School of Molecular and Life Sciences

Role of natural productivity in growth performance and health of marron (*Cherax cainii,* Austin 2002) reared under semi-intensive aquaculture system and controlled laboratory conditions.

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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 acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Animal Ethics:

Animal ethics approval from proposed research study was not required as is not mandatory for the invertebrate animal studies at Curtin University, Australia. However, all the required protocols were followed while handling the animals, as per the guidelines of Animal Welfare Act, Western Australia and the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (NHMRC, 2013).

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Date:

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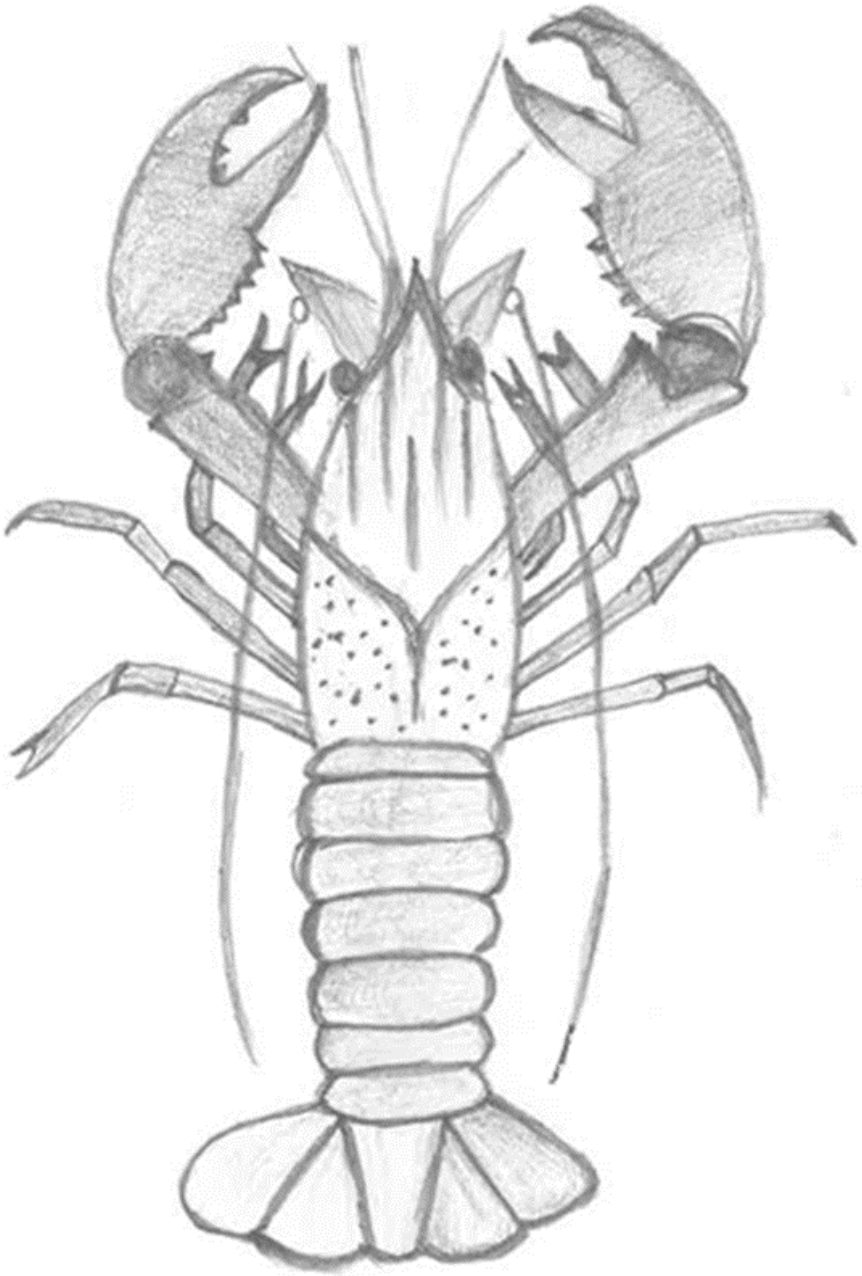

Smita Sadanand Tulsankar

This thesis is dedicated to my Aajji Parvati Mahadev Tulsankar and Shantabai Narkar, two significant women of my life whom I lost in April, who taught me to dream big and work hard to achieve them. To my parents, Mr. Sadanand Mahadev Tulsankar and Mrs. Rukmini Sadanand Tulsankar, I respect their sacrifices in helping me to accomplish my dreams. My elder brother, Prakash who spent his savings on my education, so I could continue my academic journey. My younger siblings Samidha, Shabari, Harshal, Pranay and Prajakta their everyday calls kept me stronger and sane during this time away from home. I am extremely grateful for their constant support, encouragement and for never giving up on me.

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PREAMBLE

The aim of this research was to evaluate the impact of dissolved 12 pre-selected trace elements, their correlations with plankton under semi-intensive marron (*Cherax cainii*, Austin 2002) culture ponds, and importance of plankton abundance and diversity in growth, survival rate, pigmentation, health indices and diet of marron. To fulfil the aim, research was conducted in two phases: Phase one evaluated the dissolved concentration and seasonal fluctuations of pre-selected trace elements and plankton productivity, and the correlations between trace elements and plankton under commercial semi-intensive ponds, stocked with different life stages of marron. Phase two focused on the impact of pre-selected trace element supplementation on plankton density, species diversity and plankton role in growth, survival, health indices and diet of marron.

The thesis contains ten chapters. Chapter one, introduction, provides an overview of the thesis with brief description on the marron farming in Australia and the importance of marron in aquaculture industry of Australia. The chapter also covers aim, objectives and significance of the study.

Chapter two reviews the published literature on marron farming in Australia and crayfish farming around the world. The review also focuses on the importance of trace elements as a fertilizers in aquaculture practices to improve plankton density and species diversity. It also covers the importance of plankton in marron growth, survival, health indices and diet. Impact of abiotic factors, seasonal variations and pond age on dissolved concentrations of trace elements, plankton density, species diversity, plankton succession is also explored.

Chapter three presents a general methodology used during the research, it explain the materials and methods common in field and laboratory experiments. It also covers the details of the site used for field data collection.

Chapter four is based on field experiment, and explored the effect of seasonality and pond age on water parameters, dissolved concentrations of 12 pre-selected trace elements, species diversity as well as plankton abundance, and plankton wet and dry weight under commercial semi-intensive marron culture ponds, stocked with three life stages 1. juveniles, 2. grow-out and 3. brood stock termed as brooder marron. It also investigates the correlations between dissolved trace elements and plankton density, species diversity, plankton wet and dry weight.

Chapter five, outlays the research on temporal variations and the influence of pond age on water quality, plankton density, species diversity, species richness, plankton succession under commercial semi-intensive marron culture ponds over the six noongar seasons. It also explores the correlations between plankton density, species diversity and marron yield.

Chapter six describes the experiment conducted under controlled laboratory conditions using five different weight groups of marron ranging from < 15 g to 100 g. Based on the outcomes from two field trials the feeding behaviour and feed preferences of different weight groups of marron were analysed using two formulated diets such as fish meal and black soldier fly meal (*Hermetia illucens*), and frozen copepod.

Chapter seven is a laboratory experiment, based on the findings from chapter four, five and six and did explore further different concentrations of pre-selected trace elements and their effects on plankton density, species diversity, and the follow-on effects on marron growth, survival rate, health indices and gut microbiota abundance and diversity.

Chapter eight describes the influence of feeding juvenile marron exclusively with mixed live planktons on growth, survival rate, pigmentation and health indices in the absence of formulated diet under controlled laboratory conditions.

Chapter nine includes the experiment on the effects of solo live phytoplankton and zooplankton with formulated feed and mixed on the growth, survival rate, health indices, gut microbiota abundance and diversity, histology of hepatopancreas, integrity of gut and abdominal tissue, and the contribution of plankton towards the growth of muscle tissue and whole juvenile marron.

In Chapter ten the outcomes of all experiments are discussed in a comprehensive review along with final conclusions, limitations of the study and recommendations for future research.

ABSTRACT

Australia is home to more than 110 species of freshwater crayfish, out of which marron (*Cherax cainii*, Austin 2002), a native to south-west of Western Australia, is of interest to aquaculture. Marron are mainly cultured in extensive systems as a hobby and for commercial purposes the farming is conducted under the semi-intensive farming systems. Natural productivity of the culture system plays a key role in the diet of polytrophic omnivorous marron. To date there has been a limited number of studies conducted on the relationship between ponds natural productivity and marron growth and survival under semi-intensive culture conditions. The studies on the quantity and quality of dissolved trace elements in marron pond water, seasonal fluctuations of these trace elements, plankton density, plankton community structure, the correlations between the trace elements and plankton productivity, and the importance of plankton in marron diet and health are lacking.

The main aim of the study was to understand the correlations between 12 pre-selected trace elements (Al, Ca, Co, Cu, Fe, Mn, Mg, S, Se, Si, P and Zn), and plankton density, species diversity, plankton wet and dry weight and the influence of plankton density and species diversity on the growth, survival and health of marron. To fulfil this aim, two field and four laboratory experiments were conducted. The field work took place in south-west of Western Australia near Manjimup (34°18′75″S, 116°06′61″E) and laboratory experiments were conducted at Curtin Aquatic Research Laboratory (CARL), Perth. The results from field studies showed that, the pond age and seasonal variations of environmental parameters affected the dissolved concentrations of trace elements, phytoplankton-zooplankton abundance, species diversity, and fluctuations in trace elements influenced the plankton productivity. In pond water Co and Se concentrations were below the detectable levels. Mn, Si and P were positively correlated to the plankton abundance, species diversity and plankton wet and dry weight. The research showed that twenty-six phytoplankton and seven zooplankton genera were present in marron ponds and abundance of copepod adults and copepod nauplii were recorded through-out the year. Overall, it was demonstrated that Mn, Si and P played a crucial role in plankton abundance and species diversity, and need to be maintained at optimal levels in aquaculture ponds to foster the marron growth and yield mediated through plankton.

The study on feeding behaviour and feed preference of marron showed that, fish meal was consumed by all weight groups in the least amount of time. Juvenile marron spent longer time consuming frozen copepods and rejected the black soldier fly (*Hermetia illucens*) meal diet (BSF). In addition, a higher number of frozen copepod was consumed by juvenile marron.

Under laboratory conditions, supplementation of Si and P at high concentration improved the plankton density and species diversity, resulting in better growth and health of marron. The P at higher concentration showed an increase in abundance of phosphorus solubilizing bacteria in marron gut. Mixed live phytoplankton and zooplankton achieved a significantly higher growth rate, total haemocyte count and showed an enhancement in pigmentation of juvenile marron compared to the marron solely fed on formulated feed. On feeding juvenile marron solo phytoplankton-zooplankton with formulated feed and mixed, zooplankton and mixed plankton supplementation improved the specific growth rate, survival, total haemocyte count and granular cell percentage, and reduced the haemolymph clotting time. Zooplankton supplementation improved the lysozyme activity, tail muscle dry weight indices, midgut epithelium height and tail muscle tissue myofibres in juvenile marron. In addition, the stable isotope analysis revealed that juvenile marron consumed higher percentage of zooplankton and detritus. Live plankton especially zooplankton improved the growth and health of juvenile marron under laboratory conditions.

It can be concluded that the live plankton especially zooplankton and their static availability can be an important dietary ingredient of juvenile marron and can assist in improving the growth performance. Evaluating the species specific zooplankton impact on growth, survival, health and proximate composition of juvenile marron will provide more insight into the nutritional benefits of zooplankton. This research has a capacity to contribute to a better understanding of the effect of plankton on growth, survival and health of marron cultured for human consumption, which in turn will assist in improving the yield of marron.

LIST OF ABBREVIATIONS STATED IN THIS STUDY

Al	Aluminium
ANOVA	Analysis of Variance
APHA	American Public Health Association
API	Aquarium Pharmaceuticals ®
ASVs	Amplicon Sequence Variants
B-cells	Blasenzellen cells
BM	Biomass Gain
Ca	Calcium
CARL	Curtin Aquatic Research Laboratory
ClustalO	Clustal Omega
Co	Cobalt
Cu	Copper
DHC	Differential Haemocyte Count
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
DP	Phytoplankton Predominant Index
DZ	Zooplankton Predominant Index
FAO	Food and Agriculture Organization
Fe	Iron
HCL	Hydrochloric Acid
Hid	Hepatopancreas Dry Index
HiW	Hepatopancreas wet Index
HM	Hepatopancreas Moisture Content
H'P	Phytoplankton Diversity Index
HTS	High Trace Elements Supplementation
H'Z	Zooplankton Diversity Index
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
IUCN	International Union for Conservation of Nature
JP	Phytoplankton Evenness
JZ	Zooplankton Evenness
L	Litre

LDA	Linear Discriminant Analysis
LSD	Least Significant Difference
LTS	Low Trace Elements Supplementation
Mg	Magnesium
MGA	Marron Growers Association
Micca	Microbial Community Analysis
mL	Millilitre
Mn	Manganese
MTS	Medium Trace Elements Supplementation
NCBI	National Centre for Biotechnology Information
ND	Not Detectable
NF	Not Found In Literature
NHMRC	National Health and Medical Research Council
OCL	Orbit Carapace Length
OTUS	Operational Taxonomic Units
P	Phosphorus
PCR	Polymerase Chain Reaction
Phyto	Phytoplankton
QIIME	Quantitative Insights into Microbial Ecology
S	Sulphur
Se	Selenium
SGR	Specific Growth Rate
Si	Silica
SIA	Stable Isotope Analysis
SIAR	Stable Isotope Analysis in R
SPSS	Statistical Package for the Social Sciences
SRP	Phytoplankton Species Richness
SRZ	Zooplankton Species Richness
SWCC	South-West Catchment Council
TAN	Total Ammonia Nitrogen
THC	Total Haemocyte Count
TM	Tail Muscle Moisture Content

TMid	Tail Muscle Dry Weight Index
TMiw	Tail Muscle Wet Weight Index
USA	United States of America
WA	Western Australia
WABC	West Australian Biochemistry Centre
WG	Weight Gain
Zn	Zinc
Zoo	Zooplankton

LIST OF SPECIES NAMES MENTIONED IN THIS THESIS

Scientific name	Common name
Astacus astacus	Noble crayfish
Aulacoseira subarctica	NA
Boeckella sp.	NA
Cherax albidus	White yabby
Cherax cainii	Smooth marron
Cherax destructor	Common yabby
Cherax quadricarinatus	Red claw crayfish
Cherax tenuimanus	Hairy marron
Chlamydomonas gigantean	Green algae
Chlamydomonas reinhardtii	Green algae
Chlorella vulgaris	Green alga
Chlorella zofingiensis	Green alga
Closterium subprotumidum	NA
Daphnia magna	NA
Dunaliella salina	NA
Epistylis	NA
Eriocheir sinesis	Chinese mitten crab
Euchlanis dilatata	NA
Euglena gracilis	NA
Gambusia holbrooki	Eastern gambusia
Haematococcus pluvialis	Green alga
Hermetia illucens	Black soldier fly
Hydromys chrysogaster	Water-rat
Hyriopsis bialatus	Freshwater pearl mussel
Hyriopsis bialatus	Pearl mussel
Keratella cochlearis	NA
Keratella quadrata	NA
Litopenaeus vannamei	White leg shrimp
Lupinus albus	Lupin
Macrobrachium rosenbergii	Freshwater prawn

Micrococcus lysodeiktikus	NA
Nannochloropsis oculata	NA
Nitzschia pelliculosa	Diatom
Orconectes immunis	Calico crayfish
Orconectes punctimanus	Spothanded crayfish
Orconectes rusticus	Rusty crayfish
Oreochromis niloticus	Nile tilapia
Pacifastacus leniusculus	Signal crayfish
Pandorina morum	Green alga
Panulirus cygnus	Western rock lobster
Paralithodes camtschaticus	Red king crab
Paranephrops zealandicus	Koura crayfish
Pediastrum boryanum	Green alga
Penaeus monodon	Tiger shrimp
Poecilia reticulata	Guppy fish
Pontastacus leptodactylus (Formally known as *Astacus leptodactylus*)	Narrow clawed crayfish
Procambarus clarkii	Red swamp crayfish
Procambarus spp.	White river crayfish
Scenedesmus abundans	NA
Selenastrum minutum	NA
Sphaerocystsis schroeteri	NA
Temnocephala	NA

CHAPTER 1: INTRODUCTION

1. 1. Background

Global aquaculture production has been increasing steadily and more than tripled in live weight volume from 34 million tonnes (Mt) in 1997 to 112 Mt in 2017 (Naylor et al., 2021). The Australian aquaculture industry is largely based in regional Australia and it includes the farming of three *Cherax* species such as red claw crayfish (*C. quadricarinatus*), yabbies (*C. destructor/albidus*) and marron (*C. cainii,* Austin 2002) (ABARE, 2003). Freshwater crayfish production for Australia was first reported by Food and Agriculture Organization of the United Nations (FAO) in 1984, with the production for red claw and yabby being nil while 8 tonnes of marron were produced that year (Figure 1.1) (FAO, 2021). For the year 2018 the Australian freshwater crayfish production (in quantity; tonnes) was red claw crayfish- 48.8, yabby- 51.5 and marron- 65.8 (valued at USD $2.30 million) tonnes (FAO, 2021).

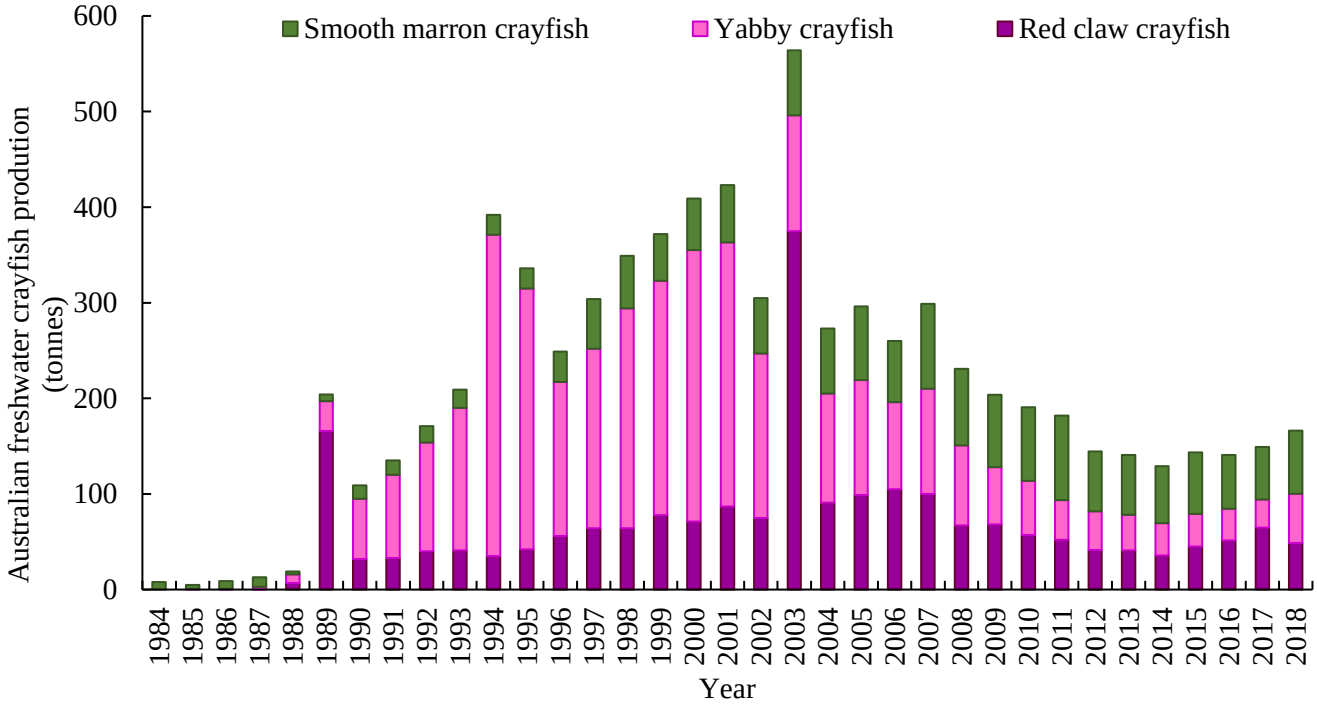


Figure 1. 1. Annual Australian freshwater crayfish production from 1984-2018 (FAO, 2021).

Due to high market value, status as gourmet product, disease free and simple life cycle marron farming is gaining in popularity (Machin et al., 2008). Australia does not have ample sources of freshwater aquatic bodies compared to several countries, however the available dams in some areas are used as a water source to produce aquatic animals. In this regards, marron is an ideal species for culture in dams and ponds, as they do not require continuous water exchange. Currently the majority of 120 g and above marron are sold domestically live and insignificant

percentage of marron are exported live (Andermel, 2016). The key challenge is how to increase the marron production (Lawrence, 2007).

To improve the yield by increasing the growth rate and reducing the size variations of juvenile marron, selective breeding was conducted by Lawrence (2007). Similarly, various studies have been conducted to improve marron growth rate and health, by analysing the effects of organic selenium, different protein sources and addition of probiotics through formulated diets on growth and survival of juvenile marron under laboratory or through aquaculture practices (Jussila and Evans, 1996, Fotedar, 2004, Sang and Fotedar, 2010b, Sang et al., 2011, Nugroho and Fotedar, 2013, Saputra et al., 2019, Morrissy, 1979, Morrissy, 1989). However, no study has been conducted to understand the effects of live plankton on growth, survival, pigmentation, health indices and gut microbiota of marron except two studies by Morrissy (1979) and Sommer et al. (1991) where authors used powdered or fresh algae *Dunaliella salina* to understand the effect on growth, survival and pigmentation of juvenile marron. The current trend in marron farming requires 2-3 years of grow-out time to achieve the market size. Maintaining beneficial plankton density may boost the growth and survival of juvenile marron, which may reduce that time period. It is an important to focus on the growth and survival of young or juveniles to achieve better yield or brooders of marron. Improving ponds plankton productivity by the use of organic or inorganic fertilizers prior stocking the juvenile marron could improve the growth and survival rate. A study by Sáez-Royuela et al. (2007) and Gonzalez et al. (2012) showed that the live food improved the growth and survival rate in juvenile signal crayfish (*Pacifastacus leniusculus*).

There is lack of scientific knowledge on various aspects of marron farming such as nursery rearing, brood stock selection and breeding protocols. To improve the marron production, farmers have shown an interest in analysing water quality of commercially farmed ponds with an emphasis on trace elements concentrations and their effects on ponds plankton productivity, as better pond management can help to improve marron production (Lawrence, 2007). In the current study the correlations between the selected trace elements and plankton density, species diversity, plankton wet and dry weight under the semi-intensive marron culture system and effects of selected trace element supplementation on plankton density and species diversity were explored. In addition, effects of dietary inclusion of plankton was analysed on growth, survival and health indices of marron under laboratory conditions. The main hypothesis is that the trace elements can influence the plankton density and communities and in turn can help to improve growth, survival rate and health status of marron.

1. 2. Aim and objectives of the study

1.2.1. Aim of the study

This study aims to increase the knowledge on the dissolved concentrations of trace elements, seasonal variations of trace elements, plankton density and plankton species diversity in commercial marron culture systems. In addition, it aims to investigate the correlations between trace elements and plankton density, species diversity, and to evaluate the importance of plankton in growth, survival rate, health indices and diet of marron. It is expected that the findings of this research will be of great importance to the marron industry. The flow of this thesis is illustrated in Figure 1.2.

In the process of achieving the above aim, the following objectives are considered:

1.2.2. Objectives

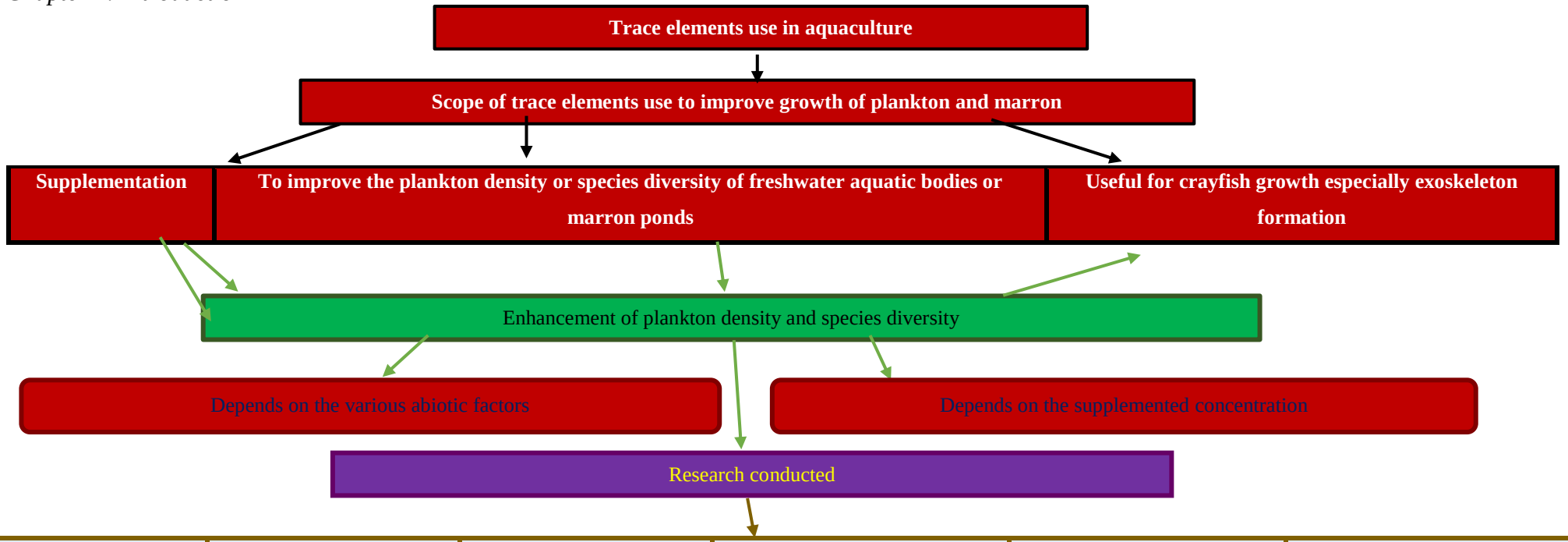
1. To determine the correlation between the dissolved quantities of pre-selected trace elements and natural productivity (phytoplankton and zooplankton) under commercial marron culture conditions.
2. To investigate the effects of seasonal, temporal variations of environmental factors and pond age on plankton density, species diversity, and plankton community composition.
3. To describe the plankton density, species diversity, plankton succession, and plankton community in semi-intensive aquaculture system of marron in south-west of Western Australia.
4. To determine the correlations, if any, between natural productivity and marron yield at various life stages.
5. To investigate the feeding behaviour and feed preference of different weight groups of marron (<15 g to 100 g) when supplied with formulated diets and copepod (based on the outcomes of objectives 2 and 3).
6. To investigate the effects of pre-selected trace element supplementation (based on the outcomes of objectives 1, 2, 3 and 4) on water quality, plankton density, species diversity as well as on growth, survival, health indices and gut microbiota of marron.
7. To evaluate the effects of mixed live plankton in the absence of formulated diet on growth, survival, pigmentation and health indices of marron.

8. To study the effects of live plankton mixture and individual taxon on growth, survival, health indices, gut microbiota, histology of hepatopancreas, muscle tissue, midgut and the plankton contributions towards the marron growth.

1.3. Significance

The significant outcomes of this research will provide information on the importance of plankton in juvenile marron growth, survival and health indices. The specific findings of this research project aim to contribute to improve knowledge of marron farming and their nutrition as below:

1. The research will help to quantify and establish underlying mechanisms of the relationship between trace elements, natural productivity and marron production under commercial farming conditions.
2. The collected information on dissolved concentrations of pre-selected trace elements will provide a baseline to understand their optimal concentrations in aquaculture systems in Western Australia.
3. Will establish the need to understand the trace elements and their correlations with natural productivity in aquaculture systems and the need to be studied considering the risk of aquatic pollution.
4. The research will provide information on feed preference and feeding behaviour of different weight groups of marron.
5. The research will not only impart new knowledge to aquaculture researchers on the ecology of farming operations of freshwater crayfish but will also assist marron farmers of Western Australia to improve marron production.
6. The research will generate new knowledge on the benefits of trace element supplementation to improve the plankton productivity in the marron industry.
7. The research will help to understand the need to reconsider the feeding management activities in commercial marron farming.
8. The research will provide information on importance of live phytoplankton and zooplankton in marron diet.



➢ **Chapter 4**	➢ **Chapter 5**	➢ **Chapter 6**	➢ **Chapter 7**	➢ **Chapter 8**	➢ **Chapter 9**
❖ Under semi-intensive farming system ❖ Seasonal variations of dissolved concentrations of trace elements and plankton abundance. ❖ Correlation between trace elements and plankton abundance, species diversity, plankton wet weight and dry weight.	❖ Under semi-intensive farming system- ❖ Seasonal and temporal variations of plankton and correlation between plankton and marron growth rate.	❖ Under laboratory conditions- ❖ Different weight groups of marron were provided with different diets to analyse the feeding behaviour and feed preference.	❖ Under laboratory conditions- ❖ Effects of Mn, Si and P supplementation on plankton density, species diversity. ❖ Effect of trace elements on marron growth, survival and health indices	❖ Effect of mixed live plankton in the absence of formulated feed on marron growth, survival, pigmentation, moulting and health indices.	❖ Effect of phytoplankton, zooplankton separately and mixed on marron growth, survival rate, immune-organosomatic indices, gut microbiota abundance and histology of hepatopancreas, gut and abdominal tissue muscle.

Figure 1.2. Conceptual diagram outlining the main theme of the study (red), and the research experiments conducted with the experimental conditions (blue).

CHAPTER 2: LITERATURE REVIEW

This chapter deals with the information on world aquaculture and role of crayfish farming in freshwater aquaculture. It also includes information on marron farming, seasonality and pond age impact and their interactions with trace elements, primary (phytoplankton) and secondary production (zooplankton) in commercial earthen marron ponds and on cultured marron in experimental tanks under controlled laboratory conditions. The information on effects of trace elements and plankton on marron growth, survival rate, immune and health indices, gut microbiota abundance, histology of marron organs and contribution of plankton towards the growth of marron has been explained.

2.1. Aquaculture

2.1.1. World Aquaculture

As the human population continues to expand beyond 7 billion, its reliance on fish production as an important protein source will increase (FAO, 2015) and wild caught fisheries alone cannot meet the demand. The total global marine catch declined in 2016 from 81.2 to 79.3 million tonnes relative to the previous year (FAO, 2018b). The decline in worldwide capture of fishery stocks has provided an opportunity for culture fisheries in terms of fish and shellfish farming or aquaculture. Aquaculture is providing substantial benefits to humanity; including food production, nutritious aquatic products, employment and potential high value trade of aquaculture products (Bostock et al., 2010, Martinez-Porchas and Martinez-Cordova, 2012, Martinez-Porchas et al., 2014). Aquaculture is one of the fastest growing food sectors worldwide, with its production growing at 7.5 % per year since 1970 (FAO, 2020b). It's contribution to the global aquaculture and capture fisheries production has risen from 25.7 % in 2000 to 46.8 % in 2016 (FAO, 2018b).

2.1.2. Freshwater aquaculture

With increasing exploitation of marine waters, scientists have been turning to freshwater aquaculture too as an important source of fish protein. Although the freshwater ecosystems cover only about 1% of the earth's surface, the ecosystems are a rich source of biodiversity providing habitat for over 40% (13,000) of the world's freshwater fish species (Funge-Smith, 2018). Freshwater ecosystems are an important source of food fish with 51.3 million tonnes harvested from inland aquaculture and 11.6 million tonnes from inland capture fisheries worldwide in 2018 (FAO, 2018b, FAO, 2020b). Freshwater aquaculture products, especially crustaceans such as shrimp, crab and freshwater crayfish, are gaining importance along with

finfish culture, especially in Asia (FAO, 2020b). In addition to providing a protein source, aquaculture practices add diversity to the economic base of regions, creates demand for training and educational services, as well as for locally produced goods (FAO, 2015). Farming of finfish, bivalves and crustaceans accounts for the three quarters of global aquaculture production by weight (Naylor et al., 2000). Global aquaculture production of 80 million tonnes in 2016 included 7.9 million tonnes of crustaceans (FAO, 2018b). The freshwater crustaceans production was higher in the early 2000s and mid-1990s, however since 2010 the production has been stable at approximately 0.45 million tonnes (FAO, 2018b).

2.1.3. Aquaculture in Australia

Aquaculture is the fastest growing primary sector industry in Australia (FAO, 2015). In 2017-18, aquaculture production of Australia was worth $1.42 billion, a 5% increase from 2015-17 (ABARE, 2020). The Australian aquaculture industry is spread throughout Australia from the tropical north to the temperate south. The industry is largely based in regional Australia and makes a positive and significant contribution to regional development (ABARE, 2020). Three freshwater crayfish species belonging to the genus *Cherax* comprising red claw, yabbies and marron are farmed in Australian aquaculture ventures. Crayfish can be exported live, and the main export markets for Australian aquaculture produces are United States, Hong Kong, China, Japan, New Zealand and Thailand. South Australia and Western Australia are the main exporters of freshwater crayfish (ABARE, 2020).

2.1.4. Freshwater crayfish distribution

Freshwater crayfish are considered as monophyletic in origin (Scholtz and Richter, 1995), and ecologically an important aquatic species, found in most parts of the world, and are present in a variety of aquatic ecosystems, mainly in temperate, subtropical water bodies and wetlands (Longshaw and Stebbing, 2016). Crayfish are dominant keystone invertebrates in many temperate aquatic ecosystems in North America, South America, Europe, Eastern Asia, Australia and New Guinea. Crayfish are native to Europe, Americas, Australia and East Asia, but naturally absent from the African (except Madagascar) and Antarctic continents, the Indian subcontinent, and much of Asia (Holdich, 2002). Crayfishes are exploited through natural fisheries wherever they are found, moreover commercial or recreational fisheries are conducted only in Australia, USA, Spain, New Guinea, Africa, Scandinavia, Turkey and mainland China (Huner, 1994, Madzivanzira et al., 2020).

With 3 families, 33 genera and over 640 species, freshwater crayfish comprise a diverse group of ecologically and commercially important species (Crandall and Buhay, 2007). The higher taxa of crayfish called superfamilies comprise; the Astacoidea and the Parastacoidea (Hobbs Jr, 1988). Astacoidea encompasses Astacidae and Cambaridae, and are restricted in their distributions to the Northern hemisphere; while Parastacoidea consist of only one family, the Parastacidae, whose distribution is restricted to the Southern hemisphere including Madagascar, southern South America, and Australasia. The family Parastacidae comprises 14 genera and approximately 139 species (Holdich, 2002).

Crayfish farming outside of Australia mainly includes Cambarid culture in North America and China and Astacid culture in Europe (Huner et al., 1988, Ding et al., 2012). Cambarid species such as *Procambarus clarkii* commonly known as red swamp crayfish or crawfish, are fast growing and found in warm waters, whereas Astacid species such as *Astacus astacus* and *Pacifastacus leniusculus* are slow growing and found in cooler temperate waters. Freshwater crayfish farming is mainly conducted in China, the southern states of the USA, Europe and Australia (Harlioğlu and Holdich, 2001, Wickins and Lee, 2002, Kozák et al., 2015).

2.1.5. Freshwater crayfish culture

The global freshwater crayfish production for the year 2019 was 2,162,159 tonnes (FAO, 2021). Freshwater crayfish industry has been developing rapidly in different regions of the world and are cultured in dams, lakes, and extensive or semi-intensive pond systems; still the majority of crayfish farming is conducted in extensive or semi-intensive systems (Harlioğlu and Farhadi, 2017). To enhance the freshwater crayfish yield five main techniques have been used 1. Management of wild populations, 2) extensive productions in natural or man-made ponds, 3) semi-intensive cultivation in ponds and raceways, 4) intensive production in tanks, basins or raceways, 5) systems that rotate crayfish with plants (Ackefors, 2000).

In Eastern Europe and Middle East (Turkey, Iran), crayfish production is based on management of wild populations (Harlioglu et al., 2004, Kozák et al., 2015). For many crayfish spp. it is not economical to rear juvenile crayfish under intensive systems until they reach the market size (Wickins and Lee, 2002). One of the economical ways to improve crayfish production, is to produce juveniles in controlled conditions and rear them in external systems including dams, lakes, natural ponds or lakes. Although majority of freshwater crayfish culture follows extensive or semi-intensive management practices, a minor percentage of culture is also conducted in tanks or raceways for commercial crayfish production for example intensive cultivation of red swamp crayfish in USA and China (Wickins and Lee, 2002, Kozák et al.,

2015). In Europe several hatcheries produce over two million crayfish annually for restocking in natural waters (Wickins and Lee, 2002). Due to the lower growth and survival rate post hatching or early days of independent life makes crayfish culture unsuccessful under controlled conditions mainly because of lack of natural productivity as feeding plays a key role in the growth and survival (Sáez-Royuela et al., 2007).

With the well-established crayfish farming systems in Europe, and the United States, while industry is still under developed in Australia (Hollows, 2016), there is an increase in demand for juvenile crayfish in aquaculture industry (Harlioğlu and Farhadi, 2017). Due to their high economic value they are considered as valuable products, and are consumed as a luxury food in many parts of the world. Crayfish are an important traditional food in Sweden and Finland, and because of their high commercial value there is considerable interest in crayfish farming (Harlioğlu and Farhadi, 2017).

Australia has a rich and diverse crayfish fauna with nine endemic genera, though *Cherax* and *Euastacus* are the two most widespread (Austin and Ryan, 2002, Beatty et al., 2004, Fotedar et al., 2015). Only three main *Cherax* species, red claw, yabbies- (common yabby -*C. destructor* and white yabby -*C. albidus*), and marron have been identified as ideal candidate species for aquaculture and are cultured for commercial purposes in Australia (Lawrence and Jones, 2002, Meakin et al., 2008). Red claw is cultured in northern Australia, in warm subtropical ponds, with a relatively fast growth rate. Yabbies (common yabby and white yabby) are cultured in Eastern Australia; and marron (*Cherax cainii*), formerly known as *Cherax tenuimanus* (Smith, 1912), is a species native to south of WA (Figure 2.1**)** and a target species for aquaculture. Marron are also cultured in Southern Africa as an introduced species; but Australia is the only significant producer of marron (Machin et al., 2008, FAO, 2017).

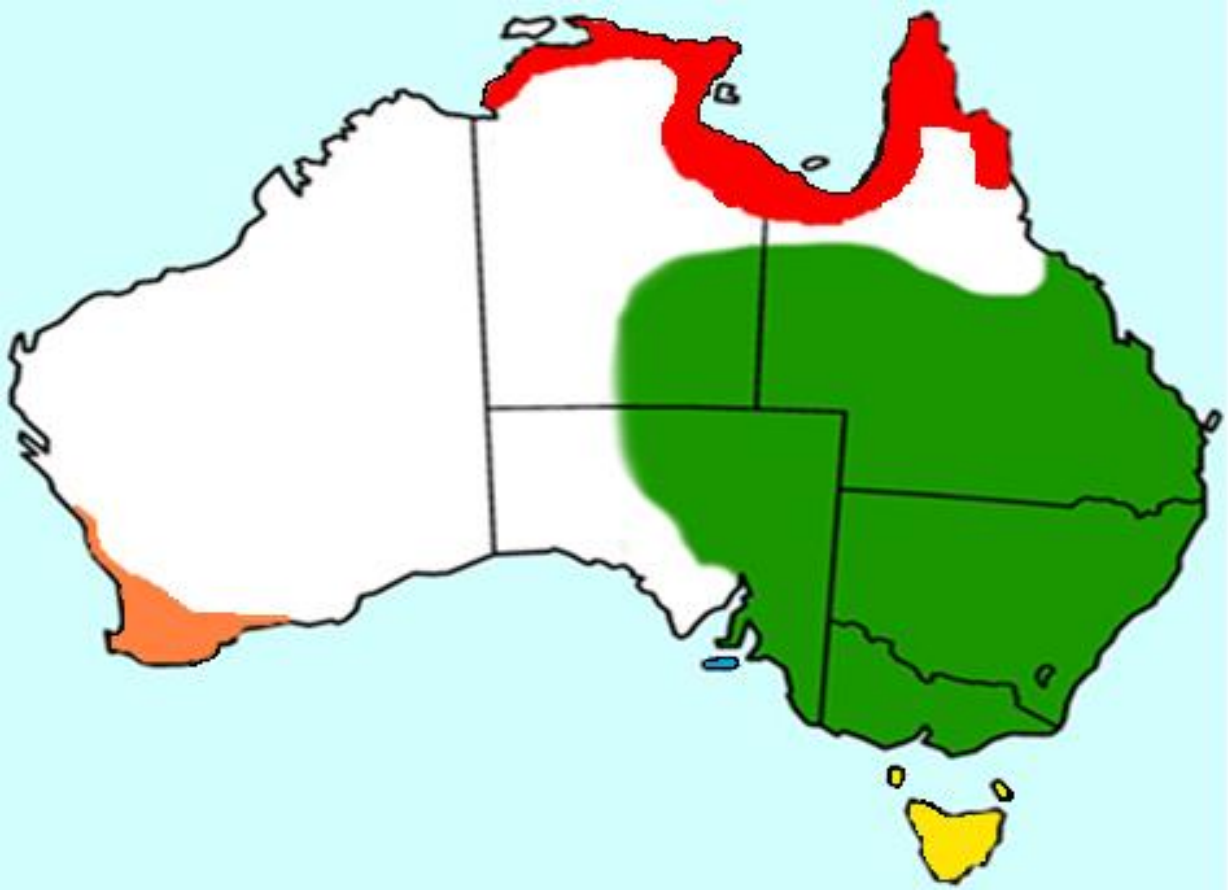


Figure 2. 1. Approximate natural distribution of common freshwater crayfish species in Australia. Marron (*Cherax cainii*) are found in the south west of Australia (orange), Redclaw (*C. quadricarinatus*) are found in the Northern Territory and Queensland (red), and Yabbies (*C. albidus*) are found in eastern and central Australia (green). Tasmania (yellow) is home to various endemic freshwater crayfish species. Several species, including marron and white yabby have been translocated to the other parts of Australia such as Kangaroo Island (Blue).

2.2. Marron production

Marron belongs to family Parastacidae; the main family of crayfish in Australia and the southern hemisphere. Marron are the third largest freshwater crayfish, and are native to south west of WA, around 75% of Australian marron production comes from WA farms mainly situated between the south coast and Geraldton (Lawrence, 2007).

2.2.1. Marron taxonomy, distribution and biology

Cherax species taxonomy is complex, and the status of a number of species is still in dispute. Hairy marron was known as *Cherax tenuimanus* since 1912, until a generic study by Austin

and Ryan (2002) demonstrated that *C. tenuimanus* is not a homogenous species and its comprised of two genetically distinct forms. Originally marron (*C. tenuimanus*) was based on the specimens from Margaret River (Australia), native to Australian Rivers and are declared as Critically Endangered in the IUCN red list (https://www.uucnredlist.org/apps/redlist/details/4618/0. 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 tenuimanus* (Smith 1912)
- *Cherax cainii* (Austin and Ryan 2002).

Hairy marron are restricted to a single river, the Margaret River (Lawrence, 2007) in the south-west of WA. Hairy marron, is distinguished by the tufts of hair-like setae on the cephalothorax and often the abdomen (Austin and Ryan, 2002) and are restricted to Margaret River. Smooth marron (*C. cainii*) are far more common and widespread, and are the only species of marron that are cultured. Smooth marron are native to the main permanent rivers in the forested, high rainfall areas in the southwest of WA. Smooth marron can be identified from other freshwater crayfish species by their dark colour, narrow pincer-like chelipeds, five dorsal keels, and two small spines on the telson.

In WA, Parastacid crayfish are restricted to the southwest coast drainage division, and have been translocated as far north as the Hutt River (Beatty, 2006) and do not occur naturally in the Pilbara or Kimberley areas. The southern distribution of marron extends as far east as Esperance, and as far as north as Geraldton, WA. Marron are endemic to the south west of WA and have been introduced to other parts of the world, notably Kangaroo Island (Australia) and South Africa, for aquaculture practices. The international interest in marron aquaculture has resulted in the species being introduced to Chile, Ecuador, Great Britain (indoor intensive culture), Malawi, New Zealand, South Africa, Zambia, Zimbabwe, Japan, south-eastern and

Central USA, China, the Caribbean and other Australian states (Morrissy et al., 1990, Alderman and Wickins, 1996). Although few industries outside of Australia have been successful, in the late 1990s marron farming expanded rapidly in both WA and South Australia. Marron is currently farmed in WA, South Australia and New South Wales. The areas in Australia potentially suitable for marron farming are determined by the temperature, water supply and the presence of clay soil suitable for aquaculture ponds (Lawrence et al., 1995).

Marron are larger than yabbies and red claw crayfish but are relatively slow growing. Marron are the third largest freshwater crayfish in the world and are capable of growing up to 2.5 kg in weight (Morrissy, 2002, Holdich, 2002). They reach market size at 30 months with an average weight size of 150-250 g, while yabbies reach the minimum market size of 30 g in less than six months (Fisheries, 2013). Unlike yabbies, marron are poor burrowers and can only survive in permanent waters such as lakes and rivers in nature, for example the Black wood and Margaret Rivers.

Still relative to the other freshwater crayfish, marron has many advantages for commercial culture (Morrissy, 1979, Ackefors, 2000), such as simple life cycle without larval stages and an ability to grow to a large size. Because of this characteristics as well as their delicate flesh, high tolerance to being transported alive, and being free from major diseases, marron are a sought after species in Western Australian aquaculture (Morrissy, 1979, Alonso, 2009b). Marron reach maturity at two or three years old. Reproduction begins in spring when the water temperature rises, a favourable season will produce more eggs ranging from 200 to 800, with clutch size also depending on the size of female marron. The female carries the fertilized eggs under the tail until they hatch late spring, during which time they are commonly known as a berried female. The hatched larvae hang around the mother's tail, by clinging to the fine hairs, for many weeks, feeding on yolk sac and moulting several times. After 14-16 weeks by mid-summer these juveniles will drop away from their mother and are ready to actively feed. Small juveniles are highly vulnerable to predation and cannibalism. After approximately six months during the early winter the juvenile are harvested and restocked in separate ponds.

Originally, marron were restricted to the rivers in the forested, high rainfall regions between Albany and Perth (Beatty, 2006), but now they are grown in both the region of their original distribution and in other parts of the state (Alonso, 2009b). Due to the decline in marron wild populations catch and size limits have been imposed (Alonso, 2009a) to secure the stock's sustainability (Alonso, 2009a). One way to utilize marron's commercial potential is through marron farming (Alonso, 2009b). The commercial production is largely based in south-west of

WA, and there is an expanding base of production in South Australia especially on Kangaroo Island (Piper, 2000).

There is renewed interest in marron farming; the iconic industry produced 55 tonnes during the year 2017 (FAO, 2017). Out of 470 active licence holders 39% are actively involved in extensive and semi-intensive marron farming with the production valued at approximately AUD $ two million a year from 2014-15 to 2016-17; higher than yabby and red claw crayfish (Fisheries, 2013, ABARE, 2018). The majority of marron farms in WA are run as an additional or marginal activity to other agriculture ventures (Alonso, 2009b). Though the farming is often conducted through extensive and semi-intensive culture systems in farm dams or ponds (Fotedar et al., 2015), commercial marron production is almost entirely based on semi-intensive purpose built aquaculture ponds (Piper, 2000).

Semi-intensive farming has helped to improve the marron production in WA (Lawrence, 2007). This interest has resulted in an increase in large numbers of scientifically designed and constructed marron farms with the current investment estimate of $ 15 million in marron farms, indicating the medium term potential for production in WA is 1000 tonnes. Around 75% of Australian marron production comes from the farming in WA (Lawrence, 2007).

In earthen ponds, marron are grown with artificial submerged shelters and are fed with commercial formulated feed as a supplement to the natural food generated in the pond, and intermittent aeration is often provided in the ponds with paddle wheel aerators. Shelters are essential in order to minimise cannibalism, and facilitates the partial harvesting while providing refuge from predators such as darters (Anhingidae), cormorants (Phalacrocoracidae), water-rat (*Hydromys chrysogaster*) and tortoises which are among the common predators of marron. Netting is used for additional protection from birds. Although Australian crayfishes are disease free, marron can be exposed to potential diseases, parasites or surface fouling caused by bacteria, fungi, worms and protozoans (Fotedar et al., 2015). Marron farming involves little or no water exchange, with water only being added or removed as necessary to compensate for rainfall and evaporation. Also the water is exchanged only once during the harvest. This approach makes up for a scarcity of water resources, and is environmentally sustainable (Alonso, 2009b).

Marron are generally considered a luxury or gourmet food and yield high prices. Compared to other crayfish their tail muscle to body ratio and meat yield are quite high (Holdich, 2002). Marron tail is 42% of the total body weight with the flesh accounting for around 31% of the total body, where in Western Rock Lobster (*Panulirus cygnus*) it is 40 % (Morrissy, 1976). Similarly, the tail meat recovery from marron is comparatively higher than *Procambarus* spp.

(red swamp crayfish and white river crayfish) with 11-25% and rusty crayfish (*Orconectes rusticus*) and calico crayfish (*Orconectes immunis*) with 6-16% (Huner, 1993); signal crayfish (*Pacifastacus leniusculus*) 11-15% and narrow clawed crayfish (*Pontastacus leptodactylus* formally known as *Astacus leptodactylus*) 9-1% (Harlioğlu and Holdich, 2001).

2.2.2. Marron food and feeding habitat

Marron are polytrophic, detritivores in feeding habit, and more carnivorous in their early life stages preferring to feed on a variety of animal matter including zooplankton (Jones, 1990, Momot, 1995, Garza de Yta et al., 2011, Brown, 1995, Nyström, 2002). Marron can consume anything existing in the pond ecosystem including microbial-enriched detritus, phytoplankton, diatoms, zooplankton, macrophytes, and plant and animal matter (Alonso, 2009b). Marron comprises an important part of freshwater 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. In natural environment the common habitats for marron are naturally clear, deep running waters with natural shelters (Merrick and Lambert, 1991, Mosig, 1998, Wingfield, 1998, Molony et al., 2004).

2.2.3. Food preference and feeding behaviour

Freshwater crayfish have been widely used for behavioural studies as they offer many advantages over other invertebrates because of their high level of social interactions in both field and laboratory conditions (Gherardi, 2002). In crayfish, antennules and antennae act as chemoreceptors, and aesthetacs located on the outer flagellum of the antennules are used for the food detection, food particles are picked up with pereopods and are then transferred to the mouthparts where they are ingested or brushed clean (Figure 2.2.) (Thomas, 1970). Mandibles are used for gripping, tearing, crushing and biting food before entry into the oesophagus (Giri and Dunham, 1999).

While searching for food pereopod one and two are constantly probing the substrate, the walking leg pincers possess rows of teeth which are specialised for food collection, the biting sides are thickened and flared to form a blade that is crenulated to provide extra grip (Holdich, 2002). In red swamp crayfish the removal of eyes, chelae, antennules, antennae, and dactyls of the first and second walking legs did not affect the crayfish ability to find food, showing that the crayfish mouthparts also possess chemoreceptors (Ameyaw-Akumfi, 1977).

By sweeping serrate setae of the distal segments over or through the substratum, 3rd maxillipeds can acquire food directly (Holdich, 2002). Third maxillipeds also help to shred the large food

items into fine particles. The food is shredded by gripping it between the mandibles and third maxillipeds. Mandibles help in gripping of food, tearing, crushing and to bite the item before entry into the oesophagus. As the diet of crayfish varies the form of mandibles changes. Some crayfish, especially juveniles are thought to be filter feeders (Holdich, 2002).

Marron feed on variety of food items and their feed preference may change with the age (Goddard, 1988). Juvenile marron are generally more active and planktivorous than adults (Sierp and Qin, 2001). The number of copepods eaten by marron decreased with an increase in weight (Tulsankar et al., 2021c).

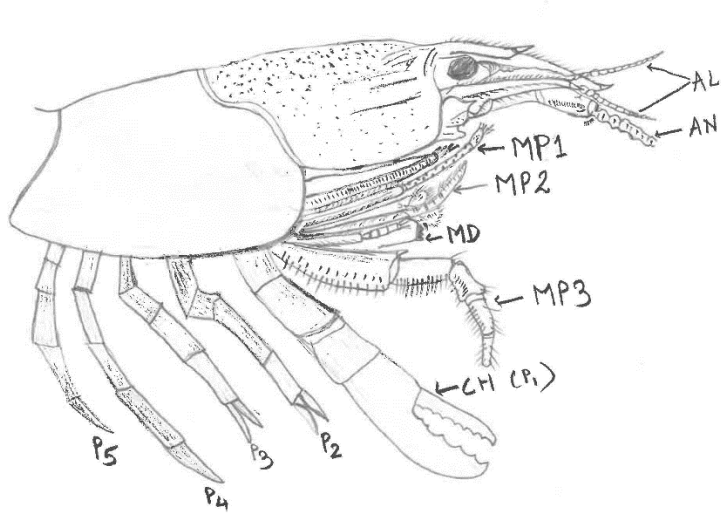


Figure 2. 2. Illustrates the lateral view of marron mouthparts and pereopods used in feeding activities. AL= flagella of antennule, AN= flagella of antenna, MP3= endopod of 3rd maxilliped, MD= mandibles, CH= chelate, P1-P5= pereopods. Modified from background and functional morphology of crayfish chapter from a book by, Holdich (2002).

2.2.4. Marron growth and moulting

As marron inhabit cool waters they are relatively slow growing compared to tropical species, such as red claw crayfish. Growth is affected by seasonal temperature and can be very slow between April and August (Australian winter) and increase from spring through to autumn. Crustacean's growth including marron, occurs via moulting, similar to other temperate crayfish (Holdich, 2002). Moulting (also known as ecdysis) is dependent on various environmental and endogenous factors such as temperature (Hughes et al., 1972, Chittleborough, 1975), photoperiod, water quality (Quackenbush and Herrnkind, 1983), stress levels (Weis et al., 1992), food quality (Chittleborough, 1975), developmental stage and reproductive maturity

(Skinner et al., 1985, Cheng and Chang, 1994). In females moulting is limited during the reproduction process. Moulting frequency can be reduced in marron entirely fed on artificial diet compared to the marron fed on natural food (Morrissy et al., 1984). Also, lack of natural food can cause the natural pigmentation loss from exoskeleton mainly due to the lack of carotenoids and asthaxanthin (Jussila and Mannonen, 1997, Tulsankar et al., 2021a). Apart from controlling growth in crustaceans, moulting also regulates the physiological changes in haemolymph and hepatopancreas (Richard, 1980, Durliat and Vranckx, 1982).

2.2.5. Marron physiology

Haemolymph is a medium of transport for nutrients and wastes in the open circulatory system of marron. During moulting, the haemolymph undergoes qualitative and quantitative changes in its constituents (Chang, 1995). Haemolymph includes various types of haemocytes. The three main known types are granulocytes, semi-granulocytes and hyalinocytes, which differ in their morphology and functions. The number of haemocytes and the proportion of granulocytes are considered as indicators of stress and health in crustaceans (Jussila and Mannonen, 1997). 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 and Zeng, 2012). In several crustacean species the haemocyte counts can be affected by various factors such as DO, pH, temperature, salinity and ammonia (Sang and Fotedar, 2009).

Hepatopancreas, the digestive gland of crayfish, serves as a pancreas and a liver, also acts as a main energy and nutrient reserve for the moulting and growth of crayfish (Lindqvist and Louekari, 1975, Holdich and Reeve, 1988). The size and moisture concentration of the hepatopancreas is used as an index of condition (Jussila and Mannonen, 1997), stress level (Haefner and Spaargaren, 1993), health and nutritional status (Evans et al., 1992, McClain, 1995b, McClain, 1995c). Hepatopancreas can detoxify foreign compounds in the haemolymph and stores Ca, Cu and other nutrients which are required for moulting and growth (Jussila and Mannonen, 1997, Depledge and Bjerregaard, 1989). The hepatopancreas size and moisture content is also impacted by diet. The organosomatic indices of tail muscle can be used to monitor the physiological condition of the animals (Prangnell and Fotedar, 2006). In marron, hepatopancreas and tail muscle are used to store energy and their wet and dry wet indices are health indicators and are often related to the quality of marron nutrition received during their growth phases.

Marron growth is largely dependent on suitable environmental parameters, they can grow and survive in the temperature range from 11.5 to 30°C (optimum 24°C) (Morrissy, 1990), however at temperatures above 27°C survival can be affected (Rouse and Kartamulia, 1992), while temperature below 13°C can inhibit growth (Morrissy, 1990). The dissolved oxygen (DO) level for marron growth should be >5 mg L^{-1} (Morrissy et al., 1984).

2.3. Water quality

Marron growth and survival is dependent on water quality. Poor water quality especially low oxygen level and excessive organic matter build up can cause a presence of *Epistylis* and *Temnocephala* (Ambas et al., 2013). *Epistylis* is a stalked protozoan that uses the animal as an attachment substrate thus appears as a fluffy growth on the exoskeleton. *Epistylis* is harmless to the cultured animal, unless they grow in large numbers to restrict water flow to the gills, causing asphyxiation. *Temnocephala* are free living flatworms (0.5-1mm) that lay their eggs in the marron gills and on the exoskeleton. Adult worms rove over the surface of the crayfish, eating algae and other microfauna (Fisheries, 2013). These two parasites do not cause any direct health risk to marron.

Ammonia and nitrite can cause physiological problems in crayfish (Jensen, 1996, Harris et al., 2001), though their concentrations are generally low in semi-intensive pond culture (Cole et al., 2019). Ammonia can be present as ionised ammonium (NH_4^+) or toxic unionised ammonia (NH_3), and is oxidised into nitrite and then into nitrate. In intensive culture there is a higher possibility of higher levels of ammonia, nitrite or nitrate which may impact the grown crayfish health and may inhibit their growth (Jussila and Evans, 1996).

The effects of acidification on marron growth and health are not well studied, however a pH range of 7 to 9 is thought to be optimum for marron culture. Low pH level (<5) can negatively affect the crayfish (Holdich, 2002); as in acidic water it can be difficult to maintain the calcium (Ca) and oxygen levels (Ackefors, 2000). Marron are sensitive to fluctuations in oxygen levels, where low oxygen levels can cause hypoxia which is a result of excessive feeding (Morrissy et al., 1984). Overloading of organic matter in ponds may lead to oxygen depletion causing low survival of marron (Morrissy, 1979), and can also increase water turbidity. Organic matter build up in sediment can create anaerobic conditions and release toxic substances such as ammonia, nitrite and hydrogen sulphide. The main cause of organic matter in ponds can be uneaten feed, faeces and senescence of microalgae (Li and Boyd, 2016).

There have not been any serious disease outbreaks in marron culture, though appearance of these parasites may impact negatively on the consumer appeal reducing its market value. To control their presence or to decimate these parasites, improving water quality, container sanitization, decreasing bacterial and nutrient density, decreasing animal density and improving nutrition will help (Fisheries, 2013). Also aeration is recommended for moderate to high levels to improve water quality and prevent anoxic conditions. Another important measure of water quality in aquaculture is water turbidity which relates to the soil type, pond water constituents, nutrient concentrations, and presence of plankton density (Culver and Geddes, 1993, Gamboa-Delgado, 2014) which can be an important food source. Turbidity can affect photosynthesis at the benthic level, while clear water may increase crayfish vulnerability to predation especially by birds. Higher turbidity may cause lack of light penetration leading to the decrease in ponds plankton productivity.

2.4. Freshwater plankton

All living organic particles which float freely, independently and involuntarily in water are known as plankton (Hensen, 1887). The planktonic community is comprised of plants and animals, based on their source of nourishment, plankton are divided into two groups termed as phytoplankton and zooplankton. Phytoplankton are the photosynthetic plants acting as primary producers, whereas zooplankton are micro and macro animals feeding on phytoplankton as primary consumer and secondary producers in aquatic ecosystems. Phytoplankton are distinguished based on their abundance and sizes for example plankton of lakes are known as Limnoplankton, while plankton of ponds as Heleoplankton and plankton of Rivers as Potamoplankton (Reynolds, 1984).

2.4.1. Plankton growth and environmental parameters

Phytoplankton growth and survival is dependent on various biotic and abiotic environmental factors such as temperature, DO, pH, ammonia, light intensity, sunlight availability, turbidity and nutrient concentration (Ndebele-Murisa et al., 2010, Tulsankar et al., 2021b, Tulsankar et al., 2020). The environmental variables directly influence phytoplankton and helps to understand the dynamics or the dominance of one or other species (Sanders et al., 1987). Ammonia levels can be influenced by the water pH, where higher pH can increase the amount of toxic unionised ammonia. In general pH is considered an important key parameter in freshwater aquaculture productivity. In pond culture high nitrate and phosphate levels can trigger an algae bloom and growth of filamentous algae (Blaas and Kroeze, 2016). Excessive

algal blooms can also influence the pH levels. Controlled laboratory conditions such as continuous saturating light, near constant temperature and ample accessibility of nutrients provide favourable conditions for plankton to grow to their highest potential. Light intensity plays an important role in the photosynthesis process; insufficient light intensities may limit the phytoplankton growth rates. Continuously illuminated plankton cultures grow better than those under specific photoperiods (Li et al., 2017a). Apart from all these factors, temperature and nutrient concentrations are known as the main primary abiotic factors controlling the plankton productivity of freshwater aquaculture ponds.

2.4.2. Temperature

Temperature is one of the most important abiotic factors controlling the growth of phytoplankton and zooplankton (Cieplinski et al., 2018, Butterwick et al., 2005). Temperature not only affects the plankton density but the species diversity and activities in space (altitude, latitude) and time (seasonal periodicity) (Butterwick et al., 2005) and influences the phytoplankton species richness (Stomp et al., 2011). The temperature requirement for the growth of plankton varies from species to species (Table 2.1), however most of the phytoplankton are able to grow at the temperature range of 10-25°C (Reynolds, 1984, Butterwick et al., 2005). Chlorophyceae abundance have been recorded in every season in aquaculture ponds and is a dominant class of phytoplankton in freshwater aquaculture ponds. On the other hand, cladoceran abundance has been recorded in colder seasons (<20°C). It is difficult to maintain the plankton densities in outdoor semi-intensive aquaculture ponds but understanding their availability during the different seasons can help to manage the growth of beneficial plankton in aquaculture ponds.

Table 2.1. Shows the required or optimum temperature range for the growth of freshwater plankton observed in semi-intensive marron culture ponds.

Study site	Class/Family	Phytoplankton	Findings	References
Laboratory conditions	Chlorophyceae	*Eudorina* sp.	Sustained growth at 2°C-30°C and showed lack of growth at >30°C.	(Butterwick et al., 2005, Yamada et al., 2008)
Laboratory conditions	Chlorophyceae	*Cosmarium* sp.	Sustained growth at 7°C, 25°C and 30°C (Butterwick et al., 2005). Temperature >32°C caused growth inhibition (Stamenković and Hanelt, 2013).	(Butterwick et al., 2005, Stamenković and Hanelt, 2013)
Laboratory conditions	*Cyanophyceae*	*Microsystis* sp.	Growth was observed at 2°C, 25°C and 30°C. Non-sustained increase at 5°C; capacity for sustained growth at 25°C and 30°C and lack of growth at 35°C was observed.	(Butterwick et al., 2005)
Laboratory conditions	Chlorophyceae	*Haematococcus* sp.	The optimum temperature for *Haematococcus pluvialis* was between 25°C and 28°C (Fan et al., 1994) and 14°C - 28°C(Evens et al., 2008).	(Fan et al., 1994, Evens et al., 2008)
Laboratory conditions	Chlorophyceae	*Monoraphidium* sp.	*Monoraphidiu*m spp. sustained growth at only 2°C, 7°C, 25°C and 30°C.	(Butterwick et al., 2005, Hawrot-Paw et al., 2020)
Laboratory conditions	Chlorophyceae	*Chlamydomonas* spp.	Can grow well in temperature range of 20°C -25°C; can tolerate as low as 15°C and as high as 32°C -34°C (Messerli et al., 2005). The optimum temperature for *Chlamydomonas reinhardtii* was 25°C, temperature < 17°C and >30°C decreased the growth (Carr et al., 2017).	(Harris, 2009, Messerli et al., 2005, Carr et al., 2017)
Laboratory conditions	Chlorophyceae	*Scenedesmus* spp.	Optimum temperature for the growth of *Scenedesmus abundans* was 20°C, cell cycle length was prolonged with decreased temperature.	(Mandotra et al., 2016)
Laboratory conditions	Chlorophyceae	*Selenastrum* spp.	The growth of *Selenastrum minutum* was determined over the wide range of temperature 15-35°C and maximum growth rate was observed at 35°C.	(Singh and Singh, 2015)

Freshwater lake	Chlorophyceae	*Sphaerocystis* sp.	In Mediterranean climate, highest abundance of *Sphaerocystsis schroeteri* was recorded between 19.1°C -26.7°C.	(Varol, 2019)
Freshwater lake	Chlorophyceae	*Tetraedron* sp.	*Tetrahedron* spp. growth observed at minimum of 14 and maximum of 30°C.	(Grover and Chrzanowski, 2006)
Laboratory conditions	Chlorophyceae	*Volvox* spp.	The optimal growth of *Volvx* sp. was observed at 32°C.	(Umen, 2020)
Controlled conditions	Chlorophyceae	*Pandorina* sp.	*Pandorina morum* was grown at the temperature of 24°C.	(Othman et al., 2018)
Microcosm conditions	Chlorophyceae	*Pediastrum* sp.	Growth rate of *Pediastrum boryanum* for different life stages was observed at 10°C and 20°C and growth rate was higher at 20°C.	(Park et al., 2014)
Laboratory conditions	Bacillariophyceae	*Navicula* spp.	Optimum temperature for the growth of *Navicula* sp. was 30°C.	(Li et al., 2017b)
Microcosm	Bacillariophyceae	*Nitzschia* spp.	*Nitzschia pelliculosa* was grown at 10°C, 15°C and 25°C; and growth was higher at 25°C.	(Chalifour and Juneau, 2011)
Laboratory condition	Trebouxiophyceae	*Actinastrum* sp.	*Trebouxia* sp (Trebouxiophyceae) were cultured <10°C and 15°C; optimum temperature for growth was <15°C.	(Balarinová et al., 2013)
Laboratory conditions	Coscinodiscophyceae	*Aulacoseira* spp.	*Aulacoseira subarctica*'s sustained growth was observed at 7°C, 20°C -25°C. Lack of growth was observed at >25°C.	(Butterwick et al., 2005)
Laboratory conditions	*Cyanophyceae*	*Chroococcales* spp.	Mean optimum growth temperature for *Cyanophyceae* spp was 29.2°C.	(Lürling et al., 2013)
Laboratory conditions	Euglenoidea	*Euglena* spp.	Optimum temperature for high multiplication rate of *Euglena gracilis* was 27°C -31°C.	(Ekelund and Danilov, 2001)
Wetland	Euglenoidea	*Peranemopsis* sp.	NF.	
Laboratory conditions	Eustigmatophyceae	*Nannochloropsis sp.*	The optimal temperature for the growth of *Nannochloropsis oculata* was 20°C, temperature <20°C and >25°C decreased the growth rate.	(Converti et al., 2009)
Laboratory conditions	Trebouxiophyceae	*Chlorella* sp.	Sustained growth of *Chlorella* sp. was observed at 7°C, 25°C and 30°C (Butterwick et al., 2005). *Chlorella vulgaris* growth	(Butterwick et al., 2005, Khalil et al., 2010, Liu et al., 2014)

			was decreased at >30°C(Khalil et al., 2010) and *Chlorella zofingiensis* grows well at 20°C –30°C (Liu et al., 2014).	
Controlled conditions	Trebouxiophyceae	*Micratinium* sp.	Optimal temperature for the maximum growth of *Micratinium* sp. was 25°C.	(Abu-Ghosh et al., 2020)
Laboratory conditions	Zygnematophyceae	*Cosmarium* spp.	In tropical conditions *Cosmarium* sp can grow at higher optimum temperature of 35°C, in temperate at 25°C -30°C and arctic species can grow faster at 7°C while 25°C can decrease their growth.	(Stamenković and Hanelt, 2013)
Laboratory conditions	Zygnematophyceae	*Closterium* spp.	Optimum temperature for maximum growth rate of *Closterium subprotumidum* was 35°C.	(Singh and Singh, 2015)
Laboratory conditions	Zygnematophyceae	*Mesotaenium* sp.	Maximum growth rate of *Mesotaenium* sp was observed between 15°C - 32°C and low at 5°C.	(Moss, 1973)
Controlled conditions	Zygnematophyceae	*Micrasterias* spp	*Micrasterias* spp can grow under wide range of temperature (Lütz-Meindl, 2016); optimum temperature range is 10- 20°C (Weiss et al., 1999). <9°C and >30°C affect their growth negatively (Lütz-Meindl, 2016).	(Lütz-Meindl, 2016, Weiss et al., 1999)
Laboratory conditions	Zygnematophyceae	*Spirogyra* spp.	Optimum temperature for the growth of *Spirogyra* spp. was 10°C to 17°C.	(Singh and Singh, 2015)
Controlled conditions	Branchionidae	*Branchionus plicatilis*	*Branchionus* spp. can tolerate the temperature ranges between 15°C to 31°C.	http://www.fao.org/3/W3732E/w3732e0h.htm#:~:text=Brachionus%20calyciflorus%20and%20Brachionus%20rubens,waters%20of%20various%20ionic%20composition.
Laboratory conditions	Branchionidae	*Keratella cochlearis*	The optimum temperature range is 1-28°C, however, their abundance was observed at 20 °C.	(Galkovskaja, 1987)
Laboratory conditions	Branchionidae	*Keratella quadrata*	Optimum temperature for the growth of *Keratella quadrata* was 15°C.	(Walz, 1983)
Laboratory conditions	Euchlanidae	*Euchlanis dilatata*	Can grow under wide range of temperature from 14°C to 26°C.	(Wenjie et al., 2019)
Lake	Cyclopoida	*Cyclops spp*	Prefers relatively lower temperature <10°C.	(Johnson et al., 2007)

Laboratory conditions Outdoor culture	Daphniidae	Daphnia *sp.*	Optimum temperature range was 10°C -23°C, (Hoefnagel et al., 2018); temperature >26°C affected *Daphnia magna* growth negatively and >29°C was lethal (Müller et al., 2018).	(Müller et al., 2018, Hoefnagel et al., 2018)
Laboratory conditions	Moinidae	Moina *sp.*	*Moina sp.* can tolerate the wide range of temperature from 5-31°C; optimum temperature for the growth is 24-31°C.	(Rottmann et al., 1992)
Lake	Calanoid	*Boeckella* spp.	*Boeckella* spp. abundance was recorded when temperature ranged between 4°C to 15°C.	(Hall and Burns, 2001)

NF= Optimum temperature range not found in literature.

2.5. Plankton and nutrients

2.5.1. Fertilization in aquaculture

Semi-intensive aquaculture practices are performed in static water bodies, where limnological production principles and aquatic ecology are used to artificially produce natural productivity of the pond through management activities (Soderberg, 2012). The principle management strategy to improve the natural productivity and yield of cultured animals involve the application of organic or inorganic fertilizers commonly known as chemical fertilizers or combination of both (Soderberg, 2012). The energy originally captured through photosynthesis is consumed by zooplankton and ultimately by the cultured animal. Marron can attain partial nutrition either by direct phytoplankton consumption or through microbial decomposition, resulting in production of microbial biomass and particulate organic matter which is consumed by zooplankton and finally by marron. To increase marron yield farmers use different types of formulated feed and occasionally fertilisation is conducted to improve pond natural productivity.

In the marron industry the use of organic fertilizers is a rare practice, mainly due to the labour cost and the risk of eutrophication, leading to algal blooms. In such a scenario the application of trace elements in suitable quantities and during the suitable season could help to improve the ponds natural productivity, without adverse effects. For example, silica supplementation can increase diatom abundance and marron can feed on diatoms. With the increase in the use of formulated feed, there is a decrease in fertilization practices, yet with high expenses and variable yields there is a growing need to focus on improving the naturally occurring plankton biota of marron ponds.

2.5.2. Inorganic (Chemical) fertilizers

Fertilizers containing phosphorus and silicate are used in aquaculture to improve plankton productivity (Boyd, 2018). Similarly Al, Ca, Mg, Si, Mn, Zn, Co, Cu, Fe, Se, P and S in micro or macro quantities are considered as essential for the growth and functioning of aquatic plants and animals (White and Brown, 2010, Alloway, 2013). These trace elements can be used to improve the density, and species diversity of plankton and growth and physiology of cultured animals in aquaculture ponds (Goldman, 2010, Tulsankar et al., 2021d). Trace element supplementation and fluctuations in their concentrations can influence plankton productivity in aquatic ecosystems (Nwankwegu et al., 2020, Tulsankar et al., 2020, Tulsankar et al.,

2021d). Moreover, excess amount of trace elements can be a reason for the growth of harmful planktons (Nwankwegu et al., 2020).

2.5.3. Advantages and disadvantages of fertilizers

Farmers wish to improve the marron productivity (Lawrence, 2007) and to achieve it they use organic or inorganic fertilizers. However, there are some advantages and disadvantages of using fertilizers such as inorganic fertilizers that are nutrient rich and soluble providing nutrients for immediate uptake by primary producers. Organic fertilizers are easily available but are nutrient-poor. Inorganic nitrogen is often added to organic fertilizers to improve their effectiveness. In developed countries, the cost of labour makes organic fertilization expensive. In developing countries where the labour costs are lower, organic fertilization is generally less expensive than purchase of manufactured inorganic fertilizers (Soderberg, 2012). With the use of inorganic fertilizers there is a chance of oxygen depletion in ponds from plankton respiration, but dissolved oxygen problems are much more prevalent in ponds fertilized with organic matter due to the oxygen requirement for decomposition.

The application of fertilizers should be conducted in suitable quantities with the required nutrients and during the suitable season to avoid the development of algal bloom (Nwankwegu et al., 2020) and anecdotes from the farmer about algal bloom due to the addition of nutrients such as nitrogen and phosphorus during the autumn.

2.5.4. Trace elements and their importance in plankton growth

Extensive research has been done on nutrient supplementation and plankton productivity in natural lentic freshwater bodies (O'Brien and de Noyelles, 1974, Downs et al., 2008, Nwankwegu et al., 2020), with less focus on trace element concentrations and their impact on plankton productivity of aquaculture farming systems. In aquatic ecosystems the trace element input is a result of natural processes such as precipitation and runoff, soil or rock disintegration, atmospheric deposition and anthropogenic activities such as over exploitation, mining, urban, agricultural and industrial activities (Khatri and Tyagi, 2015, Saha et al., 2017). Aquatic organisms assimilate dissolved trace elements directly from the environment (Zhang et al., 2018), including phytoplankton (Baeyens et al., 1998, Hassler et al., 2004), while zooplankton can acquire them through aqueous absorption and dietary ingestion of phytoplankton (Yu and Wang, 2002) ultimately providing nutrition to top predators in aquaculture ponds as shown in Figure 2.3.

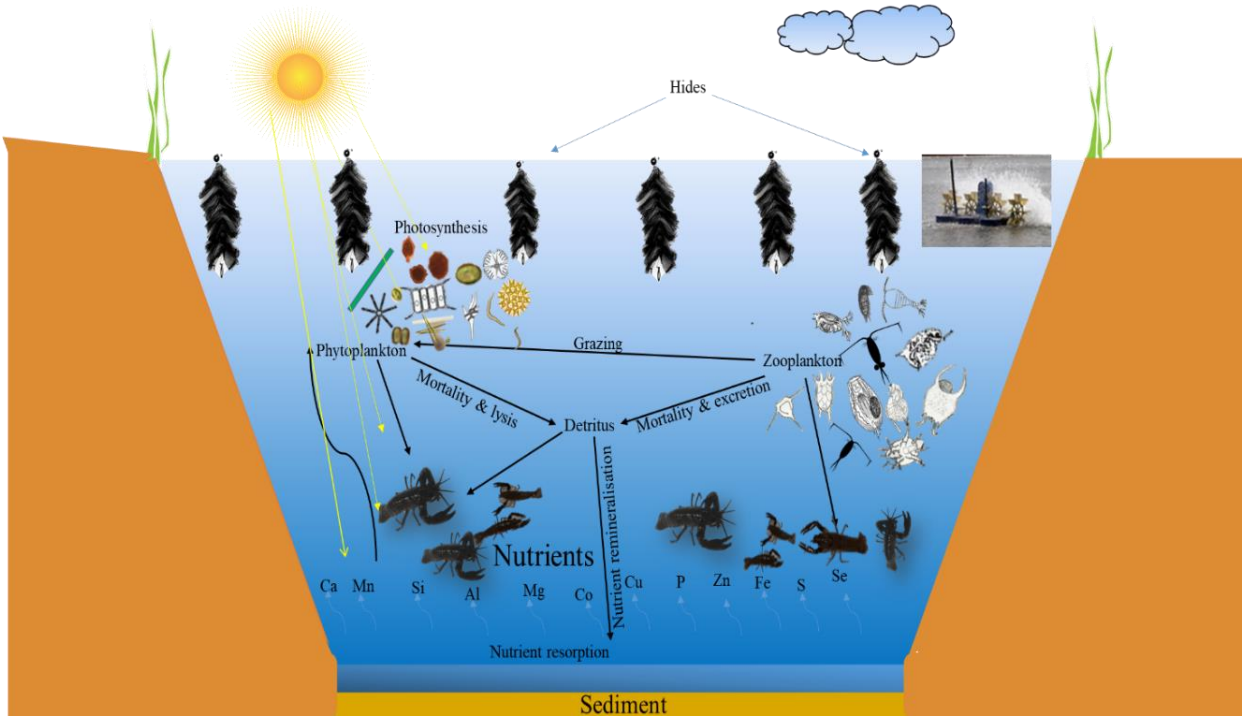


Figure 2. 3. A typical structure of semi-intensive marron cultures pond ecosystem, where phytoplankton utilize the nutrients for growth through photosynthesis and zooplankton graze on phytoplankton. Phytoplankton and zooplankton are eaten by marron in fresh form or through detritus (Tulsankar et al., 2021d) (Tulsankar et al., 2021 under review- Chapter 9).

Trace element deficiencies can limit the growth of phytoplankton (Nwankwegu et al., 2020). Phytoplankton utilise the trace elements from aquatic ecosystem for various biological functions. The following information describes the importance of pre-selected trace elements in this study and their importance in plankton growth and photosynthesis:

2.5.4.1. Aluminium (Al)

Al plays a significant role in the aquatic ecology in relation to speciation and cycling of transition metals (Achterberg et al., 1997), complexing with organic materials (Clarke et al., 1995, Nierop et al., 2002) and binding to the plankton surfaces (Crist et al., 1981). Al is highly soluble at low (<6.0) and high (>9.0) pH and potentially toxic to freshwater biota if the pH decreases below 6.0 (Gensemer and Playle, 1999, Quiroz-Vázquez et al., 2010). Al concentration between 0.05 to 0.5 mg L^{-1} slowed the growth rate of phytoplankton species *Chlamydomonas gigantea* (Quiroz-Vázquez et al., 2010); some Chlorophyceae species were able to tolerate Al exposure up to 4 mg L^{-1} at pH 5.5 (Hörnström et al., 1984). Al toxicity is dependent on water pH (Gensemer and Playle, 1999).

2.5.4.2. Calcium (Ca)

Ca influences the supply of photo-synthetically available carbon and water's capacity to buffer fluctuations in pH; both mechanisms play an important part in controlling phytoplankton's photosynthetic activity and their species composition (Reynolds, 1984). In crayfish pond culture, water having a high calcium concentration (above 20mg L^{-1}) is favourable for plankton production (Ackefors, 2000).

2.5.4.3. Cobalt (Co)

Co is essential for the synthesis of vitamin B-12 in various algae (Goldman, 2010), although high Co levels can be toxic for some phytoplankton species. Co solubility is not affected by the water pH but Co absorption by algae is impacted by water pH, for example in some *Chlamydomonas* species pH has an impact on cobalt absorption (Goldman, 2010). In freshwater Co is generally found in Co^{2+}, carbonate, hydroxide, sulphate and adsorbed forms, as well as in the form of oxide coatings and crystalline sediments (Smith and Carson, 1979). Co toxicity to freshwater plankton depends on individual species, their physiological and environmental conditions (Nagpal, 2004). For agricultural irrigation water the long term (up to 100 years) trigger value is >0.05 mg L^{-1} and for a short term (up to 20 years) is >0.1 mg L^{-1} (ANZGFMWQ, 2018). For the protection of freshwater aquatic life, Australia has developed low reliability trigger value of Co at 0.0014 mg L^{-1} (ANZGFMWQ, 2018).

2.5.4.4. Copper (Cu)

Cu helps in respiration and photosynthetic reactions in plants and animals (Goldman, 2010). Cu bioavailability and solubility is impacted by water pH, wherein higher pH under oxic conditions forms $Cu(OH)_2$ and insoluble CuO (Goldman, 2010). While at lower pH insoluble CuO can form complexes with organic compounds. These complexes contribute to the Cu loss by absorbing to organic detritus or humus (Goldman, 2010). Cu toxicity to algae depends on individual species, their physiological and environmental conditions and the chemical form of Cu in the medium (Sunda and Guillard, 1976). Cu concertation at 0.5 mg L^{-1} was injurious to algae growth (Fargašová et al., 1999). Cu toxicity for aquaculture animals may vary with the water hardness and can be toxic at >0.005 mg L^{-1} (ANZGFMWQ, 2018).

2.5.4.5. Iron (Fe)

Fe can be a major limiting factor for algae growth, it is involved in fundamental enzymatic activities such as a cofactor in photosystem I, and photochemistry in photosystem II, nitrogen consumption and chlorophyll synthesis (Hutchins and Bruland, 1998, Wan et al., 2014). Fe can

influence the ability of the photosynthetic chain reactions to use light energy hence, it's deficiency can cause reduction in photosynthetic activity (Wan et al., 2014). Different phytoplankton have different threshold for the Fe concentration (Imai et al., 1999, Raven et al., 1999), however Fe concentration >0.1 mg L^{-1} can be toxic to the aquatic ecosystem (ANZGFMWQ, 2018).

2.5.4.6. Magnesium (Mg)

Mg concentration influences the formation of photochemical Fe (II) and uptake of Fe (Fujii et al., 2015). In aquatic environment, Mg is often associated with Ca and its concentration generally remains lower than Ca (Venkatasubramani and Meenambal, 2007). However, Mg >15 mg L^{-1} can be toxic to freshwater aquaculture species (ANZGFMWQ, 2018).

2.5.4.7. Manganese (Mn)

Mn helps to increase the photosynthesis rate in some algae, as it triggers the enzyme that contributes to the formation of chlorophyll (Goldman, 2010). Mn absorption by algae is impacted by water pH, where its absorption decreases as the pH declines below 5 (Goldman, 2010). Mn concentration in freshwater aquatic ecosystem >0.01 can be toxic to the biota (ANZGFMWQ, 2018).

2.5.4.8. Phosphorus (P)

In the natural aquatic environment P availability is mainly due to the geochemical sources (Reynolds, 1984), but in aquaculture ponds feed and fertilizer addition is an main source of P input. As a part of nucleic acid and adenosine triphosphate, P is essential for the growth of plankton in aquatic ecosystems (Ikem and Adisa, 2011, Reynolds, 1984). Dissolved orthophosphate is a major source of P for phosphorus deficient phytoplankton cells and it is taken up by phosphorus deficient cells until very low concentrations (<1µg L^{-1}) remain in the water (Rigler, 1966). It is a macronutrient and plays a crucial role in phytoplankton production and species composition in pond water bodies (Lund, 1965), for example a decline in phytoplankton abundance was observed in Lake Erie when P concentration decreased (Hartig and Wallen, 1984).

2.5.4.9. Selenium (Se)

Plankton accumulate Se from the water column and partially transform it into organic Se before it is transferred to higher organisms by ingesting preys. Se is essential to many plankton to protect against oxidative damage (Ekelund and Danilov, 2001). The essential nutritional function of Se is because of the action of selenoproteins containing Se in the form of

selenocystein; selenoproteins play essential roles in maintaining cell viability (Brown and Arthur, 2001, Kim et al., 2006). In freshwater ecosystem Se >0.01 mg L^{-1} can be toxic to the aquatic animals (ANZGFMWQ, 2018).

2.5.4.10. Sulphur (S)

S is one of the main components of plankton cells, in aquatic ecosystems it's mostly available in the form of Sulphate and acquired in the same form by plankton (Giordano and Prioretti, 2016). In natural waters S concentration at ~4.8 mg (SO_4^{2-}) L^{-1} is high in comparison to the plankton requirements (Reynolds, 1984).

2.5.4.11. Silica (Si)

Phytoplankton require Si in trace amount for the carbohydrate and protein synthesis. Diatoms are dependent on the presence of Si in the water body to grow, as diatoms have siliceous cell walls (frustule). Diatoms have a high nutritional value as compared to other phytoplankton (Boyd, 2014). Si is an important nutrient for the diatoms growth and its concentration fluctuations can cause the variations in diatom density (Nwankwegu et al., 2020, Tulsankar et al., 2020).

2.5.4.12. Zinc (Zn)

Zn is a cofactor for the carbonic anhydrase enzyme, an essential enzyme for carbon uptake during the photosynthesis process (Goldman, 2010). Its concentration above 0.05 mg L^{-1} is considered as toxic which may weaken the photosynthesis (Goldman, 2010).

2.5.5. Seasonal variations of trace elements

The dissolved concentrations of trace elements vary with seasons, and it can be largely credited to the environmental factors including temperature, rainfall, evaporation and the plankton dynamics (Adhikari and Ayyappan, 2004, Sierp and Qin, 2001, Zhang et al., 2013), changes in environmental conditions may lead to absorption of some of the sediments-bound metals into the water (Khatri and Tyagi, 2015, Saha et al., 2017, Wang et al., 2018, White and Liber, 2018). In case of aquaculture ponds the fluctuations can be related to the management practices such as liming, water source and water addition, feeding, fertilization, cleaning of pond bottom soil at harvest (Tulsankar et al., 2021b, Abu Hena et al., 2018). P build-up in pond bottom sediment can occur during the culture time as a result of feeding or plankton essences (Tulsankar et al., 2020, Tulsankar et al., 2021d, Abu Hena et al., 2018). Heating up of upper layer sediments by overlying water during the summer can result into the increase in dissolved concentrations of Zn P, Si, and Cu in autumn (Tulsankar et al., 2020, Hecky et al., 1986). Increased temperatures,

pH and invertebrate activities promotes the dissolution of diatom silica in summer, which can increase the dissolved Si levels by autumn (Gibson et al., 2000). Reduction in P concentration during winter was observed by EL-Saharty (2014). There is a least possibility of trapped elements getting discharged into the water during winter, and a decline in exchangeable Ca concentration from soil can cause a decrease in the Ca concentration in water (Jeziorski et al., 2008).

Various nutrients uptake in chemical form as they naturally occur in aquatic ecosystem is necessary before planktons can be assembled into a living cells, similarly for all plants and animals trace elements are essential for homeostasis and general metabolism (Goldman, 2010). Fluctuations of dissolved trace element can affect the phytoplankton growth as well as species diversity (Goldman, 2010, Shi et al., 2013, Giordano and Prioretti, 2016, Nwankwegu et al., 2020, Tulsankar et al., 2020).

2.5.6. Seasonal variations of plankton

The plankton density, species diversity and community structure is dependent on seasonal variations of environmental parameters and trace element concentrations (Tulsankar et al., 2020, Tulsankar et al., 2021b, Sampaio et al., 2002). Seasonal variations of plankton and the instigating factors in freshwater reservoirs and lake ecosystems have been studied by various researchers (Li et al., 2019, Garcia et al., 2016, Grover and Chrzanowski, 2006, Ndebele-Murisa et al., 2010), with the limited literature available on aquaculture managed ponds (Tulsankar et al., 2021b, Prapaiwong, 2011, Tulsankar et al., 2020, Affan et al., 2005). It is not easy to illustrate relationships between the phytoplankton community structure and environmental factors (Varol, 2019), though understanding plankton communities and their seasonal variations causing factors can help to improve the abundance of beneficial plankton communities in aquaculture ponds. As in aquaculture rearing systems, plankton can provide nutrition to the farmed species. Studying the plankton communities and the effects of seasonal variations on them can help to manage the aquaculture ponds ecosystems and to improve the management practices as plankton communities are also a great indicator of aquaculture pond water quality (Casé et al., 2008). The seasonal changes in plankton communities in lentic freshwater bodies can be related to various factors such as temperature, evaporation, rainfall, nutrient deposition but in the case of aquaculture ponds, the pond management can play an important role such as feed addition, fertilization or manuring (Tulsankar et al., 2020).

The favourable season for plankton density and species diversity for marron culture ponds is autumn (Tulsankar et al., 2020) and summer (Tulsankar et al., 2021b), which can be related to the lower volume of water present in the ponds due to the evaporation during the summer, causing higher dissolved concentrations of correlated trace elements. Increased phytoplankton density improved the zooplankton abundance (Tulsankar et al., 2020), as the zooplankton abundance is entirely dependent on phytoplankton availability (Martinez-Cordova et al., 1998, Preston et al., 2003). Low water volume, high solar radiation, rising temperature and suitable levels of nutrients was the reason for increase in phytoplankton growth during the autumn season in aquaculture ponds (Bangladesh) (Affan et al., 2005). In both the studies on marron ponds (Tulsankar et al., 2020, Tulsankar et al., 2021b), plankton density and species diversity were lower during the winter, which can be linked to the rainfall, farming practices such as emptying the ponds, restocking and low temperature.

In cool temperate climate, the changes in plankton diversity or species succession was something like presence of flagellates or diatoms during the winter, herbivores during the late spring or early summer, and the dominance of nanoplankton, diatom, cyanobacteria during the summer stratification and late summer had dominance of pennate diatoms or cyanobacteria as the winter starts (Grover and Chrzanowski, 2006). These phytoplankton variations were strongly correlated to the temperature and thermal stratification (Reynolds, 1984, Sommer et al., 2012). Temperature variations can cause a shift in plankton species, more likely due to the change in density of water with the change in temperature, also photochemical reactions and physical processes in plankton cells such as transport and viscosity, are temperature sensitive (Talling, 2012). For example, increased water temperature can cause decrease in surface water viscosity, which can promote the sinking of larger non-motile phytoplankton species, and the capacity of adjusting buoyancy helps Cyanophyceae to dominate in plankton communities (O'neil et al., 2012). Similar to the changes in environmental parameters the changes in the dissolved concentrations of trace elements can influence the presence of plankton communities (Tulsankar et al., 2020, Tulsankar et al., 2021d, Calijuri et al., 2002, Sommer et al., 2012). In aquaculture ponds, environmental factors as well as fertilization or manuring and feed can cause changes in trace element concentrations (Tulsankar et al., 2020).

2.5.7. Effect of pond age on trace elements and plankton productivity

Aquaculture practices are mostly conducted in earthen purpose built ponds. Over a period, the pond bottom soil act as a reservoir of nutrients and therefore, the dynamics of water-soil

interface has a significant impact on water quality under the influence of various extrinsic variables (Boyd, 1995). Similar to the trace element fluctuations, pond age and seasonality also impacts on the plankton production and species diversity in marron ponds (Tulsankar et al., 2020). Also, pond age can affect the dissolved concentration of trace elements (Tulsankar et al., 2020, Abu Hena et al., 2018). The ponds productivity might be affected by the construction time, sediment types and practices conducted at the aquaculture venture (Tulsankar et al., 2020, Tulsankar et al., 2021b). As newer ponds may take time to become 'established' in terms of nutrient concentration, to develop beneficial plankton and microbial communities (Allan et al., 1995, Correia et al., 2002).

2.5.8. Trace elements and crayfish

Aquatic animals can accumulate trace elements directly or indirectly from the water (Farombi et al., 2007). The farmland, directly-indirectly supplied water in the aquaculture ponds or the water source contains different amounts of trace elements which may have an impact on aquatic plant and animals (Hossain et al., 2021). Crayfish can survive in polluted water but the excess concentrations or deficiency of trace elements may adversely affect the growth and survival (Hossain et al., 2021). Freshwater ecosystems are destined to have different levels of trace elements due to the natural and anthropogenic activities conducted (Khan and Nugegoda, 2007), and in case of aquaculture ponds the pond management activities such as feeding, fertilization and manuring can add trace elements (Tulsankar et al., 2020). The dissolved concentration of pre-selected trace elements observed in marron ponds around Australia is shown in Table 2.2. The available trace elements can accumulate in crayfish via plankton ingestion or transfer along food web through to humans (Ikem et al., 2021).

Freshwater crustaceans accumulate and store Ca and Cu in haemolymph (Wilder et al., 2009). More than 50 % of the whole body Cu load is stored in haemolymph (Depledge and Bjerregaard, 1989). Cu is an essential micronutrient and is an integral part of the respiratory pigment haemocyanin (Alcorlo et al., 2006). Haemocyanin maintenance requires the accumulation of Cu in relatively large quantities, for its transport and storage within the body (Taylor and Anstiss, 1999). The Cu accumulation in decapod crustaceans is regulated only up to the physiological threshold levels (Alcorlo et al., 2006). Besides the important role of Cu in living organisms, its elevated levels can cause deleterious effects on fish (Dethloff et al., 1999). Co and Zn can unfavourably affect the immunocompetence in invertebrates (Cheng, 1988, Truscott and White, 1990). P is a necessary element for plant and animal growth, rain wash

phosphate from the farm soil into the water. Se is essential for the growth of fish and crayfish (Dörr et al., 2008, Nugroho and Fotedar, 2013). S is an essential trace element for living organisms, having several roles in nutrition, it is most important component for some amino acids. Plankton use sulphate to synthesize sulphur containing amino acids that passes onto the top predators via the food chain (Boyd, 2007). Freshwater crustaceans are also dependent on bioavailable dissolved Ca in water bodies, as it is required to grow their calcified exoskeleton (Cairns and Yan, 2009). The studies on importance of Mn, Si, Mg, Fe and Al on health of aquatic invertebrates are rare, and the main focus of related studies has been to analyse the toxic effects of these trace elements on crayfish (Tulsankar et al., 2021d). Table 2.3 shows the required range and toxic concentrations of pre-selected trace elements for freshwater crayfish or crustaceans.

Table 2.2. Shows the dissolved concentrations of pre-selected trace elements observed in different marron culture systems.

Region	**Dissolved concentrations of trace elements (mg L^{-1})**												**References**
	Al	**Co**	**Cu**	**Fe**	**Mn**	**S**	**Se**	**Si**	**Ca**	**Mg**	**Zn**	**P**	
Commercial marron farm (Manjimup), Perth	0.17	ND	0.0007	0.07	0.004	5.9	ND	0.65	24.9	17.6	0.007	0.02	(Tulsankar et al., 2020)
Cement tanks at Marron Force Farm, Perth	-	-	-	0.06	<0.01	40	-	-	80.0-95.2	17.7	0.05	0.25-0.80	(Fotedar et al., 1999)
Farms from different regions of Australia- I. Pinjarra	-	-	<0.005	0.25	-	8.1	-	-	45	114	<0.005	0.006	(Lawrence, 2007)
II.Denmark	-	-	0.026	8.4	-	16	-	-	11	2	0.02	0.009	
4. Kangaroo island	-	-	0.022	12.0	-	19	-	-	5	14	0.048	0.005	
5. Pemberton	-	-	<0.005	2.3	-	22	-	-	9	12	0.007	0.003	
6. Mount Baker	-	-	<0.005	1.1	-	99	-	-	51	72	<0.005	0.029	
Maximum safe concentration	<0.03 (pH>6.5)[1]	NF	<0.005 (varies with hardness)[2]	<0.01[1]	<0.01[1,3]	NF	<0.01[1]	NF	NF	<15[1]	<0.005[1]	<0.1[2]	(Meade, 2012, DWAF, 1996, Zweig et al., 1999)

Abbreviations: ND- concentration was not detectable, NF- information not found in literature. [1](Meade, 2012)[2](DWAF, 1996)[3(]Zweig et al., 1999[)]

Table 2.3. Describes the available literature on the optimum and toxic concentration ranges of pre-selected trace elements for freshwater crayfish or crustaceans.

Trace elements	Findings	Reference
Manganese (Mn)	Mn concentration >0.5 mg L^{-1} of was toxic for freshwater crayfish (Ackefors, 2000) and concentration <0.05 mg L^{-1} cause physiological stress and behavioural difference which may impact the survivability of red swamp crayfish (Lambert, 2019).	(Lambert, 2019, Ackefors, 2000)
Silica (Si)	Red swamp crayfish was treated with 10, 100 and 1000 mg L^{-1} silicone for 72 hours; it reduced survival rate, increased malondialdehyde content in muscle and caused stress response in crayfish at biochemical and molecular level.	(Hossain et al., 2021)
Aluminium (Al)	Al at pH 5.5 can reduce the Ca uptake causing stress in crayfish (Gensemer and Playle, 1999). Signal crayfish could cope with the short-term Al contaminated food at 3-420 µg/g without any negative impact on haemolymph.	(Gensemer and Playle, 1999) (Woodburn et al., 2011)
Phosphorus (P)	Phosphate has no toxic effect on aquatic crustaceans, excessive phosphate can cause algal bloom.	(Kim et al., 2013)
Selenium (Se)	Organic Se at 0.4 mg Kg^{-1} improved the growth, survival and disease resistance of marron (Nugroho and Fotedar, 2013). Maximum Se concentration permitted in the diets for aquaculture animals is 0.5 mg Kg^{-1} (Dörr et al., 2008).	(Nugroho and Fotedar, 2013, Dörr et al., 2008)
Calcium (Ca)	Ca concentration at >10 mg L^{-1} increased growth and survival of Koura crayfish (*Paranephrops zealandicus*) and mortality was observed at 0 to 10 mg L^{-1}.	(Hammond et al., 2006)
Magnesium (Mg)	Mg toxicity is depends on the Ca concentration and can significantly increase if the Mg to Ca ratio is >9:1.	(Department of Agriculture, 2021)
Sulphur (S)	S in the form of hydrogen sulphide was toxic to red swamp crayfish at 6-13 µg L^{-1}.	(Government, 2000)
Cobalt (Co)	Aquatic invertebrates are more sensitive to chronic Co concentration compared to fish; for crustacean (*Daphnia magna)* the chronic Co value was 0.032 mg L^{-1}.	(Stubblefield et al., 2020)
Zinc (Zn)	Zn concentration at 0.02 - 0.15 mg L^{-1} was toxic for marron.	(Bennet-Chambers and Knott, 2002)
Copper (Cu)	Cu >0.1 mg L^{-1} inhibit the feeding in crayfish.	(Burba, 1993)
Iron (Fe)	Crayfish species are sensitive to the Fe concentration >0.5 mg L^{-1}.	(Ackefors, 2000)

2.5.9. Live food used in aquaculture practices

The live food production is an important step in intensification of aquaculture systems. During the rearing of juvenile fish or crustaceans, supplementation of live plankton commonly called green water is a very common practice (Tamaru et al., 1994). As live food contains all the essential nutrients required for juvenile nutrition they are called "living capsules of nutrition" (Das et al., 2012). Availability of live plankton to young signal crayfish improved growth and survival (Sáez-Royuela et al., 2007, Gonzalez et al., 2012). Improved growth and survival rate can be achieved by maintaining and feeding cultured animals with live food along with the supplemented formulated feed. Formulated feed cannot meet all the required nutrients for better growth and survival of crayfish, they must be provided with live food (Fotedar et al., 1999). Juvenile culture with higher survival and better growth rate is an crucial stage in marron farming, as there is significant size variation but it could be a most profitable stage (Lawrence, 2007), and pond management strategies can help to achieve the higher growth and survival rate of marron (Lawrence, 2007).

The green alga *Chlorella* were observed around the year in marron ponds (Tulsankar et al., 2021b), which has been popular for feeding fish or shellfish juveniles. Not only due to the nutritional qualities but due to their size range from 5 to 25 micron are ideally suitable for the early life stages of various aquatic animals. Live food, as an essential food source is used for rearing of early life stages of fish and crustaceans in aqua hatcheries (Das et al., 2012). The plankton nutritional value is dependent on cell size, digestibility and biochemical composition.

Phytoplankton production is sustained to improve the abundance of zooplankton such as *Nannochloropsis* and *Chlorella* are used to enhance the growth of rotifers (Maruyama et al., 1997). Zooplankton is required as a main source of protein or first food in many fish or crustaceans (Gonzalez et al., 2012) in early days of post hatch on consuming the nutrients from yolk sac the juveniles cannot feed on formulated feed due to the feed size, palatability etc. and they require micro or macro sized live food for better nutrition. Cladocerans and copepod supplementation has shown to improve the growth in red claw and yabbies (Verhoef et al., 1998, Jones et al., 1995, Duffy et al., 2011). Following literature describes the information on zooplankton prominently observed in marron ponds.

2.5.9.1. Rotifers

Rotifers are used in various aqua hatcheries as a live food for the juveniles of fish and shellfish (Das et al., 2012). *Keratella* spp. were observed more often in marron ponds (Tulsankar et al.,

2020, Tulsankar et al., 2021b), which has been identified as ideal live food for juvenile stages of fish and prawn species in marine and freshwater ecosystems (Das et al., 2012). Rotifer use depends on the mouth size of cultured animals, small (50 to 110 micron length) or large (100 to 200 micron length). The nutritional value of rotifers for larval fish depends on the rotifers food source (Das et al., 2012). The protein percentage of rotifers ranged between 52 to 59 % as per their food source (Awaiss et al., 1992, Øie and Olsen, 1997).

2.5.9.2. Copepods

Copepods are common zooplankton of freshwater. They are natural food for larvae and juveniles of many finfish and crustaceans (Evjemo et al., 2003). Calanoid and cyclopoida copepod adult and nauplii were observed in marron ponds in all seasons (Tulsankar et al., 2020, Tulsankar et al., 2021b). Research with several species, such as the turbot and red snapper, has shown that when offered mixed plankton diets, young larvae consume more copepod nauplii than rotifers (Das et al., 2012). Juvenile marron preferred to feed on frozen copepod in laboratory experiment (Tulsankar et al., 2021c). Herbivorous copepods are primarily filter feeders and typically feed on very small particles. But they can feed on larger particles, which give them an advantage over the rotifers. Copepods can also eat detritus (Das et al., 2012). The protein content of copepods ranged between 52.4 % to 57.6 % (Das et al., 2012).

2.5.9.3. Cladocerans

Cladocerans are generally called the ‘water fleas’. Two cladoceran genera, namely Daphnia and Moina, are important live zooplankton. Daphnia is found in freshwater ponds, tanks and lakes. Daphnia contains a broad spectrum of digestive enzymes such as proteases, peptidases, amylase, lipase and cellulase which serve as exoenzyme in the gut of fish and crustaceans (Das et al., 2012). Moina are primarily inhabitants of temporary ponds or ditches. Moina are smaller in size (0.5 to 2 mm) compared to Daphnia. With the protein content of 70% Daphnia can replace Artemia in aqua hatcheries (Das et al., 2012). Moina has also been extensively used as a live food in many aqua hatcheries to maintain the culture of aquarium and commercially important fishes (Martin et al., 2006). The protein content of cladocerans also depends on the food source, and usually averages 50% of their dry weight (Das et al., 2012).

2.6. Natural productivity and marron nutritional requirements

The natural productivity of ponds includes the primary and secondary plankton production, macrophytes, insects, detritus and other natural food sources. Freshwater crayfish are known to feed on formulated feed and natural sources of such as macrophytes, benthic invertebrates,

bacteria, plankton and animal detritus (Saoud et al., 2012, Brown et al., 1992). The natural diet of marron includes fine particulate organic matter, smaller amounts of plants, insects, detritus of plant and animal matter (O'Brien, 1995, Beatty, 2006, Fotedar et al., 2015). In a semi-intensive marron pond, a combination of natural productivity and formulated feed provide sufficient nutrition to marron (Fotedar et al., 2015), however the availability of natural feed is seasonally dependent (Cole et al., 2019, Tulsankar et al., 2020, Tulsankar et al., 2021b). The maximum growth rate of marron was achieved with food containing 20 % to 30 % protein (Tsvetnenko et al., 1995).

2.6.1. Plankton and marron growth, survival, moulting, health indices and pigmentation

In aquaculture systems, the availability of live feed during the juvenile life stage is crucial for achieving high growth and survival rates (Sáez-Royuela et al., 2007). Improved growth rate of marron and yabbies was observed on feeding with a live plankton mixture (Austin et al., 1997, Jones et al., 1995, Verhoef et al., 1998, Tulsankar et al., 2021a). Improved growth rates of marron fed plankton mixture reflects the constant supply of nutrients, whereas formulated feed has a tendency to leach out most of the water-soluble nutrients once immersed in pond water (Smith et al., 2002). Also, plankton are rich in protein, amino acids, lipids, fatty acids, minerals, chlorophyll, carotenoids, trace elements, enzymes and vitamins (Kibria et al., 1997, Napiórkowska-Krzebietke, 2017) which are essential for marron growth (Fotedar et al., 2015). Also, a constant supply of plankton would have allowed for continuous feeding through filter feeding and scavenging for detritus (Van den Berg et al., 1990). Survival rate of marron with the use of mixed live plankton was >70 % and >80 % ((Tulsankar et al., 2021a); Tulsankar et al., 2021 under review- Chapter 9)) where it was 53.3% in juvenile signal crayfish when fed with mixture of phytoplankton and zooplankton and only 10% when fed with phytoplankton alone (Celada et al., 1989). In another study by Carreño-León et al. (2014), the survival of juvenile red claw crayfish was (~70%) when fed with microalgae.

The ideal crayfish diet should improve growth, survival and pigmentation (Verhoef et al., 1998), however various studies have found that marron formulated diets were often lacking in carotenoids, which cause loss of exoskeleton pigmentation, poor growth, survival and health (Jussila and Mannonen, 1997, Sommer et al., 1991, Tulsankar et al., 2021a). Carotenoids are common in algae and are necessary for good pigmentation, suggesting that farmed marron obtain at least some of their nutrition from natural sources (Goddard, 1988). The pigmentation

of juveniles fed with live plankton mixture was dark brown compared to those provided with formulated diet (Verhoef et al., 1998, Jones et al., 1995, Tulsankar et al., 2021a).

Diet impacts not only the growth of marron, but also their pigmentation, physiology, and gut microbial community (Jussila and Evans, 1998, Sang et al., 2011). The food intake and nutritional status affects the haemocyte count in terms of quantity and quality (Le Moullac and Haffner, 2000). Higher total haemocyte count (THC) represents a higher immune status (Sharma et al., 2009), and higher THC was observed in juvenile marron fed with live plankton mixture (Tulsankar et al., 2021a). Studies analysing the plankton effect on the immune and organosomatic indices of freshwater crayfish are scarce, majority studies deals with the formulated feed and crayfish immune-organosomatic indices. Improved immune parameters were observed in freshwater Pearl mussel (*Hyriopsis bialatus*) and Chinese mitten crab (*Eriocheir sinesis*) (Supannapong et al., 2008, Jiang et al., 2020), with the supplementation of plankton. Authors suggested that the natural astaxanthin improved the immunity of the animals. Mixed plankton supplementation improved the moulting in terms of weight increment at moult compared to marron fed with formulated feed alone (Tulsankar et al., 2021a).

Hepatopancreas and tail muscle moisture content have been used as indicators of freshwater crayfish health (Jussila and Mannonen, 1997, McClain, 1995a). Hepatopancreas serves as sensitive indicator for metabolism, nutritional status and health condition in freshwater crayfish, as it is a site for the digestion, synthesis of immune factors, nutrient absorption and storage (Jussila and Mannonen, 1997, Mai and Fotedar, 2017, Calvo et al., 2011). Well organised, tightly arranged tubules showing different types of cells are linked with better health status of red swamp crayfish (Xiao et al., 2014). Zooplankton and mixed live plankton supplementation significantly improved the health of juvenile marron with increased THC count and lower moisture content of hepatopancreas indicating better health condition (Jussila, 1999); Tulsankar et al., 2021 under review- Chapter 9). Marron in good condition had 6-9% of dry tail weight muscle indices (Fotedar et al., 1999) and 6.46 to 8.22 dry tail weight muscle indices was observed in juvenile marron fed with zooplankton (Tulsankar et al., 2021 under review- Chapter 9). The supplementation of live copepods, daphnia and rotifers may have provided protein and essential vitamins and minerals for the juvenile marron (Manickam et al., 2020, Duffy et al., 2011), contributing to their nutrition and improving their tail muscle condition.

2.6.2. Plankton and stable isotope analysis in crayfish

Ecologists commonly use naturally occurring stable isotopes to identify the food sources assimilated by the consumers (Stenroth et al., 2006). Stable isotope analysis (SIA) helps to analyse the food web connections and to assess the physiological conditions of consumers (Gannes et al., 1997). With the help of the stable isotope mixing model (SIAR) the relative contribution of each possible food source to the animal's tissue formation can be estimated (Phillips and Gregg, 2001). SIA combines the benefits of both the trophic level and food web paradigm in food web ecology (Post, 2002), to evaluate the structure of ecological communities.

Understanding the contributions of different food sources towards the growth of tail muscle and whole marron can provide information on the importance of provided food sources, and can help to manage the feeding strategies in order to manage the pond's natural productivity. Research measuring SIA in crayfish (Table 2.4) has revealed that food sources of animal origin are more important for crayfish nutrition than detritus and plant material (Alcorlo et al., 2004). While marron can be provided with natural and commercial food, the actual consumption of different food types under controlled conditions has never been explored. Information on the prey items consumed by marron grown in semi-intensive ponds would benefit marron farmers by optimising the use of formulated feeds. Marron exhibited considerable variability in feed sources, indicating feeding on a variety of diets ranging from the periphyton grown on tank edge, supplied plankton and the detritus formed at the tank bottom (Tulsankar et al., 2021 under review- Chapter 9). Furthermore, when provided with a mixture of phytoplankton and zooplankton, the SIA revealed that phytoplankton had the lowest contribution towards the tail muscle of all provided feeds, which indicates juvenile marron preferred animal protein over the phytoplankton or plant protein and in the absence of zooplankton juvenile marron fed on phytoplankton (Tulsankar et al., 2021 under review- Chapter 9). This finding aligns with the outcome of a study conducted by Beatty (2006), where the isotope analysis of marron collected from the Hutt river showed that the marron assimilated higher proportion of animal matter than plant matter. Detritus and zooplankton were the most important food sources for crayfish due to their nutritional composition and their availability ((Brown et al., 1992, Jones, 1995a, Gamboa-delgado et al., 2003); Tulsankar et al., 2021 under review- Chapter 9)).

Table 2.4. Presents the literature on studies conducted on stable isotope and crayfish feeding.

Culture conditions	Cultured species	Finding	References
Purpose built plastic pond	Signal crayfish and noble crayfish	Crayfish were top consumers and received core nutrition from invertebrates and less from primary producers	(Nyström et al., 1999)
In-stream enclosures and laboratory conditions	Signal crayfish (juvenile and adult)	Both stages of crayfish relied primarily on detrital biofilms for the nutrition.	(Bondar et al., 2005)
Lake	Rusty crayfish	When more desirable prey are rare of inaccessible rusty crayfish will consume energy-poor food resources.	(Roth et al., 2006)
Hutt river, WA	Yabby and marron	Both species had similar predatory trophic position and during summer large proportion of *Gambusia holbrooki* was consumed by both species. During winter yabby preferred to feed on herbivorous matter and marron continued to feed on animal matter.	(Beatty, 2006)
Ponds and Lakes,	Signal crayfish	Adult crayfish were least carnivorous compared to juveniles.	(Stenroth et al., 2006)
Taieri River (South island of New Zealand), MacRae creek, North Col creek, Powder creek, Canton creek	Kouri crayfish *(Paranephrops zalandicus)*	Snails, nymphs and chironomid larvae are more important compared to detritus for assimilation and incorporation into the crayfish biomass.	(Hollows et al., 2002)
Commercial ponds	Marron	Marron have an ability to assimilate carbon and nitrogen different food sources. Marron fed on algae, phytoplankton, zooplankton, macrophytes, insects and formulated feed.	(O'brien and Davies, 2002)
Artificial ponds (with and without natural food items)	Yabby	Yabby preferred to consume naturally occurring food items and zooplankton had the largest contribution towards the growth of tissue.	(Duffy et al., 2011)

Laboratory conditions	Noble crayfish and signal crayfish	To understand the effects of the nutrition on the tissue stable isotope content, it requires the crayfish to moult.	(Jussila et al., 2015)
Commercial farms	Red claw crayfish	Naturally occurring pond biota and formulated feed contributed to the tissue composition of red claw. Also, the contribution of feed towards the growth of tissue varied with the availability of food source.	(Joyce and Pirozzi, 2016)
Outdoor mesocosm	Red swamp crayfish	Plant material dominated the gut content with several sediment-dwelling invertebrates and feeding was more aligned with detritus.	(Rudnick and Resh, 2005)
River	Rusty crayfish	Crayfish consume both phytoplankton and benthic invertebrates that relies on phytoplankton	(Kautza and Sullivan, 2016)
Lake Kariba (Zimbabwe)	Red claw crayfish	Red claw predominantly fed on macrophytes, macroinvertebrates and detritus	(Marufu et al., 2018)
Nýrsko reservoir (Czech Republic)	Noble crayfish	Crayfish consumed food from several trophic levels and the food source varied between the crayfish age class. Similarly food source varies with the season and habitat.	(Veselý et al., 2020)
Lowland British rivers (Evenlode, Cherwell, Rother and Chad brook)	Signal crayfish	Crayfish was omnivorous in feeding at multiple trophic levels.	(Wood et al., 2017)
Jacks Fork River, Missouri	Golden crayfish (*Orconectes luteus*) and Spothanded crayfish (*O. Punctimanus*)	Diet of both crayfish included the filamentous algae, diatoms, animal matter and terrestrial plant detritus.	(Whitledge and Rabeni, 1997)
Laboratory conditions	Marron	Marron fed on all available food sources such as periphyton, formulated feed, phytoplankton, detritus and zooplankton. The zooplankton had highest contribution towards the growth of tail muscle followed by detritus.	(Tulsankar et al., 2021 under review- Chapter 9)

2.6.3. Plankton and crayfish histology

The histological analysis of aquatic animal organs has been considered as a good indicator of the nutritional conditions (Rašković et al., 2011, Vogt et al., 1985, Foysal et al., 2021). A healthy structure of hepatopancreas cells and proper orientation of midgut with good epithelium height are associated with efficient nutrient absorption, higher growth rate and improved immunity (Dimitroglou et al., 2009). Longer intestinal folds and epithelium height are associated with higher nutrient absorption efficiency with improved health and immune functions, while on the other hand shorter intestinal folds and low epithelium height has been considered to reflect poor nutrient utilization and absorption (Rašković et al., 2011). The histology of hepatopancreas is considered to be a most important tool used to analyse the nutritional studies in crustaceans (Vogt et al., 1985). A healthy hepatopancreas has an ability to absorb nutrients, store lipids and produce digestive enzymes (Johnson, 1980). Juvenile marron fed with a mixture of plankton showed increase in the number of B cells with large vacuoles (Tulsankar et al., 2021 under review- Chapter 9). B-cells are considered as the main site for the synthesis of digestive enzymes (Xiao et al., 2014). An increase in numbers and size of B-cells suggests abundant synthesis and excretion of the digestive enzymes, which enables crayfish to enhance the digestion of ingested preys and to derive more energy from the food intake (Xiao et al., 2014). Nutrient uptake in the intestine mainly relies on its epithelium height (Duan et al., 2019). The intestinal epithelium height was improved in marron fed with the zooplankton (Tulsankar et al., 2021 under review- Chapter 9). Currently there is no peer reviewed work on the impact of phytoplankton or zooplankton on the histomorphology of marron hepatopancreas, gut or tail muscle tissue, however studies with formulated feed have shown that the diet impacted the gut morphology in crayfish (Xiao et al., 2014).

2.6.4. Gut microbiota

Gut microbiota communities play an important role in digestion and immunity of aquatic animals. The communities can be altered by the use of feed type, feed additive and protein sources (Foysal et al., 2021, Tulsankar et al., 2021d, Parrillo et al., 2017); Tulsankar et al., 2021 under review- Chapter 9). Supplementation of mixed manganese, silica and phosphorus showed significant changes in abundance of gut microbiota communities with higher abundance of phosphorus solubilizing bacteria (Tulsankar et al., 2021d). The literature availability on the impact of live plankton supplementation on marron gut microbiota is scarce. Most of the previous studies focused on the impact of various formulated feeds on gut

microbiota of marron (Foysal et al., 2019a, Saputra et al., 2019, Foysal et al., 2021). Abundance of the *Candidatus* Bacilloplasma was observed in juvenile marron fed with phytoplankton (Francis et al., 2019, Paver et al., 2013); Tulsankar et al., 2021 under review- Chapter 9). Other significantly different bacteria such as *Lactovum* and *C*. Hepatoplasma were observed in gut of juvenile marron fed with zooplankton, and *Citrobacter*, *Acinetobacter* and *Hafnia* Obesumbacterium in marron fed with mixed plankton (Tulsankar et al., 2021 under review- Chapter 9).

2.6.5. Formulated feed in marron culture

To improve marron yield, survival, immunity and overall health many studies have been conducted to analyse the effects of food formulated with inclusion of different dietary protein sources (Fotedar, 1998, Jussila, 1999, Jussila and Evans, 1996, Morrissy, 1989, Saputra et al., 2019, Saputra and Fotedar, 2021, Sommer et al., 1991, Fotedar, 2004, Tulsankar et al., 2021d, Foysal et al., 2019a, Tulsankar et al., 2021a). The main protein source used so far is fishmeal and most of the research was conducted under laboratory conditions (Table 2.5). The feed requirements of marron often differ under pond condition; the difference is thought to be the availability and consumption of naturally occurring food items in addition to the formulated pelleted food may differ (Duffy et al., 2011). Fishmeal provides amino acid balance for crayfish (Jones et al., 1996) and plankton can provide the carotenoids and asthaxanthins which improves the pigmentation and health in crustaceans (Daly et al., 2013, Tulsankar et al., 2021a).

Inclusion of formulated diet with added powder or fresh slurry of microalgae *Dunaliella salina* improved the growth, survival and pigmentation of marron (Morrissy, 1989, Sommer et al., 1991). However, studies on the dietary inclusion of either live plankton (phytoplankton or zooplankton) or mixed plankton and their nutritional contributions on the growth, survival, pigmentation, health indices and gut microbiota of marron are lacking.

Table 2.5. Shows the studies conducted on effects of different formulated feed and microalgae on growth, survival, immune and health indices of marron.

Environment	Feed protein type	Findings	Reference
Purpose-built ponds	– Supplementation of cod liver oil and sunflower oil – Fish protein and fish oil	– Improved the total biomass of juvenile marron. – Resulted in higher survival and higher wet tail muscle to body ratio. – Overall protein free and plant protein inclusion diet had lower survival and lower protein levels in hepatopancreas.	(Fotedar, 1998)
Tanks and intensive crayfish culture system	– Fish meal- stable and unstable diet	– Stable diet improved weight at moult, shorter intermoult period and higher specific growth rate in both culture systems.	(Jussila and Evans, 1998)
Laboratory conditions	– Fish meal (FM) – Poultry-by-product meal (PbM) – Feather meal (FeM) – Lupin meal (LM) – Meat and bone meal (MbM)	– Improved the neutral red dye retention technique, phagocytic rate and haemolymph bacteraemia. – PbM improved the total haemocyte count and microvilli length. – FeM improved the survival rate – MbM improved the phagocytic rate.	(Saputra et al., 2019)
Indoor tanks	– Halifax crustacean reference diet with powdered *Dunaliella salina* (PDS). – Halifax crustacean reference diet with fresh slurry of *Dunaliella salina* (FsDS)	– PDS improved the mean beta carotene levels in marron. – FsDS improved the growth rate of marron	(Sommer et al., 1991)
Purpose-built commercial ponds	– Lupin (*Lupinus albus*) protein meal (D1) – Fish meal (D2) – Fish oil (D3) – Diet without protein source (D4)	– D1, improved the tail body ratio; plant protein had negative impact on pond water quality. – D2, hepatopancreas moisture (%) was lower. – D3, improved the wet tail muscle to body weight ratio – D4 showed higher survival rate	(Fotedar, 2004)
Laboratory conditions	– Fish meal (FM; D1) – Poultry by-product meal (PbM;D2) – FM+ black soldier fly (D3)	– D3 and D4 improved the haemolymph osmolality, lysozyme activity, total haemocyte count (THC), protein and energy content of tail muscle.	(Foysal et al., 2019a)

	– PbM+black soldier fly (D4)		
Laboratory conditions	– Fish meal with the plankton supplementation	Presence of diatom improved marron specific growth rate (SGR), weight gain percentage (WG ;%) and hepatopancreas moisture content (%).	(Tulsankar et al., 2021d)
Laboratory conditions	– Fish meal pellet only (FMP) Mixture of live plankton only (MLP)	– Survival rate and organosomatic indices were similar with FMP and MLP. – MLP improved the growth rate, total haemocyte count and pigmentation of juvenile marron.	(Tulsankar et al., 2021a)
Laboratory conditions	– Fish meal pellet (FMP) – Phytoplankton (P+FML) – Zooplankton (Z+FMP) – Fish meal pellet + phytoplankton + zooplankton (FMP+P+Z)	– FMP had highest contribution towards the growth of marron tissue in the absence of live plankton supplementation. – P+FMP improved the weight at moult, and can be a source of nutrition for juvenile in the absence of zooplankton or animal protein source. – Z+FMP improved SGR, WG %, and survival rate, THC, Lysozyme activity, haemolymph clotting time and tail muscle dry weight index. – P+Z+FMP improved the survival rate, THC, Lysozyme activity, haemolymph clotting time, tail muscle moisture content (%), and had highest contribution towards the growth of tissue muscle of juvenile marron.	Tulsankar et al., 2021 under review- Chapter 9
Laboratory conditions	– Fish meal (FM) – Poultry-by-product meal (PbM) – Feather meal (FeM) – Lupin meal (LM) – Meat and bone meal (MbM)	– FM improved the carapace length – PbM improved the SGR – All protein sources showed no negative impact on marron.	(Saputra and Fotedar, 2021)

Above table contains only studies, involved with the use of fish meal and fish meal replacement protein sources in marron.

2.7. Conclusion

Thinking about the food costs in aquaculture production which account for between 50-70 % of operating cost (Fotedar, 2004, Cortés-Jacinto et al., 2005), adequate pond management in terms of the amount of natural food use and improving pond natural productivity is essential for improving the economic returns of marron farming. Therefore, research on marron pond ecology focusing on trace elements, plankton density and their species diversity, and their effects on marron growth, survival and health will provide more insight for effective marron culture. Understanding the feed and feeding preference of marron at different weight groups or life stages, or as per the seasonal changes will also help to optimise the feeding strategies. For example, availability of live plankton during the initial life stages and providing preferred formulated feed to the grow-out or adult marron could achieve higher productivity as well as help to improve the overall health of cultured marron. Though natural productivity has been shown to be an important source of nutrition in prawn farming, where fertilizers are used to stimulate the growth and abundance of beneficial natural food sources combined with the supplementation of formulated feed (Hena and Hishamuddin, 2014, Gamboa-Delgado, 2014), similar studies in marron farming are lacking.

CHAPTER 3. GENERAL METHODOLOGY

This chapter briefly describes the common materials and methods used in field and laboratory experiments. The field experiments were conducted on a semi-intensive marron culture farm in Manjimup (34°18′75″S, 116°06′61″E), WA and the laboratory work was conducted at CARL, Perth. It also includes an overview of the farm site.

This chapter also presents the methodology followed to conduct the analysis of water quality, plankton preservation and counting, dissolved trace element concentration analysis, plankton collections and preparations for outdoor stock cultures, formulated feed preparations, haemolymph collection, marron growth, survival rate and moulting analysis.

3.1. Water quality

An Oxyguard® digital DO meter (Handy Polaris 2, Norway) was used for DO and temperature measurements, and an Ecoscan pH 5 meter (Eutech instruments, Singapore) was used to record pH. A DR/890 portable colorimeter with Permachem reagents (Hach, USA) were used to analyse the total ammonia nitrogen (TAN), nitrite (NO_2-N) and nitrate (NO_3-N). Turbidity was measured with a Secchi disc. The detail methodology has been described in chapters.

3.2. Trace elements

The dissolved concentrations of pre-selected trace elements in pond water, tank water and marron haemolymph was analysed at Murdoch University Perth, Australia. The water samples were pre filtered with 0.45 µm Millipore filters to eliminate the suspended particles and were analysed by using inductively coupled plasma optical emission spectrometry (Agilent, ICP-OES, spike recovery limit 80%–120%) and standard methods described in APHA (2012) to the different detection limits.

3.3. Plankton

Plankton were analysed visually by using dissection microscope to observe zooplankton at 20x magnification, and compound microscope for phytoplankton at 400x magnification. Plankton samples were settled and viewed under a compound microscope at 400×. The plankton abundance (no/ml) was estimated using a haemocytometer. For each phytoplankton sample, 1 mL subsample was transferred to the haemocyte counter and cells were counted within four randomly selected squares, four counts per sample. For zooplankton 1 mL a sub-sample was transferred to a petri dish and four counts per sample was conducted. The phytoplankton and zooplankton species were identified to the lowest possible taxonomic level using keys from the

manual by Ingram et al. (1997), and a book by Canter-Lund and Lund (1995). The same methodology was used for all chapters except chapter 6.

3.4. Outdoor plankton culture

Plankton were collected from commercial marron pond water and were cultured in 300 L water capacity tanks as a stock culture in outdoor conditions at the Curtin Aquatic Research Laboratory (CARL), with a supply of vigorous aeration and direct sunlight. A continuous culture system was used to culture the plankton and Aquasol® by Yates Pty Ltd was added to the tanks to boost and maintain the phytoplankton density.

3.5. Formulated feed

Fish meal as a main animal protein source was used to prepare the formulated pelleted diet and the preparation was conducted at CARL. The ingredients were collected from Glenn Forest, WA.

3.6. Haemolymph collection

The haemolymph samples were collected using 1 mL syringe containing sodium citrate anticoagulant (100mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 15.5 mM NaCl and 10 mM EDTA) inserted in between the third and fourth pair of pereopods.

3.7. Marron health indices

The health indices such as total haemocyte count (THC) and differential haemocyte count (DHC), were counted using a haemocytometer (Neubauer, Germany) by observing under 40x magnification using a compound microscope. To obtain the moisture content, wet and dry weight index of hepatopancreas and tail muscle, marron samples were weighed and dried at 105ºC in an oven until the constant weight was achieved.

Figure 3.1. Overview of the site used for the field experiments. A semi-intensive marron culture farm in Manjimup (34°18′75″S, 116°06′61″E), WA.


New ponds
Old ponds
Google
Imagery ©2016 Google, Map data ©2016 Goog

CHAPTER 4. Effects of seasonal variations and pond age on trace elements and their correlations with plankton productivity in commercial freshwater crayfish (*Cherax cainii,* Austin, 2002) earthen ponds.

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Abstract

Seasonal variations can affect the concentration of trace elements, and the change in their concentrations can affect the natural productivity of freshwater aquaculture ponds. Hence, we studied the seasonal variations of the 12 pre-selected trace elements (Co, Cu, Fe, Mn, Zn, Se, Ca, Mg, P, S, Al, Si) and their relationships with primary and secondary productivity in two aged ponds, stocked with three different life stages of marron (*Cherax cainii*), for a period of one year. Trace element analysis were performed by using Inductively Coupled Plasma (Agilent, ICP-OES) technique. Except Co and Se, all trace elements, primary and secondary productivity were influenced by seasonal variation. The pond age significantly influenced the concentrations of some trace elements. On a seasonal basis, trace elements were positively correlated with the plankton abundance, species diversity, wet and dry plankton weights. Seasonal variations and pond age affected the dissolved concentrations of trace elements and plankton productivity.

Keywords: Trace elements; Phytoplankton; Zooplankton; ICP-OES; Seasonal variations, Semi-intensive marron ponds.

4.1. Introduction

In the south-west of WA marron (*Cherax cainii,* Austin, 2002) is a commercially important species for aquaculture with high market value. Like other aquaculture industries, crayfish farming is also dependent on good water quality (Ackefors, 2000). With other optimum ranged physico-chemical water parameters such as temperature, DO, pH, and ammonia, crayfish have a high requirement for certain minerals (Merrick and Lambert, 1991). Trace elements are necessary for crayfish growth, although some could be growth inhibitory (Maguire, 1979) under certain concentrations. In the aquatic environment trace elements are added via natural processes such as, precipitation input, soil degradation, rock disintegration, atmospheric deposition and anthropogenic activities such as mining, urban, agricultural and industrial

activities (Arain et al., 2008, Ahmed et al., 2011, Reimer, 1999, Khatri and Tyagi, 2015). Many aquatic organisms assimilate dissolved trace elements directly from the environment (Zhang et al., 2018), including phytoplankton (Baeyens et al., 1998, Hassler et al., 2004), while zooplankton can acquire them through aqueous and dietary ingestion (Yu and Wang, 2002). Several researchers have explained the importance of trace elements in plankton growth and their role in photosynthesis (Anderson et al., 1978, Egna and Boyd, 1997, Goldman, 2010, Bruland et al., 1991, Wang et al., 2010, Twining and Baines, 2013). Trace element concentration can influence the plankton productivity in aquatic ecosystems (Wallen, 1979, Goldman, 2010, Jeziorski et al., 2008, Giordano et al., 2005, Ikem and Adisa, 2011, Giordano and Prioretti, 2016). Moreover, their fluctuations can affect the phytoplankton (Zhang et al., 2013, Downs et al., 2008). There is a lack of public and scientific information on the concentrations of trace elements in water, their seasonal variations and effects on plankton productivity in freshwater crayfish ponds. Within a farm, ponds are often treated similarly throughout the season, yet huge differences result in the water colour and yield (personal communication with farmers). These variations in production are not well understood (Abu Hena et al., 2018). Studies that define the relationships among the physical and chemical characteristics of water and plankton in freshwater crayfish ponds are scarce, as is the information regarding the impacts of pond age on water chemistry and plankton productivity. A better understanding regarding the impacts of seasonal variations on trace elements, and their effect on different aspects of the planktons can help to increase the natural productivity of the freshwater crayfish ponds and in turn improve the growth rate of the cultured animal. As marron are largely detritivores and polytrophic in feeding habit (Morrissy, 1979), and natural food produced in ponds may be considered to provide most of marrons micro-nutritional requirements (Morrissy, 1979, Fotedar et al., 2015). With the objective of filling this research gap we investigated the seasonal variations of 12 pre-selected trace element concentrations, plankton abundance, species diversity, their wet and dry weight and the seasonal correlations between trace elements and plankton productivity during four different seasons and in two different aged commercial marron culture ponds.

4.2. Materials and methods

4.2.1. Sampling site

A commercial marron farm was selected in collaboration with the Marron Grower Association (MGA) of Western Australia (WA) and South West Catchment Council to analyse the dissolved concentration of selected trace elements and their correlations with the natural

productivity over the period of one year. The commercial farm, with a capacity of 60 ponds for marron culture is located in the south-west of WA near Manjimup (34°18'75" S, 116°06'61" E). A subsample of 28 ponds was identified based on their location and time of pond construction and utilization. The older ponds were constructed and were under operation in the year 2000 and new ponds was constructed in the year 2004-05. Of these, 12 were defined as older ponds and 16 as new ponds. Each pond had an area of 1000 m^2 with a water depth of approximately 1.5m. Ponds were stocked with three different life stages of marron: nine ponds were stocked with juveniles (3500), nine ponds with brooders (250 male and 800 females with new born juveniles) and the 10 ponds with grow-out: monosex with 40 to 90g adult marron (n~3750), 95 to 130g adult marron +1000 juveniles (n~2700**)** and 135 to 180g adult marron +1000 juveniles (n~2111). This stocking protocol was a commercial protocol followed by the farmer. Marron were stocked in ponds six months before the commencement of sampling.

4.2.2. Sampling and analysis

4.2.2.1. Water parameters

Sampling was performed seasonally, once every three months, from January to December 2016. Physical and chemical parameters including pH, dissolved oxygen, turbidity, and temperature were recorded on site. For pH an Ecoscan pH 5 meter (Eutech) was used. Turbidity was measured with a secchi disc, with clear ponds defined as having 1.5m visibility. For dissolved oxygen and temperature an Oxyguard digital dissolved oxygen meter (Handy Polaris 2) was used. The water samples for trace elements and plankton analysis were collected in 100mL containers, with three replicates, and transported in cold storage to the Curtin Aquatic Research Laboratory (CARL), Curtin University, Bentley, Australia. The trace elements samples were collected separately in 100 mL containers directly from pond water. For phytoplankton samples 1 L of pond water was filtered through a 25-micron mesh net and a concentrated volume of 100 mL was collected. For zooplankton samples 15 L of pond water was filtered through a 60-micron mesh net to obtain a concentrated volume of 100 mL. The water samples for trace elements and plankton studies were filtered and preserved within 24 hours and analysed within a week.

4.2.2.2. Trace elements

The dissolved concentrations of trace elements in pond water (pre-filtered with 0.45µm Millipore filters to eliminate the suspended particles) were analysed by using Inductively Coupled Plasma Optical Emission Spectrometry (Agilent, ICP-OES, spike recovery limit 80-

120%) and standard methods described in APHA (2012) to the different detection limits of <0.01mg L^{-1} (Al), <0.005 mg L^{-1} (Ca), <0.002 mg L^{-1} (Co), <0.001 mg L^{-1} (Cu), <0.002 mg L^{-1} (Fe), <0.005 mg L^{-1} (Mg), <0.0002 mg L^{-1} (Mn), <0.02 mg L^{-1} (P), <0.05 mg L^{-1} (S), <0.02 mg L^{-1} (Se), <0.02 mg L^{-1} (Si) and <0.002 mg L^{-1} for (Zn) at Murdoch University, Perth, Australia.

4.2.2.3. Plankton sampling

Upon collection the phytoplankton samples were preserved with acid Lugol's iodine, and preserved plankton samples were settled and viewed under a compound microscope at 400X. The plankton abundance (no/mL) was estimated using a haemocytometer. The zooplankton samples were filtered and preserved with ethanol, settled and viewed under the dissection microscope to assess the zooplankton abundance (no/mL). The phytoplankton and zooplankton species were identified to the lowest possible taxonomic level using a keys from the manual by Ingram et al. (1997) and a book by Canter-Lund and Lund (1995). To find the wet weight of phytoplankton and zooplankton, a pump vacuum rocker 300 (SPARMAX TC-501) and Whatman filter papers (GF/C type) having a millipore diameter of 0.47µm were used. The empty filter papers were weighed individually (W_0) and the plankton samples (100ml volume) from the pond were filtered while avoiding contamination to get the wet weight (W_1). Dry weight was obtained by placing the same filter papers with the sample in crucibles which were kept in an oven at 60°C overnight. The next day the crucible was removed from the oven and placed immediately into the desiccator for half an hour, and then the filter paper reweighed until a constant weight (W_2) was achieved. The plankton abundance was calculated by using the equations from Cole et al. (2019).

Phytoplankton abundance (ind. L^{-1}) = ((No. X 1000) / (Volume of grid (0.1 mm^3) / No. of grid squares counted) X (Concentrated Volume /1 mL)) / Total Volume.

Zooplankton abundance (ind. L^{-1}) = (No. X (Concentrated volume / Sub Volume)) / Total volume.

Where,

No. = Mean number of individuals counted, Total Volume = Total volume of water (L) collected from pond, Concentrated Volume = Volume of water (mL) containing concentrated zooplankton after sieving (100ml), Sub Volume = Sub sample of water (mL) from concentrated volume in which plankton is counted (1mL).

The plankton weights were calculated by the following equations;

Wet weight= $W_1 - W_0$

Dry weight = $W_2 - W_3$

Where, W_0 is wet filter paper weight, W_1 wet filter paper and plankton weight, W_2 is dry filter paper and plankton weight and W_3 is dry filter paper weight.

4.2.3. Animal ethics statement

No animal ethics approval was required as marron is an invertebrate species.

4.2.4. Analysis of data

Data were analysed using SPSS version 23 and are expressed as means and standard errors. One-way analysis of variance (ANOVA) with LSD post hoc tests were used to determine the significant difference in trace element distribution and plankton parameters between the seasons. Independent t-tests were used to find the significant difference in dissolved trace element concentrations and plankton productivity between older and new ponds. Means of individual trace elements in 12 older aged ponds were compared with the mean of individual trace element in 16 new aged ponds for each season. Kruskal-Wallis test was used when data did not conform to the assumptions of normality or homogeneity of variance. Where necessary the data were base 10 log transformed to normalise their distribution. The correlations were analysed with Pearson and linear regression. Tests were considered statistically significant at $p<0.05$. Multivariate general linear model tests were used to determine the interactions and value with $p<0.05$ considered as significant.

4.3. Results

4.3.1. Trace element fluctuations in pond water over the seasons

The dissolved concentrations of trace elements in pond water were analysed for four seasons. When two different aged ponds (older and new) were pooled together there was a relatively high variation in the average dissolved concentrations of some of the trace elements from summer to spring. Mg, Cu and Fe showed the greatest variations over the four seasons (Table 4.1). Co and Se were below detectable levels; Ca and Mg showed the highest concentrations in summer and autumn. There was a significant ($p<0.05$) interaction between seasons and pond age for S and Cu only.

Table 4.1. Fluctuations of trace elements in all ponds during different seasons (mg L^{-1}).

Trace elements	Summer	Autumn	Winter	Spring
Ca	29.6 ± 1.1^{b}	29.9 ± 0.8^{b}	21.0 ± 0.7^{a}	19.2 ± 0.8^{a}
Mg	21.7 ± 0.40^{c}	21.9 ± 0.40^{c}	16.2 ± 0.50^{b}	10.6 ± 0.40^{a}
S	6.9 ± 0.5^{b}	5.9 ± 0.5^{ab}	5.8 ± 0.3^{a}	5.1 ± 0.3^{a}
Si	0.52 ± 0.08^{a}	0.98 ± 0.12^{b}	0.64 ± 0.09^{a}	0.45 ± 0.09^{a}
Al	0.05 ± 0.01^{a}	0.08 ± 0.01^{a}	0.20 ± 0.05^{a}	0.40 ± 0.07^{b}
Fe	0.03 ± 0.03^{a}	0.05 ± 0.05^{a}	0.08 ± 0.02^{b}	0.1 ± 0.02^{c}
P	0.01 ± 0.001^{a}	0.05 ± 0.001^{b}	0.01 ± 0.001^{a}	0.01 ± 0.005^{a}
Cu	0.008 ± 0.0020^{b}	0.020 ± 0.0020^{c}	0.001 ± 0.0003^{a}	0.001 ± 0.0002^{a}
Mn	0.003 ± 0.001^{ab}	0.005 ± 0.001^{b}	0.002 ± 0.001^{a}	0.005 ± 0.001^{b}
Zn	0.002 ± 0.0003^{a}	0.02 ± 0.0200^{b}	0.001 ± 0.0001^{a}	0.005 ± 0.0005^{b}
Se	ND	ND	ND	ND
Co	ND	ND	ND	ND

The values in the same row with different superscripts shows the significantly difference ($p<0.05$) in dissolved concentrations of trace elements over the four different seasons. Data represented as (Mean ± SE), n=28.

4.3.2. Fluctuations in primary and secondary productivity of the ponds over the seasons

The plankton abundance was significantly higher during the autumn than other seasons (Table 4.2). The higher phytoplankton species diversity was found in autumn and spring. Throughout the study time, Chlorophyta such as, *Closterium* spp., S*cenedesmus* spp., *Spirogyra* sp., *Volvox* spp., *Pandorina* sp., *Pediastrum* sp., *Chlorella* spp.; Euglenoid *Euglena* spp.; Bacillariophyta *Navicula* sp., *Aulacoseira* sp.; and unidentified dinoflagellate species, were found in the pond water samples. Three species of Rotifera: *Branchionus plicalitis*; *Keratella quadrata* and *Keratella cochlearis,* Cladoceran *Daphnia* sp.; *Moina* sp; and Calanoid copepods (both nauplii and adult) were recorded in the pond water samples.

Table 4.2. Plankton abundance, species diversity, wet weight and dry weight in different seasons.

Plankton factors	Summer	Autumn	Winter	Spring
Phytoplankton abundance (10^4 individuals L^{-1})	24.1 ± 7.85^{a}	290.8 ± 41.3^{b}	90.8 ± 21.2^{a}	136.9 ± 14.7^{a}
Phytoplankton species diversity (Number of species mL^{1})	2.64 ± 0.22^{a}	3.32 ± 0.27^{b}	2.54 ± 0.24^{a}	4.00 ± 0.32^{b}
Phytoplankton wet weight (g $100mL^{-1}$)	0.04 ± 0.01	0.05 ± 0.01	0.02 ± 0.01	0.06 ± 0.01
Phytoplankton dry weight (mg $100mL^{-1}$)	2.0 ± 0.4^{ab}	2.0 ± 0.4^{a}	3.0 ± 0.4^{b}	2.0 ± 0.6^{a}
Zooplankton abundance (Ind L^{-1})	88.7 ± 16.5^{b}	277.9 ± 27.3^{d}	192.8 ± 26.0^{c}	26.5 ± 4.4^{a}
Zooplankton species diversity (Species number mL^{-1})	2.6 ± 0.24^{b}	3.2 ± 0.10^{c}	3.3 ± 0.14^{c}	1.9 ± 0.15^{a}
Zooplankton wet weight (g $100mL^{-1}$)	0.01 ± 0.0004^{ab}	0.01 ± 0.0004^{a}	0.01 ± 0.0005^{b}	0.004 ± 0.0007^{c}
Zooplankton dry weight (mg $100mL^{-1}$)	0.3 ± 0.0^{bc}	0.4 ± 0.1^{c}	0.2 ± 0.0^{ab}	0.03 ± 0.1^{a}

Changes in plankton productivity of marron ponds during different seasons, the values in the same row with different superscripts are significantly different ($p<0.05$). Data represented as (Mean ± SE), n=28.

4.3.3. Difference in trace element concentrations between pond age

Mn, Si and S varied significantly between pond age over one year of sampling, wherein S was higher in new ponds and Mn and Si were lower in new ponds (Table 4.3). The trace elements concentration was the same in all ponds irrespective of stocked life stages of marron.

Table 4.3. Trace elements concentrations in different aged pond water (mg L^{-1}).

Trace elements	Older ponds	New ponds
Ca	23.6 ± 0.9^{a}	25.9 ± 1.0^{a}
Mg	18.5 ± 0.9^{a}	17.0 ± 0.6^{a}
S	4.6 ± 0.2^{a}	6.9 ± 0.3^{b}
Si	0.86 ± 0.1^{a}	0.49 ± 0.1^{b}
Al	0.16 ± 0.03^{a}	0.19 ± 0.03^{a}
P	0.03 ± 0.01^{a}	0.01 ± 0.001^{a}
Fe	0.08 ± 0.01^{a}	0.06 ± 0.01^{a}
Cu	0.006 ± 0.001^{a}	0.007 ± 0.001^{a}
Mn	0.006 ± 0.000^{a}	0.002 ± 0.000^{b}
Zn	0.003 ± 0.000^{a}	0.009 ± 0.006^{a}
Se	ND	ND
Co	ND	ND

Dissolved concentration of trace elements in two aged ponds, the values in the same row with different superscripts are significantly different ($p<0.05$) between pond age. Data represented as (Mean ± SE), n=12 for older and n=16 for new ponds.

4.3.4. The dissolved trace elements and their safe concentrations to aquatic life

The dissolved concentrations of pre-selected 12 trace elements were compared with their safe concentrations for aquaculture species over the long-term exposure (Table 4.4).

Table 4.4. Means (mg L^{-1}) and standard error of total trace elements in water and their safe concentration for freshwater aquaculture species. Dissolved concertation of pre-selected trace elements was compared with the safe concentrations for aquaculture purpose.

Trace elements	Average mean (mg L^{-1}) ± Std. error	For aquaculture (mg L^{-1})
Ca	24.9±0.6	NF
Mg	17.6±0.5	<15[1]
Si	0.65±0.05	NF
Al	0.17±0.025	<0.03 (pH>6.5)[1]
P	0.02±0.006	<0.1[2]
Fe	0.07±0.007	<0.01[1]
Mn	0.004±0.001	<0.01[1,3]
Zn	0.007±0.004	<0.005[1]
Cu	0.007±0.001	<0.005 (varies with hardness)[2]
S	5.9±0.20	NF
Se	ND	<0.01[1]
Co	ND	NF

ND= Not detectable (Dissolved concentration were below the detectable level); NF= Not found in literature; [1]Meade (2012); [2]DWAF (1996), [3]Zweig et al. (1999).

4.3.5. Trace elements and productivity in marron ponds

The correlations between the trace elements, primary and secondary productivity in different seasons were evaluated based on pond age and different life stages of stocked marron (Table 4.5). Zooplankton abundance and phytoplankton abundance showed a positive correlation during summer. Ca, Mg and Zn were negatively correlated with the natural productivity of the ponds.

Table 4.5. Correlations between trace elements and plankton abundance, species diversity, wet weight and dry weight in marron ponds. This table shows only the seasons where significant correlations were observed.

Plankton parameters	**Seasons**	**Pond age & Stocked life stage**				
Phytoplankton abundance		**Older**	**New**	**Juvenile**	**Grow-out**	**Brooder**
	Summer					
			Mn** & Fe ($^{+}$) *	Mn ($^{+}$) *	Mn*, Fe*, Al** & ZA ($^{+}$) *	Zn* & Cu ($^{+}$) *
	Autumn					S ($^{+}$) **
	Winter				P*, Zn* & Cu ($^{+}$) **	Mn (+) **
Phytoplankton species diversity	Summer			Mg* & Zn ($^{-}$) *		
	Autumn			P ($^{+}$) **		
	Winter				Si ($^{+}$) **	
Phytoplankton wet weight	Autumn	Cu ($^{-}$) *				
	Winter				Mn ($^{+}$)*	P ($^{+}$) **
Phytoplankton dry weight	Autumn					Al ($^{+}$) *
	Winter					Mn ($^{+}$) **
Zooplankton abundance	Summer	Zn ($^{-}$) * & PA ($^{+}$) **		S ($^{+}$) *	Si ($^{+}$) *	P ($^{+}$) *
	Autumn					P ($^{+}$) *
Zooplankton species diversity	Summer			PSD ($^{+}$) **		PSD ($^{+}$) *
	Autumn	P ($^{+}$) **				
	Winter				Mg ($^{-}$) *	Ca ($^{-}$) *
Zooplankton wet weight	Winter			Mg ($^{-}$) *	Mn ($^{+}$) *	
Zooplankton dry weight	Autumn	P ($^{+}$) *			P ($^{+}$) *	Al* & PDW ($^{+}$) *
	Spring					P ($^{+}$) *

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The same row content shows positive ($^{+}$) and negative ($^{-}$) correlations in the different seasons ($p<0.05$* and $p<0.01$** level) and significant correlations ($r^2 \geq 0.72$), n=12 for older, n= 16 for new; n= 9 for juvenile, n= 9 for brooder and n= 10 for grow-out ponds. Where, PA= Phytoplankton abundance, PSD= Phytoplankton species diversity, ZA= Zooplankton abundance, PDW= Phytoplankton dry weight

4.3.6. Physical and chemical parameters

The water quality parameters were recorded (Table 4.6). The turbidity of ponds with a value of 1.5m was essentially very low, with the bottom being clearly visible.

Table 4.6. Physical and chemical parameters measured over the study period for all ponds.

Seasons / **Parameters**	**Summer**	**Autumn**	**Winter**	**Spring**
Temperature (ºC)	25.8 ± 0.2^{d}	13.4 ± 0.4^{b}	10.2 ± 0.2^{a}	17.8 ± 0.6^{c}
Dissolved oxygen (mg L^{-1})	11.2 ± 0.2^{b}	10.4 ± 0.2^{a}	11.3 ± 0.2^{b}	11.0 ± 0.1^{b}
pH	7.9 ± 0.07^{ab}	8.0 ± 0.03^{bc}	8.1 ± 0.06^{c}	7.8 ± 0.07^{a}
Turbidity (m)	0.76 ± 0.09^{a}	0.70 ± 0.07^{a}	0.89 ± 0.09^{a}	1.20 ± 0.08^{b}

The values in the same row with different superscripts shows the significant difference ($p<0.05$) in water physical and chemical parameters over the different seasons. Data represented as (Mean ± SE), n=28.

4.3.7. Pond age and primary and secondary productivity of all ponds

Plankton producticity was compared on the basis of pond age. Plankton abundance, phytoplankton species diversity and wet weight were higher in older ponds than in new ponds (Figure 4.1). There was a significant interaction between season and pond age for zooplankton abundance ($p<0.05$). Plankton producticity was compared on the basis of pond age. Plankton abundance, phytoplankton species diversity and wet weight were higher in older ponds than in new ponds (Figure 4.1).

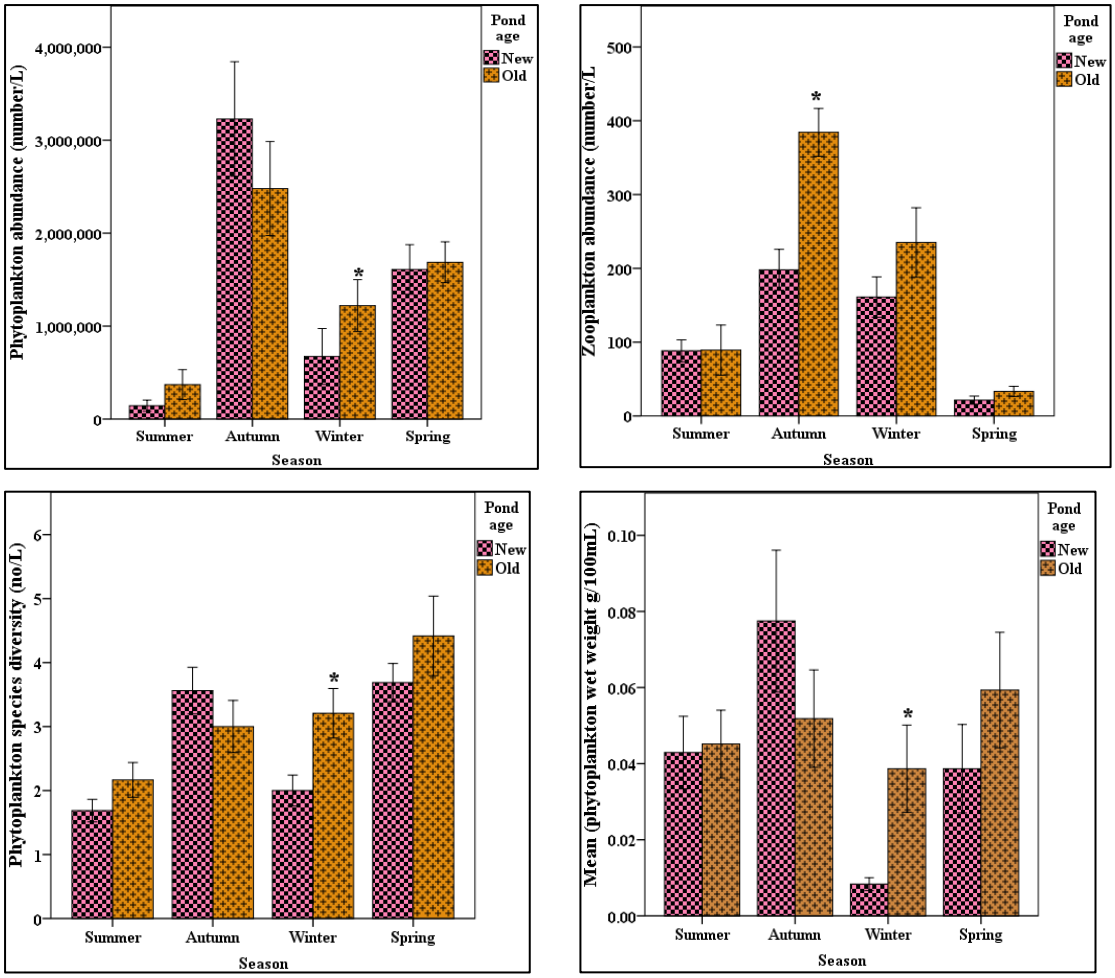

Figure 4.1. Plankton abundance, phytoplankton species diversity and wet weight in older and new ponds over the seasonal variation. *shows the significant difference ($p<0.005$) between pond age, n=12 for older and n=16 for new ponds.

4.4. Discussion

Seasonality had a significant effect on most of the pre-selected trace elements. Seasonal changes can affect trace element concentrations in the aquatic environment (Akcay et al., 2003, Turgut, 2003). Out of the 12 trace elements studied, only Se and Co were below the detection limits while the other 10 trace elements showed seasonal fluctuations (Table 4.1). The seasonal fluctuations of trace elements can largely be credited to environmental factors, including temperature, evaporation and rainfall and plankton dynamics (Dickson, 1975, Gibson et al., 2000, Hecky et al., 1986, Kimball, 1973, Sierp and Qin, 2001, Zhang et al., 2013). Farming practices, such as liming, water sources, feeding, fertilization, harvesting, cleaning the pond bottom soil at harvest and overnight drying may also have an effect (Adhikari, 2003, Egna and Boyd, 1997, Borg et al., 2001, Christos et al., 2018, EL-Saharty, 2014, Abu Hena et al., 2018, Shaked et al., 2004). Furthermore, the fluctuations in plankton abundance, and phytoplankton

species diversity (Table 4.2) can relate to changes in the concentrations of trace elements, sunlight hours, and temperature (Calijuri et al., 2002, Sommer et al., 2012). Van Nguyen and Wood (1979) and Papst et al. (1980), suggested that the phytoplankton communities collapse with nutrient depletion, as algal production is dependent on nutrients, and environmental changes such as low water temperature and cloudy weather. The south-west of WA has four distinct seasons. The summer and winter can be considered extreme weathers, whereas autumn and spring can be grouped together in terms of temperature regimes. In south-west of WA, where the majority of marron farms in Australia are located, also experiences winter rain and high evaporation during the summer months. Seasonal changes in dissolved trace elements concentrations due to rainfall and evaporation, and changes in temperature, may partly explain the seasonal fluctuations in plankton productivity of marron ponds.

There appeared to be the build-up of macronutrients, such as phosphorus, over the culture period, before decreasing after the refilling of ponds. The build-up of uneaten feed on the pond bottom can cause increases in phosphorus concentration, for example, in tiger shrimp (*Penaeus monodon*) culture ponds (Malaysia). The influx of P is result of decaying process of organic matter in the ponds, with feed being the key source of P (Abu Hena et al., 2018). During the autumn, the increased influx of P, Si, Zn and Cu in relatively shallower water bodies is caused by heating up of upper layer of sediments triggered by warmer water column above, similar result was observed with shallow lakes in central Canada (Hecky et al., 1986). High temperatures, pH and increased invertebrate activities promotes the dissolution of diatom silica in summer, which contributes to high dissolved Si levels by autumn (Dickson, 1975, Gibson et al., 2000). Research suggests that an increase in silicate concentration corresponds approximately with that in phosphate concentration (Heron, 1961). Contrary to our findings, EL-Saharty (2014) found a reduction in phosphate concentration during the autumn, due to an increase in uptake of phosphate by phytoplankton. P was decreased due to the presence of phytoplankton in a study by Sierp and Qin (2001). In the current study, P was positively correlated to plankton abundance, species diversity, phytoplankton wet weight and zooplankton dry weight.

In the aquatic environment, Ca and Mg are naturally available and can also be added through feed. The literature on the seasonality of Mg in freshwater ponds is scarce compared to other studied elements. As Ca and Mg concentrations are related, lower Mg concentration was reflective of Ca concentrations over the seasons (Venkatasubramani and Meenambal, 2007). Lower temperature results in decrease in flow of trapped elements from pond sediment into the

water column. A decline in exchangeable Ca concentration from pond soil can cause a decrease in the Ca concentration in water (Jeziorski et al., 2008). Harvesting and refilling of the ponds with freshwater may have reduced the Ca concentration. Also, crustaceans absorbs the Ca directly from water rather than feed, which may result in drop in Ca concentration in water (Robertson, 2002). To meet Ca demand freshwater crayfish rely on direct absorption of Ca from their surrounding water (Edwards et al., 2016).

Irrespective of pond age and life stage of marron, no trace elements exhibited any relationship with natural productivity, possibly due to the high inbuilt variability caused by farm management procedures. However, when ponds were categorised into two age groups and three life stages, natural productivity showed significant relationships with various trace elements (Table 4.5). Zn and Cu showed a similar pattern in correlations with the natural productivity, likely due to its synergistic behaviour with Cu to inhibit photosynthesis in plants and phytoplankton species (Goldman, 2010). Both trace elements showed positive and negative correlations with the natural productivity. Cu plays a dual role in the metabolism of plants or algae, as a micronutrient and a toxicant (El-Shiboshi, 1991, Downs et al., 2008). Cu was positively correlated to phytoplankton abundance, but negatively correlated to the wet weight of phytoplankton, relating to its higher concentration during autumn, as it inhibits algae cell division and photosynthesis at high levels (Goldman, 2010). Cu concentration during the autumn may be high enough to be toxic for some plants (Riley, 1939). High levels of free Cu and low levels of Fe are harmful for phytoplankton (Sunda et al., 1981) and can reduce growth and reproduction in plants (EL-Saharty, 2014).

High Fe, Al and Mn concentration in water can be harmful for crayfish farming. In Sweden the crayfish ponds with >0.5 mg^{-1} Fe and Mn were forced to reduce the concetration in supplying water (Ackefors, 1996). In the current study the concentration of Fe and Mn ranged between 0.03 to 0.1 mg^{-1} and 0.002 to 0.005 mg^{-1} respectively. Phytoplankton abundance showed a positive correlation with Fe during summer. The average concentration of Fe fluctuated greatly, being higher during winter and spring. Which could have been due to the Fe leaching from pond bottom sediments during the draining-down of these ponds. When the ponds were emptied a red coloured silt was observed at the bottom, indicating high Fe content in the sediment. In aquatic environments, leaching of bedrock is one of the main reasons for release of trace elements into the water (Ahmed et al., 2011, Khatri and Tyagi, 2015). Al average concentration was higher during the spring than the safe concentration for aquatic life or aquaculture (Table 4.1 & 4.4). But the concentration was not toxic to pond biota as Al is more

soluble and potentially toxic to freshwater biota if the pH decreases below 6.0 (Gensemer and Playle, 1999), and the pH of marron pond water was higher than 7.8 throughout the year. All the physical and chemical water parameters showed seasonal variations (Table 4.6) and were within the optimum range for marron (Cole et al., 2019).(Castillo-Soriano et al., 2010)

The information regarding the effects of Mn concentration on primary productivity and it's relationships with plankton in aquaculture ponds is scarce. Mn naturally occurs in rocks, soil, and sediment, and is weathered into ground and surface water; resulting in various levels of Mn in natural waters (Reimer, 1999). Phytoplankton abundance, wet and dry weight and zooplankton wet weight were positively correlated with Mn. Mn in the form of superoxide dismutase is common in diatoms (Wolfe-Simon et al., 2005). The growth of diatoms is dependent on Si, to grow the siliceous cell walls (Boyd, 2014). Si was positively correlated to phytoplankton species diversity and zooplankton abundance. Also, the Mn and Si concentrations were higher in older ponds (Table 4.3). Hena and Hishamuddin (2014) observed a higher Mn concentration in older ponds (tiger shrimp, Malaysia), and the author suggested the cause was age of ponds, liming activities and sediment leaching. In the case of the marron ponds the limestone was added only into the new ponds at the start of pond utilization.

Ca and Mg, vital for aquatic plants and animals, showed negative correlations with the plankton species diversity and zooplankton wet weight. Our output of these negative correlations can be supported by the study of Nasser and Sureshkumar (2013) which demonstrated an interaction between microalgal species richness and environmental variables, where Chlorophyceae showed a negative correlation with Mg and Ca. Hulyal and Kaliwal (2009) showed that the abundance of Cyanophytes was negatively correlated with Ca and Mg. In a study by, Castillo-Soriano et al. (2010) chlorophyll *a* was negatively correlated with the Ca concentration in intensive culture of white leg shrimp (*Litopenaeus vannamei*). More research is needed to clarify the impacts of Ca on different aspects of primary production (Shi et al., 2013).

The seasonal variations of trace elements can significantly influence algal growth (Talling, 1986, Fayissa, 2013). Our study suggests that the favourable season for plankton abundance and species diversity was autumn (March) (Table 4.2), which could be due in part to the lower volume of water present in the ponds due to the evaporation during the summer, causing higher dissolved concentrations of correlated trace elements. The phytoplankton abundance ranged from 24.1 x 10^4 to 290.8 x 10^4 L^{-1}, showing the highest abundance after the warmer days, during autumn (Table 4.2 & 4.6). Affan et al. also observed that February to March was the favourable

time for phytoplankton growth in ponds (Bangladesh), because of the low volume of water, high solar radiation, rising temperature and suitable levels of nutrients favouring rapid cell multiplication (Affan et al., 2005). The plankton abundance, and phytoplankton species diversity were lower during the winter, which may be due to the draining of ponds in winter or the calm weather, less sunlight and lower temperatures. Phytoplankton often die with calm weather (Boyd, 1982) that reduces mixing with bottom water, which is an important nutrient source for phytoplankton growth in stratified systems (Margalef, 1978). The highest zooplankton abundance during the autumn shows that the phytoplankton abundance could have supported the zooplankton abundance. The phytoplankton abundance and chlorophyll *a* concentration are important factors for zooplankton abundance, zooplankton abundance is entirely dependent on phytoplankton abundance (O'Brien and de Noyelles, 1974, Martinez-Cordova et al., 1998, Preston et al., 2003).

The plankton productivity was significantly higher in the older ponds (Figure 4.1), possibly due to the higher concentrations of Mn and Si (Table 4.3). Also, the phytoplankton abundance, species diversity and wet weight were significantly higher in older ponds. A significant interaction ($p<0.05$) in zooplankton abundance between seasonal changes and pond age was observed, wherein the zooplankton abundance and dry weight were significantly higher in older ponds during autumn and spring respectively. These results were similar to the findings of Cole et al. (2019) where the authors found the phytoplankton abundance, species diversity and zooplankton abundance were higher in older ponds. In contrast to our result, Abu Hena (2005) reported that the new culture ponds had greater phytoplankton growth than older ponds, attributed to the higher Ca concentration in older ponds, leading to a higher rate of Ca-phosphate precipitation, in turn resulting in less availability of phosphate in the water to support plankton growth (Abu Hena et al., 2018).

4.5. Conclusions

The results demonstrated the effects of seasonal variations and pond age on trace elements and natural productivity, and the effects of seasonal variations of trace elements on the abundance of plankton in freshwater crayfish ponds. Dissolved concentrations of Co and Se were below the detectable limit, while Fe showed the most seasonal fluctuation among the trace elements. The plankton productivity was higher during the autumn and was also higher in older ponds than new. The plankton productivity of the ponds was mostly influenced by three trace elements, Mn, Si and P. These trace elements play a major role in the primary and secondary productivity of ponds as higher phytoplankton abundance can likely support higher numbers

of zooplankton. The statistical analysis demonstrated that interaction between zooplankton and seasonality is strongly related to the pond age. Laboratory experiments with the trace elements addition should be performed to better understand their effect on plankton growth and species diversity.

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Conflict of interest

The described work is a part of PhD course of Miss. Smita Sadanand Tulsankar, with no potential conflicts of interest related to this article.

Data availability statement

The data is confidential, farm owner does not want to disclose it.

CHAPTER 5. Temporal variations and pond age effect on plankton communities in semi-intensive freshwater earthen aquaculture ponds: study of marron (*Cherax cainii,* Austin 2002) aquaculture in Western Australia

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Abstract

The abundance and diversity of the plankton community represents the health of the aquatic ecosystem, and plays an important role in the growth of cultured animals under aquaculture conditions. The temporal variations of plankton abundance, taxonomic composition, diversity, evenness and species richness were studied in three older and three new semi-intensive marron (*Cherax cainii,* Austin, 2002) ponds. Water parameters such as temperature, DO, pH, turbidity, TAN, nitrite, nitrate and reactive phosphate were recorded, and plankton samples were collected every two months, for one year of juvenile production cycle. A total of twenty-six phytoplankton and seven zooplankton genera were recorded. Chlorophyceae was the dominant class of phytoplankton throughout the year, followed by Trebouxiophyceae. Rotifera comprised 49.8% of the total zooplankton community (individuals L^{-1}) the largest proportion of any group. Temporal variations impacted the plankton abundance and community structure, and plankton abundance was more abundant during summer. The pond age did not influence the phytoplankton abundance, whereas zooplankton abundance was higher in older ponds.

Keywords: Freshwater pond ecology; Freshwater crayfish; Plankton indices; Cyanophyceae; Copepoda; Aquaculture**.**

5.1. Introduction

Natural lentic water bodies and earthen aquaculture ponds are unique examples of freshwater ecosystems supporting communities of various aquatic organisms, including plankton and top predators such as fish and decapod crustaceans. Both environments are subject to an excess of nutrient loadings, entering from natural and anthropogenic sources (Nõges et al., 2016). However, while natural lentic water ecosystems receive considerably higher amounts of nutrients through runoff, nutrient loading in aquaculture ponds is largely sourced from fertilizers, feed input and cultured animal excreta (Boyd et al., 2010) as part of aquaculture management practice. Fertilization is applied to increase the pond production by promoting the

natural productivity (Boyd, 2018), consisting of phytoplankton as primary producers, and zooplankton, the primary consumers and secondary producers. Phytoplankton being an integral component of freshwater ecosystems, including aquaculture ponds, contribute to the succession and dynamics of zooplankton (Li et al., 2019), that in turn provides a food source for cultured aquatic animals (Li et al., 2017c), including marron.

Western Australia's (WA) marron (*Cherax cainii*, Austin, 2002) industry produces approximately 50 tonne per year, in year 2016-17 marron production from WA was 51 tonne valued at AUD $ 1.6 million (ABARE, 2018). Numerically, its producers comprise the state's largest aquaculture sector (Fisheries, 2015). In the south-west of WA marron farming is conducted in semi-intensive ponds. As the marron breed during the spring, farmers harvest the ponds in winter (June/July) and segregate the marron into different weight groups and restock them as juveniles, grow-out (+1) or brooders. Due to the wet weather in winter and chance of raining the drained-out ponds are left empty for only a short duration, and then refilled for restocking without liming and fertilization (Fotedar et al., 2015). The amount of formulated feed broadcasted into these semi-intensive ponds varies with the season and stocked life stages, for example during the winter the feed quantity is reduced to half than summer and ponds with adult marron are fed higher quantity than juveniles (anecdotal evidence from growers). Marron also attain nutrition from natural productivity, however the ponds plankton communities structure and their variations are poorly studied. Marron pond age is also shown to impact plankton abundance (Cole et al., 2019, Tulsankar et al., 2020); as the pond ages the natural productivity increases (Correia et al., 2002).

Marron are polytrophic and can attain additional nutrition from ponds natural productivity and thus marron farmers in southwest of WA apply fermented barley straw to post-stocked marron ponds (Fotedar et al., 2015). This practice is believed to balance the nutrient and bacterial loadings over a period of time in marron ponds, it also aids in managing natural productivity and improving the water quality for marron farming. Bacteria help to reduce excessive nitrogenous wastes, provide feed for bacterivorous protozoans that in turn are consumed by macroscopic zooplankton, and contribute to the diet of freshwater crayfish (Brown et al., 1992, Rautio and Vincent, 2006, Martinez-Porchas et al., 2014).

The plankton growth is dependent on various environmental parameters such as temperature, light intensity, and nutrient concentrations (Ndebele-Murisa et al., 2010). These can also affect the pattern of phytoplankton succession, as different species have different thresholds towards

nutrients and temperature ranges (Affan et al., 2005). For example, increased temperature and eutrophication promotes Cyanophyceae abundance (Kratina et al., 2012). The temporal variations of plankton communities are good indicators of water quality and trophic status in freshwater ecosystems, and for sustainable aquaculture (Reynolds, 1996, Affan et al., 2005). A better understanding of the temporal variations of plankton communities in freshwater ecosystems may help to improve the productivity of marron farms.

Researchers have studied Australian natural lentic freshwater ecosystems to determine water quality and plankton productivity (Casanova et al., 1997, Markwell and Fellows, 2008). However, there is a lack of information on plankton communities in Australian aquaculture facilities (Pearson and Duggan, 2018). This is an exploratory study to investigate the temporal variations in plankton communities in marron ponds of different ages. Moreover, a potential relationship between the plankton abundance, community diversity and the marron yield in each pond is investigated over a time period of one year.

5.2. Materials and methods

The study was conducted at a commercial marron farm with the capacity of 60 purpose-built earthen ponds each with a maximum depth of 1.5m and water area of 1000m^2, situated in the south west of Western Australia (WA) near Manjimup (34°18’75” S, 116°06’61” E). Out of 60, six ponds were randomly selected to study temporal variations in plankton abundance, diversity, composition and their community structure. Based on construction and number of years in use, the ponds were classified as older and newer (around 15 and 11 years respectively) ponds. Six ponds, with the ratio of three older and three new were selected for the study. The farmers have treated these ponds on the basis of their personal experiences with the addition of gypsum or barley hay straw. Gypsum was added only to the new ponds at the end of construction. Hay straw additions were conducted in the new ponds after five to six months of marron stocking. The paddle wheel aerators were not used in any ponds, the feeding was conducted manually in the evening. Both the ponds were supplied same amount of feed, water level and stocked marron weights.

Before collecting first water samples in July 2016 (winter), the ponds were stocked with grow-out male and female marron with the approximate weight of 150kg/pond from the same farm with the same weight group on different days but same month before starting the sampling. The harvested ponds were left emptied for very short duration of 1 or 2 days and filled from the nearby dam owned by the farmer and was only topped up the loss due to evaporation.

Stocking was conducted immediately after filling without grading, liming or fertilization. The number of culture days differed for each pond, ranging from 300 to 397. Water samples were collected every two months, once per each Noongar season (in southwest of Australia the Noongar seasonal calendar includes six different seasons. The flowering of different plants, hibernation of reptiles and moulting of swans are used as an indicator for seasonal changes). Dissolved oxygen (DO), pH and temperature were measured at approximate depth of 15-20 cm of pond water at the same location of each pond over every sampling. An Oxyguard® digital dissolved oxygen meter (Handy Polaris 2, Norway) was used to measure dissolved oxygen (DO) and temperature, and an Ecoscan pH 5 meter (Eutech instruments, Singapore) was used to measure pH in the sampled ponds. 100 mL water samples from each pond were collected from 15-20 cm deep height to analyse total ammonia nitrogen (TAN), nitrite (NO_2-N), nitrate (NO_3-N) and reactive phosphate (PO_4) and transported with ice in esky. A DR/890 portable colorimeter with Permachem reagents (Hach, USA) were used to analyse the TAN, NO_2-N, NO_3-N and PO_4 (Downs et al., 2008). TAN, NO_2-N, NO_3-N and PO_4 were analysed at Curtin Aquatic Research Laboratory (CARL; Perth) on the same day. Turbidity was measured with a secchi disc, with clear ponds defined as having 150 cm visibility.

5.2.1. Plankton collection and analysis

Five litres of water was collected from five different stations (1 L/station; four corners and from the middle) of each pond using 20 L plastic beaker and poured through a plankton net of 20 micron mesh to obtain 100 mL phytoplankton samples. For zooplankton, 15 L of water was collected from five different stations of each pond (3 L/ station) same as phytoplankton using a beaker and collecting it in 50 L water capacity plastic bucket and poured through 60 micron mesh zooplankton net to obtain 100 mL sample. Throughout the study time all the samples were collected after four to five hours of sunrise and were transported to the CARL on ice. Phytoplankton were preserved with 3% acetic acid Lugol's within eight hours. Phytoplankton species identification was conducted using a compound microscope at 400X magnification; quantitative analysis was done using a haemocytometer. For each phytoplankton sample, a 1mL sub-sample was transferred to the haemocyte counter and cells were counted within four randomly selected squares, four counts per sample. Phytoplankton were analysed further to obtain the plankton indices. Zooplankton samples were fixed with 70% ethanol and were counted using a dissection microscope at 20x magnification. A 1mL sub-sample was transferred to a petri dish, two counts were carried out per sample. Phytoplankton and zooplankton were identified to the genus level by using the keys from a book by Canter-Lund

and Lund (1995) and manual by Ingram et al. (1997). The phytoplankton abundance (cells L^{-1}) and zooplankton abundance (individual L^{-1}) was calculated by using the following equations as described by Ingram et al. (1997) and Tulsankar et al. (2020).

$$\text{Phytoplankton abundance} = \frac{\left(\frac{\text{Number X 1000}}{\text{Volume of grid (0.1mm3)}}\right) \text{X} \left(\frac{\text{Concentrated Volume}}{\text{Sub Volume}}\right)}{(\text{Total Volume X Number of squares counted})}$$

$$\text{Zooplankton abundance} = \frac{\text{Number X} \left(\frac{\text{Concentrated volume}}{\text{Sub Volume}}\right)}{\text{Total Volume}}$$

Where,

Number = Mean number of cells or individuals counted, Total volume = Total volume of water (L) collected from pond, Concentrated Volume = Volume of water (mL) containing concentrated zooplankton after sieving (100ml), Sub. Volume = Sub sample of water (mL) from concentrated volume in which plankton is counted (1mL).

To calculate the plankton species diversity (H'), species richness (SR), evenness (J) and predominant indices (D) following formulas by Shannon and Wiener (1949); Margalef (1958); Pielou (1966) and Berger and Parker (1970) were used.

$$H' = -\sum_{i+1}^{s} \frac{N_i}{N} \log_2 \frac{Ni}{N} \qquad SR = \frac{S-1}{\log N}$$

$$J = \frac{H'}{log_2 S} \qquad D = \frac{N_{max}}{N}$$

Where,

N_i is the number of individuals of the *i'th* species; N_{max}, is the number of individuals of the most abundant species; N is the total number of individuals; S is the total number of species.

H' value can range from 0 to infinity, with a higher value showing that the ecosystem is healthier. The J value shows a single-species dominance in water body, where, 1 indicates the equal abundance of species or maximum evenness. The D value is the measure of the most abundant species in a water body; it ranges from 0 to 1, where higher value indicates that the

species accounts for a higher proportion of total individuals. SR is the number of different species observed in a sample.

5.2.2. Marron yield data

The marron yield data were collected from the farm records to calculate the growth indices; specific growth rate (SGR g % /day) and Biomass gain percentage (BG; g %) by using following equations:

SGR (g % /day) = 100 X ($\ln W_t$ - $\ln W_0$)/t

BG (%) = 100 X (W_t – W_0)/W_0

W_t is the final weight of marron, W_0 is initial weight and t is experimental time (days).

The marron survival rate was not calculated as the farm records did not include the precise number of marron at stocking and harvest times, only the yield in kilograms

5.2.3. Marron health indices

Six marron per pond were collected at the time of harvest and were transported in thermocol box with ice packs. Marron were collected with average weight of 66.5 to 73.1; average orbit carapace length (OCL) of 54.4 to 65.2 and average total length of 135.6 to 143.2. The individual marron weight, OCL and total length were recorded before dissecting for health indices analyses. The health indices analyses were performed at CARL within three days until then were stored at -75°C freezer. The tail muscle and hepatopancreas from individual marron were weighted and to obtain dry weight the samples were dried in crucibles at 105°C in the oven for 24 hrs. The organosomatic indices and moisture content were analysed using the following equations as described by Fotedar (2004),

Tail muscle moisture (TM %) = (T_w-T_d) x 100/T_w

Hepatopancreas moisture (HM %) = (H_w- H_d)/H_w

Wet tail muscle indices (TMi_w) =T_w x100/BW

Wet hepatopancreas indices (Hi_w) =H_w x100/BW

Dry tail muscle indices (TMi_d) =T_d x100/BW.

Where, T_w: Tail muscle wet weight; T_d: Tail muscle dry weight; H_w: Hepatopancreas wet weight; H_d: Hepatopancreas dry weight; BW; Body weight.

5.2.4. Statistical analysis

The data were processed using statistical software IBM® SPSS 25. One-way ANOVA with LSD post hoc tests were used to analyse temporal variations of water parameters and plankton. Kruskal-Wallis tests were used when data did not conform to the assumptions of normality and homogeneity of variance. Independent t-tests were used to compare the significance of physical and chemical parameters and plankton between older and new ponds. Pearson test was used to observe correlations. Tests were considered statistically significant at $p<0.05$**.**

5.3. Results

5.3.1. Water quality parameters and plankton in two aged ponds

The water quality parameters were within the suitable range for marron as shown by Morrissy et al. (1984); Morrissy (1990); Villarreal and Peláez (1999); Policy (2003) and Cole et al. (2019). Temporal variations significantly affected all environmental parameters (Table 5.1). DO was higher in colder months (<20°C; July and September). Nutrient concentrations and pH fluctuated over the sampling time. Pond age influenced the turbidity, being lower in new ponds than older (Table 5.2). Total phytoplankton abundance (cells L^{-1}) did not show significant difference between older and new ponds, while Trebouxiophyceae abundance (cells L^{-1}) was higher in older ponds than new. Overall, phytoplankton diversity was higher in newer ponds and zooplankton abundance in older.

Table 5.1. The comparison of water quality parameters of older and new aged marron ponds over sampling months, conducted on commercial marron farm in Manjimup (means ± S. E; n=6).

Parameters	**July**		**September**		**November**		**January**		**March**		**May**	
	Older	**New**	**Older**	**New**	**Older**	**New**	**Older**	**New**	**Older**	**New**	**Older**	**New**
DO (mg L^{-1})	12.4 ± 0.56	11.9 ± 0.41	11.3 ± 0.06	11.1 ± 0.66	7.38 ± 0.55	7.85 ± 0.59	7.33 ± 0.21	8.80 ± 0.11	7.24 ± 0.11	7.86 ± 0.24	8.65 ± 0.42	9.63 ± 0.37
Turbidity (cm)	61.7 ± 21.3	91.7 ± 38.1	70.0 ± 15.3	118 ± 31.7	68.3 ± 16.4[a]	150 ± 0.00[b]	34.0 ± 5.57	96.7 ± 26.7	26.7 ± 3.33	68.3 ± 6.01	36.7 ± 6.01	71.7 ± 13.6
Temperature (°C)	11.5 ± 0.21	10.6 ± 0.41	17.4 ± 0.23	18.6 ± 0.52	23.0 ± 0.03	24.0 ± 0.52	21.0 ± 0.15	23.1 ± 1.94	23.3 ± 0.12	22.3 ± 0.27	22.4 ± 0.17	21.2 ± 0.15
pH	8.41 ± 0.11	8.41 ± 0.11	7.43 ±0.09	7.63 ± 0.12	7.83 ± 0.13	8.01 ± 0.22	8.22 ± 0.22	8.29 ± 0.03	8.42 ± 0.09	8.54 ± 0.01	8.29 ± 0.04	8.37 ± 0.06
NH_3N (mg L^{-1})	0.12 ± 0.08	0.15 ± 0.03	0.05 ± 0.01	0.05 ± 0.03	0.19 ± 0.04	0.15 ± 0.02	0.02 ± 0.00	0.01 ± 0.01	0.54 ± 0.09	0.23 ± 0.03	0.33 ± 0.003	0.03 ± 0.03
NO_2N (mg L^{-1})	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.13 ± 0.08	0.02 ± 0.01	0.04 ± 0.01	0.01 ± 0.00
NO_3N (mg L^{-1})	0.50 ± 0.15	0.80 ± 0.55	0.80 ± 0.10	0.47 ± 0.18	1.23 ± 0.29	0.87 ± 0.27	0.73 ± 0.38	0.50 ± 0.29	0.97 ± 0.03[b]	0.53 ± 0.13[a]	4.00 ± 1.82	1.00 ± 0.64
Reactive phosphate (mg L^{-1})	0.24 ± 0.07	0.23 ± 0.15	0.17 ± 0.17	0.20 ± 0.15	0.04 ± 0.03	0.01 ±0.01	0.18 ± 0.00	0.09 ± 0.88	0.26 ± 0.06	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.02

The values in the same row with different superscripts letters represent the significant difference ($p<0.05$) between older and new pond.

Table 5.2. The comparison of water quality parameters and plankton abundance, diversity between the older and new aged marron ponds from a commercial marron farm in Manjimup (means ± S. E; n=3).

Parameters	**Older**	**New**
pH	8.10 ± 0.10	8.20 ± 0.10
DO (mg L^{-1})	9.00 ± 0.50	9.50 ± 0.40
Temperature (°C)	20.1 ± 1.10	20.3 ± 1.20
Turbidity (cm)	49.6 ± 6.20^{a}	96.9 ± 10.9^{b}
NH_3N (mg L^{-1})	0.16 ± 0.05	0.10 ± 0.02
NO_2N (mg L^{-1})	0.04 ± 0.02	0.01 ± 0.00
NO_3N (mg L^{-1})	1.20 ± 0.40	0.60 ± 0.20
Reactive Phosphate (mg L^{-1})	0.20 ± 0.03	0.13 ± 0.04
Phytoplankton abundance ($X10^4$ cells L^{-1})	278 ± 3.80	233 ± 1.70
Zooplankton abundance (Ind. L^{-1})	180 ± 24.4^{b}	77.1 ± 9.70^{a}
Phytoplankton Species diversity	5.28 ± 0.50^{a}	7.40 ± 0.30^{b}
Zooplankton species diversity	3.10 ± 0.20	3.30 ± 0.30
Chlorophyceae ($X10^4$ind. L^{-1})	101 ± 11.4	109 ± 12.5
Trebouxiophyceae ($X10^4$ind. L^{-1})	65.7 ± 9.70^{b}	41.7 ± 4.70^{a}
Eustigmatophyceae ($X10^4$ind. L^{-1})	70.3 ± 22.2	46.9 ± 17.0
Zignematophyceae ($X10^4$ind. L^{-1})	72.9 ± 25.7	37.5 ± 8.10
Euglenoidea ($X10^4$ind. L^{-1})	6.30 ± 1.80	4.40 ± 0.80
Bacillariophyceae ($X10^4$ind. L^{-1})	35.7 ± 7.50	26.0 ± 2.70
Cyanophyceae ($X10^4$ind. L^{-1})	85.6 ± 30.4	25.0 ± 4.20
Coscinodiscophyceae ($X10^4$ind. L^{-1})	62.5 ± 24.1	20.1 ± 0.00
Rotifera (ind. L^{-1})	13.9 ± 3.00^{b}	6.4 ± 1.40^{a}
Cladocera (ind. L^{-1})	1.00 ± 0.50	1.00 ± 0.50
Copepod adults (ind. L^{-1})	3.80 ± 0.90	2.80 ± 0.80
Copepod nauplii (ind. L^{-1})	8.40 ± 1.20^{b}	4.10 ± 0.70^{a}

The values in the same row with different superscript letters represents the significant difference ($p<0.05$) between older and new ponds. Abbreviation: Ind= Individuals.

5.3.2. Plankton taxa

A checklist of the plankton recorded in marron ponds (Table 5.3). The identified phytoplankton community were composed of eight groups namely Chlorophyceae, Trebouxiophyceae, Euglenoidea, Eustigmatophyceae, Bacillariophyceae, Zygnematophyceae,

Coscinodidiscophyceae and Cyanophyceae. The zooplankton community were composed of three groups namely, Rotifera, Copepoda and Cladocera. A total of twenty-six different genera of phytoplankton, six of zooplankton and unidentified species of Copepod nauplii was recorded. The highest number of different genera was recorded in the class Chlorophyceae.

Table 5.3. The total plankton taxa found in commercial marron ponds over the sampling time of a year (N=6).

Phytoplankton groups	**Class**	**Genus**	**Zooplankton groups**	**Family**	**Genus**
Chlorophyta	Chlorophyceae	*Eudorina* spp.	Rotifera	Branchionidae	*Keratella quadrata*
Chlorophyta	Chlorophyceae	*Monoraphidium* spp.	Rotifera	Branchionidae	*Keratella cochlearis*
Chlorophyta	Chlorophyceae	*Scenedesmus* spp.	Rotifera	Euchlanidae	*Euchlanis dilatata*
Chlorophyta	Chlorophyceae	*Tetraedron* sp.	Copepoda	Cyclopoida	*Cyclops* sp.
Chlorophyta	Chlorophyceae	*Volvox* spp.	Copepoda	Centropagidae	*Boeckella* sp.
Chlorophyta	Chlorophyceae	*Selenastrum* spp.	Cladocera	Daphniidae	*Daphnia* spp.
Chlorophyta	Chlorophyceae	*Haematococcus* sp.	Cladocera	Moinidae	*Moina* spp.
Chlorophyta	Chlorophyceae	*Sphaerocystis* sp.			
Chlorophyta	Chlorophyceae	*Pandorina* sp.			
Chlorophyta	Chlorophyceae	*Pediastrum* sp.			
Chlorophyta	Chlorophyceae	*Chlamydomonas* spp.			
Chlorophyta	Trebouxiophyceae	*Chlorella* spp.			
Chlorophyta	Trebouxiophyceae	*Actinastrum* sp.			
Chlorophyta	Trebouxiophyceae	*Micratinium* sp.			
Euglenozoa	Euglenoidea	*Euglena* spp.			
Euglenozoa	Euglenoidea	*Peranemopsis* sp.			
Ochrophyta	Eustigmatophyceae	*Nannochloropsis* sp.			
Ochrophyta	Bacillariophyceae	*Navicula* spp.			
Ochrophyta	Bacillariophyceae	*Nitzschia* spp.			
Ochrophyta	Coscinodiscophyceae	*Aulacoseira* spp.			
Charophyta	Zygnematophyceae	*Spirogyra* spp.			
Charophyta	Zygnematophyceae	*Cosmarium* spp.			
Charophyta	Zygnematophyceae	*Micrasterias* spp.			
Charophyta	Zygnematophyceae	*Closterium* spp.			
Charophyta	Zygnematophyceae	*Mesotaenium* sp.			
Cyanobacteria	Cyanophyceae	*Chroococcales* spp.			

5.3.3. Plankton abundance and their temporal variations

The phytoplankton abundance fluctuated from 208.3 ± 17.8 x 10^4, 180.5 ± 31.7 x 10^4, 218.8 ± 37.6 x 10^4, 368.1 ± 80.6 x 10^4, 225.7 ± 14.6 x 10^4 and 329.9 ± 58.5 x 10^4 (cells L^{-1}) from July to May respectively and was maximum in January (summer) (Figure 5.1). Plankton abundance and phytoplankton species diversity were affected due to the temporal variations, where no significant difference in zooplankton species diversity was observed.

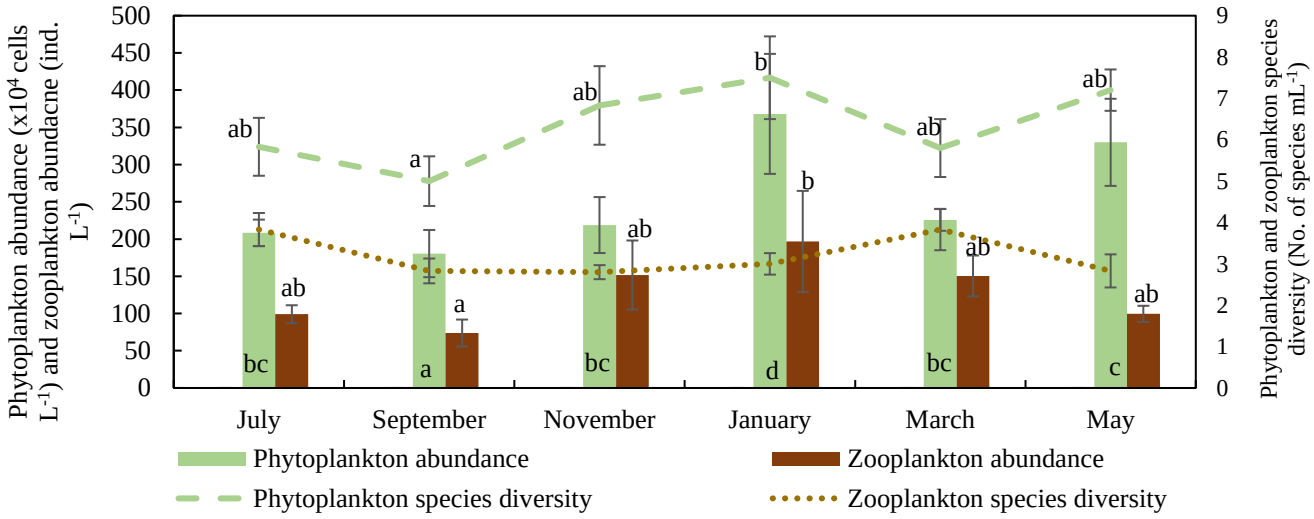

Figure 5.1. Temporal variations of the phytoplankton (x10^4 cells L^{-1}), zooplankton abundance (L^{-1}) and species diversity (Number of species/1mL) in a commercial marron ponds (means ± S. E.; n=3). Letters a, b, c shows the significant difference in plankton abundance and species diversity over the different sampling times.

5.3.4. Plankton succession

Temporal phytoplankton succession showed that Chlorophyceae were the only taxonomic group recorded during all sampling months. Cyanobacteria and Ochrophyta were observed during January to May (Figure 5.2a). *K. quadrata* and copepod nauplii abundance were recorded in most of the months (Figure 5.2b). Cladoceran's abundance was pre-dominantly observed only when the temperature were below <20°C; *Daphnia* spp. was recorded during the July and November sampling only in new ponds, though *Moina* spp. were recorded during July to November sampling in both aged ponds**.** The well-established zooplankton communities were, Rotifers (*Keratella* spp.) and Calanoid copepods. Rotifera spp. (*K. quadrata, K. cochlearis*) and Copepoda nauplii abundance was significantly (<0.05) higher in older ponds (Table 5.2), while *E. dialata* was observed only in newer ponds.

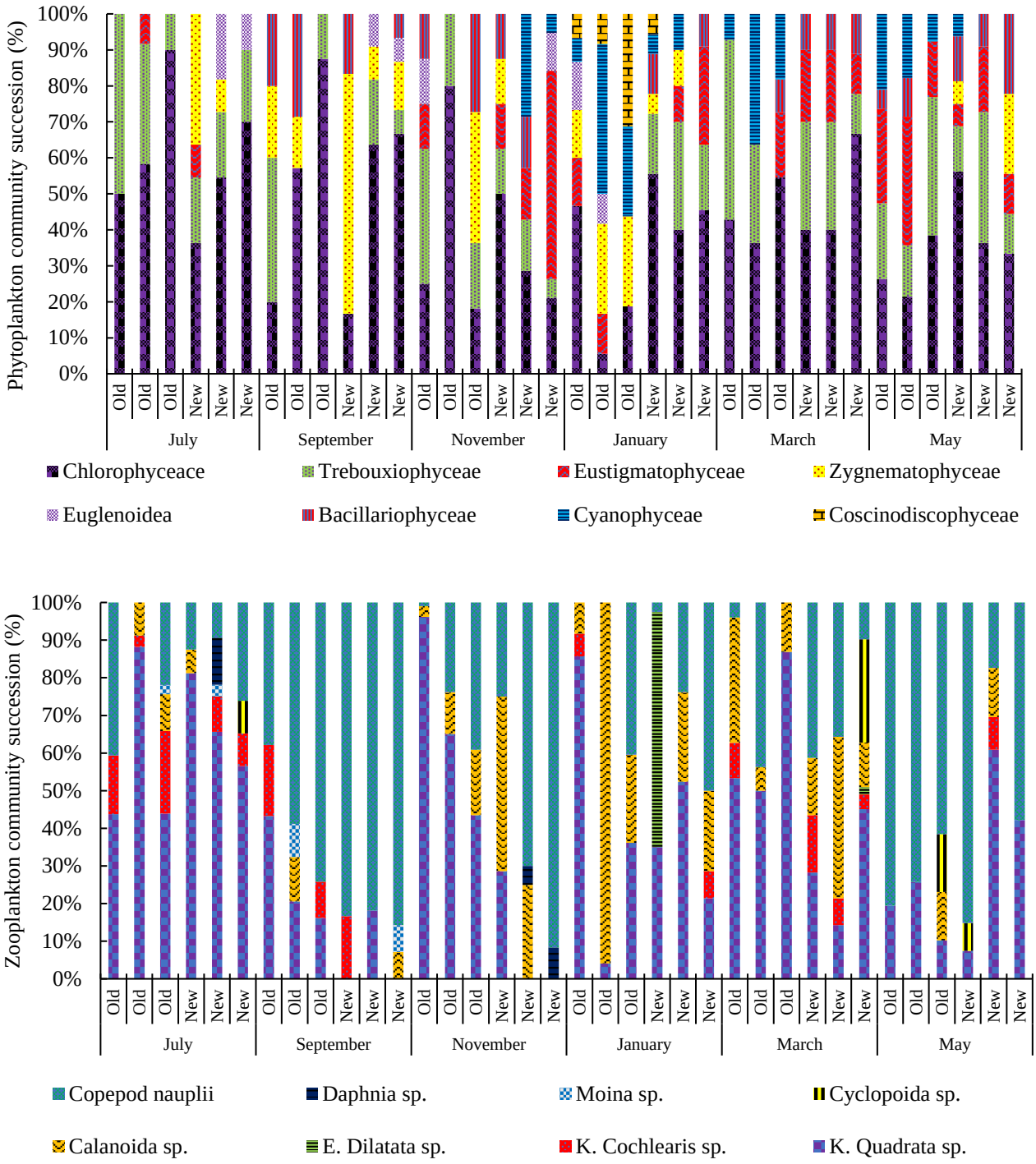

Figure 5.2. a) Phytoplankton community succession in older and new aged commercial marron ponds from Manjimup over the variations in temperature around the year (n=3).

Figure 5.1. b) Zooplankton community succession in older and new aged commercial marron ponds from Manjimup over the variations in temperature around the year (n=3).

5.3.5. Plankton community composition

Phytoplankton community composition (%) in both aged ponds was dominated by Chlorophyceae followed by Trebouxiophyceae and Eustigmatophyceae (Figure 5.3a and b). Rotifera dominated zooplankton composition while cladoceran's had the lowest abundance of individuals (Figure 5.3c and d). Rotifera and Copepoda were more abundant groups. *Keratella quadrata, K. cochlearis* and Copepoda nauplii were most abundant in older ponds, while *Euchlanis dilatata* was observed only in newer ponds.

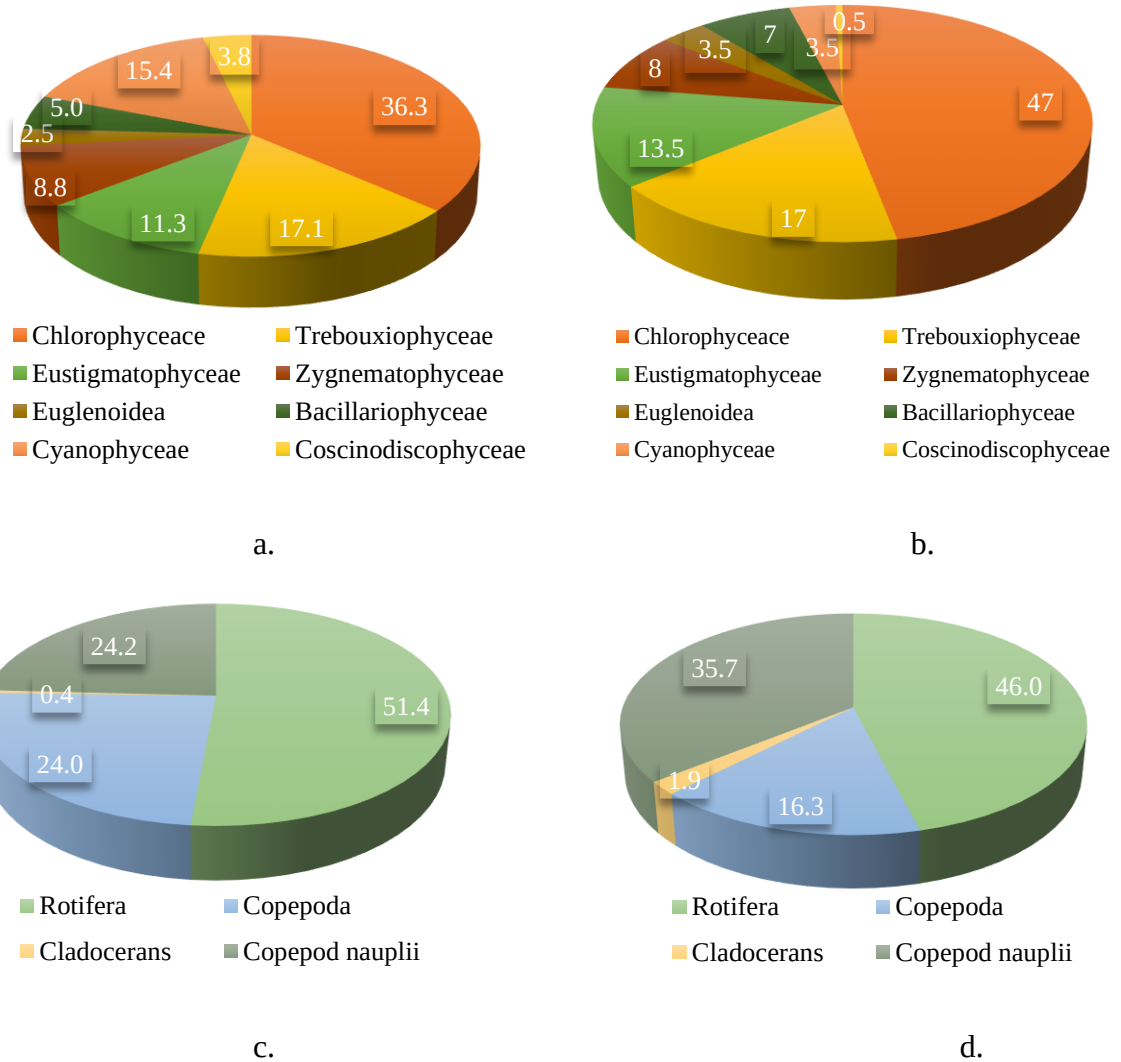

Figure 5.3. Phytoplankton (a and b) and zooplankton (c and d) community composition (%) recorded for older (a and c) and new (b and d) commercial semi-intensive marron ponds (n=6).

5.3.6. Plankton indices

Planktons diversity (H'), predominance index (D), species evenness (J), and species richness (SR) were compared between older and new ponds (Figure 5.4). The average values (mean ± S. E.) of phytoplankton H', D, J and SR for older ponds were 2.10±0.1, 0.38±0.0, 0.90±0.0 and 2.0±0.2 respectively, and for newer ponds 2.66±0.1, 0.30±0.0, 0.92±0.0 and 3.0±0.2 respectively. The phytoplankton species dominance was significantly higher in older ponds, but phytoplankton diversity and species richness were higher in newer ponds. Zooplankton species richness was higher in newer ponds (Figure 5.4).

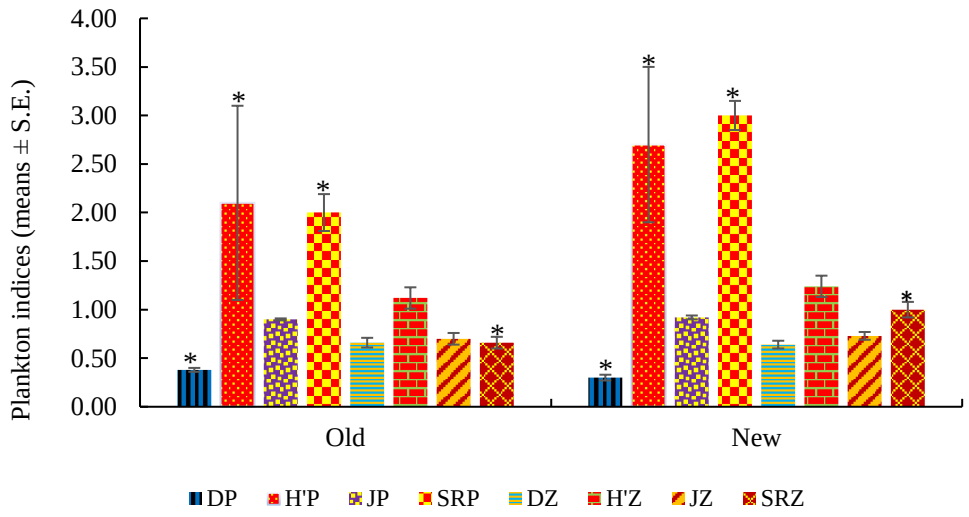

Figure 5.4. Mean ± S. E. of different plankton indices for older and new aged semi-intensive commercial ponds. Where, DP, H'P, JP and SRP represents the phytoplankton indices and DZ, H'Z, JZ and SRZ represents the zooplankton indices. Abbreviations: DP = predominant indices of phytoplankton, H'P = Shannon Wiener diversity index of phytoplankton, JP= phytoplankton evenness, SRP= phytoplankton species richness; DZ= predominant indices of zooplankton, H'Z= Shannon Wiener diversity index of zooplankton, JZ= zooplankton evenness and SRZ= zooplankton species richness. *represents the significant difference of plankton indices between the older and new ponds ($p<0.05$; $n=3$).

5.3.7. Marron weight and health indices

No significant differences ($p<0.05$) were observed in marron growth and health indices between older and new ponds. Weight gain was slightly but not significantly higher in older ponds (Table 5.4).

Table 5.4. Marron growth SGR (g; %/day), biomass gain (%) and health indices hepatopancreas moisture content (HM %), hepatopancreas wet weight indices (Hiw %), hepatopancreas dry weight indices (Hid %), marron tail muscle moisture content (TM; %), tail muscle wet weight indices (TMiw %) and tail muscle dry weight indices (TMid %) grown in a commercial marron ponds for a year (means ± S. E.; n=3).

Pond age	SGR	BG	HM	Hiw	Hid	TM	TMiw	TMid
Older	0.21 ± 0.02	111.6±22.6	66.3 ±2.40	4.00 ±0.34	1.30 ± 0.12	79.2 ± 0.96	29.3 ± 1.18	6.10 ± 0.41
New	0.19 ± 0.04	98.9 ±26.0	62.4 ±2.40	3.50 ±0.27	1.30 ± 0.10	78.3 ± 0.50	29.2 ± 0.97	6.30±0.31

5.4. Discussion

This study was a first attempt to understand the plankton abundance, diversity, evenness and richness in two distinctly aged semi-intensive earthen ponds and their responses to the changes in environmental parameters. Generally in Australian ponds, Rotifera and Copepoda are more abundant than Cladocera (Boon et al., 1994, Casanova et al., 1997) whereas, Cladocerans are more common in northern hemisphere (Boon et al., 1994). Rotifera is the most important zooplankton group in the food web of lentic water bodies, providing a food source to Copepoda (Wallace et al., 2006, Lokko et al., 2017, Wen et al., 2017). The plankton abundance and their community structure is influenced by water quality and various biotic and abiotic environmental factors.

Turbidity, zooplankton and Trebouxiophyceae abundance were lower in newer ponds. Addition of gypsum and fermented barley straw to the new ponds, made them relatively clear (Hargreaves, 1999). Zooplankton abundance was significantly higher in older ponds, as observed by Cole et al. (2019) on the same farm but different ponds and different sampling times. The stabilization of fewer more abundant plankton such as Trebouxiophyceae, rotifers and copepod nauplii adapted in the long term to the stable environmental conditions showed a higher DP in older ponds. An ecosystem with low plankton diversity indicates the presence of dominant species (Yanuhar and Arfiati, 2018, Kulabtong et al., 2019), whereas these conditions may have not yet stabilized in new ponds. Newly established plankton communities showed ephemerous high diversity in newer ponds before stabilizing species numbers and relative abundance. Markwell and Fellows (2008), observed higher macrophyte diversity in newer ponds than older (Australia). Shannon diversity index in marron ponds was moderate ($1<H'<3$), which shows that the pond's ecosystem was in good condition (Yanuhar and Arfiati, 2018).

The total plankton abundance was highest in warmer months as observed by Elakkanai et al. (2017) and Cole et al. (2019). The increased daylight hours and water temperatures can trigger growth which leads to increased phytoplankton abundance (Sommer et al., 2012), in turn providing a source of nutrition for zooplankton (Coman et al., 2003). Plankton abundance was lower during the colder months due to less sunlight, calm weather and low temperatures (Falkowski and Raven, 2013, Li et al., 2019). The highest recorded zooplankton abundance in the marron ponds was similar to the finding of Abdel-Tawwab (2006) in freshwater ponds (Sharqia, Egypt).

The varied environmental conditions also influenced the plankton community structure, as during the colder months Chlorophyceae, Trebouxiophyceae and Zygnematophyceae were more abundant, especially Chlorophyceae. Whereas, during the warmer months (>20°C) Cyanophyceae dominated, indicating that they thrive at higher temperatures than eukaryotic phytoplankton (Kosten et al., 2012), however, Coscinodiscophyceae were observed only in January (Figure 5.2a). Increased water temperature causes a decrease in surface water viscosity, promoting the sinking of non-motile phytoplankton and the capacity of adjusting buoyancy helps Cyanophyceae to dominate plankton communities (O'neil et al., 2012). Decreased NO_3-N and increased phosphorus concentrations during the January and March may have also boosted the Cyanophyceae abundance (Li et al., 2019). Cladocerans were observed only during the colder months. Favourable temperature ranges hastens the abundance of certain Cladocerans, such as Daphnia (Li et al., 2019). Daphnia from a Mediterranean climate (Castelló d'Empúries, Spain) were found to be at optimum filtration capacity when the temperature was 20°C (Müller et al., 2018). Gillooly and Dodson (2000), observed the maximum daphnia abundance when the mean water temperature was 18.5°C in freshwater lakes from Florida to Alaska. Daphnia was recorded only when lakes water temperatures were below 20ºC (Lake Chilwa and Lake Chad, Africa) (Kalk, 1979, Dumont, 1994). These results support our finding of observing Daphnia spp. in colder months. Copepoda abundance was dominated by the copepod nauplii and adults Calanoida around the year. The presence of copepod nauplii can relate to recruitment through reproduction in the ponds over time as the adult copepods were recorded in all months. Phytoplankton abundance can support copepod nauplii growth as they are herbivorous unlike the more carnivorous adults (Razak and Sharip, 2019).

The phytoplankton composition was dominated by Chlorophyceae (48.7%), as observed by Kobayashi et al. (2015) in experimental outdoor freshwater ponds (Kyoto University, Japan). Rotifera dominated the zooplankton composition as observed by Mathias (1991) in a tropical

freshwater lake (India), followed by Copepoda while Cladocerans had the lowest abundance of any zooplankton group. Copepoda abundance was dominated by Calanoida, which have vital implications for the structure and productivity of freshwater bodies in energy transfer from primary producers to consumers in aquatic ecosystems (Kobayashi et al., 2018).

Studies have shown that crayfish growth is a result of consumption of algae and zooplankton (Jones et al., 1995, Kreider and Watts, 1998, Lawrence, 1998, Duffy et al., 2011). Correia et al. (2002), recorded higher prawn (*Macrobrachium rosenbergii*) weight gain and biomass in older ponds than newer, due to the availability and consumption of natural food by prawns. In our study, the pond age did not affect the SGR, weight gain or organosomatic indices of cultured marron, likely due to the marron ponds being managed differently for each pond causing high variations. Our results about HM and TM were similar to the findings of Fotedar (2004). Our study showed similar SGR to the findings of Qin et al. (2001) for adult marron in commercial marron ponds. Feeding contributes to the health of marron and our results of marron HM % were similar to the findings of Foysal et al. (2020a), for adult marron of 63.5 ± 2.24 %.

The variations in environmental parameters affected the plankton abundance and their community structures, however the impacts of the farming practices were not studied in details. Over the time, plankton communities among the aquaculture ponds fluctuates, partly due to the reactive or experience based pond management that results in every pond being fertilized at a different time, with different doses or different feed types with varied nutritional compositions, and stocked animals (Coman et al., 2003, Casé et al., 2008, Boyd, 2009). A detailed study on addition of this locally available and used organic fertilizers such as fermented barley straw and its impact on plankton productivity and marron could provide definitive information about the semi-intensive freshwater culture ponds ecosystems in Australia.

5.5. Conclusion

Temporal variations in environmental factors and pond age influenced the plankton abundance and their species composition in freshwater earthen semi-intensive marron ponds. The temperature had a high influence on the plankton abundance and community structure. Plankton abundance and species diversity were highly variable.

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Conflict of interest-Authors declares there is no conflict of interest.

CHAPTER 6. Time spent in post-feeding activities including feed preference by different weight groups of marron (*Cherax cainii*, Austin 2002) under laboratory conditions.

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Abstract

The current study examined time spent on different post-feeding activities and food preference of five weight groups (<15g, 15-29.9g, 30-44.9g, 45-59.9g and 60-100g) of marron (*Cherax cainii*, Austin 2002) fed two formulated feed pellets and frozen copepods under controlled laboratory conditions. The experimental design consisted of housing an individual marron representing a weight group per 20 L glass aquarium. Each weight group was replicated five times, thus using 25 aquaria. All marron were fed with three different food types; fishmeal (FM) and black soldier fly (BSF) (*Hermetia illucens*) based formulated feed at the rate of 2% of respective body weight and frozen copepods at the rate of approximately 300 individuals per aquarium. Time spent in six selected post-feeding activities was measured in seconds. These activities included resting, walking, searching for food, handling and ingestion of copepods, handling and ingestion of formulated feed, and rejection of food. Results showed that the least amount of time was spent on handling and ingestion of FM by all the weight groups. The handling and ingestion of food in weight group >60 g marron stopped after half an hour post-feeding. Where, <15 g and 15-30 g marron spent significantly longer time consuming frozen copepods. Weight groups >45 g marron spent the longest time resting. FM and frozen copepods were consumed by all weight groups, however, weight groups <15 g and 15-30 g rejected the BSF. The number of frozen copepods consumed by marron was significantly higher for <30 g marron and lowest in 60-100 g marron weight group. In conclusion, the post feeding activities and feed preference were marron weight dependent.

Keywords

Black soldier fly meal, feeding behaviour, food preference, food rejection, frozen copepod, marron.

6.1. Introduction

Freshwater crayfish are widely used for behavioural studies as they offer advantages over many other invertebrates due to their high level of social interactions in both field and laboratory conditions (Gherardi, 2002). Feeding behaviour is an important aspect of animal production as

it provides the link between food being provided, the time required to consume the food and what is consumed. Measuring feeding behaviour can be used to understand how animals perceive the food provided (Nielsen et al., 2016). In crayfish, aesthetascs located on the antennules are used for food detection, food particles are picked up with pereopods and are then transferred to the mouthparts where they are ingested. Mandibles are used for gripping, tearing, crushing and biting food before entry into the oesophagus. While searching for food pereopod one and two are constantly probing the substrate (Holdich, 2002). Food preference is an important parameter for determining the growth and survival of a cultured species and its intake is regulated by the hormones and neurotransmitters that induce the feeding and terminate food ingestion upon satiation (Tierney et al., 2020).

Marron (*Cherax cainii*, Austin 2002) are native to Western Australia (WA) and are a commercially important freshwater crayfish species for aquaculture practices and a recreational fishery, and are farmed in extensive and semi-intensive systems. Marron are known to feed on detritus and zooplankton (Beatty, 2006, Meakin et al., 2009), however the feeding biology of *Cherax* spp. is poorly understood. The studies on zooplankton consumption by *Cherax* spp. under controlled conditions can provide more insight into understanding the trophic role and nutrient partitioning of freshwater crayfish (Meakin et al., 2008).

Although in commercial marron farming in semi-intensive ponds the use of formulated feed is a common practice to increase the marron production, the feed quantity is dependent on environmental factors and marron weight (Fotedar et al., 2015, Tulsankar et al., 2020). Understanding the cultured species weight dependent food preference under culture conditions is of vital importance as the efficient feeding can be crucial for profitable aquaculture (Luna et al., 2019); for example, knowing the feed preference of cultured animals at different weight groups may help to enhance their growth rate, improve the feed conversion ratio and reduce the amount of residual feed pellets which may cause significant water quality issues in aquaculture systems (Glencross et al., 2007).

A study by Meakin et al. (2008) has shown that the juvenile marron weight groups of less than 15 g (1.0-2.9 g, 3.0-7.9 g, and 8.0-15.0 g) are avid feeders of *Daphnia*. However, the feeding behaviour and feed preference of different weight groups of marron is to date unexplored. In this study we used three different foods: two formulated feed pellets with fishmeal and black soldier fly based proteins, and frozen copepods to investigate whether the time spent on post-feeding activities, feeding behaviour and food preference of marron is weight dependent. To

test the hypothesis, an experiment was conducted under controlled indoor laboratory conditions with five different weight groups of marron ranging from <15 to 100 g. The study will provide novel information on the post-feeding activities, feeding behaviour and weight dependent feed preference of marron, allowing the comparison of activities displayed by other species.

6.2. Materials and methods

6.2.1. Experimental design

Five different weight groups of marron ranging from <15 g to 100 g were used to evaluate their post-feeding activities, including feeding behaviour and their food preference between two formulated feed pellets using fishmeal (FM) and black soldier fly (BSF) meal, and frozen copepods. Each weight group had five replicates and marron were stocked individually.

6.2.1.1. Experimental animal collection

Marron were collected from an extensive culture commercial farm in Dwellingup (32.7143°S, 116.0665°S), WA. In the dam these marron were fed with a mixture of commercial formulated feed (Western Premium Marron Pellets™ with 22% crude protein, ingredients being fishmeal, edible oil, salt, cereal grains, vegetable protein meals, vitamins and minerals), crushed lupin and leftovers from the local sardine processing plant (Communication with Mickel Mitchell, Aquanat, Dwellingup). Marron were transported in a thermacol box with a wet hessian bag to the Curtin Aquatic Research Laboratory (CARL) within 90 minutes of sampling. On reaching CARL, marron were divided into five weight groups <15 g (10.1±0.78); 15-29.9 g (19.5±0.83); 30-44.9 g (37.9±0.69), 45-59.9 g (55.0±0.84) and 60-100 g (80.9±1.60) and were stocked individually with one marron per tank. Twenty five glass aquaria (36cm x 22cm x 26 cm) with a 20 L water capacity were filled with 8 L of freshwater to acclimatise the marron to laboratory conditions and feed for three weeks. The treatments were applied in a randomized complete block design. Customised mesh screen was used as aquaria lids to prevent marron from escaping. Continuous aeration was provided to each tank and 25% water exchange was conducted once a week. 12:12 photoperiod was provided to the entire set up and constant water temperature was maintained at 21°C by using automatic submersible glass aquaria heaters (Aqua One, Australia).

6.2.1.2. Copepod collection and culture

Zooplankton were collected from Blue Gum Lake (32.0374° S, 115.8482° E), Booragoon, WA using a 60-µm mesh by dragging on the lakes' surface water. Collected zooplankton were screened and cleaned by using 2-mm mesh to achieve >2 mm size zooplankton mostly

copepods and to remove unwanted smaller zooplankton. Separated zooplankton were stocked in 20 L glass tank and grown to achieve a pure copepod culture which had *Calanoid* copepods under laboratory conditions in CARL. Copepod cultures were started at the density of approximately 289 individuals L^{-1} three weeks prior to the commencement of the experiment to achieve the required abundance, as 7500 individuals per day were needed to feed the marron. A continuous culture system was used to culture copepods. Green algae *Chlorella* spp. was cultured to feed copepods and to maintain their growth and density. *Chlorella* spp. were grown under laboratory conditions with continuous aeration and 24 hours light conditions in CARL.

6.2.1.3. Feed preparation and zooplankton availability

The formulated feed pellets were prepared at CARL. The fishmeal formulated feed pellet had fishmeal as a main animal protein source and BSF formulated feed had BSF meal as a main animal protein source, the dry ingredients were acquired from Speciality Feeds Company, Glen Forrest, WA. To acclimatise marron to the formulated feed pellets, marron were fed with pellets at 2% of their body weight and frozen copepod (thawed) at a density of approximately 300 individuals per tank per day in the evening for three weeks. An hour following the introduction of the food in the tank, the uneaten feed and zooplankton were removed from the tank, using 25 micron mesh.

In order to maintain similar copepod density in each tank, copepods were counted using Sedgwick rafter by following the procedure described by Meakin et al. (2008) with some modifications. Copepods were counted by using Sedgwick rafter and placed into twenty five 500 mL containers filled with 250 ml of distilled water. A sub-sample of 10 mL were taken from 500 mL container, to recount the copepods, and the counting was repeated 10 times to calculate the average numbers of copepod in 500 mL container. After counting, the copepods were screened and frozen until used for feeding.

6.2.2. Water quality

The water parameters including temperature, dissolved oxygen (DO) and pH were checked daily. An Oxyguard® digital DO meter (Handy Polaris 2, Norway) was used for DO and temperature measurements, and an Ecoscan pH 5 meter (Eutech instruments, Singapore) was used to record pH. All water parameters were maintained in an optimum range for the growth of marron (Morrissy, 1990).

6.2.3. Post-feeding activities and food preference observations

Chapter 6: Time spent in post-feeding activities including feed preference by different weight

On the completion of the acclimation period all marron were starved for 48 hours before the post-feeding observations. FM and BSF pellets at the rate of 2 % of respective marron weight and an average of 300.2 ± 0.02 frozen copepods (2-4 mm size) were introduced into the front right corner of the aquarium at the start of post-feeding observations. All feeds were introduced into the tank water at the same time. Feeding observations were conducted for one hour immediate post-feeding. The observations were made visually over three days by two people and were categorised as: resting- staying still at same place for more than a 30 seconds; walking- including on the tank surface, climbing on tank walls and backward walking; searching for food- continuously moving antenna, first pereopod and maxillipeds; handling and ingestion of copepods- picking up copepods with the 2nd pair of pereopods and pushing in mouth through maxillipeds; handling and ingestion of formulated feed pellets- picking up the feed pellets and pushing in mouth through maxillipeds; Rejection of feed- picking up the feed, pushing it towards the maxillipeds and dropping down without ingestion. The time invested on each activity was recorded in seconds. At the end of the feeding observations, the leftover feed and zooplankton were filtered with fine mesh and stocked in separate bottles to count the numbers of leftover copepods.

6.2.4. Post-feeding activities including food handling and their characteristics

The description of post-feeding activities and their significant characteristics observed during the post-feeding observations are explained in Table 6.1.

Table 6.1. The activities conducted by different weight groups of marron during one hour of post-feeding observations.

Resting	Marron staying still at same place for more than a 30 seconds.
Walking	Moving across the substrate on walking legs including on tank surface, climbing on tank walls and backward walking.
Searching for food	Continuously moving antenna, first pereopods and maxillipeds.
Handling and ingestion of copepods	Probing the ground with walking leg, picking up and conveying the frozen copepods with the 2nd pair of pereopods in mouth through maxillipeds.
Handling and consumption of pellet food	Probing the ground with walking leg, picking up and conveying the food particles with the 2nd pair of pereopods in mouth through maxillipeds.
Rejection of food	Attracted and picking up feed pellet, pushed towards maxillipeds and dropped down multiple times without ingestion.

6.2.5. Copepod consumption

Copepod consumption (CC) was analysed by using the following formula;

$$CC = Number\ of\ copepods\ added\ to\ the\ tank - Number\ of\ copepods\ collected\ from\ the\ tank\ after\ feeding$$

6.2.6. Statistical analyses

All the numerical data were analysed using SPSS version 26 (IBM®) and the results are presented as mean ± S. E. A one way ANOVA with Duncan's multiple-range test was used to find the significance between the treatments for water quality parameters, time spent on different activities, copepod consumption by marron. A Kruskal-Wallis test was used when data lacked homogeneity. All tests were considered statistically significant at $p<0.05$.

6.2.7. Animal ethics

The animal ethics approval was not required as the marron are invertebrate aquatic crustaceans.

6.3. Results

6.3.1. Water quality

Temperature, dissolved oxygen and pH were maintained at an optimum level for marron growth throughout the experimental time as shown in Table 6.2.

Table 6.2. Water quality parameters dissolved oxygen (D.O.; mg L^{-1}), temperature (°C) and pH ranges maintained in marron tanks during the experimental days (mean ± S. E.; n=5).

Water parameters/weight groups	<15 g	15-29.9 g	30-44.9 g	45-59.9 g	60-100 g	Min./Max
D. O.	7.23 ± 0.01	7.25 ± 0.02	7.29 ± 0.02	7.27 ± 0.01	7.26 ± 0.02	7.21-7.32
Temperature	21.5 ± 0.06	21.3 ± 0.15	21.4 ± 0.12	21.2 ± 0.10	21.5 ± 0.09	20.9-21.7
pH	7.95 ± 0.06	7.78 ± 0.10	7.85 ± 0.14	7.72 ± 0.11	7.76 ± 0.10	7.60-7.79

6.3.2. Post-feeding time spent on different activities by marron

Out of the total time spent on different activities, consumption of fishmeal formulated feed pellet was conducted at the least amount of time by all weight groups of marron. The longest time was spent on eating frozen copepods by weight group <15 g ($p<0.05$). Resting activity time (%) were significantly longest in >45 g weight groups than other weight groups (Figure

6.1A, B, C, D, E, F.). Weight groups <15 g and 15 - 30 g spent a significantly longer amount of time on investigating BSF pellets. The feeding activity in weight group 60-100 g stopped after 30 minutes.

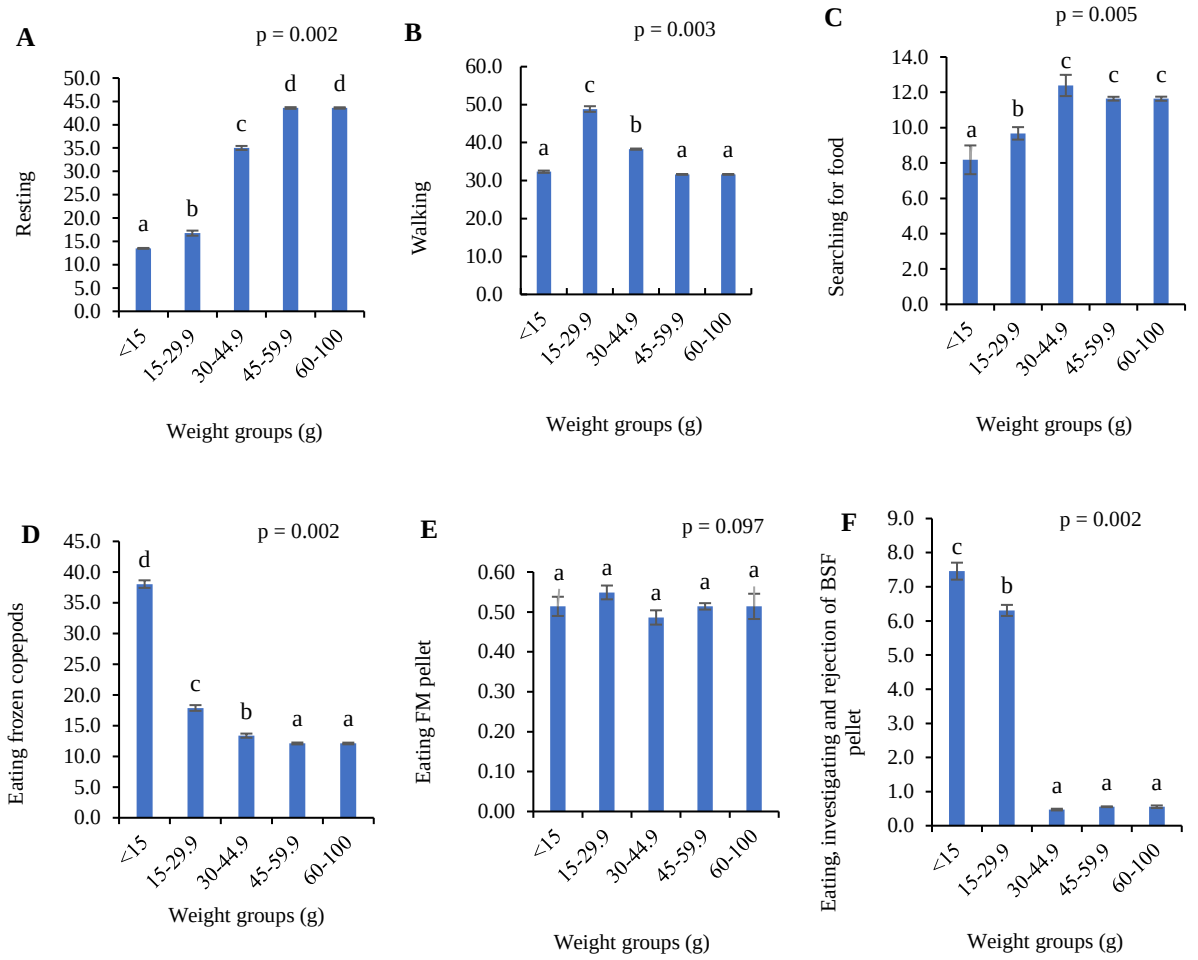

Figure 6.1. A-F. The total time spent (%; mean ± S. E.) on post-feeding activities by five different weight groups (n=5 per group) of marron ranging from <15 g to 100g: A) time spent on resting, B) the time spent on walking, C) time spent on searching for food, D) the time spent on eating frozen copepods, E) time spent eating FM pellets and F) time spent eating, investigation and rejection of BSF pellets. One way ANOVA (Duncan's test) was used for the Figure 6.1A, C, D and E and Kruskal-Wallis test was used for Figure 1B and F. No significant differences ($p<0.05$) were observed between the weight groups for time spent on feeding on the FM pellet. Letters a, b, c and d represents the significant differences between the weight groups. **Abbreviations:** FM: fishmeal formulated feed pellet, BSF: black soldier fly formulated feed pellet.

6.3.3. Marron preference for the provided feeds

Chapter 6: Time spent in post-feeding activities including feed preference by different weight

Feed preference of different weight groups of marron were recorded and were chronologically ranked based on the marron response towards the provided feed and frozen copepods (Table 6.3). FM was the first preference for the <45 g marron and second for the >45 g marron. BSF was the first preference for the >45 g marron and second for 30-45 g marron. Frozen copepod was the second preference for <45 g marron and third for >45 g marron.

Table 6.3. Marron feed preference based on the feeding behaviour observations (n=5 per group).

Marron weight groups	**FC**	**FM**	**BSF**
<15 g	2nd preference (5/5 R)	1st preference (5/5 R)	Rejected (5/5 R)
Qualification	Searching and feeding on copepods after consuming FM pellet.	Picking up and consuming FM pellet immediate post-feeding within least amount of time of feed addition.	Attracted and picking up BSF feed pellet, pushed towards maxilliped and dropped down multiple times without ingestion.
15-29.9 g	2nd preference (5/5 R)	1st preference (4/5 R)	Rejected (5/5 R)
Qualification	Searching and feeding on copepod after consuming FM pellet.	Picking up and consuming FM pellet within least amount of time of feed addition.	Attracted and picking up BSF feed pellet, pushed towards maxilliped and dropped down multiple times without ingestion.
30-44.9 g	3rd preference (4/5 R)	1st preference (5/5 R)	2nd preference (4/5 R)
Qualification	Feeding on frozen copepods after consuming FM and BSF pellets.	Picking up and consuming FM pellet within least amount of time of feed addition.	Feeding on BSF pellet after consuming FM pellet.
45-59.9 g	3rd preference (5/5 R)	2nd preference (5/5 R)	1st preference (5/5 R)
Qualification	Feeding on frozen copepods after consuming FM and BSF pellets.	Picking up and consuming FM pellet after consuming BSF pellet.	Attracted and picked up BSF pellet first within least amount of time of feed addition.
60-100 g	3rd preference (5/5 R)	2nd preference (5/5 R)	1st preference (5/5 R)
Qualification	Feeding on frozen copepods after consuming FM and BSF pellets.	Picking up and feeding on FM pellet after consuming BSF pellet.	Attracted and picked up BSF pellet first within least amount of time of feed addition.

Abbreviations: FC- frozen copepods, FM-fishmeal formulated feed pellet, BSF- black soldier fly feed pellet; R- replicates.

6.3.4. Food consumption in different weight groups of marron

Venn diagram describes the food consumption by different weight groups of marron observed during the one hour of post-feeding observations. Fishmeal formulated feed (FM) pellets and frozen copepod were consumed by all weight groups of marron (Figure 6.2). However, BSF pellet was consumed only by >30 g marron weight groups.

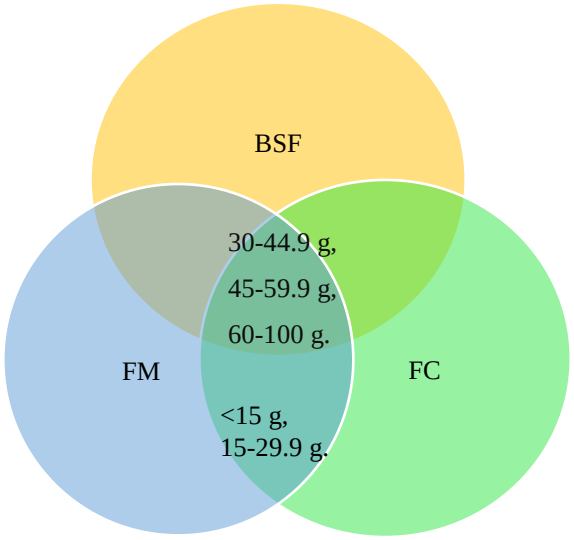

Figure 6.2. The FM, BSF and frozen copepods consumption by different weight groups of marron (ranging from <15 g to 100 g) over the one hour of post-feeding observations (n=5). **Abbreviations:** FC- frozen copepods, FM-fishmeal formulated feed pellet, BSF- black soldier fly feed pellet.

6.3.5. Frozen copepod consumption

The number of frozen copepods eaten by different weight groups of marron during the one hour of post-feeding observations was significantly different among the weight groups (Figure 6.3). The copepod consumption decreased significantly as the marron size increased between < 15 g and 100 g. The numbers of copepods consumed by <15 g marron were significantly highest than the other weight groups.

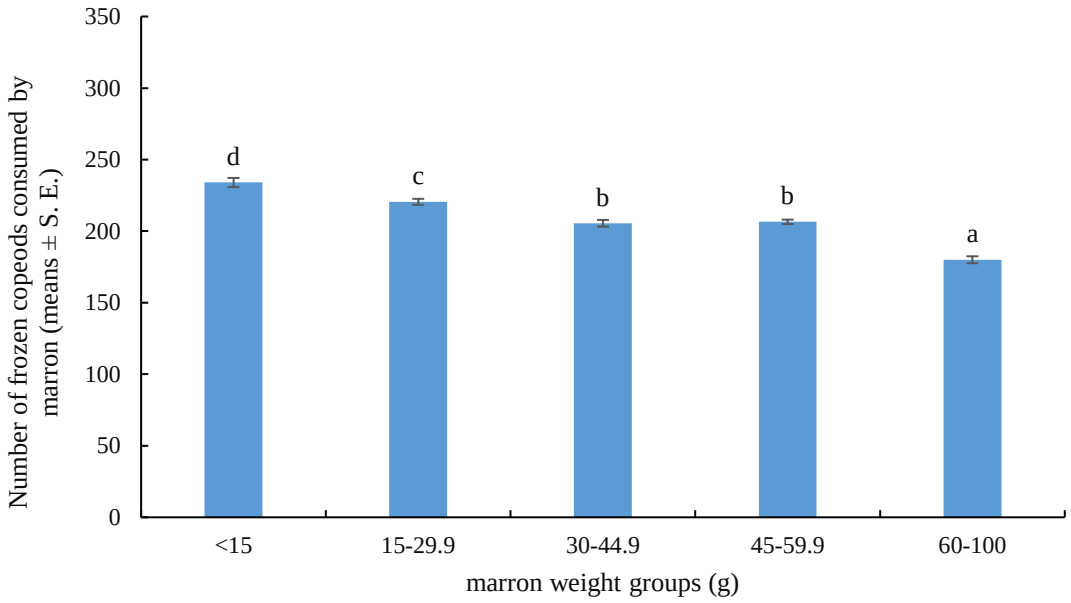

Figure 6.3. The number of frozen copepods consumed by different weight groups of marron during the one hour of feeding observation (mean ± S. E.; n=5). The letters a, b, c and d indicate significant difference ($p<0.05$) in the number of frozen copepod consumption between the different weight groups of marron.

6.4. Discussion

The current study describes the post-feeding activities, feeding behaviour and weight dependent feed preference of marron and allows the comparison of activities displayed by other species. In marron ponds the abundance of copepod adults and copepod nauplii was recorded through-out the year (Tulsankar et al., 2021b). Based on those findings, the current experiment was conducted to better understand the feeding preference of different weight groups of marron ranging from <15 g - 100 g, under controlled laboratory conditions when they are provided with frozen copepods and two different formulated feeds.

This is the first attempt to analyse the feed preference and quantifying copepod consumption by the different weight groups of marron in controlled conditions, and it could provide new insights into the role of formulated feed and zooplankton in marron diets. The data showed that the juvenile marron (<15 g) spent more time feeding on copepods, similarly to a study by Alcorlo et al. (2004) where copepods were observed in the stomach contents of red swamp crayfish (*Procambarus clarkii*) with zooplankton consumption being higher in juveniles than in adults.

Marron's ability to consume copepods indicates that copepods may play an important role in the marron diet in pond systems and may provide a source of nutrition especially during the early life stages. Similar observations were made by Meakin et al. (2009) where juvenile marron were able to capture and consume a large quantity of *Daphnia* in a short period of time i.e. <1 hour. In the case of yabbies, animals up to 45 g were efficient in capturing 400-500 individuals of live *Daphnia* (Meakin et al., 2008). The present study does not suggest any "suppression" of copepod densities, but simply that all weight groups ate frozen copepods. In a study by Sierp and Qin (2001) in a field experiment, the adult marron were unable to supress the zooplankton. Another reason for a lower number of copepods eaten by adult marron may indicate the feeding until satiation (Momot, 1995), as marron groups >30 g preferred to feed on formulated feed pellets first, and copepods were their last preference. Feeding until satiation on formulated feed pellets in a short time may have resulted in the significantly longer time on resting by the weight groups >30 g marron, and also caused suspension of feeding in weight group >60 g after 30 minutes of post-feeding as they appeared to not indulge in consuming copepods for a longer time.

As crayfish grow, the precise moment required to capture the zooplankton may decline; they may lose the dexterity (Abrahamsson, 1966), however, we did not find any impaired ability of adult marron movement to feed on frozen copepods. The size of copepod (2-4 mm) may have been easy to sight and to capture. All weight groups were equally capable of collecting and feeding on copepods. The observations made on the feeding behaviour indicated that marron feeding on copepods may not be depend on the size, as zooplankton capture and consumption relies on the mouth parts (Meakin et al., 2008). We observed that large marron could effectively use their mouth parts to feed on copepods, despite the overall increase in size, though juvenile marron (<30g) showed greater preference for frozen copepods over formulated feed pellets by spending more time feeding on zooplankton. Our results showed that the marron weight groups ranging from <15 - 100 g can effectively consume frozen copepods at an initial prey density of 170- 243 individuals per aquarium.

Frozen zooplankton feed improved the growth performance of red swamp crayfish (Sonsupharp and Dahms, 2017) and feeding on fresh or frozen copepod resulted in similar growth and survival in juvenile yabbies when compared with the formulated diet (Jones et al., 1995, Verhoef et al., 1998). Marron can consume any food available within a pond ecosystem, including plant and animal material (Alonso, 2009a). The direct impact of crayfish on pelagic plankton populations is considered to be relatively weak due to their benthic nature (Sierp and

Qin, 2001). In the case of copepods, they migrate vertically to avoid predation and to find shade during the sunny hours. This vertical migration towards the pond bottom would allow marron to feed on copepods as marron are benthic animals. During the juvenile stage copepods can be a source of nutrition for marron, even after death as a part of zooplankton rain in the detritus.

A typical trend of choosing fishmeal formulated feed by all weight groups was observed and BSF formulated feed was not eaten by <30 g marron. Juvenile marron were attracted towards the BSF, but did not consume it, despite spending significant time on exploring the food. BSF has a strong odour which may have caused a negative chemosensory response in <30 g marron (Corotto and O'Brien, 2002). Though BSF was the first preference for >45 g weight groups; the taste of BSF was not attractive to <30 g marron. In aquaculture, the use of formulated feed is also more important to achieve high growth rates in larger sized animals, whereas during their early life stages marron may rely largely on naturally occurring food items. Juvenile marron are generally more active and planktivorous than adults (Sierp and Qin, 2001). Marron in weight group 15-30 g spent more time on walking where the <15 g spent more time on eating copepods and exploring BSF diet. On the other hand, weight groups >30 g consumed the formulated feed within least amount of time and spent more time searching for it.

Our results showed that supplemented formulated feed is utilized by year one (>30 g) marron though their preference was weight dependent. BSF can be an alternative animal protein source to fishmeal based feeds for grow-out marron. In this study we did not investigate the capture efficiency of marron due to the use of frozen copepods. However, using live copepods to examine the capture efficiency of marron may provide more information on marron feeding biology including capture efficiency.

6.5. Conclusion

The post-feeding activities and feed preference are weight dependent in marron. Copepod consumption was highest in juvenile marron and the consumption decreased as weight of marron increased. Larger marron preferred to feed on formulated feed including insect based feed. Further studies investigating feed preference and impact of feed on water quality in outdoor culture systems will provide beneficial information on husbandry management of aquaculture species. Understanding species specific feeding behaviour and nutritional requirements is important for the assessment of animal welfare and ecology. It may also help to solve many feeding related problems and allow animals to maximize the potential of the nutritional value of the feed provided.

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CRediT Author Contributions

Smita Sadanand Tulsankar: Conceptualization, designing and set up of experiment, feeding observations, data collection, data analysis and writing of manuscript. **Anthony J. Cole**: Copepod counting, feeding observation, reviewing and editing manuscript. **Marthe Monique Gagnon:** Supervision, reviewing and editing manuscript. **Ravi Fotedar:** Methodology validation, supervision, review and editing manuscript. The article submission has been approved by co-author.

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CHAPTER 7. A mixture of manganese, silica and phosphorus supplementation alters the plankton density, species diversity, gut microbiota diversity and improved the health status of cultured marron (*Cherax cainii,* Austin and Ryan, 2002)

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Abstract

Trace element supplementation to the freshwater environment can influence the plankton density and species diversity, contributing to the nutrition of aquaculture species, especially during the juvenile stage. An experiment was conducted under laboratory conditions to evaluate the effects of supplementing different mixtures of manganese, silica and phosphorus on the plankton density and species diversity and their impact on cultured juvenile marron (*Cherax cainii*, Austin and Ryan, 2002). Manganese, silica and phosphorus in concentrations of 0.0024, 0.41, 0.05 mg L^{-1}; 0.0041, 0.82, 0.12 mg L^{-1}; and 0.0058, 1.26, 0.25 mg L^{-1} respectively termed as low, medium and high were supplemented to tank water containing a phytoplankton density of 3.77 ± 0.16 x 10^6 cells L^{-1} and 292.9 ± 17.6 individuals L^{-1} of zooplankton, and plankton growth was observed every 24 hrs for six days. Afterwards a three month trial was conducted studying the effects of these trace element concentrations and resulting plankton densities on marron growth, survival, haemolymph, moulting, gut microbiota and health indices. Higher concentration supplementation of silica and phosphorus resulted in a significant increase in plankton density and species diversity, leading to improved marron health indices than the control and the tanks receiving a low concentration. Silica supplementation at higher concentration increased diatom abundance. Marron specific growth rate, weight gain and dissolved copper concentration in haemolymph were significantly higher in tanks with higher supplementation and higher plankton density. Marron survival, moult interval and total haemocyte count were not affected by the supplementation. Marron gut microbiota at higher trace element concentration supplementation showed a significant increase in abundance of phosphate solubilizing bacteria.

Keywords: Aquaculture; trace elements; phytoplankton; zooplankton; freshwater crayfish; gut microbiota.

7.1. Introduction

Among the various aquaculture species farmed in Western Australia (WA), marron (*Cherax cainii*, Austin and Ryan, 2002) is a commercially important freshwater crayfish due to its high

value, distinct taste and disease-free status (Machin et al., 2008). Marron can consume any available food including plant and animal matter within a pond system (Alonso, 2009a). The effects of trace elements supplementation on fish production has been studied previously (Abdel-Tawwab et al., 2002, Adhikari, 2003, Drenner et al., 1989, Tew et al., 2006). To increase the marron production through plankton productivity, marron farmers use different organic including fermented barley straw and inorganic fertilizers, a common practice in aquaculture (Azim and Little, 2006, Boyd, 2018). Several studies have shown that, manganese (Mn), silica (Si) and phosphorus (P) are vital for plankton productivity in freshwater ecosystems (Boyd, 2014, Goldman, 2010, Li et al., 2017c, Nwankwegu et al., 2020, Pace and Lovett, 2013, Tulsankar et al., 2020, Wetzel, 2001). Their deficiency can diminish the phytoplankton growth (Goldman, 2010), and their supplementation can increase plankton biomass initiating a modulation of community structure (Goldman, 2010, Nwankwegu et al., 2020).

Phosphate fertilizers are widely used (Boyd, 2018) to increase the plankton productivity and in turn to improve the growth performance of cultured aquatic animals (Duffy et al., 2011, Jones et al., 1995, Tew et al., 2006). Under controlled laboratory conditions and in the absence of plankton, juvenile signal crayfish (*Pacifastacus leniusculus*) culture remains largely unsuccessful, mainly due to the low survival and growth rates during the early life stages (Gonzalez et al., 2012, Sáez-Royuela et al., 2007). Whereas, higher survival and growth rate of juvenile signal crayfish was obtained with a supply of live zooplankton (Sáez-Royuela et al., 2007).

Although Mn, Si and P are an essential trace elements for the growth of plankton and crustaceans, limited research has investigated their effect on the plankton density, species diversity as well as on the growth, survival, health and gut microbiota of freshwater crayfish (Sierp and Qin, 2001). Sierp and Qin (2001), where authors observed the effect of adult marron (*C. tenuimanus*) on plankton and nutrient dynamics in ponds having high water hardness. The main focus of related studies has been to analyse different concentrations of Mn, Si and their toxic effects on decapods (Lambert, 2019, Jussila et al., 1995, Hossain et al., 2021, Ackefors, 1996, Oweson et al., 2006). Trace elements have the ability to reshape the gut microbiota of guppy fish (*Poecilia reticulata*) for improved digestion, immunity and adaptation (Kayath et al., 2019). No research has been reported investigating the effects of different concentrations of Mn, Si and P supplementation on water quality, crayfish growth, survival, health indices and gut microbiota mediated through plankton density or species diversity. We hypothesized that

trace element supplementation will positively influence the plankton density and community structure and the plankton densities will improve marron growth, survival, health indices and gut microbiota. To test this hypothesis we conducted an indoor laboratory experiment for 96 days under controlled conditions.

7.2. Materials and Methods

7.2.1. Experimental design

The experiment was designed based on the outcomes of our previous field trial in commercial earthen marron ponds (Tulsankar et al., 2020), wherein, 12-pre-selected trace elements were measured, of which manganese (Mn), silica (Si) and phosphorus (P) were found to be strongly correlated with plankton abundance and species diversity over the seasons. The mean dissolved concentrations of Mn, Si and P of 28 ponds over four seasons were used to determine low 0.0024, 0.41 and 0.05 mg L^{-1}, medium 0.0041, 0.82 and 0.12 mg L^{-1} and high 0.0058, 1.26 and 0.25 mg L^{-1} concentrations respectively. These concentrations were used to evaluate the influence of the trace elements on plankton density and diversity.

7.2.1.1. Plankton collection and preparation for stock culture

Plankton were collected from the commercial marron farm in Manjimup (34°18'75" S, 116°06'61" E) WA. Pond water was filtered through a phytoplankton net to obtain 20 L of phytoplankton sample, and zooplankton net was used to obtain 20 L of zooplankton sample separately. Collected plankton were cultured as a stock culture for the experiment in outdoor conditions at the Curtin Aquatic Research Laboratory (CARL), using 300 L water capacity plastic tanks, filled with 200 L of freshwater, with a supply of vigorous aeration and direct sunlight. A continuous culture system was used to culture the plankton and Aquasol® by Yates Pty Ltd was added to the tanks to boost and maintain the phytoplankton density at 11.45 x 10^6 cells L^{-1}. Daily observations of tank conditions and plankton were made and the planktons were counted three times a week. The phytoplankton were fed to zooplankton to maintain the density.

7.2.1.2. Trace element supplementation and plankton culture

Under controlled laboratory conditions, sixteen 300 L water capacity tanks were filled with 150 L of water and were stocked with phytoplankton and zooplankton at a rate of 3.77 ± 0.16 x 10^6 cells L^{-1} and 292.9 ± 17.6 individuals L^{-1} respectively. On the same day Mn in the form of manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$), silica- sodium metasilicate nonahydrate ($Na_2SiO_3.9H_2O$) and phosphorus in the form of potassium hydrogen phosphate (K_2HPO_4) in

three different concentrations as described in Table 7.1, were prepared by dissolving in distilled water in 100 mL beakers and were supplemented to the twelve treatment tanks. Mn, Si and P were supplemented only once at the start of the experiment. The experimental design included four treatments with four replicates 1. Only plankton (Control; CTL); 2. Plankton + low trace element supplementation (LTS) 3. Plankton + medium trace element supplementation **(**MTS) and 4. Plankton + high trace element supplementation (HTS). Continuous aeration and light, diffused through air stones and Osram Lumilux 36W 4000K (one tube light per tank) respectively were used to grow the plankton. After trace element supplementation, the plankton were counted at 24, 48, 72, 96, 120 and 144 hrs to evaluate the plankton density and species diversity (Supplementary data #1). Trace elements supplementation was conducted in the absence of marron in order to avoid any inadvertent phosphorous addition through feed.

Table 7.1. Three different mix concentrations of Mn, Si and P were prepared using manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$), sodium metasilicate nonahydrate ($Na_2SiO_3.9H_2O$) and potassium hydrogen phosphate (K_2HPO_4) respectively to achieve the required three different concentrations (mg L^{-1}) of Mn, Si and P.

Chemicals/ Trace elements (mg L^{-1})	**Low supplementation (LTS)**	**Medium supplementation (MTS)**	**High supplementation (HTS)**
$MnCl_2.4H_2O$	0.0087	0.0148	0.0209
$Na_2SiO_3.9H_2O$	0.8771	1.7541	2.6954
K_2HPO_4	0.2812	0.6748	1.4058
Mn	0.0024	0.0041	0.0058
Si	0.4100	0.8200	1.2600
P	0.0500	0.1200	0.2500

Table shows the total weight of $MnCl_2.4H_2O$, $Na_2SiO_3.9H_2O$ and K_2HPO_4 used to achieve the required concentration of Mn, Si and P (mg L^{-1}).

7.2.1.3. Introducing marron to tanks under laboratory conditions

Marron were collected from the commercial marron farm in Manjimup. A total of 190 juvenile marron (weighing average initial weight 6.13 ± 0.23 g, average orbital-carapace length (OCL) 2.95 ± 0.07 cm and total length of 6.26 ± 0.11 cm) were collected and transported to the CARL. Marron were stocked in 300 L water capacity tanks to acclimate to the laboratory conditions at the CARL for 15 days. After six days (144 hrs) of trace elements supplementation and on plankton counting, all tanks were stocked with nine marron per tank in individual holding cages made up of plastic containers and top covered with mesh, to avoid cannibalism and escape of

marron. The cages had a volume of 2000cm^3 (170 mm x 115 mm x 135 mm) with four gaps of 4-5 mm on each sides to allow the water exchange directly from the tank water into the cage. Marron were fed at 2% of their body weight with fish meal based formulated feed pellet (Tulsankar et al., 2021c), once a day in the evening. The uneaten feed and faeces were removed, one hour after the feeding.

7.2.2. Water quality analysis

All water parameters were kept in an optimum range for the growth of marron (Morrissy, 1990). The water parameters including temperature, dissolved oxygen (DO) and pH were checked daily. An Oxyguard® digital DO meter (Handy Polaris 2, Norway) was used for DO and temperature measurements, and an Ecoscan pH 5 meter (Eutech instruments, Singapore) was used to record pH. A DR/890 portable colorimeter with Permachem reagents (Hach, USA) were used to analyse the total ammonia nitrogen (TAN), nitrite (NO_2-N), nitrate (NO_3-N) and reactive phosphate (PO_4) once a week. The experiment was static i.e. no water exchange was made. Tank water level was maintained at 150 L throughout the experiment by adding water to compensate for losses due to the evaporation.

7.2.3. Plankton and trace elements analysis

Throughout the experiment, plankton density was analysed three times a week and was maintained at the same density as recorded after 144 hours by either addition of plankton or removing by filtering plankton out, using respective plankton nets. For phytoplankton analysis, 2 L of tank water was filtered to obtain 100 mL of sample. For zooplankton analysis, 5 L of tank water was filtered to obtain 100 mL of sample. The filtered water was re-stocked into the same tank. The plankton species were identified to the lowest possible taxonomic level using keys from a manual by Ingram et al. (1997) and a book by Canter-Lund and Lund (1995). The plankton density (cells L^{-1}) was calculated by using the equations from Ingram et al. (1997) and Tulsankar et al. (2021b). The dissolved trace element concentration in tank water at initial, after supplementation, at the end of the experiment and in marron haemolymph were analysed at Murdoch University, Perth, WA. The water samples were collected in 100 mL plastic containers directly from the tanks and were filtered through 0.45 µm Millipore filters to eliminate suspended particles. The haemolymph samples were collected using 1 mL syringe containing 0.2 mL of sodium citrate anticoagulant (100mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 15.5 mM NaCl and 10 mM EDTA) inserted in between the third and fourth pair of pereopod and were kept on ice during the sampling and transportation. Inductively

Coupled Plasma Optical Emission Spectrometry (Agilent, ICP-OES, spike recovery limit 80-120%) with the standard methods described in APHA (2012) was used to analyse the dissolved concentrations with the detection limits of Mn (<0.0002mg L^{-1}); P (<0.02mg L^{-1}); Si (<0.02mg L^{-1}) in water and Ca (<5mg L^{-1}); Cu (<0.1mg L^{-1}); Fe (<0.5mg L^{-1}); Mn (<0.05mg L^{-1}) and P (<5mg L^{-1}) in haemolymph. The results of dissolved trace elements in haemolymph was calculated based on the dilution factor (Baden and Neil, 1998).

7.2.4. Marron growth analysis

Marron growth data were recorded fortnightly, and mortality was recorded daily. Marron specific growth rate (SGR; g % /day), weight gain percentage (WG; g %) and survival rate (SR; %) was calculated by using the following equations

Specific growth rate (SGR, g % /day) = 100 X (Ln (W_t) -Ln (W_i))/T,

Weight gain (WG, g %) = 100 X ($W_t - W_i$)/W_i.

Survival rate (SR, %) = 100 X (n_t/ n_0)

Where, W_t is final weight (g), W_i is initial weight (g), n_t is the number of marron alive at (T) days and n_0 is the number of marron stocked initially.

Marron moulting data such as dates and times were recorded daily. Moult interval (Tm; days) were measured on the basis of days required to moult, between two successive moults using the following equation;

Moult interval (Tm; days)

$T_m = T_{n+1} - T_n$

Where, T_n= date of n moult, T_{n+1}= date of n+1 moult.

7.2.5. Marron health indices

Marron health indices were analysed at the end of the experiment, by testing haemolymph for total haemocyte count (THC) and differential haemocyte count (DHC). Hepatopancreas wet and dry weight indices moisture content (HM %), wet weight (Hiw), dry weight (Hid), tail muscle moisture content (TM %), wet weight (TMiw), and dry weight (TMid) indices. Haemolymph samples were collected from one randomly selected marron per tank, the haemolymph was drawn by using a 1 mL syringe inserted in between the third and fourth pair of pereopods. THC and DHC were analysed according to Nugroho and Fotedar (2013). One

marron per tank was collected randomly, and hepatopancreas and tail muscle from each individual was collected and weighed. To obtain the dry weight, the samples were dried in crucibles at 105ºC in the oven for 24 hrs. The health indices were calculated as described by Fotedar (1998).

7.2.6. Marron hindgut microbiota analysis

At the end of the experiment, a total of 24 marron, six per treatment were randomly selected. Gut content collection and separation of hindgut was performed inside a biosafety cabinet and the gut contents with mucosa were immediately lysed using Tissue Lyser II (Qiagen, Hilden, Germany). A subsequent pool of two marron gut contents from each respective tank was created by homogenization and transferred into 1.5 mL Eppendorf tube. Bacterial DNA was extracted using Blood and Tissue Kit (Qiagen, Hilden, Germany). DNA concentration was measured in Nanodrop Spectrophotometer (Thermo Fisher Scientific, USA) and diluted into 50 ng/µL for PCR. Fifty microliters of PCR master mix was prepared for each sample contained 25 µL Hot Start 2X Master Mix (New England BioLabs Inc., Ipswich, MA, USA), 2 µL of bacterial DNA, 1 µL of each forward and reverse primers (V3-V4) and 21 µL of DEPC treated water. Forty cycles of PCR amplification was completed in a thermal cycler (BioRad S100, Bio-Rad Laboratories, Inc., Foster City, California, USA). PCR products clean-up and amplicon barcoding was performed with a secondary PCR according to the Illumina standard protocol (Part # 15044223 Rev. B). Samples were then sequenced on an Illumina MiSeq platforms (Illumina Inc., San Diego, California, USA) using a v3 kit (600 cycles).

7.2.7. Bioinformatics and statistical analysis

Raw sequences were checked for initial quality in FastQC pipeline (Andrews, 2010), trimmed for quality reads (parameters: -q 20 –l 200) in Sickle (Joshi and Fass, 2011) and merged in MeFiT program (Parikh et al., 2016). MICCA pipelines used for filtering, open reference clustering and picking of OTUs at 97% similarity threshold (Albanese et al., 2015). SILVA 1.32 release used for phylogenetic assignment of operational taxonomic units (OTUs) at different taxa level (Quast et al., 2012). Multiple sequence alignment, FastTree (version 2.1.8) GTR+CAT phylogenetic tree were performed and constructed in PASTA algorithms (Mirarab et al., 2015, Price et al., 2010). Rarefaction depth value was set to 32,996 bp and alpha-beta diversity was calculated using QIIME (v1.9.1) and R packages. Alpha diversity was calculated in terms of observed species, Shannon and Chao1 measurements. Beta ordination was calculated as Bray-Curtis dissimilarity of weighted UniFrac while permutational multivariate

ANOVA (PERMNOVA) and non-metric multidimensional scaling (NMDS) analysis were performed to calculate and visualise the clustering of samples. Non-parametric statistical distance metric was calculated using ANOSIM with 1000 permutations. Relative and differential abundance of bacterial communities were calculated using phyloseq (McMurdie and Holmes, 2013) and LEfSe (Segata et al., 2011) respectively.

7.2.8. Data analysis

All the numerical data were analysed using R software (v3.5.1) and are presented as mean ± standard error (SE). One-way analysis of variance (ANOVA) with Turkey's HSD post hoc tests were used to determine the significant differences between treatments. Paired t-test was used to determine the dissolved trace elements concentrations in water before and after supplementation. All tests were considered statistically significant at $p<0.05$.

7.3. Results

7.3.1. Water quality and plankton abundance

Temperature, DO and pH were observed at the constant level throughout the experiment (Table 7.2). On trace element supplementation, plankton density was significantly ($p<0.005$) highest in HTS tanks.

7.3.2. Plankton community

The plankton stock culture included Chlorophyceae consisting *Cladymonas* spp., *Scenedesmus* spp., *Haematococcus* spp., *Eudorina* spp., *Selenastrum* spp., *Scenedesmus* spp., and *Volvox* spp.; Trebouxiophyceae: *Chlorella* spp.; Zygnematophyceae consisting *Closterium* spp. and Bacillariophyceae consisted of *Navicula* spp., *Fragilaria* spp., *Pinnularia* spp., *Nitzschia* spp., *Gyrosigma* spp., *Cymbella* spp. and *Gomphonema* spp.. Copepoda adults and nauplii, *Keratella quadrata, Keratella cochlearis* and *Daphnia* spp. and were also observed in juvenile marron tanks.

Table 7.2. Water parameters and plankton abundance in four treatment tanks throughout the experiment (n=4).

The temperature and pH in tank water were similar to that found in marron ponds in autumn which was the season with the highest plankton abundance (Tulsankar et al., 2020, Cole et al., 2019).

Parameters	**CTL**	**LTS**	**MTS**	**HTS**
Temperature (°C)	21.5 ± 0.10	21.5 ± 0.10	21.5 ± 0.09	21.5 ± 0.11
DO (mg L^{-1})	8.34 ± 0.04	8.42 ± 0.05	8.36 ± 0.05	8.37 ± 0.06
pH	7.59 ± 0.04	7.62 ± 0.04	7.60 ± 0.04	7.60 ± 0.04
Phytoplankton abundance (x 10^6 cells*L^{-1})	3.77 ± 0.16^a	4.05 ± 0.22^a	6.05 ± 0.42^b	7.38 ± 0.64^c
Zooplankton abundance (ind. L^{-1})	293 ± 17.6^a	357 ± 16.2^b	413 ± 18.7^b	493 ± 25.7^c

^{a,b,c} indicates the significant differences among all treatments $p<0.05$; ind. is individual. Abbreviations: CTL- control tanks; LTS- low trace elements supplementation tanks; MTS- medium trace elements supplementation tanks; HTS- high trace elements supplementation tanks.

The ammonia, nitrite and nitrate fluctuated within acceptable range for marron (mean ± S. E.) as shown in Figure 7.1A, B, C and D. Nitrate-nitrogen (NO_3-N) concentration exponentially increased over the study time, whereas reactive phosphate (PO_4) concentration was highest in HTS tanks (n=4).

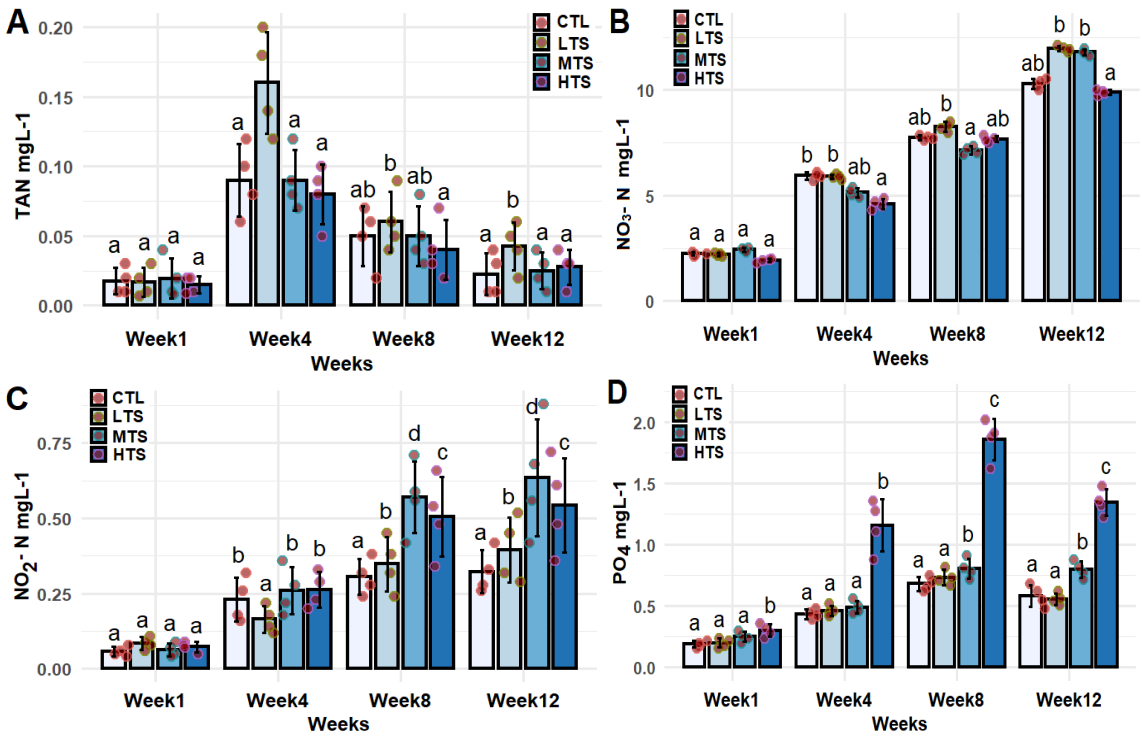

Figure 7.1. Water quality parameters of four different treatments. A) TAN, B) NO_3-N, C) NO2-N and D) PO_4. Different letters a, b and c indicates significant differences between treatments ($p<0.05$). Abbreviations: CTL- control tanks; LTS- low trace elements

supplementation tanks; MTS- medium trace elements supplementation tanks; HTS- high trace elements supplementation tanks.

7.3.3. Trace element concentrations in water

Before supplementing trace elements, the mean concentration of pre-selected trace elements in tank water were: Mn 0.0006 ± 0.0001, P 0.03 ± 0.00 and Si at 2.05 ± 0.07 mg L^{-1}. On supplementation and throughout the experiment the dissolved concentration of some trace elements increased, for example Mn in MTS, where others either decreased or increased as depicted in Table 7.3. All of the trace elements that were supplemented showed an increase in concentration except Si under LTS. At the end of the experiment the P concentration showed a progressive increase in concentration, whereas Si decreased, and Mn didn't change.

Table 7.3. Dissolved trace element concentrations (mg L^{-1}) in experimental tank water at different times (mean ± S. E.; n=4).

Treatments	CTL	LTS	MTS	HTS
After supplementation				
Mn	^{2}0.0006 ± 0.0001	^{2}0.0007 ± 0.0001	^{2}0.0013 ± 0.0001	^{2}0.0009 ± 0.0002
P	^{1}0.03 ± 0.00^{a}	^{1}0.03 ± 0.01^{a}	^{1}0.05 ± 0.00^{b}	^{1}0.19 ± 0.01^{c}
Si	^{2}2.05 ± 0.07^{a}	^{2}2.37 ± 0.15^{b}	^{2}2.97 ± 0.09^{c}	3.50 ± 0.23^{d}
At the end of the experiment				
Mn	^{1}0.0001 ± 0.0004	^{1}0.0002 ± 0.0001	^{1}0.0003 ± 0.0001	^{1}0.0003 ± 0.0001
P	^{2}0.42 ± 0.02^{a}	^{2}0.43 ± 0.02^{a}	^{2}0.46 ± 0.04^{a}	^{2}0.63 ± 0.05^{b}
Si	^{1}0.46 ± 0.03^{a}	^{1}0.49 ± 0.17^{a}	^{1}2.10 ± 0.35^{b}	3.53 ± 0.23^{c}

$^{a, b, c, d}$ shows the significant differences between the treatments; $^{1, 2}$ shows the significant differences over the time ($p<0.05$). Abbreviations: CTL- control tanks; LTS- low trace elements supplementation tanks; MTS- medium trace elements supplementation tanks; HTS- high trace elements supplementation tanks.

7.3.4. Marron growth, survival and moulting days

Individual marron was weighed every fortnight, where total weight (g), OCL (cm), and total length (cm) were recorded for each marron. There were no significant differences in survival rate among the treatments. Marron SGR and WG was highest for HTS tanks (Table 7.4).

Table 7.4. SGR (g; % /day), weight gain (g; %), survival (%) and moult intervals (Tm; days) of juvenile marron cultured for 90 days (mean ± S. E.; n=4).

Parameters	CTL	LTS	MTS	HTS
SGR				
0- 30 days	[4]0.29 ± 0.04	[4]0.32 ± 0.06	[3]0.40 ± 0.11	[4]0.50 ± 0.08
30-60	[1]0.02 ± 0.03	[1,2]0.04 ± 0.02	[1]0.03 ± 0.01	[1,2]0.08 ± 0.04
60-90	[1,2]0.05 ± 0.01	[1]0.02 ± 0.01	[1,2]0.10 ± 0.03	[1]0.03 ± 0.01
0-60	[3]0.15 ± 0.01[a]	[3]0.17 ± 0.02[a]	[2]0.21 ± 0.05[ab]	[3]0.29 ± 0.03[b]
0-90	[2,3]0.12 ± 0.01[a]	[2,3]0.13 ± 0.01[a]	[1,2]0.17 ± 0.03[ab]	[2,3]0.20 ± 0.03[b]
Weight gain				
0- 30 days	[2]9.01 ± 1.39	[2]9.94 ± 1.92	[2]13.0 ± 3.63	[2]16.4 ± 2.78
30-60	[1]0.44 ± 0.99	[1]1.12 ± 0.56	[1]0.68 ± 0.41	[1]2.60 ± 1.34
60-90	[1]1.51 ± 0.46	[1]0.58 ± 0.40	[1]3.00 ± 1.02	[1]0.72 ± 0.44
0-60	[2]9.46 ± 0.85	[2]11.1 ± 1.40	[2]13.6 ± 3.51	[2]19.4 ± 2.58
0-90	[2]11.1 ± 1.10[a]	[2]11.8 ± 1.39[a]	[2]17.0 ± 2.69[ab]	[2]20.2 ± 2.89[b]
Survival				
0- 30 days	94.5 ± 5.50	[2]97.2 ± 2.78	100 ± 0.00	97.2 ± 2.78
30-60	94.4 ± 5.55	[2]100 ± 0.00	97.2 ± 2.78	91.3 ± 5.39
60-90	80.9 ± 3.94	[1]85.4 ± 3.47	91.3 ± 5.39	87.3 ± 4.63
0-60	88.9 ± 6.41	[2]97.2 ± 2.78	97.2 ± 2.78	88.9 ± 6.41
0-90	72.2 ± 7.17	[1]83.3 ± 5.55	88.9 ± 6.41	77.8 ± 7.86
Tm (1st)	20.1 ± 1.11	20.5 ± 1.55	19.6 ± 1.46	19.6 ± 1.40
Tm (2nd)	17.5 ± 0.65	16.5 ± 0.29	16.0 ± 0.71	16.3 ± 0.63

[a, b] and [c] shows the significant differences between treatments; [1, 2, 3, 4] shows the significant differences over the culture days ($p<0.05$). Abbreviations: CTL- control tanks; LTS- low trace elements supplementation tanks; MTS- medium trace elements supplementation tanks; HTS- high trace elements supplementation tanks.

7.3.5. Marron health indices and trace element concentrations in haemolymph

The percentage of granular cells was significantly lower in LTS tank marron. HTS tank marron had significantly improved haemolymph indices compared to CTL and LTS (Table 7.5). Total concentrations of Fe and Mn in marron haemolymph were below the detectable level, whereas the Cu concentration was significantly higher in HTS tank marron compared to other treatments. Hepatopancreas moisture content and dry weight indices showed that the marron in HTC tanks were healthy.

Table 7.5. Total haemocyte count (x10^6 cells mL^{-1}), differential haemocyte count (%), hepatopancreas and tail wet weight and dry weight indices, and trace elements concentrations mg L^{-1}) in haemolymph of the marron juvenile at the end of the experiment (mean ± S.E.; n=4).

Parameters	**CTL**	**LTS**	**MTS**	**HTS**
THC (10^6cells mL^{-1})	1.54 ± 0.27	1.78 ± 0.30	1.69 ± 0.36	1.58 ± 0.16
Hyaline cells (%)	55.1 ± 0.55	58.5 ± 1.24	54.5 ± 0.41	55.3 ± 0.66
Granular cells (%)	35.4 ± 1.33^{b}	30.4 ± 0.94^{a}	35.5 ± 0.20^{b}	35.1 ± 0.55^{b}
Semi-granular cells (%)	9.50 ± 0.79	11.1 ± 0.38	10.0 ± 0.20	9.63 ± 0.52
CaH	483 ± 12.0	463 ± 12.5	443 ± 36.3	350 ± 14.6
CuH	2.00 ± 0.30^{a}	2.77 ± 0.40^{a}	3.67 ± 0.30^{a}	6.17 ± 1.00^{b}
PH	12.8 ± 0.60	11.8 ± 1.90	14.6 ± 1.40	13.4 ± 0.10
HM (%)	78.1 ± 3.60^{c}	78.0 ± 3.00^{c}	67.2 ± 2.58^{b}	57.5 ± 2.09^{a}
Hiw (%)	7.35 ± 0.40	6.26 ± 0.67	6.21 ± 0.40	7.39 ± 0.17
Hid (%)	1.65 ± 0.34^{a}	1.36 ± 0.23^{a}	2.06 ± 0.30^{ab}	3.14 ± 0.17^{b}
TM (%)	80.6 ± 0.94	81.2 ± 0.91	79.1 ± 1.36	80.6 ± 0.42
TMiw (%)	33.1 ± 2.58	36.0 ± 0.61	34.3 ± 1.03	35.2 ± 0.87
TMid (%)	6.36 ± 0.35	6.78 ± 0.38	7.17 ± 0.46	6.82 ± 0.22

$^{a, b}$ and ^{c} show the significant difference between the treatments. Where, CaH, CuH, PH represents the calcium (Ca), copper (Cu) and phosphorus (P) in haemolymph. HM % (Hepatopancreas moisture), Hiw (Hepatopancreas wet weight), Hid (Hepatopancreas dry weight), TM % (tail muscle moisture), TMiw (tail muscle wet weight), and TMid (tail muscle dry weight). LTS- low trace element supplementation, MTS- medium trace element supplementation, HTS- high trace element supplementation.

7.3.6. Sequence quality and alpha-beta diversity of the gut microbiota

After quality trimming, an average of 98,486 sequences and 358 OTUs per samples were obtained, ranging from 78,786-126,446 reads and 288-448 OTUs from 12 samples, respectively. Phylogeny assignment of quality reads obtained 15 phyla, 78 families and 126 genera. Approximate saturation rarefaction plot revealed that each sample was sequenced at enough depth to capture most of the microbial diversity (Figure 7.2A). Among the alpha

diversity indices (observed species, Shannon, Chao1), Chao1 diversity was found significantly higher in LTS group, compared to MTS and HTS. Bray-Curtis beta-dispersion on basis of relative abundance (weighted UniFrac) revealed distinct clustering of samples, PERMANOVA identified significant (P = 0.0233) impacts of trace element on gut microbiota.

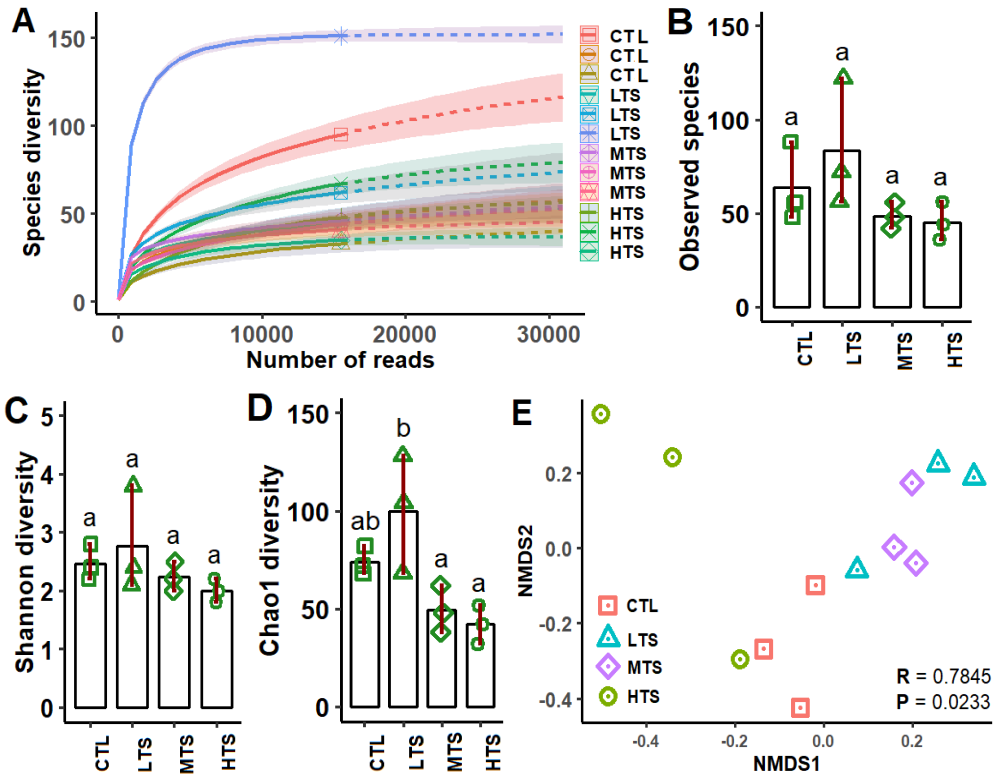

Figure 7.2. Alpha-beta diversity of microbial communities in the marron hindgut at the conclusion of the experiment. (A) Rarefaction curve in terms of sequence depth; (B) observed species; (C) Shannon diversity; (D) Chao1 diversity; (E) Beta ordination plot showing clustering of samples based on relative abundance of OTUs. Abbreviations: CTL, control tanks; LTS, low trace elements; MTS, medium trace elements; HTS- High trace elements supplementation.

7.3.7. Relative and significantly abundant bacterial communities

At phylum level, the overall relative abundance was almost similar for all treatment groups and 12 samples with same dominant phylum, Proteobacteria. High abundance of *Tenericutes* was only observed for the LTS group, compared to all other treatments (Figure 7.3A). At genus

level, *Vibrio* was the most abundant bacteria in all samples, followed *by Aeromonas*, *Candidatus bacilloplasma* and *C. hepatoplasma* (Figure 7.3B). The number of shared and unshared genera was found higher in LTS group, followed by HTS, MTS and CTL, respectively (Figure 7.3C). Differential abundance at 0.05 level of significance revealed that *Vibrio, C. Bacilloplasma and Aeromonas* in CTL, LTS and MTS were replaced by *Citrobacter, Acinetobacter and Pseudomonas* in the HTS treatment (Figure 7.3D).

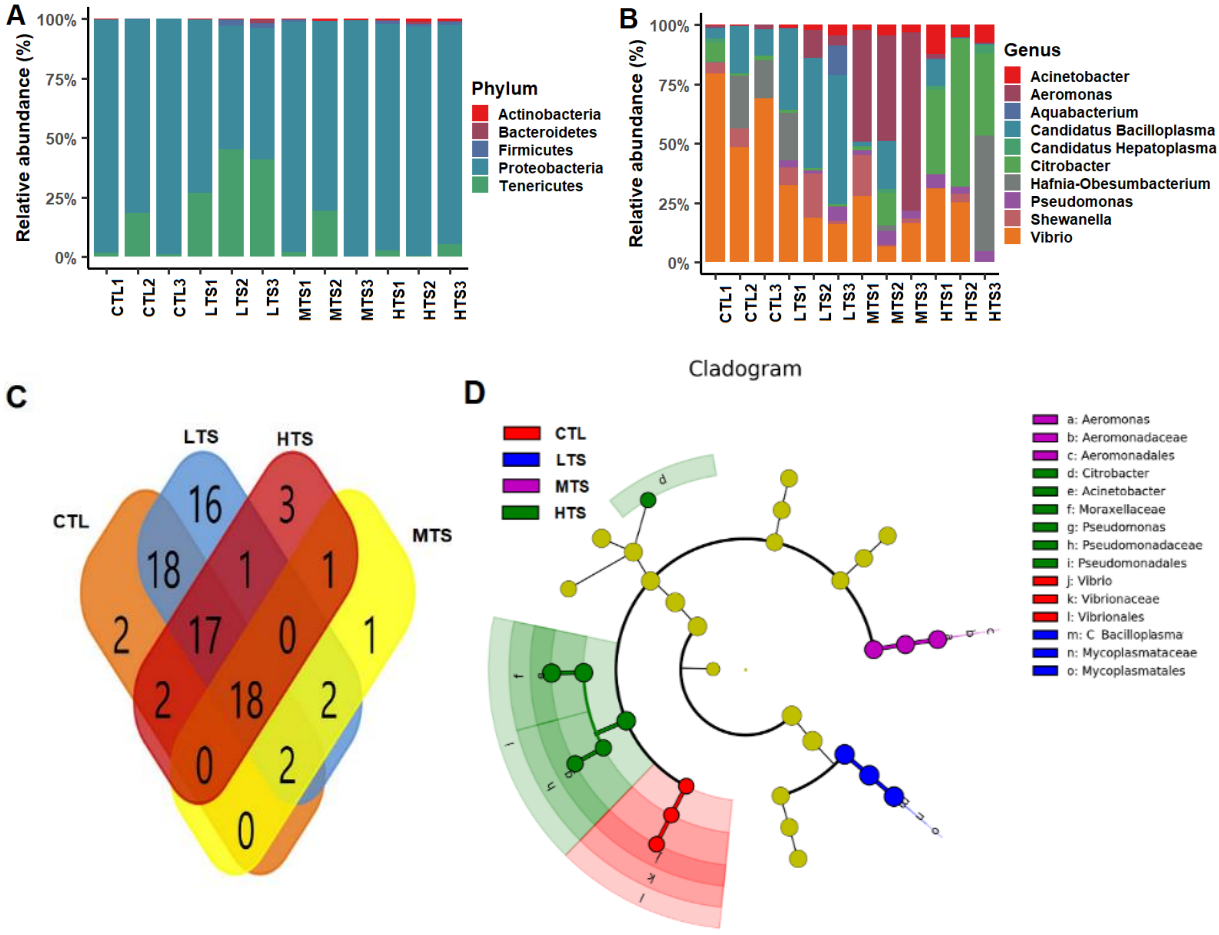

Figure 7.3. Relative and differential abundance of microbial communities in four different groups of marron at the conclusion of the experiment. (A) Relative abundance at phylum level; (B) Relative abundance at genus level; (C) Number of shared and unshared genus in different treatments; (D) Statistically significant bacteria at various taxa level in four different treatments. Abbreviations: CTL, control tanks; LTS, low trace elements; MTS, medium trace elements; HTS- high trace elements supplementation.

7.4. Discussion

This is the first study that analyses the effects of trace elements supplementation on plankton and growth, health indices and intestinal flora of marron cultured in the same body of water under laboratory conditions. Maintaining favourable water quality conditions allowed for optimum plankton growth in the tanks, similar to autumn season (Tulsankar et al., 2020). Also, maintaining plankton density at a static density throughout the experiment, by regular harvesting and addition prevented the results from being influenced by various phases of phytoplankton growth or nutrient influx due to plankton crashes. The constant input of nitrogen and P from marron feed, waste and senescence of phytoplankton, would have helped to promote growth of phytoplankton, as nitrogen and P can both frequently be limiting to primary productivity (Downs et al., 2008). However, fluctuations in TAN are common in aquaculture systems (Thakur and Lin, 2003), as nutrient concentrations build up until bacterial populations can be established to oxidise ammonia and nitrite.

Phytoplankton require P to build biomolecules such as proteins and nucleic acids (Pace and Lovett, 2013), which are essential for their growth. Addition of P resulted in an increase in reactive phosphate in the HTS treatment, which yielded the highest abundance of plankton (Li et al., 2017c, Shrestha and Lin, 1996). Similarly, higher fertilizer inputs resulted in increased primary productivity and tilapia (*Oreochromis niloticus*) production in ponds (Diana et al., 1991). In our study medium and high concentration supplementation of trace element triggered the phytoplankton growth. The HTS tanks had the highest plankton density, may be due to higher concentrations of phosphate and Si providing greater quantities of sustenance for the zooplankton community. Potassium, sodium and chloride were also added as part of chemical compounds of Mn, Si and P, which are essential nutrients for plankton growth, and may have improved marron growth and health mediated through planktons in HTS tanks (Padrão et al., 2016, Boyd, 2014, Raven, 2016, Civitello et al., 2014). The added P may have been utilized by the phytoplankton, periphyton grown on tank edges and eventually by zooplankton or be returned as detritus on the tank bottom (Drenner et al., 1989). The quantity of P and Si added in the HTS treatment tanks was high enough to increase plankton density, while this response was much reduced in the LTS and MTS tanks. On P addition in three different concentrations (0.2, 0.1 and 0.05 $g*m^{-3}$), Shrestha and Lin (1996) obseved no significant effect of different P levels on chlorophyll-α in tilapia ponds also higher P addition did not increased the tilapia yield.

The phosphate concentration increased over the experiment time, likely due to the marron feed, plankton senescence and marron waste. The plankton biomass and species composition are regulated by the availability of nutrients, while most planktons are limited by P and nitrogen, diatoms are often limited by silica (Drenner et al., 1989, Pace and Lovett, 2013). The diatom presence was more abundant only in HTS (supplementary data #1), and similar results of increase in diatoms on Si enrichment were observed by Nwankwegu et al. (2020). Addition of trace elements, such as Mn, to water may enrich phytoplankton, rotifers and other zooplankton that feed on them, and in turn improve the diet of cultured animals such as marron (Nordgreen et al., 2013).

Improved growth rate and health indices of marron in HTS can be associated to the higher plankton density, the plankton may have provided a food source in a fresh form or in the form of detritus, improving the marron nutrition. Natural productivity plays an important role in the production of white leg shrimp (*Litopenaeus vannamei*) and yabbies (*C. albidus*) (Gamboa-Delgado, 2014, Jones et al., 1995). A study on red claw (*C. quadricarinatus*) juveniles showed the greatest increase in weight (%) and highest harvest mean weight in tanks with the use of zooplankton (Jones, 1990). Comparatively, in a diet of zooplankton, unidentified bacteria and the macrophyte fed to juvenile red swamp crayfish (*Procambarus clarkii*), the higher growth rate was achieved with zooplankton (Brown et al., 1992). It is likely that the marron grown in HTS obtained added nutrition from the high plankton density therein, although it is unclear whether they obtained nutrition from the phytoplankton, zooplankton or detritus. Past research has shown that zooplankton are an important food source for juvenile crayfish, while plant matter from phytoplankton and macrophytes may be less important (Brown et al., 1992). However, plant material may provide nutritional elements not available in animal matter such as carotenoids (Jones et al., 1995). Copepods, cladocerans, and rotifers present in the tank water may have improved the health and growth of marron, as formulated feed often lacks important nutrients that the natural feeds contain (Jussila and Mannonen, 1997). Juvenile marron are thought to be poor filter feeders, so any phytoplankton probably would have been of value after settling on the cage bottom. Adult copepods or cladocerans may have been actively caught by the marron with their chelae or picked up off the cage bottom, while smaller zooplankton such as rotifers and copepod nauplii may have only been ingested as part of the detritus.

The SR and THC remained similar between treatments; however, these parameters are less associated with nutrition, and the results showed that all the treatment tanks had similar tank conditions. The HM (%) was significantly lower in MTS and HTS while Hid were significantly

higher in HTS which suggests that marron from tanks MTS and HTS had better health condition compared CTL and LTS tank marron. The hepatopancreas is an important digestive gland and is used for storage of energy and nutrients (Jussila and Mannonen, 1997) and maybe a good indicator of crayfish condition (Jussila, 1999). Higher plankton density in MTS and HTS may have increased the amount of nutrients and energy stored in the hepatopancreas reflecting an improvement in overall health of the juveniles.

The trace elements presence in tank water were not similar to their supplemented concentrations, the aquatic environmental dynamics are complex, the presence of plankton or bacteria may have absorbed or converted the trace elements. Also the supplementation of Mn and P and their presence in tank water did not affected their concentrations in marron haemolymph; Cu concentration was higher in marron from treatment HTS. Freshwater crustaceans accumulate and store the trace elements such as Ca and Cu in haemolymph (Wilder et al., 2009). More than 50 % of the whole body Cu load is stored in haemolymph (Depledge and Bjerregaard, 1989). It is an essential micronutrient and is an integral part of the respiratory pigment haemocyanin (Alcorlo et al., 2006). Haemocyanin maintenance requires the accumulation of Cu in relatively large quantities than trace levels, for its transport and storage within the body (Taylor and Anstiss, 1999). The Cu accumulation in decapod crustaceans is regulated only up to the physiological threshold levels (Alcorlo et al., 2006).

The gut bacterial community has been reported to play a key role in digestion and immunity of aquatic animals. Although the effects of dietary supplementations of probiotics, feed additives and protein sources on gut microbial communities of crayfish have been investigated (Foysal et al., 2020b, Parrillo et al., 2017), effects of trace elements and plankton on marron are still unexplored. Different P levels had no effect on the alpha diversity measurements, however the inclusion of different P levels on water influenced the gut microbial communities. Particularly, higher P level induced the growth of *Acinetobacter, Pseudomonas* and *Citrobacter,* the genera identified previously from marron gut (Foysal et al., 2019a) and widely reported as phosphate solubilizing bacteria in water (Qian et al., 2010, Wan et al., 2020). Bacteria can be transmitted from water to aquatic animals through feeding and the symbiotic relationship between water and gut microbiota of crayfish has been established in some studies (Foysal et al., 2019a, Liu et al., 2020). The phosphate solubilising bacteria in the gut of marron in our study were likely sourced from the water in the culture tank. P supplemented water had less *Vibrio* abundance, a genus commonly regarded as pathogenic for crayfish (Bean et al., 1998). Though marron is a disease-free species (Ambas et al., 2013), yet environmental pollution and habitat change

constantly increase the chance of infections by emerging pathogen like *Vibrio*. Overall, different trace elements and plankton densities influenced marron growth, health and gut microbiota.

7.5. Conclusion

Trace elements influenced plankton growth with the addition of Si resulting in an increased diatom abundance. Increased plankton abundance was associated with improved juvenile marron growth, health indices and a more diverse gut microbiota. The individual roles of phytoplankton or zooplankton on marron growth and health cannot be isolated at this point. Feeding trials with phytoplankton or zooplankton separately may provide more insight on their overall effect on marron.

Acknowledgements

Authors are thankful to Mr. and Mrs. Hall for allowing the collection of plankton and marron from their farm.

Availability of data and material

The experimental data will be provided on request and the raw data for marron gut microbiota in FASTQ files has been deposited to National Centre for Biotechnology Information (NCBI) BioProject under the accession number PRJNA682157.

Ethics approval

Animal ethics approval is not mandatory for the invertebrate animal studies at Curtin University, Australia. However, all the required protocols were followed while handling the animals, as per the guidelines of Animal Welfare Act, Western Australia and the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

CHAPTER 8. Feeding juvenile marron (*Cherax cainii* Austin, 2002) exclusively on live mixed plankton improves growth, total haemocyte count and pigmentation

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Abstract

The present study aimed to compare the nutritional effects of a mixture of phytoplankton and zooplankton and a formulated pelleted feed on the growth, survival, moulting, immune and organosomatic indices of juvenile marron (*Cherax cainii* Austin, 2002) cultured for 90 days, under controlled laboratory conditions. The experiment was conducted with two quadruplicated dietary treatments, a pre-selected plankton density and formulated feed pellet (Control). Seventy two juvenile marron of an average weight of 3.73 ± 0.16 g and average total length of 2.57 ± 0.04 cm were collected from a local farm and reared in eight 300 L water capacity tanks. In treatment tanks, the phytoplankton and zooplankton density was maintained at the average of 2.81 ± 0.02 x 10^6 cells L^{-1} and 228 ± 5.15 individuals L^{-1} respectively. Marron in the control group were provided with formulated feed at 2% of marron's body weight per day during the evening. Marron provided with mixed live plankton achieved a significantly higher growth rate, total haemocyte count and improvement in pigmentation of juvenile marron. Survival rate and organosomatic indices of juvenile marron were similar with the use of mixed plankton, and formulated feed. Therefore, from these results, it can be concluded that the live plankton can be a main source of nutrition for an early stage of juvenile marron, if the plankton density is maintained.

Keywords: Freshwater crayfish, Growth and Survival, Marron immune and organosomatic indices, Plankton, Pigmentation.

8.1. Introduction

Freshwater crayfish farming is well established worldwide particularly in Europe, and the United States, with the industry still under development in Australia (Hollows, 2016). The global freshwater crayfish production for the year 2019 was 2,162,159 tonnes (FAO, 2021). Australia is home to more than 110 species of freshwater crayfish, of which three *Cherax* species, yabbies (*Cherax destructor*), red claw (*C. quadricarinatus*) and marron (*C. cainii* Austin, 2002), have been identified as ideal candidate species for aquaculture (Lawrence and

Jones, 2002, Meakin et al., 2008). The total freshwater crayfish production in Australia for the year 2018 was 166.1 tonnes; marron contributed 65.8 tonnes valued at USD $2.30 million, red claw and yabbies contribution was 48.8 and 51.5 tonnes respectively (FAO, 2018a). Marron production showed a marginal increase by ~10 tonnes from 2017-18 (FAO, 2020a). Australia is the only significant producer of marron (Machin et al., 2008, FAO, 2021). Native to south west of Western Australia (WA), high market value, and status as a gourmet product, disease free and simple life cycle makes marron an ideal species for aquaculture (Machin et al., 2008).

In aquaculture, there is more emphasis on the use of formulated feed than managing the plankton productivity to achieve greater production (Boyd, 2018) despite the drawbacks associated with pelleted feed. The formulated feed may account for 40-60% of the total cost of production in semi-intensive aquaculture systems (Correia et al., 2003, Fotedar, 2004). Formulated feed composition is inconsistent as the inclusion of feed ingredients and their percentages depend on various factors such as availability of locally available ingredients, demand and species cultured. The composition, quality and cost of marron feeds also varies greatly, there is no consistent formulated diet that supplies the full nutritional requirements to the marron (Fotedar et al., 2015). Pelleted feeds lose stability after exposure to water, causing loss of nutrition value until consumed by marron, and having a negative impact on the water quality (Smith et al., 2002). Moreover, marron ignore the formulated pelleted feed once it has disintegrated into the water (Jussila and Evans, 1998). The main protein source of formulated feeds is fishmeal, and higher demand for fishmeal worldwide has increased the fishmeal cost (Boyd et al., 2007, Olsen and Hasan, 2012).

In the natural environment juvenile narrow clawed crayfish (*Pontastacus leptodactylus* formerly known as *Astacus leptodactylus*) and red swamp crayfish (*Procambarus clarkii)* are known to feed mainly on plant, organic detritus and zooplankton (Gutiérrez-Yurrita et al., 1998, Tcherkashina, 1977). Similarly, in a semi-intensive marron pond, a combination of natural productivity and formulated feed provides sufficient nutrition to marron (Fotedar et al., 2015). However availability of natural feed is season dependent (Tulsankar et al., 2020, Tulsankar et al., 2021b). Therefore, analysing the effects of mixed plankton in the absence of formulated feed could be an important research focus not only to minimise the use of fishmeal but also to reduce the formulated feed use by improving the plankton productivity of the ponds, mainly in early juvenile stages as observed in white leg shrimp (*Litopenaeus vannamei*) (Sánchez et al., 2014); and yabbies (Jones et al., 1995, Verhoef et al., 1998, Duffy et al., 2011).

There has been intensive research conducted on marron nutrition using different protein sources under pond culture and controlled laboratory condition (Morrissy, 1976, Morrissy, 1979, Morrissy, 1989, Sommer et al., 1991, Jussila and Evans, 1996, Jussila and Evans, 1998, Van den Berg et al., 1990, Tsvetnenko et al., 1995, Fotedar et al., 1999, Fotedar, 1998, O'brien and Davies, 2002, Fotedar, 2004, Tulsankar et al., 2021d, Saputra and Fotedar, 2021), however there is less knowledge on the nutritional requirements of marron. Few studies have been by conducted to determine the benefits of dietary zooplankton compared to formulated feed focusing on the growth and survival of Parastacid juvenile crayfish (Morrissy, 1989, Austin et al., 1997, Verhoef et al., 1998, Duffy et al., 2011)**,** but the majority of these studies have been focused on yabbies or red claw. A study by Morrissy (1989) was conducted using marron, the experimental protocol only used powdered algae extract (*Dunaliella salina*), and the measured parameters were limited to the growth and survival. So far no comparative study has been conducted to analyse the effects of a mixture of both phytoplankton and zooplankton versus formulated diet on the growth, survival, moulting, immune and organosomatic indices of juvenile marron. We hypothesized that the continuous availability of mixed plankton will improve the growth, survival, pigmentation, immune and organosomatic indices of juvenile marron, compared to when the animals are provided with formulated feed.

This is the first study to investigate the effects of a controlled and static density of live plankton mixture on juvenile marron growth, survival, moulting, immune and organosomatic indices, and pigmentation in the absence of formulated feed. The current experiment was a follow-up from the previous indoor trial conducted for 96 days to investigate the impact of supplementing Mn, Si and P in water on plankton density, diversity, and their effect on marron growth, survival, immune and organosomatic indices and gut microbiota (Tulsankar et al., 2021d). The experiment presented here was conducted to assess if the juvenile marron could receive the required nutrition from mixed live planktons alone in the absence of formulated feed. As the study focused on the nutritional role played by direct comparison between exclusive dietary planktons and formulated feed, a mixture of these two diets was not considered necessary.

8.2. Materials and Methods

8.2.1. Plankton and marron collection

Plankton and experimental animals were collected from a semi-intensive commercial marron farm in Manjimup (34°18'75" S, 116°06'61" E), Western Australia. Pond water was filtered separately using plankton net for phytoplankton and zooplankton to obtain the total quantity of

15 L of sub-sample for phytoplankton and zooplankton each. The plankton samples were brought to the Curtin Aquatic Research Laboratory (CARL) and cultured in outdoor conditions to use in the current experiment. For outdoor plankton culture, planktons were stocked in six 300 L capacity tanks topped up with 200 L of freshwater. The tanks were placed in direct sunlight and aerated continuously using airstones. Aquasol® by Yates Pty Ltd., fertilizer was used to boost and maintain the phytoplankton density at approximately 9.34 X 10^6 cells L^{-1}. Phytoplankton was used to feed zooplankton at a rate of 10 L of phytoplankton water added every second day to the zooplankton tanks.

Approximately 100 juvenile marron of an average weight of 3.73 ± 0.16 g and average total length of 2.57 ± 0.04 cm were collected and transported to CARL on the same day of collection. On arrival the marron were stocked in 300 L plastic tanks to acclimatise them to laboratory conditions for two weeks.

8.2.2. Formulated feed composition

All feed ingredients were procured from Speciality Feeds Company, Glen Forrest, Western Australia and the feed formulation was conducted at CARL (Table 8.1).

Table 8.1. Ingredients and proximate composition (in percent) of formulated feed used in this study.

Ingredients	**Basal diet**
Fish meal	40.00
Wheat (10 CP)	22.14
Corn/wheat starch	10.00
Lecithin- Soy (70%)	1.00
Vitamin C	0.05
Dicalcium Phosphate	0.02
Vitamin Premix	0.29
Canola oil	8
Cholesterol	0.5
Fish oil	10
Barley	8
Total	100
Crude Protein (%)*	28.5
Crude Lipid %*	10

*The proximate composition (Crude protein and crude lipid) of final diet (% dry matter).

8.2.3. Experimental set up

A 90 days experiment was conducted under controlled laboratory conditions in eight 300 L water capacity tanks. Each tank included 9 cages made up of plastic containers with open top for feeding covered by mesh (6 mm size) to stock the marron individually. The cages had a volume of 2 L (170 mm x 115 mm x 135 mm) with two small gaps of 5 mm on sides to allow the plankton exchange directly from the tank water into the cage as described by Tulsankar et al. (2021d). The cages were submerged in tank water up to the half of their height by mounting them on PVC pipes. Nine marron were selected randomly and placed individually into the cages. The mixture of live plankton as a feed in treatment tanks was maintained at approximate mean density of 2.73 to 2.92 x 10^6 cells L^{-1} and 213 to 238 individuals (ind.) L^{-1} of phytoplankton and zooplankton respectively, similar to the highest density found in semi-intensive marron ponds (Tulsankar et al., 2020). Plankton density was maintained by either addition or removal of plankton. Marron in control tanks were provided with formulated pelleted feed at 2% of their body weight once a day in the evening. The left over feed was collected and removed the next morning. Both dietary treatments were carried out in four replicates. Continuous aeration was supplied to the tanks.

8.2.4. Water quality analysis

The water quality parameters for plankton growth such as dissolved oxygen (DO), pH and temperature were maintained close to the range observed in marron ponds during summer, the season with highest phytoplankton abundance (Tulsankar et al., 2021b). Water parameters such as DO, pH and temperature were monitored daily. An Oxyguard® digital DO meter (Handy Polaris 2, Norway) was used to record DO and temperature, and an Ecoscan pH 5 meter (Eutech instruments, Singapore) was used to record pH. A DR/890 portable colorimeter with Permachem reagents (Hach, USA) was used to analyse total ammonia nitrogen (TAN), nitrite (NO_2-N), nitrate (NO_3-N), and reactive phosphate (PO_4) once a week. The experiment was conducted with no water exchange. Tank water level was maintained at 150 L throughout the experiment by adding water to compensate for losses due to the evaporation.

8.2.5. Plankton analysis

During the experiment the plankton density was measured every two days and maintained at the constant preselected density. Plastic containers (100 mL volume) were used to collect and store the samples. For phytoplankton samples, 1 L of tank water was filtered through plankton net to obtain 100 mL of sample. The zooplankton samples were collected by filtering 5 L of

tank water through a 60 micron net to obtain 100 mL of sample. The plankton species were identified to the lowest possible taxonomic level using keys from a book by Canter-Lund and Lund (1995) and a manual by Ingram et al. (1997). The phytoplankton density (cells L^{-1}) and zooplankton density (individuals L^{-1}) was calculated by using the equations from Tulsankar et al. (2021b) and Ingram et al. (1997) respectively.

8.2.6. Marron growth, survival and moulting analyses

The marron growth data were recorded fortnightly, by weighing and measuring individual marron. Marron mortality was recorded on a daily basis. Marron specific growth rate (SGR) and survival rate (SR) was calculated by using the following formulae as described by Evans and Jussila (1997)

$SGR = 100 X (Ln (W_t) - Ln (W_i))/T,$

$SR = 100 x (n_t / n_0)$

Where, W_t is final weight (kg), W_i is initial weight, n_t is the number of marron alive at (T) days and n_0 is the number of marron stocked initially.

Weight, length and moulting date of marron was recorded to calculate the weight increment (Wm, %), length increment (Lm, %) and moult interval (Tm, days) by using the following equations;

Moult interval (Tm, days):

$T_m = T_{n+1} - T_n$

Weight increment at a moult (Wm %):

$Wm = (W_a - W_b) \times 100/W_b$

Length increment at moult (Lm %):

$Lm = (TL_a - TL_b) \times 100/TL_b$

Where, T_n= date of n moult, T_{n+1}= date of n+1 moult, W_a = total weight after second moult (g), and W_b= total weight after first moult (g), TLa - total length after second moult; TL_b - total length after first moult.

8.2.7. Marron immune and organosomatic indices analyses

The immune indices such as, total haemocyte count (THC) and differential haemocyte count (DHC) were analysed at the end of the experiment. Three marron per replicate were randomly selected to collect the haemolymph samples. The haemolymph was drawn by using a 1 mL syringe inserted ventrally in between the third and fourth pair of pereopods. A method described by Nugroho and Fotedar (2013) was used to analyse THC and DHC.

At the end of the experiment a total of 12 marron per treatment (three per replicate) were randomly selected to analyse the hepatopancreas and tail muscle moisture content, wet weight and dry weight indices. The hepatopancreas and tail muscle samples from every individual marron were weighed; to obtain the dry weight the samples were dried at 105ºC in an oven until a constant weight was achieved. The moisture content and organosomatic indices were calculated as described by Lindqvist and Louekari (1975), Jussila and Mannonen (1997) and (Fotedar, 2004) using the following equations:

Tail muscle moisture (TM %) = (Tw-Td) X 100/Tw

Hepatopancreas moisture (HM %) = (Hw- Hd) X 100/Hw

Wet tail muscle indices (TMiw) = Tw X 100/BW

Wet hepatopancreas indices (Hiw) = Hw X 100/BW

Dry tail muscle indices (TMid) = Td X 100/BW

Dry hepatopancreas indices (Hid) = Hd X 100/BW.

Where, Tw: Tail muscle wet weight; Hw: Hepatopancreas wet weight; Td: Tail muscle dry weight; Hd: Hepatopancreas dry weight; BW: Body Weight.

8.2.8. Pigmentation observations

At the end of the experiment, the pigmentation of the individual marron was visually assessed as previously reported by Jussila and Evans (1998). Colour for individual marron was recorded and the most common colour of marron per tank was considered as the colour representation for each tank.

8.2.9. Statistical analyses

All the data were analysed using statistical IBM® SPSS version 26 and are presented as mean ± standard error (S. E.). Independent t-test was used to determine significant differences

between treatments. Mann-Whitney U test was used when the data lacked normality. All tests were considered statistically significant at $p < 0.05$.

8.3. Results

8.3.1. Water quality parameters of juvenile culture tanks

All the water quality parameters were maintained at an optimum range for marron growth and survival (Morrissy, 1975, Morrissy et al., 1984, Morrissy, 1990, Fotedar et al., 1999, Villarreal and Peláez, 1999, Policy, 2003) (Table 8.2).

Table 8.2. The water quality parameters dissolved oxygen (mg L^{-1}), temperature (°C), pH, total ammonia nitrogen (TAN; mg L^{-1}), nitrite (NO_2-N; mg L^{-1}), nitrate (NO_3-N; mg L^{-1}), and reactive phosphate (PO_4; mg L^{-1}) of the tank water holding juvenile marron throughout the culture under controlled laboratory conditions for 90 days (mean ± S. E.; n=4).

Treatments	Mixed plankton	Control
DO	9.00 ± 0.01	9.01 ± 0.01
Temperature	21.5 ± 0.01	21.4 ± 0.01
pH	7.67 ± 0.00	7.69 ± 0.00
TAN	0.02 ± 0.00	0.02 ± 0.00
NO_2-N	0.03 ± 0.00	0.03 ± 0.00
NO_3-N	1.29 ± 0.02	1.27 ± 0.02
PO_4	0.23 ± 0.00	0.22 ± 0.00

8.3.2. Plankton density and community

The plankton density was maintained at an average of 2.73 to 2.92 x 10^6 cells L^{-1} and 213 to 238 ind. L^{-1} for phytoplankton and zooplankton respectively (Table 8.3). No significant difference in plankton density over time was observed, to avoid a plankton crash a constant density was maintained by either addition or removal of plankton. The culture of phytoplankton in the outdoor tanks consisted of *Scenedesmus* spp.; *Chlorella* spp.; *Closterium* spp.; *Volvox* spp.; *Navicula* spp.; *Nitzschia* spp. while zooplankton tanks had *Calanoida* spp.*; Cyclopoida* spp.*; Keratella quadrata; Keratella cochlearis* and *Daphnia* spp. These plankton species were observed in the marron tanks during the experiment.

Table 8.3. Plankton densities controlled in tanks to culture juvenile marron for 90 days under controlled laboratory conditions (mean ± S. E.; n=4 per treatment).

Week	Phytoplankton abundance (x10^6 cells L^{-1})	Zooplankton abundance (Ind. L^{-1})
1	2.73 ± 0.03	213 ± 12.5
2	2.92 ± 0.10	238 ± 23.9
3	2.78 ± 0.06	225 ± 10.2
4	2.73 ± 0.03	238 ± 12.5
5	2.92 ± 0.09	225 ± 20.4
6	2.78 ± 0.06	219 ± 6.3
7	2.86 ± 0.06	231 ± 21.3
8	2.75 ± 0.06	225 ± 10.2
9	2.81 ± 0.05	238 ± 23.9
10	2.93 ± 0.05	239 ± 6.70
11	2.85 ± 0.07	235 ± 5.70
12	2.89 ± 0.06	215 ± 10.2
13	2.90 ± 0.07	231 ± 0.98

8.3.3. Marron growth, survival and moulting

SGR and WG (%) of juvenile marron were significantly higher for marron cultured in tanks provided with mixed plankton than marron provided with formulated pelleted feed (Figure 8.1). The SR was similar for the marron from both treatment tanks ($p>0.05$) (mean ± S. E; n=4). The increment in weight and length at moult was significantly higher in juvenile marron provided with the mixed live plankton than those provided with the formulated feed (Figure 8.2). A mixture of live plankton or formulated feed did not affect the moult intervals. No specific pattern of moulting time was observed as marron moulted during morning, afternoon and during the night.

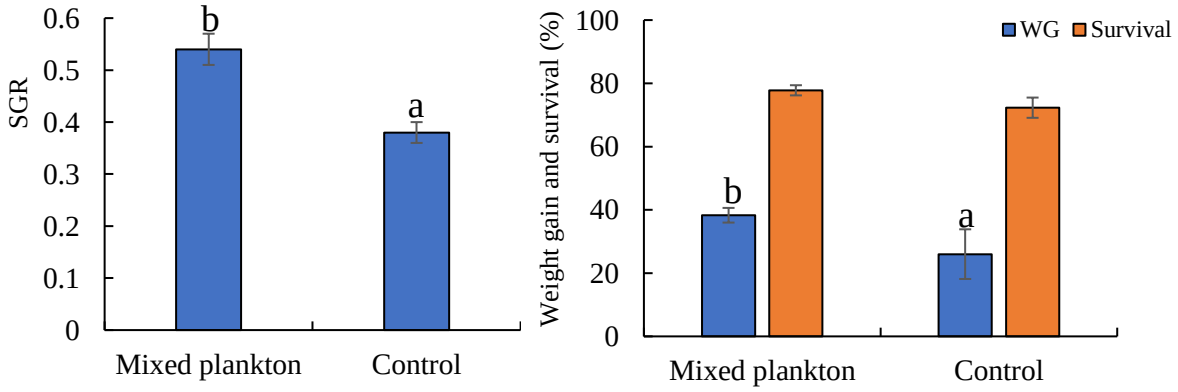

Figure 8.1. SGR, WG and SR (%) of the juvenile marron cultured under controlled laboratory conditions feeding mixed plankton or formulated feed (control) for 90 days (mean ± S. E.).

Letters a and b shows the statistically significant difference between the treatments ($p<0.05$).

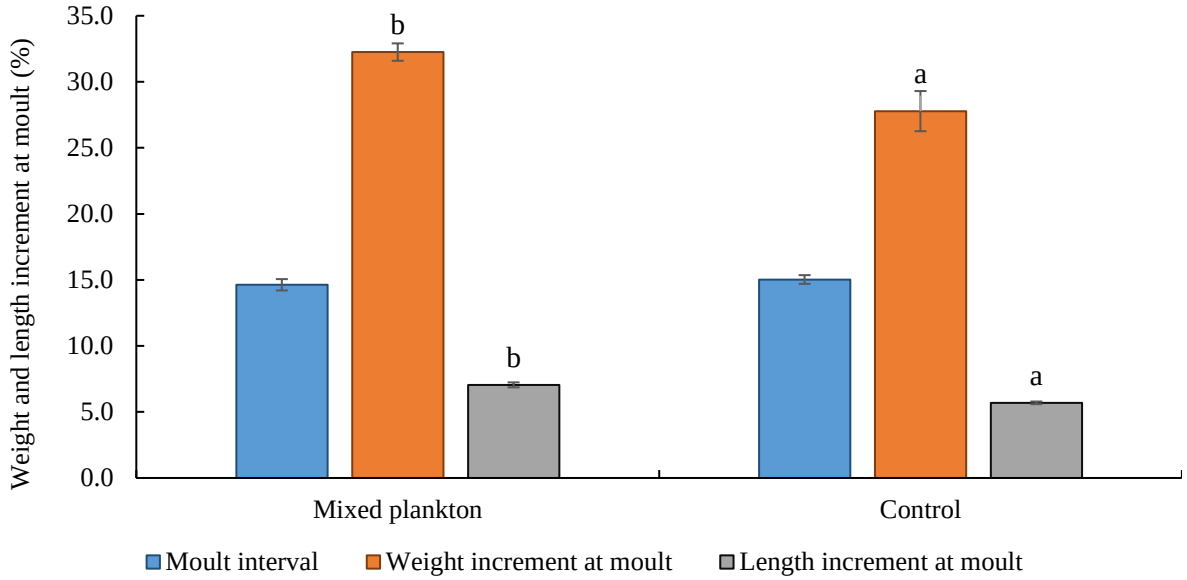

Figure 8.2. Weight and length increment of juvenile marron on moulting cultured for 90 days under controlled laboratory conditions. Letters a and b show significant differences in weight and length increments of juvenile marron when provided with mixed live plankton or formulated feed (control) (mean ± S. E.).

8.3.4. Marron immune and organosomatic indices

THC was higher in juvenile marron provided with live plankton mixture however the organosomatic indices did not show any significant differences between the two dietary treatments (Table 8.4) (mean ± S. E.; n=4).

Table 8.4. Immune and organosomatic indices of juvenile marron provided with mixed live plankton or formulated pelleted feed for 90 days under controlled laboratory conditions (mean ± S. E.; n=12 per treatment).

Treatments	Mixed plankton	Control
THC (million cells mL^{-1})	2.70 ± 0.01^{b}	1.81 ± 0.01^{a}
Hyaline (%)	63.3 ± 0.76	62.2 ± 1.65
Granular cells (%)	31.0 ± 1.05	31.7 ± 1.16
Semi-granular cells (%)	5.65 ± 0.72	6.23 ± 0.66

TM (%)	79.25 ± 1.18	78.75 ± 1.11
TMiw	20.7 ± 0.90	19.9 ± 0.87
TMid	4.21 ± 0.11	4.26 ± 0.37
HM (%)	80.0 ± 1.94	79.1 ± 1.53
Hiw	7.90 ± 0.24	7.81 ± 0.55
Hid	1.59 ± 0.17	1.63 ± 0.13

Superscript [a] and [b] show the significant difference between the treatments within a row ($p<0.05$).

8.3.5. Pigmentation

At the end of the experiment marron pigmentation was affected by the dietary treatment (Table 8.5 and Figure 8.3) where plankton provided marron had a dark brown colour and marron provided with formulated feed were bluish.

Table 8.5. Colour representation of marron of each replicate tank provided with mixed plankton and formulated pelleted feed for 90 days under controlled laboratory conditions. (n=16 per treatment).

Treatments	**Mixed plankton**	**Control**
Tank 1	Reddish Brown	Blue
Tank 2	Dark brown	Light Purple
Tank 3	Dark brown	Light Blue
Tank 4	Dark brown	Light Blue
Tank 5	Reddish Brown	Blue
Tank 6	Dark brown	Blue
Tank 7	Dark brown	Light Blue
Tank 8	Dark brown	Light Blue

Mixed Plankton Formulated feed

Figure 8.3. Difference in pigmentation of juvenile marron provided with formulated diet and live mixed plankton separately under controlled laboratory conditions for 90 days.

8.4. Discussion

At the end of this study, live plankton mixture significantly improved the SGR and WG % of marron. Similar results of improved SGR were observed in yabbies provided with zooplankton (Jones et al., 1995, Austin et al., 1997, Verhoef et al., 1998). Improved growth rate of marron fed on plankton mixture reflects the constant supply of nutrients, whereas the formulated feed has a tendency to leach out most of the water-soluble nutrients once immersed in pond water (Smith et al., 2002). Plankton are a rich source of protein, amino acids, lipids, fatty acids, minerals, chlorophyll, carotenoids, trace elements, enzymes and vitamins (Kibria et al., 1997, Napiórkowska-Krzebietke, 2017) which are essential for marron growth (Fotedar et al., 2015). A constant supply of plankton would have allowed for continuous feeding through filter feeding and scavenging of detritus (Van den Berg et al., 1990). SGR >0.6 is widely accepted for commercial marron farm (Evans and Jussila, 1997), however the current work was conducted under controlled laboratory conditions, limiting marron to feed on only plankton entering the cage which may have restricted the growth. Use of cages may have restricted the marron from feeding on detritus accumulating on the tank bottom. Use of containers and artificial diet under controlled laboratory conditions can supress growth (Geddes et al., 1988).

Although the static plankton density provided to marron was enough to improve growth and health under controlled laboratory conditions, it should be noted that it would be difficult to maintain a continuous plankton productivity in semi-intensive aquaculture facilities. In both dietary treatments the SR was high (>70%), compared to the SR observed by Celada et al. (1989), where 53.33 % SR was observed for juvenile crayfish (*Pacifastacus leniusculus*) fed

phytoplankton and zooplankton. However, similar SR as our study (~70%) was observed by Carreño-León et al. (2014) for juvenile red claw crayfish fed with microalgae.

Immune and organosomatic parameters have been used as an indicator of immune health in crustaceans (McClain, 1995b, McClain, 1995c, Fotedar et al., 2001, Haefner and Spaargaren, 1993). Higher THC represents the higher immune status (Sharma et al., 2009), with higher THC being observed in juvenile marron provided with live plankton mixture, relative to those provided with the formulated diet. In decapod crustaceans the food intake and nutritional status affects the haemocyte count in terms of quantity and quality (Le Moullac and Haffner, 2000, Persson et al., 1987). Studies analysing the plankton effect on the immune and organosomatic indices of crayfish have not been reported yet except in a study by Tulsankar et al. (2021b), where plankton supplementation improved hepatopancreas condition. Marron given fish meal formulated feed showed similar immune and organosomatic indices as observed by Sang and Fotedar (2010a).

In red swamp crayfish (*Procambarus clarkii*) and noble crayfish (*Astacus astacus*) moulting was observed predominantly during the daytime (Culley and Duobinis-Gray, 1987, Franke et al., 2013), and in yabbies mostly at night (Sokal, 1988). In juvenile marron no specific pattern of moulting time was detected during the moulting process. The mean moulting cycle was 14.6 ± 0.43 days for those provided with live plankton and 15.0 ± 0.33 days for the marron provided with formulated feed. While the moulting cycle was reported at 35.4 ± 2.2 days in a study conducted by Mai and Fotedar (2017). The moulting days in our study ranged from 14-16 days, similar to the findings of Ackefors et al. (1995) for noble crayfish. The moult interval decreased significantly in plankton fed marron, indicating that they had better nutrition (Lemos and Weissman, 2021).

The ideal crayfish diet should improve growth, survival and pigmentation (Verhoef et al., 1998), however various studies have found that marron formulated diets were often lacking in carotenoids, resulting in pigmentation loss (Jussila and Mannonen, 1997). Carotenoids are common in algae and are necessary for good pigmentation in crustaceans, suggesting that farmed marron obtain at least some of their nutrition from natural sources (Goddard, 1988). The pigmentation of juveniles provided with live plankton mixture was dark brown but the pigmentation of those provided with the formulated pelleted feed were light blue, indicating pigmentation loss. This reflects the presence of carotenoids and astaxanthin in the plankton

mixture which improved the pigmentation. Increased dietary asthaxanthin through feed for red king crab (*Paralithodes camtschaticus*) achieved darker pigmentation (Daly et al., 2013).

The micro-nutritional requirements of marron under semi-intensive or extensive farming can be partly fulfilled from natural productivity with the presence of healthy phytoplankton and zooplankton communities, and detritus, but their macronutritional requirements should be fulfilled by the supplementation of formulated feed (Fotedar et al., 2015). While plankton are rich in vitamins and minerals can sustain juvenile marron, they may not provide the bulk of nutrients needed for the adult marron to grow, for example marron cannot synthesise cholesterol and it should be included in formulated feed (Fotedar et al., 2015). Juveniles are less benthic and more motile than adults, therefore they have greater ability to capture planktonic prey (Goddard, 1988), whereas adults are less active and can capture larger zooplankton and pelleted feed with pereopods and can achieve their nutritional requirements from formulated feed. Juvenile marron spent longer time in feeding on frozen copepods (Tulsankar et al., 2021c), while yabbies <15- 45 g fed zooplankton and formulated feed spent significantly longer time feeding on zooplankton (Meakin et al., 2008); which suggests that juveniles find live-feed more attractive than formulated feed.

The inclusion of live plankton in juvenile marron diet may provide essential nutrition improving the growth rate and survival in aquaculture systems. Better management of the plankton density and diversity will help to lower the use of formulated feed reducing the operational cost. Evaluating the impact of each individual contribution of phytoplankton and zooplankton to the marron growth will put more light on marron nutritional gain from each source.

8.5. Conclusion

In this study, it was possible to maintain a constant plankton density, as the experiment was conducted under controlled laboratory conditions. While recreating the experiment under outdoor conditions the environmental parameters should be considered. Continuous availability of plankton in treatment tanks may have allowed marron to consume the plankton anytime resulting in improved growth rate, total haemocyte count and pigmentation of juvenile marron when compared with the marron provided with formulated feed only. In conclusion, juvenile marron growth rate, total haemocyte count and pigmentation of juvenile individuals was dependant on the supplied feed type.

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Animal ethics statement

Animal ethics approval was not required to conduct the experiment as marron are invertebrates.

CRediT Author statement

Smita Sadanand Tulsankar: Conceptualization, designing and set up of the experiment, day to day feeding, data collection, data analysis and writing of the manuscript. **Anthony J. Cole:** Plankton data collection, reviewing and editing manuscript. **Marthe Monique Gagnon:** Supervision, editing and reviewing the manuscript. **Ravi Fotedar:** Conceptualization**,** supervision, editing and reviewing the manuscript.

Declaration of Competing Interests

This work was conducted as a part of PhD course of Mrs. Smita Sadanand Tulsankar, and the authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

CHAPTER- 9 Live plankton supplementation improves growth, survival, health status and influences the gut microbiota and histology of juvenile marron (*Cherax cainii,* Austin 2002)

This chapter is under review

Abstract

In the present study commonly found phytoplankton and zooplankton communities in Western Australian aquaculture ponds were cultured, and provided separately or mixed with formulated feed to juvenile marron (*Cherax cainii* Austin, 2002) under controlled laboratory conditions for 90 days. Dietary treatment effects on growth, survival, moulting, immune and health indices and microbiota present in water, detritus, marron gut, and on histology of the hepatopancreas, midgut, tail muscle tissue, as well as their contributions towards the growth of tail muscle and whole juvenile marron were assessed by modelling a freshwater food web of water, plankton and marron. Zooplankton and mixed plankton supplementation improved the specific growth rate, weight gain (%) and survival rate of juvenile marron. Increased total haemocyte count, granular cell percentage and decrease in time required to clot the haemolymph were observed in juvenile marron fed with live plankton in comparison to those provided with formulated feed alone. Improved lysozyme activity, tail muscle dry weight indices, midgut epithelium height and tail muscle tissue myofibres were observed in marron fed with zooplankton. According to C and N stable isotope signatures, juvenile marron consumed a higher percentage of zooplankton followed by detritus. In control tanks, formulated feed represented the most significant input towards the diet of marron, but resulted in lower growth and survival rate. The microbiota DNA analysis and sequencing of water, gut and detritus showed that *Candidatus* Bacilloplasma, *Dinghuibater*, and *Aeromonas* were most predominant bacteria in the gut, detritus and tank water, respectively in terms of relative abundance. The highest alpha diversity of midgut bacteria was observed in marron fed with mixed plankton. Two novel bacteria *Nocardioides* and *Hyphomicrobium* were detected in marron midgut fed with zooplankton.

Keywords: freshwater crayfish, phytoplankton, zooplankton, health indices, gut microbiota, isotope analysis.

9.1. Introduction

Marron (*Cherax cainii,* Austin, 2002) is a freshwater crayfish endemic to Western Australia, where they are a popular aquaculture and recreational fishing species. Their simple life cycle lacks planktonic larval stages. Initially feeding on the yolk sac, juvenile marron drop from the

mother's tail and can acquire their nutrition from naturally occurring plankton. The majority of marron farming takes place in semi-intensive purpose-built ponds, where stocked juveniles receive nutrition from both naturally occurring live foods and supplied formulated feed, with an emphasis on feed improvement (Sang and Fotedar, 2009). Plankton represents a rich source of nutrients (Kibria et al., 1997, Napiórkowska-Krzebietke, 2017) which are essential for marron growth (Fotedar et al., 2015). To improve the growth and survival rate of juvenile marron various studies have been carried out focusing on producing nutritionally balanced formulated feeds (Jones, 1995a, Austin et al., 1997, Duffy et al., 2011).

Diet impacts not only the growth of marron, but also their physiology, pigmentation and gut microbial community (Jussila and Evans, 1998, Sang et al., 2011). For example, marron fed only formulated feed may lack in carotenoids which can cause loss of exoskeleton pigmentation, poor growth, survival and health (Jussila and Mannonen, 1997, Sommer et al., 1991, Tulsankar et al., 2021d). Plankton and formulated diets containing probiotic supplementation have resulted in gut microbiota modulation and enrichment of beneficial bacteria in the marron gut (Tulsankar et al., 2021d, Foysal et al., 2020b).

Like the gut, the hepatopancreas is another important digestive organ with an additional role in immunity. The hepatopancreas serves as sensitive indicator for metabolism, nutritional status and health condition in freshwater crayfish, as it is a site for the digestion, synthesis of immune factors, nutrient absorption and storage (Jussila and Mannonen, 1997, Mai and Fotedar, 2017, Calvo et al., 2011). Well organised, tightly arranged tubules of hepatopancreas showing different types of cells are linked with better health status of red swamp crayfish (*Procambarus clarkii*) (Xiao et al., 2014).

Marron tail muscle is exclusively used for human consumption and knowing the effect of different feeds on tail muscle myofibre formation and growth can provide more insight into favourable marron nutrition. Similarly, understanding the contributions of different food sources towards the tail muscle growth and whole marron health can provide information on the importance of provided food sources, and can help to manage the feeding strategies in order to manage the pond's natural productivity. The relative contribution of each possible food source to the formation of tissue can be estimated using Stable Isotope Analysis in R (SIAR) mixing models (Phillips and Gregg, 2003, Phillips and Gregg, 2001). While marron can be provided with natural and commercial food, the actual consumption of different food type under faming conditions has never been explored. Stable isotope analysis (SIA) is a refined

and accurate method to identify the main sources of food, and the isotopic ratios can provide information on assimilated food sources over time (Bondar et al., 2005). Information on the prey items consumed by marron grown in semi-intensive ponds would benefit marron farmers by informing the development of formulated feeds.

In aquaculture systems, the availability of live food during the juvenile life stage is crucial for achieving high survival and growth rates (Sáez-Royuela et al., 2007). If maintained, naturally-occurring plankton in marron ponds may provide substantial supplementation to formulated feed during the juvenile stages of the marron life cycle, reducing the cost of feeding and improving the growth, survival rates, pigmentation and health of cultured animals (Shui et al., 2020, Jussila and Mannonen, 1997, Napiórkowska-Krzebietke, 2017, Austin et al., 1997, Jones, 1995b, Verhoef et al., 1998, Tulsankar et al., 2021a). Under laboratory conditions juvenile marron growth was improved with the supplementation of live plankton, however, the relative contribution of either phytoplankton or zooplankton to marron growth and health could not be confirmed (Tulsankar et al., 2021a).

It is believed that plankton, detritus and microalgae are important food items for marron nutrition (Beatty, 2006). However, there is a lack of data on nutrition acquired by marron from these naturally occurring food items. Moreover, little is known about marron feeding selectivity in the presence of phytoplankton and zooplankton, and the importance of plankton to various aspects of marron health, including histology of hepatopancreas, midgut structure and tail muscle tissue growth and diversity of gut microbiota. This study aims to investigate the effects of phytoplankton and zooplankton separately and together as live feed on marron growth, survival, health indices, histology of hepatopancreas, gut health, tail muscle tissue growth and gut microbiota. In addition, the plankton contributions towards the juvenile marron diet is assessed. Findings from this research will increase the knowledge of marron feeding biology and feeding preference and on the importance of dietary plankton on juvenile marron growth and health parameters.

9.2. Materials and Methods

9.2.1. Experimental design and set up

A total of 200 juvenile marron with an weight 5.75 ± 0.12 g (mean ± S.E.), orbital carapace length (OCL) of 2.90 ± 0.02 cm and total length of 6.35 ± 0.05 cm were purchased from Blue Ridge marron farm (Manjimup, Western Australia) and were transported to Curtin Aquatic Research Laboratory (CARL), Curtin University, Western Australia. Marron were acclimatised

to laboratory conditions for 2 weeks prior the start of experiment. A randomized block design experiment was conducted over 90 days under controlled laboratory conditions with four replicates per treatment. Sixteen rearing tanks (300 L capacity) were filled with 150 L of freshwater and treatment tanks were supplemented with the respective plankton densities corresponding to each treatment. Marron were then randomly distributed into the tanks with a density of nine individuals per tank. The treatments consisted of: control (formulated food only; T1), formulated food supplemented with phytoplankton (T2), formulated food supplemented with zooplankton (T3), and formulated food supplemented with a mixture of phytoplankton and zooplankton (T4). Phytoplankton density was maintained at a density of $4.41 \pm 0.10 \times 10^6$ cells L^{-1} and zooplankton at 494 ± 2.88 individuals L^{-1} while the mixture was maintained at a phytoplankton density of $4.39 \pm 0.10 \times 10^6$ cells L^{-1} and zooplankton at 498 ± 3.11 individuals L^{-1}.

9.2.2. Water quality analysis

All rearing tanks were maintained with water quality parameters that are optimum for marron growth (Morrissy, 1990) with a constant temperature of 21°C and continuous aeration provided using a submerged thermostat and air diffusers (Aqua One, Australia).

The water parameters including temperature, dissolved oxygen (DO) and pH were monitored daily. An Oxyguard® digital DO meter (Handy Polaris 2, Norway) was used for DO and temperature measurements, and an Ecoscan pH 5 meter (Eutech instruments, Singapore) was used to record pH. A DR/890 portable colorimeter with Permachem reagents (Hach, USA) were used to analyse the total ammonia nitrogen (TAN), nitrite (NO_2-N), nitrate (NO_3-N) and reactive phosphate (PO_4) once a week. The experiment was static i.e. no water exchange was made and periphyton growth on tank edges was allowed. Tank water level was maintained at 150 L throughout the experiment by adding water to compensate for losses due to the evaporation

9.2.3. Outdoor plankton culture, formulated feed preparation and feeding

Commonly found plankton in commercial aquaculture ponds were collected from Blue Gum Lake and Neil McDougall Park Lake, Perth, Western Australia using plankton net and were cultured in 300L water capacity tanks as a stock culture in outdoor conditions of direct sunlight and continuous aeration. The plankton stock culture tanks were filled with 150 L of filtered freshwater and Aquasol® by Yates Pty Ltd was added to the tanks to boost and maintain the phytoplankton density at 10.36×10^6 cells L^{-1}. Similarly zooplankton were grown at the density

of 900 individuals L^{-1} and were fed with outdoor cultured phytoplankton. Fish meal as a main animal protein source was used to prepare the formulated pelleted marron feed. For 90 days of the experiment marron were fed every evening at the rate of 2% of their body weight. Unconsumed feed was collected with a fine mesh net after an hour of feed addition.

9.2.3.1. Plankton analysis

The plankton samples were collected every second day by filtering 1 L tank water for phytoplankton and 5 L for zooplankton. On filtering for plankton, the left-over water was added to the respective tanks. Plankton samples were collected and stored in 100 mL plastic containers and were counted on the same day using compound and dissection microscope respectively. The plankton analysis was conducted as per the procedure described by Tulsankar et al. (2021b).

9.2.3.1.1. Plankton species cultured for the experiment

Freshwater phytoplankton commonly found in marron ponds were cultured: green algae: *Chlorella* spp., *Scenedesmus* spp., *Volvox* spp., *Euglena* spp., and diatoms *Navicula* spp., *Nitzschia* spp., and *Sellaphora* spp. The zooplankton species commonly found in marron ponds were also grown: *Copepod* spp., *Keratella quadrata, Keratella cochlearis* and *Daphnia* spp. Both phytoplankton and zooplankton cultures were fed to juvenile marron. There was no significant difference in plankton densities in experimental tanks over the experimental time.

9.2.4. Marron growth analysis

9.2.4.1. Marron specific growth, survival rate and weight gain percentage

Individual marron were weighed and measured every fortnight to obtain weight gain, OCL gain and total length gain. Mortality was noted on the same day as it occurred. Specific growth rate (SGR), weight gain percentage (WG) and survival rate (SR) were analysed by using the following equations,

SGR (g % /day) = [ln (final body weight)-ln (initial body weight)/days] x 100.

WG (g %) = [final body weight – initial body weight/initial body weight] x 100.

SR (%) = (Final number of marron alive /initial number of marron stocked) x 100.

9.2.4.2. Marron moulting

Moult intervals, weight and length increment at moult were measured on the basis of days required for n+1 moult and increment in weight and total length between two successive moults using the following equations-

Moult interval (Tm, days): $T_m = T_{n+1} - T_n$

Weight increment at moult (MIW; g, %) = (weight after second moult (n+1) - weight after first moult (n)) x 100/ weight after first moult.

Length increment at moult (MIL; cm %) = (length after second moult (n+1) - total length after first moult) x 100/ length after first moult.

Where, T_n= date of n moult, T_{n+1}= date of n+1 moult.

9.2.5. Marron health indices

Marron health indices were analysed at the end of the experiment, by testing the haemolymph for total haemocyte count (THC), differential haemocyte count (DHC), haemolymph lysozyme activity and haemolymph clotting time, hepatopancreas moisture content (HM %), wet weight (Hiw) and dry weight (Hid) indices, tail muscle moisture content (TM %), wet weight (TMiw), and dry weight (TMid) indices. Haemolymph samples were collected from one randomly selected marron per tank, by using a 1 mL syringe. The syringe was inserted in between the third and fourth pair of pereopods and 0.2 mL of haemolymph was extracted. To avoid the clotting process the syringes were pre-filled with 0.2 µL of anti-coagulant (100mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 15.5 mM NaCl and 10 mM EDTA). The total haemocyte count was conducted within an hour of sampling. A sub-sample of haemolymph was transferred to the haemocytometer (Neubauer, Germany) and observed under 40x magnification using a compound microscope, cells in four random squares were recorded. Three counts of haemolymph sample for each marron was conducted and the average value was recorded and expressed as THC in 1 mL by using an equation by Nugroho and Fotedar (2013). The DHC analysis was conducted as described by Nugroho and Fotedar (2013).

To evaluate the haemolymph clotting time 0.2 mL of haemolymph was drawn as for THC without using anti-coagulant and was transferred to plain capillary tubes (Chase, Scientific glass, Rockwood, TN). The capillary tube was repeatedly inverted until the haemolymph stopped moving, and the time was noted in seconds as haemolymph clotting time. Haemolymph lysozyme activity was calculated using turbidimetric assay. Haemolymph samples (50 µL)

were pipetted in duplicates, in a 96-well micro-plate (Iwaki, Tokyo, Japan). After 15 minutes of incubation at room temperature, 50 µL of *Micrococcus lysodeiktikus* (Sigma-Aldrich, St. Louis, USA) suspended in PBS (0.25 mgL^{-1}) was added to each well plate. The absorbance of the well plate was measured at two minute intervals for 20 minutes (11 times) at 450 nm wavelength with MS212 reader (Titertek Plus, Tecan, Austria) at 25°C. One unit of lysozyme activity was defined as the amount of enzyme which resulted in a decrease in absorbance of 0.001/minute. The lysozyme activity is expressed as EU/mL.

At the end of the experiment, one marron per tank was randomly selected to collect the hepatopancreas and tail muscle and was weighed for wet weight indices. To obtain the dry weight indices, the samples were dried in crucibles at 105ºC in an oven for 24 hrs. Hepatopancreas moisture content (HM %), wet weight (Hiw) and dry weight (Hid) indices, tail muscle moisture content (TM %), wet weight (TMiw), and dry weight (TMid) indices were calculated as described by Fotedar (1998).

9.2.6. Stable isotope analysis

As marron are known to feed on plant and animal matter, the live food samples of phytoplankton, zooplankton and periphyton grown on tank edges were collected for stable isotope analysis from all sixteen tanks, 15 days prior to the termination of the experiment. For plankton samples, tank water was filtered with 47 µm glass fibre filter paper using a vacuum pump and left-over water was added to the respective tanks. For detritus samples, the tank bottom water was siphoned and centrifuged for 4 minutes at 3000 rpm and the pellet representing the settled detritus was collected into Eppendorf tubes. All samples were stored at -80ºC until the analysis. Similarly, at the end of the experiment, one marron per tank was dissected to provide tail muscle and whole marron samples. Following 24 hrs oven drying the tail muscle and whole marron samples were grounded to a fine powder in a homogeniser. Whole marron samples were prepared by digesting the whole marron powder with 1% hydrochloric acid (HCL) at 50ºC using a magnetic stirrer to remove the carbonates. HCL was added drop by drop to the sample until the bubbles disappeared. Once the digestion was complete, samples were cleaned by centrifugation using distilled water until pH 7 was achieved in the sample. Following centrifugation the tail muscle and whole marron samples were freeze dried for 24 hours.

Plankton, periphyton and detritus samples were also freeze dried for 24 hrs prior to stable isotope analysis. The stable isotope analysis was conducted at the West Australian

Biochemistry Centre (WABC), The University of Western Australia, Perth. Samples were analysed for $\delta^{15}N$ and $\delta^{13}C$, using a continuous flow system consisting of a Delta V Plus mass spectrometer connected with a Thermo Flush 1112 via CONFLO IV (Thermo Fisher Scientific, Finnigan, Germany). The stable isotope ratio was expressed in the standard δ-notation (‰). Carbon and nitrogen isotope values ($\delta^{15}N$ and $\delta^{13}C$) from tail muscle, whole marron and for all food source were measured.

9.2.7. Histology analysis

At the end of the experiment, one marron per tank was dissected to collect samples for hepatopancreas, tail muscle tissue and midgut and were preserved using 10% formalin. The slide preparation was conducted at the histopathology laboratory, Department of Agriculture, Perth, Western Australia. The samples were stained with Haematoxylin and Eosin and were observed under the light microscope at 40x magnification.

2.8. Gut histomorphology

The midgut samples were prepared as described above for the hepatopancreas and the epithelium height (EH) and lumen length (Lu) was measured using digital imaging software (Adobe Photoshop version 22.4.3, Adobe System Incorporated, USA).

9.2.8. Water, detritus and gut microbiomes analysis

9.2.8.1. DNA extraction and 16S rRNA sequencing

For water samples, 1 L of water from each tank was centrifuged at 4°C, at 10,000 rpm speed for 10 minutes to obtain a pellet. Detritus samples were carefully collected with mesh net from the bottom of the tank over the 15 days previous to experiment termination. For marron gut microbiota analysis, one marron per tank (n =4) was collected at the end of the trial and the gut was collected inside a biological safety cabinet. Gut content with its mucosa was homogenised in tissue lyserII (Qiagen, Hilden, Germany) with sterile beads, and transferred in 200 mg aliquots into 1.5 ml Eppendorf tubes for DNA extraction. To achieve the consistency in sensitivity and specificity of DNA from three different sources, DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) was used for bacterial genomic DNA with extraction following the manufacturer's instructions. Extracted DNA was diluted into 50 ng/µl final concentration. PCR amplification of V3V4 bacterial hypervariable regions was carried out with 50 µl of reaction mixture containing 25 µl 2X Hot Start Taq 2X Master Mix (New England BioLabs Inc., Ipswich, MA, USA), 2µl of template DNA, 1 µl of 0.2 µM forward and reverse primers, and 21 µl of nuclease-free water. A 35 cycles of PCR amplification was carried out in an EP

Gradient Thermal Cycler (Eppendorf, Hamburg, Germany) according to conditions mentioned in Hot Start Taq 2X Master Mix with 55°C annealing temperature. Bead purification of PCR products and amplicon barcoding via an index PCR were performed according to Illumina standard protocol for 16S metagenomic sequencing library preparation (Part # 15044223 Rev. B). Pair-end (2 × 300 bp) sequencing of the barcoded amplicons was performed on an Illumina NextSeq instrument using v3 chemistry (600 cycles).

9.2.8.2. Processing of Illumina sequence

The preliminary quality of raw sequences was checked in fastQC pipeline (Andrews, 2010). BBduk was used for quality trimming and removal of adapter sequences (Bushnell, 2014) with the following parameters: qtrim=r, trimq=20, ktrim=r, k=23, mink=11, hdist=1, minlen=200, tpe, tbo. The merging, filtering, deduplicating (fastq-uniques) and picking of amplicon sequence variants (ASVs) were performed in USEARCH pipeline by implementing-UPARSE and UNOISE3 pipeline (Edgar, 2013, Edgar, 2016b, Edgar, 2010). The final set of ASVs were filtered again for chimeras using UCHIME2 (Edgar, 2016a). UNOISE3 flow was used to map all the merge reads to non-chimeric ASVs table. Each representative ASV was assigned to different taxa level against SILVA 132 release (Quast et al., 2012). Multiple sequence alignment was performed using clustal omega (ClustalO) (Sievers and Higgins, 2014). Subsequently, a rooted phylogenetic tree was constructed in micca (v1.7.0) root pipeline (Albanese et al., 2015). Each sample of gut, water and detritus was set to a uniform depth of 3669 bp, 1414 bp and 4441 bp respectively, and subsequent calculation of alpha-beta diversity and microbial community composition were performed in QIIME (v1.9.1) pipeline (Kuczynski et al., 2011) and microbiomeSeq (https://github.com/umerijaz/microbiomeSeq), phyloseq (McMurdie and Holmes, 2013) and ampvis2 (Andersen et al., 2018) R packages.

9.2.9. Statistical analysis

All numerical data were statistically analysed using software SPSS 26 (IBM®) and the results are presented as mean ± standard error. A one way ANOVA with LSD post hoc test was used to find significant differences between the treatments. Kruskal-Wallis tests were used when the data did not comply with the assumption of homogeneity. To tests the significant differences between C and N isotopic values of the potential food source in the tail muscle and whole marron an independent t-test was used. To determine the relative isotopic contribution of each food to tail muscle and whole marron, Bayesian Stable Isotopic Mixing Model was used, complemented by Stable Isotope Analysis in R (Parnell et al., 2010). Non-parametric

multivariate analysis of the distance metric was performed using ANODIS with 1000 permutations. Statistically significant bacteria at genus level in different groups were identified using Linear Discriminant Analysis (LDA) at LDA cut-off value of 2.0 (Segata et al., 2011). All tests were considered statistically significant at $p<0.05$.

9.3. Results

9.3.1. Water quality parameters

All the water quality parameters (Table 9.1) were in the optimum range for marron culture. Orthophosphate concentrations (mg L^{-1}) were significantly higher in all tanks except in tanks where marron were fed formulated feed only.

Table 9.1. Water quality parameters temperature, dissolved oxygen (DO), pH, total ammonia nitrogen (TAN), nitrite, nitrate, orthophosphate, phytoplankton, zooplankton maintained in marron rearing tanks during the 90 days of culture time (mean ± S. E.).

Parameters	**T1**	**T2**	**T3**	**T4**
Temperature (ºC)	21.6 ± 0.02	21.6 ± 0.02	21.6 ± 0.06	21.6 ± 0.02
DO (mg L^{-1})	7.24 ± 0.01	7.22 ± 0.01	7.23 ± 0.01	7.20 ± 0.02
pH	7.72 ±0.01	7.61 ± 0.02	7.56 ± 0.02	7.57 ± 0.01
TAN (NH_3-N; mg L^{-1})	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00
Nitrite (NO_2-N; mg L^{-1})	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
Nitrate (NO_3-N; mg L^{-1})	3.16 ± 0.17	3.13 ± 0.10	3.17 ± 0.13	3.22 ± 0.11
Orthophosphate (mg L^{-1})	0.28 ± 0.006^a	0.32 ± 0.001^b	0.32 ± 0.006^b	0.32 ± 0.006^b
Phytoplankton density ($x10^6$ cells L^{-1})	NA	4.41 ± 0.10	NA	4.39 ± 0.12
Zooplankton density (individuals L^{-1})	NA	NA	494 ± 2.88	498 ± 2.91

The superscripted letters ^{a} and ^{b} show the significant differences between the treatments ($p<0.05$), for the water quality parameters maintained for juvenile marron cultured for 90 days under controlled laboratory conditions feeding live plankton and formulated feed. **Abbreviations:** T1= Control (formulated feed), T2= Phytoplankton and formulated feed, T3= Zooplankton and formulated feed, T4= mixture of phytoplankton, zooplankton and formulated feed.

9.3.2. Marron survival and growth rate

Growth and survival rate of juvenile marron fed with different live planktons and formulated feed are shown in Table 9.2. Specific growth rate (SGR) and weight gain % (WG) were significantly affected by the dietary treatments ($p<0.005$), with the highest SGR and WG %

observed in juvenile marron fed with zooplankton. Survival rate was significantly highest with the use of plankton mixture (T4).

Table 9.2. Specific growth rate, weight gain, survival and marron moulting days, increment in weight and length after the moulting of juvenile marron fed with different live plankton and formulated feed (mean ± S. E.).

Treatments	**T1**	**T2**	**T3**	**T4**
SGR (g % /day)	1.97 ± 0.01^{ab}	1.89 ± 0.02^{a}	2.13 ± 0.06^{c}	2.06 ± 0.03^{bc}
Weight gain (%)	33.8 ± 2.13^{a}	35.6 ± 5.53^{ab}	49.8 ± 3.70^{c}	46.9 ± 2.42^{bc}
Survival (%)	83.4 ± 3.20^{a}	83.4 ± 3.20^{a}	90.0 ± 1.20^{b}	97.2 ± 2.78^{c}
Moulting days	14.8 ± 0.85	14.8 ± 0.63	14.0 ± 0.41	14.3 ± 0.48
Weight increment at moult (gm; %)	29.3 ± 1.14^{a}	32.3 ± 1.08^{b}	34.5 ± 0.68^{bc}	35.7 ± 0.75^{c}
Length increment at moult (cm; %)	5.61 ± 0.32^{a}	6.33 ± 0.14^{ab}	6.45 ± 0.26^{b}	6.34 ± 0.25^{ab}

Superscripted letters $^{a, b}$ and ^{c} represents the significant differences between the treatments for growth and survival of juvenile marron cultured for 90 days under controlled laboratory conditions feeding live plankton and formulated feed. **Abbreviations:** T1= Control (formulated feed), T2= Phytoplankton and formulated feed, T3= Zooplankton and formulated feed, T4= mixture of phytoplankton, zooplankton and formulated feed.

9.3.3. Haemolymph parameters

Total haemocyte count of juvenile marron ranged between 2.44 and 6.49 x10^6 cells mL^{-1} (Table 9.3). The higher granular cells % was observed in marron fed zooplankton and mixed plankton. The lysozyme activity in juvenile marron was significantly enhanced ($p<0.05$) with the use of zooplankton and mixture of plankton. The clotting time was significantly less ($p<0.05$) for marron fed with zooplankton and mixed plankton, whereas the marron haemolymph from the control and the phytoplankton treatment required > 45.5 seconds.

Table 9.3. Total haemolymph count (cells x 10^6 mL^{-1}), hyaline (%), granular (%), semi-granular (%), lysozyme (EU mL^{-1}), clotting time (seconds), wet weight and dry weight indices of hepatopancreas and tail muscle of juvenile marron cultured for 90 days and fed with different live planktons and formulated feed (mean ± S. E.).

Treatments	**T1**	**T2**	**T3**	**T4**
THC (cells x 10^6 mL^{-1})	2.44 ± 0.12^{a}	3.73 ± 0.20^{b}	5.61 ± 0.13^{c}	6.49 ± 0.18^{d}
Hyaline (%)	59.1 ± 0.84^{b}	58.3 ± 1.88^{b}	50.5 ± 0.36^{a}	51.6 ± 0.90^{a}

Granular (%)	31.3 ± 0.64^{a}	30.4 ± 2.08^{a}	36.4 ± 1.58^{b}	38.2 ± 0.79^{b}
Semi-granular (%)	8.93 ± 0.33	10.2 ± 0.85	11.9 ± 2.26	9.71 ± 1.18
Lysozyme (EU/mL)	6.65 ± 3.50^{ab}	6.03 ± 1.65^{a}	9.08 ± 0.48^{c}	7.45 ± 5.50^{b}
Clotting time (Sec)	56.5 ± 2.84^{c}	45.5 ± 0.65^{b}	33.5 ± 1.55^{a}	35.8 ± 2.17^{a}
HM (%)	78.3 ± 1.31^{b}	76.4 ± 1.09^{b}	67.3 ± 0.41^{a}	67.3 ± 0.88^{a}
TM (%)	78.9 ± 1.04^{c}	76.7 ± 1.05^{bc}	74.6 ± 1.00^{ba}	72.7 ± 0.76^{a}
HiW (%)	6.60 ± 0.18	6.58 ± 0.41	6.48 ± 0.22	6.21 ± 0.19
HiD (%)	1.69 ± 0.22	2.12 ± 0.11	2.05 ± 0.11	1.96 ± 0.14
TmiW (%)	34.7 ± 0.52	34.1 ± 0.64	35.4 ± 0.83	36.6 ± 0.42
TMiD (%)	6.46 ± 0.22^{a}	7.04 ± 0.11^{a}	8.22 ± 0.59^{b}	7.69 ± 0.07^{ab}

Superscripted letters $^{a, b, c}$ and ^{d} are showing a significant difference between the treatments for different health indices of marron fed with live plankton and formulated feed for 90 days. **Abbreviations:** T1= Control (formulated feed), T2= Phytoplankton and formulated feed, T3= Zooplankton and formulated feed, T4= mixture of phytoplankton, zooplankton and formulated feed.

9.3.4. Stable isotope analysis

One marron per replicate (4 marron per treatment) was randomly selected to determine the values of $\delta^{13}C$ and $\delta^{15}N$ of all possible food sources such as plankton, formulated feed and overgrown periphyton on tank edges. Stable isotope composition ($\delta^{13}C‰$, $\delta^{15}N‰$), elemental composition (%C, %N) and discrimination factor ($\Delta^{13}C$, $\Delta^{15}N$) of the potential food sources for juvenile marron during the experiment were measured (mean ± S.D.; Table #S1/Appendix 6). The values of $\delta^{13}C$ and $\delta^{15}N$ of tail muscle and whole marron did not differ significantly ($p>0.05$) between the treatments (Table #S1).

Dietary contributions of the different sources to juvenile marron growth with live plankton and formulated feeds are shown in Figure 9.1. Marron fed with formulated feed (T1) had a higher contribution of formulated feed (42.7%) followed by detritus (31%) towards the tail muscle and whole marron (39 % and 31 % respectively). In marron fed with formulated feed and phytoplankton (T2), formulated feed and detritus had equal contribution (27%) towards the tail muscle but phytoplankton had the highest contribution (29%) towards the whole marron. Marron fed with zooplankton and formulated feed (T3) had a highest contribution of zooplankton (39%) followed by detritus (37%) and least from formulated feed (8%) towards the tail muscle. Marron fed with mixture of phytoplankton and zooplankton with formulated

feed (T4) had higher contribution from zooplankton (25%) and detritus (24%) towards the tail muscle and least of phytoplankton (10%).

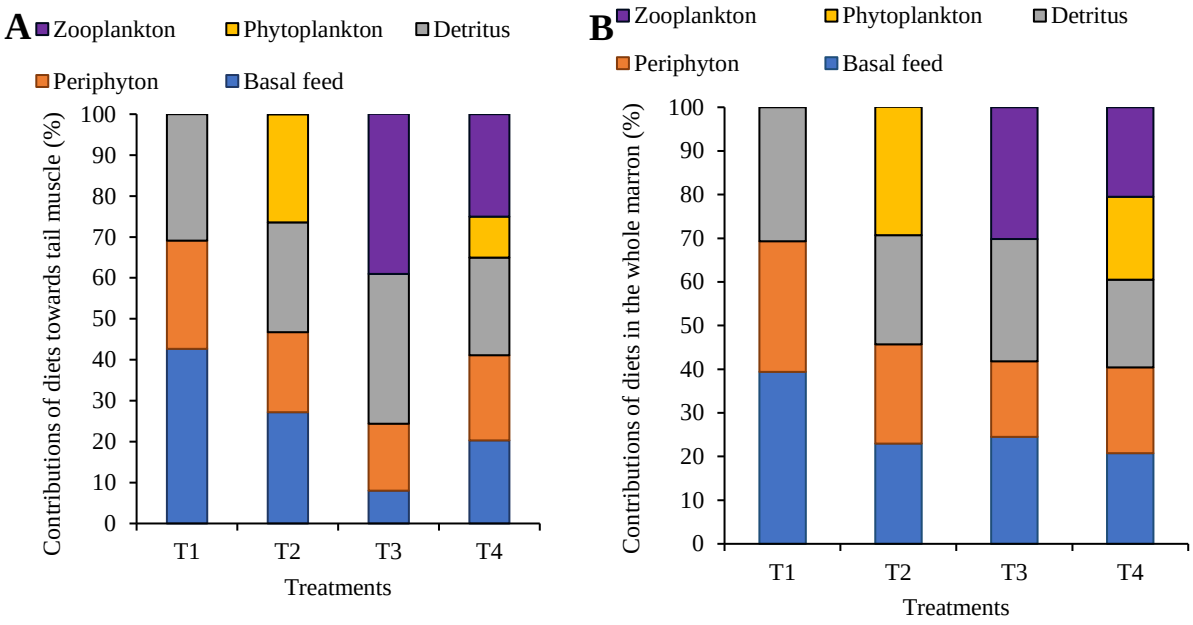

Figure 9.1. The contributions of plankton, periphyton grown on tank edges, detritus collected from tank bottom and formulated feed towards the marron tail muscle and whole marron cultured for 90 days under controlled laboratory conditions. **Abbreviations:** T1= Control (formulated feed), T2= Phytoplankton and formulated feed, T3= Zooplankton and formulated feed, T4= mixture of phytoplankton, zooplankton and formulated feed.

9.3.5. Histology analysis

The histological sections of hepatopancreas, tail muscle tissue and midgut from all treatments are illustrated in Figure 9.2. These organs exhibited organised typical structure in all treatment groups of juvenile marron. Muscle tissue provides support for the body and functions as the primary tissue, involved in locomotion, movement of appendages and are composed of striated fibres or myofibrils which are visible at 40 X (Ross et al., 2019). The muscle tissue includes several muscle fibres (containing nuclei), a typical pattern of alternate darker (anistrophic) and lighter (isotrophic) striation was observed in all treatments. Marron tail muscle tissue showed slight fractures in juvenile marron fed with phytoplankton and formulated feed, whereas in marron fed with zooplankton and mixed plankton an improved straight strands of tissue myofibres was observed. Marron midgut showed a typical structure with lumen and epithelium in all treatments (Figure 9.3), however the epithelium height was improved in marron fed with zooplankton (Figure 9.3A).

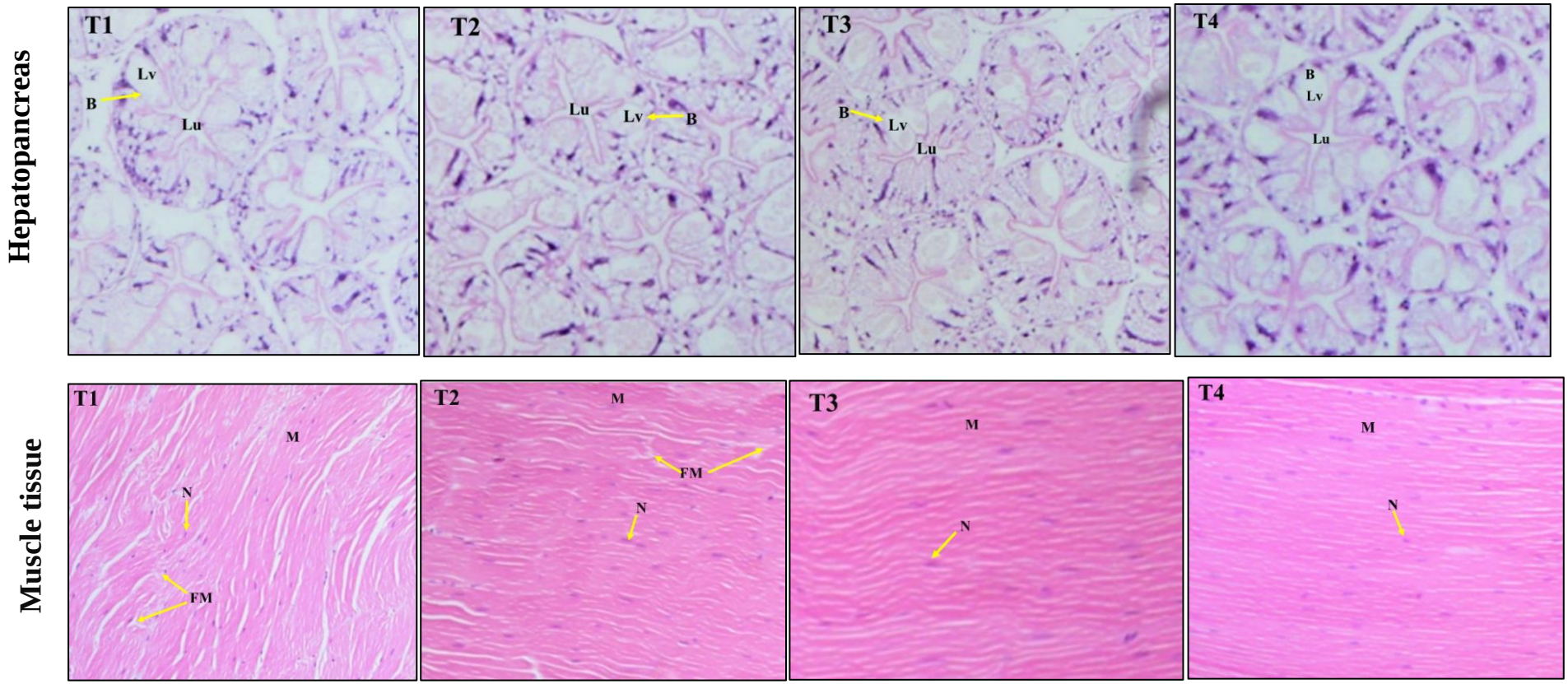

Figure 9.2. Histo-morphological structure of marron hepatopancreas and tail muscle tissue fed with different dietary treatments. Typical structure of hepatopancreas tubules with star shaped tubule lumen (Lu) was observed in marron fed with all types of feeds containing secretary cells (Blasenzellen, B-cells), Lv- large vacuole. Muscle tissue of marron with ordered myofibers (M), oval nuclei (N), and damaged muscles exhibited slight fracture (FM) in marron from T1 and T2. Haematoxylin and Eosin (40x). **Abbreviations:** T1= Control (formulated feed), T2= Phytoplankton and formulated feed, T3= Zooplankton and formulated feed, T4= mixture of phytoplankton, zooplankton and formulated feed.

Midgut

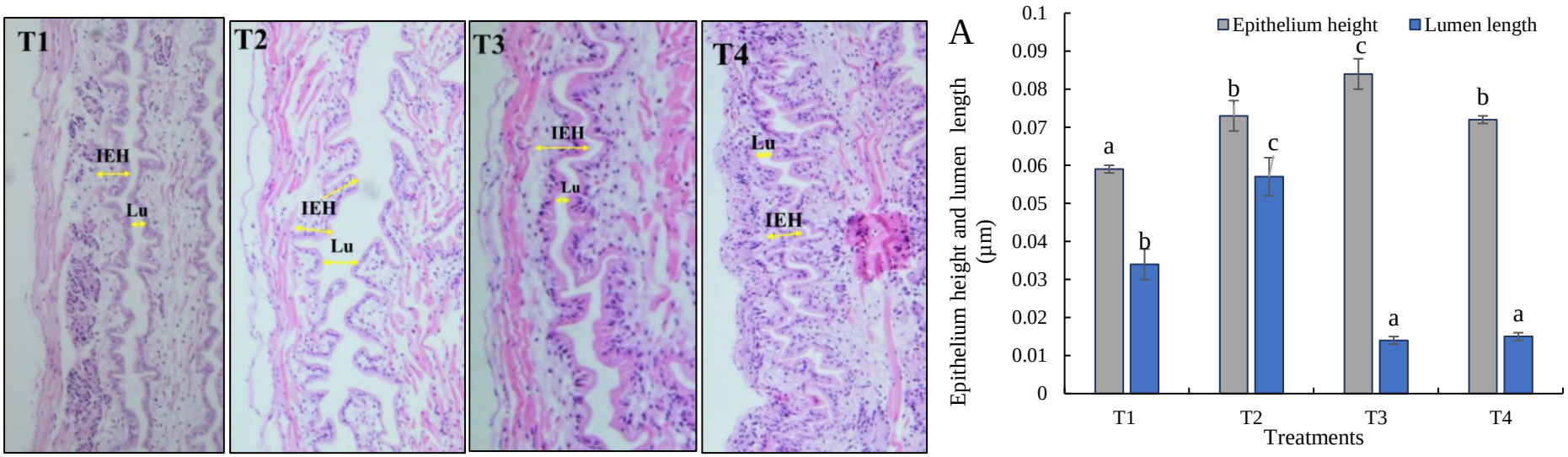

Figure 9.3. Histo-morphological structure of marron midgut fed with different dietary treatments. IEH= intestinal epithelium height, Lu=intestine lumen. Arrows indicates the intestinal epithelium height (IEH) and intestinal lumen (Lu). Variation in IEH and Lu length (µm) of marron midgut fed with formulated feed and different live planktons for 90 days (A). Different letters indicate a significant difference at α-level of 0.05. Haematoxylin and Eosin (40x). **Abbreviations:** T1= Control (formulated feed), T2= Phytoplankton and formulated feed, T3= Zooplankton and formulated feed, T4= mixture of phytoplankton, zooplankton and formulated feed.

9.3.6. Sequence statistics and alpha-beta diversity

A total of 1,263,275 high quality reads were obtained from 48 samples, ranging from 24,688 to 126,492. After quality trimming, 1,063,906 sequences were merged (84.22%) resulting in 4739 ASVs with USEARCH-UNOISE pipeline. A good coverage index value of 0.992-0.998 indicated that each sample was sequenced at high depth and up to highest saturation level. Alpha-diversity measurements showed higher Chao1 diversity only in water samples while no differences in richness and Shannon index was observed among the three different sampling matrices: gut, detritus and water (Figure 9.4 A-C). Beta dispersion ANODIS (R=0.6655, p=0.001) revealed significant differences in microbial communities between and among samples from three different sources (Figure 9.4 D). However, when comparing between treatments, only T4 in the gut showed the highest alpha-diversity in terms of richness, Shannon and Chao1 (Figure 9.4 E-G) compared to T2 and T3. Again, dispersion R value of 0.4935 and p-value of 0.002 revealed significant effects of treatments on gut microbial communities of marron. Compared to gut and water (R=0.339, p=0.002) (Figure 9.4 L), feeding treatment had the least significant effect on detritus microbial communities (R=0.3338, p=0.023) (Figure 9.4 P). Though alpha-diversity within the treatments were different for the gut, no differences in alpha diversity between treatments for the detritus (Figure 9.4 I-K) and water (Figure 9.4 M-O) samples were identified.

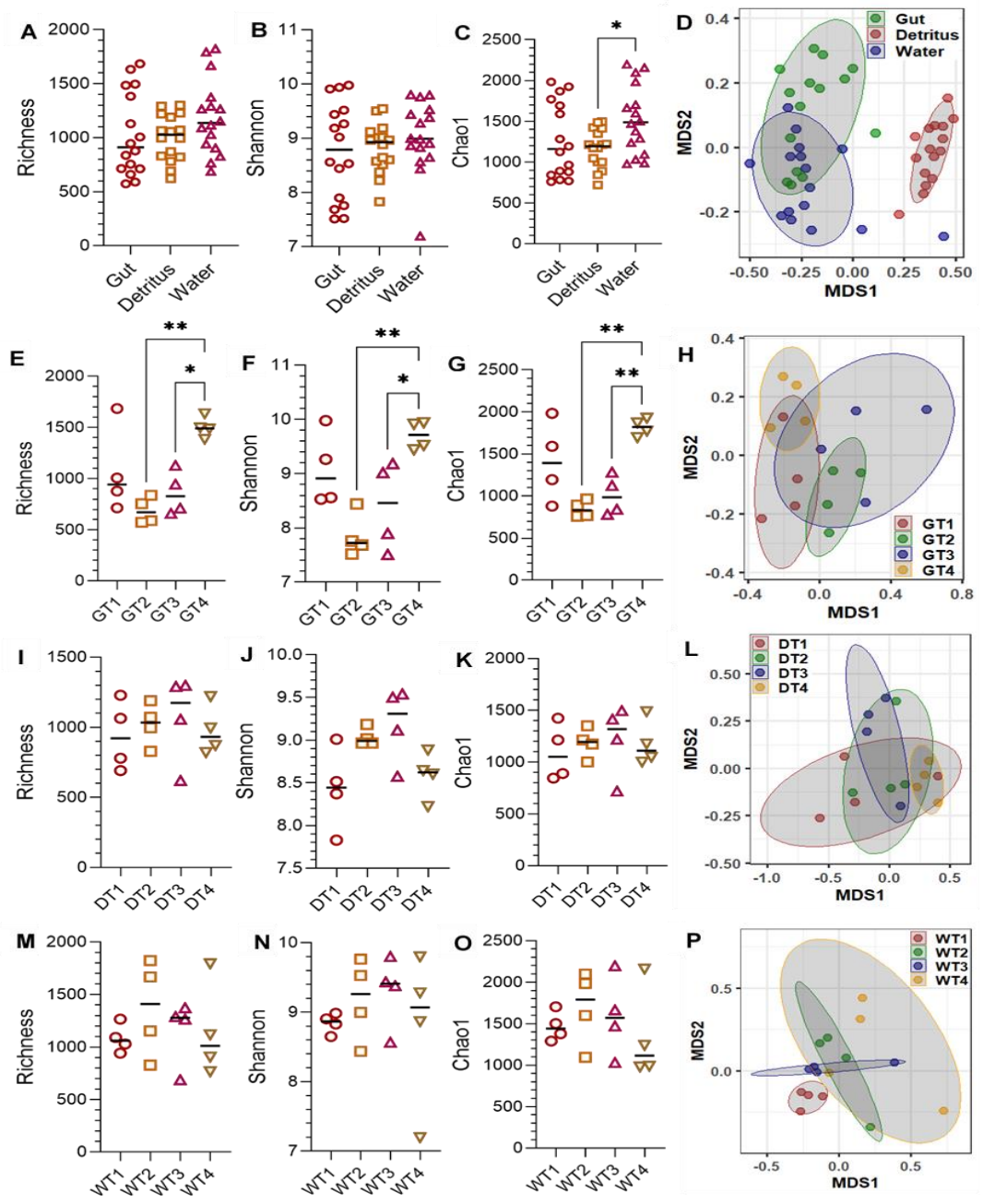

Figure 9.4. Alpha-beta diversity analysis at the conclusion of the feeding trial. (A-C) Alpha diversity measurements in three different matrices: gut, detritus and water. (D) Beta-ordination PCoA plot for gut, detritus and water samples. (E-G) Alpha diversity measurements in the gut (G) of marron for four different treatment: T1, T2, T3 and T4. (H) Beta-ordination PCoA plot for the gut samples. (I-K) Alpha diversity measurements in the detritus (D) of tank for four different treatment: T1, T2, T3 and T4. (L) Beta-ordination PCoA plot for the detritus samples. (M-O) Alpha diversity measurements in the tank water (W) for four different treatment: T1, T2, T3 and T4. (P) Beta-ordination PCoA plot for the tank water samples. GT- gut, DT- detritus, WT- water. ○- T1; □- T2; Δ- T3; ▽- T4

9.3.7. Microbial composition

Candidatus Bacilloplasma, *Dinghuibater*, and *Aeromonas* were most predominant bacteria in the gut, detritus and water representing 24.8%, 36.4% and 42.6% of the read abundance (Figure 9.5). However, the relative abundance of *C.* Bacilloplasma, *Dinghuibater*, and *Aeromonas* reached 62.2%, 41.9% and 52.7% for the T2 treatment in the gut, T4 in the detritus and T1 in the water, respectively. Beside *C.* Bacilloplasma, the higher abundance of *Aeromonas* and *Hafnia*-Obesumbacterium were detected in the marron gut of T4 groups, while higher *Vibrio* abundance was observed in the T1 treatment. With LDA score of ≥2.0, nine significantly different genera were found in the gut of marron for all four treatments, these genera being *Vibrio* in T1, *Lactovum*, *Nocardioides*, *Hyphomicrobium* and *C.* Bacilloplasma in T2, *C.* Hepatoplasma in T3, and *Citrobacter*, *Acinetobacter* and *Hafnia*-Obesumbacterium in T4 (Figure 9.6 A). On the other hand, two genera were found enriched in both detritus and water (Figure 9.6 B-C). *Gemmata* and *Phenylobacterium* were significantly different in DT1 and DT4 whereas *Aeromonas* and *Pseudomonas* had significantly higher abundance in WT1 and WT2, respectively.

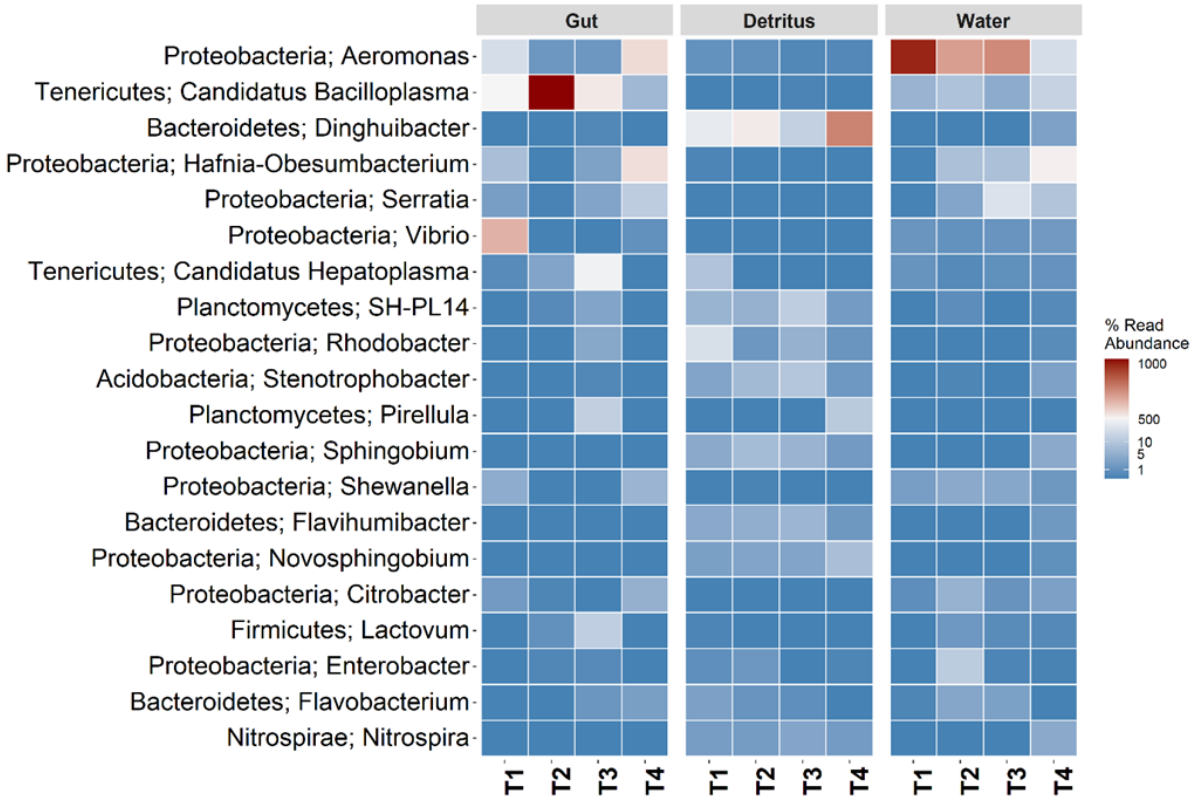

Figure 9.5. Relative abundance of bacteria at genus level in three sampling sources; gut, detritus and water (top), and four different treatments, T1, T2, T3 and T4 (bottom).

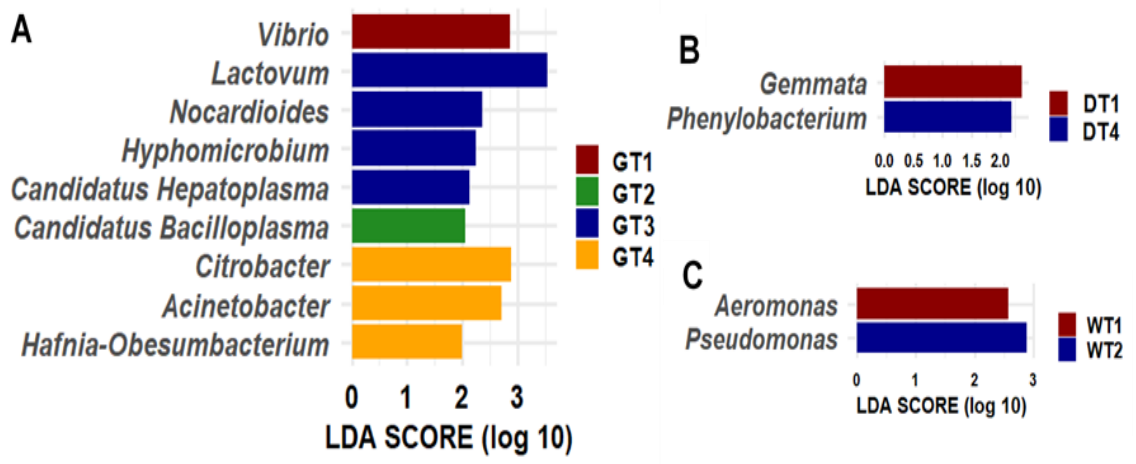

Figure 9.6. Significantly different bacteria in the gut (A), detritus (B) and water (C) at genus level at the conclusion of the trial.

9.4. Discussion

Information on the benefits of dietary plankton on health, gut microbiota and gut histology of *Cherax* spp. is sparse. The current research is first study on the benefits of supplementing dietary plankton to marron. Juvenile marron growth, survival, health indices, intestinal microbiota, histo-morphology of vital organs and plankton contributions towards the tail muscle and whole juvenile marron under controlled laboratory conditions have been described. The research will be useful to understand the nutritional requirements of juvenile marron.

Dietary supplementation of plankton had a positive impact on growth, survival and immunity of juvenile marron. Our results aligned with the findings of Sáez-Royuela et al. (2007), Jones et al. (1995), Austin et al. (1997), Verhoef et al. (1998), who observed that the supplementation of zooplankton improved the growth and survival rate of juvenile red claw (*C. quadricarinatus*), yabbies (*C. destructor*) and signal crayfish (*Pacifastacus leniusculus*). Improved growth rates of juvenile marron fed with zooplankton reflects the constant supply of nutrients. Whereas the formulated feed has a tendency to leach out most of the water-soluble nutrients into the surrounding waters (Smith et al., 2002). Zooplankton are an important component of the natural diet of freshwater crayfish. Cladocerans and copepod supplementation has shown to improve growth in red claw and yabbies (Verhoef et al., 1998, Jones et al., 1995, Duffy et al., 2011), similar to the improved growth of juvenile marron in the current study. The rapid growth rate of yabbies (*C. albidus*) was recorded on feeding with a diet solely consisting of zooplankton (Mitchell and Collins, 1989, Jones et al., 1995). In all our treatments the survival rate of juvenile marron was higher (>80%) compared to the observed

survival rate of juvenile signal crayfish at 53.3% when fed with a combination of phytoplankton and zooplankton, and only 10.7% survival when fed with a diet of phytoplankton alone (Celada et al., 1989).

Improved immune parameters were observed in freshwater pearl mussel (*Hyriopsis bialatus*) and Chinese mitten crab (*Eriocheir sinesis*) (Supannapong et al., 2008, Jiang et al., 2020), with the supplementation of plankton, where authors suggested that the natural astaxanthin improved the immunity of the animals. In our study, zooplankton and mixed plankton provided better nutrition to the juvenile marron, with similar moulting time recorded for all dietary treatments, but the weight and length at moulting were significantly higher with the supplementation of live plankton compared to formulated feed alone. A constant plankton supplementation may have allowed continuous feeding through filter feeding and scavenging of detritus (Tulsankar et al., 2021d, Van den Berg et al., 1990).

Hepatopancreas and tail muscle moisture content have been used as indicators of crayfish health (Jussila and Mannonen, 1997, McClain, 1995a). Zooplankton and mixed plankton supplementation significantly improved the health of juvenile marron, as shown by the increase in THC, % of granular cells, higher lysozyme activity and time required to clot the haemolymph. The moisture content of the hepatopancreas was also significantly lower in marron fed with zooplankton and mixed plankton, indicating better health condition (Jussila, 1999). Our results on dry weight muscle somatic indices were similar to the findings of Fotedar et al. (1999), where the authors suggested that a marron in good condition had 6-9% dry tail weight muscle index with the higher percentage measured in marron fed with zooplankton. The addition of live copepods, daphnia and rotifers may have provided a valuable source of protein and essential vitamins and minerals for the juvenile marron (Manickam et al., 2020), contributing to their nutrition and improving their tail muscle condition.

Marron exhibited considerable variability in feed source, indicating feeding on a variety of diets ranging from the periphyton grown on tank edge, supplied plankton and the detritus formed at the tank bottom. SIA showed that when provided with only formulated feed (control) as the primary diet source to juvenile marron, availability of naturally occurring sources such as the overgrown periphyton on tank edges and formed detritus appears to be a factor in the relative contribution to the marron diet. For example in the control tanks, the periphyton grown on tank edges and the bottom detritus contributed towards a significant proportion of the nutrition of juvenile marron.

In all treatments, detritus remained an important contributor to the marron diet, similarly detritus was the most frequent food item in stomach content analysis of red claw (Alcorlo et

al., 2004). Comparable results were observed in semi-intensively farmed shrimp (*Litopenaeus vennamei*) (Gamboa-delgado et al., 2003). Detritus and zooplankton are an important food source for crayfish due to their nutritional composition and their availability (Brown et al., 1992, Jones, 1995a, Gamboa-delgado et al., 2003). In between feeding, the marron may have sought additional nutrition from the plankton, periphyton and detritus. Furthermore, when provided with the mixture of phytoplankton and zooplankton, the SIA revealed that phytoplankton had the lowest contribution towards the tail muscle of all provided feeds, which indicates juvenile marron preferred animal protein over the phytoplankton or plant protein. These findings aligned with the outcome of a study conducted by Beatty (2006), where the isotope analysis of marron collected from the Hutt River showed that the marron assimilated a higher proportion of animal matter than plant matter.

However when marron received a supplementation of phytoplankton alone, phytoplankton contributed >26 % towards the tail muscle which was higher than the contribution of periphyton and highest contribution (>29%) towards the whole marron. Lack of animal prey in the phytoplankton treatment may have increased the importance of plant food sources (Alcorlo et al., 2004), as in the absence of animal prey crayfish consume plant tissues and plants (Momot, 1995). This suggest that phytoplankton can be a substitute source of nutrition for the juvenile marron in the absence of zooplankton or other sources of animal protein. Although phytoplankton supplementation provided less benefit compared to a supplementation of zooplankton and mixed plankton, dietary phytoplankton did improve growth and health conditions when compared to the control marron fed only formulated feed. Currently, the contribution of phytoplankton to marron nutrition is not defined in the published literature, with most studies focusing on using zooplankton and formulated feed as a food source for red claw, yabbies and marron (Jones, 1995b, Austin et al., 1997, Verhoef et al., 1998, Duffy et al., 2011). While phytoplankton may not provide a comprehensive suite of macronutrients needed for rapid growth of marron, they do provide micronutrients such as essential vitamins, minerals and carotenoids important for health and pigmentation (Napiórkowska-Krzebietke, 2017). Furthermore, the macronutrients needed for marron nutrition can be supplied in the form of formulated feed, while phytoplankton can provide micronutrients, and sustenance for zooplankton in aquaculture facilities.

Research applying SIA in crayfish has revealed that food sources of animal origin are more important for crayfish nutrition than detritus and plant material (Alcorlo et al., 2004). Similar results were observed in our current study, when marron were provided a diet supplemented

with zooplankton, zooplankton contributed >39% and >30% towards the tail muscle composition and whole marron body respectively, while detritus represented a slightly lower contribution with >36 % and 28 % respectively. These findings are supported by improved growth rate of marron in tanks supplemented with zooplankton. Similarly in T4, zooplankton contribution towards the tail muscle was highest followed by detritus. Similar findings were reported by Duffy et al. (2011) who concluded zooplankton was the most significant dietary contribution to the growth of juvenile yabby fed formulated diets, reared in artificial ponds. Our SIA results showed that, out of the available natural food items zooplankton had the highest contribution towards the growth of tissues. Various studies developing formulated feed for *Cherax* spp. have utilized live or dead zooplankton, and showed it supported improved growth rates (Austin et al., 1997, Verhoef et al., 1998, Duffy et al., 2011, Jones, 1995a). The improved growth performance of juvenile marron can be linked to the live plankton supplementation, due to the increased digestibility of ingredients present in live plankton (Shah et al., 2018).

Histological analysis of the hepatopancreas and midgut has been used as a practical means to assess the nutritional status in freshwater crayfish (Calvo et al., 2011, Foysal et al., 2021, Xiao et al., 2014). A healthy structure of hepatopancreas cells and proper orientation of midgut with good epithelium height are associated with efficient nutrient absorption, higher growth rate and improved immunity (Dimitroglou et al., 2009). All of these parameters improved when marron were fed with zooplankton (T3). Hepatopancreas is composed of numerous blind tubules, with each tubule consisting of different epithelial cell types, including secretary cells (Blasenzellen, B-cells) (Calvo et al., 2011, Vicentini et al., 2009). Marron fed with the mixture of plankton showed an increase in the number of B cells with large vacuoles. B-cells are considered as the main site for the synthesis of digestive enzymes (Xiao et al., 2014). The increase in numbers and size of B-cells suggests abundant synthesis and excretion of the digestive enzymes, which enables crayfish to enhance the digestion of ingested prey and to derive more energy from the food intake (Xiao et al., 2014). Nutrient uptake in intestine mainly relies on its epithelium height (Duan et al., 2019). The intestinal epithelium height was higher in marron fed with the zooplankton (T3) and the lumen length was significantly decreased in juvenile marron from T3 and T4 treatment tanks. Histological analysis of intestinal tissues showed a better morphology and orientation of gut in marron fed with zooplankton than in other treatments. Currently there is no data available on the impact of phytoplankton or zooplankton on the morphology of crayfish gut or tail muscle tissue, however it has been shown that the diet impacted the gut morphology in crayfish (Xiao et al., 2014).

Most of the previous studies on the effects of various formulated feeds on health and immunity investigated only gut microbiota of aquatic animals. Rearing environments also play a crucial role in water quality, and in shaping the gut microbial communities that have positive correlation to growth and immunity of fish (Giatsis et al., 2015) and some crustacean species (Zoqratt et al., 2018, Foysal et al., 2020c). In our study, the observed microbial communities in the gut, detritus and water were significantly different in terms of rare species. Microbiota present in water was more diverse than gut and detritus (Figure 4 A-C), similar result was observed by Giatsis et al. (2015). In accordance with previous reports on marron and other crayfish gut microbiota (Francis et al., 2019, Paver et al., 2013), abundance of the gut bacteria *Candidatus* Bacilloplasma which was a predominant bacteria in the present study appeared to be favoured by the supplementation of phytoplankton. Other significantly different bacteria including *Lactovum* and *C*. Hepatoplasma in T3, and *Citrobacter*, *Acinetobacter* and *Hafnia* Obesumbacterium in T4 are detected in abundance from the marron gut with substantial positive role in gut health (Foysal et al., 2020c). In contrast, *Nocardioides* and *Hyphomicrobium* in T3 group are uncommon contributors to the marron gut microbiome diversity, and have been detected but not in high abundance. The relevance and role of these gut microbiome bacterial groups to marron's health remains to be investigated. In our study, the bacterial population in the detritus and water are distinctly different from those of the gut, and are dominated by *Dinghuibacter* and *Aeromonas*, respectively. *Dinghuibacter*, a predominant soil and sediment bacteria (Lv et al., 2016), has also been reported in aquaculture pond water and sediment in a recent study (Dai et al., 2021). Currently, we do not have enough information for *Dinghuibacter* to correlate our results with previous reports. Nevertheless, the overwhelming abundance of *Dinghuibacter* in marron tank detritus needs further investigation to elucidate the role of these bacteria in marron health and growth. *Aeromonas*, on the other hand is the most ubiquitous bacteria in the aquatic environment (Janda and Abbott, 2010), but it has been identified as impacting fish health negatively (Foysal et al., 2019b). However *Aeromonas* has also been reported as playing differential role for crustaceans and marron (Hänninen et al., 1997, Foysal et al., 2021).

Of significance, our finding describes a reduction of *Aeromonas* in the marron gut from treatment T2 and T3 and *Vibrio* in T2, T3 and T4, two common pathogens of aquatic species. In fact, *Vibrio* species have been identified as negatively impacting gut health and biochemical composition of tail muscle in marron (Foysal et al., 2020b). In the presence of all types of commonly found plankton in semi-intensive marron aquaculture ponds, zooplankton was a more nutrient-dense food source than the phytoplankton and formulated feed for juvenile

marron. Zooplankton improved the overall health of reared juvenile marron and reduced the abundance of pathogenic bacteria in the marron gut.

9.5. Conclusion

A dietary supplementation of phytoplankton improved marron health relative to a diet composed solely of formulated feed. Moreover, our findings suggest that live plankton supplementation influenced the water, detritus and marron gut microbiota of marron. Zooplankton supplementation improved the growth, survival, health indices and epithelium height in the midgut. Stable isotope analysis revealed that the zooplankton and detritus contribution towards the tail muscle and whole marron body growth was highest compared to other food sources. These findings clarify the important role of zooplankton in marron aquaculture. Additional research is required to understand the impact of various live plankton on proximate composition of marron tail muscle. In addition, observing the effects of specific zooplankton such as copepods, rotifers and cladocerans separately on overall health of juvenile marron will provide helpful information about their effect on juvenile marron growth, survival and health.

Declarations

Funding

Not Applicable

Competing Interests

The authors declare that they have no competing interest.

Availability of data and material

The raw sequence data for gut microbiota had been deposited as fastq files in National Centre for Biotechnology Information (NCBI) and can be found under the BioProject accession number PRJNA733576.

Author's contribution

Smita Sadanand Tulsankar: Conceptualization, designing and set up of the experiment, plankton counting and analysis, day to day feeding, data collection, data analysis and writing of the manuscript. **Md. Javed Foysal:** Marron gut microbiota collection and analysis, writing and reviewing manuscript. **Anthony J. Cole**: Plankton analysis, writing, reviewing and editing of the manuscript. **Monique Marthe Gagnon:** Methodology validation, supervision, writing,

reviewing and editing of the manuscript. **Ravi K. Fotedar:** Methodology validation, supervision, writing, reviewing and editing of manuscript.

Ethics approval

Animal ethics approval is not mandatory for the invertebrate animal studies at Curtin University, Australia. However, all the required protocols were followed while handling the animals, as per the guidelines of Animal Welfare Act, Western Australia and the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

Submission declaration

The manuscript has not been published previously, accepted for publication elsewhere or it is not under consideration for the publication elsewhere. The submitted manuscript has been approved by all authors.

CHAPTER 10. General discussion, conclusions and recommendations

10.1. Introduction

This chapter discusses the findings of two field trials and four laboratory based experiments. The main conclusion of the study on the trace elements concentrations in marron pond water, their correlations with plankton, and their effect on juvenile marron growth, survival and health mediated through plankton under laboratory conditions are also provided, followed by recommendations for future research. The plan of field and laboratory experiments and their outputs are presented in diagram 10.1. Chapter 1. General introduction, 2. Literature review and 3. General methodology are not presented in the diagram 10.1 as the diagram 10.1 is mainly focused on research chapters.

Trace elements, Plankton productivity and marron

Effects of trace elements on water quality, plankton density and diversity, and on marron

Chapter 4	Chapter 5	Chapter 6	Chapter 7	Chapter 8	Chapter 9
• Experiment 1-Field trial-1. • 28- Commercial marron ponds. • Dissolved concentrations of 12 pre-selected trace elements • Ponds natural productivity	• Experiment 2- Field trial-2. • Seasonal variations of plankton density and species diversities over the six Noongar seasons. • Grow-out marron ponds • Marron growth and health indices	• Experiment- 3- • Indoor laboratory • Marron feeding behaviour • Marron weight groups • Feed preference • Copepods • Black soldier fly meal	• Experiment-4 • Indoor laboratory • Supplementation of pre-selected trace elements. • Outdoor plankton culture. • Phytoplankton, zooplankton. • Marron growth, survival, gut microbiota.	• Experiment-5 • Indoor laboratory • Formulated basal feed. • Mixed plankton • Marron growth, survival. • Pigmentation	• Experiment-6 • Indoor laboratory. • Outdoor plankton culture • Solo phytoplankton, zooplankton and mixed plankton. • Marron growth, survival • Gut microbiota • Histology

Main Findings

Chapter 4	Chapter 5	Chapter 6	Chapter 7	Chapter 8	Chapter 9
➢ Dissolved concentration of trace elements fluctuated over the seasons. ➢ Positive and negative correlations between trace elements and plankton density, species diversity, plankton wet and dry weight.	➢ Chlorophyceae was dominant throughout the year. ➢ Plankton abundance higher during summer. ➢ Copepod abundance were observed around the year ➢ Daphnia abundance were seasonal	➢ Marron feed preference was weight dependent ➢ Time spent on different activities post-feeding observations was weight dependent. ➢ Fish meal was 1st preference for juvenile marron.	➢ Si supplementation increased the diatom density. ➢ Si and P at higher concentration increased plankton density, species diversity and influenced the gut microbiota of marron.	➢ Mixture of live plankton improved growth rate and total haemocyte count of juvenile marron ➢ Plankton improved the marron pigmentation. ➢ Survival was similar in both treatments.	➢ Zooplankton and mixed plankton improved marron growth and survival. ➢ Zooplankton and detritus had highest contribution towards the diet of juvenile marron. ➢ Live plankton influenced the gut microbiota.

Figure 10.1. (Diagram). Illustration of the study plan, presenting the different objectives and parameter measured in each chapter and the main findings.

10.2. Fertilizers and plankton in aquaculture

In aquaculture farming systems, the use of organic and inorganic fertilizers to improve the plankton productivity of ponds is a widespread practice (Boyd, 2018). In the South-West of WA, marron (*Cherax cainii*, Austin 2002) are mainly cultured under extensive and semi-intensive farming systems using supplementation of formulated feed (Tulsankar et al., 2020, Cole et al., 2019, Fotedar et al., 2015). With the aim of improving yield, organic fertilizers such as chopped fermented barley hay straw and lupin are commonly used in marron farming (Tulsankar et al., 2021b, Tulsankar et al., 2020, Cole et al., 2019). In addition, various studies have shown that the use of inorganic fertilizers can enhance the phytoplankton abundance improving the zooplankton growth (Middleton and Reeder, 2003, Tew et al., 2006, Nwankwegu et al., 2020, Tulsankar et al., 2021d).

Plankton are used as live feed in aquaculture especially during the juvenile stages of crustaceans as they can improve growth, pigmentation and enhance the digestive processes (Austin et al., 1997, Lubzens et al., 1989, Verhoef et al., 1998, Jones, 1995a, Shah et al., 2018). Zooplankton has been shown to improve growth and survival rate in juvenile red claw crayfish (Jones, 1990) and yabbies (Austin et al., 1997), however only a few researchers have investigated the effects of dietary plankton on marron growth, survival and pigmentation (Morrissy, 1989, Sommer et al., 1991, Tulsankar et al., 2021a).

It is believed that under semi-intensive culture management practices marron are known to feed on both supplied formulated feed and natural food sources including detritus, phytoplankton and zooplankton (Fotedar et al., 2015). However, no peer reviewed research has been conducted to understand the ecology of marron ponds in terms of dissolved trace element concentrations, plankton abundance, species diversity, species richness and the importance of plankton in marron growth, survival, health, and diet.

To fulfil this gap the current study aimed to investigate the dissolved concentrations of 12 pre-selected trace elements, their correlations with plankton density, species diversity, plankton wet and dry weight, and role of plankton in marron growth, survival and overall health. This aim was achieved by conducting the series of field trials on semi-intensive marron farm in Manjimup, WA (34°1807500 S, 116°0606100 E) complemented by laboratory experiments under controlled conditions at CARL, Perth. Initially, the water samples were collected to analyse the dissolved concentrations of pre-selected trace elements in pond water and establish correlations with plankton density, species diversity, and with wet and dry weights of plankton under semi-intensive

farming system (Chapter 4; Objective 1). Seasonal and temporal variations of plankton density, species diversity and plankton communities, and their correlations with marron yield at three life stages (juvenile, grow-out and brooders) in a semi-intensive marron farming system were analysed (Chapter 4 and 5; Objectives 2 and 3 respectively). Afterwards feeding behaviour and feed preference of different weight groups of marron in the presence of two formulated diets and copepods under controlled laboratory conditions were explored (Chapter 6; Objective 4). Based on the outcomes of objective 1, 2, 3 and 4, an experiment was designed to explore the impact of supplementation of pre-selected trace elements (Mn, Si and P) on tank water quality, plankton density, species diversity, juvenile marron growth, survival, health indices and gut microbiota under controlled laboratory conditions (Chapter 7; Objective 5). Based on the outcomes of objective 5 a follow up experiment was conducted to evaluate marron growth, survival, health and pigmentation in the presence and absence of live plankton mixture under laboratory conditions (Chapter 8; Objective 6). Finally, an evaluation of the effects of live solo phytoplankton community, zooplankton community and their mixture on marron growth, survival, immune and organosomatic indices, histology of hepatopancreas, tail muscle tissue and midgut, and plankton contributions towards the tail muscle and whole marron was conducted (Chapter 9; Objective 7).

Results from the investigations showed that the dissolved concentrations of trace elements fluctuated due to seasonal changes (Tulsankar et al., 2020). Similarly, seasonal changes in environmental parameters such as, temperature, daylight and dissolved concentrations of pre-selected trace elements significantly affected the plankton density and species diversity. It was also shown that live plankton positively influenced the growth, pigmentation, immune-organosomatic indices and gut microbiota of juvenile marron. Marron yield was not significantly co-related with plankton density, species diversity, and plankton wet and dry weight under the semi-intensive marron farming system which may be due to the variations in farming practices and pond management (Tulsankar et al., 2020, Cole et al., 2019). The seasonal and temporal variations of temperature, sunlight and rainfall significantly impacted the plankton density, species diversity and plankton communities (Chapter 4 and 5). While analysing the plankton abundance and species diversity in marron ponds copepods were observed in all seasons (Chapter 5). The feeding behaviour and feed preference of different weight groups of marron ranging from <15 g to 100 g were evaluated under laboratory conditions. The results showed that, juvenile marron rather than adults consumed the highest number of copepods. Supplementation of trace elements showed that a higher trace element concentration increased plankton density, while enhanced levels of Si improved the diatom abundance, improving marron health. Dissolved concentrations

of pre-selected trace elements influenced the plankton density and species diversity under field (semi-intensive system marron ponds) (Chapter 4) as well as under laboratory conditions (Chapter 7). Rearing juvenile marron for 90 days under laboratory conditions in the presence and absence of live plankton mixture collected from the marron ponds (Chapter 8) showed that the live plankton mixture improved specific growth rate, total haemocyte count and pigmentation of juvenile marron. Supplementation of live phytoplankton and zooplankton separately and mixed to juvenile marron (Chapter 9), showed that the juvenile marron consumed higher percentage of zooplankton and detritus. Also, zooplankton improved the growth and health indices of marron.

10.3. Effects of formulated feed on marron

To investigate the effect of natural productivity on juvenile marron growth, survival, immune and organosomatic indices, formulated diet was used as a control. Control treatments from two laboratory experiments#5-Chapter 8 (plastic cages were used to rear juvenile marron) and experiment #6-chapter-9 (marron were reared without using plastic cages) was used to compare the effects of fish meal based formulated feed on growth, survival and health indices of juvenile marron (Figure 10.2).

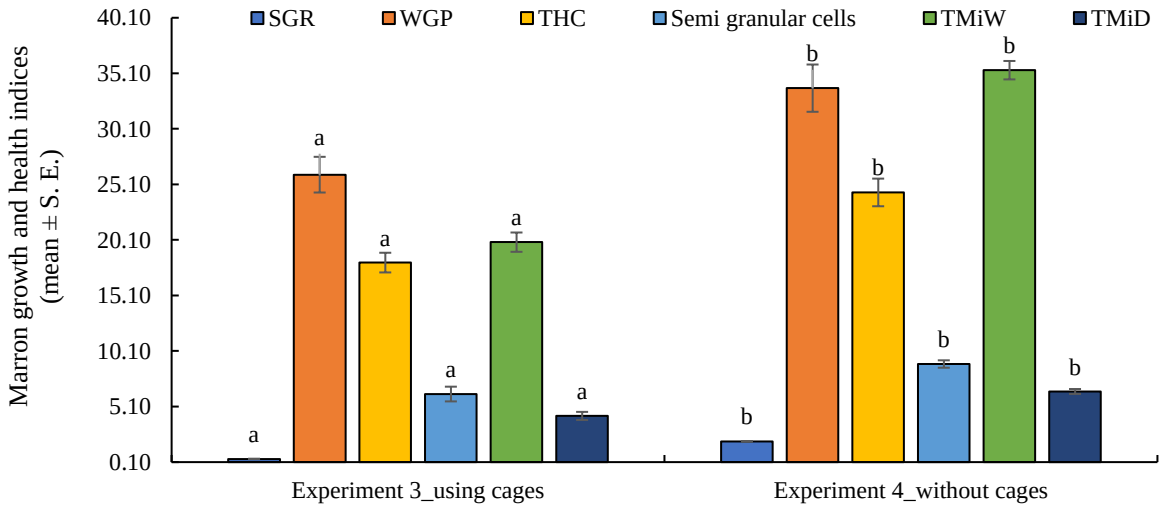

Figure 10.2. Shows the effect of formulated diet on juvenile marron reared for 90 days under controlled laboratory conditions. Letters a and b shows the significant difference between two experiments. Survival, moulting days, weight and length increment at moult, hyaline and granular cells, HM, TM, HiW, HiD did not show significant difference and are not included in this Figure. **Abbreviations**: SGR- specific growth rate; WGP- weight gain percentage; THC-total haemocyte count; TMiW- tail muscle wet weight indices; TMiD- tail muscle dry weight indices; HM-

hepatopancreas moisture; TM- tail muscle moisture; HiW- hepatopancreas wet weight indices; HiD- hepatopancreas dry weight indices (mean ± S. E.).

Significantly higher specific growth rate (SGR), weight gain percentage, improved total haemocyte count (THC), tail muscle wet weight (TMiW) and tail muscle dry weight (TMiD) indices were observed in juvenile marron reared without cages. The survival rate was not significantly different between the caged and non-caged marron. However, the percentage mean of survival was higher in marron reared without cages. SGR was 0.39 for juveniles reared in cages and 1.97 for non- caged marron. Foysal et al. (2019a) and Foysal et al. (2021), observed the SGR of 0.51- 0.53 and 0.69 by using same formulated diet for grow-out marron reared without using cages. The difference in SGR can be due to the different life stages of marron. Another reason for the significantly lower growth and health indices in experiment#5 (chapter-8) compared to experiment #6 (chapter-9), could be the use of plastic cages to rear the juvenile marron, which would have restricted access to periphyton grown on tank edges or detritus as periphyton and detritus play an important role in crayfish nutrition (Alcorlo et al., 2004, Gamboa-delgado et al., 2003). Also, it was observed that juvenile marron spent the longest time eating frozen copepods from the tank floor where grow out marron preferred to feed on the formulated diet and spent more time in resting (Tulsankar et al., 2021c). Similarly, it was observed that periphyton as well as detritus had nutrient contributions towards the growth of tissue and whole marron (Tulsankar et al., 2021 under publication- Chapter 9).

The ideal crayfish diet should improve growth, survival and pigmentation (Verhoef et al., 1998), however various studies have found that marron formulated diets were often lacking sufficient carotenoids, causing loss of pigmentation (Jussila and Mannonen, 1997). The observed SGR of juvenile marron provided with formulated feed only, in both experiments (experiment 5 & 6) was significantly lower than the marron provided with dietary plankton. Juvenile marron grown with natural food sources had better growth performance as compared to the marron fed with formulated diet; marron preying on plankton also had better pigmentation (Jussila and Evans, 1998, Tulsankar et al., 2021a). The micronutrional requirements of marron can be partly fulfilled from natural productivity with the presence of healthy phytoplankton and zooplankton communities, and with detritus, but their macronutritional requirements should be fulfilled by the formulated feed (Fotedar et al., 2015). Though plankton are rich in vitamins and minerals, they may not provide the bulk of nutrients needed for the marron to grow, for example marron cannot synthesise cholesterol and it should be included in formulated feed (Fotedar et al., 2015).

The formulated feed is amongst the highest of the operational costs in marron farming, and alone doesn't fulfil all the nutritional needs of marron. We have demonstrated that practices that will promote the abundance of plankton in semi-intensive aquaculture ponds are likely to result in a higher growth and survival rate of farmed marron, with healthier animals.

10.4. Effects of plankton on marron

Differences in plankton densities and species diversities in different experiments showed a significant effect on growth and health indices of juvenile marron in the indoor laboratory experiments (Table 10.1). Highest SGR, weight gain percentage, improved immune and organosomatic indices were observed in marron fed with zooplankton experiment #6; treatment T3 (chapter-9). Survival rate was highest with the use of zooplankton (T3) and mixed plankton (T4), compared to the marron fed with solo phytoplankton (chapter-9). Health indices of juvenile marron grown with different plankton densities under laboratory conditions are shown in Figure 10.3 and 10.4. Juvenile marron growth, immune and organosomatic indices were improved in experiment #6 (chapter-9- no cages used), when compared to all three 90 days indoor laboratory experiment outcomes. One reason for the lower growth, immune and organosomatic indices in experiment #4 (chapter-7) and #5 (chapter-8) could be the confinement of marron in plastic cages refraining them the access to detritus and free floating plankton.

Table 10.1. Plankton densities, species diversity, marron growth and survival rate observed in different laboratory experiments.

Treatments	Plankton density		Plankton diversity		SGR	Weight gain Percentage	Survival	Weight increment at Moult	Length increment at Moult	Moulting Days
	Phytoplankton ($x10^4$ cells L^{-1})	Zooplankton density (individual L^{-1})	Phytoplankton diversity	Zooplankton diversity						
Expt.2_NTS	377 ± 11.2^{b}	292.8 ± 12.6^{b}	6.22 ± 0.10^{b}	2.53 ± 0.14^{a}	0.12 ± 0.01^{a}	11.1 ± 1.10^{a}	75.0 ± 9.48	29.3 ± 1.14^{a}	5.61 ± 0.32^{a}	16.3 ± 0.63^{b}
Expt. 2_LTS	405 ± 9.04^{bc}	357 ± 13.9^{c}	6.22 ± 0.06^{b}	2.86 ± 0.08^{ab}	0.13 ± 0.01^{ab}	11.8 ± 1.39^{a}	80.6 ± 5.31	29.3 ± 1.17^{a}	6.33 ± 0.14^{a}	16.8 ± 0.48^{b}
Expt. 2_MTS	605 ± 17.5^{e}	413 ± 6.20^{d}	6.67 ± 0.32^{b}	2.89 ± 0.08^{ab}	0.20 ± 0.03^{b}	20.2 ± 2.89^{b}	88.9 ± 6.41	34.5 ± 0.68^{b}	6.45 ± 0.26^{a}	17.0 ± 0.91^{b}
Expt. 2_HTS	738 ± 7.78^{f}	493 ± 9.90^{e}	6.70 ± 0.05^{b}	2.75 ± 0.37^{ab}	0.17 ± 0.03^{ab}	17.0 ± 2.69^{ab}	80.6 ± 8.33	34.2 ± 1.14^{b}	6.34 ± 0.25^{a}	16.8 ± 0.85^{b}
Expt. 3	281 ± 16.4^{a}	228 ± 6.43^{a}	4.53 ± 0.19^{a}	3.44 ± 0.05^{b}	0.54 ± 0.03^{c}	38.3 ± 2.32^{bc}	77.8 ± 7.85	33.7 ± 1.77^{b}	7.56 ± 0.41^{b}	13.5 ± 0.29^{a}
Expt. 4_ Phyto (T2)	442 ± 9.93^{d}	NA	6.13 ± 0.31^{b}	NA	1.89 ± 0.02^{d}	35.5 ± 5.54^{c}	83.4 ± 3.20	29.3 ± 1.17^{a}	6.33 ± 0.14^{a}	14.8 ± 0.63^{a}
Expt. 4_Zoo (T3)	NA	495 ± 2.26^{e}	NA	5.25 ± 0.48^{c}	2.13 ± 0.06^{e}	49.8 ± 3.70^{d}	90.0 ± 1.20	34.5 ± 0.68^{b}	6.45 ± 0.26^{a}	14.0 ± 0.41^{a}
Expt. 4_Phyto+Zoo (T4)	422 ± 2.70^{cd}	498 ± 5.51^{e}	6.00 ± 0.41^{b}	5.00 ± 0.41^{c}	2.06 ± 0.03^{e}	46.9 ± 2.42^{cd}	97.2 ± 2.78	34.2 ± 1.14^{b}	6.34 ± 0.25^{a}	14.3 ± 0.48^{a}

Letter $^{a, b, c, d, e}$ and ^{f} in columns (vertically) shows the significant differences ($p< 0.05$) in plankton density, species diversity and marron growth parameters in different experiments and treatments conducted for 90 days under controlled laboratory conditions (n=4; mean ± S. E.). **Abbreviations**: Expt- experiment; NTS- No trace elements supplementation; P+Z phytoplankton + zooplankton; LTS- low trace element supplementation; MTS- medium trace element supplementation; HTS- high trace element supplementation; Phyto- phytoplankton; Zoo- zooplankton.

The immune parameters such as total haemocyte count (THC) and differential proportions of granular cells in haemolymph has been used as an indicator of immune health in crustaceans (Fotedar et al., 2001). In decapod crustaceans the food intake, nutritional status and environmental conditions affects the haemocyte count in terms of quantity and quality (Le Moullac and Haffner, 2000, Persson et al., 1987). Higher THC corresponds to a higher immune status (Sharma et al., 2009), and higher THC were observed in juvenile marron fed with zooplankton (experiment#6; T3- chapter-9). Granular cell counts were also increased in marron from medium and high trace element supplementation tanks and; solo phytoplankton and zooplankton tanks (chapter-7 and 9). Studies analysing plankton effects on the immune indices of crayfish have not been reported yet. Organosomatic indices such as hepatopancreas and tail muscle wet and dry weight indices are indicators of nutritional status in crayfish (Haefner and Spaargaren, 1993, McClain, 1995b, McClain, 1995c, Jussila and Mannonen, 1997). Organosomatic indices such as hepatopancreas and tail muscle wet and dry weight indices are indicators of nutritional status in crayfish (Haefner and Spaargaren, 1993, McClain, 1995b, McClain, 1995c, Jussila and Mannonen, 1997). The moisture content of the hepatopancreas was also significantly lower in marron fed with zooplankton and mixed plankton (chapter-9), indicating better health condition (Jussila, 1999).

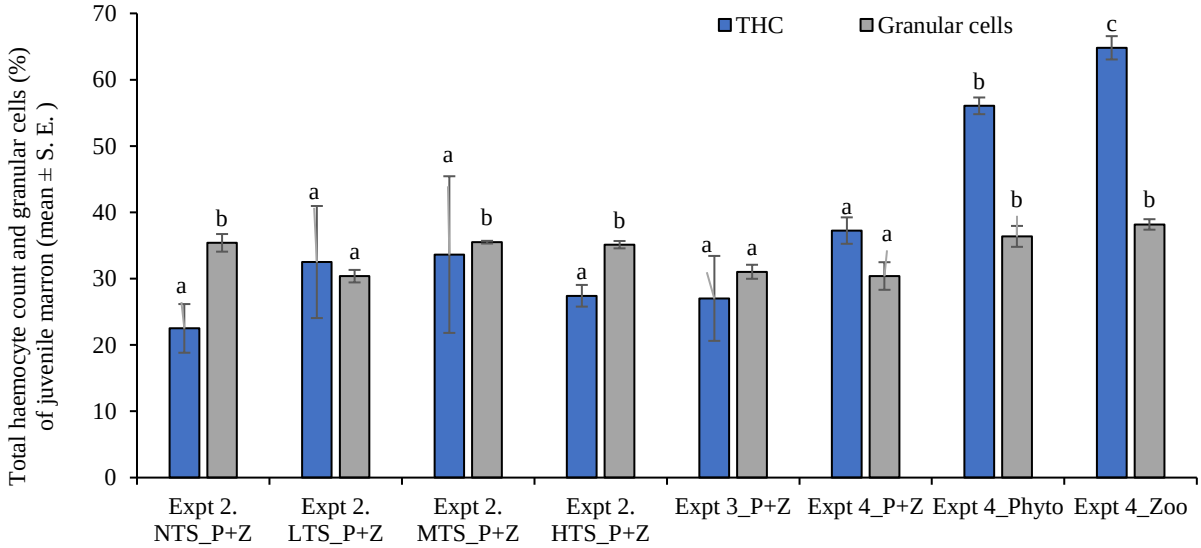

Figure 10.3. Shows the total haemocyte count (THC) and percentage of granular cells recorded in marron haemolymph reared with different dietary plankton densities for 90 days under controlled laboratory conditions. Latter a and b shows the significant differences in THC and granular cells (%) between the experiments (n=4; mean ± S. E.). **Abbreviations**: Expt- experiment; NTS- No trace elements supplementation; P+Z- phytoplankton + zooplankton;

LTS- low trace element supplementation; MTS- medium trace element supplementation; HTS- high trace element supplementation; Phyto- phytoplankton; Zoo- zooplankton; P- phytoplankton; Z- zooplankton.

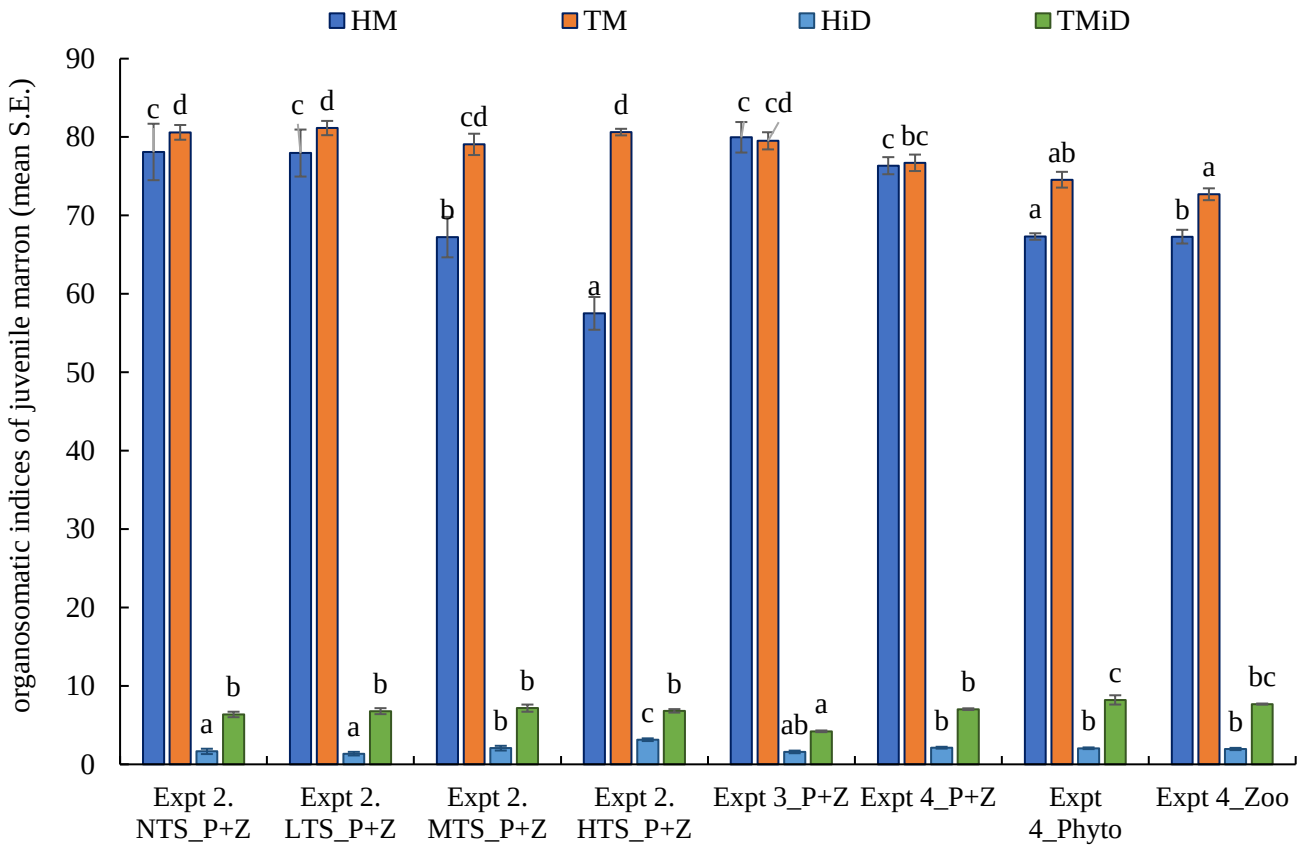

Figure 10.4. Shows the organosomatic indices of juvenile marron (only significantly different between the experiments) reared feeding different plankton densities for 90 days under controlled laboratory conditions. Letter a, b, c and d shows the significant differences in organosomatic indices between the experiments (n=4; mean ± S. E.). **Abbreviations**: Expt- experiment; NTS- No trace elements supplementation; P+Z Phytoplankton + Zooplankton; P- phytoplankton; Z- zooplankton; LTS- low trace element supplementation; MTS- medium trace element supplementation; HTS- high trace element supplementation; HM- hepatopancreas moisture (%); TM- tail muscle moisture (%); HiD- hepatopancreas dry weight indices; TMiD- tail muscle dry weight indices.

The lower plankton density in experiment #4 (chapter-7)- control and low trace element supplementation (LTS) tanks can be the reason for a reduced marron SGR and weight increment at moult relative to medium trace element supplementation (MTS) and high trace element supplementation (HTS). Another reason for improved growth, survival, immune and

organosomatic indices in experiment #4 compared to previous two experiments (Chapters 7 and 8) could be due to the plastic cages used to stock juvenile marron individually. Furthermore, the effect on growth, survival and health of marron cultured for 90 days with and without cages fed with live zooplankton are assessed in section 10.3.

10.5. Performance of marron under laboratory conditions for 90 days.

To compare the growth rate, immune and organosomatic indices of juvenile marron reared with similar zooplankton density from two different experiments: Experiment #4- HTS (Chapter-7) using the plastic cages to stock the juvenile marron, to avoid cannibalism and escape of marron, and Experiment #4- T4 (chapter-9) plastic cages were not used to rear the juvenile marron under controlled laboratory conditions (Figure 10.5).

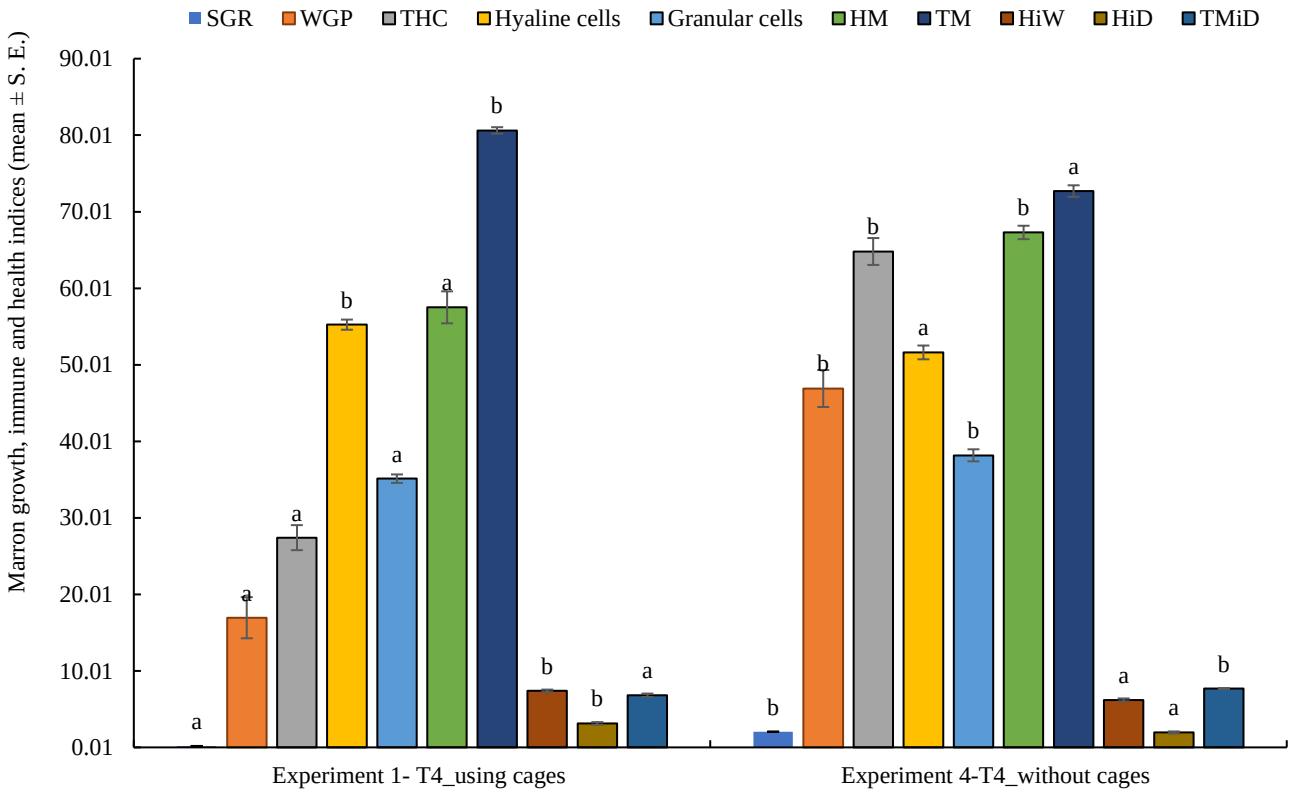

Figure 10.5. Shows the significant differences in marron growth rate, immune and health indices of juvenile marron reared under controlled laboratory conditions using cages and without cages when fed with similar zooplankton density (n=4; mean ± S. E.).

Survival, increment in weight and length at moulting, semi-granular cells, TMiW did not show significant differences and are not included in this Figure.

Plankton supplementation and rearing juvenile marron without using cages significantly improved juvenile marron growth, immune and organosomatic indices. Improved growth and health indices of juvenile marron can be mainly linked to the available access to the periphyton grown on tank edges, plankton availability and accumulated detritus at tank bottom. The use of cages may have restricted feeding on periphyton or detritus. The used cages may have allowed the growth of periphyton or detritus which may have contributed to the marron diet, but it may not have been enough to support the requirements of juvenile marron. Results from experiment #4 (Chapter 9) showed that juvenile marron fed on zooplankton as well as detritus, confirmed that detritus consumption, in a similar way as zooplankton was an important part of marron diet. In addition the cages used had a gap of 4-5 mm on each sides, which may have restricted the entry of larger zooplanktons. Also animals reared in floating cages have no access to sediments, which can provide living prey to cultured animals, influencing the growth positively (Chim et al., 2008).

Taking these limitations into account, trials with floating cages will be more suitable for semi-intensive pond conditions compared to laboratory conditions and floating cages provide a powerful tool to assess the results observed under laboratory conditions (Chim et al., 2008). The periphyton development on substrates involves positive and negative interactions between phytoplankton, autotrophic periphyton, heterotrophic microbial communities, and heterotrophic periphyton (Milstein et al., 2003) with plankton communities depending on the substrate used for cages (Fernandes and Esteves, 2003) and on the intensity of grazing on periphyton (Azim et al., 2005). In a study by Sumithra et al. (2019) periphyton grown on bamboo cages improved the weight gain of Nile tilapia (*Oreochromis niloticus*) raised in a brackish water farm. Several studies on feeding ecology of juvenile marron have shown that plankton or plankton derived detritus comprises the bulk of their gut content (Alcorlo et al., 2004, Momot, 1995).

Considering various factors, live feed remains the most practical input to improve the growth and survival of juvenile marron (Das et al., 2012). However, maintaining an adequate and secure supply of live plankton under semi-intensive farming is challenging due to the various environmental variables. Plankton are subjected to collapse or crash which makes them unreliable. Additionally, capital and operational cost to maintain a quality culture of live plankton is both expensive and cumbersome. Supplying live feed to juvenile marron could improve their growth rate and survival. On the other hand, live feed can act as a carrier of disease to the fish and shellfish, therefore maintaining the good hygiene is very important

during live feed production (Das et al., 2012) and this may not be economically viable. Healthy juveniles can mature into highly fecund broodstock capable of producing robust next generation.

There is a need to improvise and standardised the culture techniques to increase the live food production of semi-intensive aquaculture systems used for marron culture. The pond fertilization prior to stocking the nursery ponds or juvenile marron can improve the abundance of natural productivity, for example use of trace elements such as silica can increase the diatom density (Tulsankar et al., 2021d). Growing plankton in pond conditions by fertilization can reduce the cost of culturing live food in intensive set-up, which will be beneficial in terms of profits. Also, trace elements are required for the growth of marron which can be achieved through plankton or via water.

This is the first study to demonstrate the efficiency of plankton as dietary food for juvenile marron. In conclusion, this study demonstrated the potential for plankton based intensive and semi-intensive culture of juvenile marron native to WA.

10.6. Conclusions

1. Correlations between the pre-selected trace elements (Mn, P, Si, Al, Ca, and S) and ponds plankton productivity under semi-intensive farming conditions were established.
2. Seasonal variations and pond age influenced the dissolved concentrations of pre-selected trace elements and plankton productivity in commercial semi-intensive marron ponds.
3. The dissolved concentrations of Co and Se in commercial marron ponds were below the detectable levels.
4. Plankton productivity including plankton density, species diversity and plankton wet weight and dry weight were correlated to most of the trace elements, except Co and Se.
5. Trace elements played an important role in natural productivity of commercial ponds.
6. The plankton productivity of the ponds was most influenced by three trace elements, Mn, Si and P.
7. Temporal variations and pond age influenced the plankton density, species diversity, species richness and their community composition.
8. Cladoceran abundance was temperature dependent.
9. Trace elements supplementation influenced the plankton density, species diversity; Si improved the diatom abundance under controlled conditions.
10. Trace element availability in tank water influenced the gut microbiota of juvenile marron under controlled conditions.

11. The feed preference of marron was weight-dependent.
12. Larger marron spent a longer time resting.
13. Plankton supplementation improved the growth rate, pigmentation and health indices of juvenile marron.
14. Immunological indicators such as total haemocyte count, and percentage of granular cells improved with the supplementation of mixed live plankton in comparison to formulated diet.
15. Rearing juvenile marron without the use of cages improved the growth and survival rate of juvenile marron.
16. Marron midgut health was influenced by the supplementation of formulated feed, phytoplankton, and zooplankton separately, and mixed together.
17. Zooplankton and detritus were the primary dietary sources for juvenile marron, even in the presence of formulated diet.
18. Marron juveniles fed on variety of dietary sources but achieved better nutrition from zooplankton.

10.7. Limitations in this study

1. The results we observed under laboratory conditions couldn't applied to commercial ponds due to the time limitations.
2. Trace elements supplementation and their effects on plankton density and diversity were analysed under controlled conditions such as temperature, light and aeration, however applying our results under semi-intensive or commercial practices the abiotic factors will vary and could result in different outcomes to those obtained in our study.
3. In our research project plankton densities were stable as the experiments were conducted under laboratory conditions and on small scale, however extrapolation of our results at a commercial scales needs to consider the risk of plankton crash.

10.8. Recommendations for the future research

1. Further research on trace element supplementation and its effect on plankton productivity under commercial farming conditions needs to be pursued.
2. Future research should focus on increasing the zooplankton abundance in semi-intensive marron culture practices and effect of zooplankton on marron growth, survival and health indices.
3. Future research should focus on observing the feed preference of different weight groups of marron under semi-intensive farming conditions.

4. Future research should focus on understanding the effects of specific zooplankton species on marron growth, survival and health indices.

5. Future research should focus on understanding the effects of mixed live plankton on proximate composition of marron tail muscle.

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APPENDIX 1

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Effects of seasonal variations and pond age on trace elements and their correlations with plankton productivity in commercial freshwater crayfish (cherax cainii austin, 2002) earthen ponds

Author: Ravi Fotedar, Marthe Monique Gagnon, Anthony John Cole, et al
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Time spent in post-feeding activities including feed preference by different weight groups of marron (Cherax cainii, Austin 2002) under laboratory conditions

Author: Smita S. Tulsankar,Anthony J. Cole,Marthe Monique Gagnon,Ravi Fotedar
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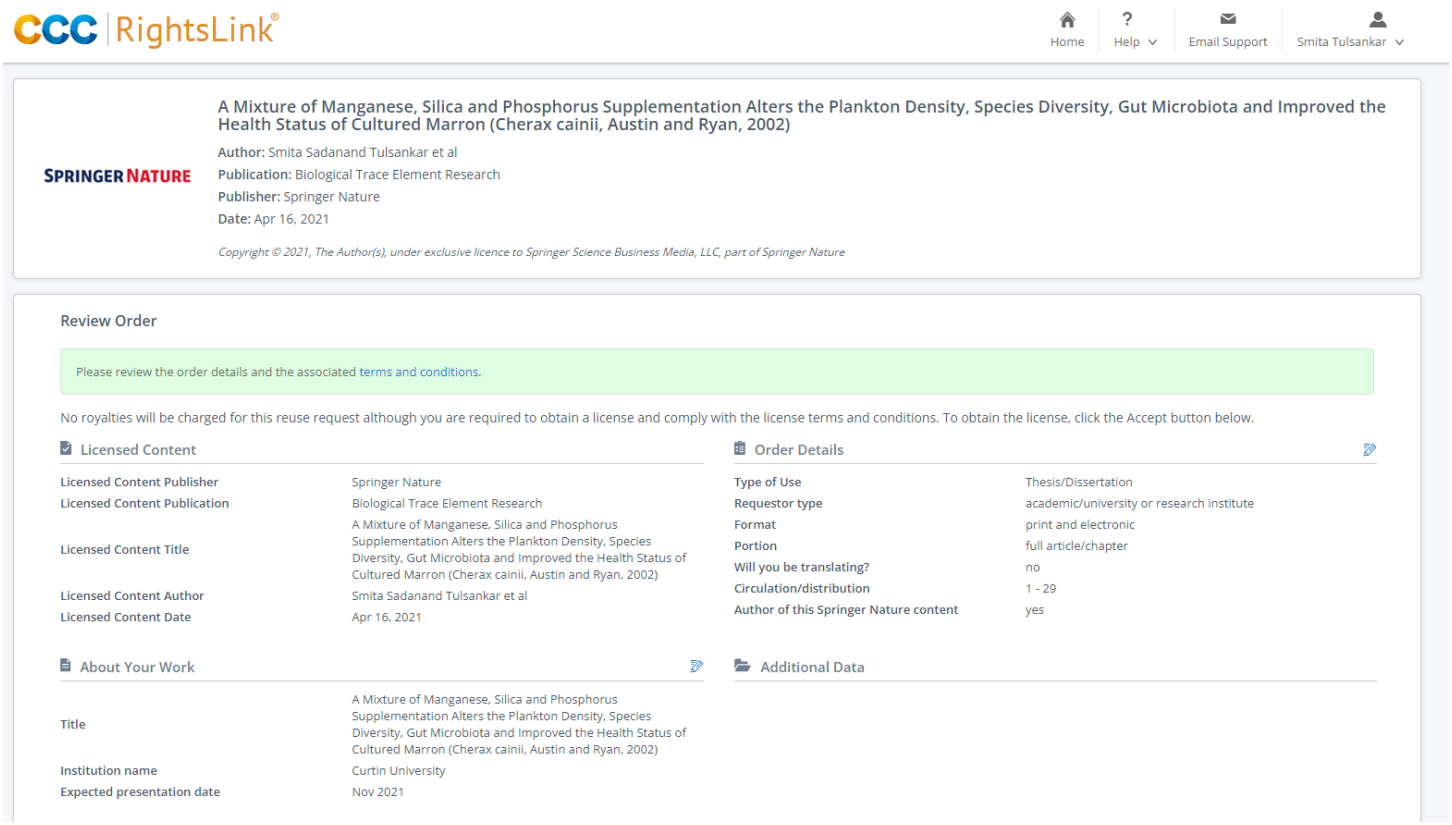

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A Mixture of Manganese, Silica and Phosphorus Supplementation Alters the Plankton Density, Species Diversity, Gut Microbiota and Improved the Health Status of Cultured Marron (Cherax cainii, Austin and Ryan, 2002)

Author: Smita Sadanand Tulsankar et al
Publication: Biological Trace Element Research
Publisher: Springer Nature
Date: Apr 16, 2021

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Institution name	Curtin University
Expected presentation date	Nov 2021

Additional Data

APPENDIX 2

List of publications produced from this research are stated below:

Chapter 4: Manuscript- 1 Published- Effects of seasonal variations and pond age on trace elements and their correlations with plankton productivity in commercial freshwater crayfish (*Cherax cainii* Austin, 2002) earthen ponds. *Aquaculture Research*, 2020, 51: 1913-1922. https://doi.org/10.1111/are.14542

Chapter 5: Manuscript- 2 Published- Temporal variations and pond age effect on plankton communities in semi-intensive freshwater marron (*Cherax cainii,* Austin and Ryan, 2002) earthen aquaculture ponds in Western Australia. *Saudi Journal of Biological Sciences*, 2021, 28: 1392-1400. https://doi.org/10.1016/j.sjbs.2020.11.075

Chapter 6: Manuscript- 3 Published- Time spent in post-feeding activities including feed preference by different weight groups of marron (*Cherax cainii,* Austin 2002) under laboratory conditions. *Applied Animal Behaviour Science*, 2021, 241: 1-6. https://doi.org/10.1016/j.applanim.2021.105376

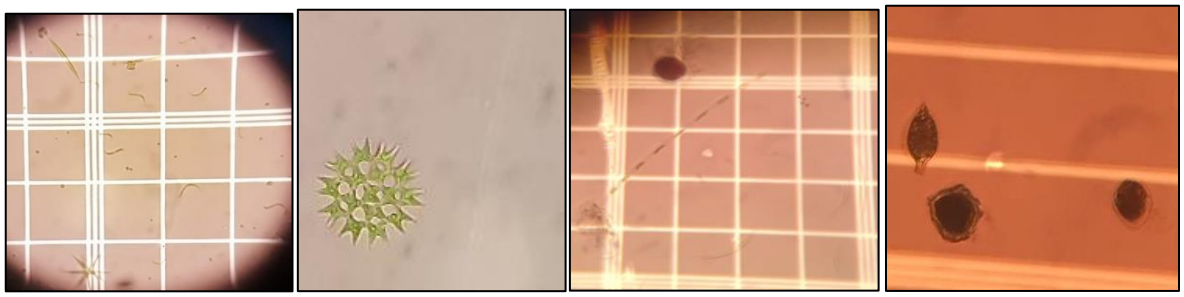
Chapter 7: Manuscript- 4 Published- A mixture of manganese, silica and phosphorus supplementation alters the plankton density, species diversity, gut microbiota and improved the health status of cultured marron (*Cherax cainii,* Austin and Ryan, 2002). *Biological Trace Element Research*, 2021, 1-12. https://doi.org/10.1007/s12011-021-02721-2

Chapter 8: Manuscript- 5 Feeding juvenile marron (*Cherax cainii* Austin, 2002) exclusively on live mixed plankton improves growth, total haemocyte count and pigmentation. *Aquaculture Reports*, 2021, 21:1-7. https://doi.10.1016/j.aqrep.2021.100792

Chapter 9: Manuscript- 6 Live plankton supplementation influences the gut microbiota, improves growth, survival and health status of juvenile marron (*Cherax cainii*, Austin 2002) – under review.

APPENDIX 3

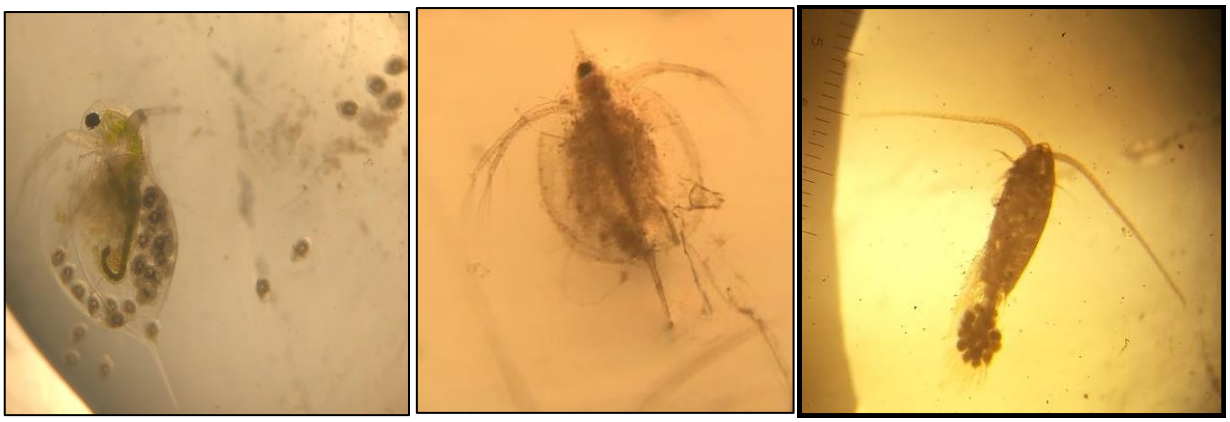
1. Images of some of the observed phytoplankton and zooplankton species in grow-out ponds (Supplementary images fom chapter-5).



i ii

iii iv

Phytoplankton- i. *Scenedesmus, monoraphidium, closterium, chlorella* and other green algae ii. *Pediastum* and iii. & iv *Haematococcus,* and filamentous algae.



Zooplankton- Cladocerans, Rotifera and Copepoda.

APPENDIX 4

Feed ingredients and formulation of pelleted feed used in laboratory experiments.

All feed ingredients were procured from Speciality Feeds Company, Glen Forrest, Western Australia and the feed formulation was conducted in CARL (Table 4). Copepods were collected from Blue Gum Lake in Booragoon, Perth. (Supplementary table for chapter-6).

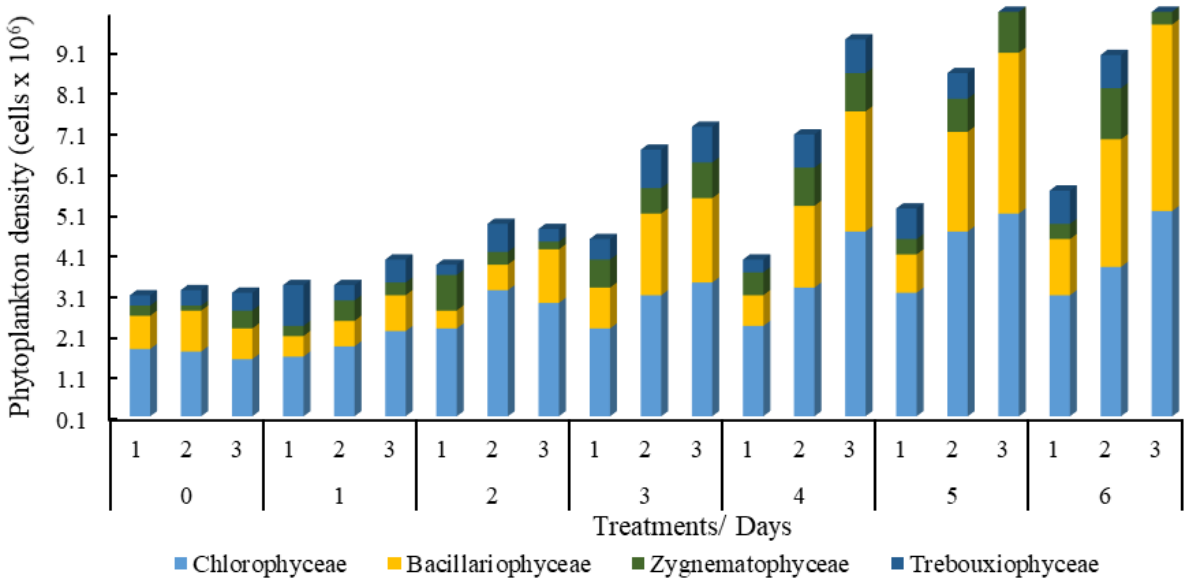
Table 4. Ingredients and protein content (in percentage) of formulated feeds and frozen copepods used to fed marron in this study. The copepod samples were collected for three days in a row to analyse the crude protein.

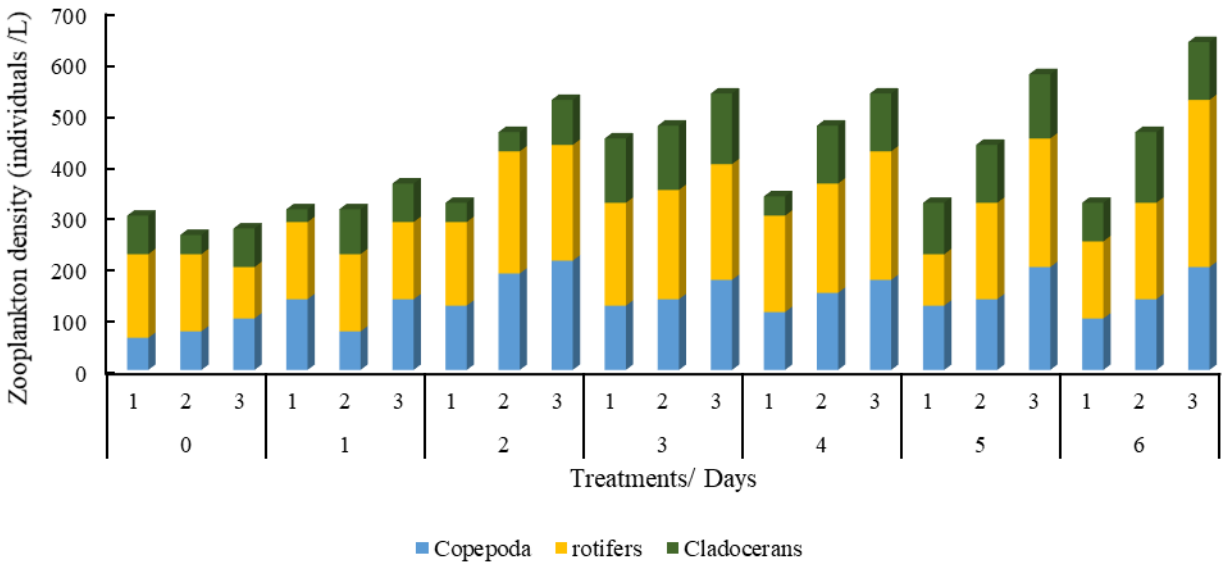
Ingredients	BSF	Basal diet	Zooplankton
Fish meal	-	40.00	-
BSF	71.00	-	-
Wheat (10 CP)	23.14	22.14	-
Corn/wheat starch	4.00	10.00	-
Lecithin- Soy (70%)	1.00	1.00	-
Vitamin C	0.05	0.05	-
Dicalcium Phosphate	0.02	0.02	-
Vitamin Premix	0.29	0.29	-
Canola oil	-	8	-
Cholesterol	0.5	0.5	-
Fish oil	-	10	-
Barley	-	8	-
Protein content (% dry weight)*			
Crude protein	31.2±0.3	28.5±0.4	50. 5 ± 0.50

*The crude protein of final diet and frozen copepods (% dry matter)

APPENDIX 5

Plankton density and diversity on Mn, Si and P supplementation (Supplementary graph for chapter-7).





APPENDIX 6

Table #S1. Stable isotope composition ($\delta^{13}C‰$, $\delta^{15}N‰$), elemental composition (%C, %N) and discrimination factor ($\Delta^{13}C$, $\Delta^{15}N$) of the potential food sources for juvenile marron (mean ± S.D.). (Supplementary table for chapter-9).

Treatments	Sources	$\delta^{13}C‰$	$\delta^{15}N‰$	%C	%N	$\Delta^{13}C$ (Tail muscle)	$\Delta^{15}N$ (Tail muscle)	Δ13C (Whole marron)	Δ15N (Whole marron)
Control	Formulated feed	-25.9 ± 1.29	10.3 ± 0.38	47.0 ± 0.10	3.43 ± 0.23	2.20 ± -0.5	-1.80 ± -0.01	1.14 ± -0.58	-2.50 ± 0.11
	Periphyton	-25.3 ± 0.96	8.69 ± 0.98	32.7 ± 3.49	4.83 ± 0.23	1.60 ± -0.67	-0.22 ± -0.61	-0.01 ± -0.77	-0.92 ± -0.49
	Detritus	-24.5 ± 1.34	8.29 ± 0.54	30.2 ± 8.58	5.47 ± 1.91	0.77 ± -1.06	0.18 ± -0.17	-0.83 ± -1.16	-0.51 ± -0.05
Phytoplankton	Basal feed	-25.9 ± 1.29	10.3 ± 0.38	47.0 ± 0.10	3.43 ± 0.23	2.0 ± -0.7	-2.1 ± 0.04	0.52 ± -0.77	-2.60 ± 0.24
	Periphyton	-25.6 ± 0.55	11.9 ± 1.59	33.7 ± 6.15	5.17 ± 1.68	1.72 ± -0.13	-3.72 ± -1.17	0.24 ± -0.24	-4.23 ± -0.97
	Detritus	-24.3 ± 0.89	11.6 ± 0.89	32.6 ± 5.17	6.17 ± 1.08	0.43 ± -0.48	-3.40 ± -0.38	-1.05 ± -0.59	-3.91 ± -0.18
	Phytoplankton	-26.0 ± 0.51	11.3 ± 1.20	36.1 ± 2.75	4.02 ± 1.21	2.06 ± -0.10	-3.07 ± -0.78	0.58 ± -0.21	-3.58 ± -0.59
Zooplankton	Basal feed	-25.9 ± 1.29	10.3 ± 0.38	47.0 ± 0.10	3.43 ± 0.23	2.7 ± -0.4	-1.8 ± 0.3	1.33 ± -0.29	-2.6 ± 0.3
	Periphyton	-25.1 ± 0.59	9.27 ± 2.24	30.2 ± 3.38	4.43 ± 0.93	1.32 ± -0.27	-0.82 ± -1.59	-0.05 ± -0.12	-1.62 ± -1.55
	Detritus	-24.2 ± 0.33	8.70 ± 0.76	30.7 ± 0.94	5.26 ± 1.37	0.50 ± 0.00	-0.26 ± -0.10	-0.87 ± 0.15	-1.05 ± -0.06
	Zooplankton	-24.6 ± 0.59	11.6 ± 0.69	32.9 ± 2.70	6.02 ± 0.61	0.82 ± -0.27	-3.13 ± -0.04	-0.55 ± -0.12	-3.93 ± 0.00
Phyto+zooplankton	Basal feed	-25.9 ± 1.29	10.3 ± 0.38	47.0 ± 0.10	3.43 ± 0.23	2.6 ± -0.5	-2.3 ± 0.02	0.66 ± -0.17	-2.81 ± 0.88
	Periphyton	-25.2 ± 0.65	10.7 ± 0.79	36.1 ± 0.67	5.45 ± 0.45	1.36 ± -0.37	-2.66 ± -0.38	-0.59 ± 0.05	-3.20 ± 0.47
	Detritus	-24.9 ± 0.55	11.2 ± 0.84	32.0 ± 1.59	5.15 ± 0.28	1.04 ± -0.27	-2.87 ± -0.51	-0.91 ± 0.05	-3.40 ± 0.35
	Phytoplankton	-26.0 ± 0.25	9.95 ± 0.42	37.0 ± 1.19	4.12 ± 0.75	2.21 ± 0.03	-1.95 ± -0.01	0.26 ± 0.35	-2.48 ± 0.84
	Zooplankton	-25.4 ± 0.86	12.0 ± 1.39	38.6 ± 4.40	6.37 ± 0.31	1.54 ± -0.58	-4.03 ± -0.98	-0.42 ± -0.26	-4.57 ± -0.13