Potential Application of Poultry Eggshell in Giant Freshwater Prawn, (*Macrobrachium rosenbergii*) Larvae Culture.

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DECLARATION

I hereby declare that the work embodied in here is the result of my own research except for the excerpt as cited in the references.

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List of Symbols and Abbreviations

ANOVA	Analysis of variance
Ca	Calcium
DO	Dissolved oxygen
g	Gram
L	Litre
Mg	Magnesium
mg/L	Miligram per litre
ppm	Parts per million
ppt	Parts per trillion
psu	Practical salinity unit
SD	Standard deviation

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ABSTRACT

Giant freshwater prawn or also known as- *Macrobrachium rosenbergii*, and locally famous as udang galah. This native species inhabiting Indo Pacific region and one of the most prawn species that had a great demand in global market. *M.rosenbergii* is a fast grow species, though requires optimum nutrient supply. Among the main nutrients require for moulting is macro-elements including calcium and magnesium. Previous studies reported application of minerals derive from poultry eggshell as supplement to livestock animals such as chicken, cattle, sheep and swine with various amount (cattle: 35g, swine: 13g and sheep: 2g to 4g). This present study aims to determine the optimal ratio of calcium and magnesium from poultry eggshell in *M. rosenbergii* larvae culture. Different calcium:magnesium ratio were prepared in the culture water for newly hatch *M.rosenbergii* larvae. Each treatment group have three replicates. Culture water containing dolomite was used as control. Data on the survivability and growth rate of experimental prawns was collected and analysed using statistical analysis which showed comparison of three replicate of three treatments. Finding from this present study suggested fundamental scientific evidence of the optimal calcium to magnesium ratio needed for *M. rosenbergii* larvae culture derived from poultry eggshell.

Keywords: Macrobrachium rosenbergii larvae, macro-element ratio, moulting, poultry eggshell.

Potensi Applikasi Kulit Telur Unggas dalam Kultur Larva Udang Galah

(*Macrobrachium rosenbergii*)

ABSTRAK

Macrobrachium rosenbergii juga dikenal sebagai udang galah di Malaysia. Spesies ini berhabitat di kawasan Indo Pasifik dan daripada pelbagai jenis spesies udang, udang galah ialah salah satu spesies yang mempunyai permintaan tinggi di pasaran global. Pertumbuhan *M. rosenebrgii* adalah agak laju, malah memerlukan zat optimum yang secukupnya. Zat yang penting diperlukan untuk persalinan kulit adalah makro-elemen yang juga merangkumi kalsium dan magnesium. Penyelidikan yang lepas menyatakan applikasi mineral-mineral daripada kulit telur unggas sebagai zat tambahan diberi kepada ayam, lembu, kambing dan khinzir dalam kuntiti yang berbeza (lembu: 35g, khinzir: 13g dan kambing: 2g-4g). Tujuan penyelidikan ini adalah untuk menentukan nisbah optimal kalsium dengan magnesium untuk kultur larva *M. rosenebrgii* daripada sumber kulit telur ayam. Nisbah kalsium:magnesium yang berbeza telah disediakan menggunakan air untuk larva *M. rosenebrgii* yang baru menetas. Tiga replika air rawatan telah disediakan dan rawatan kawalan disediakan mengguanakn dolomite. Data kebolehan hidup dan tahap pertumbuhannya telah dikumpul dan dianalisis munggunakan analisis statistik yang telah menunjukkan perbezaan antara tiga replikasi dan tiga rawatan. Pencarian daripada penyelidikan ini telah cadangkan nisbah optimal kalsium denagn magnesium daripada kulit telur yang diperlukan untuk kultur larva *M. rosenebrgii*.

Kata kunci: Larva *Macrobrachium rosenebrgii*, nisbah makro-elemen, persalinan kulit, kulit telur unggas.

CHAPTER 1

INTRODUCTION

The giant freshwater prawn, *Macrobrachium rosenbergii*, is locally known as udang galah or Malaysian freshwater prawn (Jee, 1998). This species is becoming an important targeted crustacean species and well known as the largest cultured freshwater prawn globally (Banu, 2016). A good quality broodstock will results a better growth performance of *M. rosenbergii* larvae. *M. rosenbergii* larvae undergo 11 stages of molting before transforms into post larvae (PL) (D'Abramo, 1995). The moulting stages which represents different stages of metamorphosis requires optimum amount of macro-elements. Among the most crucial macro-elements are calcium and magnesium (Greenway, 1993). These two elements have significant contribution on *M. rosenbergii* moulting frequency and larval survival (Wilder et al. 2009). These macro-elements can be found in eggshell of avian or poultry species. Generally, bird eggshell is a porous bio-ceramic formed at body temperature in a cell-free environment (Yves, 1999). By going through a calcifying process, the eggshell is mineralized and the shell contains about 96% calcium carbonate. Other components are organic matrix (2%), magnesium, phosphorus and other trace elements (Yves, 2004). The carbonates of calcium and magnesium comprises a major portion of the exoskeleton of crustaceans. The minerals

are absorbed from the water for the moulting period (Greenway, 1993). Moulting is an inherent habit of crustaceans, which periodically the old exoskeleton is totally replaced, including appendages and forms a new one. This allows further growth or weight increment (Saravanan, 2008). The main problem is the fluctuation of calcium and magnesium level during the moult cycle which indicates the need for these minerals for exoskeleton formation. Basically, crustaceans have different degrees of demand for macro-elements that changes depending on the moulting stages (Biju, 2008). The main objective of this research is to determine the optimum ratio of calcium to magnesium in *Macrobrachium rosenbergii* larvae culture. Previous research on effects of water hardness and calcium to magnesium ratios on reproductive performance and offspring quality of *M. rosenbergii* by Rezaei et al. (2016) where the optimum ratio of Ca:Mg is determined on the reproductive performance, but in this study the objective is to determine the optimal ratio of Ca:Mg for *M.rosenbergii* larvae for its continuous growth during larvae stages. Information on optimum ratio of Ca:Mg derived from eggshell into *M.rosenbergii* larvae culture, will determine the potential application of poultry eggshell towards the *M. rosenbergii* larvae. This helps in increasing survival growth rates of *M. rosenbergii* larvae and reduces cost of purchasing expensive calcium and magnesium source, thus enhance future development of this industry.

1.1 Background of Study

M. rosenbergii are greatly found in Indo-Pacific region like Malaysia, Thailand, Philippines, India, Sri Lanka, Bangladesh, Myanmar, Indonesia and Vietnam which are tropical and sub-tropical. The early life of the *M. rosenbergii* is spent in brackish water and as they reach post-larvae stage they migrate to freshwater. Most of the *Macrobrachium* species lives in freshwater and only few lives in brackish water. It is found to be there are 150 types of species of *Macrobrachium* found globally, where 49 of it are commercial and 27 of the commercial species are found in Asia and Pacific.

1.2 Problem statement

The important macro-elements needed by *M. rosenbergii* are Calcium (Ca) and Magnesium (Mg). A fluctuation of calcium and magnesium level occurs during the moult cycle which indicates the importance of these minerals for their exoskeleton formation. Different degrees of demand for macro-elements that changes for crustaceans depending on the moulting stages. What is the optimum ratio of calcium and magnesium needed for the *M. rosenbergii* larvae culture?

1.3 Objective of Study

To determine the optimal ratio of calcium to magnesium ratio (Ca:Mg) of *M. rosenebrgii* larvae culture.

1.4 Scope of Study

This study focuses on obtaining the optimum ratio of calcium and magnesium of *M. rosenbergii* larvae culture for its continuous growth and survival through moulting. In this study, poultry eggshells are used to culture the larvae.

1.5 Limitation of Study

This experiment has stopped on the 15th day as most of the larvae were dead. This study could not be repeated due to cost of purchasing egg berried female *M. rosenbergii* and the cost of transportation to transport the live species safely to research place.

1.6 Significance of Study

Determination of the optimum ratio of calcium and magnesium for *M. rosenebergii* larvae culture can contribute the farmers for continuous culture of this species and by using poultry eggshells as the macro-element source as it is eco-friendly by reducing waste of eggshells and use them for aquaculture.

1.7 Hypothesis

The optimum ratio of calcium and magnesium could be obtained by using poultry eggshells as growth potential.

1.8 Expected Outcome

An optimum ratio of calcium and magnesium to be determined from this study. The growth and survivability is also assessed with the optimum ratio of Ca:Mg by using poultry eggshells in water treatments.

CHAPTER 2

LITERATURE REVIEW

2.1 History of *Macrobrachium rosenbergii*

Macrobrachium rosenbergii (de Man, 1879), giant freshwater prawn is the popularly known palaemonid in worldwide. Previously, the taxonomy, morphology, development, anatomy, physiology, biochemistry, ecology behaviour and the nomenclature history of *M. rosenbergii* (Holthius, 2000). A problem occurred in the naming of the *M. rosenbergii* species. A debate started to justify whether the *M. rosenbergii* is only one type. Surprisingly, two subspecies of *M. rosenbergii* was found by Johnson, (1960) which are the western and eastern species based on the morphology aspect. Holthius (1998) recommended the suitable name for western subspecies as *M. rosenbergii dacqueti* (Sunier, 1925) and the eastern subspecies as *M. rosenbergii rosenbergii* (de Man, 1879). The purpose of this study is to distinguish differences between the two subspecies and if they are to be accepted as a species by using morphometric measurements and multivariate method of Multiple Discriminant Analysis (MDA). The number of specimen used to research were 373 *M. rosenbergii* species group. The specification of *M. r. rosenbergii* taxonomy was described using

two adult male specimen and *M. r. dacqueti* is three adult male specimen. The description of characters are sex, number of dorsal and ventral teeth of rostrum, position of rostrum tip, height of base rostrum and type and spine on second pereiopod. As a result, the Root 1 of MDA analysis with within group classified *M. r. rosenbergii* 100% and *M. r. dacqueti* 100% for combined sex and males only. The ROSTBASE, VENTEETH and DORTEETH classifies *M. r. rosenbergii* 100% and *M. r. dacqueti* 98.5%. Based on morphometric analysis, the two subspecies are distinct taxa and two different species. A taxonomy is provided by Rafinesque (1818), previously named palaemonidae, *Macrobrachium Bate* (1868) and present *Macrobrachium rosenbergii* (de Man, 1879). The other subspecies (*M. r. dacqueti*) taxonomy is also provided by Sunier (1925) together with diagnosis, remarks and distribution information. The separating of *M. r. rosenbergii* and *M. r. dacqueti* is based on height of rostral base and number of ventral rostral teeth. However, rostral is not reliable for distinguishing the subspecies as it is only suitable for adult, but a present finding showed the *M. r. rosenbergii* rostrum grows slower than *M. r. dacqueti.* The tip of rostrum, spine on pereiopods is not a key to totally rely on. As a conclusion, the name "*M. rosenebrgii*" was used for a long time which is also known as *M. dacqueti*. Some papers were prepared to remain the name "*M. rosenbergii*" and the Australian, Philipine and Papuan species should have other name.

2.2 General introductions on *M. rosenbergii*

The four phases of *M. rosenebrgii* are eggs, larvae, post-larvae and adults (New, 2002; Hung, 2013). The female adult becomes fertile through mating (copulation) with male adult prawns and starts egg production. Usually, the female prawns produces eggs from 80 000 to 100 000 eggs (New, 2002) during a spawning. The larvae hatches after an average of 20 days. The larvae are let free from the abdominal appendages of the female prawn and requires brackish water to survive. According to Nandlal and Pickering (2005), the larvae has 11 moulting stages to be completed between 22 to 35 days. Larvae turns into post-larvae (PL) and crawls. They start swimming in forward direction also. The post-larvae becomes an adult and live in benthic habitat (New, 2002; Hung, 2013). In 3 to 6 weeks they migrate to freshwater from brackish water (Ling, 1969; New and Singholkan, 1985; New, 2002) and prefers staying in a hidden place. Adult *M. rosenbergii* are omnivorous that feed on small clams, shrimps, filamentous algae and fish flesh same as post-larvae (New, 2002; Hung, 2013). When it is time to breed, the female prawn returns to brackish water.

In terms of larval development, *M. rosenbergii* (Ausralian strain) larvae have shorter developing duration than Malaysian strain (*M. dacqueti*) from the experiment done by Sarver et al., (1979). This can be due to niches and biological needs. Australia, Philipines aand Indonesia are the countries which have *M. rosenbergii*. *M. r. dacqueti* is reared in Indonesia but not in Australia. It is also popularly cultured and can be found in Hawaii which is originally from Malaysia (Karplus et al., 2000). Besides, in Malaysia the state of Sabah showed more morphological difference than the state of Perak and Kelantan (Harun, 2013). This is more likely due to the geographical change and climate

changes. Furthermore, the state of Perak runs a major *M. rosenbergii* broodstock from wild population beginning with hatchery stage with more genetic variability (New et al., 2010). Variability in genetic of *M. rosenbergii* is useful to raise the aquaculture industry and for the goodness of local broodstock sustenance.

Morphologically, the body length of male *M. rosenbergii* can go up to 320 mm, whereas female body can reach up to 250 mm (de Man, 1879). In addition, Harun et al., (2014) reported male *M. rosenbergii* of same age as female is huge than female. The head (chephalotorax) of male is larger than narrow abdomen and has long and massive chelipeds. According to Ling, (1969), the female prawn can be distinguished by small head and claws. The most prominent part of *Macrobrachium* is the second pair of periopod and it is recorded in most morphological findings (Munasinghe and Thushari, 2010). Often the periopods is utilised in reproductive and aggressive behaviour. This study's objective is to state the morphological characteristics of wild *M. rosenbergii* population from various genetic background in Malaysia. Samples (adult *M. rosenbergii*) from Sungai Kelantan, Sungai Perak and Sungai Papar (Sabah) was collected. A total of ten morphometric, three meristic parameters and sex distinguishing was measured and recorded. Analysis such as ANOVA, Turkey HSD and Discriminant Function Analysis (DFA) was carried out. Based on DFA, highest value was observed for Sabah (91.7%), whereas decreased value of population for Kelantan (66.7%) and Perak population (70.4%). Kelantan and Perak population was observed to be blue colour with 73.8% and 96.3% each, while Sabah population had high percentage of orange colour (83.3%). Most prominent sex was female from 3 populations. Wowor and Ng (2007) stated, rostral length is suitable to be studied for adult prawn and not young prawn though in this study rostrum length was second prominent morphometric.

Based on meristic parameters of *M. rosenebrgii*, the 3 populations (Kelantan, Perak and Sabah) did not show any significant.

Various morphotype of male freshwater prawn are found in *M. rosenebrgii* population which are small male (SM), orange claw (OC) and blue claw (BC), whereas female has one morphotype (Kuris et al., 1987; Kurup et al., 2000; New, 2002; MacGibbon, 2008; Silva-Oliveira et al., 2011). MacGibbon (2008) and Mandal et al., (2013) stated the growth rate, successful mating and behaviour is different among the three male morphotypes. Though the female consist of one morphotype, there are virgin female, berried female (egg carrying female) and open brood chamber female which does not have specific territory which could vary their growth. A female develops in duration of 6 to 20 weeks and weighs around 12g to 32g. Mondal et al., (2013) stated variety of size could be also caused by environmental conditions.

2.3 Important water quality in *Macrobrachium rosenbergii* larvae culture.

Hazardous amount of ammonia concentration in water is toxic to aquatic organisms (Chin and Chen, 1987; Daniels et al. 1987; Noor-Hamid et al. 1994; Ostrensky and Wasielesky, 1995). The parameters in charge of balancing NH^3-NH_4^+ is pH, temperature and salinity. Mainly, pH gives the most effect to the pattern of NH^3-NH_4^+ value (Spotte, 1979; Alabaster and Loyd, 1982; Tomasso, 1994). Mallasen and Valenti (2005) carried out a study to determine *M. rosenbergii* larval development based on ammonia concentration (0, 1, 2, 4, 8) mg/L and pH values (7, 8 and 9) was tested for larval development and larval respiration. The survival rate was observed to be lowest in pH 9 compared to pH 7 and 8, while mortality recorded for ammonia concentration 4 and 8 mg/L. The respiration rate in all the treatments, except 4 and 8 mg/L ammonia concentration where no difference found. It is found that the ammonia in water aids in ion and gases transportation. Lignot et al. 2000 stated cell damage is caused by high ammonia which can disrupt the cell that is in charge of Na^+, K^+-ATPase activity in crustaceans. This rise of ammonia concentration can also reduce oxygen transportation, however can be adopted by a decapod larvae depending on surrounding (Jacobi and Anger, 1985). A pH from 7.0 to 8.5 is recommended for *M rosenbergii* hatchery (Lee and Wickins, 1992; Correia et al. 2000; New, 2002). For pH lower than 6.5 or higher than 8.5 for *M. rosenbergii* larvae is observed to have slow growth rate and increasing mortality. Keeping record on ammonia concentration and pH throughout whole process of rearing larvae is necessary to maintain water quality (Mallasen and Valenti, 2005).

Rezaei Tavabe et al. (2015) studied on effects of water hardness and Ca:Mg ratios on reproductive performance and offspring quality of Macrobrachium rosenbergii.

According to Davis et al. 1993; Greenway, 1993; Roy et al. 2009; Tavabe et al. 2013 there are two macroelements (calcium and magnesium) which is important for crustaceans ecophysiological activities. The aim of the study is to determine the optimal ratio of calcium to magnesium for broodstock as control, 150 mg/L and 190 mg/L. The calcium and magnesium are absorbed by crustacean species which present in the aquatic surroundings as required during molting. However, from the experiment carried out by Rezaei Tavabe et al. (2015) showed high and low calcium to magnesium ratio does not show positive effects on offspring quality at 150 mg/L water hardness, whereas there is negative effect at 190 mg/L. This research shows that reproductive result is great at water hardness of 150 mg/L and ratio of 50% Ca to 50% Mg. Rezaei Tavabe et al. (2015) stressed that there is no studies that determines the reproductive performance of *M. rosenbergii* at different water hardness or calcium/magnesium concentrations.). In this research it is proved that water hardness of hatchery and Ca:Mg ratio has direct and measurement effect on the reproductive performance and offspring quality of *M. rosenbergii*.

2.4 Larval quality and Na/K ATPase activity.

Kamran Rezaei Tavabe, Gholamreza Rafiee, Michael Frinsko, and Harry Daniels (2016) carried out a study on effect of macro-elements and various sodium potassium adsorption ratio (SPAR) medium on Na/K ATPase activity of *Macrobrachium rosenbergii* larvae. *M. rosenbergii* early stage larve has difficulties surviving after hatching (Huong et al. 2004; Brown et al. 2010) because their osmoregulation system is not completely formed (Huong et al/ 2004). Osmoregulation carries out Na/K ATPase activity by ATP hydrolysis which is necessary for *M. rosenbergii* early stage larval development (Huong et al. 2004) prior hatching and important for euryhaline species (Wilder et al. 2010). A Na/K ATPase enzyme is a carries protein, where Na+ ions exports from cytosol and imports K+ ions into the cell by ATP hydrolysis (Lucu & Towle, 2003) against concentration gradient of it. Besides, the four macro-elements (sodium, potassium, calcium and magnesium) is also important as it effects *M. rosenbergii* larval osmoregulation, quality and growth (Tavabe et al. 2013; Rafiee et al. 2015; Rezaei Tavabe et al. 2015b). The main objective of this study is to determine the effect of different Na^+ vs K^+ and Ca_2^+ vs Mg_2^+ concentration on the activity and also the different ratio $(Na^+ + K^+)/(Ca_2^+ + Mg_2^+)$ (SPAR) on both larval quality and Na/K ATPase activity. The broodstock is obtained from a private farm in Kenly, North Carolina. Kamran Rezaei Tavabe (2016) stated that this experiment should cover mainly in three areas of NA/K ATPase activity and larval condition index (LCI) at larval stage (1^{st}, 4^{th}, 7^{th} and 11^{th} stage) which is comparison between each two different concentration of sodium vs potassium, comparison between magnesium and calcium, and comparing of various medium (20, 30, 40 and 50) of SPAR. The experimental treatment each conducted in triplicate. The larvae is collected

and counted to stock them in various treatment container. Thus, two parameters is evaluated in which is for Na/K ATPase activity and larval quality. There is three results that is obtained from three experiment. Huang et al. (2004) showed that Na/K ATPase activity elevates during the early larval stages of *Macrbrachium rosenbergii*, then decreases throughout the larval development process. This relates with the result of this study which demonstrates the activity of Na/K ATPase has measurable effect on larval quality and dry weight of *M. rosenbergii*. It is also confirmed by Towle et al. (1976) that Na/K ATPase activity is highest prior to molting and during pot-molt stages. Third experiment on SPAR is to further understand the significance of combine macro-elements effect on Na/K ATPase activity. Kamran et al. 2016 recommends larval hatcheries of *M. rosenbergii* operated to SPAR 30 on early larval stage and SPAR 40 on late developmental. The conclusion is the activity of Na/K ATPase activity is different during larval development and it is affected by interaction of four macro-elements for larvae osmoregulation system.

2.5 Importance of Calcium (Ca) and Magneisum (Mg) for *M. rosenbergii*

Kamran et al. (2013) reported effect of different calcium and magnesium concentrations separately, followed by combination on *M. rosenbergii* larviculture. According to New and Kutty (2010), *M. rosenbergii* is first introduced to Iran in 1991 from Bangladesh. Principally, this species native is at south east Asia before it is introduced to other countries (New, 2010). Tavabe, (2013) stated that hatchery process is important for larval production as it requires brackish water to be reared. New (2003) explains that suitable brackish water can be manmade using fresh water brine, commercial "sea salt" or mineral supplements mixed with locally available salt sources. This case is to reduce the cost of production of high quality post larvae and be efficient in production practices without limitations. Providing suitable brackish water is equally important as preparing it with correct amount of macro-elements content in inland hatcheries. Concentrations of macro-elements like calcium and magnesium is important too, as it is part of the water quality and also for larval shell hardening, osmoregulation and growth. The objective Kamran's study is to determine the optimum concentration and Ca/Mg ratio for *M.rosenbergii* larviculture in brackish water. This experiment is carried out in two stages, first is effect of various concentration of calcium and magnesium separately to compare the fresh water larval quality and developments.Second stage is by combining both the macro-element in the larviculture. The broodtsock is brought from Ghasreshirin freshwater prawn hatchery in western Iran and from private farm in Kenly, North Carolina state for the first and second stage experiment respectively. The egg-bearing female with greyish masses is selected and moved to hatching tanks to be hatched into larvae followed by placing in plastic container for counting of first stage (Menasveta & Piyatiratitivokul, 1980). The second

stage is carried out in simple water reuse system (New, 2003). Menavesta, 1980 suggested three methods of *M. rosenbergii* larviculture to maintain a stable concentration of macro-elements with minimum water exchange and critical brackish water with 12 ppt salinity for each triplicate treatment conducted (New, 2003). The variety soluble salts that used are $CaCl_2.H_2O$ and $MgSO_4.7H_2O$ $MgCl_2 + 6H_2O$ with equal weight. First stage of study, the triplicate treatment of calcium is (120, 180, 240 and 300 ppm) and magnesium is (300, 400, 500 and 600 ppm). In second stage, the two of the best concentrations of macro-elements is used in combined form for triplicate treatments. There are 2 parameters evaluated which are broodstock general and reproductive parameter and larval quality parameter. For the combined calcium-magnesium effects, the two best concentration of calcium (180 and 240 ppm) and magnesium (300 and 400 ppm) from the first stage is applied as it showed high significance for larval survival. Hence, the growth, survival, developmental and larval quality from the results is strongly affected by different concentration of calcium and magnesium separately and in combination. Hangsapreuke et al. (2008) stated that *Macrobrachium rosenbergii* larvae needs higher magnesium concentration around 174 ppm in environmental water, while low survival occurs during final larval stage that causes magnesium depletion due to different aquatic culture environment. Zhuang and Ahearn, (1996) showed calcium is main factor affecting *M. rosenbergii* larvae's molting frequency and hemolymph osmolality. The combination of the two elements greatly effects larval quality. Thus research concludes that appropriate concentration of calcium and magnesium elements important for larval development.

2.6 Source of calcium and magnesium from eggshell.

According to Maxwell T. Hincke (2012) the avian egg represents the advanced amniotic egg in oviparous vertebrates. The eggshell carries out the exchange of metabolic gases, water and serves as calcium storage. This study covers about eggshell biomineralization where the chicken eggshell is used as a reference and emphasises on its structure, function, mineralization and calcification. M.T Hincke (2008) described components of an egg which consists of central yolk surrounded by albumen (egg white), eggshell membranes, calcified eggshell and cuticle. The calcium in the eggshell is formed by bathing of incomplete shell during mineralization phase in uterine fluid containing 6-10 mM of ionized calcium and 70 mM of bicarbonate ions which have concentrations of 80-120 time higher than soluble product of calcite (Y Nys, J Zawadzki, J Gautron & AD Mills, 1991). The mammillary layer or mammillae has an important region which serves as calcium reserve body that contains microcrystals of calcite. It has spheruliitical texture that facilitates mobilization of calcium to nourish embryo. Besides, the completely formed eggshell contains about 6g of mineral. The composition of eggshell is 96% calcium carbonate, while the remaining components includes 2% organic matrix as well as magnesium, phosphorus and other trace elemnets (Nys Y, J Gautron, J.L Arias, J. M Garcia-Ruiz & S Solomon, 1999). A. B Rodriguez-Navarro, O Kalin, Y Nys and J.M Garcia-Ruiz (2002) states that the ultrastructure of chicken eggshell is extreme regular and it is also a polycrystalline calcium carbonate ceramic that consists only one polymorph, calcite. There are different ultrastructure for reptiles and avian, but the physiological process related to their ultrastructure are not known. The microstructure is also equal important where the eggshell composition which is smaller and less mutually aligned has stronger calcite crystals than those

formed with large and high oriented crystals. Eggshell matrix protein constituents differ between three stages of calcification process which are initial, growth and terminal (J Gautron, M. T Hincke & Y Nys, 1997). This results incorporating into mineralized eggshell and caused differential distribution throughout the eggshell zones (M.T Hincke et al. 2008). There are several terms discussed on eggshell matrix protein which includes transcriptomics, genomics, ovocleidin-17, ovocleidin-116, ovocalyxin -32, ovocalyxin-36 and osteopontin. This information might be useful for comparative studies of organic constituents of avian eggshell and their functional implication as stated by Maxwell et al. (2012).

2.7 Salinity and temperature tolerance on *M. rosenbergii*

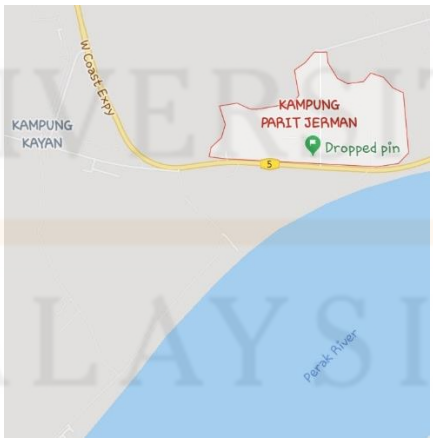
The early life cycle of *M. rosenebergii* larvae is the most crucial stage as their needs have to be maintained to optimum level. Salinity and temperature are important factors that influences the survival percent and growth of larvae. Mohanty et al. 2016 recorded from their studies that under 6 ppt. salinity number of larvae survived was zero at temperature (25°C, 31°C, 35°C). The maximum survived larvae was at 12 ppt. salinity at 31°C and 35°C. *M. rosenbergii* larvae survived in 12 ppt. and 15 ppt. salinity which the survival rate was high. There is a study indicates *M. rosenbergii* can tolerate salinity until 21 ppt. (Mukhopadhay and Sorangi, 1985). The growth of the *M. rosenebergii* larvae increased as the temperature increase according to Mohanty et al. 2016 studies. This is likely caused by high feed intake at high temperature. According to Staples and Heales (1991), increased temperature could increase moulting and growth of freshwater prawn. The tolerance of salinity is possible for *M. rosenebrgii* larvae because a several studies stated it carries out osmoregulatory process in freshwater when the salinity is at iso-osmotic point (14-15ppt). Woo and Kelly (1995) agreed that mild brackish water (10-12 ppt) decreases the energy loss to replace the salt loss and promotes growth. Monitoring salinity and temperature of *M. rosenbergii* larvae culture could make freshwater prawn as an ideal species to be reared.

CHAPTER 3

METHODOLOGY

3.1 Sampling

The berried females (egg carrying female), *M. rosenbergii* about thirty were obtained from a farmer at Kg. Parit Jerman, Perak on 6th to 7th August 2019 that was obtained from Sungai Perak, Perak. The samples were then brought in a polystyrene box with water temperature of 20-22°C to reduce the activity of *M. rosenbergii* and salinity of 0 ppt. The water were aerated with two portable aerator. The water were treated with anti-chlorine before placing the egg carrying *M. rosenbergii*. The transport duration was 5 hours to reach to aquaculture area near animal lab of University Malaysia Kelantan, Jeli Campus. Perak is majorly known for giant freshwater prawn's top production, especially locations near to coastal area which is nearby river of Perak.



Coordinate:
4°02'41.7"N
100°49'03.3"E

(A)

Figure 3.1 Maps showing berried female *M. rosenbergii* obtained (A)

3.2 Apparatus and materials

The apparatus that was used in this study were aerator, larval rearing tank (50 L), 1000 mL beaker, conical flask, and calcium and magnesium hardness checker, ammonium checker and multiparameter (HI 98194 pH/EC/DO Multiparameter-Hanna Instruments). The materials were used are berried female *M. rosenebergii* about 20g to 25g, poultry eggshells, dolomite (CaMg(CO3)2), *artemia nauplii*, Magnesium oxide (MgO)

3.3 Research Method

The process of this study started by obtaining berried female *M. rosenbergii* until analysing data collected from the experiment carried out and presented them in table and chart form.

3.3.1 Preparation of calcium:magnesium solution

The treatments groups will be contained different ratio of calcium to magnesium: T1 (18Ca:300Mg), T2 (240Ca:400Mg) and T3 (300Ca:500Mg). About 15 g of dolomite with chemical composition of $CaMg(CO_3)_2$ used as control.

3.3.2 Hatching of *M. rosenbergii* larave

Berried female of *M. rosenbergii* was placed in water mixed with formalin solution for 12-24 hours til the colour of the water changes yellowish-brown, then the water is replaced with 10 ppt water for *M. rosenebrgii* with dark greyish egg mass for hatching in larval rearing aquarium (50 L). The water was prepared by mixing dolomite of 250g in 100 L brackish water with 10 ppt. salinity, pH 7.0. The temperature set at optimum of 28°C and 5 ppm. The water was distributed into two tanks of each 45 L prepared water.

3.3.3 Larvae collection

Using 1000 mL beaker, the larvae prior hatching were collected and counted before being distributed to 12 experimental tanks. Each aquarium tank contains 250 larvae.

3.3.4 Preparation of *artemia nauplii*

In a conical flask (1L), filled up with 1 L of water (brackish water), then 2 g of brine shrimp (*artemia*) is added and aerated for 12 hours. The hatched *artemia* is filtered using fine mesh net and washed with pipe water. The process is repeated. *Artemia* was given to newly hatched larvae for 10 days, twice daily. After 10 days, *artemia* was fed only once a day.

3.3.5 Preparation of egg custard.

Four eggs of Grade A was used to prepare egg custard. The eggs were beaten in a beaker, then poured to measuring cylinder (250ml). On the other hand, the skim milk powder was measure about 50g in four small plastic container which is suitable for steaming. The beaten egg is poured to each container and mixed with milk powder. The batter is flattened and steamed for 10 minutes. After steaming, it is left to cool and grinded in a mesh net with 2 different size (starter 10-13 days and 14-post larvae stage). The egg custard was stored in refrigerator (4°C).

3.3.6 Preparation of poultry egg shell.

The eggshells were collected and cleaned using running water. It is then boiled in water for 10 minutes and laid on a baking tray. The egg shells were let to dry overnight. The grounding of eggshell was carried out by blender.

3.3.7 Experimental design

The water treatment was prepared for three different ratios by measuring and adding poultry eggshells and magnesium oxide to the water and dolomite for control treatment. For T1 (180ppm Ca:300ppm Mg), (240ppm Ca:400ppm Mg) and (300ppm Ca:500ppm Mg), (5.4 g, 7.2g, 9.0g)of chicken eggshell powder and (9.0g, 12.0g, 15.0g) of magnesium oxide added to 30 L of three containers of water respectively. The water were left for 24 hour for the particles to set down and it was prepared one day before the transfer of larvae to treatment study. For conducting the treatment, 12 aquarium tanks was prepared with aeration tube fixed and the calcium and magnesium water solution which were prepared was poured into each tank according to their treatments. Control and the three treatments was set in triplicate which gives a total of 12 aquarium tanks. On the day 11 after hatching, the larvae was counted and put into the aquariums. Precisely, 250 larvae was put in each aquarium tank which consist of 10 L of treatment water for the triplicates of three treatments (T1, T2 and T3) including control. The larvae were only fed artemia nauplii from the day it hatched until day 10.

Day 11 onwards, *M. rosenbergii* larvae fed with nauplii only once at night and egg custard will be given twice a day during day time. Water parameters such as salinity (12 ppt), pH (7.0 – 8.5) and dissolved oxygen level was kept optimum (5 ppm) and

these were recorded for 5 times using multiparameter (HI 98194 pH/EC/DO Multiparameter-Hanna Instruments), refer to (Appendix 1). Ammonia were measured once in three days to maintain the water quality (ammonium-nitrogen below 0.2 mg/L and nitrite nitrogen below 0.1 mg/L) using ammonia checker (Hanna brand). The dead *M. rosenbergii* larvae or unconsumed nauplii was removed to avoid disrupting of water quality. When the water turns yellowish, about 5 L of the prepared Ca:Mg water was added to each treatments. This process took place once every three days. The third time of water adding was replaced by changing new water. The *M. rosenbergii* larvae was transferred to a small container before changing new water and transferred to the aquarium tank again. The water replacement was kept minimum to reduce mortality of *M. rosenebrgii* larvae. The aquarium tanks were kept in a place where there is no direct sunlight. Direct sunlight gathers the larvae at one place in the aquarium which can lead to death.

$$\text{Survival rate (\%)} = \frac{Final\ number\ of\ larvae}{Total\ Number\ of\ larvae} \times 100$$

3.3.8 Calcium and magnesium measurement.

The calcium and magnesium measurement was measured once in a week using calcium and magnesium checker of Hanna brand.

3.3.9 Data analysis

Data on survivability was observed and collected every twice a day. After collection of data, was analysed using Microsoft Excel for one-way analysis of variance (ANOVA) version 2013.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Parameters (pH, Dissolved oxygen, salinity and Temperature).

Parameters of water (pH, Total dissolved solid (mg/L), salinity (ppt) and temperature (°C).

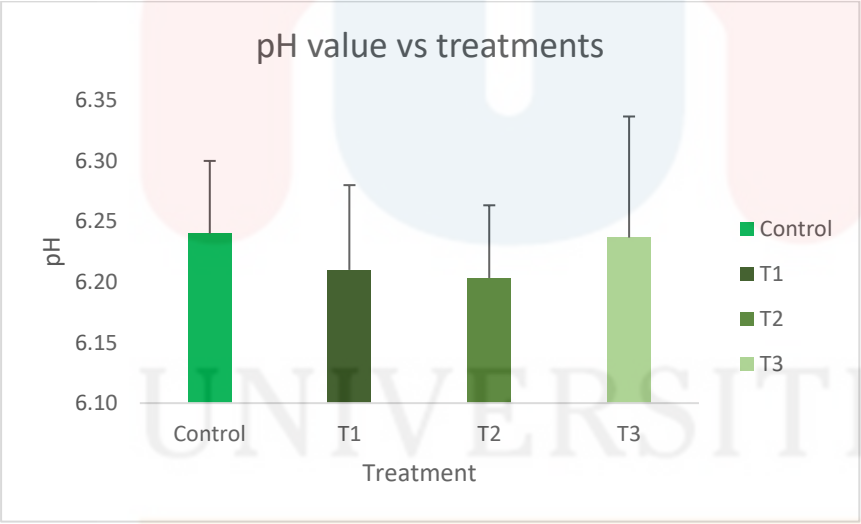


Figure 4.1 The average pH value presented is Mean ± SD for the ratio of calcium to magnesium used for each treatment and dolomite in control. The pH value was not significant (P>0.05).

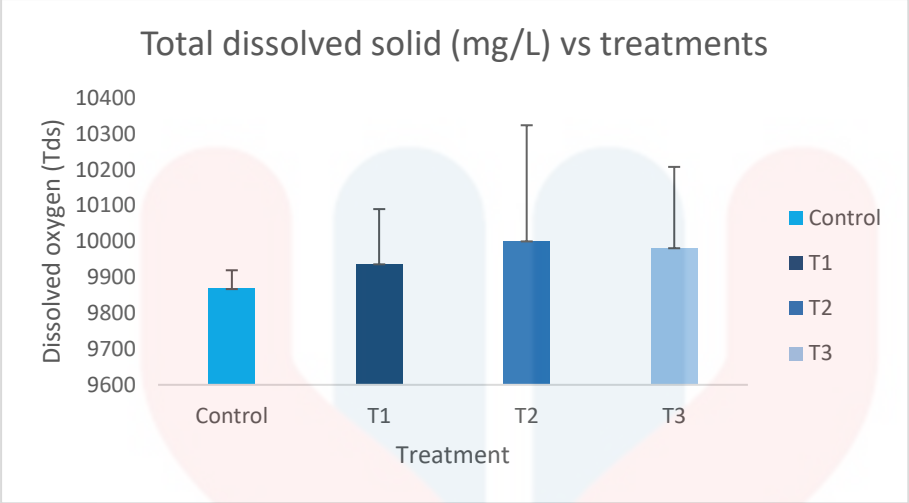


Figure 4.2 The average dissolved oxygen measurement presented in Mean ± SD value for the ratio of calcium to magnesium used for each treatment and dolomite in control. The value of total dissolved solid was not significant (P>0.05)

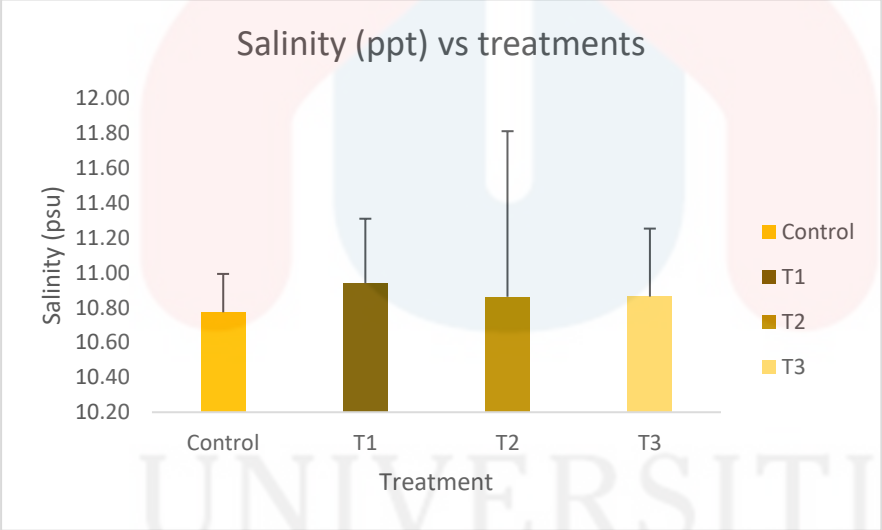


Figure 4.3 The average salinity (ppt) measurement presented in Mean ± SD value for the ratio of calcium to magnesium used for each treatment and dolomite in control. The value of salinity was not significant (P>0.05)

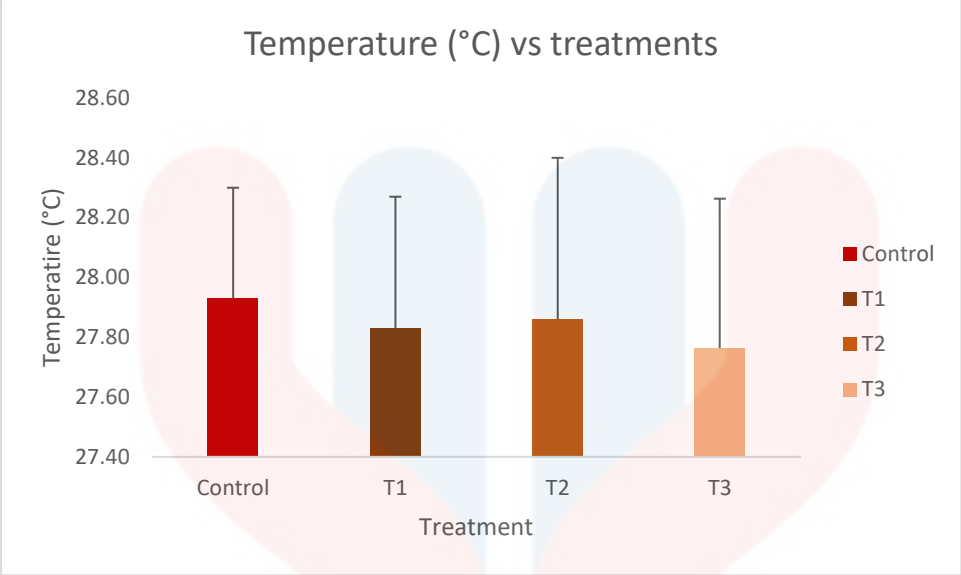


Figure 4.4 The average temperature (°C) measurement presented in Mean ± SD value for the ratio of calcium to magnesium used for each treatment and dolomite in control. The temperature was not significant (P>0.05)

4.2 Ammonia, Calcium and Magnesium test value

Ammonia (ppm NH_3-N), Calcium and magnesium test (ppm) with mean and SD value included for the parameters for all 4 treatments and the replicates.

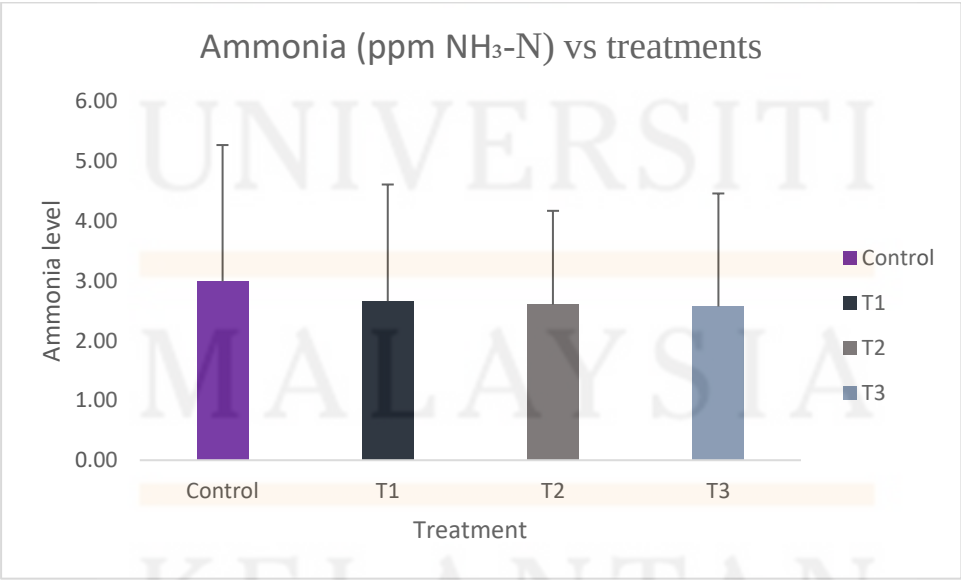


Figure 4.5 Value of ammonia recorded and presented in Mean ± SD value for the ratio of calcium to magnesium used for each treatment and dolomite in control. The value was not significant (P>0.05).

4.3 Survival rate

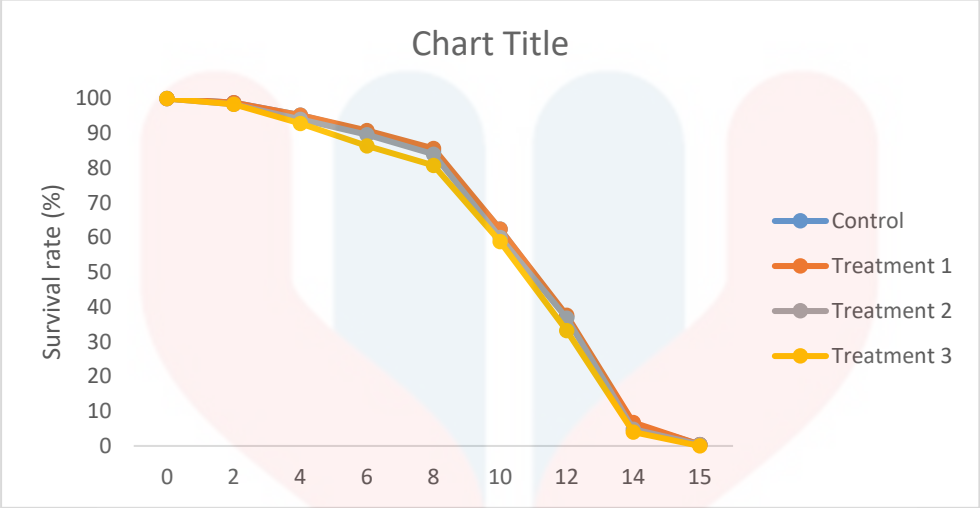


Figure 4.6 Survival rate of M. *rosenbergii* larvae for four treatments. Mean ± SD value is presented. The survival rate was not significant (P>0.05).

4.4 Contribution of calcium (eggshell) and magnesium (magnesium oxide) towards number of larvae survived

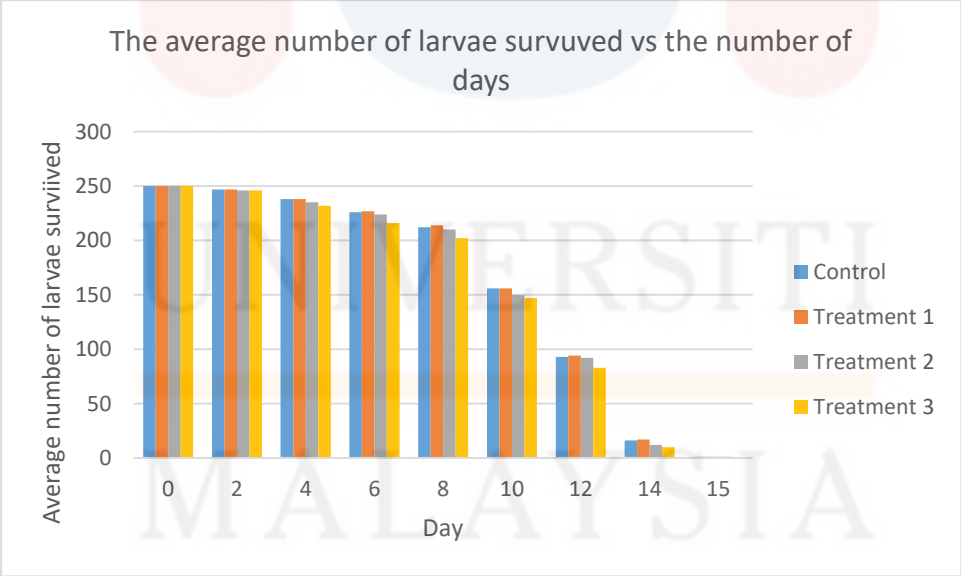


Figure 4.7 The number of larvae survived in 4 treatments and the triplicates for 16 days.

4.5 Cost of Benefit

Table 4.1 Price of eggshell and dolomite (commercial) used in this study

Materials	**Price (RM)**	**Total (RM)**
Eggshell	2 carton × 6.00	**12.00**
Dolomite (commercial)	400.00	**400.00**

The price of eggs for this study was cheaper compared to the commercial dolomite used for water treatment. The eggs were purchased and only the eggshells were used. The number of eggs in a carton is 10-12. Only 9-15 g of eggshell powder was used for treatment from the eggs that were grinded. The culture water using eggshell in this study was changed about 1 time throughout the duration of the experiment by replacing the water, eggshells and magnesium oxide (MgO), whereas the water culture using dolomite was not changed.

Based on the experiment that was carried out, there were several parameters used and included for tabulating data for results. The *Macrobrachium rosenbergii* larvae tolerated high range of ammonia, where to be exact, the larvae from Control treatment tolerated an average range of 2.99±2.28 ppm which is considered to be high compared to other treatment larvae. However, exceeding level of ammonia prescribed can be harmful to living organisms as it can lead to death or slows down the growth rate of aquatic livings. For *M. rosenbergii*, it is recommended for ammonia not to exceed 0.5 mg/l or 0.05 ppm NH_3-N. The lowest range of ammonia was observed in Treatment 3 with average range of 2.57±1.89. There is no significance difference ($P>0.05$) between Control treatment, Treatment 1, Treatment 2 and Treatment 3. Treatment 1 and 2 had moderate level of ammonia level (2.66±1.95 ppm and 2.60±1.57 ppm) respectively.

The value of ammonia still could not be maintained within 0.5mg/l or 0.05 ppm because of the turbidity in water from a physiological observation. A high turbidity is caused by suspended particle such as silt, faecal matter, unconsumed feed and dead larvae and it is by a journal author MacGibbon, (2008). From the observation of Mallasen and Valenti, (2005) experiment, they have recorded an increase in pH causes formation of NH_3 and excreting high concentration of ammonia which is toxic to *M. rosenbergii* larvae. Absence of significance difference is obtained ($P>0.05$) in all the treatments that proved ammonia is not the main factor affecting survival of larvae. However, the

readings of data of ammonia and pH (Appendix 3) respectively shows fluctuation, where there is no linear pattern in terms of their average values. It can be fairly seen in this comparison is not similar to the findings stated by Mallasen and Valenti, (2005). The fluctuation is estimated to be caused by the high turbidity of the treatment water.

The recommended level of pH is between (7.0-8.5) for *M. rosenbergii* (Lee and Wicking, 1992; Correai et al. 2000; New, 2002). Furthermore, high turbidity if found to effect the rate of dissolved oxygen, where this parameter is also are of the important parameter of treatment water for *M. rosenbergii* larvae. The lowest mean of dissolved oxygen was found in Control treatment (9868±51.50 mg/L) and highest average value is found in Treatment 2 (10001±322.99 mg/L). There is no significance ($P>0.05$) between all the four treatments because the survival rate found to be highest in Treatment 1. The value recorded for Tds (total dissolved solid) also did not synchronise with the statement by Aquaculture, (1999) which states high turbidity increases the rate of dissolved oxygen and water with low turbidity has the opposite effect.

According to MacGibbon, (2008), the effect in dissolved oxygen is caused by respiration of organisms, water temperature, faeces and unconsumed feed particles. Temperature is an essential factor which can affect the feeding rates and growth. The optimum temperature for survival of *M. rosenbergii* larvae is 29-31°C (New, 1990). However, in this current study, the range of temperature in four treatments was within 27-28°C which is lesser than the optimum range. Despite the issue of low temperature, a few number of larvae in all treatments survived and tolerated in 27-28°C. Though a few survived, but throughout the experiment a number of larvae was found to be declining gradually as the temperature recorded was below optimum. On the other hand, dissolved oxygen is also a factor that involves in controlling temperature of the water. The lowest temperature was recorded for Treatment 3 with average of 27.76±0.50°C and the highest temperature was found in Control Treatment with average of 27.93±0.37°C. This comparison was not relatable with their respective value of dissolved oxygen (Table 1). In addition, there was no significance ($P>0.05$) difference in all the treatments proving dissolved oxygen not much involved in altering the temperature in this study. The temperatures for the rest of the treatments also dropped below optimum during 4^{th}, 7^{th}, 10^{th}, 13^{th}, and 14^{th} sampling day. Though the temperature was slightly below optimum level, but remained within non-lethal limits which is 14-35°C close to *M. rosenbergii* optimal range stated by New, (1990).

In this study, the *Macrobrachium rosenbergii* larvae adapted the salinity range between 10-12 ppt though the optimum range set in this was 12 ppt. The highest average salinity was recorded in Treatment 1 (10.94±0.37 ppt) and the lowest average was recorded in Control treatment (10.77±0.22 ppt) with no drastic significance ($P>0.05$) difference. According to Subramaniam et al. 1980, the survival in salinity between 5-15 ppt is of the best. The salinity range of this study is acceptable because it matches with the findings of Subramaniam et al. 1980. However, the salinity in all the treatments is within the range of 10-12 ppt and it is unlikely to be the reason for declining survival rate.

During hatchery period of *M. rosenbergii* female adult, the larvae hatched out with the water salinity of 12 ppt and the larvae was transferred to nursery aquarium with salinity of 12 ppt also. The sampling of water was vary for every treatment.

The current study's main focus is on effect of Calcium (Ca) and Magnesium (Mg) hardness on the *M. rosenbergii* larviculture, though there are other parameters in consideration. Four treatments was used in this study including control treatment. The control treatment used dolomite ($CaMg(CO_3)_2$) and the other three treatments used different ratio of calcium to magnesium, where T1(180Ca:300Mg), T2(240Ca:400Mg) and T3(300Ca:500Mg). The calcium and magnesium source used in this study is from eggshell (powdered) and synthetic Magnesium Oxide (MgO). This study mainly focuses on the potential of eggshell towards the larviculture, which is narrowed down to *M. rosenbergii* larvae growth and survival. On the last day of treatment (day 16), the average number of larvae survived was one (1) in Control treatment, Treatment 1 and Treatment 2 out of total 3000 larvae with significance difference ($P>0.05$). In Treatment 3 only an average of zero larvae survived on the last day of experiment with survival rate of 0.00%. The highest survival rate recorded is from Treatment 1 and Control treatment throughout 16 days of experiment (Figure 8). Larvae in Treatment 2 and Treatment 3 had lower than Control and Treatment 1 survival rate. At the end of experiment, Treatment 1, Treatment 2 and Control treatment lasted with larvae survival rate of 0.40% and (1±1.00, 1±0.58 and 1±0.58) average number of larvae respectively.

The adaptation to synthetic water treatment is likely to be the reason for the declining survival rate and no significance ($P>0.05$) present. Calcium source is important for moulting and hemolymph osmolality (Wilder et al. 2004) which the source is naturally

available in brackish water and freshwater. Adhikari et al. 2007 stated that calcium concentration lower than 92 ppm can effect moulting frequency which can delay shell hardening after moulting. Similarly, magnesium content in water important to avoid inhibition of cellular ATPs activities (Morsit and Spicer, 1993). However, a high concentration of magnesium necessary for good moulting of larvae as they inhabit in brackish water. As per observation, the increasing magnesium ratio showed a negative response on the survival rate, where the average number of larvae declined. Increasing ratio of magnesium influences the survival rate of the larvae as the magnesium source used is synthetic chemical, magnesium oxide (MgO). From the calcium(Ca) and magnesium (Mg) test taken once in a week, the highest content of calcium and magnesium was recorded for Control treatment (0.49±0.17 ppm) and (0.37±0.32 ppm) respectively, whereas the lowest average content was recorded for Treatment 3, (0.19±0.04 ppm) calcium(Ca) and (0.16±0.04 ppm) magnesium (Mg). The calcium value has a regression of R square (0.65) and magnesium of R square (0.74). The regression value is related between the average value of calcium and magnesium test and average number of larvae survived.

The ratio of calcium and magnesium for *M. rosenbergii* is important especially for larvae, as they need large amount than the adult needs. The results presented from the study carried out shows that parameters such as (pH, total dissolved solid, salinity and temperature) are equally important as the calcium and magnesium concentration. The pH, dissolved oxygen and temperature affected the most on survivability of the larvae, followed by the ratio of calcium and magnesium. The suggestion of optimum calcium to magnesium ratio for *M. rosenbergii* larvae using eggshell was Treatment 1(180Ca:300Mg) similar to Control treatment as the calcium and magnesium hardness was higher and believed to be aided survival of larvae. Eggshell powder as a calcium source for larvae is one of the way to rear *M. rosenbergii* larvae in eco-friendly way by recycling the eggshell rather than dumping it. However, this method needs to be more improvised by doing research and finding the best way to maintain the quality of the water to prevent decaying of the eggshell powder. In this study, the water treatment that contains eggshell powder needs to be changed once in a week because of the change in the colour of water. It is likely to be the decomposition process taking place. On the other hand, dolomite is kept for 2 weeks regardless the changes in the colour of the dolomite particles because it is not made up of organic waste and it is chemically

processed product which is used by most aquaculture farmers. The magnesium oxide (MgO) in water treatment was seen to be difficult for the larvae to adapt. A few trial of this study is required to prove the optimum ratio needed to carry out this rearing technique by local aquaculture farmers. A better source than magnesium oxide (MgO) should be researched before recommending and it must be easily available to farmers or research students. This is to enhance the aquaculture industry of giant freshwater prawn (*Macrobrachium rosenbrgii*) to grow constantly. Utilising wastes such as poultry eggshells can be an eco-friendly method to sustain environment.

CHAPTER 5

CONCLUSION & RECOMMENDATION

The optimum calcium to magnesium ratio that was suggested suitable for growth and survivability in this study was Control treatment and 180ppm Ca:300ppm Mg as the survival rate was almost similar. The optimum ratio was determined using the number of larvae survived throughout the days of the experiment carried out. The effectiveness of the eggshell towards *M. rosenbergii* could not be observed since only one trial was attempted and short duration of study due to most numbers of larvae were dead. The optimum ratio suggested above is not suitable for small scale farming as this experiment ended at a short period of time.

For recommendation, this study needs to be carried out several times and longer duration to determine the optimum ratio of calcium and magnesium using poultry eggshells. A significant result is important to recommend this technique to aquaculture farmers. The level of calcium and magnesium ratio varies according to the stages of *M. rosenebrgii* life cycle and also other crustacean species. The studies conducted in future should include the information for an alternative magnesium source. There are less information regarding this study using poultry eggshells in local journals. Through

certain studies a valid optimum ratio of calcium and magnesium that will be helpful for small scales farmers to culture *M. rosenbergii*. This will improve the aquaculture industry locally and gives rise economically too. A survey should be conducted to determine which source is more convenient to be used either dolomite or eggshells.

CHAPTER 6

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APPENDICES

Appendix 1 Average and SD value of water treatment parameters.

	Parameters			
Treatment	pH	Total Dissolved Solid (mg/L)	Salinity (ppt)	Temperature (°C)
Control	6.24±0.06	9978±51.50	10.77±0.22	27.84±0.37
T1	6.21±0.07	10011±153.14	10.94±0.37	27.71±0.44
T2	6.20±0.06	9935±322.99	10.86±0.95	27.88±0.54
T3	6.24±0.10	9982±225.86	10.86±0.39	27.76±0.50

Appendix 2 Parameters of water treatment.

Date	Treatment	pH	Total Dissolved Solid (mg/L)	Salinity (ppt)	Temperature (°C)
23/8/19	**C1**	**6.25**	**9849**	**10.88**	**27.75**
	C2	**3.16**	**9822**	**10.75**	**27.85**
	C3	**6.18**	**9830**	**11.15**	**27.70**
	T1a	**6.22**	**9818**	**10.85**	**27.95**
	T1b	**6.24**	**9875**	**11.07**	**27.33**
	T1c	**6.21**	**9964**	**10.99**	**27.45**
	T2a	**6.20**	**9832**	**11.16**	**27.54**
	T2b	**6.32**	**9812**	**11.18**	**27.34**
	T2c	**6.24**	**9883**	**11.21**	**27.63**
	T3a	**6.57**	**9968**	**11.17**	**27.35**
	T3b	**6.35**	**9814**	**10.92**	**27.41**
	T3c	**6.20**	**9877**	**10.31**	**27.33**

Date	Treatment	pH	Total Dissolved Solid (mg/L)	Salinity (ppt)	Temperature (°C)
26/8/19	C1	6.11	9763	10.69	28.97
	C2	6.17	9778	10.74	28.29
	C3	6.30	9761	11.03	28.33
	T1a	6.18	9753	10.58	28.54
	T1b	6.15	9795	10.97	28.19
	T1c	6.11	9712	10.23	28.63
	T2a	6.17	9724	10.85	28.91
	T2b	6.23	9757	11.01	28.59
	T2c	6.19	9743	11.14	28.80
	T3a	6.20	9781	11.01	28.63
	T3b	6.15	9778	11.11	28.65
	T3c	6.17	9763	11.03	28.54

Date	Treatment	pH	Total Dissolved Solid (mg/L)	Salinity (ppt)	Temperature (°C)
29/8/19	C1	6.11	9975	10.63	28.15
	C2	6.18	10120	10.67	27.92
	C3	6.10	9863	10.56	27.97
	T1a	6.20	9954	11.02	28.13
	T1b	6.17	10340	11.10	28.13
	T1c	6.11	9967	10.95	27.64
	T2a	6.18	9934	10.25	27.85
	T2b	6.27	9978	10.18	27.98
	T2c	6.13	10110	10.21	27.66
	T3a	6.15	9987	11.07	27.94
	T3b	6.20	9981	10.86	27.51
	T3c	6.22	9984	11.03	27.63

Date	Treatment	pH	Total Dissolved Solid (mg/L)	Salinity (ppt)	Temperature (°C)
1/9/19	C1	6.28	9857	10.42	27.57
	C2	6.31	9822	10.72	27.86
	C3	6.24	9875	11.61	27.63
	T1a	6.27	9958	11.43	27.39
	T1b	6.36	10280	12.02	27.54
	T1c	6.30	9985	11.15	27.47
	T2a	6.33	10580	11.37	27.29
	T2b	6.21	10620	11.16	27.66
	T2c	6.29	10440	10.56	27.63
	T3a	6.30	10370	10.38	27.52
	T3b	6.25	10240	11.22	27.54
	T3c	6.29	10460	11.31	27.58

Date	Treatment	pH	Total Dissolved Solid (mg/L)	Salinity (ppt)	Temperature (°C)
2/9/19	C1	6.19	9938	10.40	27.64
	C2	6.18	9821	10.69	27.57
	C3	6.11	9917	10.63	27.63
	T1a	6.22	9889	10.85	27.87
	T1b	6.15	9863	11.07	27.51
	T1c	6.17	9895	10.85	27.47
	T2a	6.12	9871	11.02	27.68
	T2b	6.18	9914	10.56	27.52
	T2c	6.24	9825	10.25	27.92
	T3a	6.21	9858	10.31	27.83
	T3b	6.11	9891	10.21	27.55
	T3c	6.18	9972	11.01	27.49

Appendix 3 Average ammonia measurement of 3 samplings

Treatment	Ammonia	Calcium and Magnesium test	
		Calcium	Magnesium
Control	2.99±2.28	0.49±0.17	0.37±0.32
T1	2.66±1.95	0.28±0.04	0.26±0.10
T2	2.60±1.57	0.36±0.04	0.24±0.21
T3	2.57±1.89	0.19±0.04	0.16±0.10

Appendix 4 Ammonia measurement of 3 samplings

Treatment	**Ammonia**		
	Day		
	4	**7**	**9**
C1	4.29	4.5	0.18
C2	4.35	4.2	0.32
C3	4.11	4.38	0.56
T1a	3.2	4.11	0.46
T1b	3.16	4.28	0.24
T1c	3.5	4.29	0.73
T2a	3.45	4.02	1.24
T2b	3.22	3.58	0.61
T2c	3.18	3.55	0.58
T3a	3.17	4.39	0.44
T3b	3.25	4.31	0.65
T3c	3.17	3.49	0.26

Appendix 5 Calcium and Magnesium test of 2 samplings

Treatment	Calcium and Magnesium test			
	Day		Day	
	7	14	7	14
	Calcium	Magnesium	Calcium	Magnesium
C1	0.29	0.21	0.18	0.23
C2	0.43	0.58	0.25	0.19
C3	0.48	0.96	0.1	0.01
T1a	0.11	0.09	0.21	0.27
T1b	0.35	0.22	0.16	0.24
T1c	0.44	0.47	0.18	0.25
T2a	0.56	0.65	0.13	0.05
T2b	0.28	0.33	0.2	0.1
T2c	0.16	0.18	0.11	0.1
T3a	0.1	0.04	0.19	0.11
T3b	0.18	0.13	0.25	0.21
T3c	0.31	0.36	0.21	0.08

Appendix 6 Number of larvae survived recorded for 16 days

Treatment	Ratio of Ca:Mg	Number of larvae survived								
		Day								
		0	2	4	6	8	10	12	14	15
C1	Control	250	248	237	225	210	154	94	18	1
C2		250	245	239	225	215	158	93	15	0
C3		250	247	238	227	212	157	91	15	1
T1a	180:300	250	247	238	227	215	155	95	16	2
T1b		250	247	237	226	213	158	92	18	1
T1c		250	246	238	227	216	155	94	18	0
T2a	240:400	250	248	235	223	213	153	94	15	1
T2b		250	245	236	225	210	150	93	12	1
T2c		250	246	237	225	208	147	90	10	0
T3a	300:500	250	245	233	220	201	149	83	11	1
T3b		250	247	229	215	200	145	85	10	0
T3c		250	245	235	214	205	148	81	10	0

Appendix 7 Number of larvae survived in each treatment and survival rate for 16 days with mean and SD value included.

Treatment	Ratio of Ca:Mg	Average number of larvae survived								
		Day								
		0	2	4	6	8	10	12	14	15
Control										
	Control	250±0	247±1.53	238±1.00	226±1.15	212±2.52	156±2.08	93±1.53	16±1.73	1±0.58
Survival rate (%)		**100.00**	**98.80**	**95.20**	**90.40**	**84.80**	**62.40**	**37.20**	**6.40**	**0.40**
T1										
	180:300	250±0	247±0.58	238±0.58	227±0.58	214±1.53	156±1.73	94±1.53	17±1.15	1±1.00
Survival rate (%)		**100.00**	**98.80**	**95.20**	**90.80**	**85.60**	**62.40**	**37.60**	**6.80**	**0.40**
T2										
	240:400	250±0	246±1.53	235±1.00	224±1.15	210±2.52	150±3.00	92±2.08	12±2.52	1±0.58
Survival rate (%)		**100.00**	**98.40**	**94.00**	**89.60**	**84.00**	**60.00**	**36.80**	**4.80**	**0.40**
T3										
	300:500	250±0	246±1.15	232±3.06	216±3.21	202±2.65	147±2.08	83±2.00	10±0.58	0±0.58
Survival rate (%)		**100.00**	**98.40**	**92.80**	**86.40**	**80.80**	**58.80**	**33.20**	**4.00**	**0.00**

Appendix 8 Adult female *M. rosenbergii* (berried female).

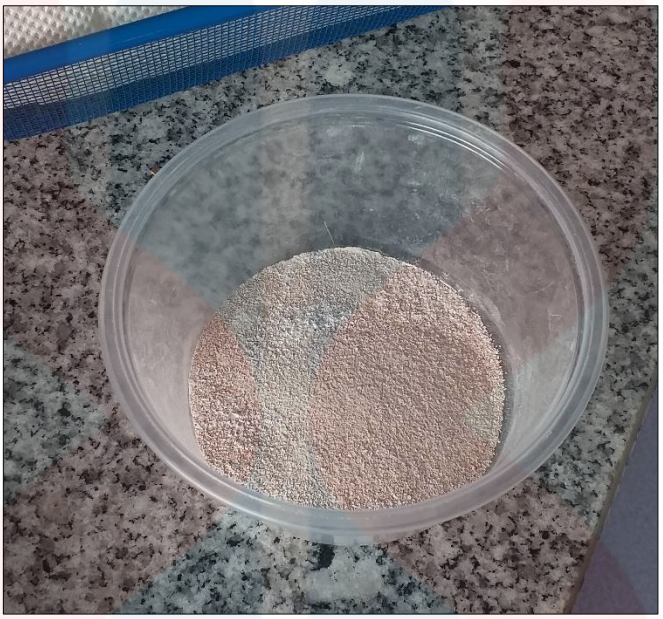
Appendix 9 *M. rosenbergi* separated into hatchery container.

Appendix 10 *M. rosenbergii* separated into hatchery container.

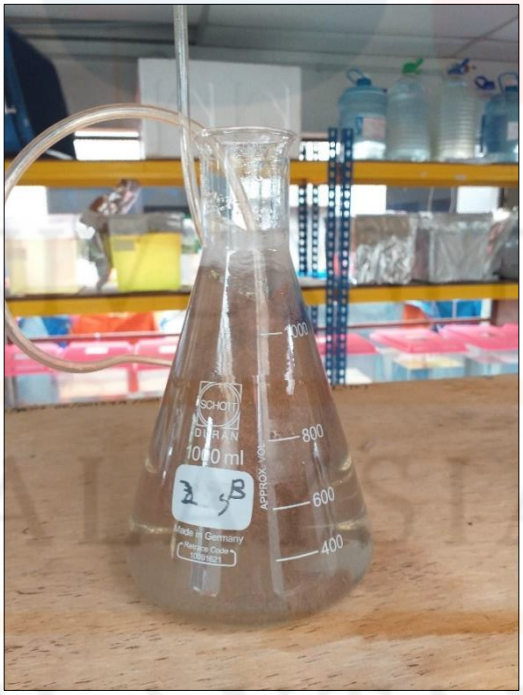
Appendix 11 Sampling (counting) of *M. rosenbergii* larvae.

Appendix 12 Preparation of egg custard.

Appendix 13 Drying process of eggshell.

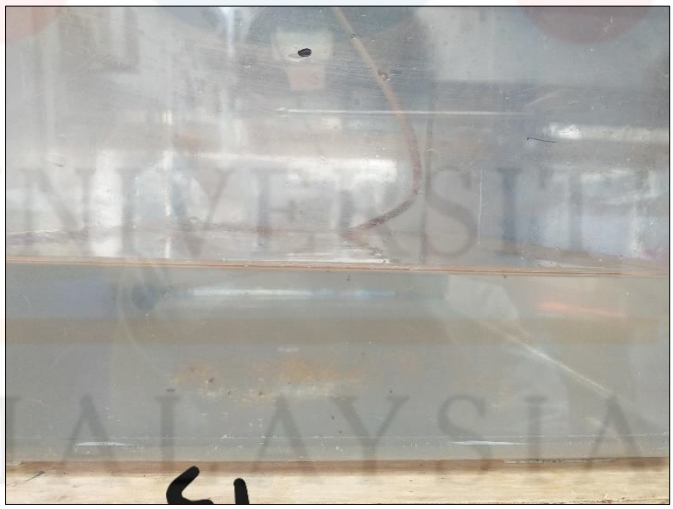


Appendix 14 Grinded form of egg shell.



Appendix 15 Preparation of *artemia nauplii*.

Appendix 16 Preparation of treatment water.

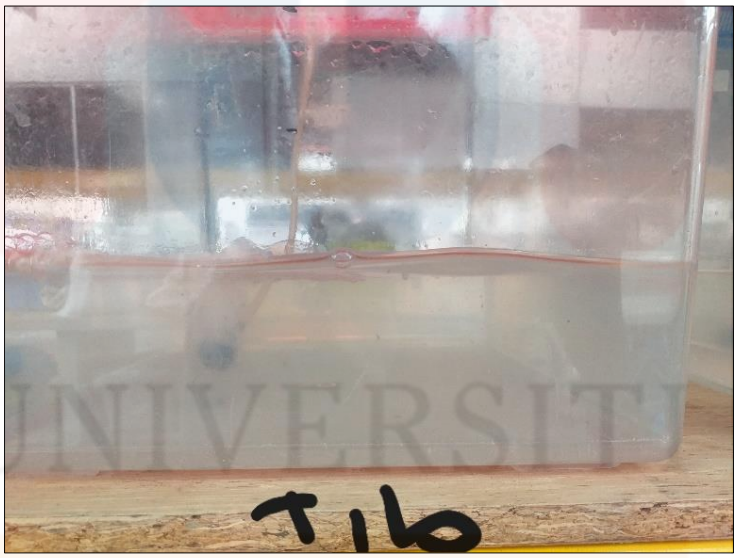


Appendix 17 Treatment (Control 1)

Appendix 18 Treatment (Control 2)

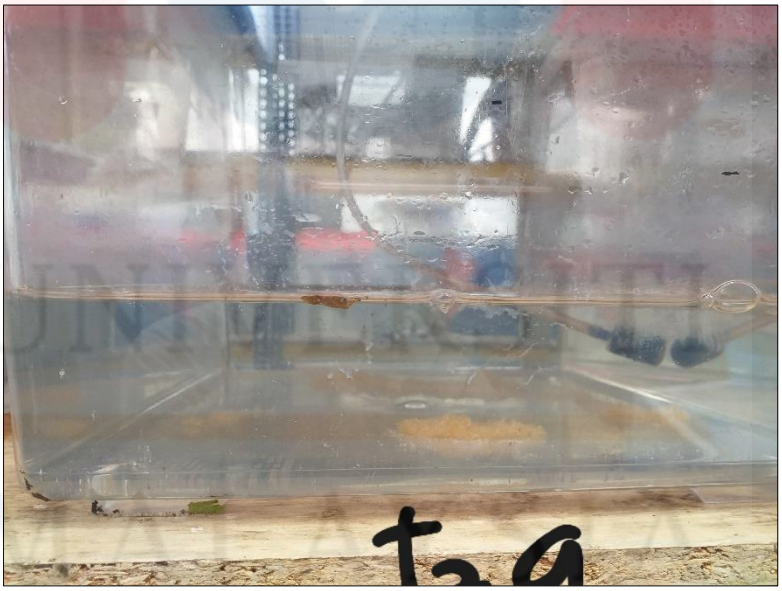
Appendix 19 Treatment (Control 3)

Appendix 20 Treatment (Treatment 1a)



Appendix 21 Treatment (Treatment 1b)

Appendix 22 Treatment (Treatment 1c)

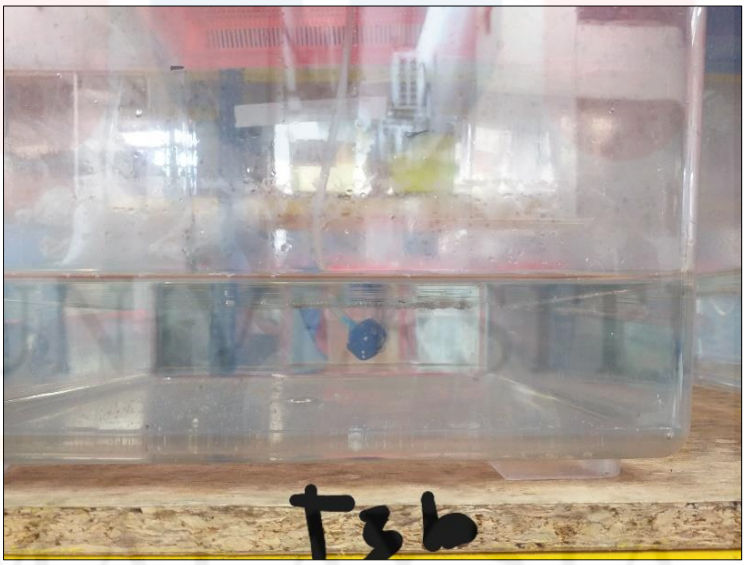


Appendix 23 Treatment (Treatment 2a)

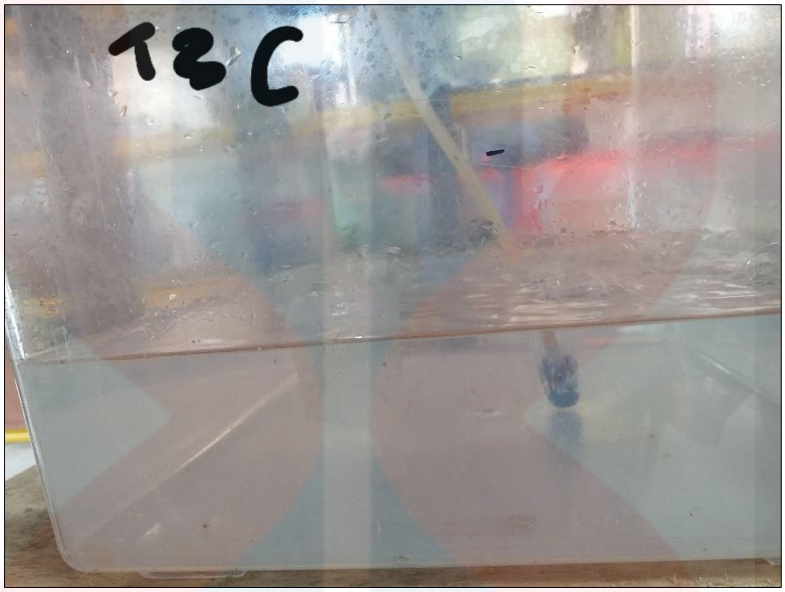
Appendix 24 Treatment (Treatment 2b)

Appendix 25 Treatment (Treatment 2c)

Appendix 26 Treatment (Treatment 3a)



Appendix 27 Treatment (Treatment 3b)



Appendix 28 Treatment (Treatment 3c)

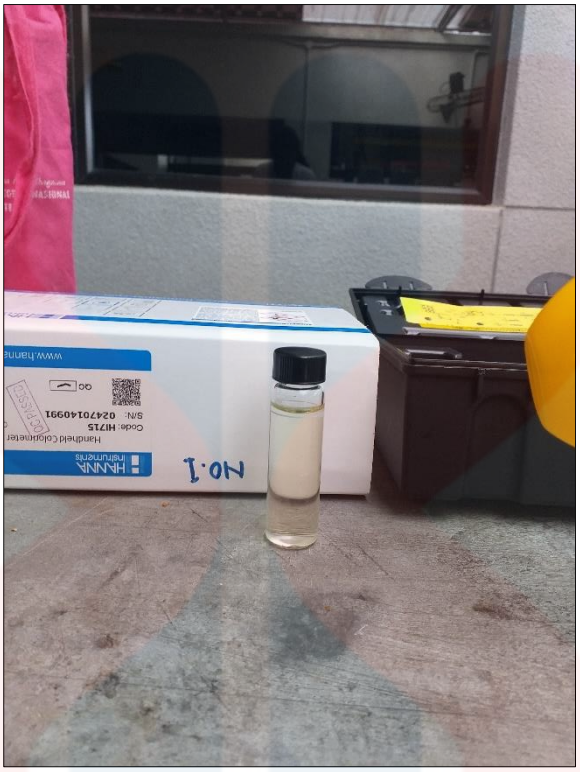
Appendix 29 Magnesium testing kit (Hanna brand).

Appendix 30 Magnesium tested for water treatment

Appendix 31 Calcium testing kit (Hanna brand)

Appendix 32 Calcium tested for water treatment

Appendix 33 Ammonia tester (Hanna brand)



Appendix 34 Ammonia tested for water treatment