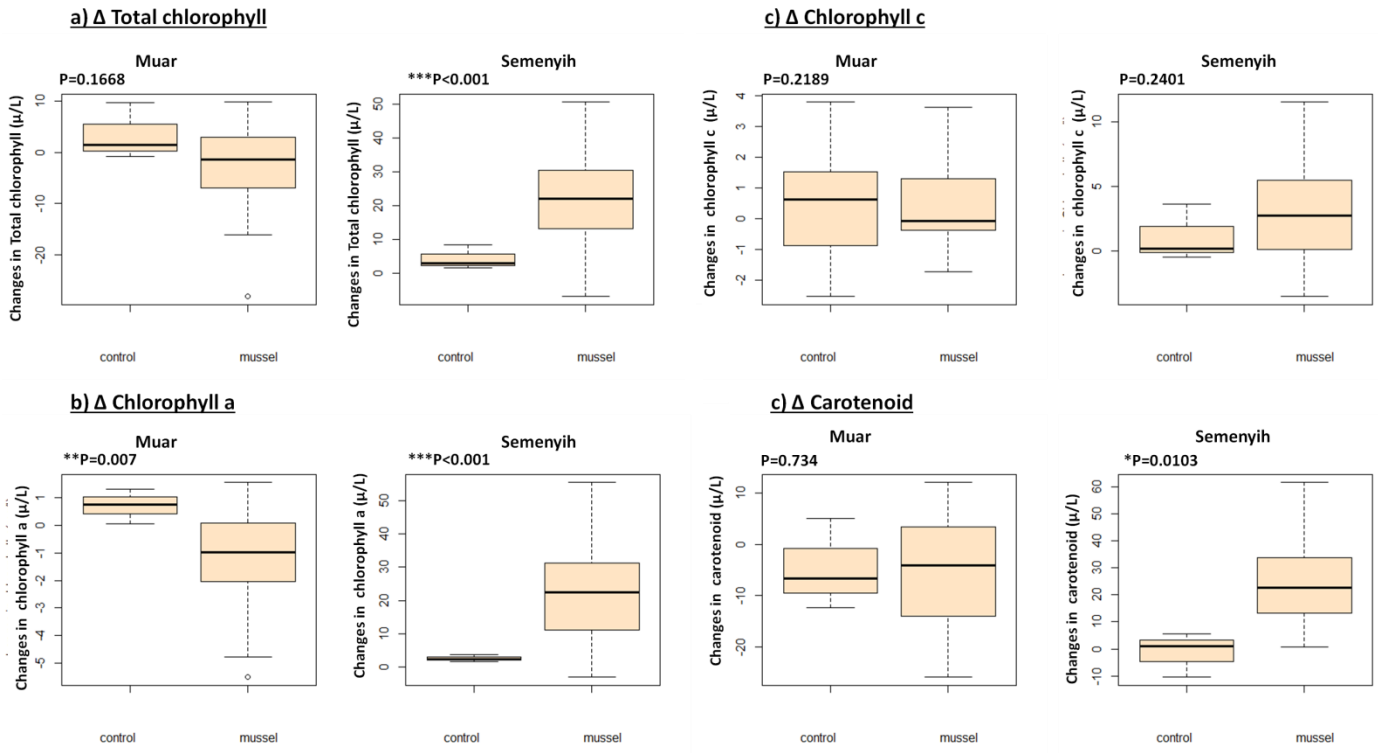

Figure 11: Box plots of the changes in concentration of pigments after 3h in 3L of water without (control) and with one freshwater mussel (mussel) for Muar River (n=33) and Semenyih Lake (n=23), and P-values of Welch Two Sample t-test, (*p<0.05, **p<0.01, and ***p<0.001).

The most interesting result lies in the changes of concentration of Chl a for Semenyih Lake mussels (Fig.12b). Instead of a decrease in the concentration of Chl a in mussel jars as observed in the Muar River, mussel presence in Semenyih Lake water led to a strong increase in the concentration of Chl a. The other pigments (Chlorophyll c and Carotenoid) also had shown an increase in concentration, leading to an increasing total chlorophyll concentration for the mussels which is an opposite of the declining trends observed in Muar River system. The effects of mussel presence in this respect on nutrients (Fig.11) and on pigments (Fig.12) indicate clear differences in the effect of the invasive species *S. woodiana* in Lake Semenyih compared to the native species *P. vondembuschianus.* and *C. contradens* in Muar River. Mussels apparently exhibit contrary effects with respect to a number of parameters, most notably NH_4 (Fig. 10a) and Chl a (Fig. 11b).

In Muar River, the effect of mussels on SRP was further confirmed by a strong correlation between mussel shell length and ΔSRP (P-value= 0.05, adj. r^2= 0.0901) in each mussel jar (Fig. 13a). General linear model with mussel species as co-variate also had shown significant difference between the two species *P. vondembuschianus* and *C. contradens* (P-value=0.0194, n=30, F=0.09484) that were not found in any response variables (Fig. 13b). There is also an increase in TP change; again this effect is not statistically significant and not visible in mesocosm.

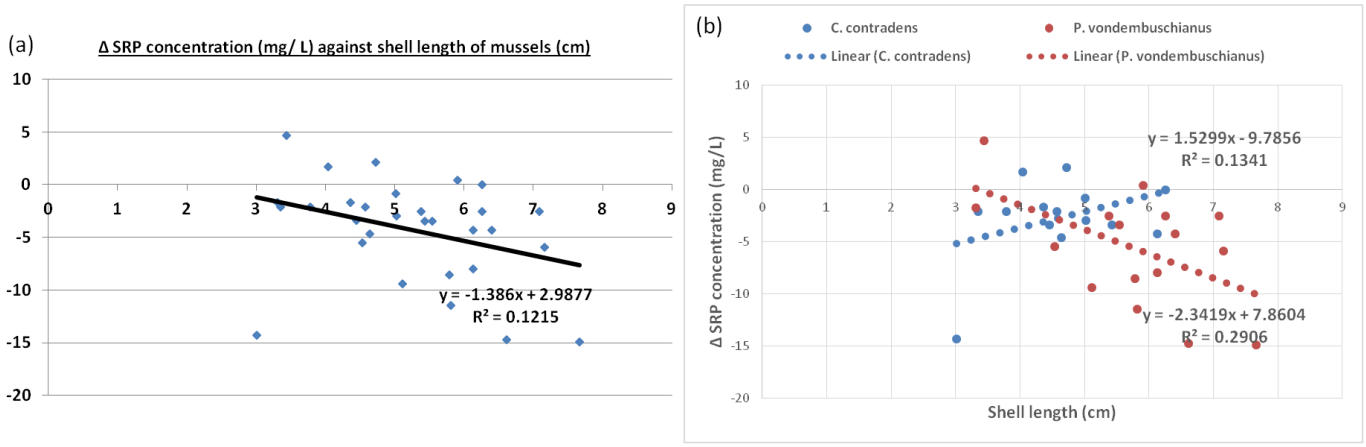

Figure 12: a) Linear Regression test for the correlation between the changes in concentration of nutrients with the shell length of mussels during the ex situ filtration of Muar River mussels (n=30)(P=0.05. adj. r^2=0.0901). b) General Linear Model of correlation between mussel shell length and Δ SRP for two mussel species *C. contradens* and *P. vondembuschianus* in ex-situ filtration experiments on Muar River water (n=30) (F= 0.09484, adj. r^2=-0.1033, P=0.0194).

In Semenyih Lake ex situ experiment, the changes of concentration in all of the parameters with significant differences are also strongly correlated with the mussel length in each jar (Fig. 14). In the changes of nutrient concentration, the shell length of mussels had shown to be significantly correlated with ΔNH_4 (P-value<0.001, adj. r^2=0.6634) (Fig.14a) and ΔTP (P-value<0.001, r^2= 0.5205) (Fig.14b). For changes in pigment concentration, the shell length of mussels had been shown to be significantly correlated with ΔTotal chlorophyll (P-value<0.001, adj. r^2= 0.326) (Fig.14c) , ΔChlorophyll a (P-value=0.002, adj. r^2= 0.3909) (Fig.14d) and ΔCarotenoid (P-value=0.014, adj. r^2=0.3909) (Fig.14e).

During the filtration, the water was visibly clearer towards the end of the 3-hour filtering period with some deposits forming around the mussels as compared to the water clarity at the start of the experiment. This change in water clarity was visible in the mesocosm as well, although the effects were not as obvious due to the vast difference in water inside respective sealed containers.

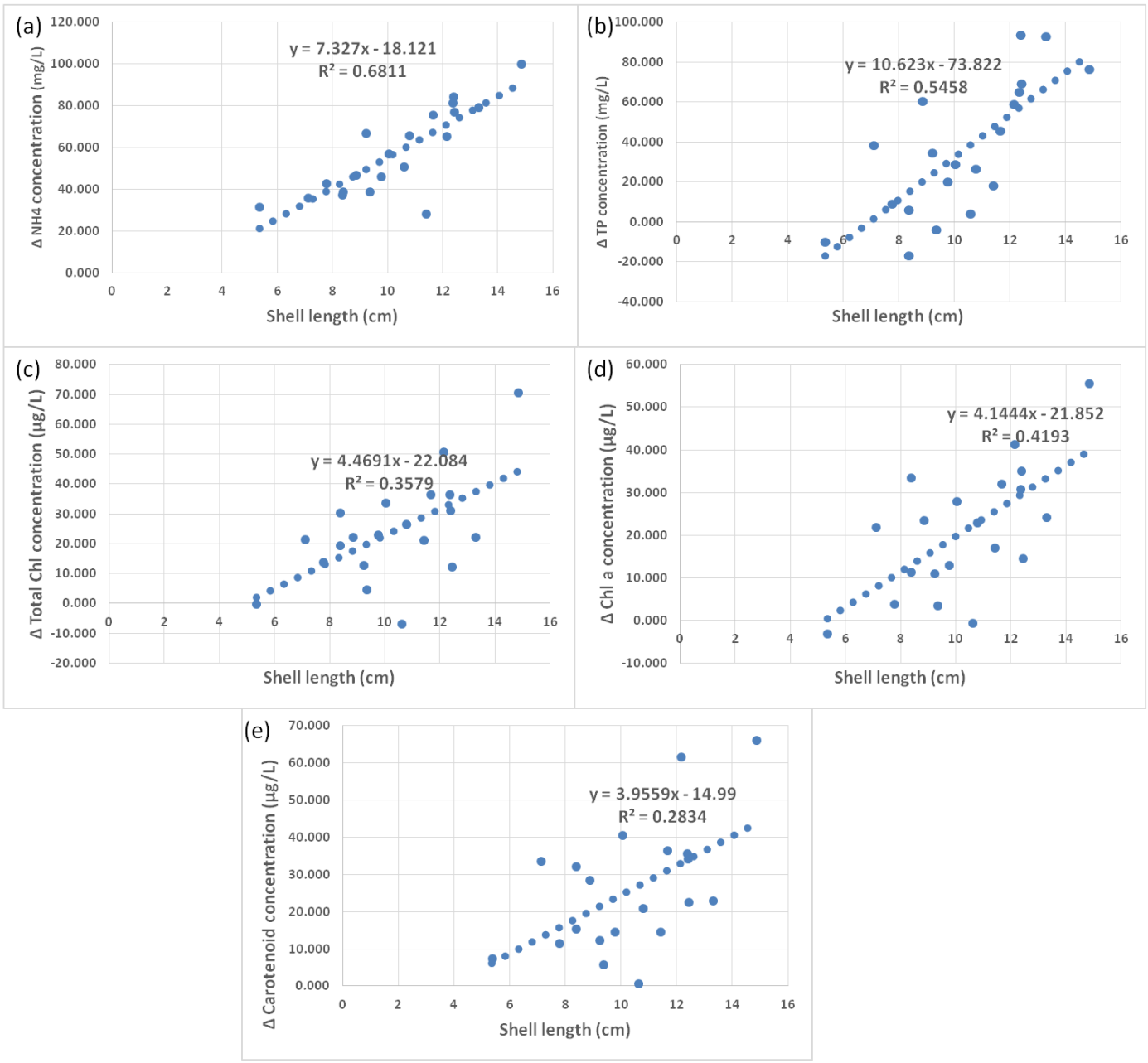

Figure 13: Linear Regression test for the correlation between the changes in concentration of nutrients and pigments with the shell length of mussels during the ex situ filtration of Semenyih Lake mussels (n=20). a) ΔNH4 (P<0.001, adj. r^2=0.6634) b) ΔTP (P<0.001, adj. r^2=0.5205,), c) ΔTotal Chlorophyll (P<0.001, adj. r^2=0.326), d) ΔChlorophyll a (P=0.002, adj. r^2=0.3909), and e) Δcarotenoid (P=0.014, adj. r^2=0.2517).

2.4 Discussion

The effects of large, widespread increases in nutrient loads, and the general relationships between mussels and nutrients have not received as much attention from mussel ecologists (Strayer, 2014).The present study revealed considerable differences in the functional ecology of different Peninsular Malaysia freshwater mussel species in different ecosystems. Whilst native mussels in the Muar River significantly decreased Chl a and SRP concentrations in their environment, the non-native *S. woodiana* appeared to affect Lake Semenyih by excreting high amounts of NH4 and depositing undigested pigments and TP. The clearance of chlorophyll from the water column were often measured as corresponding to the clearance rate of which mussels filter materials from the water column (Vaughn *et al*. 2004).

In situ clearance rates (field experiments)

Although no significant effect of mussel presence was observed during in situ experiments assessing mussel clearance rates in both Muar River and Semenyih Lake, there are some observable trends in the change of nutrient and pigment concentration. Based on my analyses, the concentration of Chl a and Carotenoids both significantly decreased with increasing number of mussels at both Muar and Semenyih. This may indicate that *C. contradens* and *P. vondembuschianus* in Muar River and *S.woodiana* in Semenyih Lake are able to alter nutrient limitations in their respective ecosystems, similar to the filter-feeding organisms observed by Atkinson *et al.* (2013) in Kiamichi, Little and Mountain Fork Rivers.

The changes of pigments indicate that the mussels in this mesocosm experiment were clearing away the phytoplanktons from the water column, although not to an extent that significant differences in paired t-tests can be observed. This is most probably due to the fact the mussels do not have enough time to filter all of the water in each experimental bins (average 42L) in 3 hours, since an average recorded freshwater mussel filtration rate is ~0.5-1 L/h (Mc Ivor, 2004) . Besides the volume, the sampled water was also taken near the top of water surface instead of from the sediment-water interface where the mussels-nutrient interaction is taking place.

Strayer *et al.* (1999) stated that the amount of materials that mussels are able to remove from the water column depends on the abundance of feeding mussels, therefore the strong correlation between mussel density and the decrease of total chlorophyll concentration in Muar River may suggest that in general, *C. contradens* and *P.*

vondembuschianus behave similarly to their counterparts in temperate regions when in natural conditions. However, there is a possibility that the decreasing concentrations of both pigments are due to the settlement of suspended particles rather than due to the effects of the filter-feeding activity of mussels.

Even though changes in concentration of several parameters in control sites were significantly correlated with mussel density, this observation is likely an outlier in this field-experiment. As the bins were placed in natural sediment, it is possible that not all mussels within the control-bins were taken out prior to the filtering time period. Freshwater mussels have been observed to bury fully for several centimetres deep in some instances (Zieritz *et al.* 2014). These fully-buried mussels may have started to emerge after we removed the semi-buried mussels in the upper layer.

Another factor of these changes in control bins could be contributed to the fact that shells of epibenthic mussels burrowed in the sediment stabilises the benthic substrate (Gutierrez *et al.* 2003). Therefore, there is also a possibility that the removal of the mussels loosens the benthic structure, causing the sediment more prone to disturbance of water flow within the control bins compared to the mussel bins where all the mussel shells are still intact in the sediment.

This is not the first time that no significant observation could be made on the filtration rate of freshwater mussels during in situ experiment. Nichols & Garling (2000) discovered that stream mussel species captures bacteria at 10 times the efficiency of lake mussel species under laboratory conditions, though no significance difference were recorded when the all the same species were used during field studies. Soto & Mena (1999) determined that *D. chilensis* combines direct consumption of particulates with biodeposition in clearing the water column by corroborating results from mesocosm tank experiments with the results from laboratory filtration experiments. These studies indicate that the integration of field studies and laboratory studies are very important in order to thoroughly study the relationship between the filter-feeding of freshwater mussels and its effect on the nutrient change in the water column.

Ex situ clearance + deposition (Laboratory experiment)

Ex situ experiments assessed the joint effects of clearance and deposition of mussels by homogenization of particles in water column and materials deposited by the mussels prior to water sampling for analysis. This homogenization of particles (instead of

just sampling the water near the surface as conducted during in situ experiment) could be the main reason that significant changes in the concentration of nutrient and pigment were more readily observable in ex situ (clearance + deposition) compared to in situ (clearance) experiment.

Mussel excretion products contribute towards an increase in the NH_4 concentration in both river mussel and lake mussel community, although the effect of a single mussel $3L^{-1}$ $3h^{-1}$ was strongly significant only for the lake mussel community. This high difference shows that *S. woodiana* is contributing a lot more to the NH_4 load in Semenyih Lake compared to the native *P. vondembuschianus* and *C. contradens* in the Muar River (6.52 mg/L against 2.93 mg/L).The increase in NO_3 observed for *S. woodiana* in Semenyih Lake is most likely the subsequent result of the denitrification of NH_4 excreted by the mussels, which explains why the increase of concentration is not as high for the river mussel community which have much lower NH_4.

The decrease in SRP concentration shows that mussels in both systems remove phytoplankton from the water column, as removal of algae from the water column reduces SRP-leakage into the water from dying algae (Bowes *et al*. 2012). The decrease however was only significant for river mussel community which suggest that the lake mussel community are not actively removing phytoplankton from system. Instead, TP increase was strongly significant ($P<0.001$) only for Semenyih Lake but not in Muar River mussel community. Increasing TP concentration reflects the mussel's biodeposition activity, in which undigested algal molecules are deposited in the form of faeces and pseudofaeces (Vaughn & Hakenkamp, 2001), which means that *S.woodiana* is more active in the biodeposition of organic materials compared to *C.contradens* and *P.vondembuschianus*.

Even though there are two different species of native freshwater mussel in Muar River, I found no significant differences between *P. vondembuchianus* and *C. contradens* in all response variables except in ΔSRP. This significance difference between each of the river mussel species however may be due to size effect instead of species effect. From my samples, *P. vondembuschianus* (average = 5.76cm) are in general have longer shell compared to than *C. contradens* (average = 4.62cm) and consequently, higher biomass. Vaughn *et al*. (2004) also found no significant species effects between *A. plicata* and *A. ligementina* in their in situ clearance mesocosm

experiment in Kiamchi River. This finding suggests that even in tropical ecosystem, biomass effect triumphs over species effects on the mussels' filtration rates.

As with differences in nutrient concentrations, the pronounced differences in pigment concentration observed between the Muar River mussels and the Semenyih Lake mussels (Fig. 12) also provides further evidence that different mussel species play different roles in different types of freshwater ecosystems. For river mussel community, the concentration of pigments decreased indicating that algae were filtered from the water column and subsequently digested by the native unionids as expected based on previous studies (Chowdhury *et al.* 2016; Soto & Mena, 1999; Vaughn *et al.* 2004),

For the lake mussel community however, all the concentration of pigments vastly increased in mussel treatment jars (Fig. 12). The average final concentration of Chl a in particular, increased almost 3-fold from the Chl a concentration at the beginning of the experiment (from ~7.6µg/L to ~26.15 µg/L). The significant increase in 3 hour period means that the predominant process taking place in the lake system is the biodeposition of undigested material instead of filtration as observed in the river system. *S. woodiana* in the ex-situ clearance excretes 19.15µg NH_4 $mussel^{-1}$ H^{-1}.

As a comparison to the biodeposition rate of other freshwater mussel species, *M. flacata*, had an average biodeposition rate of 3mg $h^{-1}mussel^{-1}$, with 25% from the total amount of biodeposited materials are organic matter (Spooner & Vaughn, 2006). Adult *D. chilensis* produced 7.9 ± 4.8 µg of NH_4 in an environment with 10-12µg of Chl a in an hour, after a day of feeding (Soto & Mena, 1999) while *E. menziesii*, excretes 4-50µg N $mussel^{-1}h^{-1}$, which increases with increasing mussel size (Cyr *et al*. 2017).

The present study represents the first time that mussels were shown to play obviously different roles in different tropical freshwater habitats. There is a possibility that this difference could be attributed to the vast differences between *S. woodiana* and other unionid species. Among invasive unionid, *S. woodiana* is the only species that have vastly different environmental requirements from other species of freshwater mussels. For example, they are the only species that actually thrive under anthropogenically modified conditions rather than being negatively-affected (Zieritz *et al*. 2016). Further experiments is needed to test whether this depositing behaviour is exhibited by all *S.woodiana* populations or just under some circumstances, and whether the behaviour is a characteristic of the species or caused by the environmental conditions instead.

Conclusion

The significant decrease of Chl a concentration and the significant increase in chl a concentration in river mussel community and lake mussel community respectively indicates the primary role undertaken by each of the freshwater mussel community, either filtration or biodeposition. Filtration and biodeposition by freshwater mussels alter the nutrient limitation in the water column of freshwater habitat and increases the availability of organic matter for the consumption of other organisms in the benthic ecosystem, a trait that earned its status as ecosystem engineers. The relationship between freshwater mussels and benthic macroinvertebrates will be discussed in the next chapter.

Chapter 3: Mussels and benthic biodiversity

3.1 Introduction

Approximately 126,000 freshwater animal species have been described, representing 9.5% of the total number of globally recognised animal species (Balian *et al.* 2008). This figure is highly disproportional when compared to other habitats, considering that freshwater habitats only take up roughly 0.01% of the total surface of the planet. Unfortunately, the disproportionate richness also means that freshwater habitats are extremely vulnerable to impacts of anthropogenic changes and environmental changes (Dudgeon *et al.* 2006). Some of the major factors contributing towards declining freshwater biodiversity are the destruction and degradation of habitat, as well as the overexploitation of both water and organisms (Vaughn, 2010). Destruction of habitat is attributed to human activities such as deforestation which is often followed by urbanisation, as well as the impacts from agricultural practices (Downing *et al.* 2010).

In Malaysia, Douglas *et al.* (1993) had observed that logging activities contributed towards increased river sediment yield by two to fifty times than the natural rate. Apart from logging, oil palm plantations also had been shown to affect the abundance and diversity of Malaysian freshwater macroinvertebrates (Mercer *et al.* 2014). In addition to the direct effects of habitat modification towards freshwater mussels in Malaysian rivers, the declining fish population also has a significant impact on the reproduction success of the mussels. This is due to the dependence of these mussels on host fish during its obligatory parasitic stage (Bogan, 2008). Similar to the pattern observed globally, the biodiversity in Malaysian freshwater habitats are at great risk, with 87% of its fish species are threatened mainly due to habitat modification (Chong *et al.* 2010).

In many freshwater ecosystems, freshwater mussels (Bivalvia: Unionida) make up the bulk of the benthic biomass, fulfilling crucial ecosystem functions (Howard & Cuffey, 2006; Spooner & Vaughn, 2006; Vaughn & Hakenkamp, 2001). The ecosystem engineering ability of freshwater mussels had been demonstrated in several studies previously, although these studies are mostly carried out in temperate ecosystems (Howard & Cuffey, 2006; Spooner & Vaughn, 2006; Vaughn & Spooner, 2006). In the rivers of Ouachita Highlands, the presence of mussels has been associated with an increase in benthic biodiversity (particularly Oligochaeta, Chironomidae, Ephemeroptera and Trichoptera) (Vaughn & Spooner, 2006). In the Kiamichi River, increasing biomass

of periphyton produced by living mussels was observed to lead to an increase in invertebrate richness and abundance significantly compared to empty shells alone (Spooner & Vaughn, 2006). This was particularly true for grazing invertebrate taxa, such as larval Chironomidae, Ephemeroptera and Trichoptera. Similarly, in Dhanmondi Lake in Bangladesh, the density of freshwater mussels was shown to have strong positive correlation with local macroinvertebrate taxon richness (Chowdhury *et al*. 2016).

The studies by Vaughn & Spooner (2006) and Chowdury *et al.* (2016) were mainly descriptive and therefore do not provide experimental evidence that mussels are causing high biodiversity of invertebrates. However, even without evidence for causation, a significant correlation between the presence of mussels and benthic biodiversity can be used for monitoring purposes by using freshwater mussels as indicator organisms as suggested by Aldridge *et al.* (2007). The use of indicator organisms can be particularly helpful for biomonitoring in developing countries where little money, expertise and tools are available for monitoring.

The patterns of biodiversity and functional ecology of different organisms in temperate regions are fairly well researched and understood (Covich *et al*. 1999; Thorp, 2015; Vaughn & Hakenkamp, 2001; Wallace & Webster, 1996). In tropical forest streams the variation in aquatic macroinvertebrate assemblage structure had been strongly attributed to stream velocity, water quality and substrate structure (Md Rawi *et al.* 2013). However, the relationship between freshwater mussels and the benthic invertebrate biodiversity in this region had not been as widely-studied as in temperate regions. The consistent exposure to sunlight, leading to continual primary production, and higher volume of rainfall causes tropical rivers to have warmer water and face large predictable floods much more frequently than temperate systems (Boulton *et al*. 2008). Therefore, the role of mussels in tropical habitats may be expected to differ considerably from that in temperate systems.

Despite the major threat towards biodiversity in Malaysian freshwater habitats, governance practices however focused mostly on management of water shortage, floods and pollution instead of conservation efforts (Weng, 2005). A management strategy geared more towards conservation of freshwater biodiversity is required in order to protect the ecosystem from further deterioration. The main key to successful biomonitoring, management and conservation of habitat is the ability to perform frequent

assessments of biodiversity using a method that has a low impact and is affordable in a long term (Lim *et al*. 2016).

Besides being time consuming, identification of bioindicator taxa based on morphology also rarely supports species-level resolution (Hajibabaei *et al.* 2011). This is especially true in the cases of organisms that are still in immature life stages. To overcome this problem, molecular genetics tools such as DNA barcoding have emerged as an alternative in recent decades, and had been noted to be effective in detecting even elusive and invasive species (Bohmann *et al.* 2014). One of the most widely applied techniques is DNA barcoding. The primary concept of DNA barcoding is the comparison between the query sequence and a DNA barcode reference library comprised of known species (Hajibabaei *et al.* 2011). The standard 'taxon barcode' for most animal groups is the mitochondrial Cytochrome c Oxidase I gene (COI), and by far the most represented in Genebank reference library (Leray *et al*. 2013).

The present paper aims at improving our knowledge on the biodiversity and processes of macroinvertebrates in tropical rivers, using rivers in Peninsular Malaysia as study systems and with an emphasis on the role of freshwater mussels of the order Unionida. Detailed objectives are to (1) determine whether mussels are associated with diversity of benthic organisms or particular groups of benthic organisms in rivers in Peninsular Malaysia; (2) gather preliminary data on benthic macroinvertebrate habitat requirements (specifically, water chemistry); (3) assess availability of DNA barcoding data for molecular identification of macroinvertebrates in the study region.

3.2 Materials and Methods

The association between mussel presence/ absence and the macrozoobenthos composition and diversity was investigated in a descriptive study described and presented in the following:

Benthic macroinvertebrates samples were taken from 28 different rivers across Peninsular Malaysia; 14 of the rivers with recorded mussel presence and another 14 with recorded mussel absence according to Zieritz *et al*. (2016) (Fig.15). Since the focus of conservation efforts should be species that are native to the Peninsular Malaysia, only the sites with the presence of native freshwater mussel population were chose for this experiment, which are mainly located in the Pahang catchment and Perak catchment of Peninsular Malaysia (Zieritz *et al*. 2016). Sampling was carried out in the states of Perak, Selangor, Negeri Sembilan, Melaka, Pahang and Kelantan from September 2016 to December 2016.

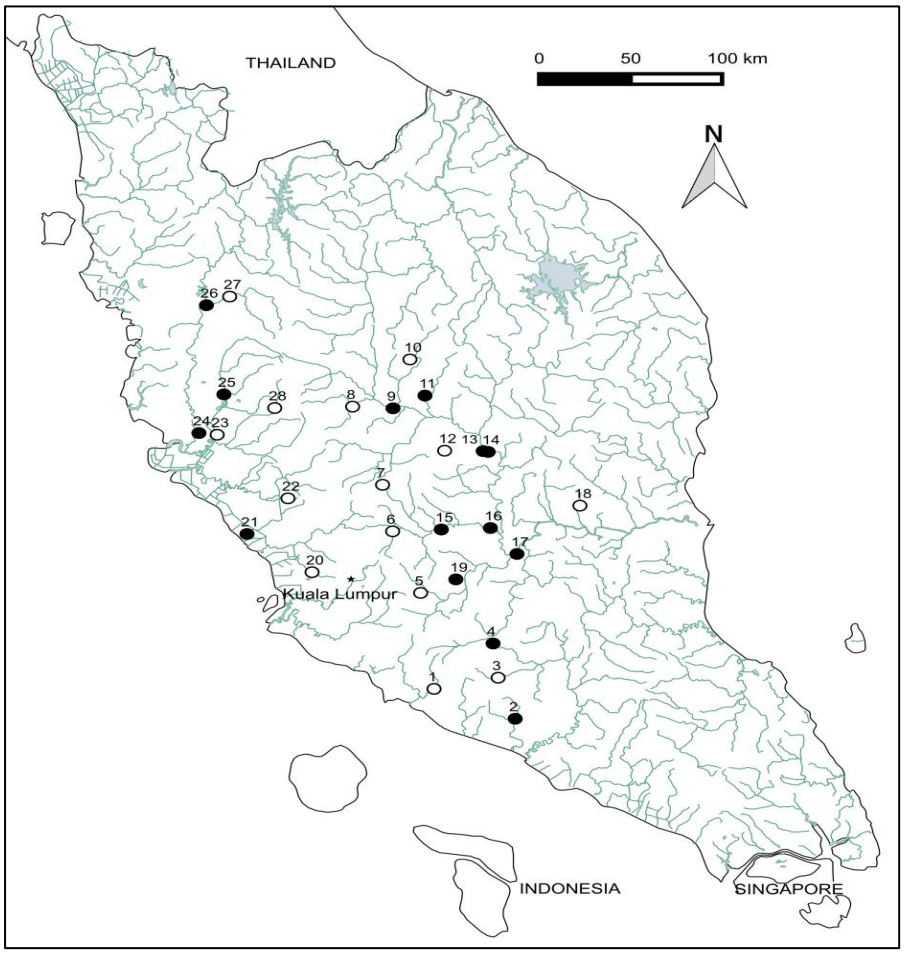

Figure 14: The 28 sampling locations in Peninsular Malaysia. 14 sites have mussel populations present (Black) and 14 sites without present mussel populations (White).

Study sites ranged from small streams in residential villages, streams flowing through farms and oil palm plantation to large rivers with bridges built for vehicles crossing (Fig.16). Some rivers have intact natural banks while some others had corroded banks or man-made structures along the banks. Sites with and without mussels did not exhibit significant differences in nutrient or ion concentrations tested (Table 2). In other words, the water chemistry in all mussel and non-mussel sites is generally similar, therefore can be ruled out as a contributing factor towards the results of the difference in species richness survey.

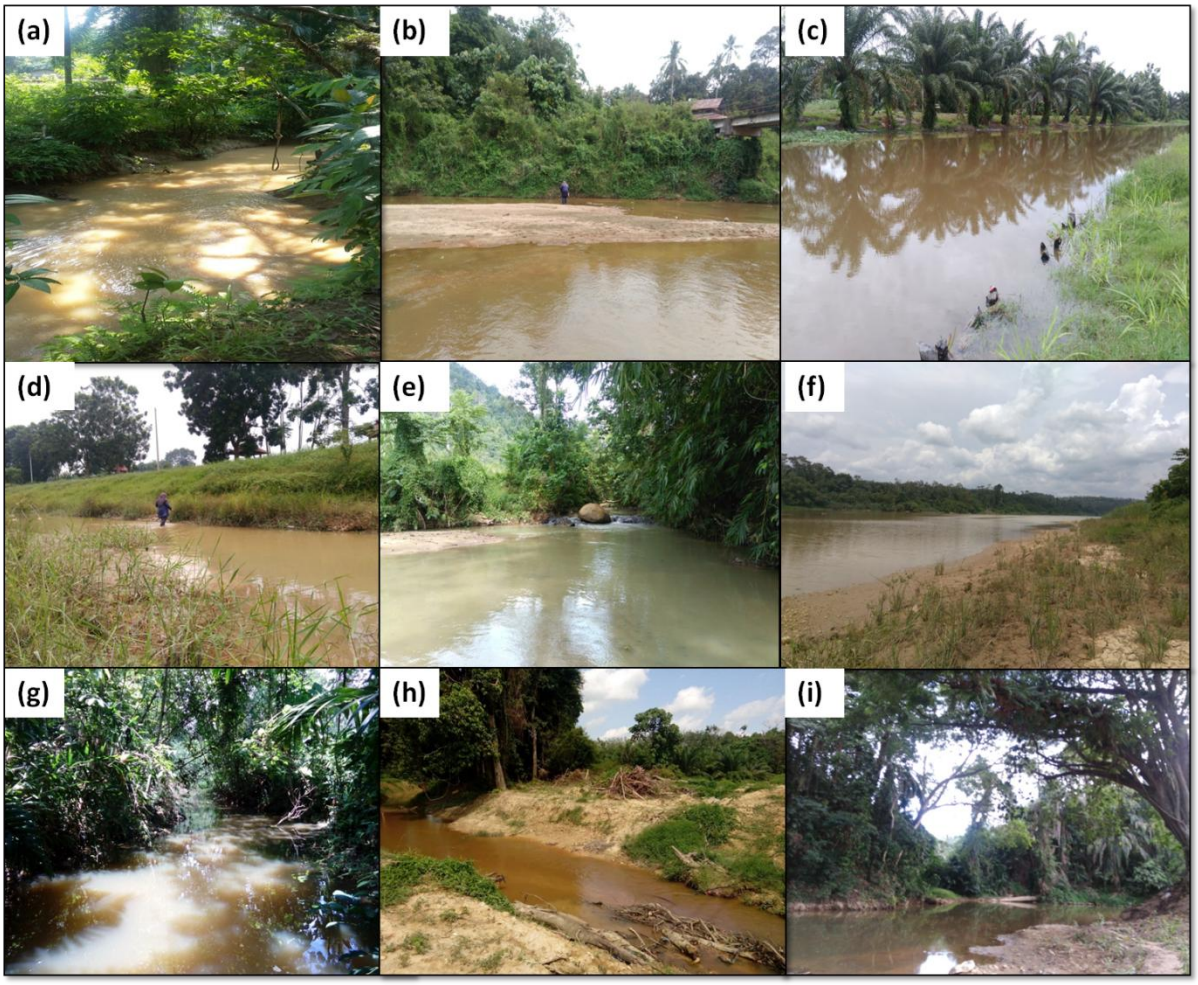

Figure 15: Some of the sites sampled for macroinvertebrates biodiversity. a) unnamed stream in Kampung Hayat, tributary of Triang River, b) Rembau River, c) water channel for paddy irrigation in Tanjung Karang, Selangor, d) Chohong River, e) Kongkoi River, f) Pahang River, g) unnamed stream in Kampung Ubi, Sungai Koyan, h) Kawang River, i) Bilut River.

Table 2: Water chemistry parameters [mean ±SD (range)] at 14 sites with and 14 sites without freshwater mussels (Unionida) in Peninsular Malaysia, including results of Welch's Two Sample T-tests. * Indicates P<0.05 and is significantly different

	Water chemistry parameter [mean ±SD (range)]		t	df	P
	Sites without Unionida	Sites with Unionida			
Silicate	7.476±2.425 (2-10)	7.543±2.290 (3-10)	-0.073	23.744	0.943
SRP	22.892±18.836 (2-47)	16.074±12.422 (7-52)	1.103	22.648	0.282
TP	82.455±123.249 (5-459)	83.254±76.924 (15-285)	-0.019	20.292	0.985
NH4	419.102±891.557 (7-2657)	47.865±81.943 (0-266)	1.550	13.256	0.145
Chloride	3.940±4.497 (0-15)	3.943±4.299 (1-13)	-0.002	19.960	0.999
Sodium	3.925±3.311 (1-13)	4.262±2.608 (0-9)	0.284	22.477	0.779
Magnesium	1.870±0.975 (0-3)	2.055±0.723 (0-3)	-0.541	22.025	0.594
Potassium	2.148±1.884 (0-7)	2.851±1.838 (0-5)	-0.943	22.921	0.355
Calcium	9.162±6.666 (2-28)	14.430±12.016 (2-49)	-1.340	16.889	0.198

At each site, a Hess benthic sampler was placed in a spot and the sediment within the enclosed area was disturbed to detach macroinvertebrates from rocks and layers of the benthic substrate. Flowing river water will push the suspended particles downstream into a catchment net, and everything that was caught in the net was considered as one replication of sampling. Three spots downstream of one another were sampled consecutively before all the sampled were pooled in a single Falcon tube. Two replicates of samples were taken at each site for molecular analysis, and two replicates of samples were taken for morphological identification. All the samples were preserved in 100% ethanol, and kept in a freezer for storage. The benthic sampler and all its nets were cleaned with 10% bleach solution in between each river site to prevent cross-contamination.

Morphological identification

Macroinvertebrate individuals were sorted from each bulk sample into individual 1.5ml tubes for each sites and observed under a microscope for defining morphological traits, identified to order and – wherever possible – lower taxonomic level based on the key by Yule & Yong (2004). These traits included size, presence/absence of wings, shape of head and torso, mouth type and arrangement of leg pairs among others.

Molecular Analysis

Eleven individual macroinvertebrates were isolated into 1.5ml tubes labelled with site number and individual number. The DNA from each of the individuals was extracted

using Macherey-Nagel Nucleospin Tissue DNA extraction kit following the manufacturer's instruction. For 25µL PCR reaction, MyTaq Red Mix PCR premix was used in order to minimize pipetting thus reducing the risk of contamination. 12.5µL of the premix was pipette into 0.2ml PCR tubes, followed by 0.25µL of forward primer mlCOIintF –GGWACWGGWTGAACWGTWTAYCCYCC (Leray *et al*. 2013), 0.25µL of reverse primer HCO2198-TAAACTTCAGGGTGACCAAAAAATCA (Folmer *et al*. 1994), 9µL of ultrapure water and 3µL of DNA template. The preparation was done entirely on ice, and Polymerase Chain Reaction (PCR) was later carried out in order to amplify a part of the COI region for Sanger sequencing purposes.

For the PCR process, the cycle was started with 2 minutes initial warming at 94^{o}C, followed by 30 cycles of 94^{o}C denaturation step for 30 seconds, 30 seconds of annealing step at 43^{o}C and 1 minute of extension step at 72^{o}C, and upon the completion of the 30th cycle, a final 10 minutes standing time at 72^{o}C was incorporated before dropping down to 4^{o}C to rest until the PCR products were taken out from the thermal cycler for gel electrophoresis.

The PCR products were later run with 2% agarose gel to check whether amplification of the COI region was successfully. Once adequate quality bands were obtained, the unpurified PCR products were sent to a commercial company (First BASE Laboratories Sdn Bhd) for PCR clean-up followed by Sanger sequencing. Once the sequences were obtained from the sequencing company, the chromatograms were examined and sequences cleaned up with program Chromatogram Explorer. Finally, for each sequence, a nucleotide Basic Local Alignment Search Tool (BLAST) was run in Genbank to identify the most closely matching sequence available on Genbank and, on that basis, to identify the organisms down to species or higher level.

Statistical analysis

The difference between the species count in mussel sites and non-mussel sites were tested using Welch's Two Sample T-tests. Besides the species count, I ran Welch's Two sample T-tests between the water chemistry parameters in mussel sites and non-mussel sites to see whether there were any difference between these two types of sites. I also ran a Canonical Correspondence Analysis (CCA) to elucidate the relationships between biological assemblages of species and the water chemistry parameters of mussel sites and non-mussel sites.

3.3 Results

In total, I found 127 specimens in total from four phyla Arthropoda, Annelida, Nematoda and Mollusca from all sites (Table 3). Organisms from the Insecta class are the most commonly sampled in this study (91 individuals out of 127), making up approximately 70% of the entire bulk of sampled organisms. The Insecta class is also the most diverse class among sampled organisms, with at least 7 identified orders from the total of 13 orders recorded from all sites. From the four phyla, there are 13 orders discovered; Trichoptera, Ephemeroptera, Plecoptera, Coleoptera, Hemiptera, Diptera, Odonata, Decapoda, Veneroida, and an unidentified order each from Arachnida, Nematoda, Oligochaeta, and Gastropoda.

Table 3 :Higher Taxa that were found in macrozoobenthos samples taken across 28 sites in Peninsular Malaysia

	Number of Individuals	Total for Phylum and Order
Phylum Arthropoda	-	**Phylum = 94**
Class Insecta	-	**Class = 91**
Order Trichoptera	-	**Order = 7**
Fam. Hydropsychidae	4	-
Fam. Unidentified	3	-
Order Ephemeroptera	-	**Order = 25**
Fam. Baetidae	3	-
Fam. Potamanthidae	2	-
Fam. Neophemeridae	3	-
Fam. Caenidae	5	-
Fam. Unidentified	12	-
Order Plecoptera	-	**Order = 2**
Fam. Unidentified	2	-
Order Diptera	-	**Order = 27**
Fam. Chironomidae	15	-
Fam. Nymphomyiidae	1	-
Fam. Cerratopogonidae	1	-
Fam. Dolichopodidae	2	-
Fam. Unidentified	8	-
Order Hemiptera	-	**Order = 13**
Fam. Gerridae	7	-
Fam. Mesoveliidae	4	-
Fam. Unidentified	3	-
Order Coleoptera	-	**Order = 6**
Fam. Elmidae	1	-
Fam. Dysticidae	2	-
Fam. Unidentified	3	-
Order Odonata	-	**Order =11**
Fam. Libelliliudae	3	-
Fam. Calopterygidae	2	-
Fam. Aeshinidae	3	-
Fam. Lestidae	2	-
Fam. Unidentified	1	-
Class Arachnida	-	**Class = 1**
Order Unidentified	1	-
Class Malacostraca	-	**Class = 2**
Order Decapoda	-	-
Fam. Palaemonidae	2	-
Phylum Nematoda	-	**Phylum = 2**
Class Unidentified	2	-
Phylum Annelida	-	**Phylum = 23**
Class Oligochaeta	-	-
Order Unidentified	23	-
Phylum Mollusca	-	**Phylum = 7**
Class Gastropoda	-	**Class = 2**
Order Unidentified	5	-
Class Bivalvia	-	**Class =2**
Order Veneroida	-	-
Fam. Cyrenidae	-	-
Genus Corbicula	2	-
Total Number of individuals	= **127**	

Comparing sites without and with mussels

Total species richness of benthic macroinvertebrates was significantly greater at sites with freshwater mussels compared to sites without freshwater mussels (**Error! Reference source not found.**). At the order level, the only order exhibiting a statistically significant difference between sites with mussels and without mussels was the Hemiptera, which on average counted one species per mussel site, and which were found at seven times more mussel-sites compared to non-mussel sites (Table 4). Other orders that showed a distinct though statistically insignificant association with mussel sites were Plecoptera and Decapoda, which were only present at mussel-sites, and Gastropoda, Odonata, Ephemeroptera and Diptera, which were up to four times more common and diverse at mussel-sites compared to non-mussel sites. In contrast, presence and species richness of sampled oligochaetes, nematodes and bivalves was similar between sites with mussels and sites without Unionida, and Trichoptera and Coleoptera actually were more common at sites without mussels.

Table 4: Average species richness for each macroinvertebrate order at sites with and without freshwater mussels in Malaysia, including results of Welch's Two Sample T-tests. * Indicates P<0.05 and is significantly different (M=4.536 SD=3.350).

	Number of sites with taxon		Species count [mean ±SD (range)]		t	Df	P
	Sites without Unionida	Sites with Unionida	Sites without Unionida	Sites with Unionida			
Trichoptera	4 (29%)	2 (14%)	0.286 ±0.469 (0-1)	0.214 ±0.580 (0-2)	0.359	24.923	0.723
Ephemeroptera	7 (50%)	9 (64%)	0.643 ± 0.745 (0-2)	1.143±1.167 (0-4)	-1.351	22.082	0.190
Plecoptera	0 (0%)	2 (14%)	0.143±0.363 (0-1)	0.000	-1.472	13.000	0.165
Diptera	6 (43%)	9 (64%)	0.714±0.914 (0-2)	1.214±1.122 (0-3)	-1.293	24.980	0.208
Hemiptera	1 (7%)	7 (50%)	0.0714±0.267 (0-1)	1±1.109 (0-2)	-3.045	14.504	0.008*
Coleoptera	3 (21%)	2 (14%)	0.286±0.611 (0-2)	0.143±0.363 (0-1)	0.752	21.160	0.460
Oligochaeta	7 (50%)	9 (64%)	0.786±0.893 (0-2)	0.857±0.770 (0-2)	-0.227	25.456	0.823
Odonata	2 (14%)	5 (50%)	0.214±0.579 (0-2)	0.5±0.855 (0-3)	-1.036	22.852	0.311
Arachnida	1 (7%)	0 (0%)	0.071±0.267 (0-1)	0.000	1.000	13.000	0.336
Nematoda	1 (7%)	1 (7%)	0.0714±0.267 (0-1)	0.0714±0.267 (0-1)	0.000	26.000	1.000
Decapoda	0 (0%)	2 (14%)	0.000	0.143±0.363 (0-1)	-1.472	13.000	0.165
Gastropoda	1 (7%)	4 (29%)	0.071±0.267 (0-1)	0.286±0.469 (0-1)	-1.486	20.643	0.153
Bivalvia (excl. Unionida)	1 (7%)	1 (7%)	0.071±0.267 (0-1)	0.071±0.261 (0-1)	0.000	26.000	1.000
All orders	11 (79%)	13 (93%)	3.286±2.701 (0-7)	5.786±3.56 (0-11)	-2.095	24.256	0.047*

Ephemeroptera and Oligochaeta were the most common orders sampled in our study, present at 57% of all sites (16 out of 28) (Table 4). For rare orders found, Arachnida was only found in only one site, whereas Plecoptera, Nematoda, Decapoda and Bivalvia other than Unionida were all found in two sites each. The maximum number of species found at a given site was 11 which happen to occur in a mussel site. In 4 out of the 28 sites (1 of them with Unionida populations), not a single macroinvertebrate organism was collected.

Environmental conditions and macrozoobenthos composition in study area

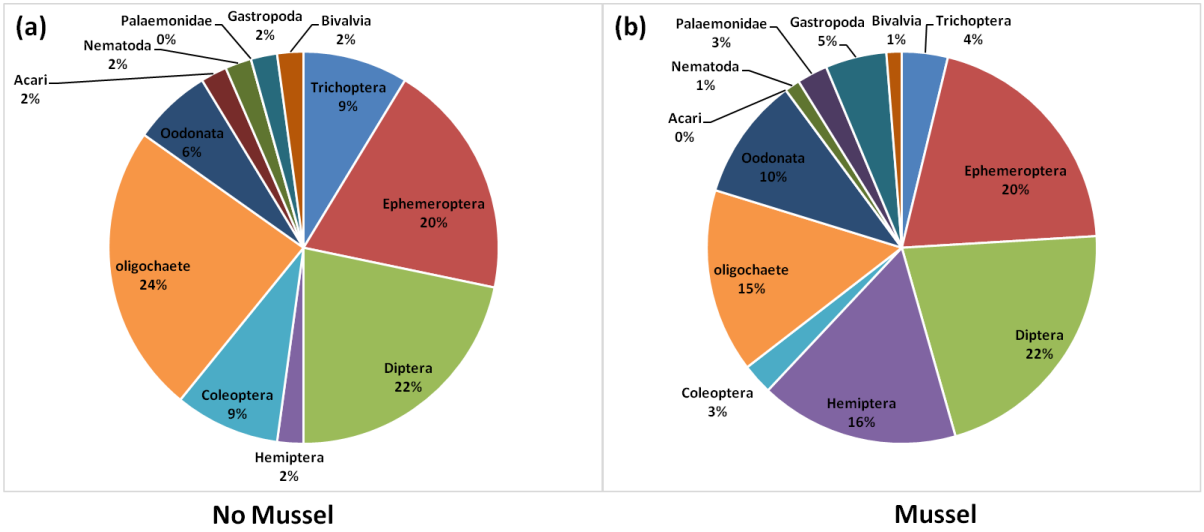

Figure 16: General distribution of species richness across macroinvertebrate orders across (a) 14 sites with and (b) 14 sites without freshwater mussels (Unionida) in Peninsular Malaysia.

Orders Ephemeroptera (20%) and Diptera (22%) made up equal percentage of species richness for both mussel sites and non-mussel sites, constituting nearly half of the total taxa found (Figure 16). The most obvious difference is in the percentage of Hemiptera in mussel sites (16%) compared to their percentage in non-mussel sites (2%). Oligochaeta, Coleoptera, and Trichoptera made up a higher percentage of species richness in non-mussel sites compared to mussel sites, whilst Odonata were more commonly found in mussel sites. Decapoda and Arachnida were both only found in mussel sites and non-mussel sites, respectively. In both mussel and non-mussel sites, Nematoda, Gastropoda and Bivalvia were all rarely present, although mussel sites have a higher percentage of Gastropoda (5%) found compared to in non-mussel sites (2%).

The associations of freshwater macroinvertebrates order with water chemistry parameters

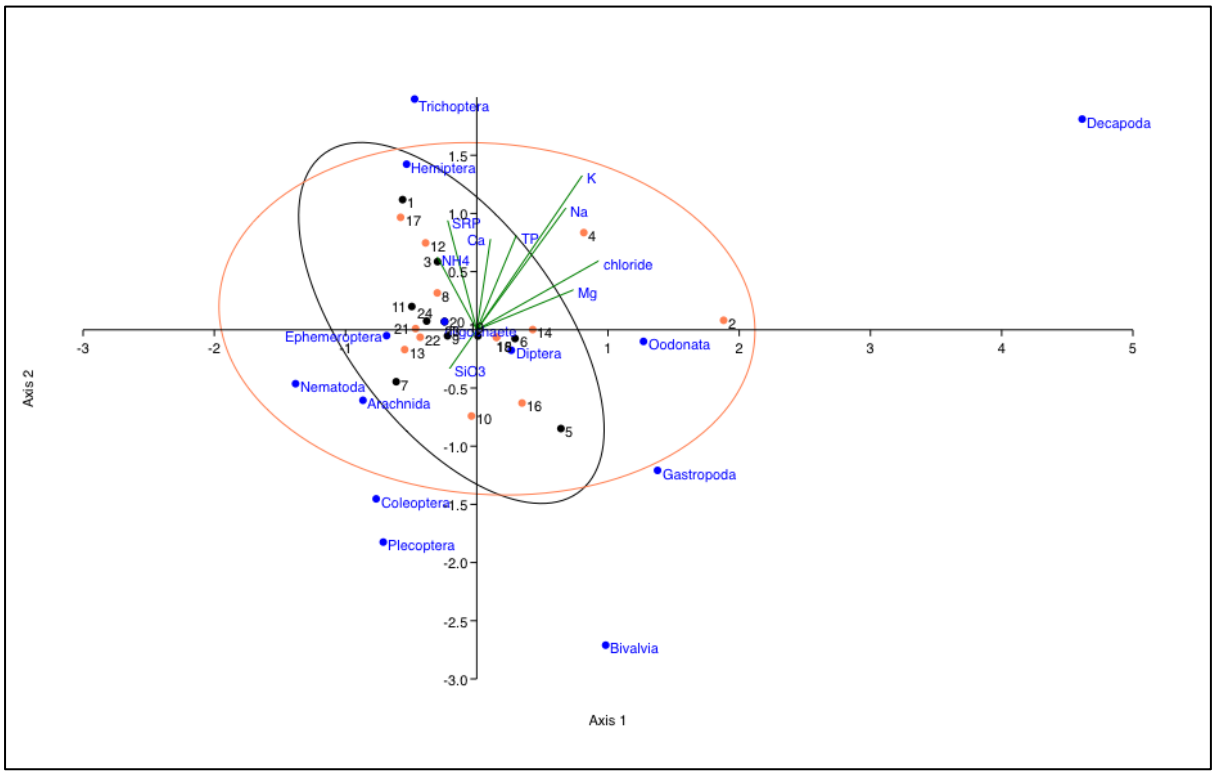

Figure 17: Canonical Correspondence Analysis (CCA) plot displaying association between water chemistry parameters and macroinvertebrate orders at sites with freshwater mussels (red dots and 95% Confidence Ellipse) and without freshwater mussels (black dots and 95% Confidence Ellipse) in Peninsular Malaysia. Abbreviations: Ca, Calcium; Cl, Chloride; Mg, Magnesium; Na, Sodium; K, Potassium; TP, Total Phosphorus; NH_4, Ammonia; SRP, Soluble Reactive Phosphorus; SiO^3, Silicate.

The CCA plot the association between freshwater macroinvertebrate orders with water chemistry parameters (Fig. 18) indicated that some macroinvertebrate orders displayed preferences for particular water chemistry conditions such as high ion concentration or high nutrient concentration. Decapoda for instance, appeared to prefer sites with high ion concentrations, whereas Nematoda, Arachnida, Plecoptera and Coleoptera were clustered together at the other side of the vectors, indicating a preference for low-ion conditions. Gastropoda, Plecoptera, Coleoptera and Bivalvia, on the other hand, showed an affiliation with sites of low nutrient concentration, whilst the opposite case was true for Trichoptera and Hemiptera, which have an environmental preference for relatively high nutrient concentrations. Ephemeroptera, Diptera and Oligochaeta did not exhibit particular preferences in terms of water chemistry within the study area.

Availability of DNA sequence data for Malaysian macroinvertebrates

Of the 11 randomly selected specimens from the samples, only two COI sequences matched with available sequences in Genbank to such an extent that is indicative of conspecificity (Table 5). These were (1) *Cheumatopsyche lucida*, a hydropsychid Trichoptera; and (2) *Paragomphus capricornis*, a gomphid Odonata (Fig.19). For eight further specimens, fairly closely matching sequences were available (85-92% similarity in COI), thus probably indicating the same genus. The best matching COI sequence available on Genbank for our Diptera specimen sequenced showed only 73% similarity, which suggests that the Malaysian genus in our sample has not yet been sequenced. For specimen 11A2, the closest matching species is aphid *Mindarus obliquus*, a terrestrial plant parasite (Foottit & Maw, 2014). The specimen might be from the same genus, though this means that it is most probably a terrestrial species as well, fallen into the river and sampled along during one of the replications.

Table 5: Results of Genbank BLAST of obtained COI sequences from macroinvertebrate specimens collected from sites across Peninsular Malaysia (numbers in code indicate site number as shown in Fig. 12); identities, gaps and taxon name of closest matched sequence available on Genbank is given.

Specimen	Identities	Gaps	Species Name	Order
1A1	258/258(100%)	0/258(0%)	*Cheumatopsyche lucida*	Trichoptera
5B2	232/236(98%)	0/236(0%)	*Paragomphus capricornis*	Odonata
28B2	259/282(92%)	0/282(0%)	*Sandracottus insignis*	Coleoptera
11A2	39/43(91%)	0/43(0%)	*Mindarus obliquus*	Hemiptera
21A2	78/86(91%)	0/86(0%)	*Dimeragrion percubitale*	Odonata
28B1	227/253(90%)	0/253(0%)	*Setodes mercurius*	Trichoptera
8A2	212/237(89%)	0/237(0%)	*Elmidae sp.*	Coleoptera
4B1	172/200(86%)	2/200(1%)	*Rhagovelia simulata*	Hemiptera
4A2	171/198(86%)	7/198(3%)	*Caridina sp*	Decapoda
21A1	217/256(85%)	0/256(0%)	*Iswaeon anoka*	Ephemeroptera
11A1	179/244(73%)	2/244(0%)	*Anopheles liangshanensis*	Diptera

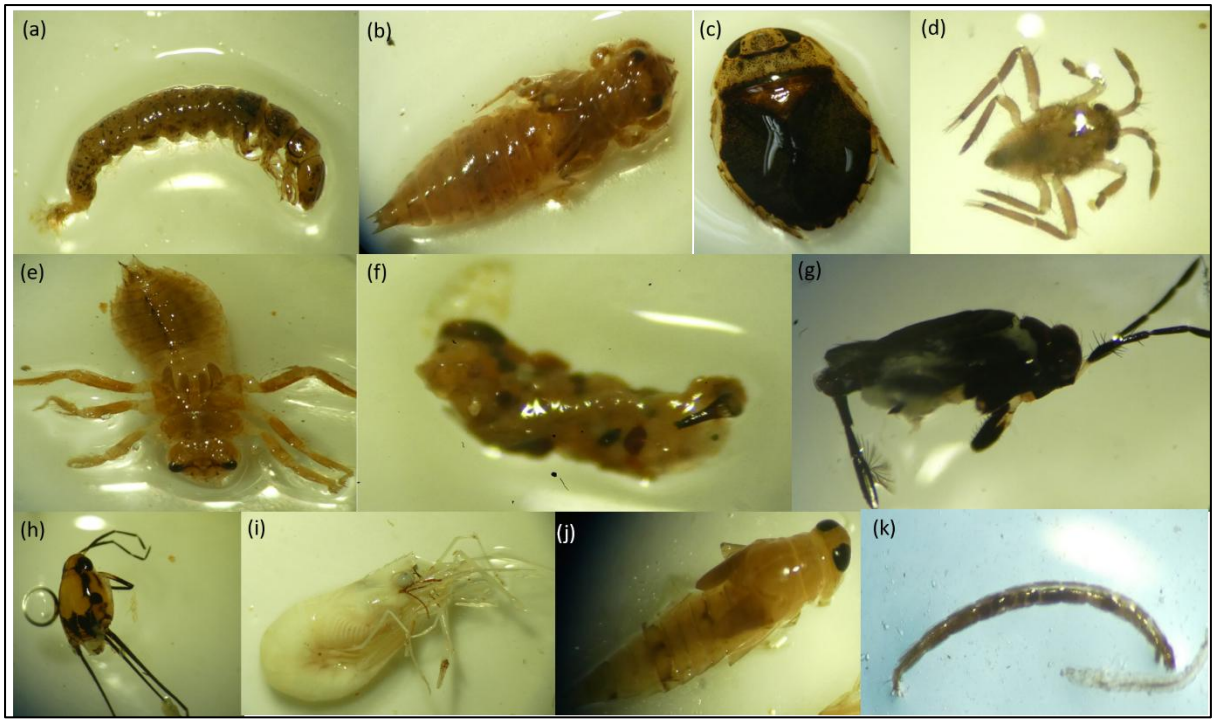

Figure 18: Freshwater invertebrates samples sequenced in our study, labelled with closest match in Genbank. a) 1A1, *Cheumatopsyche lucida.* b) 5B2, *Paragomphus capricornis* c) 28B2, *Sandracottus insignis. d)* 11A2*, Mindarus obliquus. e)* 21A2, *Dimeragrion percubitale.* f) 28B1*, Setodes mercurius.* g) 8A2, *Elmidae sp.* h) 4B1, *Rhagovelia simulate.* i) 4A2, *Caridina sp.* j) 21A1, *Iswaeon anoka.* k) 11A1, *Anopheles liangshanensis.*

3.4 Discussion

Association between freshwater mussels and macrozoobenthos

The present study showed that macroinvertebrate species richness in tropical rivers is significantly positively associated with the presence of freshwater mussels' sites (P-value=0.047). There were almost twice as many number of species found at mussel sites compared to the species found in non-mussel sites (Table 4). This confirms the patterns observed in earlier studies in temperate river (Aldridge *et al*. 2007) and tropical lake (Chowdhury *et al*. 2016), indicating that freshwater mussels in tropical rivers also affect benthic macroinvertebrates positively. Considering the lack of significant differences in water chemistry between sites with and without mussels (Table 2), it is highly unlikely that differences in macrozoobenthos species richness was caused by more favourable water chemistry at mussel sites.

While the general trend of higher total macroinvertebrate richness at sites with mussels is similar across a number of previous studies (Aldridge *et al*. 2007; Chowdhury *et al*. 2016; Vaughn & Spooner, 2006), the response by different macroinvertebrate orders towards mussel presence apparently varies between sites. In present study, Hemiptera showed the most pronounced association with the presence of mussels with 7 out of all the 8 occurrences recorded in mussel sites, and found in only one site without mussels (Table 4). In addition, we observed a positive association between mussel-presence and other groups such as Ephemeroptera, Plecoptera, Odonata, Decapoda and Gastropoda, although the results were not significant.

This finding is slightly different with observations by Vaughn & Spooner (2006) in the Ouchita Higlands, where density of Ephemeroptera and Trichoptera were significantly higher at mussel sites compared to non-mussel sites. On the other hand, in UK lowland rivers, Aldridge *et al*. (2007) recorded a significantly higher occurrences of Gastropoda and Isopoda in mussel sites as compared to non-mussel sites, with Ephemeroptera (Caenidae) and Plecoptera both being common at sites without mussels. In Lake Dhanmondi, Dhaka, Diptera (20%), Gastropoda (17%) and Hemiptera (11%) where the dominant groups at mussel sites (Chowdhury *et al*. , 2016).

Since tropical freshwater mussels tend to prefer slow-flowing, muddy habitat (Zieritz *et al*. 2016), the prominence of Hemiptera at mussel sites in our study might be due to a joint preference for slow-flowing habitats of both groups, rather than resulting from a causative relationship with the presence of mussels itself. Whilst I did not record

water flow, the results indicated that Hemiptera had an environmental preference for higher calcium concentration (Fig. 17). This is in accordance with Grinang (2013), in which Hemiptera were found to be the most abundant group (23.8%) in Padawan Limestone in Sarawak, which suggests that they survive quite well in areas with high calcium concentration. Malaysian freshwater mussels are usually found in areas with higher calcium concentration as well (Zieritz *et al*. 2016), so it might be another case of joint preference for calcium-rich sites for Hemiptera and mussels.

In temperate latitudes, Ephemeroptera and Plectoptera or also known as the EPT group are more diverse as compared to the tropics, whereas the situation is the opposite for Odonata, Hemiptera and Coleoptera, which are more diverse in the tropical region (Palmer & Menninger, 2013). The higher diversity in tropical region may be a possible reason for Hemiptera to have the most pronounced response towards the presence of mussels during our study instead of members of the EPT group as observed in other studies in the temperate regions (Vaughn & Spooner, 2006).

The same might be true for Odonata, which exhibit very high levels of species richness and endemicity in Southeast Asia, including Peninsular Malaysia, Java, Sumatra and Borneo (Kalkman *et al*. 2008). Though not statistically significant, Odonata showed a distinct association with sites with mussels in our study, and around 70% of the total odonate larvae that we sampled were obtained from sites with mussels. Since diversity and endimicity of Odonata and Hemiptera is so high in Malaysia, the fact that mussels may be supporting diversity of these taxa might be particularly important in terms of the role of freshwater mussels as ecosystem engineers.

There is a fairly good potential in using freshwater mussels as predictor organisms for tropical river diversity, specifically in the case of Plecoptera and Decapoda since both these orders were only found in mussel sites, and potentially for Hemiptera and Odonata as well as they had shown higher prevalence in mussel sites. With a lot more replication, the potential of freshwater mussels as a biodiversity predictor organism for these and other invertebrate orders can be explored and utilised in developing a conservation plan for freshwater habitats. The potential of mussels as predictor organisms in determining the biodiversity of river ecosystems is advantageous in term of reduced need of advanced sampling in order to identify “biodiversity hot spot” sites, allowing better management of rivers and disturbances (Aldridge *et al*. 2007).

Undeniably there is a possibility that other factors play a role in the correlation such as water flow which we suspected in the case of Hemiptera, or perhaps disturbances from influx of sediments from flood events. However, in most cases a causative relationship between the presence of mussels and the diversity of freshwater invertebrates seems more likely. A mesocosm experimental set-up to study the correlation between the presence of mussels and the effects that they had on benthic community structure in a controlled experimental condition may provide us with a more definite answer to this matter (see Appendix 1 for details). Coupling this field survey with mesocosm-based or lab-based experiments, as we did in our study on the effects of mussels as biofilters (Chapter 2) will generate a more comprehensive understanding of the relationship between mussels and other benthic organisms (Crowe *et al.* 2011).

Habitat requirement by tropical freshwater macroinvertebrates orders

Besides addressing the relationship between freshwater mussels and other benthic macroinvertebrates, my study also provides some interesting data on the habitat preferences of several freshwater invertebrate orders. For example, the CCA plot showed that Decapoda, exclusively represented by the family Palaemonidae, were only found in sites with high ion concentrations. A high ion concentration is commonly associated with seawater, but none of our sites were within such close proximity to the sea that they would be tidally influenced. Therefore, the high ion concentrations must have come from a different source such as pollution or agricultural run-off. The order Palaemonidae such as *Macrobrachium acanthurus, M. olfersii, M. potiuna* (Moreira *et al*. 1983) and *M. amazonicum* (Zanders & Rodríguez, 1992) are known to be strong hyperosmotic regulators in freshwater or low salinities conditions, maintaining higher salt concentrations in their body fluids in freshwater environments. My data confirms the low tolerance of Palaemonidae against low salinity.

Ephemeroptera, Plecoptera and Trichoptera (EPT) are generally regarded as sensitive to pollution and their abundance often used as a measure of water quality (Baker & Sharp Jr, 1998). However in my survey, Trichoptera was shown to be fairly tolerant against eutrophication; Ephemeroptera did not exhibit an association with neither polluted nor unpolluted sites whereas Plecoptera were associated with low nutrient concentrations. Similar to the data of macroinvertebrates richness recorded in Hulu Terengganu (Wahizatul *et al.* 2011) and Padawan Limestone (Grinang, 2013), Ephemeroptera made up the majority of the sampled individuals, around 20% of all the individuals sampled in both mussel sites and non-mussel sites.

Availability of DNA barcoding data for freshwater macroinvertebrates

A molecular-based assessment such as DNA barcoding will be easier and produces more reliable data than morphological-based assessment alone (Bohmann *et al.* 2014). Although the initial plan of using NGS methods to identify the sampled macroinvertebrates cannot be discontinued due to some failure in amplifying the number of DNA strands using PCR, we still included a molecular-based identification by sending some of our individual samples for Sanger sequencing in order to assess the availability of DNA barcoding data for molecular identification of macroinvertebrates in the study region.

COI sequences from only 2 out of 11 randomly selected species were available on Genbank, demonstrating the inadequacy of DNA barcoding data for freshwater macroinvertebrates in Southeast Asia. For most groups, a fairly closely related species that are most probably congeneric (same genus) was available on Genbank, but the divergence was still considerably. However, the genetic distance to the best-matching Genbank sequence for one Diptera specimen sequenced was 27% (Table 5). This indicates that a lot of work still needs to be done in terms of assessing the overall freshwater invertebrate biodiversity for the region, particularly for small, invertebrate taxa. My results indicate that freshwater invertebrates in the region are inadequately studied and that many taxa in are yet to be discovered. The recent discovery of a new cryptic species of Unionida, *Hydriopsis biolata* by Zieritz *et al*. (2016) in the Pahang River is an example of this.

Conclusion

Freshwater mussels in tropical region have positive effects on the biodiversity of benthic macroinvertebrates similar to their counterparts in temperate regions, although the order that showed strong preferential towards mussel presence differed between the two systems. A detailed, experimental study can provide evidence for causative relationship between the presence of mussels and the biodiversity of macroinvertebrates, thus solidifying its status as an important ecosystem engineer in a freshwater ecosystem. An in-depth understanding of the functional ecology of freshwater mussels in the tropical region and its effect on freshwater biodiversity are important for effective freshwater habitat management strategy in the future.

Chapter 4: Conclusion

Despite being highly imperilled, the conservation status of freshwater mussels in Peninsular Malaysia is still unclear and has garnered very little interest as opposed to the fate of their North American and European counterparts. The present thesis represents one of the first studies that suggest mussel communities in different freshwater ecosystems may play different functional roles.

The major role of freshwater mussels in an ecosystem lies in its filter-feeding mechanism, which in temperate systems, has been shown to significantly affect the concentration of nutrients in the water column as well as the amount of organic matter transferred towards the bottom of the sediment. The effects on the water column in nature were assessed in mesocosm experiments, which were supplemented by controlled laboratory experiments using the water and mussels from the same sites in order to simultaneously assess effects on the water column as well as the substrate and eliminate random effects due to natural causes.

River mussel community significantly decreased phytoplankton from the water column, while lake mussel community mainly affected its environment by depositing undigested phytoplankton and excreting large amounts of NH_4. In both cases, water clarity increased in mussel treatments, indicating that mussel filtration promotes water clarity and consequently, macrophyte growth. The extent of this effect as well as the effect on the benthos might, however, be vastly different in different conditions, judging from the extensive differences in deposition rates between lake and river mussels. The exact causes of these observations and in particular, the question whether these differences were a site- or species- effect, remain to be answered.

The second part of the thesis revolved around the interaction of freshwater mussels with benthic macroinvertebrates as they are commonly known as ecosystem engineers Besides providing physical shelter against predation, the abundance of organic matter excreted by freshwater mussels has been proven to provide benthic macroinvertebrates with much-needed nutrient resources in temperate systems. My results confirm these patterns across tropical rivers in Peninsular Malaysia, as species richness was considerably higher in mussel sites compared to non-mussel sites for several taxa, such as Odonata, Decapoda and Gastropoda. Despite living mainly on water surface, the diversity of Hemiptera at river sites was significantly correlated with the presence of

freshwater mussels, most probably due to a shared preference for slow-flowing, muddy habitats and areas with high calcium concentration. The main limitation of this project lies in the fact that it is a descriptive study, and the presence of mussels could therefore not be said to be directly causative of the composition in benthic invertebrates. A controlled experiment such as was initially planned (See appendix 1), testing colonisation rates by macroinvertebrates in replicated enclosures with and without mussels, would be able to address this question directly.

In Peninsular Malaysia the seasonal variation comes in the form of alternating periods of dryness and rain which often exacerbated by La Nina and El Nino events; a phenomenon that are not a common occurrence in temperate ecosystems. In this research, I encountered both of these situations which surprisingly cause a strong difference in the mussel population in sites leading to a few setbacks in the process. Since the majority of existing knowledge of the functional ecology of freshwater mussels are built upon the observation of temperate freshwater mussels behaviour, an information gap still exist in regard of native tropical species behaviour during the two tropical climate extremes (drought and flood). A better understanding of the functional ecology of tropical freshwater mussels might bring more widespread awareness of the perils faced by the freshwater species in the tropical waters and garnering the attention needed into pushing for more progressive conservation efforts in the future.

My study highlighted the role of freshwater mussels in promoting water clarity and the biodiversity of benthic macroinvertebrates. Coupled with the role of mussels in nutrient cycling due to their filter-feeding activities, which helps to balance the oligotrophy in streams and lakes, these bivalves are extremely important in maintaining a healthy, intact freshwater ecosystem. The rapidly declining freshwater population across Malaysia is a major tell-tale signs in the degrading quality of our rivers, a clear indication that more needs to be done in order to protect the biodiversity in our freshwater habitats.

Appendix 1

The initial plan of assessing the effects of freshwater mussels on benthic macroinvertebrates biodiversity involves a more experimental, mesocosm-based approach rather than independent natural sampling. In this plan, 40 mesh-wire cylindrical cages with open tops (dimension: 35cm diameter X 30 cm height) are placed along 200m stretch of a river with a healthy population of native species of freshwater mussels (a small river in Kampung Hayat, near Pahang-Negeri Sembilan border) (Fig.20). Prior to placing the cages, the sediment underneath the spot was dug, and a batch or autoclaved sediment from the same river was added once the cage was held in place. 20 of these cages would be the “controls” in which no mussels would be added, and the remaining 20 cages would be added with mussels.

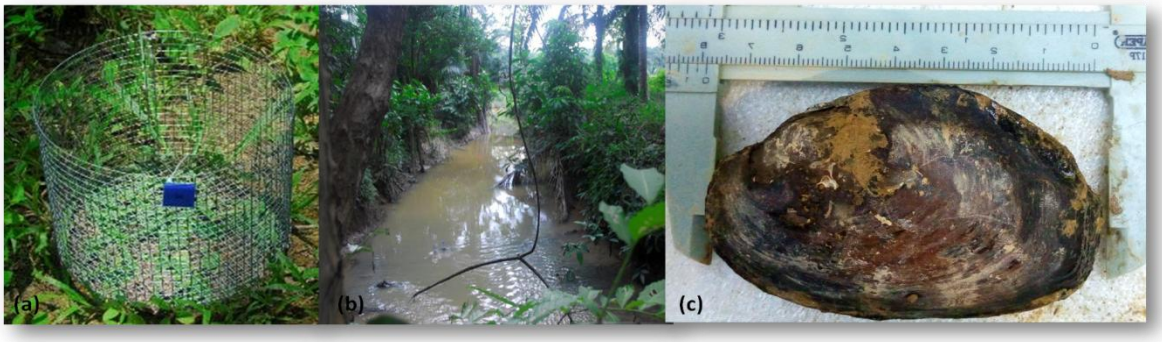

Figure 19: a) galvanized mesh cages used in our mesocosm, b) experimental site in Kampung Hayat, c) mussel species found in study site.

Upon setting up the cages, 10 randomly selected cages were sampled as the “Time 0” samples, and subsequent samplings were scheduled in the following two weeks, one month and two months. However, upon returning to the site for the second sampling, we discovered that nearly all of our cages were swept away by rising river level following a heavy rain, according to locals. Since we don’t have enough time to restart the whole experiment, we decided to proceed with natural sampling for biodiversity assessment instead.

Appendix 2 : DNA metabarcoding

Introduction

As mentioned in Chapter 3, freshwater habitats feature a disproportionally high number of species compared to terrestrial and marine habitats, as well as providing a range of precious ecosystem services (Strayer & Dudgeon, 2010). The main key to successful biomonitoring, management and conservation of habitat is the ability to perform frequent assessments of biodiversity using a method that has a low impact and is affordable in a long term (Lim *et al*. 2016).

The initial plan of my project was to utilize a molecular-based approach such as DNA barcoding in assessing the biodiversity of benthic macroinvertebrates in sites with freshwater mussels and sites without freshwater mussels. The concept of DNA barcoding is the comparison between a query sequence and a DNA barcode reference library comprised of known species (Hajibabaei *et al.* 2011). DNA metabarcoding - a term coined by Taberlet *et al*. (2012) - refers to the high-throughput identification of multispecies (or higher-level taxon) using degraded DNA extracted from an environmental sample (eDNA). According to Hajibabaeil et al (2011) DNA metabarcoding also includes the species identification of entire organisms isolated prior to analysis from bulk samples. DNA metabarcoding is the most widely used method of molecular identification , and has been shown to improve the capacity of bioassessments by reducing the cost and time required for taxonomic identification (Carew *et al.* 2013). In detail, I planned to identify organisms by DNA metabarcoding instead of traditional morphological identification

The conventional method of obtaining DNA sequence data (i.e. a DNA barcode of a specimen) involves amplification of the target region (most commonly, Cytochrome Oxidase I, COI) using Polymerase-Chain Reaction (PCR) followed by Sanger sequencing(Shokralla *et al*. 2014). Whilst Sanger-based DNA sequencing produces robust results in building DNA barcode reference libraries, it is not practical when dealing with bulk environmental samples, which can contain thousands of individuals from hundreds of species at one time, ranging from bacteria to higher eukaryotes (Hajibabaei *et al*. 2011). Although Sanger sequencing can produce up to 1kilobyte (kb) of sequence data from a single specimen, it is incapable of processing environmental samples which are far more complex (Shokralla *et al.* 2012). Environmental samples usually have a lot of debris and foreign materials which volume greatly exceeded the concentration of DNA that may be present within the bulk sample.

Next-Generation-Sequencing (NGS) can overcome this problem. Rather than long reads from a sample that was PCR-amplified, a massively parallel sequencing method produces reads of around 21 to 400 base pairs, but in large number of strands(Pettersson *et al.* 2009). This ability to read millions of DNA sequences in parallel made NGS technologies ideal for analysing large-scale biodiversity of environmental samples (Shokralla *et al*., 2012). The potential of NGS as a routine tool in environmental monitoring is considered to be high, since multiple species in many samples can be simultaneously sequenced in a single instrument run(Carew *et al*., 2013).

Apart from producing high numbers of sequences per read, NGS also facilitates biodiversity assessments of groups that are traditionally difficult to identify. The technique has been successfully applied, for example, in studying the response of the arthropod fauna to land-use changes (Beng *et al*., 2016), biomonitoring the diversity of river benthic macroinvertebrates (Hajibabaei *et al*., 2011), identifying 46 distinct species of Chironomidae across ten different field sites (Carew *et al*. 2013) and assessing the diversity of mammal diversity in tropical forest from the DNA derived from the gut contents of blowfly(Lee *et al.* 2016). NGS proofed particularly suitable in studies investigating the response in biodiversity towards environmental changes, which require multiple, repetitive, uniform sampling events.

The majority of biodiversity studies using 454 Genome Sequencer FLX (preferred NGS platform for biodiversity studies) to date have targeted prokaryotic biodiversity from various environmental samples, ranging from the ocean floor to human micro-flora (Hajibabaei *et al*. 2011). Zinger *et al*. (2012) predicted that the use of NGS methods will increase over the following years despite sequencing/cloning remaining the most widely-used method for microbial richness assessment. The third major advantage of NGS to traditional biomonitoring tools is the high detection rate. Comparing between eDNA detection and conventional surveys such as electrofishing and single emergence traps on a site-by-site basis, Lim *et al*. (2016) found that eDNA samples almost always outperform the former and frequently outperform the latter.

The shortcomings and disadvantages of eDNA metabarcoding are threefold. Firstly, one of the major challenges of eDNA metabarcoding is removing false positives that are usually caused by the false detection of eDNA from other sources instead of the intended study subject(Bohmann *et al*. 2014). Since the basis of eDNA is detecting every possible DNA that may be present within the sample including degraded ones,

even a slight contamination may be perceived as false positive. Secondly, barcoding technologies do not allow adequate resolution and identification of taxonomy(Zinger *et al*., 2012). Given the size and intricate lifecycle stages of various freshwater macroinvertebrates, the conventional method of morphological identification can be both time-consuming and costly. The study was going to compare the performance of benthic biodiversity assessment of traditional morphological identification and sampling (as described in Chapter 3) with DNA metabarcoding.

Materials and methods

Samples were dried in an oven at 56^{o}C overnight to let the ethanol used in preserving the samples to evaporate completely (Fig. 21). Next, DNA was extracted from bulk samples using Machery-Nagel Nucleospin Tissue kit, following the manufacturer's instructions, with the exception of increase in the volume of pre-lyse buffers proportional to the ratio as stated in the manual. I checked for the quantity and quality for DNA by running the extracted DNA through 0.8 % agarose gel (Fig.22a) and tested their concentration using a spectrophotometer. Polymerase Chain Reaction (PCR) was later carried out in order to amplify the COI region for DNA metabarcoding purposes.

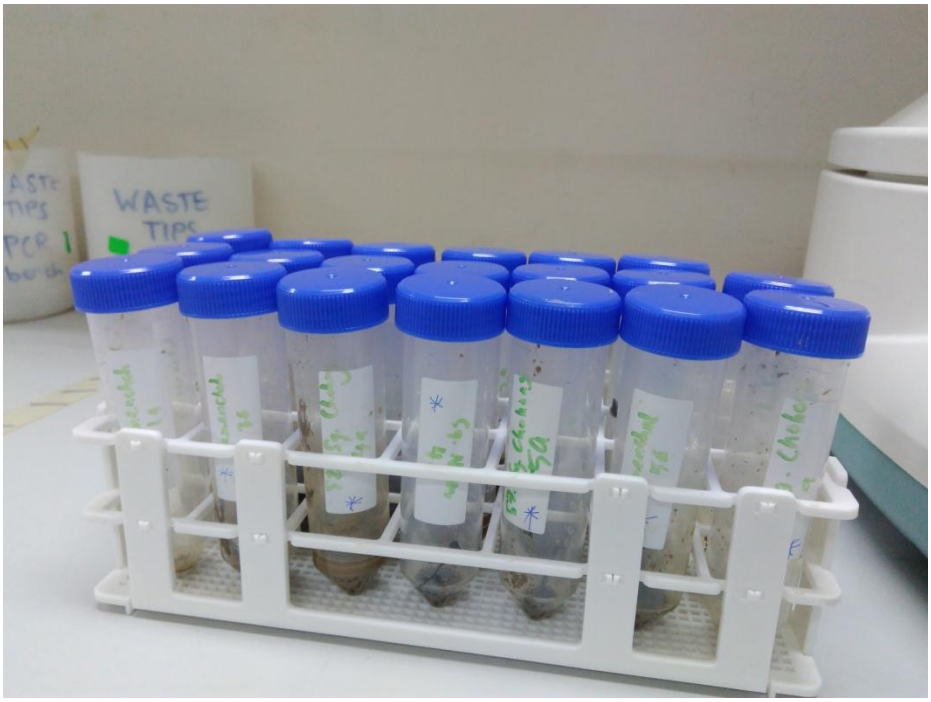

Figure 20: Samples prior after drying in the oven and ready for bulk DNA extraction.

PCRs premix were performed prepared by mixing 12.5µL MyTaq Red Mix PCR premix (used in order to minimize pipetting thus reducing the risk of contamination) with 0.25µL of forward primer mlCOIintF –GGWACWGGWTGAACWGTWTAYCCYCC (Leray

et al., 2013), 0.25µL of reverse primer HCO2198-TAAACTTCAGGGTGACCAAAAAATCA (Folmer et al., 1994) and 9µL of ultrapure water. Finally, 23 µL of the mastermix was pipette into each PCR tube, followed the addition of 3µL of DNA template, making up a total of 26µL reaction. Everything was carried out on ice prior to inserting the samples in thermo cycler machine.

PCR cycles were run in a Fisher Thermo Scientific thermocycler with 2 minutes initial warming at 94^{o}C, followed by 30 cycles of 94^{o}C denaturation step for 30 seconds, 30 seconds of annealing step at 43^{o}C and 1 minute of extension step at 72^{o}C, and upon the completion of the 30th cycle, a final 10 minutes standing time at 72^{o}C was incorporated before dropping down to 4^{o}C to rest until the PCR products were taken out from the thermal cycler for gel electrophoresis. The PCR products were later run through a 2% agarose gel to check whether the amplification of the COI region had successfully occurred.

Results and Discussion

However, none of the samples produce a good enough band to be sequenced (Fig. 22b). I tried running the PCR with a different set of primers as used by Hajibabaei *et al*. (2011) in their study on river benthos, LepF1: 59-ATTCAACCAATCATAAAGAT ATTGG-39 (Hebert *et al*. 2004) and a newly designed reverse primer, EPT-long-univR: 59-AARAAAATYATAAYAAAIGCGTGIAIIGT-39. Even when running the PCR according to their protocol in which they succeeded in using DNA metabarcoding in assessing the biodiversity of river benthos, I still failed to get bands during gel electrophoresis that indicates that the DNA strands in my sample had been amplified.

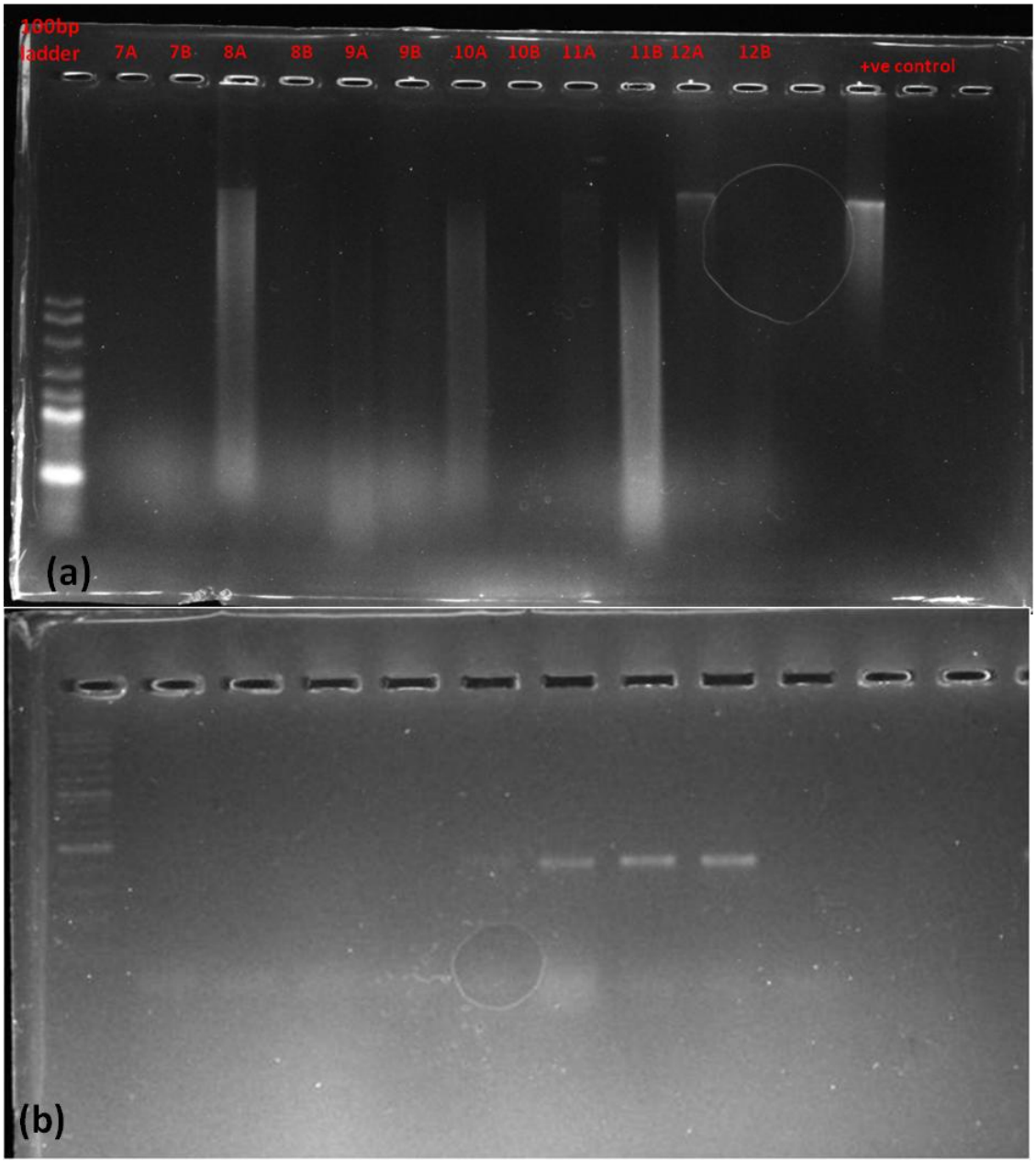

Figure 21 : a) DNA sample run on 0.8% agarose gel with positive controls. B) PCR products with the DNA samples as template run on 2.0% agarose gel. The three bands obtained are all from positive controls, no bands for any of the samples.

One possible factor may be due to the fact that the sample collected were sediment samples taken from the bottom of rivers. The concentration of DNA may be diluted as compared to eDNA collected from soil sample, fecal sample or even gut contents. Hajibabaei *et al*. (2011) had previously performed the assessment of benthic macroinvertebrate biodiversity, however in their protocol the macroinvertebrates were isolated from the bulk sample and had their DNA extracted individually. Besides individually extracting the isolated macroinvertebrates, another possible protocol example is the one conducted by Brandon-Mong *et al*. (2015) in which the insect

samples collected from a ‘malaise trap’ are mixed in large soup-like mixture and DNA was later extracted from the whole batch. The molecular protocol of capture and extraction method of eDNA from water had been shown to have a strong effect on the detection of biodiversity using Next-Generation Sequencing platform (Deiner *et al.* 2015). In freshwater habitats, DNA becomes untraceable 2 weeks after the removal of animal (THOMSEN *et al*., 2012).

For future works, my suggestion would be isolating the macroinvertebrates from bulk samples and combine all of the organisms and extract the DNA from their bulk sample. This step aims on reducing the potential inhibitory substances that are quite common in freshwater eDNA samples within the samples, thus improving the chance of a successful DNA amplification. A preliminary procedural study should be first conducted in order to establish a viable working protocol before moving further into a grander scale of things. Another possible option that can be explored is utilising preservation ethanol to obtain the eDNA that may be contained within a bulk sample as demonstrated by Stein *et al.* (2013). The addition of bovine serum albumin into the PCR mixture is another solution that had proven to work against PCR inhibitors within bulk samples. With this method, instead of isolating all the macroinvertebrates found in the bulk sample, the absolute ethanol that was used to preserve the samples upon collection was used as the source of DNA template, a method that have the potential for a more rapid, hassle-free biomonitoring of benthic macroinvertebrates.

Bibiliography

Aldridge, D. C. (1999). The morphology, growth and reproduction of Unionidae (Bivalvia) in a fenland waterway. *Journal of Molluscan Studies*, *65*(1), 47–60.

Aldridge, D. C., Fayle, T. M., & Jackson, N. (2007). Freshwater mussel abundance predicts biodiversity in UK lowland rivers. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *17*(6), 554–564.

Atkinson, C. L., First, M. R., Covich, A. P., Opsahl, S. P., & Golladay, S. W. (2011). Suspended material availability and filtration–biodeposition processes performed by a native and invasive bivalve species in streams. *Hydrobiologia*, *667*(1), 191–204.

Atkinson, C. L., Vaughn, C. C., Forshay, K. J., & Cooper, J. T. (2013). Aggregated filter‐feeding consumers alter nutrient limitation: consequences for ecosystem and community dynamics. *Ecology*, *94*(6), 1359–1369.

Baker, S. C., & Sharp Jr, H. F. (1998). Evaluation of the recovery of a polluted urban stream using the Ephemeroptera-Plecoptera-Trichoptera index. *Journal of Freshwater Ecology*, *13*(2), 229–234.

Balian, E. V, Segers, H., Lévêque, C., & Martens, K. (2008). The freshwater animal diversity assessment: an overview of the results. *Hydrobiologia*, *595*(1), 627–637.

Beng, K. C., Tomlinson, K. W., Shen, X. H., Surget-Groba, Y., Hughes, A. C., Corlett, R. T., & Slik, J. W. F. (2016). The utility of DNA metabarcoding for studying the response of arthropod diversity and composition to land-use change in the tropics. *Scientific Reports*, *6*, 24965.

Bogan, A. E. (1993). Freshwater bivalve extinctions (Mollusca: Unionoida): a search for causes. *American Zoologist*, *33*(6), 599–609.

Bogan, A. E. (2008). Global diversity of freshwater mussels (Mollusca, Bivalvia) in freshwater. *Hydrobiologia*, *595*(1), 139–147.

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., … De Bruyn, M. (2014). Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology & Evolution*, *29*(6), 358–367.

Borthagaray, A. I., & Carranza, A. (2007). Mussels as ecosystem engineers: Their contribution to species richness in a rocky littoral community. *Acta Oecologica*, *31*(3), 243–250. https://doi.org/http://doi.org/10.1016/j.actao.2006.10.008

Boulton, A. J., Boyero, L., Covich, A. P., Dobson, M., Lake, S., & Pearson, R. (2008). Are tropical streams ecologically different from temperate streams. *Tropical Stream Ecology*, 257–284.

Bowes, M. J., Gozzard, E., Johnson, A. C., Scarlett, P. M., Roberts, C., Read, D. S., … Wickham, H. D. (2012). Spatial and temporal changes in chlorophyll-a concentrations in the River Thames basin, UK: Are phosphorus concentrations beginning to limit phytoplankton biomass? *Science of the Total Environment*, *426*, 45–55.

Brandon-Mong, G.-J., Gan, H.-M., Sing, K.-W., Lee, P.-S., Lim, P.-E., & Wilson, J.-J. (2015). DNA metabarcoding of insects and allies: an evaluation of primers and pipelines. *Bulletin of Entomological Research*, *105*(6), 717–727.

Carew, M. E., Pettigrove, V. J., Metzeling, L., & Hoffmann, A. A. (2013). Environmental monitoring using next generation sequencing: rapid identification of macroinvertebrate bioindicator species. *Frontiers in Zoology*, *10*(1), 45.

Chong, V. C., Lee, P. K. Y., & Lau, C. M. (2010). Diversity, extinction risk and conservation of Malaysian fishes. *Journal of fish biology*, *76*(9), 2009-2066.

Chowdhury, G. W., Zieritz, A., & Aldridge, D. C. (2016). Ecosystem engineering by mussels supports biodiversity and water clarity in a heavily polluted lake in Dhaka, Bangladesh. *Freshwater Science*, *35*(1), 188–199.

Covich, A. P., Palmer, M. A., & Crowl, T. A. (1999). The role of benthic invertebrate species in freshwater ecosystems: zoobenthic species influence energy flows and nutrient

cycling. *BioScience*, *49*(2), 119–127.

Crowe, T. P., Frost, N. J., & Hawkins, S. J. (2011). Interactive effects of losing key grazers and ecosystem engineers vary with environmental context. *Marine Ecology Progress Series*, *430*, 223–234.

Cummings, K. S., & Graf, D. L. (2015). Chapter 19 - Class Bivalvia1 A2 - Thorp, James H. In D. C. B. T.-T. and C. F. I. (Fourth E. Rogers (Ed.) (pp. 423–506). Boston: Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-385026-3.00019-X

Cyr, H., Collier, K. J., Clearwater, S. J., Hicks, B. J., & Stewart, S. D. (2017). Feeding and nutrient excretion of the New Zealand freshwater mussel Echyridella menziesii (Hyriidae, Unionida): implications for nearshore nutrient budgets in lakes and reservoirs. *Aquatic Sciences*, *79*(3), 557–571. https://doi.org/10.1007/s00027-016-0517-9

Deiner, K., Walser, J.-C., Mächler, E., & Altermatt, F. (2015). Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. *Biological Conservation*, *183*, 53–63.

Dionisio Pires, L. M., Bontes, B. M., Samchyshyna, L., Jong, J., Van Donk, E., & Ibelings, B. W. (2007). Grazing on microcystin-producing and microcystin-free phytoplankters by different filter-feeders: implications for lake restoration. *Aquatic Sciences-Research Across Boundaries*, *69*(4), 534–543.

Douglas, I., Greer, T., Bidin, K., & Spilsbury, M. (1993). Impacts of rainforest logging on river systems and communities in Malaysia and Kalimantan. *Global Ecology and Biogeography Letters*, 245-252.

Downing, J. A., Van Meter, P., & Woolnough, D. A. (2010). Suspects and evidence: a review of the causes of extirpation and decline in freshwater mussels. *Animal biodiversity and conservation*, *33*(2), 151-185.

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z. I., Knowler, D. J., Lévêque, C., Naiman, R.J., Prieur-Richard, A.H., Soto, D., Stiassny, M.L. & Sullivan, C. A. (2006). Freshwater biodiversity: importance, threats, status and conservation challenges. *Biological reviews*, *81*(2), 163-182.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol*, *3*.

Foottit, R. G., & Maw, E. (2014). Aphids (Hemiptera: Aphidoidea) of the Prairies Ecozone of Canada. *Arthropods of Canadian Grasslands (Volume 3): Biodiversity and Systematics, Part 1.*, 347–369.

Graf, D. L., & Cummings, K. S. (2007). Review of the systematics and global diversity of freshwater mussel species (Bivalvia: Unionoida). *Journal of Molluscan Studies*, *73*(4), 291–314.

Grinang, J. (2013). Fishes and macroinvertebrates of Padawan Limestone, Sarawak, Malaysia. *Borneo Journal of Resource Science and Technology*, *3*(2), 1–14.

Gutierrez, J. L., Jones, C. G., Strayer, D. L., & Iribarne, O. O. (2003). Mollusks as ecosystem engineers: the role of shell production in aquatic habitats. *Oikos*, *101*(1), 79–90.

Haag, W. R., & Williams, J. D. (2014). Biodiversity on the brink: an assessment of conservation strategies for North American freshwater mussels. *Hydrobiologia, 735*(1), 45-60.

Hajibabaei, M., Shokralla, S., Zhou, X., Singer, G. A. C., & Baird, D. J. (2011). Environmental barcoding: a next-generation sequencing approach for biomonitoring applications using river benthos. *PLoS One*, *6*(4), e17497.

Hakenkamp, C. C., & Palmer, M. A. (1999). Introduced bivalves in freshwater ecosystems: the impact of Corbicula on organic matter dynamics in a sandy stream. *Oecologia*, *119*(3), 445–451.

Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(41), 14812–14817.

Helfrich, L. A., Neves, R. J., & Chapman, H. (2005). Sustaining America's Aquatic Biodiversity. Freshwater Mussel Biodiversity and Conservation.

Howard, J. K., & Cuffey, K. M. (2006). The functional role of native freshwater mussels in the fluvial benthic environment. *Freshwater Biology*, *51*(3), 460–474.

Jones, C. G., Lawton, J. H., & Shachak, M. (1994). Organisms as ecosystem engineers. In *Ecosystem management* (pp. 130–147). Springer.

Kalkman, V. J., Clausnitzer, V., Dijkstra, K.-D. B., Orr, A. G., Paulson, D. R., & van Tol, J. (2008). Global diversity of dragonflies (Odonata) in freshwater. *Hydrobiologia*, *595*(1), 351–363.

Lee, P.-S., Gan, H. M., Clements, G. R., & Wilson, J.-J. (2016). Field calibration of blowfly-derived DNA against traditional methods for assessing mammal diversity in tropical forests 1. *Genome*, *59*(11), 1008–1022.

Leray, M., Yang, J. Y., Meyer, C. P., Mills, S. C., Agudelo, N., Ranwez, V., … Machida, R. J. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Frontiers in Zoology*, *10*(1), 34. https://doi.org/10.1186/1742-9994-10-34

Lim, N. K. M., Tay, Y. C., Srivathsan, A., Tan, J. W. T., Kwik, J. T. B., Baloğlu, B., … Yeo, D. C. J. (2016). Next-generation freshwater bioassessment: eDNA metabarcoding with a conserved metazoan primer reveals species-rich and reservoir-specific communities. *Royal Society Open Science*, *3*(11), 160635.

Lopes-Lima, M., Teixeira, A., Froufe, E., Lopes, A., Varandas, S., & Sousa, R. (2014). Biology and conservation of freshwater bivalves: past, present and future perspectives. *Hydrobiologia*, *735*(1), 1–13.

Lopes- Lima, M., Sousa, R., Geist, J., Aldridge, D. C., Araujo, R., Bergengren, J., … Van Damme, D. (2017). Conservation status of freshwater mussels in Europe: state of the art and future challenges. *Biological Reviews*, *92*(1), 572–607.

Mackereth, F., Heron, J., & Tailing, J. (1978). Water Analysis: Some Revised Methods for Limnologists. Freshwater Biological Assoc., Scientific Pub., 36.

McIvor, A. L. (2004). Freshwater mussels as biofilters. University of Cambridge.

Md Rawi, C. S., Al-Shami, S. A., Madrus, M. R. and Ahmad, A. H. (2014), Biological and ecological diversity of aquatic macroinvertebrates in response to hydrological and physicochemical parameters in tropical forest streams of Gunung Tebu, Malaysia: implications for ecohydrological assessment. Ecohydrol., 7: 496-507. doi:10.1002/eco.1368

Mercer, E. V., Mercer, T. G., & Sayok, A. K. (2014). Effects of forest conversions to oil palm plantations on freshwater macroinvertebrates: a case study from Sarawak, Malaysia. *Journal of Land Use Science*, *9*(3), 260-277.

Moreira, G. S., McNamara, J. C., Shumway, S. E., & Moreira, P. S. (1983). Osmoregulation and respiratory metabolism in brazilian Macrobrachium (Decapoda, Palaemonidae). *Comparative Biochemistry and Physiology Part A: Physiology*, *74*(1), 57–62.

Nalepa, T. F., Gardner, W. S., & Malczyk, J. M. (1991). Phosphorus cycling by mussels (Unionidae: Bivalvia) in Lake St. Clair. *Hydrobiologia*, *219*(1), 239-250.

Nichols, S. J., Silverman, H., Dietz, T. H., Lynn, J. W., & Garling, D. L. (2005). Pathways of food uptake in native (Unionidae) and introduced (Corbiculidae and Dreissenidae) freshwater bivalves. *Journal of Great Lakes Research*, *31*(1), 87–96.

Palmer, M. A., & Menninger, H. L. (2013). Invertebrates, Freshwater, Overview A2 - Levin,

Simon A BT - Encyclopedia of Biodiversity (Second Edition) (pp. 369–378). Waltham: Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-384719-5.00077-0

Parker, B. C., Patterson, M. A., & Neves, R. J. (1998). Feeding interactions between native freshwater mussels (Bivalvia: Unionidae) and zebra mussels (Dreissena polymorpha) in the Ohio River. *American Malacological Bulletin*, *14*(2), 173–179.

Pettersson, E., Lundeberg, J., & Ahmadian, A. (2009). Generations of sequencing technologies. *Genomics*, *93*(2), 105–111.

Riisgård, H. U. (2001). On measurement of filtration rates in bivalves—the stony road to reliable data: review and interpretation. *Marine Ecology Progress Series*, *211*, 275–291.

Saraiva, S., Van Der Meer, J., Kooijman, S., & Sousa, T. (2011). Modelling feeding processes in bivalves: a mechanistic approach. *Ecological Modelling*, *222*(3), 514–523.

Shokralla, S., Gibson, J. F., Nikbakht, H., Janzen, D. H., Hallwachs, W., & Hajibabaei, M. (2014). Next- generation DNA barcoding: using next- generation sequencing to enhance and accelerate DNA barcode capture from single specimens. *Molecular Ecology Resources*, *14*(5), 892–901.

Shokralla, S., Spall, J. L., Gibson, J. F., & Hajibabaei, M. (2012). Next- generation sequencing technologies for environmental DNA research. *Molecular Ecology*, *21*(8), 1794–1805.

Soto, D., & Mena, G. (1999). Filter feeding by the freshwater mussel, Diplodon chilensis, as a biocontrol of salmon farming eutrophication. *Aquaculture*, *171*(1–2), 65–81. https://doi.org/https://doi.org/10.1016/S0044-8486(98)00420-7

Spooner, D. E., & Vaughn, C. C. (2006). Context-dependent effects of freshwater mussels on stream benthic communities. *Freshwater Biology*, *51*(6), 1016–1024.

Stein, E. D., White, B. P., Mazor, R. D., Miller, P. E., & Pilgrim, E. M. (2013). Evaluating ethanol-based sample preservation to facilitate use of DNA barcoding in routine freshwater biomonitoring programs using benthic macroinvertebrates. *PloS One*, *8*(1), e51273.

Strayer, D. L., Downing, J. A., Haag, W. R., King, T. L., Layzer, J. B., Newton, T. J., & Nichols, S. J. (2004). Changing perspectives on pearly mussels, North America's most imperiled animals. *BioScience*, *54*(5), 429–439.

Strayer, D. L., & Dudgeon, D. (2010). Freshwater biodiversity conservation: recent progress and future challenges. *Journal of the North American Benthological Society*, *29*(1), 344–358.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., & Willerslev, E. (2012). Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, *21*(8), 2045–2050.

THOMSEN, P., Kielgast, J. O. S., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., … Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*(11), 2565–2573.

Thorp, J. H. (2015). Chapter 4 - Functional Relationships of Freshwater Invertebrates BT - Thorp and Covich's Freshwater Invertebrates (Fourth Edition) (pp. 65–82). Boston: Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-385026-3.00004-8

Vaughn, C. C. (1997). Regional patterns of mussel species distributions in North American rivers. *Ecography*, *20*(2), 107–115.

Vaughn, C. C., Gido, K. B., & Spooner, D. E. (2004). Ecosystem processes performed by unionid mussels in stream mesocosms: species roles and effects of abundance. *Hydrobiologia*, *527*(1), 35–47.

Vaughn, C. C., & Hakenkamp, C. C. (2001). The functional role of burrowing bivalves in freshwater ecosystems. *Freshwater Biology*, *46*(11), 1431–1446.

Vaughn, C. C., Nichols, S. J., & Spooner, D. E. (2008). Community and foodweb ecology of freshwater mussels. *Journal of the North American Benthological Society*, *27*(2), 409–423.

Vaughn, C. C., & Spooner, D. E. (2006). Unionid mussels influence macroinvertebrate assemblage structure in streams. *Journal of the North American Benthological Society*,

25(3), 691–700.

Vaughn, C. C. (2010). Biodiversity losses and ecosystem function in freshwaters: emerging conclusions and research directions. *BioScience, 60*(1), 25-35.

Wahizatul, A. A., Long, S. H., & Ahmad, A. (2011). Composition and distribution of aquatic insect communities in relation to water quality in two freshwater streams of Hulu Terengganu, Terengganu. *Journal of Sustainability Science and Management*, *6*(1), 148–155.

Wallace, J. B., & Webster, J. R. (1996). The role of macroinvertebrates in stream ecosystem function. *Annual Review of Entomology*, *41*(1), 115–139.

Watters, G. T. (1999). Freshwater mussels and water quality: a review of the effects of hydrologic and instream habitat alterations. In *Proceedings of the First Freshwater Mollusk Conservation Society Symposium* (Vol. 1999, pp. 261–274).

Weng, C. N. (2005). Sustainable management of rivers in Malaysia: Involving all stakeholders. *International Journal of River Basin Management*, *3*(3), 147-162.

Yule, C. M., & Yong, H. S. (2004). *Freshwater Invertebrates of the Malaysian Region*. Academy of Sciences Malaysia. Retrieved from https://books.google.com.my/books?id=57o_AAAACAAJ

Zanders, I. P., & Rodríguez, J. (1992). Effects of temperature and salinity stress on osmoionic regulation in adults and on oxygen consumption in larvae and adults of Macrobrachium amazonicum (Decapoda, Palaemonidae). *Comparative Biochemistry and Physiology Part A: Physiology*, *101*(3), 505–509.

Zhu, B., Fitzgerald, D. G., Mayer, C. M., Rudstam, L. G., & Mills, E. L. (2006). Alteration of ecosystem function by zebra mussels in Oneida Lake: impacts on submerged macrophytes. *Ecosystems*, *9*(6), 1017–1028.

Zieritz, Alexandra. (2010). Variability, function and phylogenetic significance of unionoid shell characters. PhD thesis, University of Cambridge.

Zieritz, A., Geist, J., & Gum, B. (2014). Spatio-temporal distribution patterns of three stream-dwelling freshwater mussel species: towards a strategy for representative surveys. *Hydrobiologia*, *735*(1), 123–136.

Zieritz, A., Lopes-Lima, M., Bogan, A. E., Sousa, R., Walton, S., Rahim, K. A. A., … McGowan, S. (2016). Factors driving changes in freshwater mussel (Bivalvia, Unionida) diversity and distribution in Peninsular Malaysia. *Science of the Total Environment*, *571*, 1069–1078.

Zinger, L., Gobet, A., & Pommier, T. (2012). Two decades of describing the unseen majority of aquatic microbial diversity. *Molecular Ecology*, *21*(8), 1878–1896.