School of Molecular and Life Science

Substrate Dependent Microbial Bioremediation in Freshwater Marron (*Cherax cainii* Austin 2002) Aquaculture

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DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

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Signature : Anthony J. Cole

Date : 29th of March 2019

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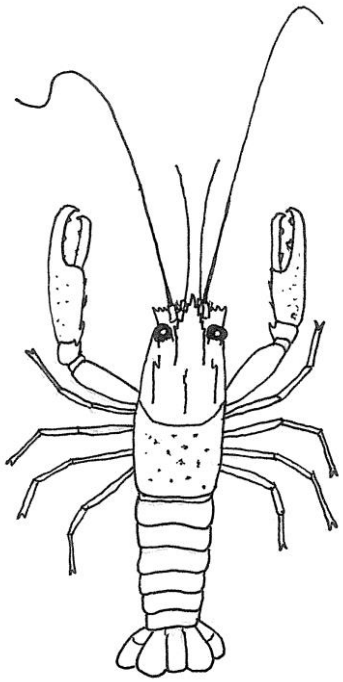

Anthony J. Cole

This thesis is dedicated to my parents, Darrel and Kathleen Cole

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
APHA	American Public Health Association
API	Aquarium Pharmaceuticals ®
BM	Bioball Media
CARL	Curtin Aquatic Research Laboratories
CFU	Colony-Forming Units
DHC	Differential haemocyte count
DO	Dissolved Oxygen
FCR	Food Conversion Ratio
Hid	Dry hepatopancreas index
Hiw	Wet hepatopancreas index
HM%	Moisture content of the hepatopancreas
HPC	Heterotrophic Plate Count
LSD	Least significant difference
N	Nitrogen
OCL	Orbital Carapace Length
P	Phosphorous
PAO	Phosphate Accumulating Organisms
RAS	Recirculating Aquaculture System
SD	Stocking Density
SGR	Standard Growth Rate
SPSS	Statistical package for the social sciences
TAN	Total Ammonia Nitrogen
Tbd	Dry tail muscle to body ratio
Tbw	Wet tail muscle to body ratio

THC	Total haemocyte count
TM%	Moisture content of tail muscle
TWC	The Water Cleanser
TWC+	The Water Cleanser Plus
TWC+B	The Water Cleanser and *Bacillus* sp.

LIST OF SPECIES NAMES USED IN THIS THESIS

Scientific Name	Common Name
Aeromonas bestarium	N/A
Aeromonas eucrenophila	N/A
Aeromonas veronii	N/A
Astacus astacus	Noble Crayfish
Astacus leptodactylus	Narrow Clawed Crayfish
Bacillus cereus	N/A
Bacillus mycoides	N/A
Bacillus subtilis	Hay/Grass Bacillus
Bacillus thuringiensis	N/A
Bacillus vietnamensis	N/A
Bidyanus bidyanus	Silver Perch
Carassius auratus	Common goldfish
Cherax cainii	Smooth Marron
(Formerly *Cherax tenuimanus*)	
Cherax tenuimanus	Hairy Marron
Cherax destructor / albidus	Yabby
Cherax quadricarinatus	Redclaw Crayfish
Skistodiaptomus pallidus	Calanoid Copepod
(Formerly *Diaptomus pallidus*)	
Gadus morhua	Atlantic Cod
Ictalurus punctatus	Channel Catfish
Lates calcarifer	Barramundi
Lemna minor	Duckweed
Macrobrachium rosenbergii	Giant Freshwater Prawn

Oncorhyncus mykiss	Rainbow Trout
Oreochromis niloticus	Nile Tilapia
Pacifastacus leniusculus	Signal Crayfish
Penaeus brasiliensis	Red Spotted Shrimp
(Formerly *Farfantapenaeus brasiliensis*)	
Penaeus indicus	Indian White Shrimp
(Formerly *Fenneropenaeus indicus*)	
Penaeus japonicus	Kuruma Prawn
(Formerly *Marsupenaeus japonicus*)	
Penaeus latisulcatus	Western King Prawn
Penaeus paulensis	Pink Shrimp
Penaeus monodon	Tiger Prawn
Penaeus vannamei	Pacific White Shrimp / Whiteleg Shrimp
(Formerly *Litopenaeus vannamei*)	
Pinctada maxima	Pearl Oyster
Pseudomonas aeruginosa	N/A
Pseudomonas anguilliseptica	N/A
Pseudomonas brassicacearum	N/A
Pseudomonas cedrina	N/A
Pseudomonas synxantha	N/A
Pseudomonas extremorientalis	N/A
Procambarus clarkii	Red Swamp Crayfish
Rheineimera soli	N/A
Salmo salar	Atlantic Salmon
Thunnus maccoyii	Southern Bluefin Tuna
Vibrio mimicus	N/A

ABSTRACT

Aquaculture is an important source of cheap protein and employment, especially with the decline of wild fish stocks and increasing demand for seafood. With limited land and water resources intensification of aquaculture is sometimes necessary, however this can negatively affect water quality and the quality of produce. The maintenance of good water quality is essential in all types of aquaculture, including that of marron (*Cherax cainii* Austin 2002). One solution to improve water quality of the culture environment and to reduce water use is bioremediation by using certain substrates that function by promoting the growth of biofilm, containing heterotrophic bacteria. This research investigated the role of oil-based substrates, The Water Cleanser™ (TWC and TWC+), and associated microbes, in the bioremediation of water in marron culture.

Two indoor laboratory experiments, an outdoor laboratory experiment and a field experiment were conducted to investigate the effects of TWC, Bioball Media, and probiotics on water quality, plankton communities, bacterial communities, marron health and productivity. TWC significantly reduced the concentration of orthophosphate in all trials. TWC and TWC+ reduced the concentrations of ammonia, nitrite and nitrate under indoor laboratory conditions, while Bioball Media reduced the concentration of nitrate only. A combination of TWC and *Bacillus* sp. was effective in bioremediation in outdoor laboratory conditions, and increased the abundance of phytoplankton. TWC had no effect on the bacterial abundance in the water column in any experiment, however commercial marron ponds with TWC resulted in a higher abundance of *Bacillus* sp. Ponds with TWC also had a significantly higher final biomass of juvenile marron. No negative effects were found on plankton communities. Meanwhile, pond age had a significant effect on water quality and natural productivity. There were no adverse effects of TWC or probiotics on the haemolymph indices (THC, DHC) or condition indices of marron. The results of this study suggest that TWC can provide habitat for heterotrophic bacteria, which maintain lower concentrations of ammonia, nitrite, nitrate and orthophosphate in aquaculture conditions. TWC also affects the bacterial composition of culture water, by promoting certain species (e.g. *Bacillus* sp.). Further research is required to assess the microbial composition on TWC surface, to better understand the mechanisms by which TWC functions, and to further determine the benefits of TWC on the health and survival rate of marron.

Chapter 1: Introduction

*This thesis presents an investigation into substrate dependent microbial bioremediation of marron (*Cherax cainii *Austin 2002) aquaculture. Substrates can have significant benefits for aquatic systems, including the improvement of water quality, and the maintenance of good water quality is essential to all forms of aquaculture. The thesis includes seven chapters, those concerning experiments are described in Figure 1.1.*

Chapter 1 presents an introduction to the thesis. This provides an overview of the main aspects of the research, as well as the rational, aim, objectives and significance.

1.1 Introduction

Aquaculture is simply the practice of farming aquatic plants and animals in water. This includes freshwater culture, and mariculture, among other forms. Aquaculture is an important industry as a source of income, fish protein and for food security (Martinez-Porchas & Martinez-Cordova 2012), in part due to declining fish stocks and increasing demand. Aquaculture may help to relieve pressure on fish stocks, though there is some controversy over this, as aquaculture often sources feeds from wild fish stocks, and can have a detrimental impact on the environment (Naylor et al. 2000; Cabello et al. 2016; Alonso 2009). The aquaculture of marron (*Cherax cainii* Austin 2002) has a lower impact on the environment than more intensive forms of aquaculture, and is considered sustainable. However, water quality is still an important issue and intensification of aquaculture may require new methods, such as the use of substrates for bioremediation, to maintain good water quality and good marron productivity and health.

Marron is a freshwater crayfish species native to, and successfully cultured in, Western Australia. Marron aquaculture is considered a sustainable practice; which has minimal water use, low environmental impact, and is mostly free of diseases (Alonso 2009). However, if the industry is to expand more intensive aquaculture may be required, with repercussions for the water quality within ponds, the ecology of ponds, the quality of marron, and the surrounding environment. Maintaining good water quality is essential in marron culture, as marron are adversely affected by changes in water quality (Ackerfors 2000). Elevated levels of ammonia and/or nitrite can cause physiological problems, growth inhibition, and mortality in freshwater crayfish (Jensen 1996; Jussila 1997[a], Harris et al. 2001). High levels of nitrogen and phosphorous lead to eutrophication. One solution to improve water quality and ecology of aquatic systems is bioremediation. This process commonly uses probiotics, such as *Bacillus* sp., microalgae or macroalgae to improve water quality and aquatic ecology (Lananan et al. 2014; Chávez-Crooker & Obreque-Contreras 2010; Perumal et al. 2015; Ardiansyah & Fotedar 2016[a]; Abdel-Tawwab 2008; Samocha et al. 2015). Artificial substrates can also be used, which encourage the growth of bacteria-containing biofilm, and periphyton (Viau et al. 2012). These can be effective in improving water quality (Schveitzer et al. 2013), while also providing a complementary food source for certain species, including *Cherax* species (Jones et al. 2002; Viau et al. 2012).

One potential tool for the bioremediation of aquaculture is The Water Cleanser™ (TWC), which is an oil and wax based substrate for microbial growth, and provides habitat and a carbon source for heterotrophic bacteria. Heterotrophic bacteria require a carbon source in order to function; and to convert nitrates to nitrogen gas via nitrite (Hamlin et al. 2008). Bacteria can enhance the nutrient cycling of an aquatic system, increasing the rates of nitrification and denitrification processes and cycling of phosphates from organic matter to dissolved particles and to and from sediments (Moriarty 1997). This could aid in the bioremediation process by removing excess organic matter, nitrogenous wastes and biologically available phosphates (orthophosphates) from water. Alternatively, the enhanced nutrient cycling may improve natural productivity by releasing nutrients from organic matter into usable forms for phytoplankton. TWC is thought to promote bacterial and biofilm growth in aquatic systems; which may in turn improve water quality and limit excessive build-up of organic matter and organic wastes. TWC may improve the health of marron and the surrounding aquatic environment, while also providing an additional food source as attached biofilm. Increased understanding of bioremediation using substrates may also help to improve other forms of aquaculture. Limited research has been conducted using TWC in aquaculture systems, therefore further research is needed to better understand microbial bioremediation using TWC in aquaculture.

1.2 Aims and objectives

This study aims to increase understanding of microbial bioremediation using TWC substrate, to identify the role of bioremediation in marron (*Cherax cainii*) aquaculture systems, and to investigate the effects on microbial ecology, natural productivity, and on marron productivity and health. The flow of this thesis in relation to this is illustrated in Figure 1.1.

The thesis sets out to address the aim above through the following specific objectives:

1. To evaluate the effects of substrates on bacterial abundance and diversity of colony types under commercial and laboratory conditions of marron aquaculture.

2. To evaluate the effects of substrates and beneficial bacteria on water quality, natural and marron productivity and marron health under commercial and laboratory conditions.

3. To assess the impact of substrates on water quality, bacterial abundance, and marron productivity and health at different stocking densities under laboratory conditions.

4. To study the relationships between water quality, natural productivity, bacterial abundance, diversity of colony types, and marron productivity under commercial and laboratory conditions.

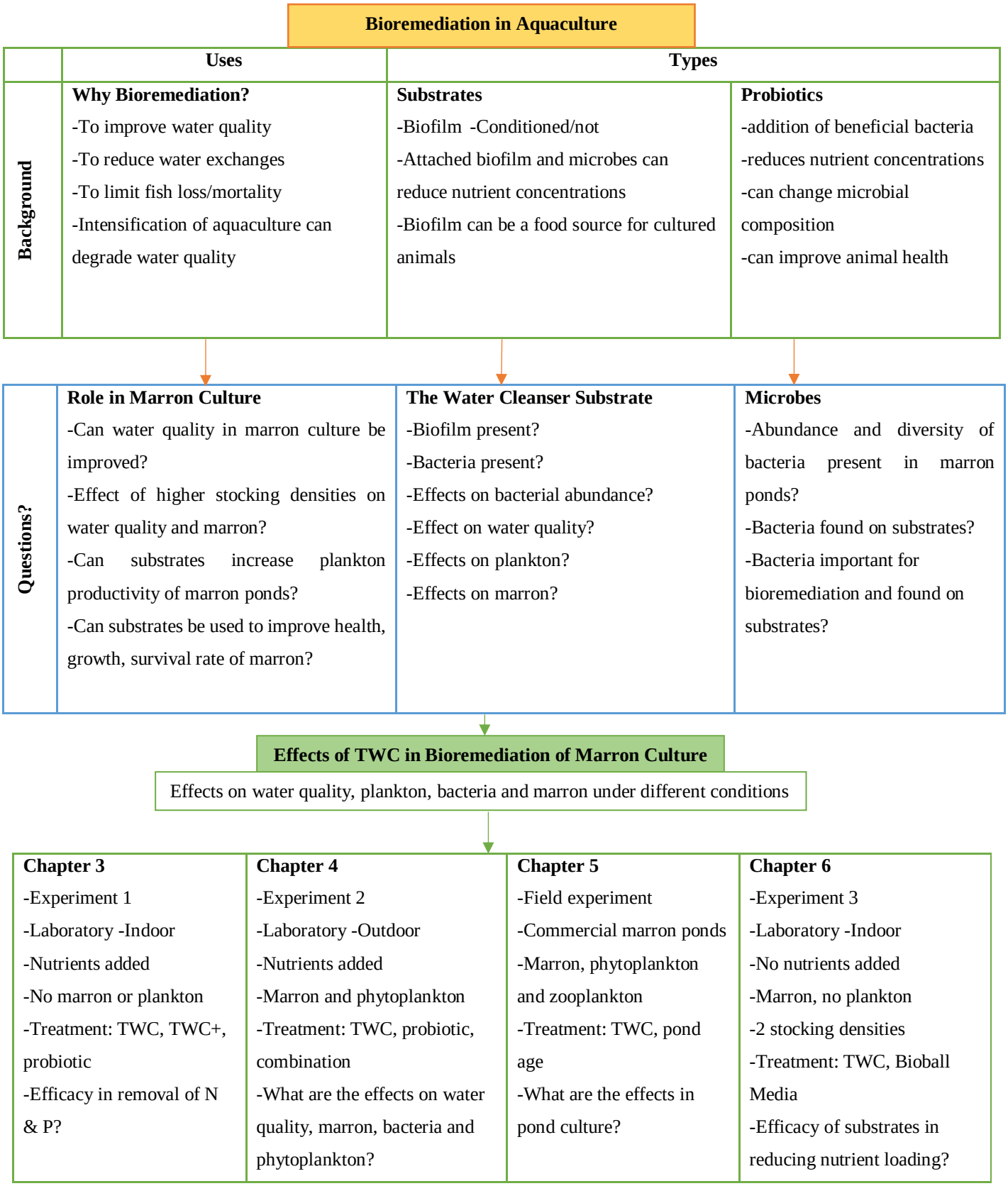

Figure 1.1: Conceptual diagram outlining the main themes of the study (orange), the main questions this study investigates (blue), and the research topic and the experiments carried out, with the conditions under which each study was done (green).

1.3 Significance

There is a growing need to improve water quality, reduce water usage, improve environmental conditions and increase productivity in aquaculture systems (Zhou et al. 2009; Samocha et al. 2002; Romano & Zeng 2012; Morrissy 1979). Sustainability is becoming increasingly important in aquaculture (Naylor et al. 2000), and this can be achieved in part by reducing inputs, such as water use (Samocha et al. 2002), and improving water quality in water bodies and effluent (Hamlin et al. 2008). Improving the microbiome of aquaculture systems via the addition of probiotics or substrates may provide a simple solution to this growing problem. Substrates can promote growth of bacteria and biofilm, which can have multiple benefits for the water quality, ecology and overall health of aquatic systems and the health of cultured species (Viau et al. 2012; Thompson et al. 2002; Santhana Kumar et al. 2017; Batvold & Browdy 2001). This research will help to increase understanding of the use of substrates in microbial bioremediation, and add to the current knowledge about bioremediation in marron aquaculture. The study may also help determine uses for substrates in marron culture and other forms of aquaculture; improving productivity, health of the environment and cultured animals, and increasing their farming sustainability.

Chapter 2: Literature Review

Chapter 2 presents a review of the literature of the main aspects of aquaculture and bioremediation discussed in this thesis. Aquaculture in general is discussed, as well as that of Australia, freshwater crayfish and specifically marron. The issues surrounding water quality and the need for bioremediation are presented, as well as the marron biology and the role of natural productivity in marron culture; relating to Chapters 3, 4 & 5. The effects of stocking density are discussed, relating to Chapter 6. An overall review of microbes in aquaculture, and the use of probiotics and substrates is given, as well as a brief background to the oil-based substrate, The Water Cleanser (TWC), investigated in this thesis, and its potential benefits for marron aquaculture.

2.1 Aquaculture

2.1.1 World Aquaculture

Aquaculture is an important industry in terms of food security and supplying seafood where fisheries alone cannot meet demand. Aquaculture has brought substantial benefits to humanity; including food produced, the nutritional quality of aquatic products, as a source of employment, and for the high trade potential of aquaculture products (Martinez-Porchas & Martinez-Cordova 2012; Martinez-Porchas et al. 2014; Bostock et al. 2010). This is increasingly become an important source of income and fish protein, with the decline of fish stocks, and increasing demand for seafood. Almost half of the global production of food fish is produced by fish farming (Nadarajah & Flaaten 2017; Perumal et al. 2015). In 2014, 44.1% of seafood was contributed by aquaculture (FAO 2016). Asia is the largest producer of farmed fishes, largely due to the favourable tropical climate conditions, and availability of suitable sites and natural water resources (Perumal et al. 2015).

Aquaculture is also one of the world's fastest growing food sectors, with an annual growth of approximately 4.7% (Perumal et al. 2015). Between 1987 and 1997 global production of farmed fish and shellfish more than doubled (FAO 1999). Aquaculture can have adverse impacts on the environment and certain fish stocks however (Naylor et al. 2000; Cabello et al. 2016; Alonso 2009) and needs to be more sustainable. There is increasing need to develop more sustainable aquaculture, with less reliance on fishmeal sourced from fisheries, excessive water use, and antibiotics. As aquaculture becomes more intensive, the high densities of animals and high inputs needed to achieve good production can cause problems in terms of water usage, water quality and health. Fish cages for example are known to increase organic matter on the bottom sediments (Holmer 1991; Karakassis et al. 1998), and cause the progressive transformation of sediments into an anoxic surface (Danovaro et al. 2003). Bioremediation can be used to improve the water quality and cleanliness of aquatic systems, and provides an alternative to filtration and water exchanges. Bioremediation may also improve the environmental health of aquatic systems. Macrophytes can be used to reduce nutrient concentrations (Abdel-Tawwab 2008; Samocha et al. 2015; Ferdoushi et al. 2008). Microbial bioremediation may be applicable to aquaculture where nutrient levels are excessive or where the environmental and ecological health

can be improved. This may also improve carrying capacity of aquaculture ponds; increasing the stocking rate of animals without impacting on growth or water quality.

Water resources are limited, therefore new systems such as recirculating aquaculture systems and biofloc are being used to limit water use without compromising on water quality (Ardiansyah & Fotedar 2016[a]; Ahmad et al. 2017; Ray et al. 2010). Meanwhile, heavy use of antibiotics has been used, particularly in shrimp culture, leading to environmental problems and growing antibiotic resistance in pathogens (Cabello et al. 2016). This has led to an increase in probiotics use in aquaculture (Balcázar et al. 2006). The use of artificial substrates, such as the Water Cleanser™ (TWC), Aquamat™, Bioballs®, bamboo or ethylene substrates, is an alternate solution which can provide a habitat for probiotic bacteria and biofilm which in turn may promote the health and growth of cultured species and help control water quality (Schveitzer et al. 2013; Thompson et al. 2002; Viau et al. 2012).

Research into bioremediation in aquaculture is largely focused on probiotics, with the use of artificial substrates in bioremediation slowly becoming more common. Limited bioremediation research has been carried out in freshwater crayfish culture. Crayfish culture is present in Australia, largely due to the several native species that are suitable for aquaculture there.

2.1.2 Australian Aquaculture

In Australia, the majority of aquaculture production is limited to five main types; Pearl Oysters (*Pinctada maxima*), Edible Oysters (various species), Atlantic Salmon (*Salmo salar*), shrimp/prawns (various species) and Southern Bluefin Tuna (*Thunnus maccoyii*) (ABARE 2003). Cage culture is common for marine fishes, while pond culture is more widely used for shrimp and freshwater species. Aquaculture in Australia involves producing high value products and fishes, often for export. There is potential for aquaculture expansion in Australia, especially in marine and brackish waters as freshwater is somewhat limited. Presently, mariculture is more common, though freshwater culture is practiced. Freshwater aquaculture species farmed include rainbow trout (*Oncorhyncus mykiss)*, silver perch (*Bidyanus bidyanus*), Barramundi (*Lates calcarifer*) and freshwater crayfish (*Cherax* sp.). There was an estimated production of 400 tonnes from crayfish aquaculture in Australia in 1999 (Ackerfors 2000).

2.1.3 Freshwater Crayfish Aquaculture

Worldwide crayfish aquaculture includes cambarid culture in North America and China, and astacid culture in Europe (Huner et al. 1988; Ding et al. 2012). The culture of crayfish is sometimes referred to as astaciculture. There are two main families of crayfish cultured outside of Australia; Cambarids and Astacids. Cambarids such as *Procambarus clarkii* are fast growing and found in warm waters, whereas most astacids such as *Astacus astacus* and *Pacifastacus leniusculus* are slower growing and inhabit cooler temperate waters.

In Australia three main crayfish species are cultured, all in the genus *Cherax* (Decapoda: Parastacidae). In northern Australia Redclaw Crayfish (*Cherax quadricarinatus)* is cultured, in warm subtropical ponds, with a fast growth rate. In Eastern Australia the Yabby (*Cherax destructor / Cherax albidus*) is cultured. Marron (*Cherax cainii* Austin 2002), formerly *Cherax tenuimanus* (Smith 1912), are a freshwater crayfish species native to south Western Australia (Figure 2.1) and a target of aquaculture there. They are also cultured in southern Africa. They are larger than the yabby (*Cherax destructor*) and redclaw (*C. quadricarinatus*), but can be relatively slow growing. They are the third largest species of crayfish in the world and are capable of reaching 2kg in weight (Holdich 2002; Morrissy 2000), though market size is approximately 70 to 200 g. Unlike yabbies and other crayfish, marron are poor burrowers and can only survive in permanent waters such as lakes and rivers in nature, such as the Blackwood and Margaret River. The cultured marron *C. cainii*, known as 'smooth marron', were formerly known as *C. tenuimanus,* which are now referred to as 'hairy marron'. Marron are a decapod crustacean in the family Parastacidae; the main family of crayfish in Australia and the southern hemisphere. There is great diversity of freshwater crayfish in Australia; there are nine endemic genera of crayfish found in Australia, though *Cherax* and *Euastacus* are the more widespread genera (Beatty et al. 2013). In Western Australia, parastacid crayfish are restricted to the southwest coast drainage division, and do not occur naturally in the Pilbara or Kimberley areas.

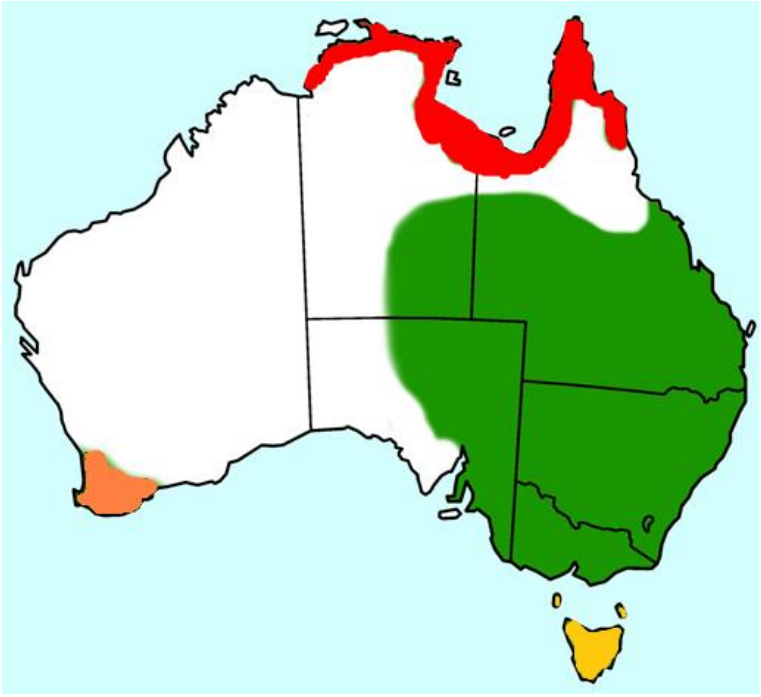

Figure 2.1: *Cherax* distribution in Australia. Marron are found in the south-west (orange), redclaw in the north (red) and yabbies in the east (green). Various crayfish species are endemic to Tasmania (yellow), but are not commercially cultured.

Marron are cultured in Australia extensively and semi-intensive, often in farm dams or ponds, in integrated farming. The majority of marron farms in W.A. are run as an additional or marginal activity (Alonso 2009). Marron are grown with artificial shelters and supplementary feeding, often with intermittent aeration depending on stocking rates of ponds. Shelters are essential in order to minimise cannibalism, allow for partial harvesting, and to provide refuge from predators such as birds, with netting providing additional protection. Marron farming involves little or no water exchange, with water only being added or removed as necessary to compensate for rainfall and evaporation. This approach makes up for a scarcity of water resources, and is environmentally sustainable (Alonso 2009). There may be differences between old and new ponds, as per construction time and sediment. Newer ponds may take time to become 'established', increase nutrient concentration, and develop microbial and invertebrate populations (Allan et al. 1995; Correia et al. 2002).

There is considerable market potential for marron (Morrissy 1979) and considerable interest in freshwater crayfish culture (Rouse & Kartamulia 1992). The marron species

has many advantages for culture, such as having a relatively large size and a simple life cycle with no larval stage. Marron breed naturally in ponds and dams, and are brooders. Reproduction generally begins in spring when the water temperature and day length begin to increase. They are the largest cultured freshwater crayfish in the world. Marron production has been stable for the past few decades at close to 50 tonnes/annum (ABARE 2003). Marron are generally considered a luxury food highly respected by chefs, and yield high prices. The tail muscle to body ratio and the meat yield are quite high when compared to other crayfish, and compares favourably with marine rock lobsters (Holdich 2002). Similarly to other *Cherax* species, Marron are also an important component of freshwater ecosystems in Australia.

2.2 Marron Biology

2.2.1 Taxonomy and Distribution

Freshwater crayfish are considered monophyletic in origin (Holdich 2002), and are native to every continent except Africa and Antarctica, though crayfish are native to Madagascar. Parastacids occur in New Guinea, New Zealand, Madagascar, South America, and Australia. The family Parastacidae comprises 14 genera and approximately 139 species (Holdich 2002). *Cherax* and *Euastacus* are the more widespread genera (Beatty et al. 2013). The natural distribution of *Cherax cainii* has been extended as far north as the Hutt River, north of Geraldton, and as far east as Esperance. Marron (*Cherax cainii*) are identified by their dark colour, narrow pincer-like chelipeds, five keels on the dorsal surface of the carapace, and two small spines on the telson. The systematic classification of marron is as follows:

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Suborder: Pleocyemata

Infraorder: Astacidea

Family: Parastacidae

Genus: *Cherax*

Species: *Cherax cainii* (Austin 2002)

2.2.2 Biology and Physiology

Marron are primarily detrivores, but are polytrophic, occupying various part of the trophic web. They can consume microbially-enriched detritus, phytoplankton, including diatoms, zooplankton, macrophytes, and other plant matter and animal matter. Marron have been found to be predatory in part, consuming small fish as part of their diet (Duffy et al. 2010). Cannibalism is also common in marron culture. In south-western rivers they are usually the dominant species due to their large biomass. They can fall prey to terrestrial birds and other animals, however, including finfish, cormorants, water rats and tortoises (Tay et al. 2007). Due to the predation by finfish polyculture is uncommon. Marron comprise an important part of aquatic ecosystems by converting plant and algal matter into consumable animal protein, and by occupying a key role in the ecosystem as both predator and prey. Juveniles are generally more carnivorous than adults, consuming more zooplankton. Shelters are important for juvenile crayfish for reducing cannibalism and providing a refuge (Jones et al. 2002).

The life cycle of marron is fairly simple and easily managed. Spawning occurs in late winter to early spring, with temperature being a probable stimulus for spawning. Reproduction generally begins when the water temperature and day length begin to increase. Females incubate from 100 to 300 berries attached to pleopods under the tail, which then hatch with young remaining attached for 3 stages before being released as juveniles. As marron are brooders they are easier to culture than marine crustaceans such as lobsters, which go through several larval stages.

The physiology of marron is similar to that of other decapod crustaceans. Haemolymph is the transport medium of the decapod crustaceans' open circulatory system. The system includes various types of haemocytes, similar to blood cells. The three main types are known as granulocytes, semi-granulocytes and hyalinocytes, which differ in morphology and function.

The number of haemocytes and the proportion of granulocytes are considered indicators of stress and health of crustaceans (Jussila 1997[b]). The total haemocyte

count (THC), essentially measuring the production or release of haemocytes into the haemolymph, is one of the most important immunological responses for crustaceans (Romano 2012). The haemocytes of several species of crustaceans can be affected by factors such as temperature, pH, salinity, dissolved oxygen and ammonia (Sang et al. 2009).

The hepatopancreas is an important organ in crayfish which acts as both a pancreas and a liver, and as a store of energy and nutrients. Hepatopancreas size and moisture concentration can be used as condition indices (Jussila 1997[b]). Changes in size and moisture content of the hepatopancreas can be affected by diet, and also indicate condition of crustaceans. The hepatopancreas can detoxify foreign compounds in the haemolymph, and store calcium and other nutrients required for moulting (Jussila 1997[b]). The organosomatic indices of the tail muscle can also be used as a monitor of physiological condition in crustaceans (Prangnell & Fotedar 2006). As the hepatopancreas and tail muscle are used as energy stores in marron, their composition is often related to the nutrition of marron.

Growth of marron is largely dependent on temperature, with optimum temperatures for marron at approximately 15-20°C, but they can be higher at 24°C, while the minimum temperature for growth is at 11-13°C (Morrissy 1990). At temperatures above 27°C survival can be affected (Rouse & Kartamulia 1992), while temperature below 13°C can inhibit growth (Morrissy 1990). As marron inhabit cool waters they are relatively slow growing compared to tropical species, such as redclaw. From the effects of seasonal temperature change growth is not constant year round, and can be very slow between April and August. Growth of arthropods, including crayfish, occurs via moulting. Similarly to other temperate crayfish (Holdich 2002), growth is generally seasonal, where moulting may slow from April to August (winter) and increase from spring through to autumn. Moulting is also limited by reproduction in females, as berried females cannot moult. Temperature is the main limiting factor for growth in colder months, while in warmer months other factors are limiting such as food availability and photoperiod. Growth of crayfish may be suppressed in laboratory conditions due to limited space, poor water quality and/or an entirely artificial diet (Geddes et al. 1988). Fed only an artificial diet moulting frequency may be depressed compared to marron fed a natural diet (Morrissy et al. 1984), while some loss of exoskeleton pigmentation may also occur, presumably because of lack of carotenoids

(Sommer et al. 1991). Other factors such as water quality, and concentrations of important trace elements such as calcium may also be limiting. Nitrogenous wastes ammonia and nitrite can have lethal and sublethal effects on crustaceans, and may also impede growth.

2.2.3 Water Quality

Marron are adversely affected by changes in water quality (Ackerfors 2000), for example parasites and surface fouling such as *Epistylis* can occur on marron due to poor water quality and excessive organic matter build up (Ambas et al. 2013). *Epistylis* is a protozoan common in crayfish aquaculture that can restrict water flow to the gills, causing asphyxiation, and also decreases value. The presence of *Epistylis* is an indicator of high organic load or excessive organic matter build up, low oxygen levels and poor water quality in dams (Department of Fisheries 2012; Ambas et al. 2013). Another common parasite is *Temnocephala,* a flatworm that can found as eggs on the tail underside. They pose no health risk, however. Disease is not currently a serious issue in marron aquaculture, and though surface fouling decreases market value it does not have lethal effects. Aeration is recommended for moderate to high densities to improve water quality and prevent anoxic conditions. Turbidity is an important measure in aquaculture, as it relates to microalgae concentrations (Gamboa-Delgado 2014), which can be an important food source and source of primary productivity. Turbidity can affect photosynthesis at the benthic level, while high concentrations of phytoplankton may increase the turbidity. Clear water may increase vulnerability of crayfish to predation by birds such as cormorants. Turbid ponds encourage daytime feeding and provide some protection from predators. This also doesn't impair their feeding abilities as crayfish rely on chemo-sensitivity. When turbidity is too high though benthic natural production will decrease due to lack of light penetration and primary production will be limited.

Toxic ammonia and nitrite can cause physiological problems in crayfish (Jensen 1996; Harris et al. 2001), though the levels are generally low in semi-intensive pond culture. Ammonia from excretion and other sources can be transformed into toxic unionised ammonia, or oxidised into nitrite; which is then oxidised into nitrate. High ammonia, nitrite and nitrate levels can occur in intensive culture, which may be a problem for health of crayfish and may inhibit growth (Jussila & Evans 1996). Inclusion in plant

protein diets has been shown to increase unionised ammonia levels in ponds culture, which has been attributed to lower survival (Fotedar 2004). High pH levels can also increase the amount of ammonia in the toxic unionised form. The pH of water in aquaculture is of key importance. The pH is considered to be important to freshwater productivity in general. Though the effects of acidification in aquatic systems and aquaculture systems containing marron is not well studied, a neutral to slightly alkaline pH is thought to be optimal for marron. Crayfish species are thought to be negatively affected at low pH, below 5.6 (Holdich 2002). In acidic waters calcium and oxygen levels in crayfish can be difficult to maintain (Ackerfors 2000). Acidified systems may inhibit reproduction. Meanwhile, excessive algal blooms can cause very high pH levels.

In tank culture ammonia and nitrite can be more problematic, while in pond culture high nitrate and phosphate levels can trigger algal blooms and filamentous algae growth (Blaas & Kroeze 2016). Eutrophication can have direct and indirect negative effects on crayfish. In natural systems, runoff from fertilizers in agriculture, and from stormwater drains can cause excess build-up of nutrients. In aquaculture runoff, excessive feeding, fertilization and overstocking can cause this. There are a host of problems caused by eutrophication including decrease in oxygen concentration as algal and bacterial blooms consume more oxygen, and toxic cyanobacteria blooms. Eutrophication can lead to high biological oxygen demand (BOD) and low dissolved oxygen in ponds, especially in summer (Huner 1994). Marron are sensitive to low oxygen levels and hypoxia brought about by excessive feeding (Morrissy 1984). Overloading of a pond with organic matter may lead to low survival due to oxygen depletion (Morrissy 1979), and may increase turbidity. Build-up of organic matter in sediment can create anaerobic conditions and slowly release nutrients and toxic substances such as ammonia, nitrite and hydrogen sulphide. The main sources of organic matter are uneaten feed, senescence of microalgae and faeces (Li & Boyd 2016). In systems with algal blooms low oxygen levels may be found in the morning, while algal die-off will further deplete oxygen levels. Meanwhile, crayfish can consume toxic blue-green algae, but consumption for an extended period of time may cause ill effects and may even be lethal. Filamentous algae can also grow to ‘choke’ eutrophic ponds. A stable, green algae bloom can be beneficial to natural productivity and in supporting natural food sources for crayfish, however. Ponds with higher

stocking rates, such as intensive systems, may increase eutrophication and impede on water quality.

2.2.4 Stocking Density in Crayfish Culture

There are many studies concerning crayfish and stocking density (Mazlum 2007; Fotedar et al. 1999; García-Ulloa et al. 2012; Jones & Ruscoe 2000; Mills & McCloud 1983). Most studies have focused on juvenile crayfish, which are generally more susceptible to mortality. Several studies have also found that size at stocking and stocking density can greatly affect production (Jones & Ruscoe 2000; Geddes & Smallridge 1993). Mazlum (2007) found that narrow-clawed crayfish (*Astacus leptodactylus*) juveniles were negatively affected by increased stocking density in terms of growth in both length and weight. Similarly, Mills & McCloud (1983) found greater growth of yabbies (*Cherax destructor)* at the lower stocking density of $10/m^2$. Geddes et al. (1988) found that *C. destructor* growth was significantly depressed in small crayfish held communally at a higher density. Growth rate of freshwater crayfish has been found to be inversely related to density (Verhoef & Austin 1999).

In aquaculture ponds, carrying capacity is often exceeded to that of a natural system. Carrying capacity is generally defined as the maximum population or biomass that can be supported sustainably, given resource constraints and other physical and biological factors (Acou et al. 2011). When carrying capacity is exceeded physical factors such as water quality may be negatively affected, and in turn growth and health of animals in the water body may be impaired. Higher stocking density may lead to stunted growth, higher levels of wastes and nutrients, lower oxygen levels, and higher mortality rates. In crayfish culture higher stocking densities can also lead to increased fighting among crayfish, cheliped damage, lower production, and lower survival rates (Mazlum 2007). In intensive culture, using individual cages may improve survival and decrease cheliped damage. Overcrowding influences oxygen, water quality, food availability, competition and negative social interaction (García-Ulloa et al. 2012). Crowding may also increase stress in crayfish. Marron are susceptible to low oxygen levels, and may require aeration for intensive aquaculture (Morrissy 1979). Various methods may be adopted to improve carrying capacity of systems. In prawn (*Macrobracium rosenbergii)* culture, carrying capacity may be improved by adding substrate; increasing growth and overall size of prawns (Tidwell et al. 1998). In marron

culture, hides or shelters are commonly used, whereas substrate may be used for detrital food formation or for microbial colonisation (Morrissy 1979; Jones & Ruscoe 2000). Using artificial substrates may also improve water quality, allowing for higher stocking densities without impacting on growth and health of animals. Higher stocking rates of animals can lead to better utilization of space, labour and water, and greater production per unit of space. The substrates present in this study provide no habitat or refuge for marron however.

In Chapter 5 of this thesis, containers have been used to house marron. Research has suggested a method for determining the area needed for unrestricted growth in a similar animal; lobsters (Geddes et al. 1988). The area, A, required for unrestricted growth has been expressed in the form $A = bC^2$, where C is carapace length. The value for b may vary from 20 to 75, with lower values limiting growth. By this method container size and sizes which may limit growth for crayfish can be determined. However, Geddes et al. (1988) found that growth, in terms of moult increments and intermoult periods, of *C. destructor* was not significantly depressed by smaller container size. The size of containers used in the current study, in Chapter 5, were determined not to limit growth using the formula $A=bC^2$. Apart from providing shelters and the maintenance of good water quality, promoting the natural food items is important in marron culture.

2.3 Freshwater Crayfish and Natural Productivity

The natural diet of marron is largely composed of fine particulate organic matter, as well as smaller amounts of plants and insects and other plant and animal matter (O'Brien 1995). Freshwater crayfish are known to feed on both artificial feed and natural sources of feed including macrophytes, benthic invertebrates, zooplankton, algae, bacteria and detritus (Saoud et al. 2012; Browne et al. 1992). Marron are opportunistic feeders but are primarily detrivorous. They also ingest large amounts of microbes living in the detritus and decomposing organic matter. Thus while they can feed on artificial feed, natural food sources also contribute largely to their diet. Feed can constitute a large proportion of the production costs associated with marron culture, thus increasing natural productivity can save on costs and potentially increase production. Natural productivity is the production of plankton and natural food sources. Natural productivity is important in prawn aquaculture, where fertilizer is

often added to stimulate the natural food sources which supplement artificial feeding (Gamboa-Delgado 2014; Abu Hena & Hishamuddin 2014). Fewer studies have investigated the role of natural productivity in marron culture, however studies have shown the benefits of zooplankton and other natural food sources in crayfish culture. Austin et al. (1997) found that juvenile *C. destructor* fed a combination of zooplankton (*Daphnia* sp.) and artificial feed had higher survival and growth rates then juveniles fed artificial feed alone. Juveniles fed zooplankton also had less variability in size then pellet fed juveniles. There is potential for ponds to be partly or wholly dried out, planted with a forage crop such as clover and incrementally flooded to provide a food source for crayfish. This method of feeding was used in one study, where extensive aquaculture with a forage crop was used to produce modest yields of *C. destructor* (Geddes & Smallridge 1993). Zooplankton were also present and may have provided a source of high protein. The use of a forage crop has not been widely tested in marron aquaculture yet however. More research is needed to establish suitable natural feeds for adult marron in aquaculture. It is recommended that zooplankton populations are well established in juvenile ponds, as they may be key in improving juvenile survival and growth. Zooplankton communities are thought to be partly dependent on phytoplankton.

Phytoplankton growth is known to be dependent on the availability of nitrogen and phosphorous, both of which are cycled by microbes. Thus altering the concentration and composition of microbes in the water, and on substrates, will likely affect the concentrations of phytoplankton and in turn zooplankton and epiphytes, and affect crayfish growth. The application of microbes may also change the concentration of cyanobacteria, which can form harmful blooms and produce toxins. Microbes may influence the natural productivity, by affecting nutrient cycles. However, while aquatic heterotrophic bacteria decompose organic matter and cycle nutrients, they may also consume inorganic phosphorous, thus competing with phytoplankton (Sorokin 1999). Bacteria and associated biofilm may also provide food directly to marron, which are known to ingest microbes in organic matter.

2.4 Microbes

2.4.1 Microbes in Aquaculture

Microbes were not known to mankind until Antonie Philips van Leeuwenhoek (1632–1723), a Dutch tradesman and scientist from Delft, The Netherlands, first found microbes in 1676. He is commonly known as the Father of Microbiology, and is considered to be the first microbiologist (Zhou et al. 2009). Microbes are an important component of all ecosystems, including lakes, rivers and seas. Bacteria have several important roles in aquatic systems; including affecting oxygen levels, oxidising ammonia and nitrite, influencing pH levels, affecting other water quality parameters, contributing to food webs, and as heterotrophic decomposers; cycling nutrients from organic matter to increase availability to primary producers (Moriarty 1997). Microbes can also convert wastes into consumable cellular protein that may provide alternative food sources for cultured aquatic animals (Bender & Phillips 2004). The use of bioflocs is common in many forms of aquaculture, where inorganic waste and carbon is converted to a food source for larvae. In nature, bacteria are often found in association with microalgae and cyanobacteria, and can influence the development or decline of algal blooms (Subashchandrabose et al. 2011). Classical methods such as plating bacteria have been used for isolating or counting bacteria colonies, but these can be fairly inaccurate and newer methods such as epifluorescence microscopy have become powerful tools for determining numbers and biomass of bacteria (Moriarty 1997).

Aquatic microbes include phytoplankton, bacteria, protozoans and microzooplankton (Sorokin 1999). The microbes that grow on the Substrate and consume organic oils include Archaea bacteria and *Bacillus* sp., though other microbes may also settle on the Substrate and contribute to its bioremediation processes (Marine Easy Clean 2015). These may include *Nitrosomonas*, *Nitrobacter* and other chemoautotrophic bacteria, though are thought to be mostly heterotrophic in nature. *Nitrosomonas* convert toxic ammonia into less toxic nitrite, while *Nitrobacter* convert this nitrite into far less toxic nitrate, which can then be used by primary producers. Nitrogenous wastes can be sourced from ammonia excretion, solid faecal waste, decaying organisms, excess feed and nitrogen fixation. Ammonia is more toxic at higher pH and temperature, which shifts the ionization equilibrium towards the unionized form (Hargreaves 1998). Ammonia transformation requires oxygen, therefore there is often a build-up of ammonia in anoxic sediments.

Heterotrophic bacteria, including *Bacillus* species, require a carbon source to function. Microbial nitrogen removal is limited by the supply of carbon as an electron donor (Robinson et al. 2018). Bacteria may assimilate nitrogen, phosphorous and other nutrients or trace elements to build biomass. They may also convert nitrates to nitrites and then nitrogen gas via denitrification, removing it from the system. Exogenous carbon is required for this process (Robinson et al. 2018).

Microscopic organisms also accomplish the basic transformation of organic matter (Sorokin 1999). Dead organic matter build up a detritus energy pool made up of particulate and dissolved organic matter, which can be used by microbes. Bacteria decompose and oxidise complex organic substances from this stock of dead organic matter, using 15 to 35% for their own biosynthesis (Sorokin 1999). This drives the self-purification process in aquatic systems. Several types of microbes decompose organic matter to meet the growth requirements of cultured species and reduce pollution from aquaculture (Zhou et al. 2009). Bender & Philips (2004) suggest that microbial ecosystems might be useful to bioremediation of environmental pollutants.

2.4.2 Probiotics

The overuse of antibiotics in aquaculture to treat disease can lead to the emergence of bacterial resistant species and environmental problems. Antibiotics may be ineffective due to overuse leading to increased disease resistance by bacteria, damage to the microflora of the environment and antibiotic residue building up in aquatic systems (Zhou et al. 2009). Probiotics can be used as an environmentally friendly alternative to disease management. Probiotics can be defined as microbial supplements which beneficially affect the host and their digestive health. The health of the aquatic environment and ecosystem can also be improved. Probiotics may also aid in removing excess organic matter, nutrient cycling, water quality improvement and potentially may improve natural productivity. They may be added in feed or added directly to the water. Probiotics can be used to alter bacterial composition and abundance in the gastrointestinal tract, improving digestive health and immunity. Several studies have investigated the use of probiotics in crustacean aquaculture (Ambas et al. 2013; Hai et al. 2009). Ambas et al. (2013) found that *Bacillus mycoides* and *Bacillus sp.* were effective in improving physiological condition, immune parameters and the level of the bacterial load in the intestine of marron (*C. cainii)* when compared to the control

diet. The total haemocyte count was similar between treatments before exposure to *Vibrio mimicus* but after there was a significant decrease. Probiotic treatments had a significant effect on differential haemocyte count, resulting in a higher granulocyte cell proportion.

Prebiotics have also been widely used in prawn aquaculture as an alternative to antibiotics and chemical treatments. Prebiotics are supplements which promote the growth of intestinal flora. Hai et al. (2009) found that immunostimulants increased the standard growth rate and survival of western king prawns (*Penaeus latisulcatus*). Commercial probiotics, Bio-mos and β-1,3-D-glucan, and customised probiotics, *Pseudomonas synxantha* and *P. aeruginosa*, were used. The bacterial load in the intestine of prawns fed probiotics also increased (Hai et al. 2009). In marron farming limited research has been done on probiotics or prebiotics. However, some bacterial additives, generally containing *Nitrosomonas* and *Nitrobacter* are used by marron farmers to improve water quality.

Microbes can also convert wastes into consumable cellular protein that may provide alternative food sources for cultured aquatic animals (Bender & Phillips 2004). The use of bioflocs is common in many forms of aquaculture, where inorganic waste and carbon is converted to a food source for larvae. Culturing microbes or biofilm on substrates can also provide a complementary food source. Probiotics can also be used to improve the microbial ecology of a system; changing the microbial concentrations and species present, and improving the health of the system and in turn the animals. Carbon sources such as fermented barley straw or humic acid may also be used. These can be rich sources of carbon for heterotrophic bacteria such as *Bacillus* species.

2.4.3 Bacillus Species

Bacterial probiotics for bioremediation include *Nitrosomonas, Nitrobacter, Bacillus, Pseudomonas* and *Acinetobacter*. Nitrifying bacteria have only one main role however, and using *Pseudomonas* and *Vibrio* strains can be dangerous as pathogenic strains present in the water may transfer genes to the probiotic forms. *Bacillus* are gram positive bacteria and are unlikely to use genes for antibiotic resistance or virulence from *Vibrio* species (Moriarty 1999). *Bacillus* sp. are commonly associated with improvement of water quality. Archaea and certain *Bacillus* species are thought to consume organic oils including hydrocarbons, and cycle nutrients. *Bacillus* sp.

compete for nutrients with other bacteria, thereby limiting the growth of pathogenic and resistant bacteria (Hong et al. 2005; Moriarty 1999). *Bacillus* which produce an antibiotic may also outcompete *Vibrio*, even if only a fraction are killed the *Bacillus* may then outnumber the *Vibrio* and there will be a shift in dominance. Research into probiotics such as *Bacillus* in aquaculture is mostly focused on addition to feed, with its use in bioremediation slowly becoming more common.

2.5 Bioremediation

2.5.1 Bioremediation in Aquaculture

As aquaculture becomes more intensive, the high densities of animals and high inputs needed to achieve good production can cause problems in terms of water usage and water quality. Fish cages for example are known to increase organic matter on the bottom sediments (Holmer 1991; Karakassis et al. 1998), and cause the progressive transformation of sediments into an anoxic surface (Danovaro et al. 2003). Bioremediation can be used to improve the water quality and cleanliness of aquatic systems, and provides an alternative to filtration and water exchanges. Bioremediation may also improve the environmental health of aquatic systems. Macrophytes can be used to reduce nutrient concentrations (Abdel-Tawwab 2008; Samocha et al. 2015; Ferdoushi et al. 2008), and a combination of probiotics and macrophytes may be effective in reducing organic wastes. Microbial bioremediation may be applicable to aquaculture where nutrient levels are excessive or where the environmental and ecological health can be improved. Bioremediation is the use of microbial organisms to improve water quality and remove pollutants in water. The process involves many different organisms including bacteria and microalgae (Chávez-Crooker & Obreque-Contreras 2010). Application of bacteria or probiotics can modify the bacterial composition of water and sediments which can improve various water quality parameters (Perumal et al. 2015). This can potentially replace previously established bacteria, some of which may be pathogenic, and increase populations of bacteria that oxidise toxic nutrients and heavy metals; improving environmental health of the water and sediment. With the intensification of various aquaculture farms, research is increasing with the demand for sustainable and environmentally friendly aquaculture (Wang et al. 2005). Bioremediation may improve carrying capacity of aquaculture

ponds; increasing the stocking rate of animals without impacting on growth or water quality.

Fish farmers are concerned about soil and water quality, and can use biological and chemical amendments to treat and improve this. Fertilization of ponds and excessive feeding can lead to accumulation of nutrients such as nitrogen and phosphorous in the sediment and water, which can cause eutrophication, algal blooms, poor ecosystem and animal health and other problems associated with poor water quality. The use of bacterial suspensions for bioremediation is thought to reduce blue green algae, restrict algal blooms, decrease nitrate, nitrite, ammonia and phosphate, increase dissolved oxygen levels and enhance the degradation of organic matter (Browdy & Hopkins 1995). Studies using bacterial suspensions can have varied results, however (Boyd & Gross 1998). One study showed no significant differences in inorganic nitrogen, total phosphorous, chemical oxygen demand, chlorophyll *a*, abundance of bacteria and phytoplankton, and percentages of blue green algae between ponds with and without bacterial-treatment (Boyd et al. 1984). Another found that treating northern white shrimp (*Penaeus vannamei*) ponds with commercial probiotics increased the population density of beneficial bacterial flora, which included *Bacillus* sp. and photosynthetic bacteria (Wang et al. 2005). The treatment also stabilised pH levels, and inorganic nitrogen and phosphate were significantly lower in treated ponds than the control. Shrimp in bioremediated ponds also had higher survival rate, FCR and final production yield (Wang et al. 2005). Increased *Vibrio* count in control ponds may have decreased production, as numerous *Vibrio* species can cause infections in shrimp.

Microbes are often used to treat water high in heavy metals. Heavy metals can have many toxic effects at high concentrations. Lead, for example, has been shown to reduce the ability of crayfish (*C. destructor*) to survive a hypoxic environment (Ahern & Morris 1999). While inorganic pollutants, including heavy metals, cannot be removed by microbial activity, microbes can change the electrostatic charge of these pollutants, affecting their mobility in the environment (Kirchman 2012). Microbes may improve survival and health of crayfish by treating water high in heavy metals. Microbes can breakdown organic compounds, even those toxic to eukaryotic organisms.

A large portion of research into bioremediation is focused on treatment of wastewater, or aquaculture effluent (Chávez-Crooker & Obreque-Contreras 2010). There is less

research on bioremediation within aquaculture systems; and results can vary greatly with differed reports on both positive and negligible effects. The use of bioremediation products is often not grounded on reliable research, instead coming from observations. Some positive effects of bacterial applications in prawn aquaculture have been reported, however the effectiveness of these probiotics has not been clearly established (De Paiva-Maia et al. 2013).

There has been little research into the bioremediation of freshwater crayfish aquaculture. One study used biofilm, attached to a substrate, to improve water quality and survival of redclaw crayfish, and as a food source (Viau et al. 2012). Microorganisms have an important role in the transference of organic matter through trophic chains in aquatic ecosystems (Danovaro et al. 2003; Meyer-Reil 1994), and play a fundamental role in converting organic detritus into living biomass (La Rosa et al. 2001). Viau et al. (2012) found that the control also showed a greater increase in ammonium and nitrite during the beginning of the experiment. Survival and growth of redclaw with the biofilm treatment was also much better than without the biofilm. The biofilm is an organic matrix containing micro-organisms including bacteria, cyanobacteria, microalgae, protozoans, nematodes and rotifers. Bacteria, cyanobacteria and microalgae may act as producers and bioremediators, while also providing a food source for other microorganisms and for freshwater crayfish. Substrates may be used to culture biofilm.

2.5.2 Bioremediation using Substrates

An alternative to probiotics in the bioremediation of aquaculture systems is the use of substrates. Artificial substrates include bamboo or wood substrates, plastic netting, polyethylene, Bioballs and other plastic substrates, mats such as the Aquamat™, and oil or wax based substrates such as the Water Cleanser™, which is investigated in this thesis. Substrates can harbour beneficial bacteria and periphyton, both of which improve water quality (Schveitzer et al. 2013). Substrates may provide as sites for attachment of biofilms. Biofilm cultured on substrates can influence water quality and provide a complementary food source (Viau et al. 2012). Various studies have investigated the effects of artificial substrates on water quality in shrimp aquaculture (Bratvold & Browdy 2001; Khatoon et al. 2007; Schveitzer et al. 2013; Thompson et al. 2002; Viau et al. 2013), while relatively few studies have applied substrates to

Cherax crayfish culture (Jones et al. 2002; Viau et al. 2012). Results can be varied however. Schveitzer et al. (2013) found that application of a polyethylene mesh substrate did not have any significant improvements in water quality as ammonia, nitrite, nitrate and orthophosphate. Water quality was thought to be mainly affected by bacteria present in the water column. Alternatively, Thompson et al. (2002) reported that biofilm attached to artificial substrates maintained a low level of ammonium and phosphate in shrimp culture.

Substrates can be used to provide habitat for animals, thus reducing effects of high stocking density, and can improve water quality, thereby improving carrying capacity of aquatic systems. However, the Substrate is not designed as a refuge or habitat, and is used primarily to improve water quality. Scarce research has focused on substrates and freshwater crayfish, with most studies focusing on shrimp culture. In the past the use of artificial substrates has mainly been used to improve growth of aquatic organisms and in reducing the effects of stocking density (Schvietzer et al. 2013). In one study, substrates provided shelter for crayfish (*Cherax destructor*) and improved growth, but did not increase survival (Jones et al. 2002). Survival and growth of shrimp (*Litopenaeus vannamei*) has been improved by using substrates; which increased the surface area of the tank and reduced the relative stocking density (Schveitzer et al. 2013). Substrates may also reduce the effects of high stocking density indirectly by improving water quality. While research has sought to understand effects of various substrates in bioremediation of aquaculture, no noteworthy research has applied oil-based substrates to aquaculture, including that of marron.

2.6 The Water Cleanser Substrate

The Water Cleanser™ (TWC) is an oil and wax based substrate used in the bioremediation of aquatic systems, by providing a habitat and carbon source for microbes. TWC may potentially increase the rate of decomposition of organic matter using microbes. Therefore, TWC may enhance the cycling of nitrogen within the system, or enhance removal of nitrogen as nitrogen gas, by bacterial processes, and improve water quality. The effects of substrates on disease have not been widely studied, however microbes have been applied to treat disease in aquaculture.

The crude oil within TWC may provide a carbon source for various microbes. Hydrocarbons, including crude oils, have been found to be suitable substrates for

microbial growth. More than 100 species of heterotrophic bacteria, including some *Pseudomonas sp.* and some *Bacillus sp.*, are known to utilize hydrocarbons as a carbon source (Hu et al. 2017). For example, *Bacillus subtilis* can grow on hydrocarbon substrate, and utilize waxy crude oil, model crude oil, and long chain paraffins present therein, producing biosurfactants which increase the bioavailability to other microorganisms (Sakthipriya et al. 2015). Various species of microorganisms, including micromycetes, yeasts and bacteria, are capable of hydrocarbon oxidation to utilize a wide spectrum of hydrocarbons, including aromatics, and can have a high growth rate (Astashkina et al. 2015). On the surface of TWC abundant growth can be visible, as microbial biofilm. Biofilm is a microbial consortium associated with a matrix of extracellular polymeric substances bound to submerged surfaces, which can affect water quality in aquaculture (Pandey et al. 2014). TWC acts as a habitat, and a carbon and mineral source for a microbial community living within a biofilm. Crude oil contains many impurities, including heteronuclear compounds, emulsified water and minerals (Tissot & Welt 1984).

TWC provides certain trace elements and organic oils for the microbes to thrive, and does not leach any hydrocarbons into the water (Marine Easy Clean 2015). In addition, microbial communities can be found on TWC, with the bacteria as the base of the food chain. The bacteria cultured include various *Bacillus* sp., *Acinetobacter* sp., *Mycobactelium* sp., *Nocardia* sp., *Paecilomyces* sp., *Holomonas* sp., *Lactobacillus* sp., *Tenacibaculum* sp., *Lysinibacillus* sp., *Burkholderia* sp., *Pseudomonas* sp., *Aeromonas* sp., *Rheinheirmera* sp. and *Exiguobacterium* sp. (Marine Easy Clean 2015). The cultured bacteria and other microbes are thought to consume organic matter, organic oils and wastes in the water, which may in turn improve turbidity, reduce the availability of nutrients to algae, and improve the health of aquatic systems and animals therein (Marine Easy Clean 2015). Healthier water can improve physiology of cultured species, as better water quality can improve health and in turn quality of cultured species (Li et al. 2013[a]). The beneficial bacteria cultured on TWC are thought to consume organic matter and nitrogenous wastes in the water, while some may accumulate inorganic phosphates (Figure 2.2). The microbes convert ammonia and ammonium to nitrite, and the nitrite in turn to nitrate, via nitrification. Denitrification may also increase, removing nitrogen from the aquatic system. These wastes can cause physiological problems in aquaculture species, including freshwater

crayfish (Jensen 1996; Harris et al. 2001), carp (Sun et al. 2012) and prawn species (Wang et al. 2004). Ammonia may be toxic at high levels, and often causes a sublethal reduction of animal growth or suppression of immunocompetence, while nitrite is also toxic and this toxicity is expressed through the competitive binding of nitrite to haemoglobin, reducing the capacity of blood to carry oxygen (Hargreaves 1998). The haemolymph of most crustaceans, including crayfish, does not contain haemoglobin (Jensen 2003), meaning that nitrite is less toxic to these animals. Nitrite can induce a decrease in extracellular chloride ions however (Jensen 1996). Low levels of dissolved oxygen also occur in aquaculture systems, and can be lethal to aquatic organisms (Chabot & Dutil 1999). The availability of dissolved oxygen can be limited in aquaculture systems, especially when stagnant, where bacterial action can cause hypoxia (Aji 2012). The bacteria associated with TWC are thought to consume low amounts of oxygen, as some are anaerobic (Marine Easy Clean 2015). Another macronutrient, phosphate, can cause algal blooms at high levels, and TWC is thought to reduce the availability of phosphate. TWC may help in the maintenance of water quality of aquaculture systems.

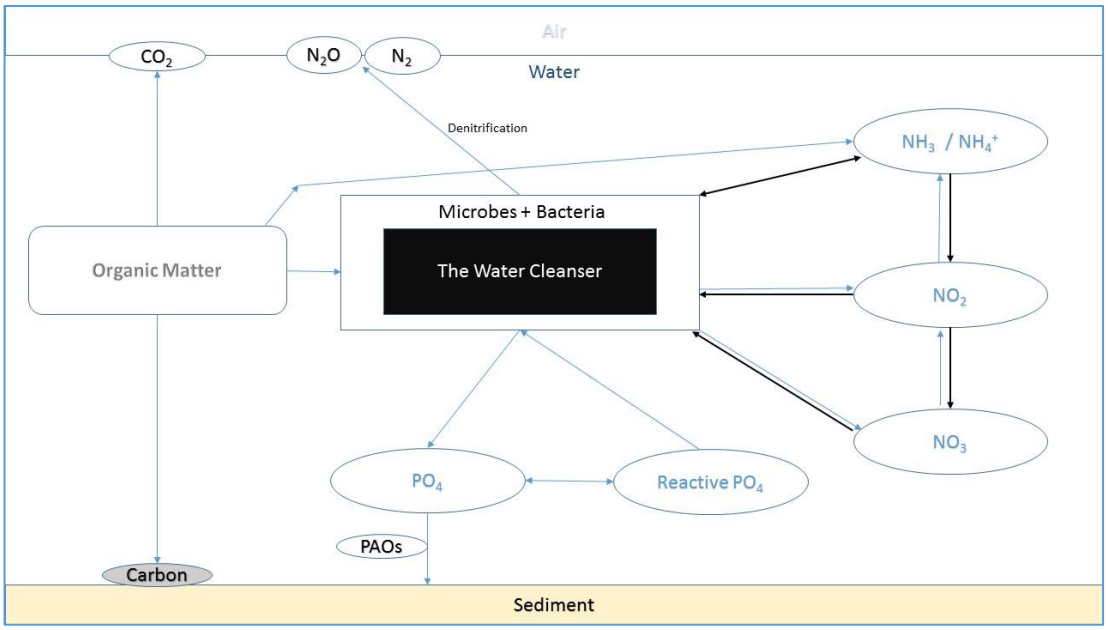

Figure 2.2: Conceptual diagram of The Water Cleanser in an aquatic environment; showing theoretical pathways between Nitrogen, Phosphorous, organic matter and TWC. PAOs = Phosphate Accumulating Organisms.

Limited research has been conducted using TWC for microbial bioremediation in aquaculture. However, a previous study has shown that TWC can improve survival

and carrying capacity of goldfish (*Carassius auratus*) in aquaria (Marine Easy Clean 2015). In a similar study on *Cyprinus carpio* culture, turbidity of ponds remained stable following application of TWC (Iguodala & Cole 2015, unpublished data).

2.7 Potential for Bioremediation in Marron Aquaculture

Freshwater crayfish are ideal species for this research as they are known to feed on plankton (Austin et al. 1997; Gamboa-Delgado 2014), and are sensitive to changes in water quality, both of which may be affected by microbial bioremediation. Very little research has investigated bioremediation in marron aquaculture. The use of bioremediation in freshwater marron aquaculture may help to maintain low levels of nitrogenous wastes and phosphates, improve environmental health and ergo improve marron health and productivity.

Research into bioremediation in marron ponds may help improve health, and in turn growth rate of marron, and could also help increase stocking density without impacting on water quality. Research could focus on using bacterial additives (Wang et al. 2005), substrates (Viau et al. 2012), macrophytes (Ardiansyah & Fotedar 2016[b]) or new systems to increase production, improve water quality and reduce water use and other costs. A better understanding of marron ecology within aquaculture systems is also needed, as it is unknown exactly how it feeds and what it feeds on. TWC may also provide a complementary food source as biofilm, though is primarily designed to improve water quality.

Chapter 3:

Efficacy of different oil-based substrates as bioremediators

Chapter 3 presents the first laboratory experiment, focusing on water quality in an indoor environment, without marron. This experiment was designed to trial two different substrates, The Water Cleanser™ and The Water Cleanser™ Plus, and a commercial probiotic, BioAid®, as bioremediators, under laboratory conditions.

3.1 Introduction

In aquatic systems, accumulation of wastes can have adverse impacts on the ecosystem (Hargreaves 1998, Jørgensen 2008). Ammonia may be toxic at high levels, and often causes a sublethal reduction of fish growth or suppression of immunocompetence, while nitrite is also toxic and this toxicity is expressed through the competitive binding of nitrite to haemoglobin, reducing the capacity of blood to carry oxygen (Hargreaves 1998, Sun et al. 2012). Nitrites can cause physiological problems for freshwater fish (Sun et al. 2012), while high nitrate and phosphate levels can increase risk of harmful algal blooms, filamentous algae growth and slight toxicity (Blaas & Kroeze 2016; Hamlin et al. 2008).

Probiotic bacteria can modify the bacterial composition of water and sediments, which can improve various water quality parameters (Perumal et al. 2015). Heterotrophic and polyphosphate-accumulating bacteria are used to remove nitrogen and phosphorous, respectively, in wastewater (Wang et al. 2015). The use of environmental probiotics may also reduce the availability of heavy metals and other toxins (Kirchman 2012) in aquatic systems and inhibit pathogenic bacteria (Hong et al. 2005); improving water quality and ecology.

Heterotrophic bacteria require a carbon source in order to function; and to convert nitrates to nitrogen gas via denitrification (Hamlin et al. 2008). Oil-based substrates can provide hydrocarbons, which can be utilised as a carbon and energy source (Astashkina et al. 2015; Hu et al. 2017), while also providing habitat for various microbes. The Water Cleanser™ is made from crude oil and waxes, and is designed primarily for bioremediation. Another form of the substrate used in this study, the water cleanser plus (TWC+), is made from a mixture of crude oil, tea-tree oil and waxes. Because of the antibacterial properties of tea-tree oil, TWC+ is thought to be more selective in terms of the bacterial species that colonise the substrate than TWC (Personal communication with Marine Easy Clean). TWC is a substrate for the growth of microbial biofilm, which has been shown to be important in nitrification; where ammonia oxidation rates can be nearly 60 times higher than in water particles (Holl et al. 2011). Biofilm attached to substrates has previously been used to improve water quality (Otoshi et al. 2006; Viau et al. 2012). This study was conducted under laboratory conditions as a preliminary trial to investigate the efficacy of oil-based

substrates and a commercial probiotic in removal of nitrogen and phosphorous from water, with limited variables.

3.2 Materials and Methods

3.2.1 Experiment Design

This experiment was conducted at the Curtin Aquatic Research Laboratory, Curtin University, Bentley, Perth, Western Australia, in a temperature controlled facility.

Twelve 15 L glass aquaria (36cm x 22cm x 26 cm) were filled with 12L of filtered freshwater, with aeration and no mechanical filtration. Ammonium chloride (NH_4Cl) and potassium phosphate dibasic (K_2HPO_4) were added to all aquaria to attain an initial concentration of 4.11mg/L TAN (total ammonia nitrogen) and 3.5 mg/L total PO_4. Four treatments were used in triplicate in a completely randomised design (Figure 3.1): 1) a control treatment with no additions of substrate or probiotic, 2) the standard water cleanser substrate by Marine Easy Clean Pty Ltd., made with crude oil and waxes, (TWC) at 22g/tank, as two spherical balls 28mm each in diameter, with a dorsal hole to provide more surface area, 3) the water cleanser plus substrate, made with tea tree oils and waxes (TWC+), at 22g/tank, as two rectangular blocks (35x34x20mm) with ridges to provide more surface area, and 4) a Bio-Aid commercial probiotic (Bio-Aid) by Aquasonic Pty Ltd.; at 2 drops per tank initially. This was the recommended initial dosage of Bio-Aid, which contains a mix of 27 different strains of heterotrophic probiotic bacteria (Aquasonic). TWC and TWC+ were compared in terms of composition, not surface area to volume ratio. The experiment ran for three months.

Dissolved oxygen (DO) and temperature, and pH and conductivity were monitored twice a week using an Oxyguard Handy Polaris DO meter and an Ecoscan pH 5 meter respectively. TAN, NO_2, NO_3, total phosphate (PO_4), and orthophosphate were measured using Permachem Reagents from HACH ® every 20 days, and a Skalar colorimeter auto analyser (Downs et al., 2008), according to the methods described in the Standard Methods for the Examination of Water and Wastewater (APHA, 1998). API test kits from Aquarium Pharmaceuticals ® were used for regular measurements twice a week, and standardised using the Permachem method.

3.2.2 Bacterial Abundance

Prior to bacteria culturing, agar plates were made by the following procedure:

Approximately 3.5g of tryptone glucose yeast agar powder (Oxoid, U.K.) was stirred in 200 mL of distilled water in a conical flask and brought to the boil. The flask of agar was placed in an autoclave for 15 minutes for sterilization. The flask was then cooled to approximately 40°C, and the agar poured into petri dishes, in a laminar flow cabinet. The dishes were left for 30 minutes to allow agar to set, and to remove condensation, before being wrapped in alfoil and placed in a refrigerator until plating. Before plating the agar plates were brought back to room temperature.

Bacterial colonies were counted in colony-forming units (CFU) after 11 weeks with a dilution of ($10^{-1.3}$), to determine bacterial abundance. A 50 µL subsample was collected from a sample of aquaria water, then transferred onto agar plates using aseptic techniques. Samples were spread using a hoop, which was flamed between samples, and then cultured on standard plate count agar (Tryptone glucose yeast agar) at 30°C for 48 hours. Viable colonies were counted and identified according to colony morphology and colour to estimate diversity.

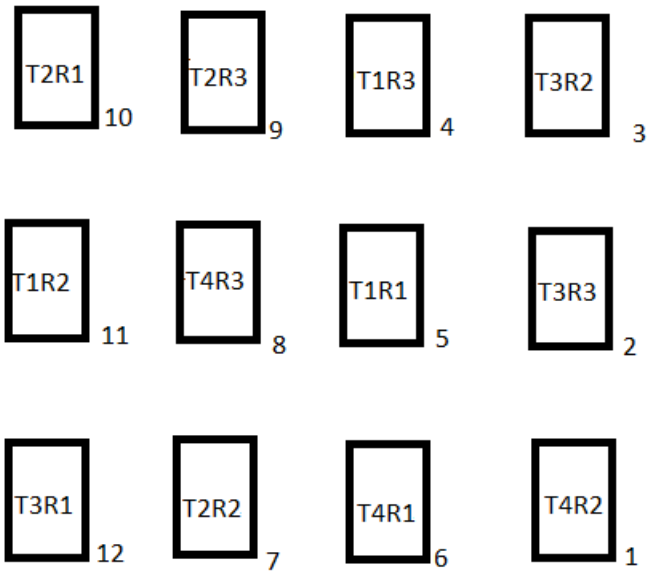

Figure 3.1: Experimental Design of Experiment 1; with experimental units represented as rectangles, T=Treatment, R=Replicate.

3.2.3 Statistical Analysis

All data were expressed as mean ± standard error. All statistical analyses were carried out using SPSS version 23.0. Significant differences among treatments and time were determined using a one-way ANOVA, for each factor. Least-significant difference (LSD) pos-hoc tests were used for multiple mean comparisons when the p value showed significance. Kruskal-Wallis tests were used when data did not have normal distribution and homogeneity of variance. Differences were considered significant at $p<0.05$ in all cases.

3.3 Results

3.3.1 Water Quality

The physico-chemical parameters, pH, temperature, DO and conductivity, were not significantly different among tanks with TWC, TWC+, Bio-Aid or control. Temperature ranged from 15.1°C to 23.1°C, with an overall mean of 18.45±0.11 °C. TWC and TWC+ changed the dynamics of nutrient concentrations in the aquaria. While in the control and Bio-Aid aquaria, TAN was oxidised more quickly than in TWC and TWC+ aquaria, the concentrations of NO_2-N and NO_3-N rose to significantly higher levels, which are toxic to some freshwater invertebrates (Soucek & Dickinson 2012). TAN decreased steadily in all treatments, but was significantly lower in the aquaria with Bio-Aid than with substrates after 7, 8, 9 and 11 weeks, and lower in control aquaria after 9 and 11 weeks (Figure 3.2). TAN fell to <0.02 mg/L in all aquaria by week 13.

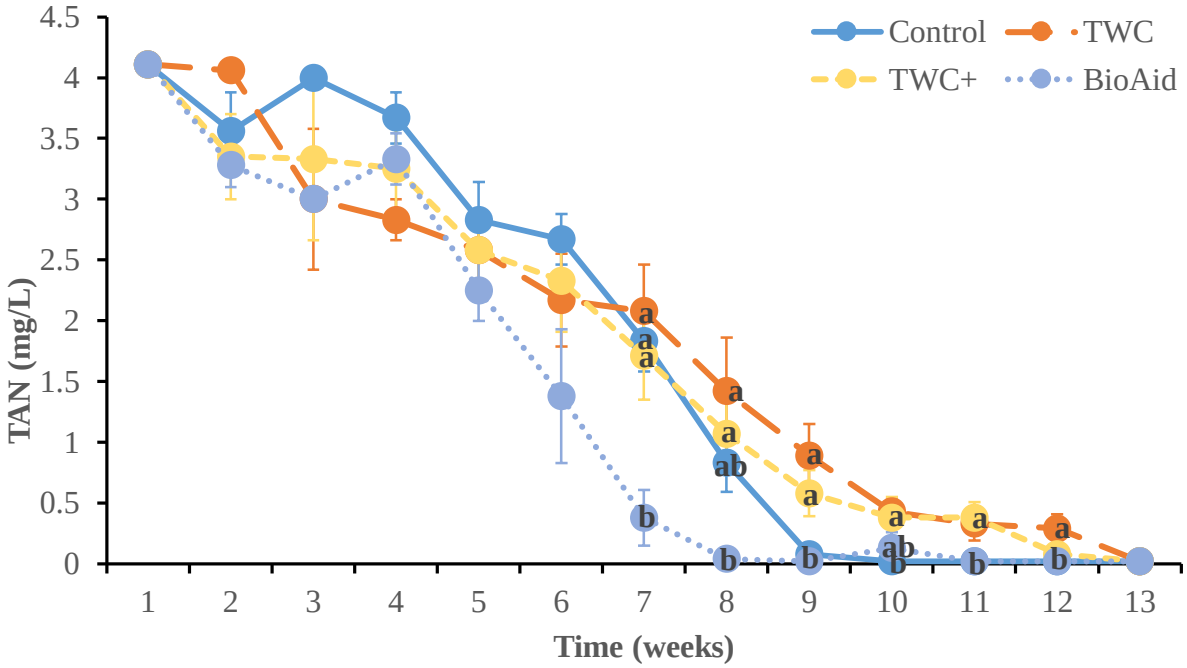

Figure 3.2: Concentrations of TAN (mg/L) in four treatments over time (weeks). Like letters (a, b) indicate that the means are statistically similar (α=0.05).

Over time, the concentrations of NO_2-N and NO_3-N were generally lower and more stable in TWC and TWC+ aquaria than in the control and Bio-Aid aquaria, which showed large fluctuations and peaks in both NO_2-N and NO_3-N (Figure 3.3). Total nitrogen was significantly lower in TWC and TWC+ aquaria than without substrates after 10 weeks.

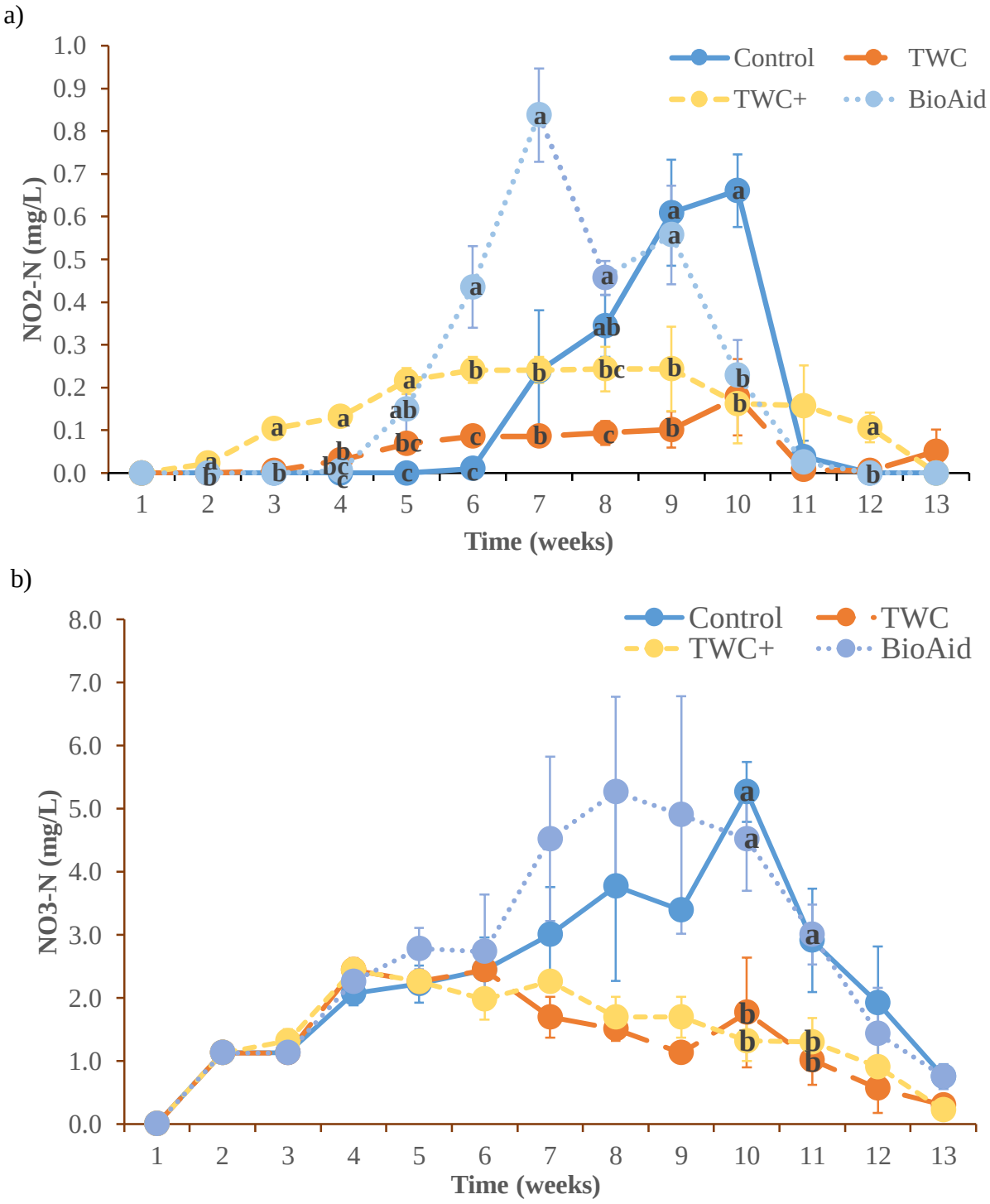

Figure 3.3: Concentrations of Nitrite-Nitrogen (NO_2-N) (a) and Nitrate-Nitrogen (NO_3-N) (b) over time. Like letters (a, b, c) indicate that the means are statistically similar (α=0.05).

The concentration of orthophosphate was significantly lower with TWC+ than with BioAid or control in week 4 (0.85±0.02, 1.01±0.03 mg/L, 0.96±0.04 respectively), and lower than with BioAid in week 7 (0.96±0.01, 1.15±0.04 mg/L respectively).

Concentrations of PO_4 and NO_3-N were lower with TWC+ than with Bio-Aid or control at the end of trial (Figure 3.4).

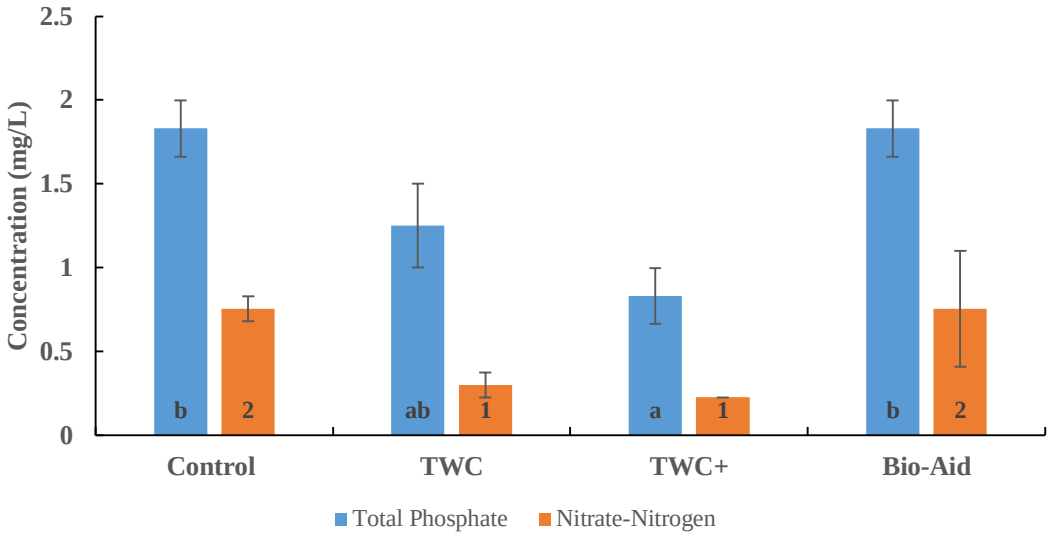

Figure 3.4: Concentrations of Nitrate-Nitrogen (NO_3-N) and Total Phosphate after 13 weeks. Different letters (a, b) show significant differences in total phosphate and numbers (1, 2) show significant differences in NO_3-N between treatments ($\alpha<0.05$).

3.3.2 Bacterial abundance

No significant differences were found among heterotrophic plate count (HPC) after ten weeks. Mean HPC was 51.6±35.2, 55.7±11.1, 25.0±7.94 and 7.87±0.29 (CFU/mL x 10^2) in control, TWC, TWC+ and Bio-Aid tanks respectively, after 11 weeks.

Biofilm grew at the bottom of aquaria in all tanks. A thick layer of biofilm developed on TWC and TWC+ after 4 weeks, varied in colour and texture (Plate 3.1). Cocci and rod-shaped bacteria were found within the biofilm, as determined by microscopy.

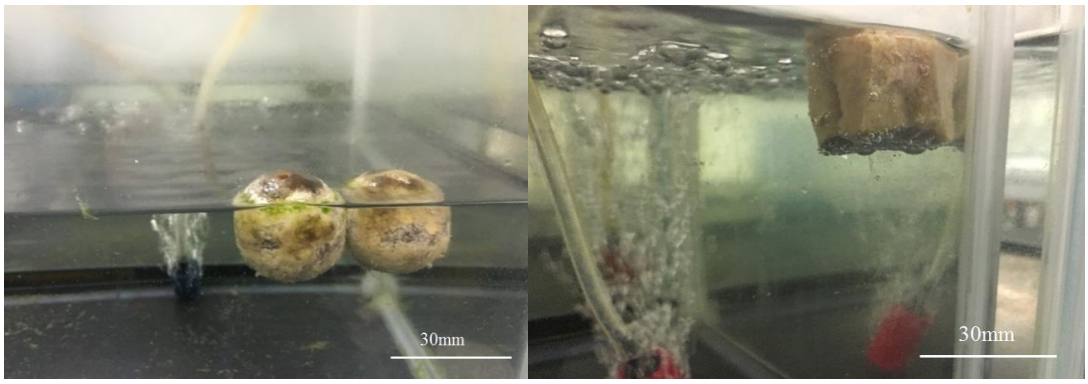

Plate 3.1: The Water Cleanser™ (TWC) (left) and TWC+ (right) after six weeks of experiment 1, with attached biofilm on the submerged surface. TWC was ball-shaped, hollow with a dorsal hole, TWC+ was in block form, with ridges.

3.4 Discussion

The Water Cleanser™ promoted the growth of biofilm and had a significant effect on water quality. The peak concentrations of nitrite and nitrate were significantly reduced with the application of TWC and TWC+. Both substrates were similarly effective as bioremediators, accelerating the nitrification of nitrite, and denitrification of nitrate. The substrates were effective in reducing nitrogenous metabolites under controlled conditions; at temperatures ranging from 15.1°C to 23.1°C, with low fluctuations. The results show TWC to be more effective in reducing the concentration of nitrite than TWC+, attributed mainly to the differences in oil composition. Addition of an artificial substrate, with attached biofilm, has been shown to maintain lower levels of ammonium and nitrite in *Cherax quadricarinatus* culture (Viau et al. 2012). Substrates can have varied effects however; for example in one study, addition of a substrate with attached biofilm resulted in a reduction of ammonium, but not of nitrite and nitrate (Thompson et al. 2002). In comparison to previously studied substrates, it is possible that TWC may function differently as a bioremediator, though this still requires validation from a comparative study. TWC and TWC+ may also help with the start-up of new aquarium or aquaculture systems by exposing animals to lower levels of nitrites and nitrates. Jones et al. (2002) reported a maximum mean nitrite concentration of 1.3 mg/L in a study using a different substrate (Aquamat™), compared to a maximum mean value of 0.58 mg/L in tanks with TWC. Meanwhile, the acclimation of a conventional biological filter using chemical addition can take up to 36 days before nitrite

concentrations are reduced to levels below 1 mg/L, exposing animals to acute toxicity during this time (Manthe & Malone 1987). In finfish, nitrite leads to the oxidation of functional haemoglobin to methaemoglobin, which cannot bind oxygen, while in crayfish, nitrite competes with chlorine and bromine ions for the same uptake mechanism within the haemolymph (Jensen 2003). Jensen (1996) has shown that nitrite levels of 1 mM are toxic to noble crayfish (*Astacus astacus*). Therefore, in crayfish aquaculture maintaining safe levels of nitrite is important, as with finfish species. Promoting the growth of bacteria on oil-based substrates within aquaculture systems may reduce nitrites, through denitrification, thus improving the health of cultured animals.

While aquaria with TWC and TWC+ exhibited a greater reduction in nitrite, nitrate and phosphate concentrations, there was a slower rate of ammonia oxidation. The Bio-Aid probiotic effectively oxidised ammonia, but was only added initially, which may explain why the nitrates and phosphates were not removed quickly. The addition of probiotics has previously given mixed results. Some studies have reported only limited effects of probiotics on water quality (Boyd & Gross 1998; Chiayvareesajja & Boyd 1993), while others have reported significant reductions in ammonia levels with probiotic treatments (Nimrat et al. 2012). The difference that we observed between the substrates and the probiotic can be partly explained by variation in the numbers of nitrifying and heterotrophic bacteria, which are often associated with changes in filter conditions (Itoi et al. 2006). In TWC and TWC+ aquaria, increased ammonia could have been sourced from mineralization of organic matter by bacteria attached to the substrates (Prabu & Santhiya 2016). Also present on the substrate surface were protozoans, which can also work as mineralizers by excreting ammonium and returning nitrogen incorporated by the bacteria to the water (Caron 1994). The degree of grazing by ciliates and other protozoans on TWC surface was not studied, though this predation pressure can affect the bacterial abundance on substrates (Thompson et al. 2002). The composition of attached biofilm and periphyton, and effects on water quality, may vary between substrates composed of different materials.

In this experiment, TWC and TWC+ were more effective in reducing the concentrations of nitrites, nitrates and phosphates than the Bio-Aid or control, though were not as effective in the oxidation of ammonia. Both substrates functioned similarly, though TWC maintained lower concentrations of nitrite than TWC+. A

biofilm developed on both substrates after several weeks of trial, containing heterotrophic bacteria, protozoans and minute nematodes. TWC provides habitat for various heterotrophic bacteria already present in the water, and hydrocarbon which can be utilised by certain bacteria. The water cleanser may function differently as a bioremediator to probiotics or substrates made up of different materials. This trial shows that TWC and TWC+ have an effect on nutrient concentrations in freshwater environments.

Chapter 4:

Effects of an oil-based substrate (The Water Cleanser™) and commercial probiotics on water quality, phytoplankton and microbial ecology, and marron (*Cherax cainii* Austin 2002) in outdoor culture

Chapter 4 presents the second laboratory experiment, focusing on water quality, phytoplankton and microbial ecology in outdoor marron tank culture. This experiment was designed to trial TWC, TWC combined with Bacillus sp., and a commercial probiotics as bioremediators in an aquaculture environment, and to investigate potential benefits or disadvantages for natural productivity, and marron health and productivity.

4.1 Introduction

In Australia, marron (*Cherax cainii* Austin) farms are run extensively and semi-intensively, and often as a secondary source of income based upon low-input self-sustaining populations (Mills & Mccloud 1983). If the industry is to expand, intensification may be required, while still maintaining good water quality and quality produce. Intensive stocking results in increases in nutrient loads from uneaten feed and metabolic waste products (David et al. 2015); leading to eutrophic conditions and algal blooms (Seymour 1980). Concomitantly, an increase in ammonium and nitrite may damage the health of aquatic organisms (Deng et al. 2014). Poor water quality and excessive organic matter can also cause epibionts such as *Epistylis* and *Temnocephala* to thrive, which can be problematic for the health and market value of marron (Ambas et al. 2013).

Microbial bioremediation is one solution to decrease the build-up of nutrients in aquaculture water and wastewater. Additions of heterotrophic and polyphosphate-accumulating bacteria have been used to remove nitrogen and phosphorous in aquatic systems (Zokaeifar et al. 2014; Wang et al. 2005; Wang et al. 2015). Microorganisms can influence the development and decline of algal blooms (Subashchandrabose et al. 2011), regulating algal populations in water bodies so as to avoid algal blooms (Zhou et al. 2009), or alternatively benefiting phytoplankton by increasing nutrient availability through mineralization of organic matter (Amin et al. 2012). Microbial communities play a major role in recycling the autochthonous nutrients accumulating within the system (Avnimelech et al. 1995). Increased abundance of phytoplankton can increase phytoremediation (Lananan et al. 2014; Labbé et al. 2017), and may also provide more food sources for freshwater crayfish, which consume phytoplankton such as diatoms in biofilm (Viau et al. 2012), and zooplankton, which depend on phytoplankton, and detritus, which may be formed by decaying plankton (Saoud et al. 2012).

Substrates can also be used for bioremediation, and provide suitable sites for the growth of biofilm, which can provide a complementary food source (Viau et al. 2012). Substrates also harbour beneficial bacteria and periphyton, both of which may improve water quality (Schveitzer et al. 2013). Various studies have investigated the effects of substrates on water quality in shrimp aquaculture (Khatoon et al. 2007; Schveitzer et

al. 2013; Thompson et al. 2002; Viau et al. 2013), while relatively few studies have applied substrates to *Cherax* crayfish culture (Jones et al. 2002; Viau et al. 2012). Artificial substrates are most commonly been made up of plastic, polyethylene mesh, mats, netting and other similar materials. Bacteria densities can be increased by maintaining organic particles as substrate in the water column (Hargreaves 2006). Furthermore, artificial substrates may create a habitat for nitrifying bacteria (Otoshi et al. 2006). Substrates may also enhance the degradation of organic matter, as this can at times be limited by the substrate, rather than the microbial biomass (Avnimelech et al. 1995).

Unlike conventional substrates, the Water Cleanser™ (TWC) is oil and wax based, and is designed primarily for bioremediation. Past trials by (Marine Easy Clean 2015) show that TWC provides habitat for bacteria present in the surrounding water, while also providing a carbon source, high surface area, and trace elements within the oils. A carbon source is essential for heterotrophic bacteria processes including denitrification (Hamlin et al. 2008). The provision of a carbon source will promote the growth of heterotrophic bacteria, influencing the bacterial population. TWC could thereby encourage the growth of *Bacillus* sp. for example, which are known to affect the water quality and microbial ecology of aquatic systems, and health of crustaceans (Nimrat et al. 2012; Ambas et al. 2013; Hai et al. 2009). However, the effectiveness of TWC in marron culture has not been investigated so far.

The first experiment showed that TWC is effective in bioremediation, however the effects in an aquaculture environment are largely unknown. No peer reviewed research has applied TWC to aquaculture. Semi-intensive marron aquaculture is dependent on maintaining good water quality, and in promoting the pond ecology to provide natural food sources, both of which may be affected by TWC. The current study evaluated the effects of TWC, TWC with *Bacillus* sp. (TWC+B), and a commercial probiotic (Eviro-3) on nutrient concentrations, bacterial and phytoplankton abundance and species composition, and marron growth and health in outdoor culture. The temporal fluctuations, and relationships between different parameters were also studied. This experiment was designed as a preliminary trial to examine the effects of the TWC on water quality, marron health, and phytoplankton productivity, and its applicability to commercial marron ponds, as investigated further in Chapter 5.

4.2 Materials and Methods

4.2.1 Experiment Design

The experiment was undertaken outdoors, with natural sunlight and subject to rainfall and evaporation. Photoperiod was close to 12:12. Twelve white plastic cylindrical tanks (46cm height and 58cm diameter) were filled to 85L ± 5L with filtered freshwater, and aerated, without mechanical filtration. Tanks were half covered with a mesh lid, as part of a mechanism to prevent escapees. Four treatments were used in triplicate in a randomised block design: 1) a control with no additions of substrate or probiotic, 2) The water cleanser substrate (TWC) as 200g rectangular floating blocks (155x107x20mm) by Marine Easy Clean Pty Ltd, 3) TWC + B (the water cleanser substrate with *Bacillus* sp. in inactive form) (Plate 4.1), and 4) E-viro 3 commercial probiotic (40mL per tank initially) by Enviroplus Pty Ltd. 1.85 g of Aquasol fertiliser by Selleys Yates Pty Ltd was added per tank initially, to increase nutrient availability. The experiment was carried out for 18 weeks.

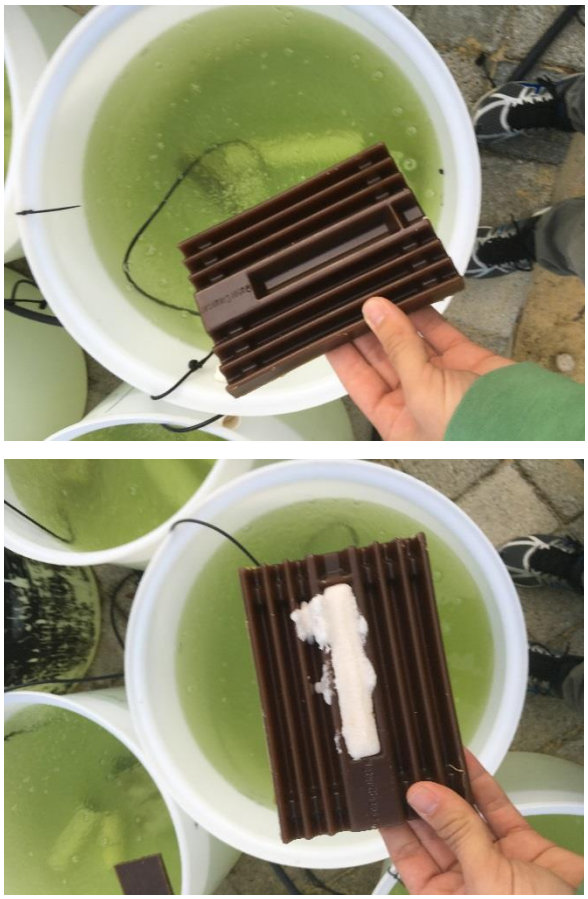

Plate 4.1: TWC (left) and TWC + B (right) upon application.

4.2.2 Water Quality

The physico-chemical parameters; DO, temperature, pH, and conductivity were measured twice a week, diurnally. TAN, NO_2-N and NO_2-N concentrations were measured every 20 days, and orthophosphate every 30 days, with Permachem tests and colorimeter by HACH ®, and a Skalar colorimeter auto analyser (Downs et al. 2008), and were determined based on the standard methods of testing Water and Wastewater (APHA, 1998). Concentrations of ammonia, nitrite, nitrate and total phosphate (PO4) concentrations were also monitored twice a week during the first month with API test kits, in case of elevated levels. Samples were kept refrigerated and processed within 7 days to prevent excessive changes in concentrations.

4.2.3 Animals and Growth Parameters

Marron were procured from Blue Ridge Marron, Manjimup, and acclimatised in stock tanks for 7 days before trial, with aeration and regular water exchange. Thirty six (51.33±1.29 g; 56.5±0.5 mm OCL), were stocked at a density of 3 marron per tank. Sufficient shelter was provided in the form of PVC pipes. Feed was provided at a rate of 0.5% of wet body weight, 3 times per week. Tanks were checked daily for mortalities or escapees, and animal condition. Marron were weighed individually every month, and measured for OCL, from the start of the carapace to the end of the rostrum, using a calliper. Growth was determined as mean monthly weight gain (mean weight from end of month – mean weight at start of month), and total individual weight gain from start to 3 months (mean weight at end – mean weight at start). Mortalities and escapees were discounted from growth data.

4.2.4 Phytoplankton

Phytoplankton species were procured from commercial marron ponds in Manjimup, WA and cultured outdoors in an aerated stock tank. Initially, approximately 10 L of microalgae stock water was added to each experimental tank in order to cultivate a population of phytoplankton. Phytoplankton abundance and species composition was measured every two weeks. Water samples were taken directly from the water surface into 100 mL plastic containers. Lugol's Iodine was added at 3% concentration for staining and preservation, and samples were counted within 3 hours. Phytoplankton were counted using a haemocytometer with a heavy cover slip, and viewed using a Motic compound microscope at 400x magnification. Phytoplankton abundance was

calculated by using equations adapted from Ingram et al. (1997) and Nugroho & Fotedar (2013):

Phytoplankton abundance (cells/L) = ((No. x 1000 / (volume of grid ($0.1mm^3$) x No. of squares in grid counted)*(Conc. Vol. / 1 mL)) / Tot. Vol.

where Tot. Vol. = Total volume of water (L) collected from the pond, Conc. Vol. = Volume of water (mL) containing concentrated plankton after sieving, Sub. Vol. = Sub-sample of water (mL) from concentrated volume in which plankton is counted, and No. = Mean number of cells or individuals counted.

Identification was carried out to genus level, with reference to Ingram et al. (1997), and the number of genera per sample counted. The species diversity was further calculated using the Shannon diversity index with the following formula: $H = - [\Sigma P_i \times \text{Ln}(P_i)]$, where H = Diversity index, P_i = the number each species in the sample/total number of samples, and $\text{Ln}(P_i)$ = the natural logarithm of this proportion.

4.2.5 Haemolymph Indices

As a measure of stress and changes in immune response, haemolymph samples were taken from marron after 20 days (following a period when nutrient concentrations were high), and after 55 and 80 days (periods when nutrient concentrations were low). Syringes were filled with 0.3 mL trisodium citrate anticoagulant and 0.3 mL of haemolymph. 27 G needles were used with 1 mL syringes. Total haemocyte count (THC) was taken within 8 hours of sampling, using a haemocytometer and a Motic compound microscope at 400x magnification. Samples were stained with Giemsa and May-Grunwald for differential haemocyte count (DHC), then granular, semi-granular and hyaline cells were counted, and DHC determined following established protocol (Sang et al. 2009).

4.2.6 Bacteria

Bacterial abundance was determined in the water column after 43 days, giving bacteria a six week period to become established, with follow up sampling four weeks later, after 70 days. Water samples were diluted by 10^{-3}; by diluting 1 mL of samples in 100 mL of sterile 0.85% saline solution, and 100 µL of diluted sample then being transferred onto agar plates using aseptic techniques. Samples were spread using a hoop, which was flamed between samples. Samples were cultured on standard plate

count agar (Tryptone glucose yeast agar) at 30°C for 48 hours. Viable colonies were counted and identified according to colony morphology and colour to estimate diversity. *Bacillus* species were identified to genus level by colony morphology and were counted. Heterotrophic Plate Count (HPC), *Bacillus* sp. count and estimated diversity was determined on TWC surface, by taking a swab of $1cm^3$ from the submerged surface and conducting a standard plate count as per the water sampling method.

4.2.7 Statistical Analysis

All data obtained were expressed as mean ± standard error. All statistical analysis was done using SPSS version 23.0. Mean differences between treatment and time (weeks) were compared using a multivariate two way analysis of variance (ANOVA). Where significant main effects were detected a least significant difference (LSD) post-hoc test was conducted. A Kruskal-Wallis test was used where data lacked normality and/or homogeneity of variance. Spearman's rank test was carried out to determine relationships between measured parameters, such as TAN and THC. Differences were considered significant at $\alpha=0.05$ in all cases.

4.3 Results

4.3.1 Water quality

The physico-chemical parameters pH, temperature, DO and conductivity were not significantly affected by treatment. Diurnal temperature ranged from 10.3°C to 19.2°C, with an overall mean of 14.8±0.1°C. Temperature remained low throughout the experiment, relative to the optimal temperature for marron growth (Morrissy 1990); (Figure 4.1). The pH ranged from 7.78-11.8 with an overall mean of 7.91±0.02. Higher phytoplankton abundance was correlated with higher pH levels ($R=0.629$, $p=0.028$). Dissolved oxygen remained high in all tanks, partly due to the high abundance of phytoplankton, with an overall mean of 10.98±0.11. A thin layer of biofilm was visible growing on the surface of TWC after 79 days.

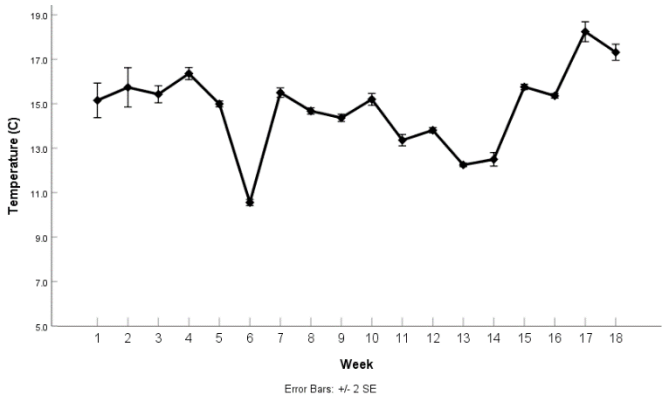

Figure 4.1: Mean temperature of all tanks over 18 weeks.

After five days nutrient concentrations had decreased significantly in TWC+B tanks. Nitrate and total phosphate were significantly lower in TWC+B treatment than all other treatments (Table 4.1). The concentration of orthophosphate was also significantly lower in TWC+B tanks in week 1. There were no other significant differences in terms of phosphates. Mean TAN and NO_2-N means remained less than 1.20 and 0.026 mg/L respectively in all treatments during the experiment, and were never significantly different between treatments. TAN levels peaked in week 3 in all treatments, possibly due to a build-up of uneaten feed and marron waste. Following week 3, TAN levels remained less than 0.10 mg/L in all treatments. NO_3-N fell to less than 0.50 mg/L after week 3, and then rose to more than 0.49 mg/L in week 13 in all treatments. NO_3-N was not significantly different between treatments, except in the E-viro 3 tanks after 10 weeks, where NO_3-N rose to 0.50±0.29 mg/L compared to 0.10±0.06, 0.13±0.03 and 0.30±0.06 mg/L in the control, TWC+B and TWC tanks, respectively. NO_3-N was negatively correlated with phytoplankton abundance ($R=-0.740$, $p<0.001$), as was TAN ($R=-0.675$, $p<0.001$).

Table 4.1: Water quality after 5 days, when concentrations of NO_3-N and PO_4 had both significantly decreased in TWC+B tanks.

	Control	**TWC**	**TWC+B**	**E-viro3**
TAN	<0.02±0.00	<0.02±0.00	<0.02±0.00	0.02±0.02
NO_2-N	<0.01±0.00	<0.01±0.00	<0.01±0.00	<0.01±0.00
NO_3-N	0.26±0.07[a]	0.30±0.04[a]	<0.10±0.00[b]	0.30±0.04[a]
PO_4	1.17±0.17[a]	1.17±0.17[a]	0.33±0.08[b]	1.33±0.17[a]

Superscript [a,b] indicate significant differences between treatment ($\alpha<0.05$).

4.3.2 Bacteria

The heterotrophic plate count (HPC) was not significantly different between treatments at any time (Table 4.2). Variance was high within treatments, as inferred from the high standard errors. Mean bacterial abundance was comparably higher in experiment 2 than experiment 1, likely due to the increase in nutrient inputs from feed. During experiment 2, where *Bacillus* sp. was added in experiment 2 (TWC+B), *Bacillus* sp. were present in water in TWC + B in all 3 tanks after 7 weeks and 2 tanks after 10 weeks. *Bacillus* sp. were also present in one of the E-viro 3 tanks after 7 weeks. On TWC surface *Bacillus* sp. was found in TWC+B tanks but not in TWC tanks. HPC on the substrate surface was not significantly different between TWC and TWC+B, at 859±133 and 392±189 CFU/cm^3 respectively.

Table 4.2: Mean Heterotrophic Plate Count (HPC) in marron culture water.

HPC ($x10^4$) (CFU/mL)	Control	TWC	TWC + B	E-viro 3
Week 7	10.7 ± 5.25	14.1 ± 3.97	9.40 ± 0.600	9.07 ± 2.74
Week 10	5.63 ± 3.19	6.80 ± 3.61	34.1 ± 29.3	6.17 ± 1.88

4.3.3 Marron

Marron survival could not be measured accurately due to escapees. One mortality was found each in the control and TWC+B tanks, and 2 in TWC tanks. Cannibalism was the most frequent cause of mortality, often occurring after moulting. The average number of marron per tank was reduced from 3.0 to 2.0 after 3 months, and then to 1.75 after 4 months, primarily due to escapees. Neither substrate nor E-viro 3 significantly influenced the marron mortality rate, or monthly weight gain. However, in the E-viro 3 tanks average weight gain was higher in the second month, than in the first or third months; at 0.773 g±0.152, -0.310±0.213 and 0.107±0.272 respectively. Mean individual weight gain from start to 3 months was 1.37±0.67 g, 1.96±1.14 g, 1.51±0.45 g, and 0.90±0.42 g for control, TWC, TWC+B and E-viro 3 respectively.

The THC and DHC were not significantly different between treatments at any time. Within the control tanks, THC was significantly higher ($p<0.05$) after 12 weeks, at 4.96±0.97 x 10^6 haemocytes/mL, than after 3 weeks and 8 weeks, at 1.13±0.17 x 10^6 and 1.69±0.43 x 10^6 haemocytes/mL respectively. Negative correlation was present

between THC and TAN (p=0.017, R=-0.670). After 12 weeks, the proportion of granulocytes was not significantly different between treatments, varying from 12.0±0.7% with TWC to 14.3±3.6% with TWC+B.

4.3.4 Phytoplankton

The phytoplankton abundance was highest in TWC+B treatment in week 5 and week 7 (Figure 4.2), with weekly fluctuations (Table 4.3). Phytoplankton abundance grew exponentially and peaked after three weeks, and then fell again after five weeks. The mean number of genera found and the Shannon-Wiener Diversity Index (*H*) were not significantly different between treatments. The number of genera increased from the start to end of experiment; from 2-4 to 5-7 overall. Within TWC tanks, the Shannon-Wiener Diversity Index fluctuated over time (Table 4.4). The stock water was dominated by *Scenedesmus sp., Chlorella sp.,* and *Chlamydosomonas sp.* (Plate 4.2). Species found throughout the experiment included *Scenedesmus sp., Chlorella sp., Chlamydomonas sp., Euglena sp., Monoraphidium sp.,* and *Tetraedron sp.*

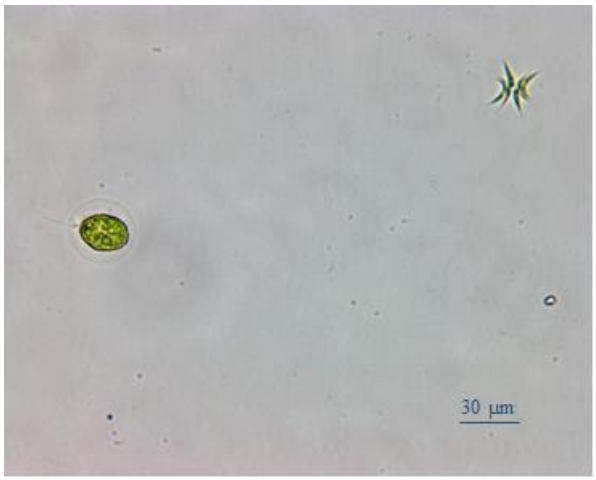

Plate 4.2: *Chlamydomonas* sp. and *Scenedesmus* sp. found in phytoplankton stock water, at 400x magnification.

Table 4.3: Mean phytoplankton abundance between four treatments over 18 weeks.

Phytoplankton abundance (cells / mL x 10^4)	**Control**	**TWC**	**TWC + B**	**E-viro 3**
Week 1	$^{w}1.8\pm0.2$	$^{x}2.8\pm1.1$	$^{x}2.9\pm0.59$	$^{x}2.3\pm0.6$
Week 3	$^{x}103.7\pm3.7$	$^{z}89.7\pm20.1$	$^{z}87.2\pm11.6$	$^{z}97.8\pm5.5$
Week 5	$^{y}20.3\pm2.6^{a}$	$^{xy}19.0\pm5.4^{a}$	$^{xy}35.3\pm1.2^{b}$	$^{x}9.7\pm2.7^{a}$
Week 7	$^{y}2.4\pm6.6^{a}$	$^{xy}2.3\pm4.6^{a}$	$^{yz}57.8\pm6.9^{b}$	$^{x}16.4\pm4.1^{a}$
Week 9	$^{y}29.0\pm3.7$	$^{xy}28.6\pm6.2$	$^{yz}67.5\pm23.0$	$^{y}55.5\pm12.2$
Week 11	$^{z}53.8\pm12.6$	$^{yz}57.8\pm8.7$	$^{yz}64.5\pm17.4$	$^{yz}69.3\pm24.0$
Week 18	$^{y}31.2\pm2.51$	$^{z}69.4\pm25.8$	$^{z}99.9\pm28.4$	$^{yz}78.7\pm1.0$

Superscript ^{a,b} indicate significant differences between treatment, while ^{x,y,z} indicate significant differences over time.

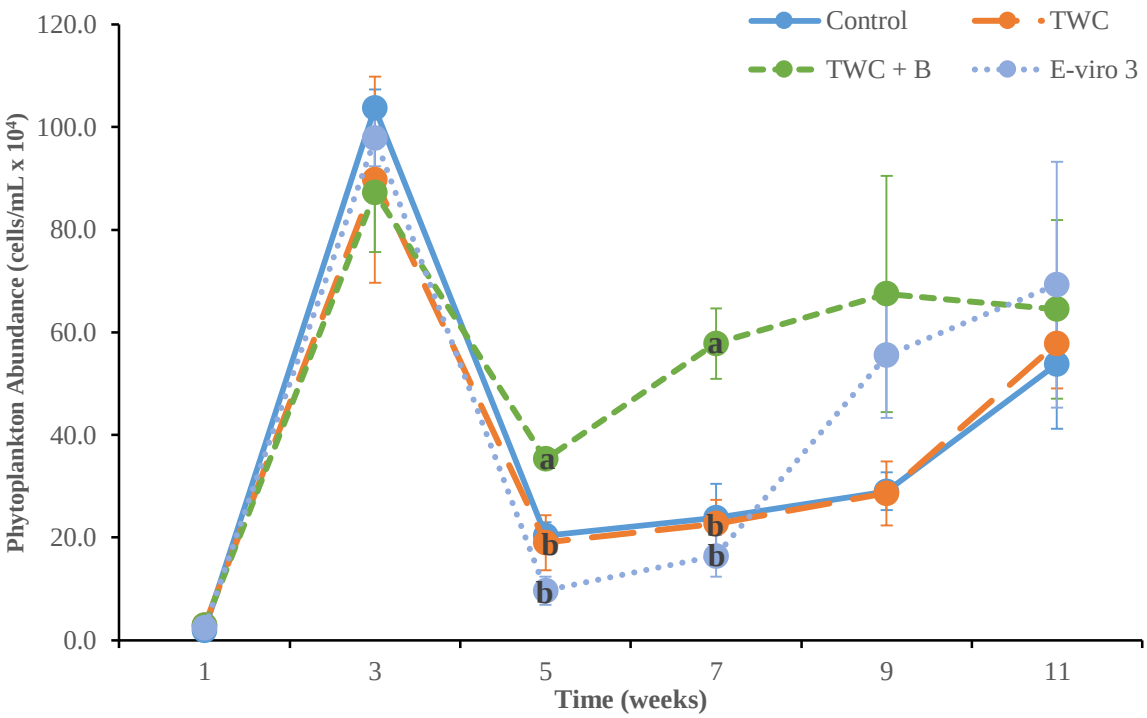

Figure 4.2: Changes in phytoplankton density (cells/mL) in different treatments over 11 weeks. Like letters (a, b) indicate that means are statistically similar ($\alpha<0.05$).

Table 4.4: Shannon Index (*H*) between four treatments over 18 weeks.

Shannon Index (*H*)	**Control**	**TWC**	**TWC+B**	**E-viro 3**
Week 1	0.91±0.13	^{yz}1.12±0.05	1.03±0.18	0.98±0.19
Week 3	1.01±0.09	^{xy}0.98±0.03	0.94±0.01	0.90±0.01
Week 5	0.92±0.12	^{z}1.21±0.04	1.29±0.08	0.95±0.25
Week 7	1.14±0.04	^{yz}1.12±0.04	1.23±0.09	0.91±0.14
Week 9	1.10±0.08	^{xyz}1.05±0.07	1.07±0.16	1.16±0.05
Week 11	1.04±0.06	^{xy}0.93±0.08	1.12±0.03	1.18±0.10
Week 18	1.22±0.16	^{x}0.90±0.10	1.18±0.05	0.77±0.19

Superscript ^{x,y,z} indicate significant differences between time (weeks).

4.4 Discussion

Past studies have found mixed results based on the effects of artificial substrates on water quality. Substrates made from mineral media have been used to culture microbial mats, which can then be used to remediate nutrient-enriched aquaculture effluent (Bender & Phillips, 2004), or used directly as filter media in aquaculture systems (Bender et al. 2004). Culturing biofilm on TWC prior to application could similarly improve the rate of bioremediation, as biofilm can take several weeks to develop on TWC surface. In contrast, a polyethylene mesh substrate was not effective in reducing concentrations of ammonia, nitrite, nitrate and orthophosphate in one study (Schveitzer et al. 2013). Similar results were found in the current experiment under outdoor culture conditions. The indoor experiment showed that oil-based substrates have a significant impact on water quality when nutrient concentrations are high and in the absence of high concentrations of microalgae, however. This was also under a controlled environment, with higher, more stable temperatures than in the outdoor experiment. The average temperature during the outdoor experiment, of 14.8°C, was lower than the optimum temperature of many nitrifying bacteria and *Bacillus* strains, at 25-35°C (Song et al. 2011; Hargreaves 1998), which may have limited the efficacy of TWC. Schveitzer et al. (2013) attributed the limited effects to a low amount of biological growth on the substrate surface. Biofilm was present on TWC in experiment 2, however the nutrient concentrations were likely to have been reduced mainly by phytoremediation from microalgae, as similar effects were observed with TWC and

without substrate. While microalgae such as diatoms and cyanobacteria can be grown on substrates to reduce nutrient concentrations in culture systems (Khatoon et al., 2007; Thompson et al., 2002), our observations suggest that TWC encourages the growth of a diverse consortium of heterotrophic rather than autotrophic microbes for bioremediation.

The oil-based substrates developed a thick layer of biofilm during both experiments, containing heterotrophic bacteria useful for water quality improvement. Biofilm growing on plastic substrates generally does not develop the same thick layer of biofilm (Personal observations), and is often dominated by cyanobacteria or microalgae rather than bacteria. A similar study suggested that TWC surface develops higher bacteria counts than the surface of plastic filter media. The study found heterotrophic bacteria counts to be 5 to 8 times higher on TWC than on standard filter media, with higher numbers of *Bacillus* sp. also present (Cole, A. unpublished data). The function in bioremediation between these different types of substrates may vary greatly, through this requires more extensive research for verification.

Substrates are rarely used in combination with probiotics to promote microbial growth. The combination of TWC and *Bacillus* sp. significantly reduced concentrations of nutrients in the first week of experiment 2 (Table 4.1). This is mainly attributed to the probiotic, as biofilm on TWC takes several weeks to become established. The commercial probiotic, E-viro 3, did not reduce the concentrations of nitrogen and phosphate, although previous studies have been found to decrease inorganic nitrogen and phosphate concentration in aquaculture water (Wang et al., 2005). TWC may help to maintain the effectiveness of probiotics for a longer time by providing habitat and a carbon source for the bacteria to thrive. In experiment 2, TWC+B maintained a population of *Bacillus* sp. on TWC surface.

The surface of TWC becomes abundant in heterotrophic bacteria after several weeks in the aquatic environment. TWC provides a habitat, and contains hydrocarbons; which is likely to provide an accessible carbon source for *Bacillus* sp. (Hu et al. 2017). *Bacillus* sp., is commonly used to improve water quality and in nitrate and phosphate removal (Hong et al., 2005, Boyd & Gross, 1998, Hu et al., 2017). The first experiment did not determine whether *Bacillus* sp. was present, though in experiment 2, *Bacillus* sp. was found on TWC surface where the bacteria was added initially. This suggests

that addition of probiotics into a system containing TWC can result in a continued population living on TWC surface. Observations showed that TWC surface also contained microalgae, but was dominated by heterotrophic bacteria.

There was a high abundance of phytoplankton and epiphytic microalgae present in the outdoor tanks, influencing the tank environment. Nitrate and phosphate levels remain low when microalgae biomass is high (Lananan et al. 2014). Nutrient levels are also a limiting factor on microalgae abundance (Zhihong et al. 2010). Nitrate and ammonia both had an inverse relationship with phytoplankton abundance, which can partly be explained by the uptake of nutrients as phytoplankton biomass increased, and the steady release of nutrients from dead algae cells (Seymour 1980). Meanwhile, TWC+B had a positive impact on phytoplankton abundance, which may be attributed to the addition of heterotrophic bacteria; which are important for nutrient mineralization in many plankton ecosystems (Caron, 1994). Addition of *Bacillus* sp. and *Lactobacillus* sp. has likewise been found to increase Chlorophyta abundance in shrimp recirculating systems (*Litopenaeus vannamei*) (De Paiva-Maia, Alves-Modesto, Otavio-Brito, Oliveira, & Vasconcelos-Gesteira, 2013). Microbial communities play a major role in recycling the autochthonous nutrients accumulating within the system (Avnimelech et al., 1995). In the current study microalgae including *Scenedesmus* sp. and *Chlorella* sp. were abundant, and it has previously been demonstrated that both are useful in the reduction of nutrients (Lananan et al., 2014, Uma Devi, Swapna, & Suneetha, 2014). Lananan et al. (2014) found that a combination of *Chlorella* and a microorganism was more effective in decreasing orthophosphate than either organism alone. A combination of microalgae and the *Bacillus* sp. added to TWC+B tanks reduced concentrations of orthophosphate effectively for the first two weeks, however this changed in the middle to end of the experiment where it was not different to the other treatments.

Phytoplankton grew exponentially at the start of experiment; as nutrients were available, and then the phytoplankton population crashed to a much lower biomass following the depletion of nutrients, reflecting patterns of an algal bloom. However, in the TWC+B tanks the phytoplankton density did not collapse to such low levels. If applied to natural systems this may help to prevent the depletion of oxygen associated with the collapse of algal blooms. The collapse of dense algal blooms often causes mortality due to depletion of oxygen by algal decomposition (Seymour, 1980). The

use of aerators prevented any depletion of oxygen in the present study. Here, as in natural systems, the 'bloom' of excess algae resulted in accumulation of dead algae (Pace & Lovett, 2013). Following the drop in phytoplankton abundance, the nutrients released from marron waste, excess feed and organic matter decomposition allowed the algae population to recover.

The slow growth of marron was mainly attributed to the low temperatures, as temperatures below 11-13°C can be limiting to growth (Morrissy, 1990). Meanwhile, there was no significant effect of any treatment on growth, and any biofilm growing on the floating TWC was physically inaccessible to marron, providing no complementary food source. Substrates can increase growth rates in shrimp and crayfish, by promoting the growth of periphyton for use as a complementary food source, or by providing habitat (Jones et al., 2002; Schveitzer et al., 2013). For example, Schveitzer et al. (2013) found an increase in growth and survival of *L. vannamei* attributed to a substrate providing increased surface area for habitat and food sources as periphyton. Meanwhile, Viau et al. (2012) have attributed improved growth and survival of *C. quadricarinatus* to maintenance of good water quality using biofilm.

Marron in this study were generally in good health. Meanwhile, there was a relationship found between the concentration of ammonia and THC. Exposure to ammonia, at a concentration of 1.37mg/L, can reduce the THCs of marron (Sang et al., 2009). During these times neither TWC, TWC+B nor E-viro 3 improved the THCs compared to the control. TWC+B might be expected to improve THC and DHC as addition of *Bacillus* sp. has improved DHC in shrimp (*Penaeus brasiliensis)* (De Souza et al., 2012), however in our study no improvement was found. Similarly, one would expect E-viro 3 to improve health and survival of animals, as previous studies have demonstrated increased survival in *L. vannamei* ponds treated with commercial probiotics (Wang et al. 2005), though no difference was found between treatments in terms of physiology or mortality rate.

4.5 Conclusions

TWC provided a habitat for heterotrophic bacteria, including *Bacillus* sp., in this study, and when combined with probiotics significantly reduced nutrient concentrations. The first experiment previously demonstrated that addition of TWC can maintain lower concentrations of nitrite and nitrate than without TWC. When applied to an outdoor culture system however, no significant effects on total phosphate, nitrite or nitrate were present. TWC had no significant negative effects to phytoplankton abundance or diversity, and had no significant effects on marron haemolymph, as THC and DHC. Meanwhile, TWC+B maintained a population of *Bacillus* sp. in the water column and the substrate surface. TWC+B also maintained a higher abundance of phytoplankton than any other treatment. The results show that TWC can be effective in bioremediation, and had no negative effects on phytoplankton or marron. This substrate is suitable for trialling in marron pond culture, and may be suitable in reducing the effects of high stocking density by improving water quality.

Chapter 5:

Effects of pond age and a commercial substrate (The Water Cleanser™) on natural productivity, bacterial abundance, nutrient concentrations, and growth and survival of marron (*Cherax cainii* Austin 2002) in semi-intensive pond culture

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(With some alterations, including Figures presented.)

Chapter 5 investigates the effects of The Water Cleanser™ (TWC) and pond age in marron pond culture. Experiments 1 and 2 showed the potential of the standard TWC in bioremediation, and demonstrated that the substrate had no detrimental effects on marron or microalgae. Therefore, TWC was selected as a suitable treatment for a field study. This field experiment investigates the effects of pond age and TWC on water quality, bacterial abundance, bacterial colony diversity, natural productivity and marron production on a commercial marron farm.

5.1 Abstract

The effects of a commercial substrate (The Water Cleanser™) (TWC), and pond age, on bacterial abundance, nutrient concentrations, natural productivity and marron production, were investigated on a commercial marron (*Cherax cainii*) farm. The farm had 21 ponds, categorised as: 10 new ponds (2 juvenile, 4 grow-out, and 4 brooder; 5 with TWC, 5 without) and 11 old ponds (5 juvenile, 4 grow-out, and 2 brooder; 5 with TWC, 6 without). At the end of 7 months, TWC had no significant effects on the concentration of total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N) and nitrate nitrogen (NO_3-N), but reduced the concentration of orthophosphate. The initial concentrations of NO_2-N, NO_3-N after 18 weeks, and orthophosphate after 12 weeks, were significantly higher in old ponds. The phytoplankton abundance, after 6 weeks and 18 weeks, and bacterial abundance after 6 weeks were significantly higher in old ponds. Water quality and natural productivity in old and new ponds showed temporal fluctuations. There were no significant effects of TWC on phytoplankton or zooplankton abundance and diversity. In old ponds, there was a significant increase in juvenile marron final biomass of 26.3% with TWC, attributed to higher survival rate. The results suggest that TWC can be used to improve juvenile marron production.

5.2 Introduction

Marron (*Cherax cainii* Austin 2002) are an omnivorous, polytrophic, parastacid crayfish and a target of fisheries and aquaculture in Australia (Molony & Bird 2005; O'Brien 1995; Rouse & Kartamulia 1992). Water quality is of key importance in all forms of aquaculture, including marron farming. Excessive dissolved concentrations of ammonia, nitrite and nitrates can have physiological impacts on crayfish (Jensen 1996). Nutrient-rich waters also stimulate growth of filamentous algae, which are an unlikely food source for crayfish and can greatly hinder crayfish mobility (Ulikowski et al. 2015). However, marron nutrition is partly dependent on phytoplankton growth, which is limited by nitrogen and phosphorous (Wallen 1979). Zooplankton are also an important food source for crayfish, especially for juveniles (Jones 1995). Seasonality can greatly affect the abundance and species composition of phytoplankton and zooplankton communities, partly due to fluctuations in solar radiation and temperature (Canovas et al. 1996; Armitage et al. 1973). The current trial

was conducted from October to April; comprising of warmer and variable temperatures in October and November (spring), high temperatures with low rainfall and a long photoperiod in January (summer), and cooler and variable temperatures in March and April (autumn), showing seasonal changes in the water quality and development of natural productivity.

The age of ponds affects water quality, including nutrient concentrations, and plankton communities; impacting on food availability, and growth of cultured animals (Correia et al. 2002; Zimba et al. 2003). Newer ponds may take time to become 'established' and develop microbial and invertebrate populations of high nutritional value (Allan et al. 1995). The age of ponds may also affect plankton composition, for example Zimba et al. (2003) found that older channel catfish ponds were dominated by cyanobacteria, compared to newer ponds which had higher numbers of centric diatoms and green algae. Growth of algae communities, associated with periphyton, can be encouraged via the addition of substrates (Santhana Kumar et al. 2017).

Substrates are used in aquaculture systems to provide sites for the development of biofilm, a microbial consortium associated with a matrix of extracellular polymeric substances bound to submerged surfaces (Pandey et al. 2014), that has a potential to improve water quality, and crustacean survival and growth (Khatoon et al. 2007; Otoshi et al. 2006; Viau et al. 2012). Various substrates may be used as habitat for heterotrophic bacteria, including duckweed (*Lemna minor)* (Ardiansyah & Fotedar 2016[b]). Heterotrophic bacteria may aid in bioremediation of nitrogenous wastes and phosphates (Li & Boyd 2016), and may enhance mineralization of organic matter, releasing ammonium into the water (Amin et al. 2012), but require a carbon source for certain functions, including denitrification (Hamlin et al. 2008). Concomitantly, addition of a carbon source may increase bacterial diversity (Hu et al. 2017). Carbon addition can change the predominant bacteria to *Bacillus* sp. over *Vibrio* sp., while use of a substrate (Aquamat®) has been shown to reduce *Vibrio sp.* counts in prawn ponds (Zhao et al. 2012; Santhana Kumar et al. 2017). Research suggests that attached biofilm, periphyton or particulate organic matter on artificial substrates can provide an additional food source for prawns and crayfish (Otoshi et al. 2006; Moss & Moss 2004; Viau et al. 2012). Substrates have been used to encourage growth of primarily

cyanobacteria and microalgae, including diatoms, to promote bioremediation and animal growth (Khatoon et al. 2007).

Oil-based substrates may provide both a habitat and carbon source, as hydrocarbon. More than 100 species of heterotrophic bacteria, including *Pseudomonas* sp. and *Bacillus* sp. are known to utilize hydrocarbons as a carbon source (Hu et al. 2017). Oil-based substrates may have multiple repercussions on the pond environment, such as improved water quality, enhanced ecology, improved animal health, and changes in bacterial composition. However, limited evidence based research has been conducted using oil-based substrates, especially in marron aquaculture.

The substrate used in this trial is an oil and wax based product known commercially as The Water Cleanser™, which acts as a substrate for the growth of heterotrophic bacteria and biofilm. TWC is thought to provide habitat to harbour bacteria, by providing a large surface area and a carbon source, maintaining populations of beneficial bacteria a prolonged period of time (Marine Easy Clean 2015). TWC is also a potential biofilter for nutrient removal. Attached biofilm may provide an additional food source for marron, as microbial productivity is known to contribute as food for crayfish growth (Morrissy et al. 1984). TWC may promote the growth of heterotrophic bacteria, certain species of which are beneficial to marron health (Ambas et al. 2013). By providing a carbon source, TWC would benefit the growth of *Bacillus* species already present in aquaculture ponds. This field experiment aimed to investigate the effects of a commercial substrate and pond age on water quality, natural productivity and marron production on a commercial marron farm.

5.3 Materials and Methods

5.3.1 Location

A field trial was conducted near Manjimup, Western Australia at a commercial marron farm (34°18’75” S, 116°06’61” E) (Figure 5.1; Figure 5.2). All laboratory based analytical work was conducted at the Curtin Aquatic Research Laboratories (CARL); Perth, Bentley, WA.

Figure 5.1: Location of study site.

Figure 5.2: Study site, near Manjimup, WA.

5.3.2 Study Design

Marron were stocked in ponds from May 2016, and harvested in May 2017 by farmer. Twenty-one ponds were selected for the study, and categorised according to life stage, pond age, and presence of The Water Cleanser™ (TWC), as: 10 new ponds (2 juvenile, 4 grow-out, and 4 brooder; 5 with TWC, 5 without) and 11 old ponds (5 juvenile, 4 grow-out, and 2 brooder; 5 with TWC, 6 without). Ponds were treated to the same commercial marron farming practices. Ten kilograms of TWC, supplied by Marine Easy Clean Pty Ltd, were added to treatment ponds, as 10 x 1 kg rectangular blocks, and dispersed evenly around the ponds. Blocks were designed to stand upright at the bottom of the ponds, as per the supplier's suggestion (Plate 5.1).

Plate 5.1: Photographs of marron ponds showing the The Water Cleanser block, and hides.

5.3.3 Animals

Marron were stocked as juveniles, monosex grow out 40-90 g, monosex grow out 95-130 g and brooders in May-June 2016. Adults were removed from brooder ponds following juvenile production in summer-autumn. Approximately 3500 juvenile marron were stocked in juvenile ponds, while 150-155 kg of 1+ marron were stocked in grow out ponds. Final biomass (kg per pond) was calculated for juvenile and grow out ponds at harvest in May-June 2017. Juvenile survival rate was estimated by counting the numbers stocked, calculating the average weight at harvest and estimating the number harvested.

5.3.4 Water Quality

Sampling was carried out every six weeks, in spring (October and November), summer (January), and autumn (March and April), over seven months. Ponds had a shallow gradient, approximate depth of 1.5 m and area of 1005 to 1325 m^2.

During sampling, pH, temperature and dissolved oxygen (DO) were taken with an Ecoscan pH meter 5 (Eutech), and Oxyguard Handy Polaris meter respectively in all ponds. Turbidity was measured using a secchi disc, with clear ponds given a turbidity reading of 150cm (approximate pond depth). 100 mL of water was taken from each ponds for nutrient concentration analysis. The concentrations of total ammonia nitrogen (TAN), NO_2-N, NO_3-N and orthophosphate were analysed using Permachem reagents from HACH® and a Skalar colorimeter auto-analyser (Downs et al. 2008). All measurements of water quality were in accordance with standard methods for the examination of water and wastewater (APHA 1998).

5.3.5 Bacteria

During daylight, 1.5 mL of pond water was collected in sterilized Eppendorf tubes for bacteria analysis. Samples were kept on ice during transportation, and were analysed for Colony Forming Units (CFU) within 24 hours of sampling. A 50 µL sample of pond water was directly cultured onto standard plate count agar (Oxoid, U.K.) using aseptic techniques. This dilution factor was adequate to attain plates with 30 to 300 colonies. After incubation for 48 hours at 30°C, colonies were counted and expressed as CFU/mL as previously described (Leonard et al. 2000); calculated by multiplying the colony count by the dilution factor. Colonies were identified according to colony morphology to estimate diversity and *Bacillus* sp. count. *Bacillus* sp. were distinguished by colony morphology. At the end of trial, 3 samples of TWC were collected from 3 randomly selected ponds and analysed for bacterial species identification by Matrix-assisted laser desorption ionization time-flight mass spectrometry (MALDI-TOF MS), by the Department of Agriculture (Western Australia).

5.3.6 Natural Productivity

Phytoplankton were sampled with a fine plankton net and concentrated from 500 mL of pond water into 100 mL plastic containers. Zooplankton samples were sampled with

a coarse plankton net of 60 micron, and concentrated from 15 L of pond water into 100 mL containers. Phytoplankton samples were preserved with 2-3% Acid Lugol's Iodine within 24 hours of sampling. Samples were then inverted several times, and a sub-sample was counted using a haemocytometer at 400x magnification. Identification was carried out to genus level, and species richness calculated, as number of genera per sample. Zooplankton samples were preserved in 70% Ethanol within 24 hours of sampling. Number and species present in 1mL sub-samples were counted in a petri dish at 20x magnification. Identification was carried out to family level, and species richness calculated, as number of families per sample. The presence of dinoflagellates, and water bug count per 100mL sample, was also measured. Plankton were identified with reference to Ingram et al. (1997). Phytoplankton and zooplankton abundance were calculated by using equations adapted from Ingram et al. (1997) and Nugroho & Fotedar (2013):

Phytoplankton Abundance (cells/L) = ((No. x 1000 / (volume of grid ($0.1mm^3$) x No. of grid squares counted)*(Conc. Vol. / 1mL)) /Tot. Vol.

Zooplankton Abundance (ind. /L) = (No. x (Conc. Vol. / Sub. Vol.)) / Tot. Vol.

where Tot. Vol. = Total volume of water (L) collected from the pond, Conc. Vol. = Volume of water (mL) containing concentrated plankton after sieving, Sub. Vol. = Sub-sample of water (mL) from concentrated volume in which plankton is counted, and No. = Mean number of cells or individuals counted.

5.3.7 Statistical Analysis

All data were expressed as mean ± standard error. Data was analysed between treatment and pond age with a multivariate two way analysis of variance (ANOVA) and independent t-tests. Least significant difference (LSD) post-hoc tests were used for multiple mean comparisons when the p value showed significant differences. For data which were not normally distributed Kruskal-Wallis and Mann Whitney tests were used. Finally, Pearson correlations were carried out to determine relations between water quality, productivity, and bacteria counts. All computations were done with SPSS version 23.0 and a p value of less than 0.05 was deemed significant.

5.4 Results

5.4.1 Water Quality

All water quality parameters were within the optimal range for marron (Morrissy 1984; Morrissy 1990; Environment Protection Water Quality Policy 2003), except TAN (Table 5.1). The maximum pH was higher than the optimum for *Cherax quadricarinatus* (Villarreal & Peláez 1999). Turbidity ranged from 20 to 150 cm (clear). There was no significant difference in water quality grand means between ponds with and without TWC over the course of the trial. However, pond age had a significant influence on water quality, bacterial abundance and phytoplankton grand means (Table 5.2).

Table 5.1: Optimum ranges for water quality compared to observed range for all ponds throughout the study.

Parameter	Optimum	Observed range
Temperature (°C)	11-30[1]	20.5-29.3
pH	6.5-9.0[4]	7.25-9.19
[a]DO (mg/L)	>5[2]	>6.3
[b]TAN (mg/L)	<0.01[3]	<0.3
NO_2-N (mg/L)	<1.0[3]	<0.089
NO_3-N (mg/L)	<10.0[3]	<2.0

[1] = Morrissy, 1990, [2] = Morrissy et al., 1984, [3] = Environment Protection (Water Quality) Policy, 2003, [4] = Villarreal & Peláez, 1999.

[a] TAN=Total Ammonia Nitrogen

[b] DO=Dissolved Oxygen

Table 5.2: Grand means and standard errors for water quality between old and new ponds (n=55, n=50 respectively).

Parameter	Old	New
pH	8.19 ± 0.05	8.30 ± 0.04
Temp (°C)	23.3 ± 0.30	23.2 ± 0.24
DO (mg/L)	7.92 ± 0.12[a]	8.46 ± 0.14[b]
Turbidity (cm)	73.53 ± 5.90[a]	97.78 ± 6.26[b]
TAN (mg/L)	0.056 ± 0.009	0.054 ± 0.008
NO_2-N (mg/L)	0.006 ± 0.002[a]	0.003 ± 0.001[b]
NO_3-N (mg/L)	0.441 ± 0.056[a]	0.296 ± 0.028[b]
Orthophosphate (mg/L)	0.35 ± 0.03	0.36 ± 0.06
HPC (CFU/mL) ($x10^3$)	3.02 ± 0.48[a]	1.78± 0.31[b]
Est. Bacteria Diversity	6.95 ± 0.50	6.16 ± 0.41
Phyto abundance (cells/L) ($x10^6$)	4.33 ± 0.48[a]	2.31 ± 0.23[b]
Phyto Diversity	6.26 ± 0.39[a]	4.94 ± 0.35[b]
Zoo abundance (ind./L)	99.7 ± 14.9	69.8 ± 10.8
Zoo Diversity	2.25 ± 0.088	2.43 ± 0.118

Superscript letters [a, b] indicate a significant difference ($p<0.05$).

There was no significant difference in pH, temperature, DO, or turbidity of ponds with and without TWC. The DO level was significantly higher in new ponds after 12 to 24 weeks. The pH and DO level were significantly higher in brooder ponds after 6 weeks, while life stage had no significant effect on the physico chemical parameters at all other times. Temperature was highest after 12 weeks (summer); with a mean of 24.60°C in old ponds and 24.81°C in new ponds (Figure 5.3). Turbidity was significantly lower in old ponds than new ponds, at 85.83 cm and 136.33 cm respectively, after 6 weeks. Pond age had no significant effect on turbidity for the remainder of the trial. No data were available for physico-chemical parameters in October.

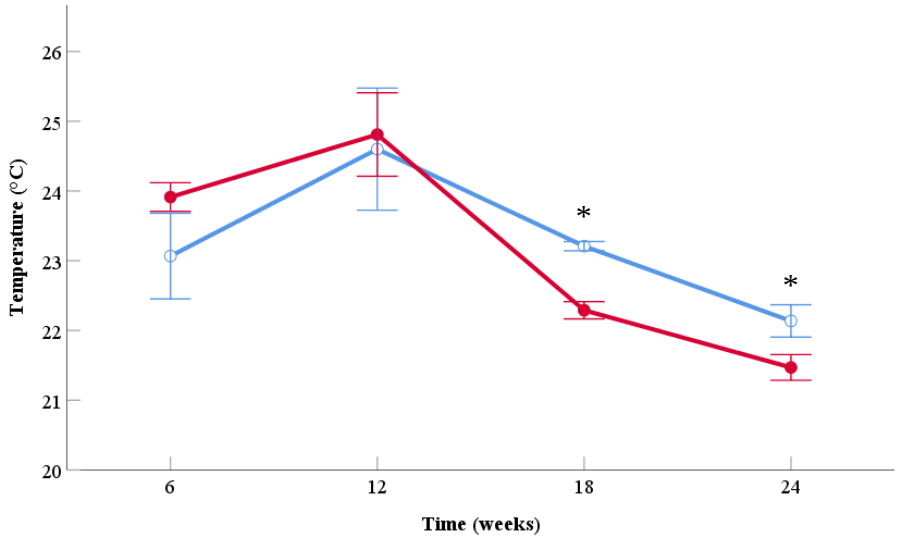


Figure 5.3: Variation of temperature from spring (6 weeks) to autumn (24 weeks), according to old ponds (open circles) and new ponds (filled circles), with mean and standard error (old ponds, n=11; new ponds, n=10). An asterisk represents a significant difference between pond age ($p<0.05$).

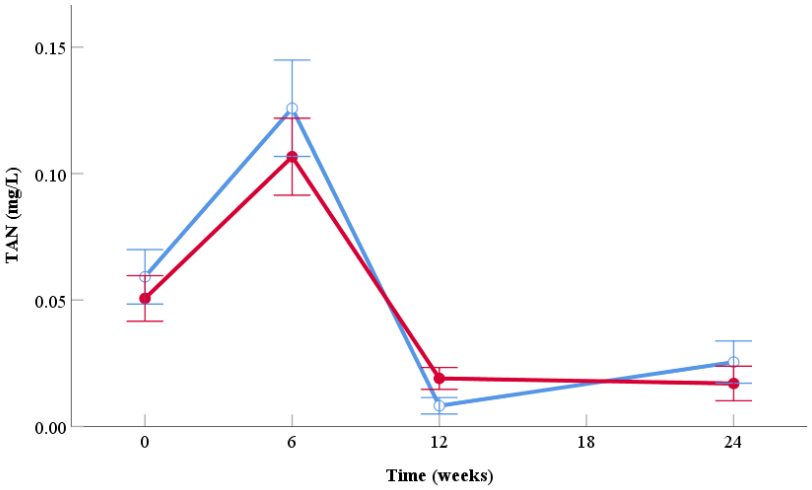
5.4.2. Nutrient Concentrations

Concentrations of TAN, NO_2-N, NO_3-N and total nitrogen were not significantly affected by TWC. After 24 weeks (April), significantly lower concentrations of orthophosphate were present in old ponds with TWC (Table 5.3). Meanwhile, pond age had a significant effect on all nutrient concentrations except TAN (Figure 5.4), and Total Nitrogen. Seasonally, TAN concentration peaked after 6 weeks, while all nitrogenous waste levels fell after 12 weeks (summer), coinciding with an increase in bacteria and plankton abundance. The concentrations of Total Nitrogen were lowest after 12 and 24 weeks. No TAN data could be obtained after 18 weeks.

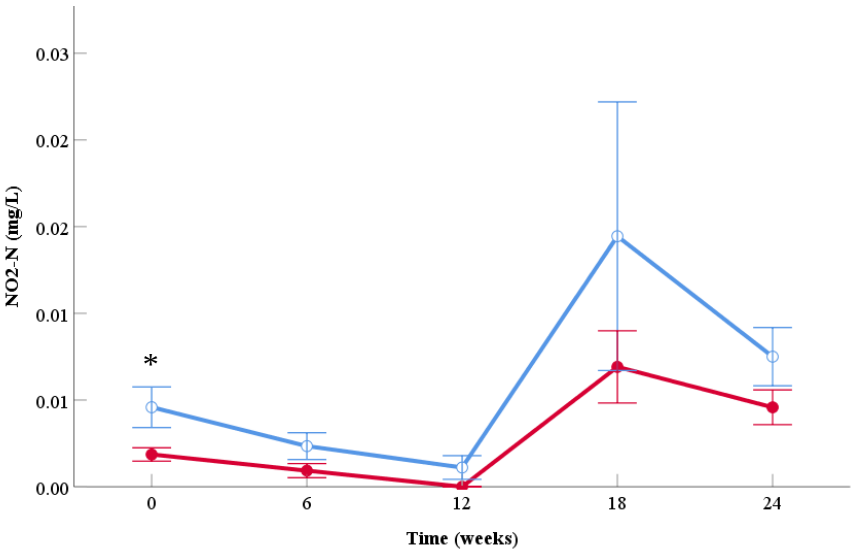
Table 5.3: Concentration of orthophosphate in old and new ponds with and without TWC.

Orthophosphate	**Control**		**TWC**		**Overall**	
	Old	**New**	**Old**	**New**	**Old**	**New**
0 weeks (Oct)	-	-	-	-	0.21 ± 0.06	0.27 ± 0.04
6 weeks (Nov)	0.09 ± 0.02	0.36 ± 0.18	0.19 ± 0.06	0.10 ± 0.04	0.13 ± 0.03	0.23 ± 0.10
12 weeks (Jan)	0.57 ± 0.07^{m}	0.12 ± 0.06^{n}	0.42 ± 0.03	0.24 ± 0.15	0.50 ± 0.05^{m}	0.18 ± 0.08^{n}
18 weeks (Mar)	0.51 ± 0.08	0.48 ± 0.07	0.65 ± 0.13	0.39 ± 0.07	0.57 ± 0.07	0.44 ± 0.05
24 weeks (Apr)	^{a}0.43 ± 0.22	1.12 ± 0.24	^{b}0.27 ± 0.24 ^{m}	0.45 ± 0.24^{n}	0.36 ± 0.04	0.79 ± 0.25

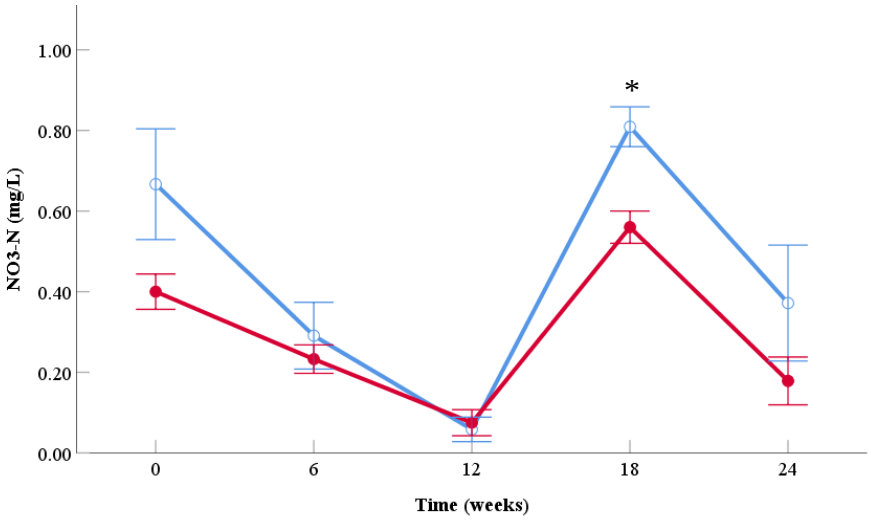
Superscript $^{a, b}$ indicate a significance between ponds with and without TWC; superscript $^{m, n}$ indicate a significant difference between old and new ponds ($p<0.05$).


TAN (mg/L)
0.15
0.10
0.05
0.00
0
6
12
18
24
Time (weeks)

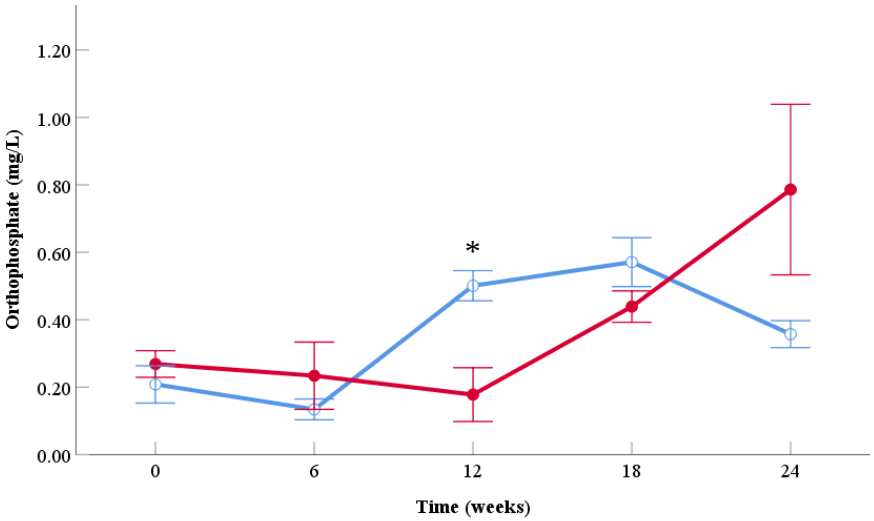
a)


NO2-N (mg/L)
0.03
0.02
0.02
0.01
0.01
0.00
*
0
6
12
18
24
Time (weeks)

b)


NO3-N (mg/L)
1.00
0.80
0.60
0.40
0.20
0.00
*
0
6
12
18
24
Time (weeks)

c)



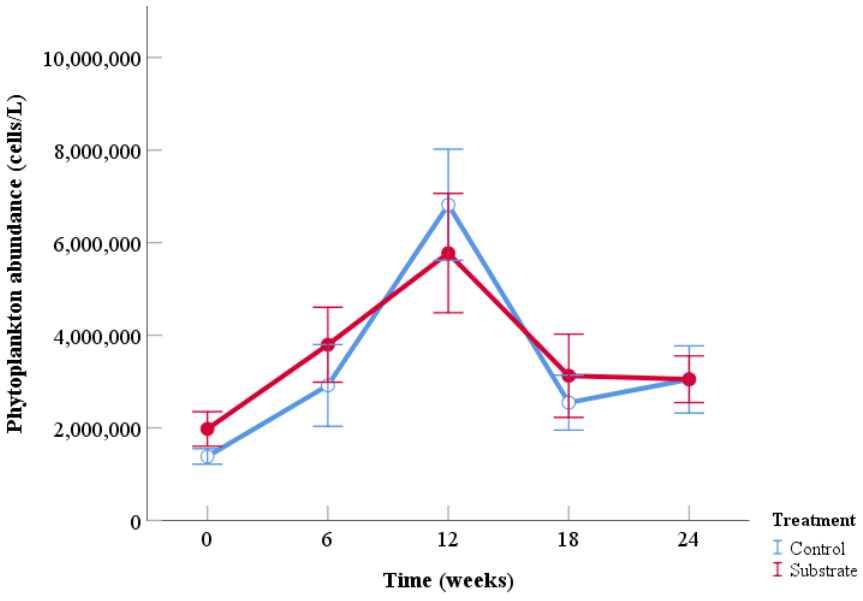
d)

Figure 5.4: Variation in concentrations of a) TAN (Total Ammonia Nitrogen), b) NO_2-N, c) NO_3-N and d) Orthophosphate, comparing old (open circles) and new (filled circles) ponds over time. No TAN data was at 18 weeks. An asterisk represents a significant difference between pond age ($p<0.05$).

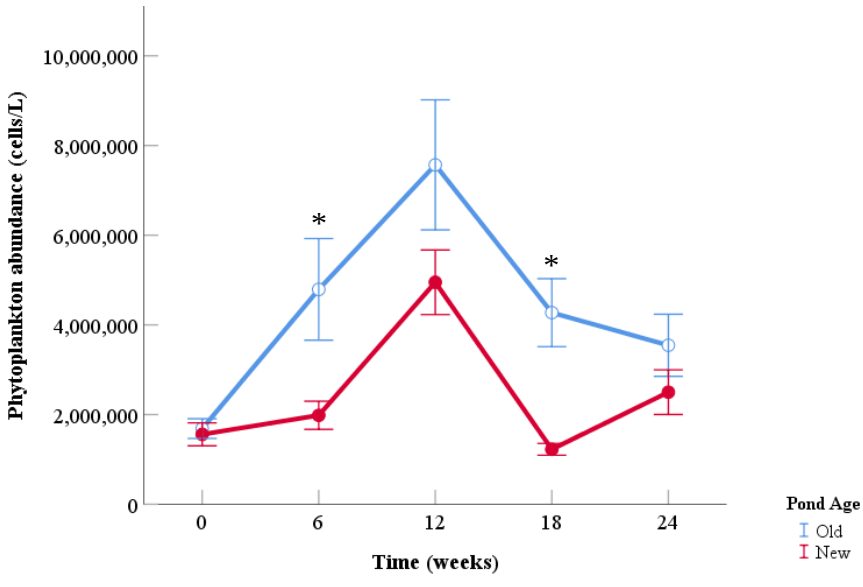
5.4.3. Natural Productivity

The Water Cleanser™ had no significant effect on the phytoplankton abundance and species richness. Meanwhile, both the abundance and species richness of phytoplankton were significantly higher in old ponds than new ponds after 6 weeks (spring) and 18 weeks (autumn). Phytoplankton abundance ranged from 500 000 to 14 500 000 cells/L. Species found during the study included *Closterium* sp., *Chlamydomonas* sp., *Scenedesmus* sp., *Monoraphidium* sp., *Spirogyra* sp., *Volvox* sp., *Pediastrum* sp., *Tetraedron* sp., *Chlorella* sp., *Pandorina* sp., *Euglena* sp., *Navicula* sp., *Aulacoseira* sp., unidentified dinoflagellates, and unidentified centric diatoms. Phytoplankton species richness was highest in the warmer seasons, after 6 weeks (spring) and 12 weeks (summer), while zooplankton species richness was highest after 18 weeks (autumn). Phytoplankton and zooplankton abundance were highest after 12 weeks (summer) (Figure 5.5). Zooplankton abundance ranged from 3 to 160 individuals/L and was not significantly affected by TWC or age of ponds, though was higher in the brooder life stage than juvenile or grow-out after 24 weeks (autumn). Phytoplankton species richness was higher than in juvenile ponds than brooder ponds after 6 weeks (spring), while in the first sampling, zooplankton species richness was higher in grow out ponds than brooder ponds, at 7.78 and 4.63 genera respectively. There was a significant interaction between substrate type and life stage, where

zooplankton species richness was higher in ponds with substrate in juvenile and grow-out ponds, but higher in ponds without substrate in brooder ponds, after 18 weeks (autumn). In ponds with TWC; zooplankton species richness was higher in new ponds after 6 weeks (spring), while phytoplankton species richness was higher in old ponds after 18 weeks (autumn). Zooplankton species richness was also higher in new ponds after 24 weeks, in ponds without TWC. The abundance of certain species appeared to be somewhat seasonal, e.g. Cladoceran abundance was greatest in spring. Zooplankton species found include rotifers; *Branchionus* sp. and *Keratella* sp.; Calanoida and Cyclopoida copepods (adult and nauplii); and Cladocerans *Daphnia* sp. and *Moina* species. Insecta (Orders Hempitera and Coleoptera) were commonly found.



a)



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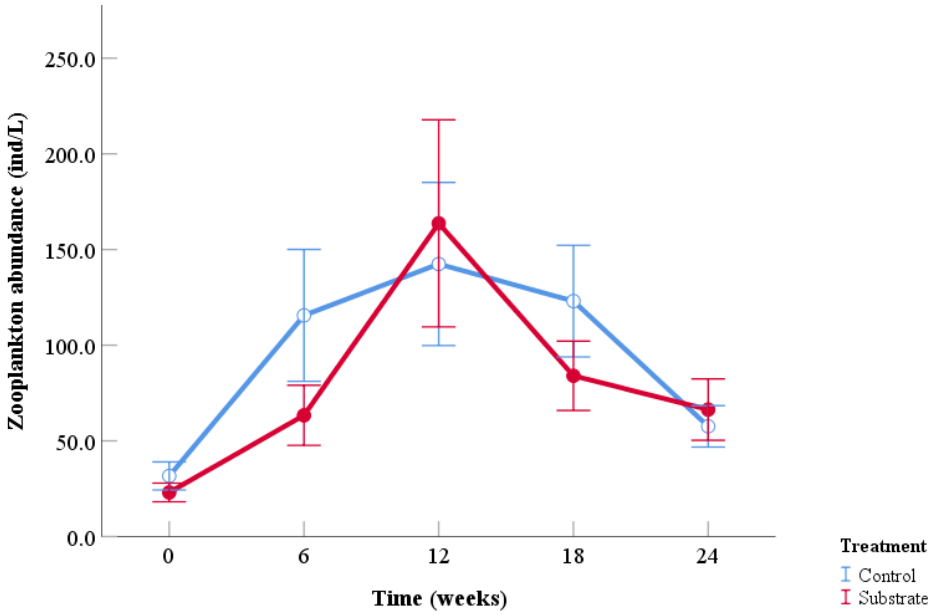


c)

d)

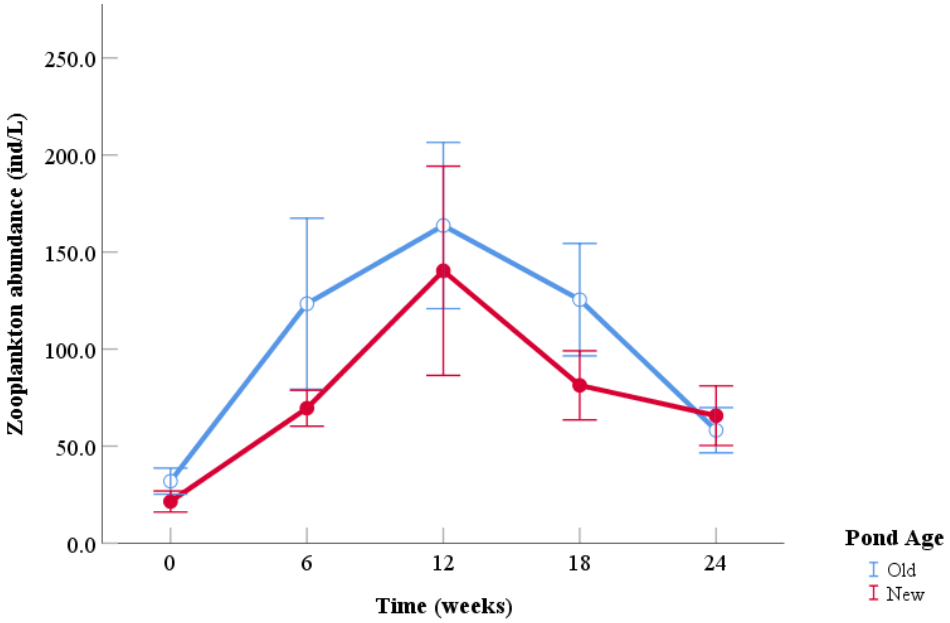


Figure 5.5: Fluctuation of phytoplankton (a, b) and zooplankton (c, d) abundance (no. / L), comparing with and without substrate (control), and pond age. An asterisk represents a significant difference between pond age ($p<0.05$).

5.4.4. Bacteria

The heterotrophic plate count (HPC), *Bacillus* sp. count and estimated colony diversity are summarized in Table 5.4. Life stage had no significant effect on bacterial abundance, while estimated colony diversity was higher in juvenile ponds than grow-

out and brooder ponds, in the first sampling. Biofilm was observed on TWC surface after six weeks. After 24 weeks, no bacteria data could be obtained. At the end of trial, TWC samples from three randomly selected ponds were analysed, using MALDI-TOF MS, and the following bacteria species were found: *Aeromonas eucrenophila, Pseudomonas brassicacearum, Bacillus cereus, Bacillus thuringienis, Pseudomonas anguilliseptica, Pseudomonas extremorientalis, Rheineimera soli, Aeromonas bestarium, Exiguobacterium sp., Pseudomonas cedrina, Aeromonas veronii,* and *Bacillus vietnamensis.*

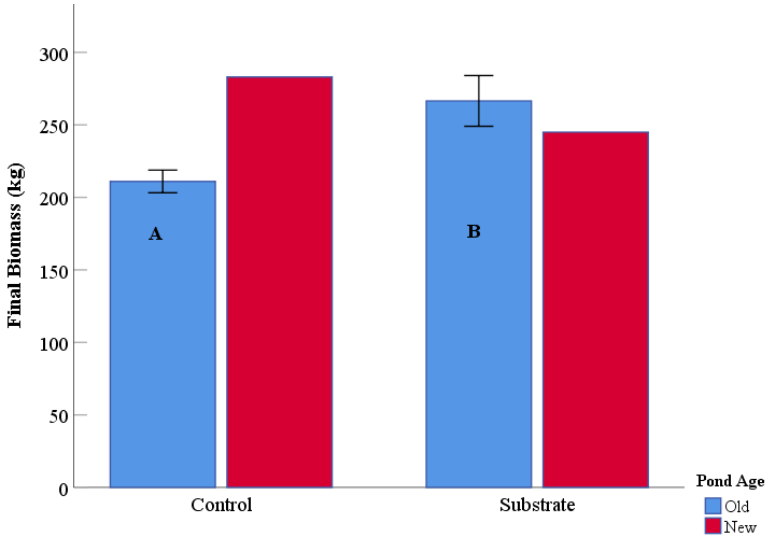
Table 5.4: The Heterotrophic Plate count (HPC) ($x10^3$) and *Bacillus* sp. count as CFU/mL and estimated diversity between old and new ponds with and without TWC.

Variable	**Control**		**TWC**		**Overall**	
HPC (CFU/mL)	**Old**	**New**	**Old**	**New**	**Old**	**New**
1st Sampling	3.58 ±	1.36 ±	3.23 ±	1.93 ±	3.40 ±	2.45 ±
(Oct)	3.03	0.62	2.26	1.69	1.75	0.92
2nd (Nov)	2.35 ±	1.20 ±	3.05 ±	1.42 ±	2.64 ±	1.30 ±
	0.90	0.23	0.88	0.17	0.62^{m}	0.14^{n}
3rd (Jan)	6.23 ±	3.94 ±	3.47 ±	3.21 ±	4.98 ±	3.58 ±
	1.30	1.70	1.10	1.00	0.93	0.94
4th (Mar)	1.17 ±	1.02 ±	1.24 ±	0.74 ±	1.20 ±	0.88 ±
	0.18	0.19	0.30	0.16	0.16	0.12
***Bacillus* sp.**						
1st (Oct)	<20.0	40.0	20.0	<20.0	20.00	40.0
2nd (Nov)	<20.0	20.0	20.0	<20.0	20.0	20.0
3rd (Jan)	36.7 ±	16.0 ±	48.0 ±	24.0 ±	41.8 ±	20.0 ±
	15.0	4.0	30.1	9.80	15.1	5.2
4th (Mar)	^{a}50.0 ±	20.0 ±	^{b}120.0 ±	20.0 ±	81.8 ±	20.0 ±
	10.0	12.7	26.1^{m}	9.43^{n}	16.5^{m}	9.4^{n}
Estimated						
colony diversity						
1st (Oct)	3.8 ±	3.6 ±	3.0 ±	2.5 ±	3.4 ±	3.3 ±
	0.5	0.7	0.7	0.3	0.4	0.4
2nd (Nov)	4.7 ±	4.9 ±	4.6 ±	5.0 ±	4.7 ±	4.9 ±
	0.5	0.4	0.3	0.3	0.3	0.2
3rd (Jan)	^{a}10.7 ±	7.4 ±	^{b}8.0 ±	9.4 ±	9.5 ±	8.4 ±
	0.6^{m}	0.7^{n}	0.3	1.4	0.6	0.8
4th (Mar)	^{a}10.8 ±	7.4 ±	^{b}8.0 ±	9.4 ±	9.6 ±	8.4 ±
	0.9^{m}	0.8^{n}	0.8	0.5	0.7	0.5

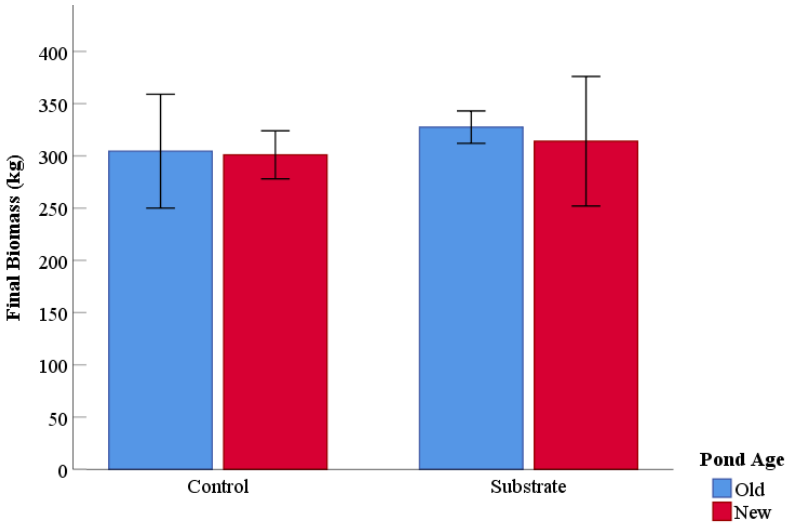
Superscript $^{a, b}$ indicate a significant difference between ponds with and without TWC; superscript $^{m, n}$ indicate significant difference between old and new ponds ($p<0.05$).

5.4.5. Marron Growth and Survival

Final biomass was calculated for grow-out and juvenile ponds at harvest. No growth data was available for brooder ponds. In juvenile old ponds, final biomass was significantly higher with TWC (Figure 5.6). There was a mean increase of approximately 26%. Final biomass was not significantly different between old and new ponds, or between ponds with and without substrate irrespective of pond age.



a)



b)

Figure 5.6: Mean final biomass for juvenile (a) and grow-out (b) ponds, between old and new ponds with substrate and without substrate (control). A and B represent significant differences ponds with and without TWC ($p<0.05$). Where no standard error bar is present n=1.

Survival rate of juvenile marron was not significantly different between ponds with or without substrate in old or new ponds, irrespective of pond age, or between old and

new ponds (Figure 5.7). However, survival rate was approximately 20% higher in old ponds with substrate than without.

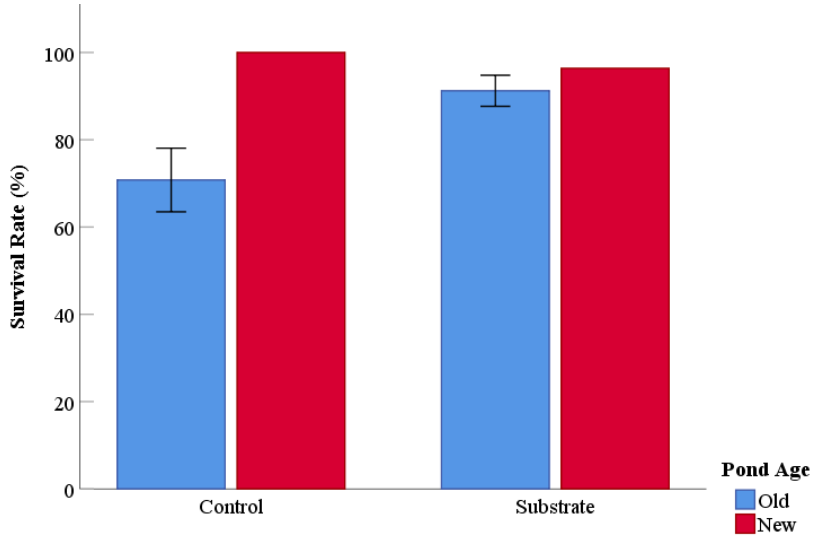


Figure 5.7: Survival rate in juvenile ponds between old and new ponds with and without substrate (control). Where no standard error bar is present n=1.

5.4.6 Correlations

No strong correlation (R>0.70) was present between phytoplankton and zooplankton abundance. Ponds with high abundance often had high species richness. There was a significant negative correlation between phytoplankton abundance and zooplankton species richness in old ponds after 6 weeks (p=0.005, R=-0.749) (Figure 5.8). There were no strong correlations found between nutrient concentrations and plankton parameters, or between the final biomass and survival rate of juvenile marron.

No strong correlations were found between bacteria abundance and other parameters. However, old ponds with higher orthophosphate also had higher bacteria diversity after 12 weeks (p=0.009, R=0.743) (Figure 5.9), where phosphorous may have been a limiting factor. Weak correlations were also found between HPC and nitrogenous nutrient concentrations. Ponds with higher HPC were also likely to have higher bacteria diversity (p<0.05). A two-way ANOVA found no significant interaction effects between pond age and substrate.

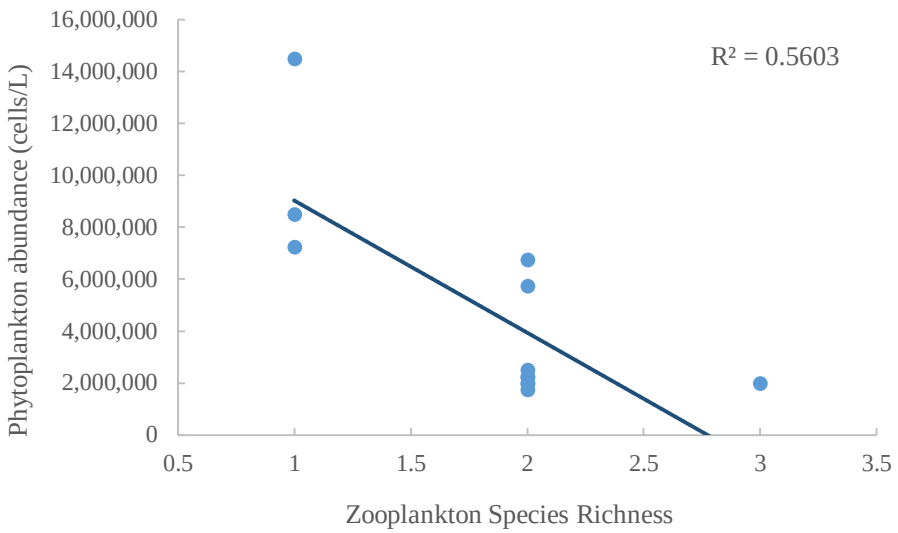


Figure 5.8: Correlation between phytoplankton abundance and zooplankton species richness in old ponds after six weeks (spring).

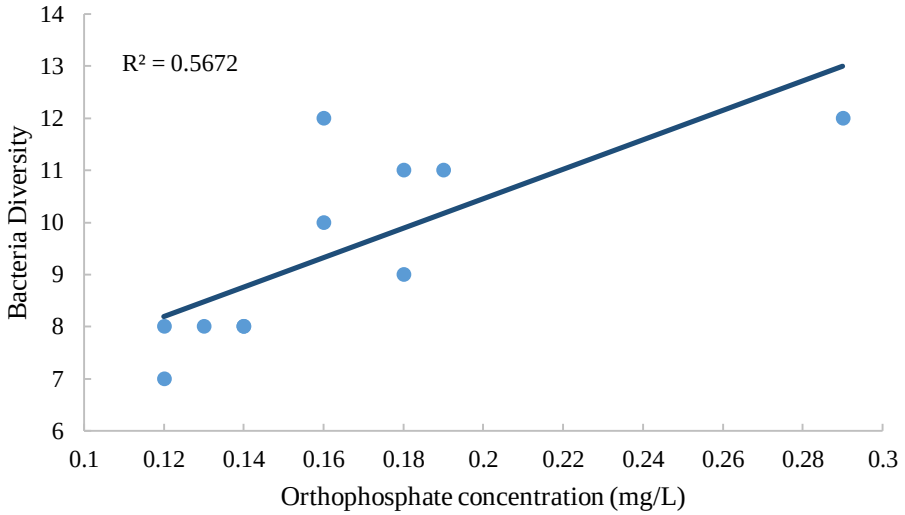


Figure 5.9: Correlation between the concentration of orthophosphate and estimated bacteria diversity in old ponds after 12 weeks (summer).

5.5 Discussion

The Water Cleanser™ had a significant effect on the juvenile marron, because this is a more critical stage of the crayfish life cycle, where mortality can be high (Ghanawi & Saoud 2012). Artificial substrates have previously improved survival rate in various prawn cultures including *Penaeus monodon* tank culture (Khatoon et al. 2007), and *Litopenaeus vannamei* pond culture (Santhana Kumar et al. 2017); both attributed to improved water quality. Viau et al. (2012) have shown that biofilm attached to artificial substrate can improve survival and contribute to growth of *C. quadricarinatus* (redclaw crayfish) juveniles, by providing a complementary food source, as well as improving water quality, which is essential for good survival. Jones et al. (2002) found that another species in the *Cherax* genus, *Cherax destructor*, consumes biofilm attached to artificial substrate. TWC also provided biofilm, containing bacteria, protozoans, nematodes, and microalgae including diatoms. Signs of biofilm utilization were present in both juvenile and grow out ponds. In *C. quadricarinatus* culture, analysis of stomach contents found that they consumed various periphyton found in biofilm, including ciliates, rotifers and nematodes (Viau et al. 2012), which can be nutritionally important (Da Silva et al. 2008). Better nutrition, provided by good natural productivity, can help to improve juvenile crayfish survival (Ghanawi & Saoud 2012).

Detritus and zooplankton are important food items due to their nutritional composition and availability (Browne et al. 1992; Jones 1995). In the present study substrate had no significant effect on zooplankton abundance, however. Uddin et al. (2009) found similar results in the zooplankton populations of tilapia (*Oreochromis niloticus*) and freshwater prawn (*Macrobrachium rosenbergii*) polyculture; where there were no significant differences between Rotifera and Crustacea numbers in ponds with and without bamboo substrate with attached periphyton. Phytoplankton composition varied however, where ponds with bamboo substrate had higher numbers of Bacillariophyceae, Chlorophyceae and Cyanophyceae than control ponds (Uddin et al. 2009).

Natural productivity generally increases in the warmer months (Armitage et al. 1973; O'Brien & deNoyelles 1974; Affan et al. 2005). The population of Calanoida copepods (*Skistodiaptomus pallidus*) in a large freshwater pond in Kansas, U.S. was

found to be more abundant in spring and autumn, and much lower in winter (Armitage et al. 1973). O'Brien & deNoyelles (1974) found a high level of chlorophyll *a*, indicating high phytoplankton abundance, in late summer, while Affan et al. (2005) found the lowest phytoplankton abundance in winter. In the current trial the highest abundance of phytoplankton and zooplankton was also in summer. While a relationship clearly exists between phytoplankton and zooplankton, no strong correlation was found between phytoplankton and zooplankton abundance in this study, as in another study (Armitage et al. 1973). McKnight et al. (1990) showed that increased zooplankton grazing can cause a decrease in phytoplankton abundance, however. In the current trial, higher zooplankton diversity was related to lower phytoplankton abundance, possibly showing enhanced grazing by certain species, such as rotifers (*Keratella* sp.). The increase in phytoplankton in summer in the current trial would have depleted the ammonia and nitrate levels, while the increased concentrations of orthophosphate in summer may have helped to trigger the increase in phytoplankton growth (O'Brien & deNoyelles, Jr. 1974).

The concentration of orthophosphate was significantly reduced in ponds with TWC after 24 weeks. A study in intensive shrimp (*Penaeus paulensis*) culture has shown the effectiveness of biofilm, attached to PVC tubes, in reducing ammonium and phosphate levels (Thompson et al. 2002). TWC had no significant effect on concentrations of nitrogen metabolites in marron ponds though, partly due to low nutrient concentrations. Fotedar (2004) reported ammonia and nitrite levels of 0.5 mg/L or less in semi-intensive marron ponds in Jurien Bay, Western Australia. In systems with higher nutrient loading, substrates have been used to improve water quality. A substrate (Aquamat) combined with sand sediment has resulted in low TAN and orthophosphate levels in shrimp (*L. vannamei*) tanks (Bratvold & Browdy 2001). Phosphorous in water can be oxidised to produce energy for microbes, and used for their growth and metabolism (Lananan et al. 2014). Phosphorous may then be converted into cellular polyphosphate and protein. Meanwhile, the application of an artificial substrate (Aquamat) into semi-intensive shrimp (*L. vannamei*) ponds has resulted in a reduction of 12% in the concentration of nitrogen in effluent, attributed to attached heterotrophic bacterial and algal biomass (Santhana Kumar et al. 2017). Observations from this field experiment and experiments 1 and 2 suggest that TWC may provide better habitat for heterotrophic bacteria than algae.

Biofilm containing heterotrophic bacteria was found attached to TWC surface in marron ponds. This could be the main cause for the higher *Bacillus* sp. count found in pond water with substrate. Another study has found a variety of heterotrophic bacteria, including *Bacillus* sp. and *Pseudomonas* sp., on duckweed substrate (Ardiansyah & Fotedar 2016[b]). Application of *Bacillus* sp. is generally used to increase *Bacillus* sp. counts in aquaculture water (Zokaeifar et al. 2014), however TWC maintains the *Bacillus* sp. population without further addition, by providing a habitat and carbon source, as hydrocarbon. *Bacillus* sp. have been shown to reduce concentrations of orthophosphate and nitrogen in aquaculture water (Wang et al. 2005; Lalloo et al. 2007; Xie et al. 2013). TWC with attached *Bacillus* sp. could affect marron via ingestion of biofilm or by altering the bacterial composition of water, potentially improving physiological condition of marron (Ambas et al. 2013). Addition of *Bacillus* sp. in water has been shown to increase survival of the Indian white shrimp (*Penaeus indicus*) in tanks and earthen ponds (Ziaei-Nejad et al. 2006). Of the species found on TWC surface; *Bacillus thuringienis* helps to maintain eco equilibrium and inhibits the proliferation of harmful organisms (Zhou et al. 2009), while *B. cereus* is a common and beneficial probiotic in aquatic animals (Deng et al. 2014; Hong et al. 2005; Balcázar et al. 2006). *Aeromonas* sp. and *Pseudomonas* sp. are common indigenous bacteria in aquatic animals, sometimes in association with animals, and ubiquitous in nature. *Pseudomonas* sp. are commonly used as probiotics, while others are pathogenic (Balcázar et al. 2006).

No discernible effect was present on bacterial abundance in the water column, largely due to the high variation in bacterial abundance. Schveitzer et al. (2013) also found no significant differences in microbial activity between tanks with and without substrate. Bacterial abundance in marron ponds was highest in summer, coinciding with a low concentration of nitrogen. The optimum temperature for denitrification, for nitrifying bacteria and for several *Bacillus* strains, is between 25°C and 35°C (Song et al. 2011; Hargreaves 1998). Phosphorous also limited the growth of certain species, as inferred by the significant correlation between orthophosphate and estimated bacterial diversity. Phosphorous is often a limiting element in microbial growth (Kirchman 2012).

Fertilization of newly-dug freshwater prawn ponds has been suggested by Correia et al. (2002), due to low biomass of microbial and macroinvertebrate

populations. Fertilization is uncommon in marron aquaculture though, due to the risk of eutrophication leading to algal blooms (Comm. with farmers). Nutrient concentrations, turbidity and dissolved oxygen levels were lower in new marron pond, resulting in lower phytoplankton abundance, bacterial abundance, *Bacillus* sp. count, and estimated bacterial diversity. In a study on channel catfish (*Ictalurus punctatus*) ponds, Zimba et al. (2003) also found that older ponds had significantly higher nitrogen and phosphorous concentrations. Pond age had no discernible effect on zooplankton abundance, but significantly higher species richness was present in new marron ponds. Abu Hena & Hishamuddin (2014) found similar results, recording no differences in zooplankton abundance between old and new ponds, and higher diversity and evenness in new ponds. Macroinvertebrate populations were more established in older ponds.

5.6. Conclusions

The trial demonstrated the significant effects of pond age on water quality, natural productivity and bacterial abundance. The Water Cleanser™ significantly improved the growth rate of juvenile marron in old ponds, attributed to a high survival rate, and to an extent to biofilm attached to the substrate providing a complementary food source. Additionally, there was higher abundance of *Bacillus* sp. in ponds with TWC. Effects on water quality were limited however, with a reduction in the concentration of orthophosphate only, in the final sampling. TWC may be applied to pond culture with no detrimental effects to natural productivity or marron, however more research is needed to determine its effects on plankton and microbial ecology, and marron health and physiology.

Chapter 6:

Comparing Bioball® Media and The Water Cleanser™ in reducing nutrient loading in marron (*Cherax cainii* Austin 2002) culture

Chapter 6 presents the third laboratory experiment conducted, comparing the effects of low and high nutrient inputs (as determined by marron stocking density) and two types of substrate (The Water Cleanser™, and Bioball® Media) in indoor marron tank culture. This may help determine if substrates can be used to reduce nutrient loading and improve marron health in aquaculture.

6.1. Introduction

In aquaculture, relatively low proportions of the nitrogen and phosphorous added through feed are retained in animal biomass with the majority wasted in water and sediment (Khoi & Fotedar 2010). For example, Thakur & Lin (2003) found that 22-30% N and 10-13% P was retained in *P. monodon* biomass. This can result in high nutrient loading; the difference between nutrient supplied, e.g. as feed, and nutrients harvested in the form of cultured species (Verdegem 2013). In marron (*Cherax cainii*) farming, eutrophication, build-up of organic matter and high biological oxygen demands can cause health issues such as hypoxia (Morrissy et al. 1984). One solution to reduce nutrient concentrations, thereby improving survival and growth of crustaceans, is bioremediation using substrates (Viau et al. 2012; Viau et al. 2013).

Artificial substrates have previously been used to lessen the effects of high stocking density, by providing additional habitat and a complementary food source in biofilm (Pandey et al. 2014; Schveitzer et al. 2013; García-Ulloa et al. 2012; Jones et al. 2002; Otoshi et al. 2006). There is thought to be an inverse relationship between stocking density, and growth and survival of freshwater crayfish (Verhoef & Austin 1999; Naranjo-Páramo et al. 2004; Jones & Ruscoe 2000; Mills & McCloud 1983; Geddes 1993). One of the reasons for a reduction in growth rate at higher stocking density could be the deterioration of water quality present at high densities (Naranjo-Páramo et al. 2004). Nutrient loading is likely to increase with increasing stocking density, due to corresponding higher feed inputs and animal waste. The addition of substrates which promote bacterial growth can be used to reduce nutrient concentrations in shrimp and crayfish culture. Substrate addition has been shown to improve the survival rate of crustaceans, by reducing the relative stocking density, providing a complementary food source, and/or improving the water quality (Schveitzer et al. 2013; Khatoon et al. 2007; Viau et al. 2012). The water cleanser (TWC) is one commercially available substrate that could be used to negate some of the negative effects of increased nutrient loading associated with high stocking density, such as poor water quality and animal health.

The previous chapters have shown that TWC can improve water quality by reducing concentrations of nitrites, nitrates and orthophosphates. Bioball® Media (BM) can also be effective in removing nutrients from water (Masłoń & Tomaszek 2015). However,

no comparison has been made between TWC and other substrates in the previous chapters, while no reduction in the concentration of ammonia, or any significant improvement in marron health has been shown with addition of TWC. Concomitantly, no peer-reviewed research has investigated the effects of TWC in marron aquaculture. The aim of this experiment was to compare the efficacy of a filter media substrate, Bioball® Media (BM), and an oil-based substrate, TWC, in reducing nutrient loading in marron culture with different rates of nutrient inputs as a result of different stocking densities. Furthermore, the experiment investigated the effects of these treatments on marron growth and health, and bacterial abundance in water.

6.2. Methodology

6.2.1. Location

All laboratory work was carried out at the Curtin Aquatic Research Laboratories (CARL), Curtin University, Bentley, Perth, Western Australia. The experiment was conducted indoors in a temperature-controlled aquaria facility.

6.2.2. Animals

For the experiment, marron (weight = 62.14±1.90g, and OCL (Orbital Carapace Length) = 59.7±0.59mm) were brought from a commercial marron farm in Manjimup, Western Australia (site of field trial). They were acclimatised for one week in three 200 L tanks with PVC pipes as shelters, aeration and daily water exchange.

During the experiment, marron were kept in individual cages made by connecting two 17 cm diameter Clearpond™ planter baskets at the open ends (Plate 6.1). The cages were designed to prevent cannibalism and escapees, and to allow for individual feeding. Marron were monitored for condition regularly and any mortalities were removed, weighed, and replaced with stock marron to maintain stocking density. Mortalities were recorded as a measure of survival rate. Individual marron weight and OCL were measured every month. Then, the monthly weight gain, weight gain for 2 months (0-60 days), and weight gain for 3 months (0-90 days) were calculated. Moults were recorded, however were too infrequent to allow for moult increments or moult frequency to be calculated.

6.2.3. Experimental Design

The experimental units were distributed in a standardised block design, with four replicates for each treatment level. Stocking density was used to provide low (SD1) and high (SD2) nutrient loadings. The three treatments were: 1) no substrate, 2) The Water Cleanser™ (TWC), as 100g rectangular floating blocks (93x93x20mm), and 3) 100g of Bioball® Media (BM) (diameter=25.4mm) (Plate 6.3). Marron were stocked at two densities: SD1) 2 marron per tank (approximately 454g/m^2) and SD2) 4 marron per tank (approximately 908g/m^2). Four replicates of each of the three substrate treatments were used for each stocking density, totalling 24 experiment units. Twenty four cylindrical 100 L water tanks (diameter=58cm, height=46cm; surface area=1.10m^2) were filled to 80L with filtered freshwater and aerated for the experiment, without mechanical filtration. Regardless of treatment, each tank contained 4 of the planter basket cages, to standardise any potential effect of the presence of the cage, as microbial substrate. The experiment ran for 3 months, from May to August 2017.

6.2.4. Feed and Nutrient Loading

Marron were fed individually at a rate of approximately 0.5% of body weight daily for the first four weeks, and three times a week for the remaining period of time. The reduction in feeding was required due to a build-up of excess feed. The feed was fishmeal-based, with an average of 30.7% protein, an energy content of 18.63 MJ/kg and 14% moisture content. The feed was manufactured at the CARL, unlike that in experiment 3 (Chapter 4) which was made commercially, and had a higher protein content. Nitrogen and phosphorous concentrations of the feed were approximately 4.91% and 1% respectively. Uneaten feed was removed twice a week. Derived from feed inputs, the estimated nutrient loading for the entire experiment was approximately: 1.513 g N and 0.312 g P at low stocking density, and 3.06 g N and 0.624 g P at high stocking density. The nutrient budget could not be calculated due to the removal of uneaten feed.

Plate 6.1: The cylindrical white tanks and black planter-basket cages used in the experiment (Cages remained horizontal during the experiment).

6.2.5. Water Quality Sampling and Analysis

The pH was measured with a pH meter (Ecoscan pH 5, Eutech Instruments, Singapore) and DO and temperature with a DO meter (Handy Polaris, Oxyguard, Denmark) twice a week, diurnally. Ammonia, nitrite, nitrate and total phosphate were measured twice a week with API test kits (Aquarium Pharmaceuticals Incorporated™, USA). Nutrient concentrations were standardised using Permachem test kits (HACH®, USA) and a colorimeter auto analyser (Skalar, The Netherlands) (Downs et al. 2008). The concentration of orthophosphate was determined after seven weeks using the molybdovanadate method.

During the first month, there was a deterioration of water quality and an increase in the concentration of nitrite (>4 mg/L) in several tanks. As there was a high likelihood that water quality would deteriorate further, to prevent marron mortalities, a 90% water exchange was carried out in all tanks after four weeks. Afterwards, the feeding rate was decreased from daily feeding to three times per week to help alleviate this. There were no further water exchanges throughout the experiment.

Observations were taken during the experiment, monitoring amounts of excess feed, waste and fungal growth in tanks, changes in marron condition, and microbial growth on the substrates.

6.2.6. Analysis of Haemolymph

Haemolymph samples from marron were taken every 2 weeks to monitor stress. A 0.3mL aliquot of haemolymph was withdrawn from the underside of marron between the 4th and 5th thoracic leg, using 27 G needles with 1 mL syringes containing 0.3 mL of anticoagulant (trisodium citrate). Marron were marked for identification and returned to cages. Haemolymph samples were then placed into an Eppendorf tube kept on ice, and analysed within 6 hours. Total haemocyte count (THC) was estimated using a haemocytometer under 400x magnification. The total haemocyte count was calculated as THC = (cells counted x dilution factor x 1000)/volume of grid (0.1 mm^3). Samples were stained with Giemsa and May-Grunwald to analyse the differential haemocyte count (DHC), which was determined following established protocol (Sang et al. 2009).

6.2.7. Analysis of Organosomatic Indices

At the end of trial, one marron was randomly selected from each tank for tissue sampling. Excess moisture was removed, marron were weighed for total weight, and OCL was measured with a calliper. Marron were dissected, the carapace was removed, the hepatopancreas was placed into a crucible and weighed, and the tail muscle was removed from the exoskeleton and placed in a crucible and weighed, to determine wet weight. Crucibles were placed in an oven (Thermotec 2000, Contherm, New Zealand) for 24 hours at 105°C, before being placed into a desiccating chamber for 30 minutes, and reweighed to determine dry weight.

The hepatopancreas moisture content (HM%), tail muscle moisture content (TM%), wet hepatopancreas index (Hiw), dry hepatopancreas index (Hid), wet tail muscle to body ratio (Tbw) and dry tail muscle to body ratio (Tbd) were calculated using established equations (Fotedar 1998):

$$HM\% = (WHwet - WHdry) \times 100 / WHwet$$

$$TM\% = (WTwet - WTdry) \times 100 / WTwet$$

Where: WHwet is the weight of the wet hepatopancreas (g)

WHdry is the weight of the dry hepatopancreas (g)

WTwet is the weight of the wet tail muscles (g)

and WTdry is the weight of the dry tail muscles (g)

Tbd = WTwet x 100 / Wt

Tbw = WTdry x 100 / Wt

Hiw = WHwet x 100/ Wt

Hid = WHdry x 100 / Wt

Where: Wt = Total weight of the animal (wet)

6.2.8. Bacteria Sampling and Analysis

Heterotrophic plate count (HPC) of the water column, in colony forming units (CFU)/mL, was determined after 2, 6 and 11 weeks; before substantial bacterial growth was thought to have developed, after substantial growth had developed, and four weeks following this. Bacteria samples were collected in 1.5mL Eppendorf tubes directly from the tank water within 30 minutes of plating. Using a pipette, 10µL of water was directly transferred onto Tryptone glucose yeast agar (Standard Plate Count Agar) using aseptic techniques. Agar plates were prepared as per Chapter 3. Samples were spread using a hoop, which was flamed between samples. Petri dishes were wrapped in alfoil and placed for in an incubator (Thermoline, Australia) for 48 hours at 30°C. Viable colonies were counted and identified by colour and colony morphology to estimate diversity after 24 hours and 48 hours under 20x magnification. *Bacillus* sp. were identified by colony morphology and counted (Plate 6.2). *Bacillus* sp. were sampled, fixed onto a glass slide and stained with crystal violet stain for further identification (Plate 5.4).

Swabs were taken from approximately 1cm^3 of TWC and BM surface, and cultured on Tryptone glucose yeast agar. Colony count and identification was carried out after 48 hours of incubation.

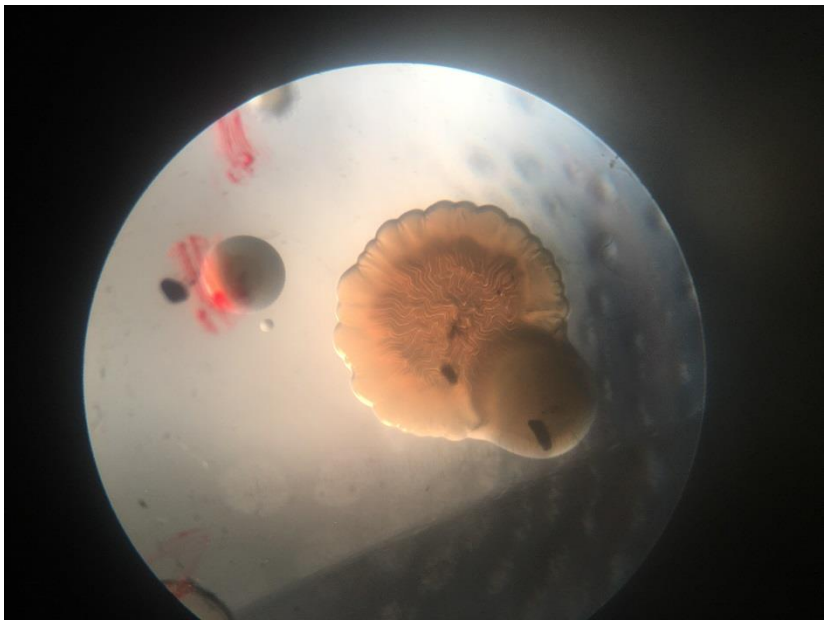


Plate 6.2: Heterotrophic bacteria colonies after 48 hours incubation, during counting procedure, under 20x magnification.

6.2.9. Statistical Analysis

Patterns in the data were expressed as mean ± standard error. Data were analysed between substrate type (Control, BM, TWC), stocking density (SD1, SD2), and week (1 to 13) with a univariate three factor mixed model analysis of variance (ANOVA) and independent samples t-tests. Least significant difference (LSD) post-hoc tests were used for multiple mean comparisons. For data which were not parametric, Kruskal-Wallis and Mann Whitney tests were used. Pearson correlations were used test for associations between measured parameters. SPSS version 23 was used for all statistical analyses and a p value of less than 0.05 was deemed significant.

6.3. Results

6.3.1. Observations

After six weeks, a thick layer of biofilm was observed on the submerged surface of TWC (Plate 6.3). No visible biofilm was visible on the surface of the BM. Microorganisms, including ciliates of varied size, other protozoans, minute nematodes, microalgae, fungi, and various unidentified bacteria (majority rod-shaped) were present in the biofilm attached to TWC, as determined by microscopy.

Marron were generally in good condition, though some turned a blueish hue towards the end of experiment; possibly due to a lack of carotenoids in the artificial diet (Sommer et al. 1991). Epibionts were found attached to marron, including *Temnocephala* eggs, indicative of poor water quality (Holdich 2002). Fungi, possibly *Saprolegnia*, grew on any uneaten feed and marron carcasses.

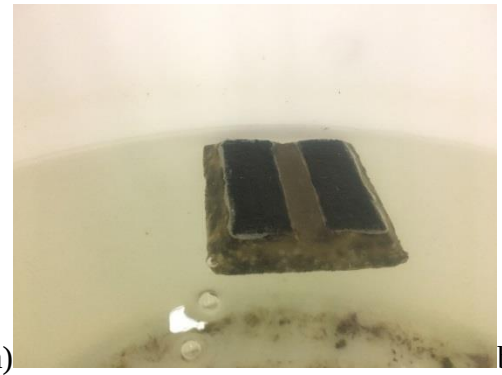
a)  b) 

Plate 6.3: TWC with submerged biofilm (a), and the Bioball® Media used (b).

6.3.2. Survival

There were 10 mortalities throughout the experiment: 2 in control tanks, 1 at low and 1 at high stocking density; 4 in BM tanks, 1 at low and 3 at high stocking density; and 4 in TWC tanks, all at high stocking density. No cannibalism or escapees occurred. Mortality often occurred after moulting, attributed to poor water quality, as inferred by the presence of fungal growth in the tank water and general observations indicating that high levels of organic matter were present.

6.3.3. Water Quality

The pH, temperature and dissolved oxygen (DO) remained within suitable ranges for marron culture (Table 6.1), and were not significantly different between substrate type or stocking density. The minimum recorded temperature of 14°C would not have been too low to prevent growth of marron, while the maximum of 20°C would have allowed for favourable growth (Morrissy 1990). In control tanks the DO was significantly lower at SD2 after 4 and 8 weeks (Table 6.2), reflecting a high biological oxygen demand.

Table 6.1: Mean ± standard error and range of the physico-chemical parameters measured in all tanks throughout the experiment.

	pH	**Temp (°C)**	**DO (mg/L)**
Mean	7.57 ± 0.01	17.0 ± 0.06	9.41 ± 0.02
Min	7.0	14.0	7.0
Max	8.0	20.0	10.0

Table 6.2: Mean DO between low (SD1) and high (SD2) stocking density, in weeks 4 and 8.

	Control		**BM**		**TWC**	
DO (mg/L)	**SD1**	**SD2**	**SD1**	**SD2**	**SD1**	**SD2**
Week 4	9.42^{m} ± 0.03	9.17^{n} ± 0.09	9.36 ± 0.06	9.26 ± 0.04	9.41 ± 0.06	9.26 ± 0.04
Week 8	9.86^{m} ± 0.09	9.72^{n} ± 0.12	9.78 ± 0.11	9.86 ± 0.12	9.86 ± 0.09	9.78 ± 0.11

Superscript letters $^{m, n}$ indicate significant differences between SD (stocking density) ($p<0.05$).

The NH_3, NO_2, NO_3, and PO_4 concentrations were periodically high during the experiment (Table 6.3).

Table 6.3: Mean ± standard error and range for nutrient concentrations in all tanks throughout the experiment.

	NH_3 (mg/L)	**NO_2 (mg/L)**	**NO_3 (mg/L)**	**PO_4 (mg/L)**
Mean	0.06 ± 0.01	0.68 ± 0.04	11.26 ± 0.36	1.07 ± 0.07
Min	<0.01	<0.01	<0.01	<0.01
Max	3.00	5.00	40.00	6.00

In terms of grand means, water quality and bacterial abundance and diversity were similar between treatments, though NO_2 concentration was significantly lower in tanks with substrate, at SD1 (Table 6.4). In comparison to SD1, SD2 resulted in a significant increase in the concentration of NH_3 in TWC tanks, NO_3 in tanks without substrate, and PO_4 in all treatments.

Table 6.4: Mean ± standard error for water quality and bacteria parameters in all tanks throughout the experiment (n=24 x no. of samplings).

Variable	Control		BM		TWC	
	SD1	SD2	SD1	SD2	SD1	SD2
NH_3 (mg/L)	0.06 ± 0.02	0.07 ± 0.02	0.05 ± 0.01	0.12 ± 0.04	0.02^{m} ± 0.00	0.05^{n} ± 0.01
NO_2 (mg/L)	^{a}0.77 ± 0.11	0.80 ± 0.10	^{ab}0.65 ± 0.10	0.74 ± 0.09	^{b}0.43 ± 0.08	0.67 ± 0.10
NO_3 (mg/L)	10.57^{m} ± 0.77	13.94^{n} ± 1.15	11.35 ± 0.79	10.30 ± 0.73	9.62 ± 0.69	11.80 ± 0.96
PO_4 (mg/L)	0.69^{m} ± 0.11	1.34^{n} ± 0.21	0.94^{m} ± 0.14	1.72^{n} ± 0.26	0.50^{m} ± 0.08	1.22^{n} ± 0.18
pH	7.57 ± 0.03	7.57 ± 0.03	7.58 ± 0.03	7.57 ± 0.03	7.58 ± 0.03	7.56 ± 0.03
Temp (°C)	16.98 ± 0.14	17.04 ± 0.14	17.01 ± 0.14	16.88 ± 0.14	16.97 ± 0.14	16.98 ± 0.14
DO (mg/L)	9.47 ± 0.05	9.33 ± 0.06	9.40 ± 0.06	9.41 ± 0.06	9.48 ± 0.06	9.37 ± 0.06
HPC ($CFU/mLx10^3$)	14.52 ± 2.07	16.88 ± 3.84	13.57 ± 2.11	13.59 ± 2.03	11.64 ± 1.91	18.76 ± 2.95
***Bacillus* sp. ($CFU/mLx10^3$)**	0.64 ± 0.54	0.20 ± 0.06	0.20 ± 0.11	0.03 ± 0.03	0.06 ± 0.03	0.14 ± 0.09
Colony Diversity	8.25 ± 0.51	8.25 ± 0.72	8.38 ± 0.55	7.36 ± 0.44	7.63 ± 0.18	8.75 ± 0.57

Superscript letters $^{a, b}$ indicate differences between treatment, letters $^{m, n}$ indicate differences between SD (stocking density) ($p<0.05$).

6.3.4. Nutrient Concentrations

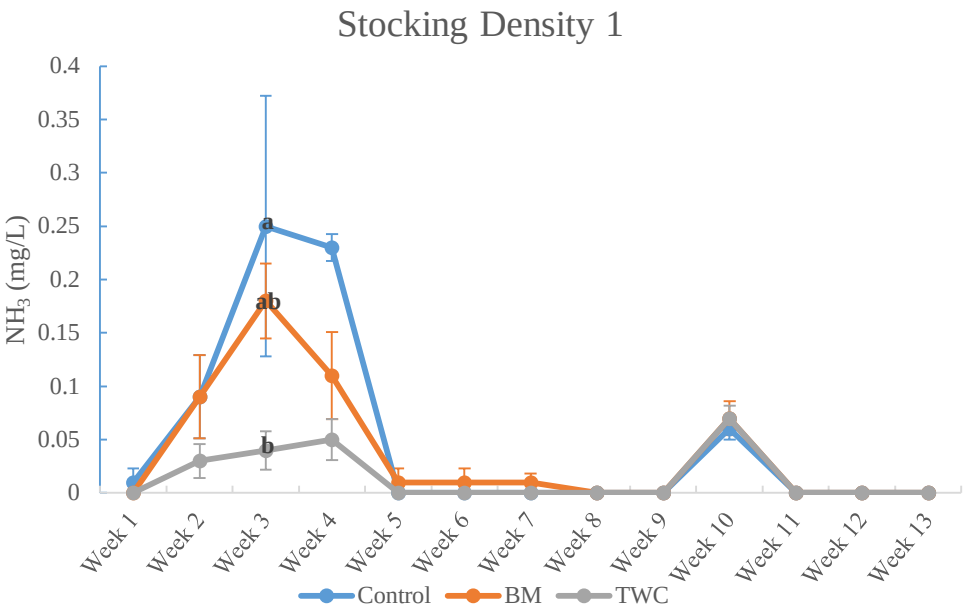
A two-way ANOVA found significant differences between substrate type and stocking density in all nutrient concentrations measured ($\alpha=0.05$). Though no interaction effects were present for NH_3, NO_2, or PO_4, a significant interaction was found for NO_3 (Table 6.5). A repeated measures ANOVA found NH_3, NO_2, and total PO_4 to change significantly over time ($\alpha=0.05$). In general, nutrient concentrations were frequently higher in tanks without substrate, and higher at high stocking density, signifying a higher rate of nutrient loading.

Table 6.5: Results of 3-factor mixed model ANOVA on concentrations of NH_3, NO_2, NO_3, & PO_4 with different substrate treatments and stocking densities over 13 weeks. All factors are fixed. Significant differences at α=0.05 are shown in **bold**.

	df	SS	*F*	p
NH_3				
Substrate Type	2	0.209	3.44	**0.033**
SD	1	0.173	5.68	**0.018**
Week	10	3.121	10.26	**<0.001**
ST*SD	2	0.091	1.49	0.227
NO_2				
Substrate Type	2	5.104	8.75	**<0.001**
SD	1	1.867	6.40	**0.012**
Week	12	295.481	84.44	**<0.001**
ST*SD	2	1.034	1.77	0.171
NO_3				
Substrate Type	2	178.422	89.21	**0.010**
SD	1	203.100	203.10	**0.001**
Week	12	9275.321	772.94	**<0.001**
ST*SD	2	313.599	156.80	**<0.001**
PO_4				
Substrate Type	2	13.971	16.16	**<0.001**
SD	1	46.189	106.88	**<0.001**
Week	12	481.641	92.88	**<0.001**
ST*SD	2	0.271	0.31	0.731

ST=Substrate Type, SD=Stocking Density, SS=sum of squares.

There were weekly fluctuations in the concentration of NH_3, and these patterns were generally similar between treatments, although the scale of fluctuation differed. Stocking density had no significant effect on the concentration of NH_3 in tanks with BM or control, however NH_3 was higher at higher stocking density in TWC tanks after three weeks. Figure 6.1 compares the effects of substrate on NH_3, at different stocking densities. The NH_3 and NO_2 levels remained <1.0 mg/L after seven weeks, signifying that nitrifying bacteria had become established in all tanks.



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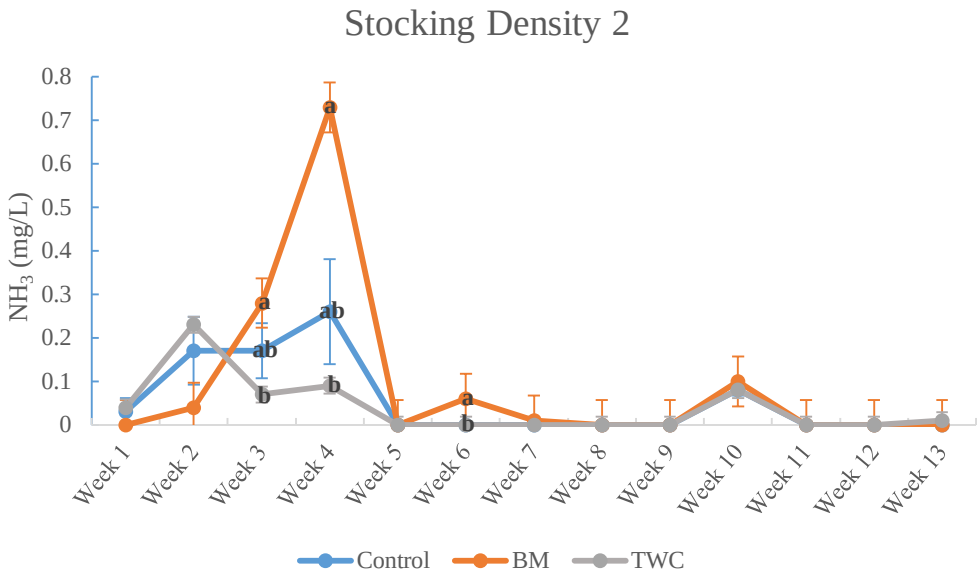
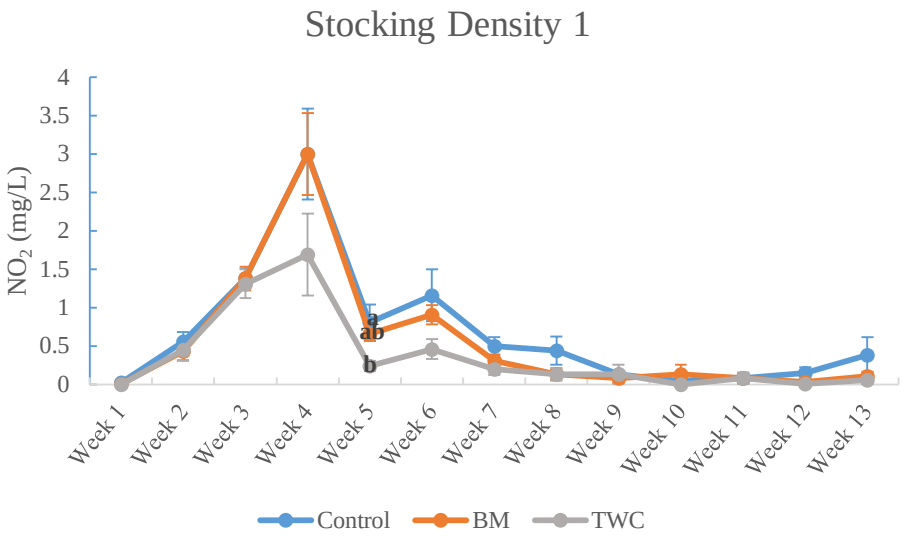


b)

Figure 6.1: Concentration of NH_3 with different substrate types, at low (a) and high (b) stocking density. Like letters (a, b) indicate that means are statistically similar (α=0.05).

In control and TWC tanks, the concentration of NO_2 was significantly higher at SD2 in week 2 only, while in BM tanks NO_2 was significantly higher at SD2 in week 7 only. Several differences were found in the concentrations of NO_2 between treatments (Figure 6.2).



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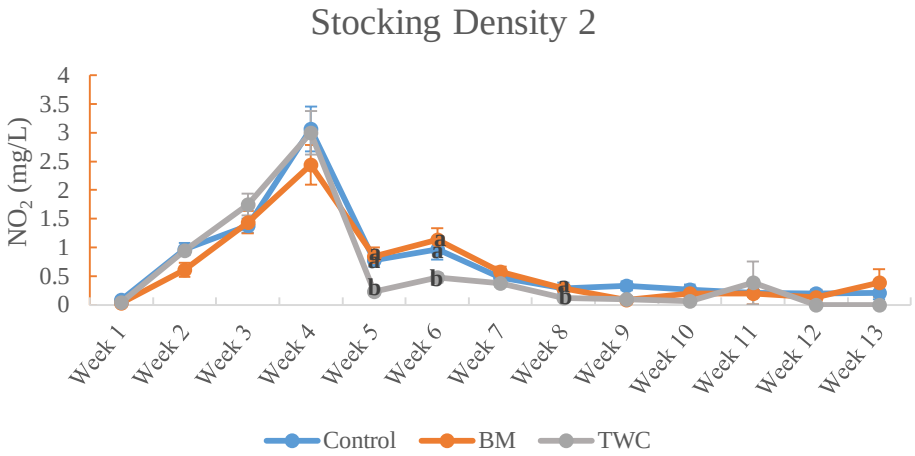
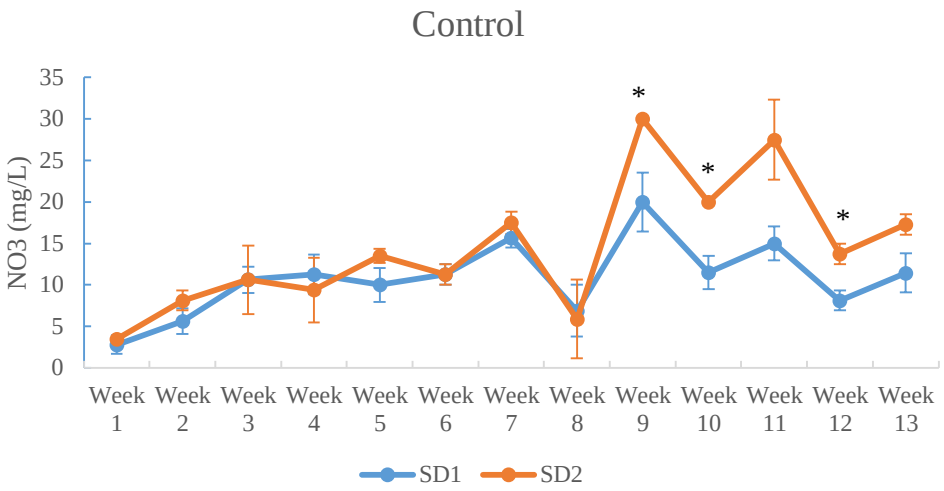


a)

Figure 6.2: Concentration of NO_2 with different substrate types, at low stocking density (a) and high stocking density (b). Like letters (a, b) indicate that means are statistically similar (α=0.05).

The concentration of NO_3 was not affected by substrate type or stocking density for the first 8 weeks. The water exchange after four weeks had no significant effect on the concentration of NO_3. NO_3 was significantly higher at SD2 than at SD1 several times throughout the experiment (Figure 6.3). The addition of substrates had a significant effect on NO_3 at SD2, but not at SD1 (Figure 6.4).



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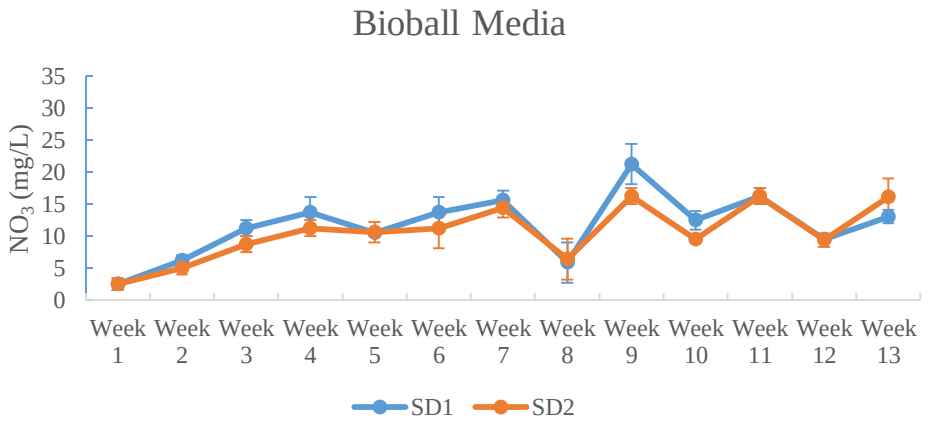

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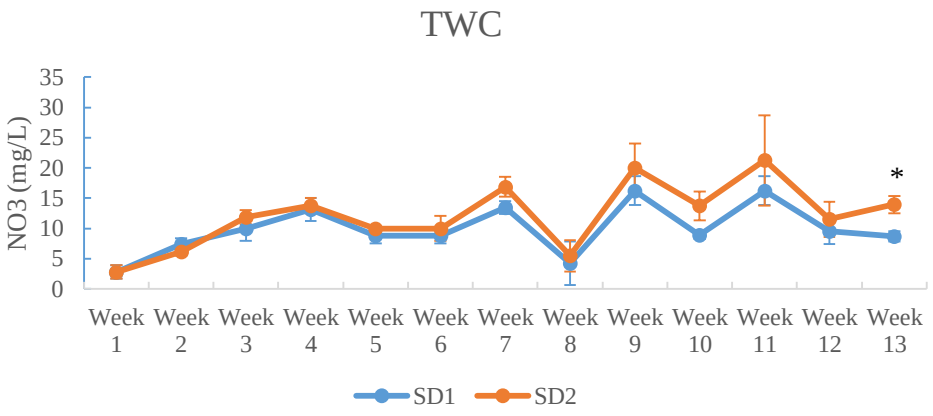
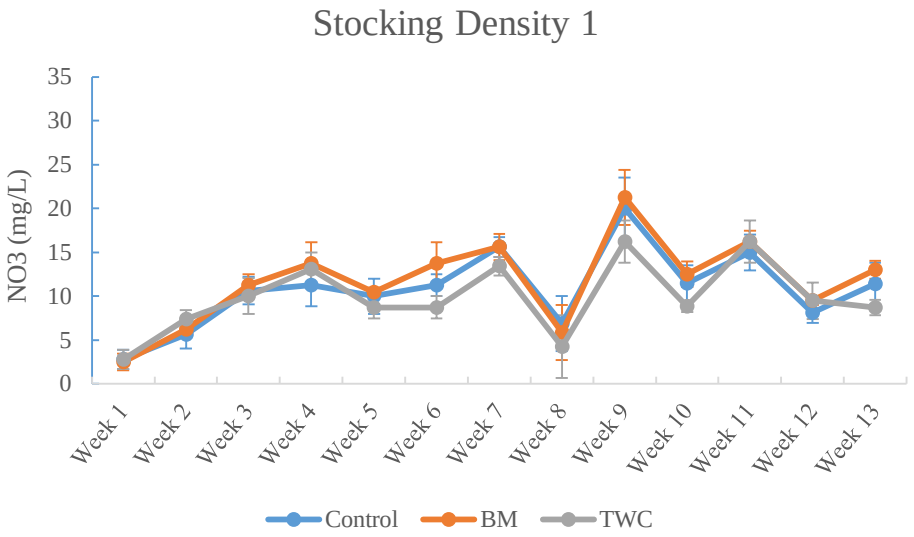


c)

Figure 6.3: Concentration of NO_3 in control (a), BM (b) and TWC (c) tanks, between two stocking densities.



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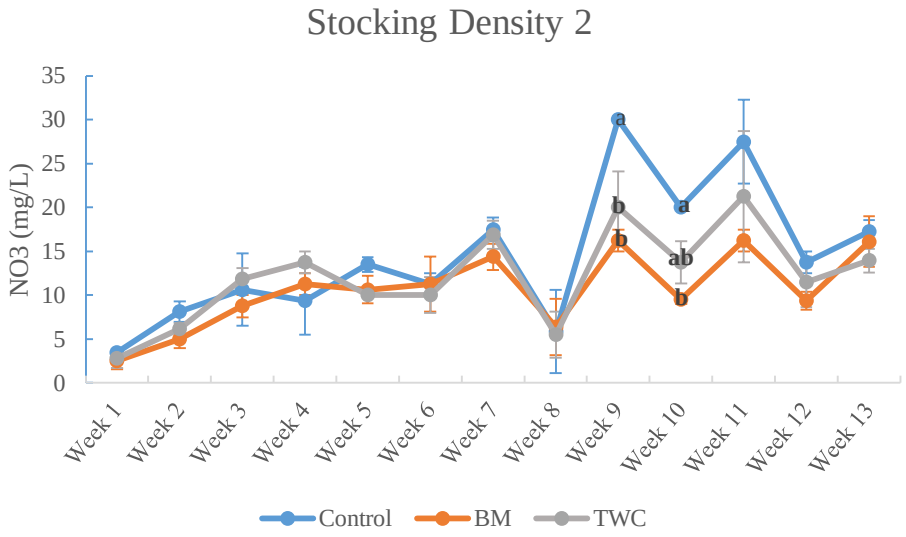
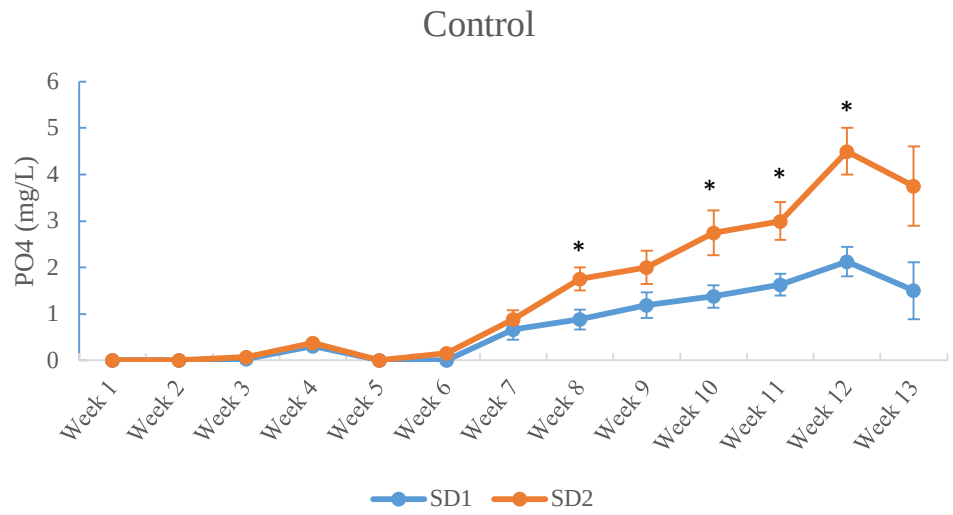


b)

Figure 6.4: Concentration of NO_3 with different substrate types at low (a) and high (b) stocking density. Like letters (a, b) indicate that means are statistically similar (α=0.05).

The concentration of total phosphate steadily increased for the first four weeks, and then fell after the 90% water exchange, before increasing once more (Figure 6.5; Figure 6.6). Substrate had a significant effect on PO_4 at low stocking density, but not at high stocking density (Figure 6.6).



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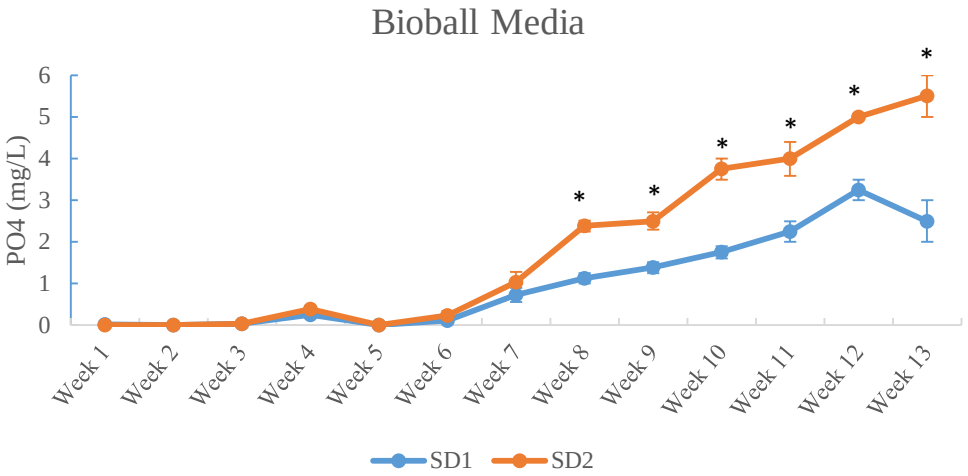

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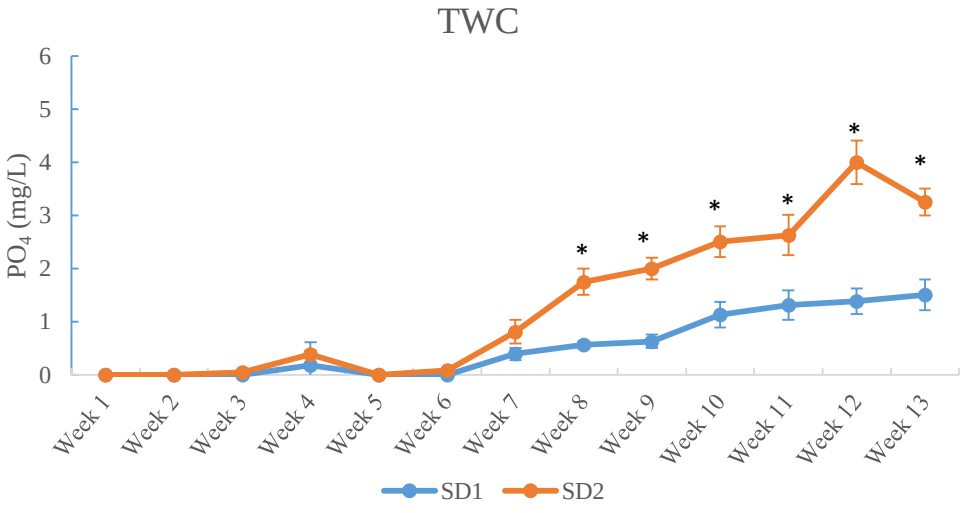
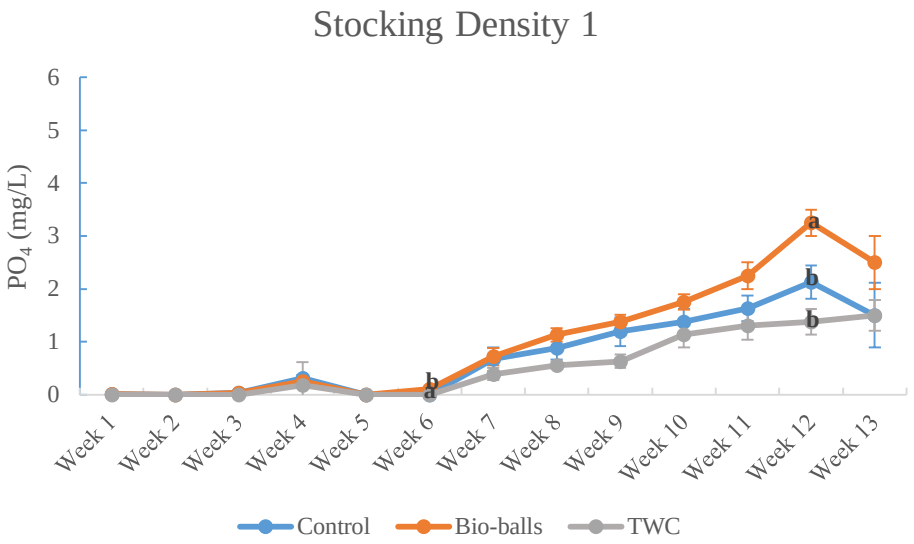


c)

Figure 6.5: Concentration of total phosphates at different stocking densities in control tanks (a), BM tanks (b) and TWC tanks (c).



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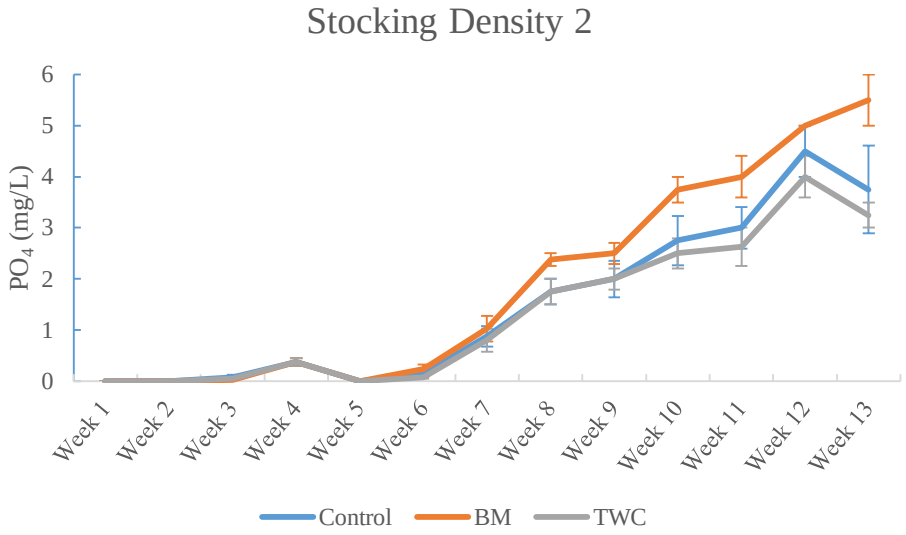

b)

Figure 6.6: Concentration of total phosphates between different substrate types at low (a) and high (b) stocking density. Like letters (a, b) indicate that means are statistically similar (α=0.05).

The concentration of orthophosphate mid-experiment, after seven weeks, was significantly lower in TWC tanks than in BM tanks at SD1 (Figure 6.7).

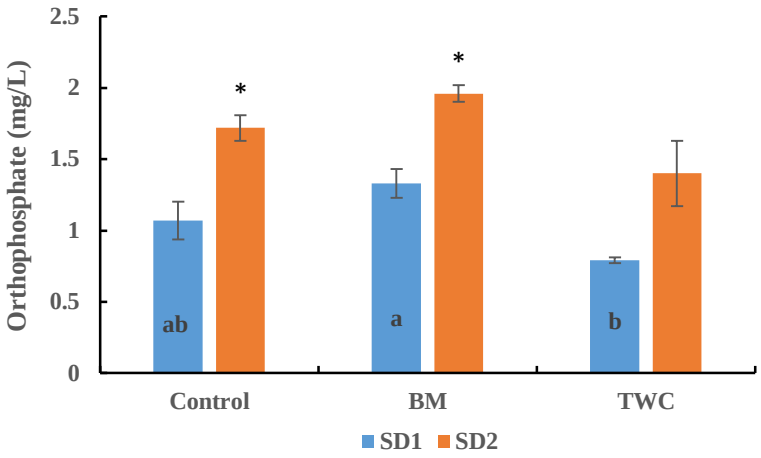

Figure 6.7: Concentrations of orthophosphate with different substrates at low and high stocking density, after seven weeks. Like letters (a, b) indicate that the means are statistically similar between treatments, an asterisk represents a significant difference between SD1 and SD2 (α=0.05).

6.3.5. THC and DHC

Initially, the total haemocyte count (THC) and differential haemocyte count (DHC) were 3.54 ± 0.437 (cells x 10^6) and 17.50 ± 2.98 (%) respectively, indicating that marron were in good condition. Throughout the experiment there were no significant differences between treatments, stocking densities, sexes, or times (Table 6.6). No strong correlations were found between THC, DHC and any water quality parameters. The THC of marron in experiment 2 was similar to that of experiment 3, the former ranging from a mean of $1.13x10^6$ to $4.96x10^6$ in control tanks. The marron THC in experiment fluctuated more in experiment 2 however.

Table 6.6: THC (cells x 10^6) and DHC (% proportion of granulocytes) between substrate type and stocking density over time.

	Control		**BM**		**TWC**	
	SD1	**SD2**	**SD1**	**SD2**	**SD1**	**SD2**
THC (x10^6)						
Week 3	2.68 ± 0.71	2.53 ± 0.28	2.28 ± 0.37	3.06 ± 0.36	1.91 ± 0.30	3.06 ± 0.67
Week 5	3.95 ± 1.53	2.98 ± 0.24	1.78 ± 0.27	3.05 ± 1.33	2.34 ± 0.40	2.02 ± 0.38
Week 7	2.87 ± 0.72	3.05 ± 0.16	2.74 ± 0.41	2.51 ± 0.38	2.54 ± 0.14	2.22 ± 0.09
Week 9	4.24 ± 1.87	2.98 ± 1.34	3.02 ± 1.11	3.64 ± 1.04	2.39 ± 0.79	2.11 ± 0.37
Week 13	3.11 ± 0.94	2.41 ± 0.38	1.74 ± 0.53	2.20 ± 0.57	2.84 ± 0.47	2.18 ± 0.43
DHC						
Week 3	14.3 ± 6.17	18.3 ± 2.06	22.4 ± 2.84	24.4 ±0.90	22.8 ± 3.15	24.1 ± 3.37
Week 5	25.5 ± 2.09	20.6 ± 3.82	25.9 ± 4.41	23.6 ± 3.78	24.6 ± 4.66	22.3 ± 2.46
Week 7	23.0 ± 3.46	24.5 ± 6.50	21.6 ± 1.95	23.0 ± 0.54	28.5 ± 1.62	21.0 ± 1.14
Week 9	-	-	-	-	-	-
Week 13	20.9 ± 0.47	22.3 ± 2.60	21.6 ± 2.70	22.5 ± 4.13	32.5 ± 4.80	24.5 ± 2.50

6.3.6. Organosomatic Indices

The hepatosomatic condition indices (HM%, Hiw, Hid) were not significantly different between substrate type or stocking density at the end of experiment. However, the mean tail moisture content (TM%), wet tail muscle to body ratio (Tbw) and dry tail muscle to body ratio (Tbd) were not the same (Table 6.7). The tail muscle may have been in poorer condition in marron in TWC tanks at SD2. The Tbw of marron was significantly lower at SD2 than at SD1, irrespective of treatment.

Table 6.7: Organosomatic indices of marron at end of experiment 3, after 13 weeks.

	Control		**BM**		**TWC**	
	SD1	**SD2**	**SD1**	**SD2**	**SD1**	**SD2**
HM%	77.6 ± 4.18	62.1 ± 2.98	68.4 ± 5.42	62.4 ± 1.73	69.0 ± 2.01	76.1 ± 4.85
TM%	80.1 ± 0.78	78.5^{a} ± 0.44	76.7 ± 1.05	78.5^{a} ± 0.27	79.4 ± 0.51	80.4^{b} ± 0.68
Tbw	32.6 ± 2.17	28.2 ± 1.31	29.9 ± 1.71	30.0 ± 1.18	31.5 ± 1.07	27.5 ± 0.68
Tbd	6.49 ± 0.49	6.06^{ab} ± 0.20	6.09 ± 0.48	6.43^{a} ± 0.29	6.49 ± 0.22	5.38^{b} ± 0.15
Hiw	4.19 ± 0.34	4.43 ± 0.31	4.16 ± 1.08	5.14 ± 1.29	5.50 ± 0.84	4.37 ± 0.66
Hid	0.970 ± 0.24	1.70 ± 0.23	1.38 ± 0.48	1.87 ± 0.40	1.69 ± 0.25	1.02 ± 0.25

Superscript letters $^{a, b}$ indicate differences between treatment ($p<0.05$).

6.3.7. Bacteria

Stocking density and substrate type had no significant effect on heterotrophic plate count (HPC), however there was a fluctuation over time (Table 6.8). Colony diversity ranged from 4 to 13, and after six weeks was significantly higher at SD1 than SD2 in BM tanks (10.50 and 8.50 respectively). *Bacillus* sp. were found in the water column in tanks with TWC, BM, and without substrate (Plate 6.4). The *Bacillus* sp. count in water was not significantly different between treatment or stocking density, due to the high variability within treatments. HPC and colony diversity were not significantly different between TWC and BM surfaces, while *Bacillus* sp. were only found on TWC surface (Table 6.9). Fungal growth on agar was found in one sample only.

Table 6.8: Heterotrophic Plate Count (HPC) and *Bacillus* sp. count between treatments and stocking densities.

	Control		**BM**		**TWC**	
	SD1	**SD2**	**SD1**	**SD2**	**SD1**	**SD2**
HPC (CFU/mLx10^3)						
Week 2	9.3 ± 1.1	6.4 ± 3.3	6.2^x ± 2.2	5.8^x ± 2.9	8.0 ± 1.5	7.9^x ± 2.4
Week 6	16.1 ± 5.4	26.0 ± 8.3	16.7^y ± 3.0	18.5^y ± 0.6	11.3 ± 3.0	25.7^y ± 5.0
Week 11	18.2 ± 1.6	18.3 ± 4.2	17.9^y ± 2.8	16.5^y ± 2.4	15.6 ± 4.4	22.7^y ± 2.2
***Bacillus* sp.**						
Week 2	50.0 ± 25.0	75.0 ± 75.0	0.0 ± 0.0	25.0 ± 25.0	0.0 ± 0.0	25.0 ± 25.0
Week 6	50.0 ± 28.9	50.0 ± 50.0	75.0 ± 47.9	0.0 ± 0.0	0.0 ± 0.0	225.0 ± 143.6
Week 11	725.0 ± 692.1	25.0 ± 25.0	125.0 ± 125.0	0.0 ± 0.0	100.0 ± 40.8	0.0 ± 0.0

Superscript letters $^{x, y}$ indicate significant differences between weeks ($p<0.05$).

Table 6.9: Bacterial plate counts and diversity on different substrates after nine weeks, n=number of samples analysed.

	BM		**TWC**	
	SD1 (n=1)	**SD2 (n=1)**	**SD1**	**SD2**
HPC (CFU/cm^3)	289.0	478.0	611.0 ± 184.1	520.0 ± 69.8
***Bacillus* sp.**	0.0	0.0	1.5 ± 1.5	2.5 ± 1.0
Colony Diversity	8.0	9.0	8.0 ± 0.6	9.0 ± 1.1

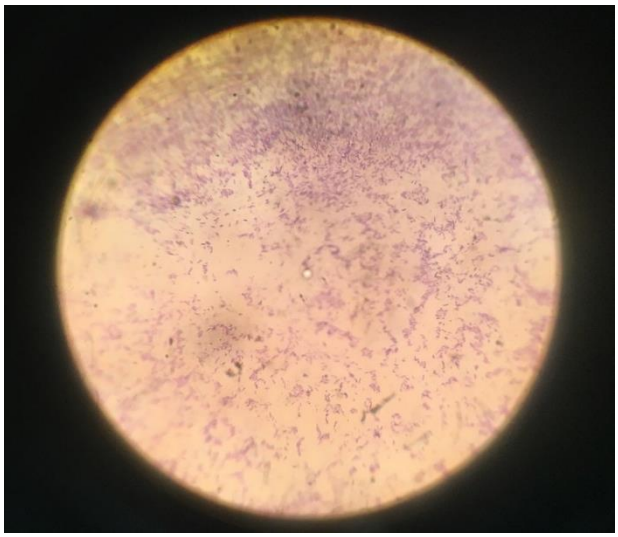

Plate 6.4: *Bacillus* sp. stained with crystal violet, at 1000x magnification.

6.3.8. Marron Growth

The specific growth rates (SGR) of marron in this study, Table 6.9, were relatively low compared to those found by Ambas et al. (2013), ranging from 0.27±0.09 to 0.51±0.35%, and by Nugroho & Fotedar (2013) of 0.19±0.01% in the control group. The highest SGR for the duration of the study was only 0.11 ± 0.03%. Meanwhile, there was no significant effect of stocking density on growth. Substrate type and time both had significant effects on SGR (Table 6.10). The reduction in feeding after the first month had no significant effect on growth. Marron in control tanks were significantly larger at SD2 initially and at the end of trial ($p<0.05$). Moults were not recorded, therefore no moult increment or moult frequency data are present.

Table 6.10: Marron specific growth rate (SGR) and mean weight between treatment and stocking density.

	Control		**BM**		**TWC**	
	SD1	**SD2**	**SD1**	**SD2**	**SD1**	**SD2**
SGR						
0-30 Days	0.03 ± 0.03	0.04 ± 0.02	^{x}0.02 ± 0.02	0.02 ± 0.01	0.06 ± 0.05	0.08 ± 0.10
30-60 Days	0.13 ± 0.07	0.20 ± 0.11	^{x}0.07 ± 0.03	0.16 ± 0.06	0.19 ± 0.11	0.12 ± 0.07
60-90 Days	-0.01 ± 0.02	-0.05^{a} ± 0.04	^{y}0.18 ± 0.23	0.16^{b} ± 0.06	0.08 ± 0.12	0.05^{ab} ± 0.03
0-60 Days	0.08 ± 0.03	0.11 ± 0.06	0.04 ± 0.01	0.09 ± 0.03	0.13 ± 0.04	0.06 ± 0.04
0-90 Days	0.05 ± 0.02	0.06 ± 0.05	0.09 ± 0.08	0.11 ± 0.03	0.11 ± 0.04	0.06 ± 0.03
Mean weight (g)						
Initial weight	51.42^{m} ± 2.89	66.60^{n} ± 3.77	53.90 ± 1.45	57.30 ± 4.27	56.30 ± 2.32	66.50 ± 3.85
Final weight	54.05^{m} ± 3.59	69.90^{n} ± 2.23	58.60 ± 3.48	63.60 ± 5.11	62.02 ± 3.18	70.20 ± 4.20

Superscript letters $^{a, b}$ indicate significant differences between treatment, letters $^{m, n}$ indicate differences between SD, letters ^{x,y} indicate differences between monthly SGR ($p<0.05$).

6.4. Discussion

The concentrations of ammonia, nitrite, nitrate and orthophosphates were significantly reduced by using The Water Cleanser™ at times where these concentrations peaked with Bioball® media (BM) or without substrate, while both TWC and BM were effective in reducing the concentration of nitrate. Substrates and filter media have previously been effective in improving the water quality of culture systems (Viau et al. 2012; Thompson et al. 2002; Lezama-Cervantes & Paniagua-Michel 2010; Masłoń & Tomaszek 2015). The use of substrates with attached biofilm has also been shown to improve the survival rate of juvenile redclaw crayfish (*Cherax quadricarinatus*), via maintenance of good water quality (Viau et al. 2012). High concentrations of ammonia, nitrite and nitrate are toxic to aquatic animals, including decapod crustaceans (Jensen 1996; Jensen 2003; Romano & Zeng 2007). However, the mortality rate of marron in this study was less than 11% overall (~89% survival rate), and neither substrate type nor stocking density had a significant effect. These factors can have varied effects on the survival rate; for example Jones et al. (2002) found that a synthetic substrate (Aquamat®) resulted in no significant improvement of crayfish (*Cherax destructor*) survival (86.3% with food and no Aquamat, and 85.0% with food

and conditioned Aquamat), while Rodgers et al. (2006) found that stocking density had no effect on the survival rate of juvenile *C. quadricarinatus* (47.8% at 4/m^2 and 44.2% at 6/m^2) in earthen ponds. Conversely, Fotedar et al. (1999) showed that survival rate of juvenile marron in tanks can be significantly lower at a high stocking density of 13/m^2 than at lower stocking densities of 3 and 6/m^2. This compares to the stocking densities of ~7.5 and 14/m^2 (adult marron) at SD1 and SD2 in the current trial. The different densities used, life stage of crayfish, and culture conditions may explain the disparity in results.

Growth of freshwater crayfish is inversely related to density (Verhoef & Austin 1999; Mills & McCloud 1983; Mazlum 2007; Morrissy 1979). There was no significant effect of stocking density or substrate on marron growth in the current trial. The presence of containers could have lessened the detrimental effects of stocking density by reducing fighting and competition between marron. Geddes et al. (1988) found that *C. destructor* had depressed growth held communally and at high density, compared with those held individually in containers. Mills & McCloud (1983) attributed lower growth of *C. destructor* held communally at high stocking density to aggressive interactions leading to stunted growth. Water quality also influences growth rate, for example Björnsson & Ólafsdóttir (2006) showed that growth of finfish (*Gadus morhua*) has been linked to deterioration of water quality at high stocking density. In addition to these factors, the marron growth rate in experiments 2 and 3 was probably influenced by the different protein concentrations in feed. In both experiments, TWC had no significant effect on growth, while in this study growth was improved with BM in the final month, compared to control. This may indicate suppressed growth in control tanks, probably relating to poor water quality. While SGR was equivalent to that found by Nugroho & Fotedar (2013) in control tanks after 30-60 days, after 60-90 days no growth was observed in control tanks, indicating a deterioration in water quality and/or marron health. Poor water quality can induce stress in aquatic animals (Mazlum 2007), affecting growth. Overall, the marron growth rate was slow, as compared to other laboratory studies (Ambas et al. 2013; Nugroho & Fotedar 2013). This may have been due to a lack of filtration, resulting in a build-up of organic matter and poor water quality, or a lower feeding rate.

The level of dissolved oxygen was higher at the higher stocking density, signifying a higher biochemical oxygen demand (BOD) (Jouanneau et al. 2014). High feeding

rates, and the resulting build-up of organic matter, could have increased the BOD (Morrissy et al. 1986). Tanks with substrates showed no significant difference in DO levels, indicative of lower concentrations of organic matter, despite the same level of nutrient loading. The substrates increased habitat availability for microbes, which play a key role in the decomposition of organic matter (Meyer-Reil 1994), while TWC also provides hydrocarbon, which can be utilised by certain bacteria as a source of carbon (Zhou et al. 2009).

Intensive culture of crustaceans requires increased feed inputs, resulting in higher nutrient loading. NH_3-N, NO_2-N, total nitrogen and total phosphorous have increased with increasing stocking density of Kuruma prawns (*Marsupenaeus japonicus*) (Li et al. 2013[b]). And in Recirculating Aquaculture Systems (RAS) with teleost fishes (*Lates calcarifer*), total nitrogen and total phosphorous have increased at higher stocking density (Ardiansyah & Fotedar 2016[a]). Similarly, the higher stocking density of marron also increased nutrient concentrations in the current experiment. TWC and the BM were unable to maintain ammonia, nitrite, and phosphate concentrations at similar levels between stocking densities, however the effect of density on nitrate concentrations was reduced. TWC may have enhanced denitrification by providing a carbon source for bacteria (Robinson et al. 2017).

Heterotrophic bacteria, including *Bacillus* sp., are effective in the oxidation of ammonia in aquaculture (Nimrat et al. 2012). The culturing of bacteria on TWC surface may have been key to its effectiveness in maintaining low ammonia concentrations during this study. A variety of heterotrophic bacteria, including *B. lecheniformis* and *B. subtilis*, have been described attached to duckweed (*Lemna minor*) in an RAS system, contributing to the removal of nutrients (Ardiansyah & Fotedar 2016[b]). Similarly, microbial mats have been shown to increase ammonia oxidation rates in shrimp (*Litopenaeus vannamei*) culture (Lezama-Cervantes & Paniagua-Michel 2010). Microscopy suggests that TWC cultures primarily heterotrophic bacteria and various microbes (Personal observations). The BM, when placed in a sequencing batch reactor, has been used in the removal of ammonium via nitrification (Masłoń & Tomaszek 2015). However, when placed freely in tanks in the current experiment, the concentration of ammonia rose to higher levels with BM than with TWC or without substrate.

TWC resulted in the highest rates of nitrite oxidation, in weeks 5, 6, and 8, probably due to the addition of a carbon source. Carbon sources can be limiting in aquaculture systems, resulting in incomplete denitrification and accumulation of nitrite (Hamlin et al. 2008). Increased denitrification would have removed nitrogen from the system, as nitrogen gas and nitrous oxide (Wang et al. 2011). Substrates often have varied results on nutrient concentrations, for example substrates with attached biofilm have been used to reduce nutrient concentrations in *C. quadricarinatus* culture (Viau et al. 2012), whereas in a similar study substrates increased the nitrite concentration compared to control, perhaps due to enhanced ammonia oxidation (Bratvold & Browdy 2001). In another study, biofilm attached to substrate increased the concentrations of nitrite and nitrate compared to the control, due to a greater release of nitrite than ammonium (Thompson et al. 2002), while microbial mats can have a similar effect (Lezama-Cervantes et al. 2010). TWC may function differently, by increasing the rate of denitrification with the addition of a carbon source.

TWC reduced the concentration of orthophosphates mid-experiment, attributed to the development of bacterial biomass, however the concentration of total phosphate was not reduced. Density effects were also more evident for total phosphate than orthophosphate in TWC tanks, while the increase in nutrient inputs led to an increase in orthophosphate in BM and control tanks. This same effect on orthophosphate was found in experiments 1 & 2, and in the field trial, probably due to an increased rate of conversion of orthophosphate into polyphosphate, by bacteria. Bacteria attached to TWC may include polyphosphate accumulating organisms, which play a key role in phosphate removal (Masłoń & Tomaszek 2015). As polyphosphates are stored in sediments and soils, the lack of sediment may have limited polyphosphate deposition (Kirchman 2012). Moreover, the biomass of polyphosphate accumulating organisms may have been too low to contain the high amounts of phosphates in intensive culture (Wang et al. 2011; Lananan et al. 2014). Substrate can be effective bioremediators of total phosphate, however. In one study, biofilm attached to substrate was used to remove 33% of phosphorous in an aquaculture system (Thompson et al. 2002). BM in a filtration system has also been used to remove phosphorous (Masłoń & Tomaszek 2015). The use of TWC and BM within filters, or in areas of greater water flow, may improve phosphate reduction. In this study, BM was the least effective treatment in reducing phosphates, as it hampered removal of excess feed. Research has shown

substrates to be ineffective in reduction of orthophosphate in certain aquaculture systems, including biofloc (Schveitzer et al. 2013), largely due to the high microbial biomass in the water column. In experiment 2 the concentrations of nutrients remained lower than in experiment 3, due to the large biomass of phytoplankton present. High nutrient loading did not cause a significant stress response of marron in either experiment.

THC and DHC are indicators of stress response, health and immunity of crustaceans, including marron (Sang et al. 2009; Nugroho & Fotedar 2014; Ambas et al. 2013; Le Moullac & Haffner 2000). The THC and DHC of marron were not significantly affected by substrate type or nutrient loading. TWC had no significant ill effects on the haemolymph of marron, similar to the results of Chapter 4. In contrast to findings from previous studies (Le Moullac & Haffner 2000; Jensen 1996; Yildiz & Benli 2004), elevated concentrations of ammonia (1mg/L), nitrite (3mg/L), nitrate, and phosphates did not have any acute toxic effects on marron, as determined by THC and DHC. However, the high nutrient loading may have caused chronic toxicity, as determined by the organosomatic indices. Marron in tanks with TWC held at high density were in poor physiological condition at the end of the experiment; determined by the high tail muscle moisture content and low Tbd. The organosomatic indices have been used as measures of physiological response in marron (Ambas et al. 2013). High stocking density also resulted in low Tbw irrespective of treatment, indicating poor physiological condition and stress (Sang & Fotedar 2004). TWC itself was unlikely to have caused loss of physiological condition, as no effect was present at low stocking density. While bacterial populations attached to TWC were diverse, any pathogenic bacteria present would face competition with less pathogenic species, and can be controlled by protozoa through grazing (Thompson et al. 2002). Ambas et al. (2013) found that application of probiotics improved physiological condition of marron, yet TWC had no similar effect, despite harbouring *Bacillus* sp. Nutrient concentrations fluctuated to periodically high levels, although no acute toxic effects were found on marron. The results suggest that there were some chronic toxic effects at higher stocking density, which neither substrate was able to mitigate.

Populations of heterotrophic bacteria, including *Bacillus* sp., developed on the surface of TWC. Bacteria are important in the control of ammonia, nitrite, nitrate and phosphate in water (Schveitzer et al. 2013; Thompson et al. 2002; Browdy & Hopkins

1995), and play an important role in nutrient cycling and decomposition (Anderson et al. 1987, Sorokin 1999, Kirchman 2012). Bacterial abundance is also related to system function (Ray et al. 2010). The increase in bacterial abundance in the water column of marron tanks was triggered by a build-up of nutrients and organic matter, while the higher nutrient inputs at SD2 resulted in higher bacterial abundance than at SD1. Populations of heterotrophic bacteria and periphyton within the biofilm are highly dependent on nutrient availability (Pradeep et al. 2004; Pandey et al. 2014). Meanwhile, biofilm on TWC surface differed greatly to that on BM, and *Bacillus* sp. was only present on TWC biofilm, probably due to the provision of a carbon source in this substrate.

6.5 Conclusions

The concentrations of ammonia, nitrite, nitrate and orthophosphate were periodically reduced in tanks with TWC compared to tanks without substrate, while BM reduced the concentration of nitrate only. In addition, the BM and TWC both resulted in a lessened effect of density on nitrate concentrations and dissolved oxygen levels. TWC was also a suitable substrate for the growth of *Bacillus* sp. However, the concentration of total phosphate was not reduced by either treatment, and increased at higher stocking density in all treatments. There was no improvement in the survival or growth of adult marron with TWC. Furthermore, the THC, DHC and organosomatic indices of marron were not improved with TWC or BM, and Tbw was negatively impacted by high stocking density. Even so, no negative effects on marron were present with the use of substrates. This study shows that TWC may be placed freely in tank culture to reduce concentrations of ammonia, nitrite, nitrate and orthophosphate, and to reduce the effects of high stocking density on water quality, with no negative effects on marron growth, survival, or physiological condition.

Chapter 7: General Discussion

Chapter 7 presents a general discussion of the study. Here the findings of the three laboratory experiments and the field experiment are compared and summarised. The main conclusions of the effects of The Water Cleanser™ on water quality, microbial ecology, natural productivity and marron health and productivity are given, followed by recommendations for further research, summarised in Figure 7.1.

7.1 Introduction

Substrates have been used in aquaculture to promote microbial growth, improve water quality and affect the growth and survival of cultured animals (Viau et al. 2012; Bratvold & Browdy 2001; Thompson et al. 2002; Schveitzer et al. 2013). Maintaining good water quality is crucial for the health, survival and growth performance of all crustaceans (Smith et al. 2002; Stumpf et al. 2014). The Water Cleanser™ (TWC) is thought to provide a habitat and carbon source for heterotrophic bacteria, including those important in the improvement of water quality (Marine Easy Clean 2015). However, no peer-reviewed research has investigated the effects of oil-based substrates, such as TWC, in aquaculture.

This study investigated the effects of TWC on water quality (Chapter 3, 4, 5, & 6; Objective 2), natural productivity (Chapter 4 & 5; Objective 2), marron growth and health (Chapter 4, 5 & 6; Objective 2 & 3), and bacterial abundance and diversity of colony types (Chapter 3, 4, 5 & 6; Objective 1) in marron culture. The impact of TWC on water quality, bacteria and marron at different stocking densities was assessed (Chapter 6; Objective 3), and the relationships between nutrient concentrations, phytoplankton, zooplankton, bacteria and marron health and productivity were studied (Chapter 3, 4 & 5, Objective 4). The conditions of each experiment in these chapter, and the main findings, are summarised in Figure 7.1.

The results of this study suggest that TWC provides habitat for a high abundance of heterotrophic bacteria, including *Bacillus* sp., and therefore may function differently from previously used substrates. *Bacillus* sp. are associated with good water quality (Wang et al. 2005), and health of marron (Ambas et al. 2013), however they require a carbon source for processes including denitrification (Hamlin et al. 2008). An oil-based substrate such as TWC would provide a carbon source in the form of hydrocarbon (Hu et al. 2017; Sakthipriya et al. 2015), and therefore promote the growth of denitrifying bacteria. Bacteria are known to affect the water quality and natural productivity of aquatic systems (Wang et al. 2015; Zhou et al. 2009), are essential in aquaculture for removal of excess nitrogen and phosphorous, and are key components of the nitrogen, phosphorous and carbon cycles (Sorokin 1999). Bacteria also play a major role in recycling nutrients locked in organic matter to stimulate primary productivity (Moriarty 1997). High concentrations of ammonia and nitrite can

cause physiological problems for aquatic animals, including crayfish (Jensen 1996; Harris 2001; Jussila & Evans 1996). In marron ponds, poor water quality can adversely affect marron (Morrissy et al. 1984), while eutrophication can trigger algal blooms (Seymour 1980). However, nutrients are required for promoting phytoplankton and zooplankton communities, to provide a natural food source for crayfish, especially for juveniles (Jones 1995). Freshwater crayfish are known to feed on both artificial feed and natural sources of feed including macrophytes, benthic invertebrates, zooplankton, algae and detritus (Saoud et al. 2012). The culture environment and in turn the health, survival and growth of marron could be impacted by the addition of an oil-based substrate, such as TWC.

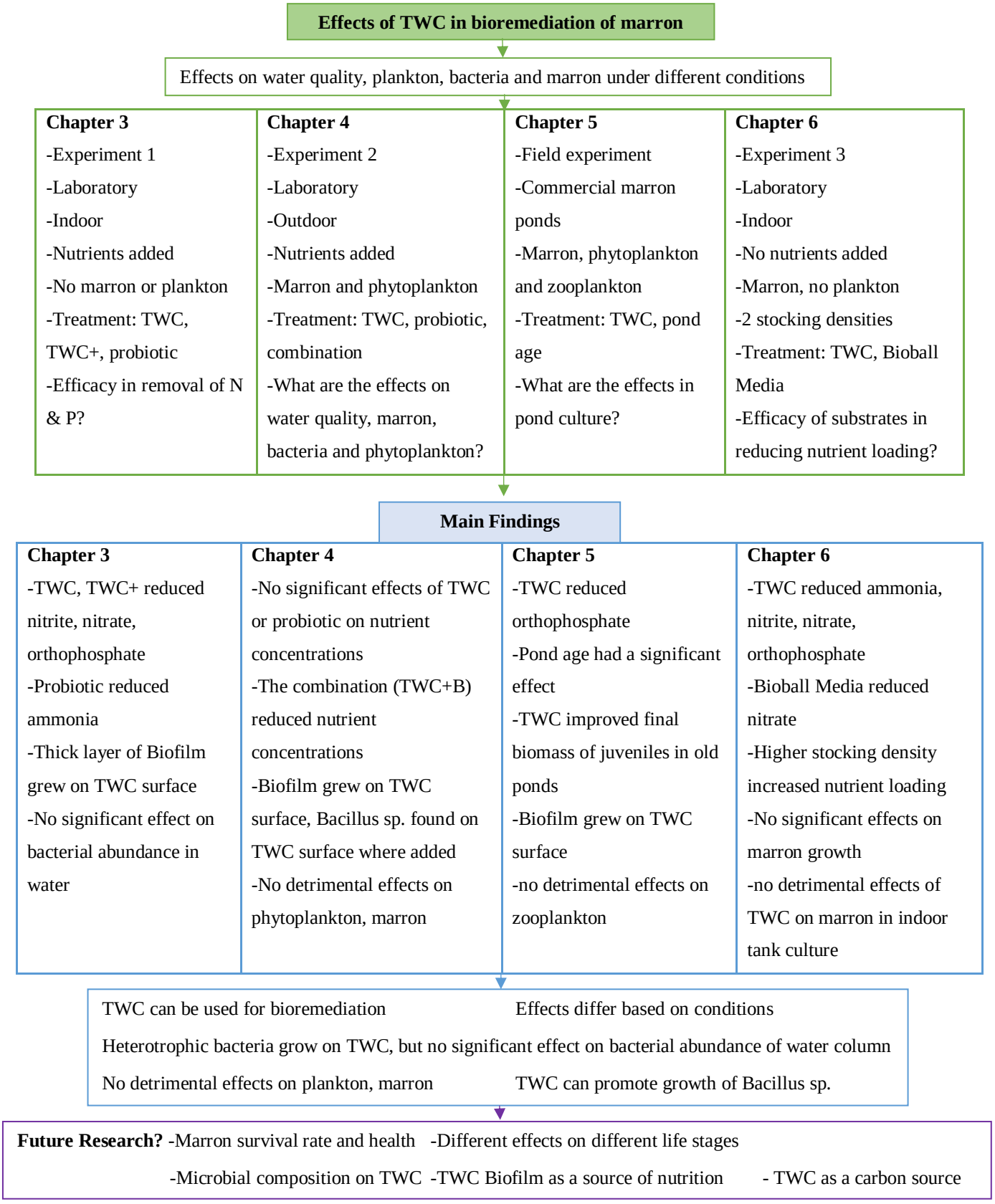

Figure 7.1: Summary diagram of the study, showing the different objectives and conditions in each Chapter, as illustrated in Figure 1.1. The main findings of each experiment are summarised, and the main recommendations for further research given.

7.2 Effects of TWC on Water Quality

The Water Cleanser™ affected the water quality in all of the experiments, including the field experiment. In the first experiment (Chapter 3), TWC and TWC+ significantly reduced nitrite, nitrate and orthophosphate compared to aquaria without treatment. TWC had a greater bioremediation effect than in following experiments, likely because it was conducted under controlled conditions, with limited variables. The temperature was also higher on average than in the second experiment (Chapter 4), with low fluctuations in temperature compared to outdoor laboratory conditions. The mean temperature in the third experiment of 17.0 ± 0.06°C was also higher than in the second experiment, and it was under controlled conditions. The temperatures in the second experiment were often lower than the optimum temperature for nitrification for nitrifying bacteria and several *Bacillus* strains (Song et al. 2011; Hargreaves 1998). The controlled conditions with higher temperatures in the first and third experiment likely allowed for increased efficiency of the substrate compared to the second experiment. In the second experiment (Chapter 4), held in outdoor conditions, concentrations of nitrite and nitrate were similar between treatments. The high abundance of microalgae could have led to enhanced phytoremediation (Lananan et al. 2014), as suggested by the lower concentrations of NO_3-N in experiment 2 and the field experiment (Table 7.1), which may have also limited the effect of TWC on nutrient concentrations. Meanwhile, in the third experiment (Chapter 6), TWC maintained lower concentrations of ammonia, nitrite and nitrate compared to tanks without substrate, while Bioball Media (BM) reduced concentrations of nitrate only. Increasing stocking density increased the rate of nutrient loading both with and without substrates. TWC reduced concentrations of nitrite and nitrate in experiments 1 and 3, and reduced the concentration of ammonia in experiment 3 only. It is possible that the addition of NH_4Cl in experiment 1 reduced the rate of biofilm growth initially, as shown in biofilm bioreactors (Singh et al. 2013), reducing the rate of ammonia oxidation. In the field experiment (Chapter 5), there were no significant effects of TWC on concentrations of ammonia, nitrite, or nitrate. The lower nutrient concentrations in ponds may account for this (Table 7.1). In all experiments, the control treatment had no addition of substrate or probiotic, though conditions differed in each experiment. The indoor conditions varied to that of the outdoor culture largely because of the high abundance of microalgae in the outdoor culture, while the marron

ponds were more similar to a natural system and contained sediment, which is an important site for organic matter decomposition by bacteria (Sorokin 1999). In all experiments, the concentrations of orthophosphate were reduced with TWC.

Table 7.1: Comparison of nutrient concentrations between all experiments, 6 weeks after application of TWC.

	Experiment 1		**Experiment 2**		**Experiment 3**		**Field Experiment**	
	Control	**TWC**	**Control**	**TWC**	**Control**	**TWC**	**Control**	**TWC**
TAN	2.67 ± 0.33^{b}	2.17 ± 0.60^{b}	0.03 ± 0.01^{a}	0.03 ± 0.01^{a}	<0.01 ± 0.00^{a}	<0.01 ± 0.00^{a}	0.12 ± 0.03^{a}	0.12 ± 0.02^{a}
NO_2-N	<0.01 ± 0.00^{a}	0.09 ± 0.04^{b}	<0.01 ± 0.00^{a}	<0.01 ± 0.00^{a}	0.09 ± 0.03^{b}	0.07 ± 0.03^{b}	<0.01 ± 0.00^{a}	<0.01 ± 0.00^{a}
NO_3-N	2.07 ± 0.68^{b}	2.82 ± 0.86^{b}	0.08 ± 0.05^{a}	0.05 ± 0.03^{a}	3.96 ± 0.21^{c}	3.67 ± 0.28^{b}	0.21 ± 0.04^{a}	0.29 ± 0.10^{a}
Orthophosphate	1.07 ± 0.04^{b}	1.06 ± 0.03^{b}	1.04 ± 0.14^{b}	0.86 ± 0.07^{b}	1.39 ± 0.14^{b}	1.10 ± 0.16^{b}	0.25 ± 0.13^{a}	0.13 ± 0.04^{a}

Superscript letters $^{a, b, c}$ indicate significant differences between experiments (α<0.05).

The Bio-Aid and E-viro 3 probiotics were used in the first and second experiment respectively. Probiotics are known to reduce nitrogenous waste concentrations and phosphate concentrations in aquaculture water (Xie et al. 2013; Wang et al. 2005; Li & Boyd 2016). The probiotics applied in this study had varied effects on the nutrient concentrations (Figure 7.2). The Bio-Aid did reduce the concentration of ammonia after seven weeks, although concentrations of nitrate and phosphate remained as high as in the control, TWC and TWC+ aquaria. E-viro 3 had no significant effect on nutrient concentrations, apart from an increase in nitrate after ten weeks. However, there were microalgae present in experiment 2, which may have reduced nutrient concentrations by absorbing bioavailable nutrients. In experiment 1, ammonium chloride and potassium diphosphate were added, while a commercial fertilizer (Aquasol) was added to experiment 2, to increase nutrient levels. A combination of TWC and *Bacillus* sp. was effective in reducing nutrient concentrations initially, due to the probiotic added, and maintained a higher phytoplankton abundance than TWC or E-viro 3, attributed to enhanced nutrient cycling by bacteria. This combination is likely to be viable in bioremediation of aquaculture, with no detrimental effects on phytoplankton or marron.

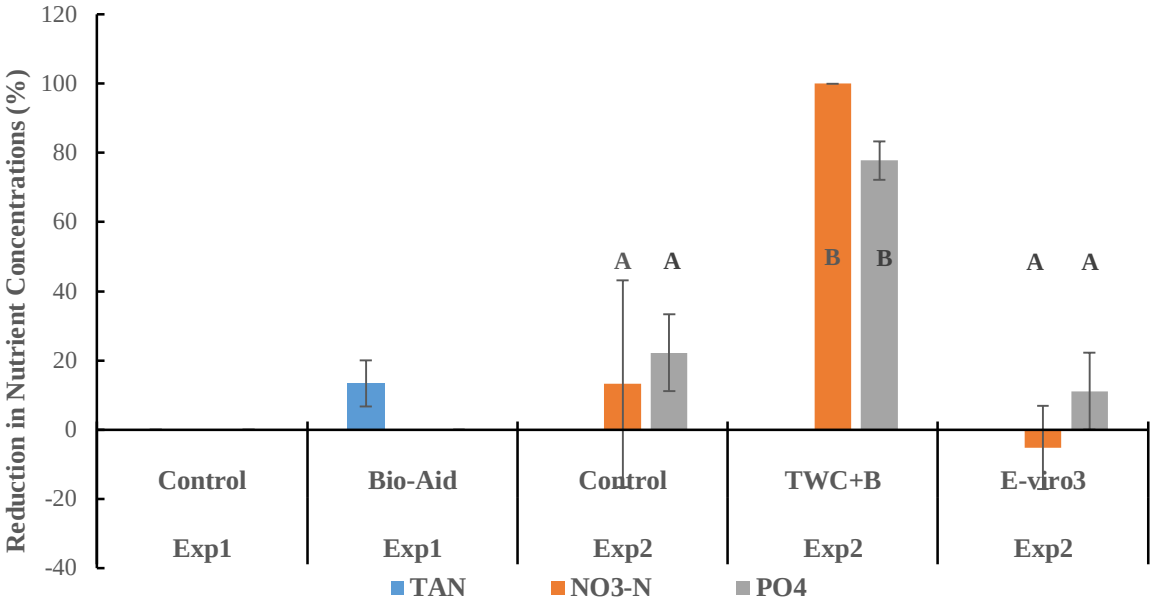

Figure 7.2: Effects of different probiotics on the reduction of nutrient concentrations in two experiments (n=3), after one week, following a period when the probiotics were most active. Different letters indicate significant differences between treatments (α=0.05). Significant reductions in concentrations of TAN in experiment 2, and NO_3-N and PO_4 in experiment 1, were not found. Error bars = Standard error of mean.

7.3 Effects of TWC on Phytoplankton and Zooplankton Communities

TWC had no positive or negative effects on plankton communities, though it likely contributed to natural productivity to some extent by providing biofilm. Meanwhile, TWC combined with *Bacillus* sp. increased the abundance of phytoplankton. The added *Bacillus* sp. may have enhanced nutrient cycling, thereby preventing collapse of the phytoplankton community after the initial consumption of nutrients. In another study, *Bacillus* sp. and *Lactobacillus* sp. added to shrimp RAS increased the abundance of Chlorophyta (De Paiva-Maia et al. 2013). *Bacillus* sp. can also play an important role in nutrient cycling in shrimp (*Penaeus vannamei*) ponds (Wang & He 2009). Bacteria produce extracellular enzymes, which are important in the environment for the degradation of macromolecular compounds in organic matter (Wang & He 2009). Further research could investigate to what extent TWC enhances the degradation of organic matter.

The phytoplankton and zooplankton abundance was not significantly affected by TWC in tank or pond culture, suggesting that TWC and attached biofilm did not inhibit or promote plankton growth. Artificial substrates often have limited effects on plankton communities. For example, Schveitzer et al. (2013) found that gross primary production was not affected by the use of substrates. Substrates can actually have a negative effect, where lower concentrations of chlorophyll *a* in water can be present in tanks with substrates than without (Schveitzer et al. 2013), while substrates can also result in lower phytoplankton biomass (Bratvold & Browdy 2001). These results were largely due to competition for nutrients between phytoplankton in the water column and algae growing on substrates. The results of experiment 2 and the field experiment did not show any strong competitive effect between the phytoplankton and the microbes growing on TWC. There is limited research investigating the effects of substrates on zooplankton, though attached periphyton can include zooplankton such as rotifers, copepods, ciliates and cladocerans (Santhana Kumar et al. 2017). Cladocerans and copepods are an important component of the diet of juvenile crayfish (Jones 1995). Increased periphyton growth on the surface of TWC could potentially provide a complementary food source for marron. In pond culture marron growth may be affected, where TWC could provide a complementary food source as biofilm (Personal observations).

7.4 Effects of TWC on Marron Health, Growth and Survival

In the laboratory experiments, there were no significant differences in the growth rate of marron following application of TWC or probiotics. In another study, Ambas et al. (2013) found no effects of growth rate with the use of a probiotic (varied species) in marron diet. Marron growth can be relatively slow under laboratory conditions (Ambas et al. 2013; Nugroho & Fotedar 2013). In experiment 3, stocking density had no effect on marron growth, while SGR was similar between experiment 3 at SD2 (at 908.4g/m^2 or 15/m^2) and experiment 2 (at 568g/m^2 or 11/m^2), despite the higher stocking density at SD2 (Table 7.2). Containers were present in experiment 3 however, limiting fighting and competition between marron. The SGR of marron was significantly higher in ponds than in laboratory conditions without substrate, partly due to the different culture conditions (Figure 7.1), while growth was similar between ponds and laboratory conditions with TWC (Table 7.2). Therefore, TWC may have had an indeterminate effect on marron growth rate.

The results on the effects of TWC on marron health were inconclusive. In experiments 2 & 3, TWC had no effect on the THC and DHC of marron. THC was higher in experiment 2 marron, irrespective of treatment, while DHC was higher in experiment 3 marron, irrespective of treatment. Within treatments, no significant differences were present (Table 7.2). The survival rate was not improved with application of TWC in tank culture, however to better understand the effects on survival rate of juveniles in pond culture requires more comprehensive research. The improvement in final biomass of juvenile marron was likely due to improved survival, though more reliable survival data may be required to confirm this effect. However, the research does suggest that TWC was not detrimental to the THC, DHC, organosomatic indices or survival rate of marron.

Table 7.2: Comparison of Growth and Physiological Parameters of adult 1+ marron from three experiments with and without TWC. SGR is after 3 months in experiments 1 and 2, and after 12 months in the field experiment; THC and DHC is after 3 months.

	Experiment 2		**Experiment 3**				**Field Experiment**	
			Stocking Density 1		**Stocking Density 2**			
	Control	**TWC**	**Control**	**TWC**	**Control**	**TWC**	**Control**	**TWC**
SGR	<0.001 ± 0.024[a]	0.030 ± 0.012	0.053 ± 0.022[a]	0.088 ± 0.083	0.057 ± 0.047[a]	0.112 ± 0.027	0.208 ± 0.023[b]	0.189 ± 0.031
THC (x10^6)	4.96 ± 0.97	7.03 ± 3.03	3.11 ± 0.94	2.84 ± 0.47	2.41 ± 0.38	2.18 ± 0.43		
DHC (%)	13.67 ± 3.36	12.87 ± 1.14	20.87 ± 0.47	32.50 ± 4.80	22.25 ± 2.60	24.50 ± 2.50		

Superscript letters [a, b] indicate significant differences between experimental conditions (α=0.05).

7.5 Bacterial Abundance and Diversity in Different Experiments

The bacterial abundance in the water column was not affected by TWC in any experiment, while the probiotics used in this study also had no effect on bacterial abundance, partly due to the high variability within treatments. Bacterial abundance was highest in outdoor marron tank culture, in experiment 2, than all other culture environments. This relates to the high abundance of phytoplankton, and the resulting high amount of organic detritus present, made up of uneaten feed, marron waste and senescent phytoplankton. In the field experiment, bacterial abundance was higher in

old ponds, where nutrient concentrations were high, than new culture ponds. The results suggest that although not increasing overall abundance, addition of TWC increases *Bacillus* sp. numbers in marron ponds, while addition of *Bacillus* sp. can maintain a continued population on TWC surface. This could be caused by TWC supplying a carbon source, as *Bacillus* sp. are known to thrive with the presence of a carbon source, and require one for many functions, such as denitrification (Hu et al. 2017; Robinson et al. 2017).

Heterotrophic plate counts revealed that high bacterial populations were inhabiting the surface of TWC in all experiments. Plate counts were high on TWC and BM surfaces in experiment 3, with no differences found between plate counts from different tank environments or substrate types (Figure 7.3). Various microorganisms, including protozoans, were found on TWC surface, which may have affected populations of heterotrophic bacteria through grazing (Thompson et al. 2002). Autotrophic bacteria, such as *Nitrosomonas* sp. and *Nitrobacter* sp., cyanobacteria, and microalgae on the surfaces were not extensively studied here, though research could investigate if these microbes are present on oil-based substrates.

Microbial mats (Lezama-Cervantes & Paniagua-Michel 2010), and other substrates with attached biofilm have previously been used for bioremediation (Da Silva et al. 2008; Pandey et al. 2014; Schveitzer et al. 2013; Viau et al. 2012). In the current study, the oil-based substrates (TWC, TWC+) had attached biofilm and microorganisms therein, the composition of which largely depended on the environment and nutrient concentrations. The taxonomic composition and biomass of periphyton is often dependent on nutrient availability (Lu et al. 2016). The bacteria and biofilm attached to TWC was promoted by the nutrient inputs in experiments 1, 2 and 3. In experiments 1 and 3, where nutrient concentrations were high, a thick layer of biofilm was present (observations), while in experiment 2 where nutrient concentrations were low, thin layer of biofilm was present (observations). The biofilm on TWC in experiment 2 contained microalgae, as determined by microscopy, though it is unclear whether algal growth was enhanced by the substrate. The composition of biofilm attached to TWC in marron ponds also differed. Overall, the substrate provided a suitable habitat for various heterotrophic bacteria, and promoted the growth of a diverse biofilm.

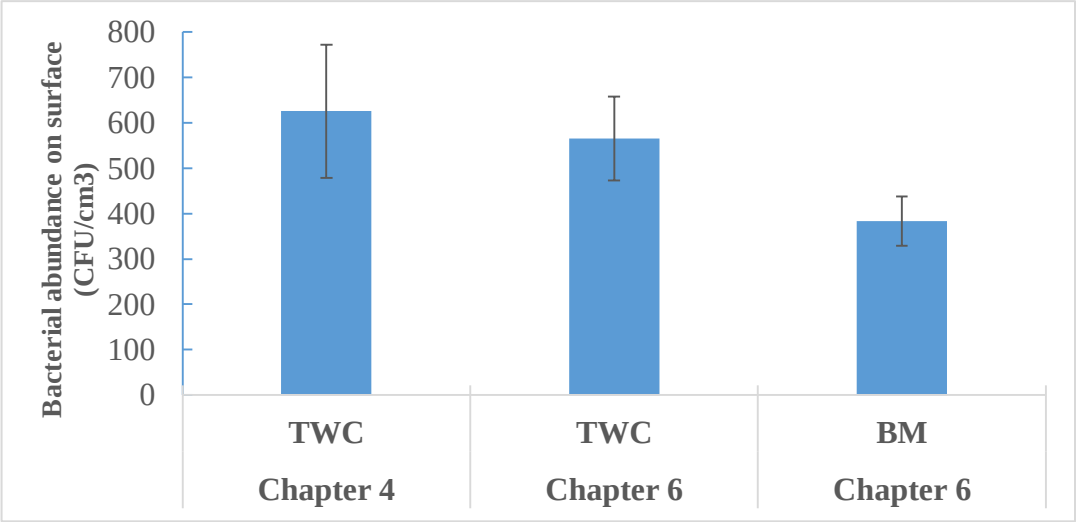

Figure 7.3: Comparison of bacterial abundance on the surface of TWC in experiment 2 (n=6) and TWC (n=8) and BM (Bioball Media) (n=3) in experiment 3. Error bars=standard error of mean.

7.6 Conclusions

This study shows that, The Water Cleanser™ influences the concentrations of ammonia, nitrite and nitrate, particularly under laboratory conditions, and reduces concentrations of orthophosphate in tank and pond culture (Figure 7.1). The substrate used in this study can be an effective bioremediator of marron culture. TWC can also provide a habitat for microbes, promoting the growth of heterotrophic bacteria including *Bacillus* species, however may not have a significant effect on bacterial abundance in the water column. From this study, it can be concluded that TWC can improve the growth of juvenile marron, with no detrimental effects to marron, phytoplankton or zooplankton. Here are the main findings:

- TWC, TWC+ and TWC+B were effective in reducing concentrations of orthophosphate. Both TWC and the probiotics used reduced the concentrations of nitrogenous metabolites under certain conditions.

- TWC was more effective in bioremediation than BM. TWC and BM both reduced the effects of density on concentrations of NO_3.

- There were no discernible effects of any treatment on the bacterial abundance in the water column.

- TWC had no detrimental effects on natural productivity, while TWC+B increased the abundance of phytoplankton.

- TWC had no significant effects on the growth rate, survival rate or haemolymph physiology of adult marron under laboratory conditions, however it increased the final biomass of juvenile marron in old culture ponds.

- Nutrient concentrations, turbidity, bacterial abundance and natural productivity were periodically higher in old ponds than new ponds.

- TAN was negatively correlated with THC, phytoplankton abundance was positively correlated with pH, but negatively with TAN and NO_3. There was a relationship found between phytoplankton abundance and zooplankton species richness, but not abundance. Experimental units with higher concentrations of orthophosphate generally had a higher diversity of bacteria.

7.7 Recommendations for Further Research

Throughout this thesis, numerous possible avenues for further research have been identified. Oil-based substrates are a relatively new topic in the field of aquaculture, with little known so far. The findings of this thesis would suggest the following for assessing the performance of substrates, including TWC, in improving water quality in marron aquaculture:

- The bio-availability of the oils present in TWC and other oil-based substrates as a carbon source for heterotrophic bacteria should be evaluated further.

- The mechanisms responsible for any improvement in marron health, growth or survival could be further studied, including changes in microbial composition, whether biofilm attached to TWC can be a viable source of nutrition, and different effects on different life stages (e.g. juvenile, grow-out).

- More research is required to determine differences in quantity and quality of bacteria cultured on the oil-based substrates compared to wooden substrates (e.g. bamboo), Bioball Media®, Aquamat®, microbial mats, and polyethylene substrates.

- A sample was drilled inside of TWC to detect bacteria but produced no aerobic or anaerobic growth. The inside of TWC is thought to contain numerous capillaries, though no electron microscopy was carried out in this study to confirm this. Further research could investigate the internal structure of TWC and whether any microbes are present.

- The role of nutrient concentrations and sources of nutrients on microbial composition and biomass on TWC could be investigated.

- Further research could investigate the effects of different bacterial species assemblages on bioremediation using TWC and similar oil-based substrates.

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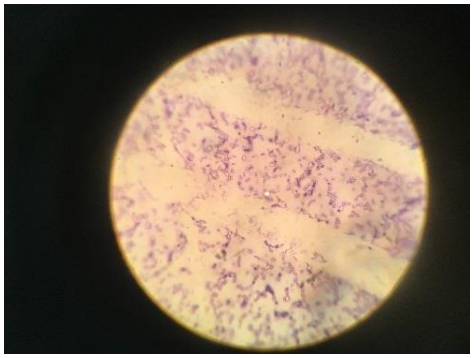
Appendix A

A) Marron tanks in experiment 2, showing presence of phytoplankton, and mesh covering.

B) Marron weighing in experiment 2.

C) Marron used in experiment 3.

D) Cocci-shaped bacteria stained with crystal violet, at 1000x magnification.



Appendix B

Statement of Author Contribution

Title of Manuscript: Effects of pond age and a commercial substrate (the water cleanser™) on natural productivity, bacterial abundance, nutrient concentrations, and growth and survival of marron (*Cherax cainii* Austin, 2002) in semi-intensive pond culture.

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Author	**Contribution to Paper**	**Contribution Percentage (%)**
Anthony J. Cole	Developed the experimental design, performed data collection, data analysis, writing and reviewing of manuscript, and acted as corresponding author.	81%
Ravi Fotedar	Supervised development of work, including experimental design, and reviewed and edited the manuscript.	10%
Smita S. Tulsankar	Helped in data collection and editing of manuscript.	7.5%
Benjamin J. Saunders	Helped in editing of manuscript.	1.5%

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