School of Molecular and Life Sciences

The role of carbon to nitrogen ratio in marron (*Cherax cainii*, Austin 2002) culture systems

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This thesis is presented for the Degree of

Doctor of Philosophy

of

Curtin University

November 2023

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:

The proposed research study did not require animal ethics approval because marron is an invertebrate species. However, while handling the animals, all required protocols were followed, as per the recommendations of the Western Australian Animal Welfare Act and the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th edition (NHMRC, 2013).

Signed: Thi Thu Thuy Nguyen
Date: November 2023

STATEMENT OF CONTRIBUTION TO THE THESIS

As per the regulations outlined by Curtin University for graduate degrees, this serves as confirmation that the thesis presented herein reflects the work of the Candidate, with contributions from the supervisory panel and collaborators as noted.

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Below are the details of the contributors for each chapter:

Contribution refers to the overall involvement of both the student and other contributors (supervisors, etc.) in writing the thesis. Marking a '√' in the remaining boxes indicates the specific aspect(s) of the thesis each individual has engaged in.

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CHAPTER 6.1 (Paper 1): The effect of two dietary protein sources on water quality and the aquatic microbial communities in marron (*Cherax cainii*) culture. Published in Microbial Ecology, 82, 299-308. The table of contribution percentages is presented in Appendix 2.

CHAPTER 6.2: Carbon/Nitrogen ratio in the sediment drives microbial diversity and composition under marron (*Cherax cainii*) aquaculture: A study with two different protein diets

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CHAPTER 7 (Paper 2): Effects of carbon source addition in rearing water on sediment characteristics, growth and health of cultured marron (*Cherax cainii*). Published in Scientific Reports. The table of contribution percentages is presented in Appendix 3.

CHAPTER 8: Supplementation of molasses increases bacterial diversity in the sediments and growth of juvenile marron (*Cherax cainii*) cultured under laboratory conditions

Contributors	**Concept Development**	**Data Collection**	**Data Analyses**	**Writing**	**Contribution (%)**
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CHAPTER 9: General discussion, Conclusions and Recommendations

Contributors	**Concept Development**	**Data Collection**	**Data Analyses**	**Writing**	**Contribution (%)**
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ACKNOWLEDGEMENTS

I would like to thank the Vietnamese Government and Curtin University for sponsoring me via a joint scholarship to undertake a PhD research at the Department of Environment and Agriculture, Curtin University, Perth, Western Australia.

Sincere thanks to my supervisor, Professor Ravi Fotedar for his support, encouragement and advice during my study. Huge thanks to Professor Marthe Monique Gagnon as my co-supervisor for support and guidance.

Huge gratitude to Peter and Steve, the owners of Blue Ridge Marron farm for allowing me to use their farm and marron for my experiments. Their hospitality and support did make my work more effective.

Thanks to all the staff and post-graduate students and research fellow at the Curtin Aquatic Research Laboratory, especially Mr. Rowan Kleindienst for his unwavering support and assistance in ordering materials and chemicals and setting up my experiments.

Thanks to all my family members for their love, support and encouragement. I acknowledge my friends for their great help and for sharing my hardship and happiness during my PhD study.

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PREAMBLE

This research aims to improve understanding of the role of carbon to nitrogen (C/N) ratio in marron (*Cherax cainii,* Austin 2002) farming systems. The thesis has emphasised on the responses of water quality, sediment characteristics, and marron to different C/N ratio inputs to the rearing environment including the earthen pond and laboratory conditions. There are nine chapters in the thesis. **Chapter 1** is an introduction that provides background information on water and sediment characteristics in aquaculture, highlights the relationship between nutrient inputs, waste accumulation, and health of aquatic cultured species, and suggests potential applications to enhance marron growth and immunity. This chapter also justifies and underlines the needs, aims, and objectives of the current research. **Chapter 2** gives an overview of the worldwide crayfish and marron industry, reviews the research on aquaculture rearing environment, specifically on C and N concentrations in sediments of various culture systems, assesses factors driving these macronutrients, and highlights the interaction of sediment with growth and health of aquatic animal species. The second part of the chapter covers several topics, with a primary focus on crayfish species, including (i) studies on the application of dietary probiotics; (ii) alternate dietary protein sources; and (iii) carbohydrate supplementation in freshwater decapod crustaceans. This review sets the context for past research on marron, emphasizing the need for evaluating carbohydrate and protein sources, as well as C/N inputs in marron culture systems. **Chapter 3** provides all general methodologies that are used in the research chapters. **Chapters 4 to 8** present research findings. Chapters 4 and 5 describe the results of trials that were conducted under commercial marron farming conditions while chapters 6 to 8 report the outcomes of laboratory experiments. Specifically, **Chapter 4** evaluates the seasonal effects of some selected water parameters and sediment quality (C/N ratio and microbial communities). In **Chapter 5**, a feeding trial is conducted to examine the impact of two commercially formulated diets with varying C/N contents of 14.52 and 12.07, with and without probiotic supplementation, on the performance of marron and the characteristics of pond water and sediment. **Chapter 6**

assesses the effect of two dietary protein sources (fishmeal and poultry-by-product meal) on water and sediment quality and marron growth and health cultured in laboratory conditions. **Chapter 7** investigates the effects of supplementing three carbohydrate sources, namely corn flour, molasses, and wheat flour, to maintain a C/N input of 12. This chapter aims to assess the influence of this application on water and sediment quality, as well as the growth and health of marron. **Chapter 8** endeavours to manipulate the input of the C/N ratio by supplementing two distinct quantities of molasses and subsequently assess the impact on water, sediment, and marron responses. **Chapter 9** presents a comprehensive discussion that emphasises the key findings and formulates future research recommendations on marron.

ABSTRACT

The marron (*Cherax cainii*, Austin 2002) is a popular species of freshwater crayfish in Western Australia, possessing several positive attributes for aquaculture. Despite some marginal improvements achieved by providing better nutrition and a variety of immunostimulants in the rearing environment at the laboratory scale, marron aquaculture production has not increased significantly in Australia. Like other benthic decapod aquacultured species, marron exhibits slow feeding behaviours and predominantly occupies the sediment-water interface as its primary habitat. Therefore, changes in water and sediment characteristics can directly affect the well-being of marron. The lack of research on the role of macronutrient inputs such as carbon (C) and nitrogen (N) in modulating health, physiology, and immunity, as well as the high accumulation of nutrient wastes in the rearing environment, can create bottlenecks to commercially viable productivity of marron culture. This current study was designed to characterize the effects of different carbon to nitrogen (C/N) ratio inputs through formulated feeds or external carbon supplementation on the rearing environment, growth and health of marron. Five experiments, including two trials in Blue Ridge Marron farm in Manjimup, Western Australia, and three in indoor laboratory conditions at Curtin Aquatic Research Laboratory, were conducted to address these research objectives.

Seasonal variations were found in the biotic and abiotic water parameters, as well as the C/N ratios of the sediment in semi-intensive outdoor marron ponds. Significant linear relationships between sediment C/N ratios and several measures of water quality were observed. By identifying the environmental conditions that drive changes in microbial communities, this study has characterized the resident microbial populations in marron pond sediments during seasonal cycles and found the highest diversity of sediment microbiota in spring. Commercial diets with variable C/N ratio inputs (12.07 and 14.52) and the inclusion or absence of probiotics had significant effects on the microbiota of the water and sediment. The diet supplemented with probiotics have reduced the abundance of some harmful bacterial groups such as *Vibrio* and *Aeromonas* in water and sediment of

marron pond. However, the diet with C/N ratio of 12.07 resulted in higher marron production and more numbers of marron in bigger size grades.

Research under laboratory conditions revealed that over the time, regular feeding had significant influences on nutrient waste accumulation in water including ammonia, nitrite, nitrate, and phosphate. There were differences in the prevalence of specific bacterial taxa in sediment and water in response to dietary protein sources derived from fishmeal (FM) and poultry-by-product meal (PBM) with a C/N ratio of 9. Significant correlations were found between various microorganisms, water quality indicators, and sediment C/N ratios. Although protease activity in marron hepatopancreas fed PBM-based diet was less than FM-based diet, similar growth and health parameters of marron were obtained, regardless of dietary treatments.

The addition of carbohydrates to marron systems resulted in maintaining water quality, retaining high C/N ratios, enriching bacterial communities in sediments, and improving marron growth and immune performances. In the experiment with marron of 11g size class, molasses was demonstrated to be a more effective external carbon source for marron culture than corn flour and wheat flour. When testing molasses supplementation on marron in the 3g size class, similar results were also achieved. However, while molasses supplementation to maintain C/N input of 12 significantly decreased the amount of *Vibrio*, a further increase in molasses quantity had no such impact, and marron growth and health responses were similar between different molasses-added treatments.

In summary, the C/N ratio plays a crucial role in marron aquaculture, influencing water quality, growth and health of marron. This research suggests the marron aquaculture industry should investigate reduced feeding rates with added carbon sources in marron culture and evaluate the texture and flavour of biofloc-cultured marron to achieve cost savings and ensure marron flesh quality.

The abstract has also been presented by a schematic diagram shown below:

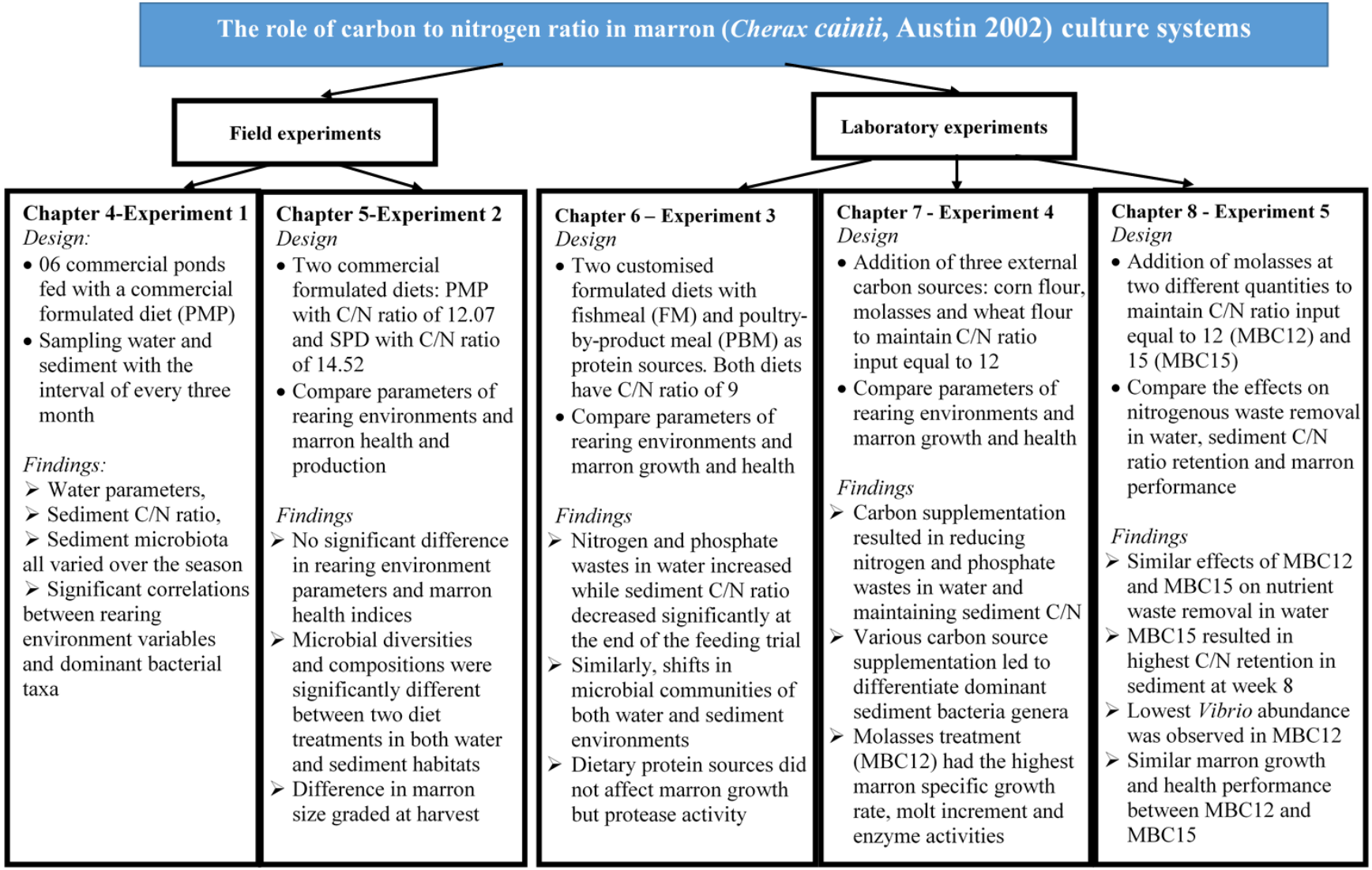


Figure 1.1 Diagram summarizing all research chapters in the thesis, illustrating various objectives and parameters measured in each experiment along with key findings

LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percent
ANODIS	Analysis of dissimilarities
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
APHA	American Public Health Association
bp	Base pair
cm	Centimeter
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
FAO	Food and Agriculture Organization
ha	hectare
HTS	High throughput sequencing
Inc.	Incorporation
kg	Kilogram
L	Litre
g	Gram
g/L	Gram per Litre
g/kg	Gram per Kilogram
GC	Granular cell
KJ	Kilojoules
KJ/g	Kilojoules per gram
LEfSe	Linear Discriminant Analysis Effect Sizes
LDA	Linear Discriminant Analysis
LSD	Least Significant Difference
µm	Micrometer
µM	Micromolar
Micca	Microbial Community Analysis
m^2	Squared meter
mg	Milligram

mg/L	Milligram per Liter
mL	Milliliter
mm	Millimeter
MSA	Multiple sequence alignment
ng/µl	Nanogram per Microliter
NCBI	National Centre for Biotechnology Information
NMDS	Non-metric multidimensional scaling Test
OTUs	Operational Taxonomic Units
PBS	Phosphate-buffered saline
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational analysis of variance
s	second
S.E.	Standard error
SPSS	Statistical Package for the Social Sciences
USA	United States of America

COMMON AQUACULTURE SPECIES NAMES MENTIONED IN THE THESIS

Scientific name	Common name
Astacus leptodactylus/ Pontastacus leptodactylus	Narrow-clawed crayfish
Cambarellus montezumae	Acocil crayfish
Carassius auratus	Crucian/Gibel carp
Cherax albidus	White yabby
Cherax cainii	Marron (Smooth marron)
Cherax destructor	Common yabby
Cherax quadricarinatus	Red claw crayfish
Cherax tenuimanus	Marron (Hairy marron)
Clarias gariepinus	African catfish
Cromileptesaltivelis	Humpback grouper
Cyprinus carpio var. koi	Koi carp
Farfantepenaeus brasiliensis	Pink shrimp
Hermetia illucens	Black soldier fly
Ictalurus punctatus	Channel catfish
Litopenaeus vannamei	White leg shrimp
Macrobrachium rosenbergii	Freshwater prawn
Moronechrysops × *M. saxatilis*	Sunshine bass
Notemigonus crysoleucas	Bait minnow
Oncorhynchus kisutch	Coho salmon
Oncorhynchus mykiss	Rainbow trout
Oreochromis niloticus	Nile tilapia
Pacifastacus leniusculus	Signal crayfish
Pangasianodon hypophthalmus	Catfish
Paranephrops zealandicus	Koura crayfish
Penaeus monodon	Tiger shrimp
Procambarus clarkii	Red swamp crayfish
Sciaenops ocellatus	Red drum crayfish

CHAPTER 1 Introduction

1.1 Background

Water and sediment are important parts of aquaculture systems (Jana and Sarkar, 2005, Boyd and Tucker, 2012), and nutrient exchange between sediment and the water column is extensive (Avnimelech, 1999, Boyd, 2017). In fact, many aquaculture species are benthic omnivores and spend most of their time in the sediment-water interface environment, even utilising organic matters in the sediment as a major diet (Tulsankar et al., 2021a, Abu Hena and Hishamuddin, 2012, Meakin et al., 2008). The water-sediment interface contributes to nutrition and is responsible for the health of the farmed aquatic species, particularly decapod crustaceans (Lemonnier et al., 2010, Ariadi et al., 2019). While maintaining suitable water quality can contribute to the success of aquatic farming (Boyd and Green, 2002, Hukom et al., 2020, Orozco-Lugo et al., 2022), little is known about the sediment characteristics as well as its role in aquaculture. In farming conditions, various inputs such as different forms of feed and additives are provided to enhance animal production. However, feeding practices often result in the increase of nutrient loading in which more inorganic metabolites are generated in the water column. Bottom sediments are the ultimate reservoir where these nutrients alongside undigested feed and animal excreta are deposited. A large proportion of carbon and nitrogen from overfeeding practices are not recovered in harvested animals but are rather incorporated and stored in the sediments (Sahu et al., 2013, Bosma and Verdegem, 2011). While carbon is not toxic and easy to be recycled, nitrogenous wastes have lethal impacts on farmed animals and are partially decomposed by the activity of microorganisms through anaerobic and aerobic processes (Moriarty, 1997, Avnimelech and Ritvo, 2003, Deng et al., 2020). This suggests a key role of microbial communities with respect to water quality and nutrient cycling, which in turn influences growth and health of aquatic animals (Zhou et al., 2009, Boyd, 2017, Moriarty, 1997). Microbial community structures and functions could be significantly driven by many factors such as physicochemical factors (Sun et al., 2021, Li et al., 2021b), nutrient inputs (Dai et al., 2018), the stages of cultured species (Zhang et al., 2020), and farming

practices (Choi et al., 2022). However, very little information is available in the literature relating water parameters and sediment quality to the microbial communities in water and sediments, especially with respect to marron aquaculture.

Marron (*Cherax cainii*) is a freshwater crayfish native to Western Australia and has a huge potential market (Beatty et al., 2019, Tuynman and Dylewski, 2022) with a higher farm gate price than any other freshwater crayfish species (Ackefors, 2000). However, due to its low farming production, it is mainly consumed in domestic markets and exported to only a few countries (Nunes et al., 2017, McClain, 2020). The species is a sought-after delicacy, has a simple life cycle, diseases free, huge size and can be transported live (Morrissy et al., 1990, Alonso, 2010, Alonso, 2009a, Alonso, 2009b). Extensive or semi-intensive culture systems using formulated feed, without any water exchange, are currently applied for most of the marron farming. These farming systems are suitable for areas that face freshwater shortage and have less impact on the environment but still provide optimum conditions to maintain marron's health (Alonso, 2009a). However, slow growth rates and poor survival are some of the challenges the industry is coping with and need to be addressed to improve marron production (Alonso, 2010). There is potential to boost marron production through maintaining optimal rearing environment conditions as well as providing appropriate diets and supplements in commercial farming. The most suitable supplementation and diet affecting sediment quality leading to enhanced growth, however, needs further research.

In designing aquaculture diets, the selection of ingredients is based not only on the farmed species performance, but also on their availability, cost, environmental and ecological implications. Supplementing or replacing fishmeal by cheaper protein sources may result in reduced costs and food security (Cottrell et al., 2020). Some alternative protein sources from plants (soybean, lupin), animals (poultry-by-product, tuna hydrolysate), and insect (black soldier fly - *Hermetica illucens*) sources have been used in the diet for many crayfish species such as signal crayfish (*Pacifastacus leniusculus*) (Fuertes et al., 2012, Fuertes et al., 2013), red claw (*Cherax quadricarinatus*) (Jiang et al., 2023, Qian et al., 2021), red swamp (*Procambarus clarkii*) (Tan et al., 2018, Lunda et al., 2020), yabby (*Cherax*

destructor) (Jones et al., 1996a) and marron (Foysal et al., 2022a). Studies evaluating the effectiveness of diets based on various protein sources on marron show the potential of poultry-by-product meals for the marron farming industry (Foysal et al., 2022a, Foysal et al., 2019b, Siddik et al., 2020). However, information on the impact of poultry-by-product meal in the diet for marron on water quality, microbial community, and marron performance is still limited.

Aqua-diets are usually rich in nutrients, however, a significant proportion of these nutrients is unassimilated by the cultured animals and is dispersed into the rearing environment (Herath and Satoh, 2022, Amirkolaie, 2005). Among the wastes, the accumulation of toxic nitrogenous compounds is a sign that the culture environment is deteriorating (Hargreaves, 1998, Abakari et al., 2021a). Toxic nitrogenous wastes can be controlled by manipulating the ratio of carbon to nitrogen entering the culture systems where various carbon sources are supplemented to provide an appropriate ratio of carbon/nitrogen to the culture environment (Avnimelech, 1999, Avnimelech et al., 2008). This practice may not only help to maintain water quality but also enable nutrient retention through the immobilization of dissolved inorganic nitrogen by bacteria in well-aerated cultured systems (Minaz and Kubilay, 2021, Abakari et al., 2021b). The bacterial biomass can be utilized as an additional food source by target cultured animals (Chen et al., 2018b, Ekasari et al., 2019, Khanjani and Sharifinia, 2020, Lunda et al., 2020). Such an approach can lower the demand for expensive protein in the formulated commercial feed, thereby improving the economic efficiency of the farming of various fish species (Azim and Little, 2008, Minabi et al., 2020) and shrimp species (Chu and Brown, 2022, Kumar et al., 2017, Pérez-Velasco et al., 2023).

The researchers have evaluated the feasibility of rearing narrow-clawed (*Astacus leptodactylus*) (Doğukan et al., 2021), red claw (Azhar et al., 2020) and red swamp crayfish (Li et al., 2023, Li et al., 2019a) in carbon supplementation systems (biofloc technology - BFT). The findings showed that in optimal aeration conditions as a required condition of BFT, narrow-clawed, red claw and red swamp crayfish all can adapt and perform well to

BFT conditions. Moreover, water quality was maintained, and survival of narrow-clawed crayfish was enhanced (Doğukan et al., 2021) while feeding on formulated feed of red claw and red swamp was more efficient (Li et al., 2019a, Azhar et al., 2020). Additionally, Li et al. (2019a) documented that digestive enzyme activities, proximate composition and growth rate of red swamp were enhanced. Those studies also revealed that in these carbon-added culture systems, red swamp and red claw may consume microbial proteins in the suspended flocs and sedimented matters as an extra food source for their growth (Azhar et al., 2020, Li et al., 2019a). However, no equivalent study has been conducted on marron.

Overall, despite efforts to improve marron production, little is known about the factors affecting water and sediment in their interaction with marron growth and health performance. This study aimed to investigate the possible seasonal variation of sediment characteristics and the effects of different commercial diets on marron production when marron are cultured in earthen ponds. The effects of dietary protein sources, carbohydrate sources and levels on water, sediment, and performances of marron aged 0^+ and 1^+ were also assessed when marron were cultured in laboratory conditions.

1.2 Specific objectives

1. To quantify changes of selected water parameters in marron ponds through seasonal cycles.
2. To assess the seasonal variation in carbon to nitrogen ratio in marron pond sediments.
3. To characterise the resident microbial communities in marron pond sediments through seasonal cycles and identify the environmental factors driving changes in microbial communities.
4. To compare the influence of different commercial diets on water and sediment parameters in relation to growth and health performances of marron cultured in ponds.

5. To evaluate how various dietary protein sources, affect water, sediment characteristics and the growth and health outcomes of marron cultivated in laboratory settings.
6. To study the effect of carbohydrate supplementation on water and sediment parameters in relation to growth and health responses of marron cultured in laboratory conditions.
7. To examine the effect of different supplement levels of carbohydrates on water, sediment parameters and marron performances cultivated in laboratory conditions.

1.3 Significance of the study

- The significance of this study will provide novel information on seasonal variations in sediment carbon/nitrogen ratio, and microbiota contents of marron pond. The information can be used for better management of commercial marron ponds.
- The current research findings will support farmers to choose the appropriate protein source in formulating marron diets to sustain marron aquaculture industry.
- The research will provide a preliminary evaluation into the potential of carbohydrate types and supplementation levels to improve water and sediment quality as well as providing improved marron immunity and productivity.
- The research outcomes will inform industry authorities in supporting proper measures for sustainable marron farming.

CHAPTER 2 Literature review

2.1 Global aquaculture

Global fisheries and aquaculture production has achieved a historic pinnacle, indicating a heightened trajectory for the sector's pivotal role in guaranteeing future food security and fulfilling nutritional requirements. In 2020, while fishery production remained stagnant, aquaculture reached a historic milestone, totaling around 123 million tons and generating a remarkable USD 281 billion in revenue (FAO, 2023).

Decapods such as crabs, crayfish and shrimps are the major species making significant economic and nutritional contributions. The global population of freshwater crayfish comprises 737 species and subspecies (Crandall and De Grave, 2017), of which approximately 15 species belong to three families (Parastacidae, Cambaridae, Astacidae) that hold noteworthy economic significance (Holdich, 1993, Mazlum, 2003). In 2021, worldwide freshwater crayfish production reached 2.7 million tons (FAO, 2023). Freshwater crayfish farming is primarily conducted in the USA, Australia, Europe, and China (Holdich, 1993, Jiang and Cao, 2021, FAO, 2023). In Australia, three *Cherax* species—red claw (*C. quadricarinatus*), yabbies (including common yabby, *C. destructor*, and white yabby, *C. albidus*), and marron, *C. cainii*—have been identified as ideal candidates for aquaculture and are commercially cultured (Lawrence and Jones, 2002, Meakin et al., 2008).

2.2 Marron biology and aquaculture

Taxonomically, marron are arthropods, crustaceans, and decapods, as known as the third-largest freshwater crayfish globally and being native to the south-west of Western Australia (Morrissy et al., 1990). According to the Fletcher and Santoro (2011), marron can attain weights of up to 2.5 kg and reach maturity in two to three years. Reproduction typically begins in spring with rising water temperatures, and a favorable season can yield 200 to 800 eggs, depending on the size of females. The females carry fertilized eggs under their tail until hatching in late spring. Hatched larvae attach to the mother's tail, feeding on the

yolk sac and undergoing multiple moults for several weeks until detaching from the mother and begin active feeding. As an omnivorous species, marron can consume a wide range of items in the pond ecosystem, such as microbial-enriched detritus, plankton, diatoms, macrophytes, and both plant and animal matter (Alonso, 2009a). Therefore, marron play a vital role in freshwater aquatic ecosystems, converting plant and algal material into valuable animal protein (Beatty et al., 2019). Nevertheless, marron food preferences may shift with age (Goddard, 1988). Compared to adults, juvenile marron are typically more planktivorous (Sierp and Qin, 2001), and their consumption of planktons decreases as they grow (Tulsankar et al., 2021a). In their natural habitat, marron typically inhabit clear waters with natural shelters (Fletcher and Santoro, 2011).

Marron is one of important cultured crayfish species, contributing significantly to global crayfish aquaculture production (Jiang and Cao, 2021, Tuynman and Dylewski, 2022, FAO, 2023). Due to its simple life cycle, absence of pelagic larval stages, potential for substantial growth, delicate flesh, robust tolerance for live transport, and resistance to several diseases, marron is a good candidate for aquaculture (Morrissy, 1979, Alonso, 2009b). Moulting in marron involves shedding the old exoskeleton and forming a new one, and their growth, unlike fish, occurs periodically with distinct increases in body weight and length (Sandeman and Sandeman, 2000, Abehsera et al., 2021). The moult cycle typically encompasses four stages, intermoult, premoult, moult, and postmoult-soft stage (Freeman, 1990). In the postmoult-soft stage, marron exhibit a vulnerable soft shell, and in conditions of limited shelters and high population density, is at increased risk of predation and cannibalism. In addition to its role in controlling growth, the moulting process also governs physiological changes in the haemolymph and hepatopancreas (Phlippen et al., 2000, Durliat and Vranckx, 1982), with associated qualitative and quantitative alterations occurring in the haemolymph (Chang, 1995).

Haemolymph encompasses diverse haemocyte types, including granular, semi-granular, and hyaline cells, each distinguished by unique morphology and functions. The proportions of haemocytes serve as indicators of stress and health in crayfish (Jussila, 1997b, Winzer,

2005). The total haemocyte count (THC), a crucial measure of haemocyte production or release into the haemolymph, represents a significant immunological parameter (Romano and Zeng, 2012). The hepatopancreas, which functions both as the pancreas and liver, as well as the tail muscle, serve as the primary energy and nutrient reservoirs crucial for marron moulting and growth (Lindqvist and Louekari, 1975, Prangnell and Fotedar, 2006). Wet, dry indices and moisture concentration of hepatopancreas and tail muscle are indicative measures of condition (Jussila, 1997a, Jussila and Mannonen, 1997), stress levels (Haefner and Spaargaren, 1993), and health and nutritional status (Evans et al., 1992, McClain, 1995, McClain, 2020). Various factors including developmental stage (Skinner et al., 1985, Aiken and Waddy, 1992), water quality (Haddaway et al., 2013, Hammond et al., 2006, Huynh, 2010) and food quality (Olsson et al., 2008, Tan et al., 2018) can affect marron moulting and physiological status. Marron and other crayfish fed entirely on artificial diets may experience reduced moulting frequency or pigmentation loss compared to those fed natural food (Morrissy et al., 1984, Jussila, 1997a). Effective management of these factors is crucial in crayfish aquaculture, as their control profoundly affects commercial viability of the venture.

In earthen pond farming condition, marron can achieve marketable size at approximately 30 months, with an average weight ranging from 150 to 250 g (Fletcher and Santoro, 2013). In Australia, the marron farming industry reached its peak at 88.3 tons, resulting in an annual production valued at approximately AUD $2.45 million in the financial year 2010-2011 (Tuynman and Dylewski, 2022). Despite demonstrating lower volatility compared to its crayfish counterparts, marron farming output has experienced a period of stagnation over the past decade, as depicted in Figure 2.1 (Tuynman and Dylewski, 2022). This stagnation can be attributed to various challenges, including inadequate supply of juveniles, production dependence on seasonal factors, and instances of failure due to poor management practices (Alonso, 2010, Luckens et al., 2015). Consequently, while multiple export opportunities are available, the industry's ability to meet demand remains

constrained (Alonso, 2010), indicating that the marron aquaculture sector has not yet fully met its potential (Beatty et al., 2019).

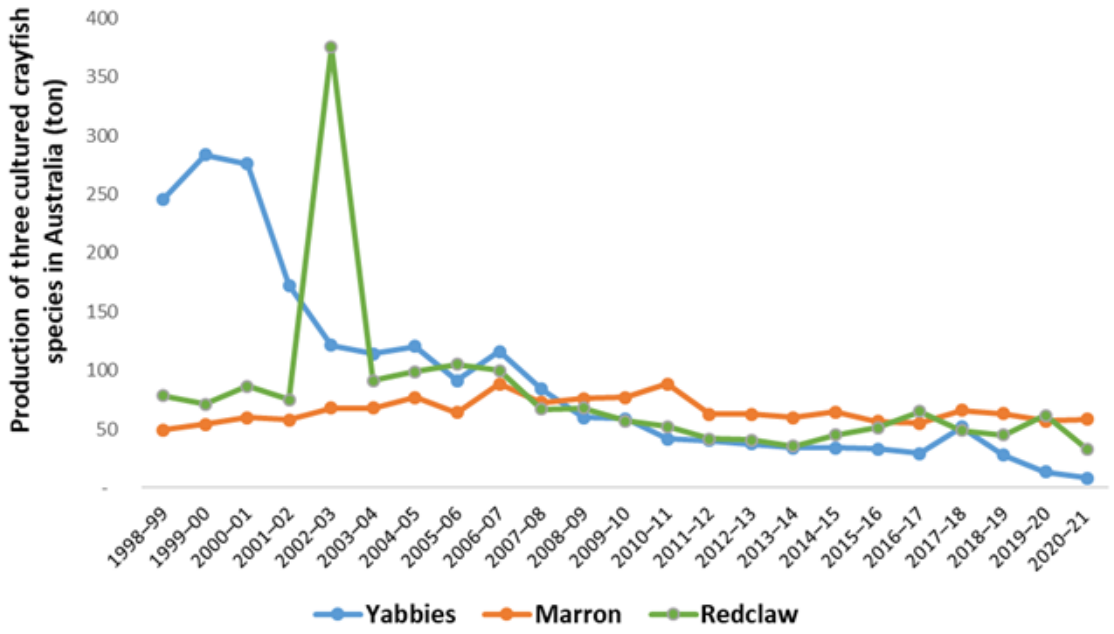


Figure 2.1 Aquaculture production of three crayfish species in Australia, 1998-2021. Source (Tuynman and Dylewski, 2022)

2.3 Water quality requirements in crayfish aquaculture

Crayfish farming, like all other aquaculture sectors, relies on high water quality standards (Ackefors, 2000). Water temperature is a significant factor in crayfish production (Westhoff et al., 2021, Westhoff and Rosenberger, 2016). Red claw juveniles have a temperature threshold ranging from 10°C to 35°C (King, 1994) and moulting frequency of this species is temperature-dependent (Meade et al., 2002). Similarly, within the tolerance temperature range of 14-22°C, the moulting frequency of crayfish (*Paranephrops zealandicus*) is positively correlated with water temperature, leading to a proportional increase in the growth rate of this species as water temperature rises (Hammond et al., 2006). Furthermore, Jin et al. (2019) documented that reproductive performance (rates of feeding, spawning and fecundity) of red swamp (*Procambarus clarkii*) were significantly

affected by water temperature. Under farming condition, it is typically necessary to maintain dissolved oxygen concentrations above 5 mg/L for most crayfish species (Ackefors, 2000). Maintaining appropriate dissolved oxygen level by mechanical aeration can stimulate the assimilation of nitrogenous wastes in the culture systems (Boyd and Tucker, 2012). Ammonia excretion is critical significance within crayfish culture systems characterized by insufficient water throughput. A portion of the excreted ammonia undergoes transformation into potentially harmful substances, notably un-ionized ammonia and nitrite which were determined to cause mortalities for red claw at 2.92 mg/L and 4.74 mg/L, respectively (Liu et al., 1995). Total ammonia nitrogen below 0.5 mg/L and nitrite concentration less than 0.3 mg/L are recommended for crayfish aquaculture practices (Masser and Rouse, 1997). Another form of nitrogen, but less toxic in the culture system is nitrate which is the final product of the nitrification process. It is reported that red claw exhibited no mortality when exposed to nitrate concentrations as high as 1 g/L for a period of five days (Meade and Watts, 1995). However, it should be noted that the conversion between nitrogen forms depends on many factors, in which temperature and pH play important roles.

The cultivation of crayfish is optimally conducted within a pH range of 7–8 (Rognerud et al., 1989, Masser and Rouse, 1997). In their natural habitats, freshwater crayfish are seldom found in waters with pH levels below 6 (Økland and Økland, 1986, Appelberg, 1985). Prolonged exposure to pH levels of 5.6 and below can impede calcium uptake in various species post-moulting, prolonging the period of exoskeletal hardness and consequently increasing mortality rates (Malley, 1980). Notably, when female noble crayfish (*Astacus astacus*) were subjected to pH 5.0 conditions, a significant loss of attached eggs was observed within 30 days after oviposition in natural environments (Appelberg, 1985).

Like other crayfish species, marron also require good water quality for their growth (Table 2.1). Poor water quality and the excessive accumulation of organic matter can lead to the occurrence of protozoa, such as *Epistylis*, on marron (Ambas et al., 2013). To enhance water quality and prevent anoxic conditions, aeration is advised for moderate to high

stocking densities (3-13 animals/m^2) (Fotedar et al., 1999, Fletcher and Santoro, 2011). As marron are practically cultured at relatively low density in semi-intensive ponds, no issue related to toxic nitrogenous compounds is reported. However, elevated levels of ammonia, nitrite, and nitrate can occur in intensive culture systems, potentially posing health risks to marron and hindering their growth (Jussila and Evans, 1996). Marron exhibit sensitivity to reduced oxygen levels, especially when hypoxia is induced by excessive feeding (Morrissy et al., 1984). The excessive accumulation of organic matter in ponds can also result in reduced survival rates due to oxygen depletion (Morrissy, 1979). It is reported that seasonal variation and pond age demonstrated a considerable influence on both the water quality, natural productivity, and the inherent productivity of marron ecosystems (Cole et al., 2019, Tulsankar et al., 2021b). Cole et al. (2019) reviewed water quality requirements for marron aquaculture, noting the tolerance of marron to water temperature between 11-30 ºC, pH levels of 6.5-9.0, dissolved oxygen exceeding 5 mg/L, total ammonia below 0.01 mg/L, nitrite concentrations under 1 mg/L, and nitrate levels below 10 mg/L.

2.4 Role of sediment in crayfish aquaculture

In crayfish aquaculture, the characteristics of soil/sediment/detritus present in the system play crucial roles, alongside water quality. The terms "sediment" and "detritus" are frequently utilized in the literature to refer to the material covering the bottom soils (Boyd, 1995). Sediments, comprising of eroded soil, decaying plankton, uneaten food, feces, and microorganisms can occupy as much as 50% of the pond bottom area (Hopkins et al., 1994, Boyd, 1995, Burford and Longmore, 2001). Although there have been advancements in enhancing the digestibility of commercial feeds for cultured fish, approximately 15% of the ingested feeds are transformed into feces (Reid et al., 2009), and about 5% are unconsumed (Bureau et al., 2003). Therefore, sediment is often rich in organic matter and nutrients (Avnimelech and Ritvo, 2003, Kouba et al., 2018).

Carbon (C) and nitrogen (N) are the major nutrients accumulated in sediment of aquaculture systems (Boyd, 1995, Avnimelech and Ritvo, 2003, Sahu et al., 2013). In general, organic C serves as an energy source for bacteria and other microorganisms in

sediment-water interface, facilitating the release of nutrients into the water column through diverse biochemical mechanisms (Moriarty, 1997, Frade et al., 2020). Aquaculture pond soils containing less than 0.5% organic carbon are categorized as unproductive, while those falling within the 0.5-1.5% and 1.5-2.5% ranges are considered to possess medium and high productivity levels, respectively (Boyd, 1995). However, an organic carbon content exceeding 2.5% may not be conducive to cultured animal production due to the potential for excessive microbe proliferation and subsequent oxygen depletion in the water (Boyd, 1995). Boyd et al. (2010) summarized the data from 233 aquaculture ponds and found that organic carbon concentration in sediment varied between 1.08% and 7.01%.

N stands as one of the pivotal nutrients essential for phytoplankton and its primary sources are inorganic fertilizers and formulated feeds (Boyd and Tucker, 2012). Therefore, appropriately providing amounts of these sources can enhance natural productivity and achieve targeted animal production goals (Boyd and Tucker, 2012). Nitrogen levels in pond bottom sediments normally can range from 0.1 to 0.3% and they do not differ with pond age (Thunjai et al., 2004).

The C/N ratio significantly impacts the activity of sediment microorganisms, which, in turn, plays a crucial role in determining the rate at which nutrients are released from decomposing organic matter (Meyer et al., 2022, Jia et al., 2023). Mineralization, the process of breakdown, is directly influenced by the C/N ratio. A review on the concentrations of C, N and C/N ratio in aquaculture pond soil and sediment is presented in Tables 2.1 and 2.2. Generally, studies on these aspects are scanty and typically, sediment C/N ratios falling within the range of 10 to 15 are favorable for aquaculture (Thunjai et al., 2004, Adhikari, 2003).

Table 2.1 Carbon and nitrogen concentrations (g/kg) in different types of aquaculture sediments

Type of sediment	TOC (g/kg)	N (g/kg)	C/N	References
Striped bass pond	398 ± 18	47.4 ± 3.4	9.1 ± 0.8	Mirzoyan et al. (2008)
Tilapia pond	429 ± 19	38.7 ± 4.0	10.3 ± 0.8	
Prawn tank in a greenhouse	318 ± 19	35.8 ± 2.7	9.9 ± 1.9	
Mixed-fish pond	31.44 ± 6.09	1.56 ± 0.09	-	Ma et al. (2018)
Crab pond	20.03 ± 3.12	1.49 ± 0.06	-	
Culturing unit outlet	482.8	28.6	16.9	Kouba et al. (2018)
The immersed biofilter	314.1	22.5	14.0	
Sedimentation pond	160.0	11.7	13.7	

Abbreviations: TOC – total organic carbon; N – nitrogen; C/N – carbon to nitrogen

Table 2.2 Percentage of carbon and nitrogen in sediment of aquaculture ponds

Species	Country	Pond age (years)	C (%)	N (%)	C/N	References
Various fish species	Alabama, USA	2	2.33 ± 0.19	0.35 ± 0.02	6.5 ± 0.8	Munsiri et al. (1995)
		23	2.2.85 ± 0.09	0.38 ± 0.05	5.7 ± 0.2	
		52	2.73 ± 0.17	0.39 ± 0.07	7.0 ± 0.3	
Tilapia	Thailand	-	1.55 – 4.96	0.10 – 0.30	5.1 – 19.2	Thunjai et al. (2004)
Freshwater prawn	Thailand	-	1.38 ± 0.43	0.14 ± 0.04	9.9 ± 1.8	Wudtisin and Boyd (2006)
	Thailand	3	1.82 ± 0.24	0.12 ± 0.02	-	Boyd et al. (1999)
		12	2.28 ± 0.05	0.17 ± 0.39	-	
Shrimp	Honduras	12	2.35 ± 0.06	0.29 ± 0.01	-	
		12	1.77 ± 0.16	0.23 ± 0.02	-	
Bait minnow	Arkansas,	7	1.71 ± 0.27	0.03 ± 0.01	-	Tepe and Boyd (2002)
	USA	20-25	1.98 ± 0.26	0.10 ± 0.01	-	
		30-35	2.16 ± 0.13	0.05 ± 0.01	-	
Carps	Thailand	-	3.02 ± 1.5	0.28 ± 0.12	10.8 ± 2.0	Wudtisin and Boyd (2006)
Clarias hybrid	Thailand	-	1.46 ± 0.8	0.18 ± 0.11	8.1 ± 3.2	Wudtisin and Boyd (2006)

Abbreviations: C- carbon; N – nitrogen; C/N – carbon to nitrogen

Despite the importance of sediment in aquaculture ecosystems, studies on the correlation of sediment characteristics and aquacultured animals are still rare (Shafi et al., 2022). Previous reports on sediment have assessed the accumulation of sediment in different areas, pond ages (Steeby et al., 2004, Boyd, 1995) and sediment types (Martinez-Garcia et al., 2015) (see Tables 2.1 and 2.2). These studies found that aquaculture inputs led to elevated levels of organic matter and nutrient concentrations in the sediment (Steeby et al., 2004, Boyd, 1995). Like other aquatic decapod species, crayfish are benthic species that mainly inhabit the sediment-water interface. Sediment can be consumed by various crayfish species such as red swamp (Alcorlo et al., 2004, Rudnick and Resh, 2005), red claw (Marufu et al., 2014) and marron (Meakin et al., 2008, Tulsankar, 2021, Beatty, 2006).

There are several factors influencing sediment nutrient dynamics in aquaculture. Mai (2016) reported that the TN concentration in bottom soil of marron ponds, ranging from 28.2 to 82.1 mg/100 g dry soil, exhibited a significant increase during summer and autumn compared to other seasons, while the organic matter levels remained stable throughout the year among the studied ponds, varying from 19.8 to 28.3 mg/100 g dry soil. Therefore, seasonal variations in terms of temperature, rainfall and water flow patterns can influence sediment transport and deposition. Aquaculture management practices, such as pond preparation, stocking species, feeding regimes and aeration can affect sediment dynamics (Drozdz et al., 2020). Overfeeding, for example, can result in excess organic matter sedimenting (Boyd and Tucker, 2012). In the absence of proper management, sediment tends to accumulate in the deeper sections of ponds, leading to a reduction in the available water volume for cultivation. This accumulation, through various processes, can have detrimental effects on water quality (Bunting and Pretty, 2007). The type of aquaculture species and stocking density can impact sediment dynamics. Some species may stir up sediment more than others, and high stocking densities can lead to increased waste production. Effective sediment management in aquaculture is crucial for maintaining water quality, preventing disease outbreaks, and optimizing the overall health and production of the system. Proper sediment management techniques before stocking (for example, use of

sedimentation basins) and after harvesting (for example, sediment removal) can control sediment accumulation to a certain level. Nonetheless, these approaches are characterized by their high cost, are time-consuming and have the potential to introduce pathogens into the systems (Devaraja et al., 2013). Moreover, monitoring and maintaining sediment quality during stocking is important for protecting the culture environment and ensuring the health and well-being of cultured organisms. Alternative methodologies have also been proposed and implemented for the improvement of water quality in the culture systems of marron and other crayfish species. These include the use of probiotics (Foysal et al., 2019d, Cole et al., 2019), sustainable aqua-feeds (Foysal et al., 2022a), or stimulating the heterotrophic microorganisms in situ through external carbon supplementation (Azhar et al., 2020). However, it is essential to emphasize that a thorough examination of the potential impacts on sediment characteristics remains unexplored in this field, highlighting the imperative for additional research.

2.5 Role of rearing environment microorganisms in crayfish aquaculture systems

Many researchers indicate that microbes can serve as beneficial allies in protecting aquatic species against infections (Brunt et al., 2007, Hai, 2015, Knipe et al., 2021). By manipulating the microbiome, the risk of infection decreases, subsequently reducing the necessity for antimicrobial treatments and lowering the development of antibiotic resistance (Bentzon-Tilia et al., 2016). Certain beneficial bacteria can generate extracellular enzymes and supply micronutrients to promote the growth of aquatic animals (Wang et al., 2020a). The microbiome of both aquatic animal gut and rearing environment plays a crucial role in determining the cultured animal well-being (Rajeev et al., 2021). This is because these aquatic animals, especially decapod species are constantly in direct contact with water and sediment, the microbiome in these habitats directly influences their health and growth (Chen et al., 2017a). The changes in water and sediment microbiota have a substantial impact on the gut microbial communities of aquatic decapod crustaceans (Dodd et al., 2020, Giatsis et al., 2015). Moreover, it is important to highlight that the connection between bacterial compositions in water and the animal gut is comparatively

less significant when contrasted with the strong correlation observed in the microbiota between sediment and the gut (Del'Duca et al., 2015, Huang et al., 2018, Wang et al., 2014). Hence, it is imperative to gain insights into not just the microbial populations within the animal but also those within the rearing environment, as this knowledge can contribute to improving the health of aqua-cultured animals by avoiding disease outbreaks (Rajeev et al., 2021).

Multiple factors, including seasons, culture conditions (ex. water exchange, water quality, culture duration), developmental stages of animals, collectively influence alterations in water and sediment microbial communities (Huang et al., 2018, Zeng et al., 2021, Giatsis et al., 2015). Previous studies revealed the fluctuations in microbial composition and abundance in white leg shrimp cultures induced by seasonal variations (Zhang et al., 2016). It was also documented that the availability of nutrients is a pivotal factor leading to substantial fluctuations in the composition and functionality of bacterial communities (Jia et al., 2020). Specifically, total nitrogen and organic matter represent primary elements that limit both bacterial growth and their abundance and activity (Jia et al., 2020). In rearing water, significant associations between nutrients and microbiota were identified (Nguyen et al., 2021). Moreover, Huang et al. (2018) demonstrated that variations in nitrogenous compounds in the rearing water can trigger changes in microbial communities in the sediment. A study on white leg shrimp revealed that 37% of the bacterial communities present in the rearing tank water originated from feed or water exchange, highlighting the beneficial impact of these factors on water bacteria (Heyse et al., 2021). Inadequately managed culture systems frequently result in the accumulation of excessive nutrients, which, in turn, can deteriorate the culture environment by promoting the proliferation of pathogenic bacterial communities (Sun et al., 2021, Lo et al., 2022).

Research into microbiota in crayfish aquaculture has primarily concentrated on the characterization of microbial composition and functions within the gut of three species including red swamp, red claw, and marron (Alvanou et al., 2023, Hernández-Pérez and Söderhäll, 2023). Hernández-Pérez and Söderhäll (2023) summarized 30 research articles

on the common core microbiome in crayfish and covered advances in manipulating gut microbiota and their impact on crayfish growth. Up-to-date research findings on how gut microbiota, disease, and environmental changes are interconnected in crayfish were also documented in that review. Another review paper by Alvanou et al. (2023) focused on numerous factors influencing gut microbiota abundance and composition such as developmental stage, culture methods, environmental conditions and diets. Of the four mentioned crayfish species, the red swamp crayfish has gained more research attention, particularly concerning various factors affecting their microbiota (Alvanou et al., 2023). Different development stages (Xie et al., 2021), seasons and environmental parameters (Liu et al., 2020a, Guo et al., 2020, Xavier et al., 2021), culture models (Liu et al., 2020b, Huang et al., 2022b), health conditions (Xue et al., 2022) and diets (Wan et al., 2022) significantly differentiated their gut microbial communities.

On red claw and marron, various factors influencing their microbiota abundance and composition have also been investigated (Alvanou et al., 2023). Nanoplastic exposure (Cheng et al., 2022) and virus infection (Zheng et al., 2023) can lead to significant changes in the microbiota of red claw gut. In marron gut, the phylum Tenericutes and the genus *Candidatus Bacilloplasma* are the core bacteria (Foysal, 2021); however, *Vibrio* can become dominant when marron are under starvation condition (Foysal et al., 2020d). The composition of the marron gut microbiota is notably influenced by the use of diverse biological filters, formulated diets containing various probiotics and protein sources (Foysal, 2021). Different protein sources in the diets for marron can also influence the microbial structure in the rearing water (Foysal et al., 2022a, Nguyen et al., 2021). Additionally, the implementation of suspended zeolite, recognized for its nitrogenous waste filtering capabilities, has been proposed as a potential method for altering the microbial communities in the sediment of marron tanks (Foysal et al., 2022b). Nevertheless, there are less studies on microbiota in water and sediment in marron culture systems and our understanding of the factors influencing these microbial communities is still limited, highlighting the need for further research in this area.

2.6 Formulated diets and dietary protein sources affect crayfish growth and health

Within the aquaculture industry, poor feeding management practices can lead to water quality deterioration and effluent discharge, issues that have become of increasing concerns (Boyd and Tucker, 2019, Ahmad et al., 2022). Developing a low protein diet with high nutrient digestibility can be seen as a proper measure that could reduce waste loads but possibly affect marron's health and production. Consequently, there exists a pressing need to identify dietary formulations capable of concurrently promoting environmental sustainability and enhancing animal performance.

To date, it has been widely accepted that probiotics play a significant role in aquaculture since dietary probiotic can improve animal immune status and growth performance (El-Saadony et al., 2021, Nimrat et al., 2013). A dietary probiotic supplement can also act as an eco-friendly method for stress and disease management in sustainable aquaculture (Wang et al., 2019, Duan et al., 2017). Previous studies have demonstrated the beneficial effects of dietary probiotic in laboratory and field conditions on growth performance of various crayfish species (Table 2.3). The probiotic approach that has been applied to marron aquaculture showed promising outcomes, especially in indoor systems with controlled environments (Cole et al., 2022, Foysal, 2021, Ambas et al., 2019). Compared to indoor, outdoor aquaculture systems are more complex, and nutrient inputs along with many other factors, including in situ nutrients of the bottom soil, can contribute to the target yield. The cultured species partially use nutrients entering the pond, most of which are suspended in water and accumulated in the pond sediments. The evaluation of ponds with varying diet inputs infusing a mixture of nutrients into the water along with assessment of microbial communities in water and sediments are lacking and needs to be studied in future research.

Table 2.3 Application of probiotics in crayfish aquaculture (Adapted from Alvanou et al. (2023))

Probiotics	Species	Administration	Responses	References
Lactobacillus plantarum	*Astacus leptodactylus*	Feed additive	Improved immunity parameters	Valipour et al. (2019)
A mixture of *Bacillus sp*., *Acinetobacter sp*., and *Chryseobacterium balustinum*	*C. quadricarinatus*	Inhibition test	Incapable of outgrowing and defeating harmful bacteria *A. hydrophila*	Hayakijkosol et al. (2017)
Micrococcus spp.	*C. quadricarinatus*	Feed additive	Improved growth and immune systems	Amrullah and Wahidah (2019)
Ecoterra® *composed of B. licheniformis, B. subtilis, Nitrobacter, Nitrosomonas, Rizobium, Saccharomyces cereviciae,*	*C. quadricarinatus*	Water additive	Increased the values of haemolymph parameters	Carreño-León et al. (2014)
Spomune© *(B. subtilis and C. butyricum)*	*C. montezumae*	Feed additive	Increased survival, weight gain and growth rate	Javier et al. (2021)
B. amyloliquefaciens spp. and *L. plantarum*	*Pontastacus leptodactylus*	Feed and water additive	No positive effects on growth Negative effects on survival and gut and hepatopancreas	Özdoğan et al. (2022)
Subtilis-C (*B. subtilis, B. licheniformis*)	*P. leptodactylus*	Feed additive	Increased survival rate and immunity	Pronina et al. (2021)

Hafnia alvei	*P. leptodactylus*	Feed and water additive	No positive effects on growth and survival Potential inhibitory activity against *Aeromonas hydrophilla*	Didinen et al. (2016)
B. amyloliquefaciens	*P. clarkii*	Feed additive	Enhanced enzyme activities and white-spot syndrome virus resistance	Xu et al. (2021)
B. coagulans and *L. lactis*	*P. clarkii*	Feed additive	Increased gut microbiota diversity, enzyme activities, haemocyte phagocytosis rate and pathogen resistance	Zhu et al. (2023)
L. fermentum GR-3	*P. clarkii*	Feed additive	Increased production, positive effects on gut microbiota	Han et al. (2022b)
B. amyloliquefaciens	*P. clarkii*	Feed additive	Enhanced enzyme activities and white-spot syndrome virus resistance Decreased apoptosis of haemocytes	Lai et al. (2020)
Bacillus subtilis CK3	*P. clarkii*	Water additive	Increased survival rate and immunity, resistance to *A. veronii*	Yang et al. (2022)

Bacillus sp., *Shewanella sp.*	*C. tenuinamus*	Feed additive	No positive effects on survival and growth Inhibit pathogenic *Vibrio mimicus*	Ambas et al. (2013)
B. mycoides	*C. tenuinamus*	Feed additive	Increased survival of live transport, gut bacterial population, total haemocyte count	Ambas et al. (2015)
Clostridium butyricum	*C. cainii*	Feed additive	Changed in gut microbiota, enhanced innate immune responses	Foysal et al. (2019d)
B. mycoides	*C. cainii*	Feed additive	Modulated the health status, gut microbiota and innate immune response	Foysal et al. (2020b)
Lactobacillus acidophilus and *L. plantarum*	*C. cainii*	Feed additive	Improved health status, modulated gut microbiota and innate immune response	Foysal et al. (2020c)
L. plantarum	*C. cainii*	Feed additive	Increased immunity parameters and gut microbiota diversity	Foysal et al. (2021)
Commercial probiotics	*C. cainii*	Feed additive		Current study

Furthermore, the utilization of diverse dietary feed components in aquaculture inherently leads to alterations in the microbial ecosystems within the rearing environment, as evidenced by Bentzon-Tilia et al. (2016) and Foysal et al. (2022a). The well-being of aquatic species exhibits a pronounced association with the composition and functions of microbial communities both within the animals' digestive systems as well as in its environment (Adamovsky et al., 2018, Huang et al., 2018). While research has probed into the influence of various dietary protein supplements on the gut microbiota and health status of numerous crayfish species (see Table 2.4), comparatively limited attention has been devoted to investigating the ramifications of divergent dietary regimens on crayfish rearing environment. The introduction of aqua-diets has been observed to expedite the establishment of microbial populations within the aquatic rearing environment (Elsaidy et al., 2015, Ibekwe et al., 2017). In this context, residual feed materials, which contain essential trace elements, vitamins, and minerals, serve as valuable nutrient sources for indigenous microbial communities, thereby promoting their proliferation (Crab et al., 2012, Cardona et al., 2016). These microorganisms not only contribute significantly to the removal of organic waste and the remediation of water quality but also produce microbial proteins that can serve as a nutritional resource for aquatic species (Bentzon-Tilia et al., 2016, Delamare-Debouteville et al., 2019). Specifically, the augmentation of phyla such as Bacteroidetes, Firmicutes, Tenericutes, as well as the presence of specific bacterial species including *Bacillus, Citrobacter, Pseudomonas*, and *Serratia*, has been documented to naturally facilitate the detoxification of aquatic bio-toxins and the decomposition of organic waste in aquaculture environments (Zhu et al., 2017, Foysal et al., 2020e).

Due to their omnivorous feeding habits, crayfish species demonstrate a moderate protein necessity, which is estimated to be around 30% protein content in formulated feeds (Fotedar, 1998, Ambas et al., 2017). Fish meal (FM) has traditionally constituted a significant source of dietary protein for cultured aquatic organisms (FAO, 2018). However, the declining production of FM observed in recent decades has spurred research efforts aimed at identifying cost-effective and sustainable alternatives to FM, while ensuring

optimal growth of various aquatic species (FAO, 2018, Eroldogan et al., 2022). Noteworthy among these alternatives is poultry-by-product meal (PBM), which has shown promise in enhancing growth rates, digestibility, and immune responses in diverse aqua-cultured crayfish species (see Table 2.4), including marron (Saputra et al., 2019, Foysal et al., 2019b, Siddik et al., 2020).

In marron aquaculture, it has been observed that both FM and PBM diets exert significant influences on the accumulation of nitrogenous compounds and their associations with microbial communities in the rearing water (Nguyen et al., 2021). On the aspects of microbiota, the study of Nguyen et al. (2021) revealed that the presence of analogous predominant bacterial genera, including *Aeromonas*, *Hafnia obesumbacterium*, *Candidatus Bacilloplasma,* and *Serratia*, in the water column, irrespective of the dietary protein sources employed. Furthermore, a separate study conducted by Siddik et al. (2020) disclosed that marron fed with fermented PBM exhibited a reduction in the abundance of *Aeromonas*, accompanied by a significant upregulation of *Lactobacillus* within their guts. Notably, the *Lactobacillus* group functioned as probiotics, contributing to improved nutrient absorption and utilization, as emphasized by Siddik et al. (2020). Subsequently, Foysal et al. (2022a) conducted a study that documented heightened diversity in bacterial communities within both the water and gut habitats of marron fed with PBM. Overall, multiple studies have demonstrated a strong correlation between microbial communities present in the water, sediments, and guts of aquatic animals (Del'Duca et al., 2015, Xiong et al., 2015). While the most dominant bacterial taxa were found to be shared between the rearing environment and the digestive tracts of the animals, there was also a notable similarity in the microbial community profiles between sediments and animal guts (Del'Duca et al., 2015, Huang et al., 2018, Wang et al., 2014). Given these findings, it is imperative to gain a comprehensive understanding of not only the microbial groups residing within the gut of aqua-cultured animals but also those present in their surrounding aquatic environment. Such knowledge is crucial in bolstering the health of aqua-cultured organisms and mitigating the risk of disease outbreaks.

Table 2.4 Studies on dietary protein sources in crayfish aquaculture

Protein sources	Species	Responses	References
Various protein sources (meat, snail, SBM, yabby meal, zooplankton)	*Cherax destructor*	No difference in survival and growth performance The strongest pigmentation in animals fed a zooplankton based diet	Jones et al. (1996a)
SBM to replace FM	*C. destructor*	A diet of 30% protein with 20% soy meal resulted in maximum animal weight	Jones et al. (1996b)
Commercial, FM-based, and SBM-based diets	*Astacus leptodacylus*	High rate of SBM diet reduced the growth, but had no negative impacts on biochemical parameters	Banaee et al. (2013)
SBM to replace FM	*C. quadricarinatus*	50% replacement fishmeal by soy meal resulted in higher lipid levels in hepatopancreas and muscle	Gutiérrez and Rodriguez (2010)
Cottonseed meal, SBM, rapeseed meal and peanut meal to replace FM	*C. quadricarinatus*	SBM, rapeseed and peanut meals were not suitable as adversely affected red claw's growth Cottonseed meal positively influenced amino acid nutrition, digestion, and immunity	Qian et al. (2021)
SBM to replace FM	*C. quadricarinatus*	25% replacement of FM by SBM in a 45-50% dietary protein resulted in the highest survival and growth	Fuertes et al. (2012)
SBM-based diets containing FM, PBM, ground peameal or distillers dried grains with soluble	*C. quadricarinatus*	SBM diet alone resulted in low survival and growth All four protein sources in SBM diets had similar effects on red claw performance.	de Yta et al. (2012)
SBM and brewer's grains with yeast to replace FM	*C. quadricarinatus*	A meal as a combination of SBM and brewer's grains with yeast can totally replace FM	Muzinic et al. (2004)
Biofloc biomass to be included into a commercial diet	*Procambarus clarkii*	Biofloc biomass elevated growth with up to 66% inclusion	Lunda et al. (2020)

SBM and/or cotton seed meal to replace FM	*C. quadricarinatus*	Total replacement of FM by SBM or cotton seed meal reduced growth performance, enzyme activities A mixture of SBM and cotton seed meal can replace 50% FM	Jiang et al. (2023)
SBM to replace FM	*C. quadricarinatus*	50% FM could be replaced by SBM without adverse effects on growth	Gutiérrez and Rodriguez (2010)
PBM to replace fishmeal	*C. quadricarinatus*	FM could be replaced with PBM at various levels without negative effects on red claw growth and survival	Saoud et al. (2008)
FM, MBM, poultry meal, SBM, canola meal, lupin meal and brewer's yeast	*C. quadricarinatus*	Red claw are capable of using nutrients from a variety of dietary protein sources.	Pavasovic et al. (2007)
PBM and BSF to replace FM	*C. cainii*	Growth performance of marron was independent of dietary treatments PBM + BSF –based diet positively affect health and intestinal microbiota of marron	Foysal et al. (2019b)
Compare BSF-based diet and BSF supplemented with probiotics *Lactobacillus plantarum* diet	*C. cainii*	Growth performance of marron was similar in both diet treatments Both diets upregulated immune gene expression in the gut	Foysal et al. (2021)

SBM, lupin, PBM, BSF and tuna hydrolysate to replace FM	*C. cainii*	Plant-based diets favoured the growth of *Flavobacterium, Aeromonas*, and *Vogesella* in the gut of marron Insect meal increased abundance for Firmicutes in rearing water PBM and BSF-based diets resulted in high bacterial diversity in water and gut.	Foysal et al. (2022a)
A mixture of abalone waste and fermented *Sargassum spp* to replace FM	*C. cainii*	A fermented waste-seaweed mixture can substitute up to 100% FM protein in the formulated diet without negatively affecting growth performance of marron	Achmad et al. (2023)
PBM, feather, lupin, and MBM to replace FM	*C. cainii*	Feather meal-based diet resulted in highest survival rate PBM-based diet positively affected total haemocyte count, microvilli length, and high temperature exposure.	Saputra et al. (2019)
FM, PBM	*C. cainii*	Varied bacterial taxa prevalence due to dietary proteins. Correlations found between dominant bacterial taxa and water quality indicators.	Nguyen et al. (2021)

Abbreviations: SBM - soybean meal; FM - fish meal; PBM - poultry-by-product meal; MBM - meat and bone meal; BSF - black soldier fly meal.

2.7 Carbon/nitrogen ratio as a vital factor for sustainable aquaculture

Traditional aquaculture systems face inherent limitations resulting from a cyclic interplay of cause and effect. Densely populated aquaculture farms lead to coastal ecosystem pollution, contaminating the rearing environment. Sustainable models have been required to produce more food from aquaculture using limited resources and with lower environmental impacts (FAO, 2018); indeed, biofloc technology (BFT) is considered highly suitable for this purpose (Bossier and Ekasari, 2017, Nisar et al., 2022, McCusker et al., 2023). BFT employs aquatic microorganisms to mitigate metabolites, such as ammonia and nitrite originating from feed remnants, excrements, and urine in the culture systems. In BFT, the provision of carbohydrates serves to stimulate the proliferation of heterotrophic microorganisms. Biofloc-based aquaculture systems enhance biosecurity by preventing the intrusion of external pathogens and are eco-friendly by reducing water usage and minimizing waste discharge (Abakari et al., 2021b, Ogello et al., 2021).

In biofloc-based systems, carbon-to-nitrogen (C/N) ratio holds a pivotal role, facilitating the conversion of waste materials into bacterial biomass (Avnimelech, 1999, Bossier and Ekasari, 2017, Crab et al., 2012). Manipulating this ratio by introducing external carbon sources into aquaculture systems can enhance nitrogen uptake by heterotrophic bacteria, resulting in decreased ammonium levels and increased microbial biomass (see review by Ahmad et al. (2017); Dauda (2020); Minaz and Kubilay (2021) and Abakari et al. (2021b). This practice confers several benefits upon the aquaculture system, including the maintenance of favorable water quality and the provision of an additional nutrient source to support the growth of various cultured species (Wei et al., 2016, Panigrahi et al., 2018). Numerous studies have substantiated that elevating C/N ratio in the water can lead to enhancements in feed utilization, bolstered innate immune responses (Miao et al., 2017b, Panigrahi et al., 2018, Panigrahi et al., 2019), as well as improvements in the health of the hepatopancreas and digestive tracts in crustaceans (Tong et al., 2020, Xu and Pan, 2013).

Carbohydrates, such as corn or wheat flour, are not only essential components of crustacean diets (Sang and Fotedar, 2010, Saputra et al., 2019) but are also utilized as exogenous carbon sources in aquaculture systems. Research has demonstrated that supplementing

with corn starch and wheat flour can yield several benefits, including the improvement of water quality, augmentation of heterotrophic bacterial biomass, and enhancement of the growth and survival rates of various brackish shrimp species, including pink shrimp (*Farfantepenaeus brasiliensis*) (Emerenciano et al., 2012) and white leg shrimp (Kim et al., 2021, Tinh et al., 2021). In contrast, molasses, although not a common ingredient in the diets of decapods, has gained widespread use as an external carbon source in the aquaculture of tiger shrimp (*Penaeus monodon*) (Kumar et al., 2014) and white leg shrimp (Samocha et al., 2007). It has been demonstrated that, compared to other exogenous carbon sources, molasses is particularly effective in ammonia removal and promoting the growth of white leg shrimp (Khanjani et al., 2017, Serra et al., 2015). Prior investigations have documented positive outcomes in the cultivation of freshwater decapod species, notably the freshwater prawn and various crayfish species, including red claw, narrow-clawed, and red swamp, within biofloc-based aquaculture systems supplemented with various exogenous carbon sources (see Table 2.5). Nevertheless, comparable investigations remain absent in the context of marron, thus necessitating additional research endeavors to elucidate potential positive impacts of carbon supplementation on the health and immune competence of marron to sustain this aquaculture industry.

Table 2.5 Summary of studies on the benefits of exogenous carbon supplementation in freshwater decapod aquaculture

Species	Initial size (g)	Carbon sources	C/N ratio	Culture duration (days)	Benefits	References
Macrobrachium rosenbergii	0.13	Molasses	-	30	Improved FCR	Ballester et al. (2017)
	11.94	Molasses	-	45	No need to include vitamin and mineral in diet	Ballester et al. (2018)
	0.03	Molasses	20	180	Higher production and lower FCR	Pérez-Fuentes et al. (2013)
	Postlarvae (14-day-old)	Acetate, glycerol and glucose	10	15	The glycerol bioflocs had highest protein content Prawn fed on bioflocs and had high survival rate	Crab et al. (2010)
	0.32	No carbon addition	-	60	High stocking density (250 prawns/m^2) resulted in higher prawn biomass Low stocking density (50 prawns/m^2) increased survival and reduced FCR	Negrini et al. (2017)
	0.26	Molasses + probiotics	10, 20	90	Enhanced prawn immunity parameters such as total haemocyte count and enzyme activities	Miao et al. (2017b)

	0.19	Molasses Glucose Sucrose	10	45	Higher gene expression levels exhibited better antibacterial response	Miao et al. (2020)
	0.01	Molasses Rice bran + *Bacillus spp.*	5	35	Rice bran combined with *Bacillus spp*. Resulted in higher prawn final weight and weekly weight gain	Santos et al. (2022)
	0.016	Corn starch	10-25	21	Lower C/N ratios (10 and 15) negatively affected prawn growth C/N ratio of 15-25 resulted in higher crude protein of prawn	Hosain et al. (2021b)
	Larvae stage 1 – postlarvae 15	Refined sugar	17.5	32	Light intensity of 20,000 lux improved larval performance cultured in a biofloc system	Tao et al. (2021)
Co-cultured *C. quadricarinatus* and *O. niloticus*	Crayfish (11.5) and tilapia (7)	Molasses	10, 15	80	Positive effects of increasing C/N ratio on water quality and feed utilization efficiency	Azhar et al. (2020)
A.leptodactylus	37.6	Molasses	15	32	Improved crayfish survival	Genc et al. (2019)

A.leptodactylus	-	-	-	45	Crayfish adapted well at high densities in biofloc conditions, represented by haemolymph indices in normal range	Doğukan et al. (2021)
P. clarkii	9.7	-	-	30	Crude protein of biofloc (36.8%) meets crayfish protein requirement Positive effects on crayfish in terms of increasing enzyme activities	Li et al. (2019a)
	0.01	Glucose	15	45	200 larvae/m^2 was optimal stocking density when cultured crayfish in biofloc systems	Li et al. (2023)
C. cainii	11 g	Corn flour, Molasses, Wheat flour	12	60		Current study
	3 g	Molasses	12, 15	60		Current study

Abbreviation: FCR – Feed conversion rate

CHAPTER 3 General methodology

Two field trials were completed at Blue Ridge marron farm (34°12′22″ S, 116°01′01″ E) in Manjimup, Western Australia (Figure 3.1). Three laboratory experiments were conducted at Curtin Aquatic Research Laboratory (CARL) wherein the diagram of the experimental tanks is shown in Figure 3.2.

Figure 3.1 Blue Ridge Marron farm. Source: Google map. Ponds A-F were used for the experiment 1 (Chapter 4) and ponds G-H were used for the experiment 2 (Chapter 5).

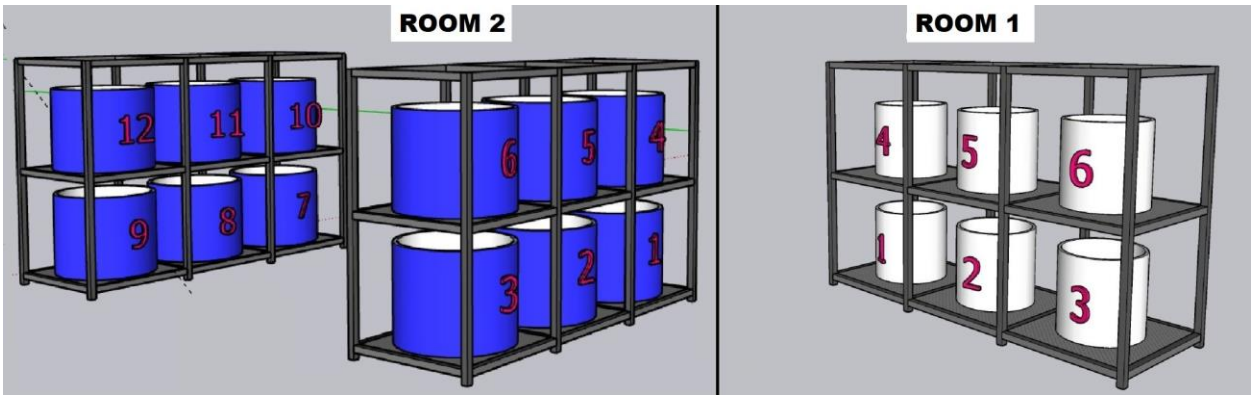


Figure 3.2 Diagram of aquatic tanks used for the experiment 3 (Chapters 6.1 and 6.2) in room 1 and the experiments 4 (Chapter 7) and 5 (Chapter 8) in room 2.

3.1 Sampling and analysing environmental factors in the water column

3.1.1 Abiotic factors

Temperature, pH, dissolved oxygen (DO) and water transparency were measured on site (field trials) or directly in tanks (laboratory trials). Water temperature in all tanks was maintained at 20±2°C within the suitable range for marron culture (Morrissy, 1990). The constant temperature and continuous aeration were maintained in the experimental tanks using digital submersible thermostats (Aqua One, Perth, Australia) (room 1) or a temperature controlled room (room 2) and air pump (Aqua One, Perth, Australia).

Water temperature and pH in ponds and tanks were determined with a digital pH/ºC CyberScan pH 300 (Eutech Instruments, Singapore), and DO was measured by a portable YSI 55 DO meter (Perth Scientific, Australia). Water transparency in ponds was measured using a Secchi disk.

For nutrient concentration analysis, water samples were taken out from ponds or tanks and analysed by using HACH® colorimeter in CARL. All reagents used in water samples analysis were obtained from ROWE Scientific Pty Ltd, Adelaide, Australia. The concentrations of ammonia, nitrite, nitrate, and phosphate were analysed by salicylate (0-0.5 mg/L), diazotization (low range 0-0.35 mg/L), cadmium reduction (high range 0-30 mg/L), and amino acid (0-30 mg/L) methods, respectively. All measurements of water quality were in accordance with standard methods for the examination of water and wastewater (APHA, 1998).

3.1.2 Biotic factors

In field trial, plankton samples were collected into 100 mL small plastic bottles by pouring 10 L of water from each sampling point and passing them through 60 μm and 25 μm mesh plankton nets to obtain zooplankton and phytoplankton species, respectively. Zooplankton samples were preserved with 70% ethanol whereas 3% acid Lugol's iodine was added to the phytoplankton bottles. The number of zooplankton in 1 mL sub-samples was counted in a petri dish at 20× magnification while phytoplankton was estimated using a

haemocytometer at 40× magnification. Plankton abundances were calculated by using equations described by Cole et al. (2019):

Phytoplankton abundance (cells/L) = ((Counted number x 1000)/(Volume of grid x Number of grids counted)) x (Concentrated volume/Total volume)

Zooplankton abundance (ind. /L) = (Counted number x (Concentrated volume/Sub-sample volume))/Total volume

3.2 Sediment collection

For field trials, sediment samples were collected by a sediment sampler (Figure 3.3). The first few centimetres of pond sediments were taken as sediment samples. For laboratory trials, sediments were sampled by siphoning. Approximately 30% of water from every culture tank with faecal waste was transferred to another tank, and after careful collecting of sediment, the water was then transferred back into the respective tank. All samples were kept under cool conditions during transportation. In the laboratory, sediment samples were centrifuged for 10 minutes at 10,000 rpm to obtain sedimentary pellets and then stored in -80ºC refrigerator for further analysis.

Figure 3.3 Sediment sampler

3.3 Sediment carbon/nitrogen ratio analysis

Sediment samples were oven-dried at 60ºC, sieved to 2mm, and ground into powder. Approximately 2 mg of dried samples were directly weighed into tin capsules using a

PerkinElmer AD-6 auto-balance. Percentages of carbon and nitrogen in sediment samples were determined by PE 2400 CHN Elemental Analyzer (PerkinElmer, USA) (Culmo and Shelton, 2013).

3.4 Analysing microbiota in water and sediment samples

3.4.1 DNA extraction, PCR amplification and amplicon sequencing

DNA extraction from water and sediment samples was performed using DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. DNA concentration and quality (260/280 ratio) were assessed in NanoDrop Spectrophotometer 2000cc (Thermo Fisher Scientific, USA). A dilution was performed to prepare an even 50 ng/µl final concentration for PCR amplification.

The bacterial V3-V4 hypervariable regions with overhang adapters (5′F-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; 5′R-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) were amplified according to Illumina 16S metagenomic sequencing protocol (Part # 15044223 Rev. B). Forty cycles of PCR amplification was performed in T100 thermal cycler (Bio-Rad Laboratories, Inc., USA) with 50 µl final volume containing 25 µl Hot Start 2X Master Mix (New England BioLab Inc., USA), 2 µl of template DNA, 1 µl (10 µM) of each forward and reverse primers and 21 µl of DEPC treated water (Thermo Fisher Scientific, USA). Positive amplification was confirmed by agarose (1.5%) gel electrophoresis. Sample purification was carried out using AMPure XP bead (Beckman Coulter Inc., CA, USA) followed by amplicon indexing using a secondary PCR. Samples were pooled to equimolar concentrations after second purification and nanodrop measurement. Paired end (2 × 300 bp) sequencing was performed in an Illumina MiSeq platform (Illumina Inc., CA, USA) using v3 kit.

3.4.2 Bioinformatics

The primary quality of raw amplicon sequence data was checked using fastQC pipelines (Andrews, 2010). Quality trimming of reads was performed using Sickle program and reads less than 200 bp were removed (Joshi and Fass, 2011). Merging and Filtering Tool (MeFiT) was used for the merging of overlapping pair-end trimmed reads with default parameters. Filtering of chimeric sequences, *de novo* assembly of reads into Operational Taxonomic Units (OTUs) at 97% similarity threshold and removal of singleton OTUs were performed using micca otu (version 1.7.0) (Albanese et al., 2015). Phylogenetic classification of representative OTUs was performed against SILVA 132 database collected at 97% similarity (Quast et al., 2012). Multiple sequence alignment (MSA) and rooted phylogenetic tree construction were performed using PASTA algorithm (Mirarab et al., 2015). Alpha and beta diversities were computed using the R packages and the QIIME pipeline (version 1.9.1), (Kuczynski et al., 2012). Alpha diversity and microbial community were calculated employing phyloseq package (McMurdie and Holmes, 2013). Beta-ordination was performed based on the Bray-Curtis dissimilarity of bacterial abundance and the distance metrics were measured using permutational multivariate analysis of variance (PERMANOVA) (Dixon, 2003). Linear discriminant analysis (LEfSe) at the 0.05 significance level was used to identify differentially abundant bacteria at the genus level (Segata et al., 2011). Using the R package microbiomeSeq (https://github.com/umerijaz/microbiomeSeq), the Pearson correlations were examined, and correlation coefficients were presented.

3.5 Feed formulation

For field trials, a commercial formulated feed (premium marron pellets-PMP) purchased from Weston™ Animal Nutrition company was used in the experiment 1. Along with this formulated feed, a Solair feed (SPD) obtained from Solair Group company were tested in the experiment 2. Table 3.1 displays the approximate components of these two commercial diets. Both diets were made of main ingredients including wheat, lupins, fishmeal, meat and bone meal, beef tallow, crystalline amino acids, vitamin and minerals. The proximate composition of two diets was analyzed at CARL and presented in Table 3.1. PMP had significantly higher crude protein, energy, and crude ash but lower carbohydrate and C/N ratio than SPD. Lipid content and protein/energy ratio were similar between the two diets, with SPD additionally receiving probiotics supplementation.

For laboratory, the designed pelleted feed was made with fish meal as the main protein source. This diet was used to feed marron in the experiments 3, 4 and 5. Poultry-by-product meal was used as a main protein source to formulate another diet which was tested in the experiment 3. The proximate compositions of these two customised diets are presented in Table 3.2.

Table 3.1 The proximate composition of two commercial diets (mean ± S.E., n=3)

Parameters	**PMP**	**SPD**	**p-value**
Crude Protein (% dry weight)	20.85 ± 0.35 [a]	19.41 ± 0.10 [b]	<0.05
Lipid (% dry weight)	7.22 ± 0.02 [a]	7.27 ± 0.01 [a]	>0.05
Ash (% dry weight)	7.64 ± 0.03 [a]	6.11 ± 0.04 [b]	<0.01
Energy (KJ/g)	19.53 ± 0.14 [a]	17.81 ± 0.06 [b]	<0.01
Protein/Energy ratio	1.07 ± 0.01 [a]	1.09 ± 0.00 [a]	>0.05
NFE (% dry weight)	64.29 ± 0.36 [a]	67.21 ± 0.14 [b]	<0.01
C/N ratio	12.07 ± 0.03 [a]	14.52 ± 0.05 [b]	<0.01
Probiotics	No	Yes	

Abbreviations PMP - premium marron pellets; SPD – Solair feed; KJ/g – kilojoule/gram

Table 3.2 Ingredients and proximate composition of feeds as per Foysal et al. (2019b)

Ingredients (g/100g dry matter)	**FM**	**PBM**
Fishmeal	41.00	-
Poultry by product meal	-	39.00
Soya bean meal	10.00	10.00
Wheat flour	37.00	38.00
Corn starch	4.80	4.80
Cod liver oil	4.20	5.20
$CaCO_3$	0.02	0.02
Vitamin premix	0.23	0.23
Vitamin C	0.05	0.05
Cholesterol	0.50	0.50
Lecithin-Soy	1.00	1.00
Betacaine	1.20	1.20
Proximate composition		
Crude Protein (% dry weight)	29.93	29.61
Lipid (% dry weight)	7.12	7.32
Gross Energy (KJ/g)	18.21	18.75

All ingredients were procured and feeds were formulated by Glen Forest Specialty Feeds, Western Australia. FM - (fishmeal diet); PBM (poultry-by-product meal diet); KJ/g – kilojoule/gram.

3.6 Proximate composition analysis of formulated feed and marron tail muscle

Analysis of crude protein (%), gross energy (KJ/g), lipid (%) and ash (%) were implemented following standard methods as described by (AOAC, 1990). Crude protein was analysed by applying the Kjeldahl method with a Tecator digestive system 20 1015 run by a Tecator Autostep 1012 controller and a Tecator Kjeltec 1030 Auto Analyser. Bomb calorimeter was used to analyse gross energy. The method of cold extraction utilizing a methanol: chloroform solvent allowed for the determination of crude lipid while samples were burned in a muffle furnace at 600ºC to determine ash.

3.7 Marron health indices

3.7.1 Haemolymph parameters

To obtain marron haemolymph, a 1 mL syringe was filled with sodium citrate anticoagulant. Anticoagulant solution was prepared as described by Tulsankar et al. (2022b) which was a combination of trisodium citrate (30 mM), glucose (100 mM), citric acid (26 mM), NaCl (15.5 mM), and EDTA (10 mM). The syringe was gently inserted to the position between 3rd and 4th pairs of marron pereopods to collect the haemolymph sample then release it into an Eppendorf tube.

Marron total haemocyte count (THC) and differential haemocyte count (DHC – granular cells, semi-granular cells, and hyaline cells) were assessed using methods described by Nugroho and Fotedar (2013b). THC was determined as a mean of numbers of cells that was counted in both grids of a haemocytometer (Neaubauer, Munich, Germany) under microscope with 40x magnification. To calculate DHC, one drop of the haemolymph sample was smeared onto a glass microscope slide. It was then air dried and fixed in 70% methanol for 10 min. The fixed smears were stained with routine May-Grunwald and Giemsa stains for 10 min each and then mounted with coverslips. The number and percentages of three major marron haemocyte types for each marron were counted using a minimum number of 200 cells on each slide.

THC (cells/mL) = *(cells counted × dilution factor × 1000)/grid volume*

Granular cell (%) = *(number of granular cell/200) × 100*

Semi-granular cell (%) = *(number of semi-granular cell/200) × 100*

Hyaline cell (%) = *(number of hyaline cell/200) × 100*

Lysozyme activity in marron haemolymph was performed in a 96-well microplate (Iwaki, Japan) using the turbidimetric method (Bowden et al., 2004) with some modifications as described previously (Le and Fotedar, 2014). Each well contained 100 µL haemolymph sample and 100 µL *Micrococcus lysodeiktikus* suspended in 0.25 mg/mL PBS (Sigma-

Aldrich, USA) with two wells for each sample. During 16 minutes, the absorbance at 450 nm was measured every two minutes. Lysozyme activity was determined as the amount of enzyme causing in a decline in absorbance of 0.001/min. Lysozyme activities were presented as units/mL of haemolymph (EU/mL).

3.7.2 Moisture content

To measure the moisture indices, marrons were dissected. All hepatopancreatic lobes and complete mass of muscle tissues from the marron's abdomen were weighed and then dried in the oven at 105°C till attained constant weight. Moisture indices were calculated using the following equations previously described by Fotedar (1998).

Tail muscle moisture (TM %)	*= (WTwet – WTdry) × 100/WTwet*
Dry tail muscle index (Tid)	*= WTdry × 100/Wt*
Wet tail muscle index (Tiw)	*= WTwet × 100/Wt*
Hepatopancreas moisture (HM %)	*= (WHwet – WHdry) × 100/WHwet*
Dry hepatosomatic index (Hid)	*= WHdry × 100/Wt*
Wet hepatosomatic index (Hiw)	*= WHwet × 100/Wt*

Where, WTwet: weight of wet tail muscles (g); WTdry: weight of dry tail muscles (g); Wt: total weight of marron (g); WHwet: weight of wet hepatopancreas (g); and WHdry: weight of dry hepatopancreas (g).

3.8 Marron growth performance

3.8.1 Survival, moult and growth calculations

Juvenile marron 0^+ and 1^+ were used as experimental animals. At the beginning and end of the experiment, all marrons were counted and weighed. Marron were also weighed on occasion in accordance with each experiment and after each moult. Survival rate, percentage weight gain, specific growth rate (SGR), and moulting indices (moult interval – MI and moult weight increment – MWI) were determined using the formulae as follows:

Survival rate (%) = *(∑harvested marron)/(∑stocked marron) x 100*

SGR (%/day) = *[(ln final weight − ln initial weight)/days] x 100*

Weight gain (%) = *(final weight − initial weight) x 100/ initial weight*

MI (day) = *the number of days to moult*

$$MWI\ (\%) = \frac{(weight\ after\ moult - weight\ before\ moult)x100}{weight\ before\ moult}$$

3.8.2 Protease activity

For laboratory trials, protease activity in marron haepatopancreas was measured. At the end of the trials, one marron from each tank was collected for hepatopancreatic protease assay. A subsample of 0.2 g of hepatopancreatic tissue was homogenised on ice in 2ml phosphate buffer saline and then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected in a fresh sterile tube and used for further enzyme activity analysis. Activities of protease were measured as the change in absorbance recorded at 450 nm using diagnostic reagent kits, following the instructions provided in the Thermo Scientific™ Pierce™ Protease Assay Kit.

3.9 Statistical analysis

Data were presented in tables and figures as mean ± S.E. Homoscedasticity and normality of data were assessed using Levene and Shapiro tests, respectively before performing any statistical tests (independent samples t tests and analysis of variance-ANOVA). Tukey's HSD post-hoc tests were used for multiple mean comparisons. For non-parametric data, Kruskal-Wallis and Mann-Whitney tests were used. Pearson correlations and linear regression models were used to test for correlations between measured parameters. Statistical analyses were conducted either using SPSS statistics version 26 for Windows (SPSS Inc.) and R-Studio at a significance level of 0.05 in most cases and 0.01 and 0.001 in some cases.

CHAPTER 4 (Experiment 1) Dynamics of carbon/nitrogen ratio and microbial ecology in the sediments of semi-intensive marron (*Cherax cainii*) culture earthen ponds

Abstract

Understanding the interaction between water quality, sediment characteristics, and sediment microbial composition is important for marron (*Cherax cainii*) culture. This study collected water and sediment samples from a commercial marron farm throughout the year with intervals of every three months, representing spring, summer, autumn, and winter seasons. The results showed that water quality, sediment carbon/nitrogen (C/N) ratio and sediment microbial communities in marron ponds varied significantly between four seasons. The sediment C/N ratio was highest in summer and lowest in winter. Sediment microbiota richness and diversity were highest in spring, compared to autumn and winter. Distinct ordination of autumn and winter samples in PCoA plot signifies the compositional differences. However, no difference in diversity and composition was observed between spring and summer. The dominant bacterial phyla in the sediment were Proteobacteria, Cyanobacteria, Bacteroidetes and Chloroflexi wherein the latter three showed compositional differences across the season. *Aeromonas, Cyanobium PCC, Dechloromonas, Flavobacterium, hgcl-clade*, and *Polynucleobacter* were predominant and showed season-specific abundance. Some of the bacterial genera responded differently to environmental changes. Significantly positive correlations were found between *Shewanella* abundance and dissolved oxygen in autumn, and *Pseudomonas* abundance and zooplankton loads in winter. *Sediminibacterium* and *Fluviicola* proliferated as water nitrite and sediment C/N ratio increased during spring, respectively. *Methyloparacoccus* was also prevalent in spring and negatively associated with water ammonia and nitrate. These results suggested that seasonal and environmental factors have strong influences on sediment bacterial communities which could play a vital role in improving water and sediment quality, thereby controlling the semi-intensive culture system.

4.1 Introduction

Aquaculture production systems, historically dominated by extensive and improved-extensive pond-based farming, have been increasingly steered toward intensification and semi-intensification using pelleted feed. This leads to accumulation of nitrogenous wastes from animal excreta and the degradation of uneaten feed, ending up settling 10-20 cm of sediments in aquaculture pond bottom (Boyd, 1995, Boyd and Tucker, 2012). The essential elements of sediment include carbon and nitrogen (Boyd, 1995, Boyd et al., 2010, Avnimelech, 1999), which has been reported as important factor in the growth and health of aquatic species in aquaculture systems (Boyd, 1995, Sonnenholzner and Boyd, 2000, Thunjai et al., 2004). Fertilisers and feed are provided to aquaculture ponds to promote natural productivity and animal production. However, part of the nutrients is unused and accumulates in sediment which can or alter the sediment quality (Sonnenholzner and Boyd, 2000, Shafi et al., 2022). Depending on culture objects, culture methods and pond management strategies, the accumulation of carbon and nitrogen could be varied (Sonnenholzner and Boyd, 2000, Shafi et al., 2022). For instance, in tilapia (*Oreochromis niloticus*) ponds, carbon concentrations ranged from 1.55% to 4.96% while nitrogen varied from 0.1% to 0.3% (Thunjai et al., 2004). The ratios of carbon to nitrogen (C/N) can be 5 to 10 in sediments of channel catfish (*Ictalurus punctatus*) ponds (Munsiri et al., 1995), up to 19 in tilapia ponds while much greater ratios between 20 and 50 were recorded in bait minnow (*Notemigonus crysoleucas*) ponds which indicated high accumulation of organic matter (Tepe and Boyd, 2002). These elements might have eventually been influenced microbial communities thereby regulating sediment functioning.

Microbial ecology is also an important factor in pond sediments which plays role as primary producers and is considered the ultimate decomposers in aquatic environments (Moriarty, 1997, Gupta et al., 2021, Rajeev et al., 2021). The presence of microorganisms in aquaculture systems could have both positive and negative impacts. The beneficial microorganisms can help increase the water quality and health status, resulting in improving the growth and survival of targeted animals, and reducing harmful pathogens

(Hai, 2015). Some beneficial bacteria can produce extracellular enzymes as well as provide micronutrients such as vitamins, fatty acids, and essential amino acids in addition to macronutrients to support the healthy growth of aquatic animals (Wang et al., 2020a). Conversely, microbes might negatively affect farmed animals' health by causing growth retardation, sporadic mortalities, and eventually cause economic losses (Tang and Bondad-Reantaso, 2019). Filamentous bacteria such as *Vibrio* spp., which could be primary or opportunistic pathogens are commonly observed in hatcheries, water, and sediment of tiger shrimp (*Penaeus monodon*) and white leg shrimp (*Litopenaeus vannamei*) grow-out ponds (Jayasree et al., 2006, Amatul-Samahah et al., 2020), and their abundance is closely linked to shrimp disease (Defoirdt et al., 2011, Xiong et al., 2014b, Zhang et al., 2014a). Hence, managing C/N ratio and understanding their interaction with microbial communities in sediment is necessary for water quality control and animal performance in aquaculture ecosystems (Xu et al., 2016, Panigrahi et al., 2018).

In addition to diet, season is one of the factors affect sediment C/N ratio and bacterial communities (Huang et al., 2018). A study reported seasonal induced changes in composition and abundance of the microbes in white leg shrimp cultures (Zhang et al., 2016). Therefore, understanding environmental factors and their possible influence on the microbial community is important to maintain sediment C/N ratio and ensure the healthy environment for decapod aquaculture (Hou et al., 2017).

Marron (*Cherax cainii*) holds regional significance due to its high nutritional value and has the added benefit of having strong recognition in the marketplace in Australia. Many studies have been done over the years to enhance the growth and health of marron raised under semi-intensive aquaculture conditions (Tulsankar et al., 2020, Tulsankar et al., 2021b, Cole et al., 2019). The seasonal variations of trace elements in the water column of marron ponds were found and the number of bacteria in the pond water was also quantified in previous studies (Cole et al., 2019, Tulsankar et al., 2020). Also, our previous study found a correlation between environmental factors and water microbiota (Nguyen et al., 2021). To the authors knowledge, a cross connection between sediment composition and microbial communities of marron culture systems has yet to be performed. Foysal et al.

(2022a) reported that microbial communities in the sediment of marron tanks shift along with the culture period rather than the type of treatment. Two trials were conducted in indoor tanks with controlled environments which were not affected by seasonal variation (Foysal et al., 2022b, Nguyen et al., 2021). The present study therefore aims to characterize the microbiota diversity in commercial marron pond sediment to improve the understanding of taxa-environmental correlations over the year under semi-intensive freshwater aquaculture ecosystems.

4.2 Materials and methods

4.2.1 Sampling site

All samples were collected from six ponds (A-F, see Figure 3.1 in Chapter 3) stocked with juvenile marron. The construction of these ponds was simple, with dimensions of approximately 40x25 m (1000 m^2). The water from the settling pond was pumped into these culture ponds. Water level of these ponds was set at 1.25 m at the commencement and was maintained at full levels (1.5 m) by constant water addition but without water exchange. Paddle wheels were provided to maintain dissolved oxygen concentration at night time. Each pond had its own outlet gate to facilitate pond harvesting.

Juvenile marrons at around 10 g were obtained on site from broodstock ponds during the period 19^{th}–30^{th} July 2018, manually graded, counted, and weighed before being stocked into the ponds. The initial stocking densities were set at three juveniles per square metre for all ponds. Marron were fed with a commercial feed (premium marron pellets, 20.85% crude protein and carbon/nitrogen ratio of 12.07) obtained from Weston™ Animal Nutrition company. During the growth period, an equal proportion 2.5 kg of the pellets per pond was used to feed marron once a day at around 03:00 pm. Feed was applied to the ponds by broadcasting evenly around the ponds by hands.

4.2.2 Sampling intervals and sampling points

Samples were collected four times in one year with interval of every three-month from October 2018 to July 2019, representing four seasons including spring, summer, autumn,

and winter. At each sampling time, samples were collected at four corners of each pond, following the sampling method mentioned by Liu et al. (2020b) for microbial analysis.

4.2.3 Assessment of environmental factors in the water column

The abiotic and biotic water parameters, namely temperature, pH, dissolved oxygen (DO), ammonia, nitrite, nitrate, water transparency, phosphate, phytoplankton abundance, zooplankton abundance were assessed as described in section 3.1 of Chapter 3.

4.2.4 Sediment collection

A sediment sampler was used to collect pond sediments. The sample from each sampling point was labelled properly and kept separately in separate containers. All samples were transferred to the laboratory under cool conditions and kept frozen -80ºC until analysis.

4.2.5 Sediment carbon/nitrogen (C/N) ratio analysis

The C/N ratio of sediment samples were analysed with the method described in section 3.3 of Chapter 3.

4.2.6 Sediment microbial ecology

DNA extraction, PCR amplification, and sequence data processing were executed according to the procedures described in section 3.4 of Chapter 3.

4.2.7 Statistical analysis and bioinformatics

Bioinformatics and statistical analysis were described in sections 3.4 and 3.9 of Chapter 3. Descriptive statistics in SPSS were utilized to analyze the dataset of water quality and sediment C/N ratio. The data were examined to ensure they met the assumptions of normality and homogeneity of variance. Following this, two-way analysis of variance (ANOVA) was used to check the interaction between pond types and seasons. Then, one-way ANOVA with Tukey's HSD was applied to explore potential variations across four seasons for water quality and sediment C/N ratio ($p<0.05$).

4.3 Results

4.3.1 Seasonal variations of water quality parameters

Several selected water quality parameters from six ponds were analysed for four seasons (Table 4.1). High variations of all these factors were recorded across seasons. The highest water temperature, pH, and plankton abundances in summer were achieved compared to other seasons. Dissolved oxygen measurements were all above 6 mg/L and highest in winter. Water transparency with Secchi disk depth value of 120 cm was measured in spring and winter while those values in summer and autumn were 65 cm and 80 cm, respectively. Although nitrogenous compounds and phosphate concentration ranged between seasons, those values were low and within the suitable range for marron aquaculture (Cole et al., 2019).

Table 4.1 Water quality parameters (mean ± S.E., n = 24) in marron ponds during four seasons

Parameters	Spring	Summer	Autumn	Winter
Temperature (°C)	17.44 ± 0.05^{a}	21.38 ± 0.04^{b}	18.20 ± 0.04^{c}	13.19 ± 0.04^{d}
pH	7.25 ± 0.03^{a}	8.20 ± 0.14^{b}	8.16 ± 0.07^{b}	7.72 ± 0.09^{c}
DO (mg/L)	7.26 ± 0.07^{a}	6.95 ± 0.08^{a}	9.13 ± 0.08^{b}	10.15 ± 0.23^{c}
Water trans. (cm)	120 ± 0.00^{a}	65 ± 3.31^{b}	80.83 ± 2.44^{c}	120 ± 0.00^{a}
Ammonia (mg/L)	0.07 ± 0.007^{a}	0.03 ± 0.004^{b}	0.02 ± 0.001^{b}	0.02 ± 0.001^{b}
Nitrite (mg/L)	0.01 ± 0.001^{a}	0.01 ± 0.001^{a}	0.02 ± 0.001^{b}	0.01 ± 0.001^{a}
Nitrate (mg/L)	0.23 ± 0.01^{a}	0.18 ± 0.01^{a}	0.36 ± 0.01^{b}	0.40 ± 0.02^{b}
Phosphate (mg/L)	0.24 ± 0.02^{a}	0.15 ± 0.01^{b}	0.32 ± 0.03^{c}	0.26 ± 0.02^{ac}
Ph.Ab. ($x10^3$ cells/L)	387.5 ± 9.52^{a}	995.8 ± 41.04^{b}	937.5 ± 39.59^{b}	491.7 ± 30.42^{a}
Zoo.Ab (ind./L)	56.7 ± 4.37^{a}	156.7 ± 7.01^{b}	115.0 ± 5.99^{c}	56.7 ± 2.99^{a}

Different superscripts in the same row show significant differences ($p<0.05$). Abbreviations DO - Dissolved oxygen; Water trans. – Water transparency; Ph.Ab. - Phytoplankton abundance; Zoo.Ab - Zooplankton abundance; ind. – individuals.

4.3.2 Seasonal variations of sediment carbon/nitrogen ratio

The results revealed a significant interaction effect between pond types and seasons on sediment C/N ratio ($F = 12.939$, $p < 0.001$).

The results in Table 4.2 showed that although there were some fluctuations, sediment C/N ratio reached its peak in summer, and declined to the lowest point in winter. The results revealed significant differences in sediment C/N ratio between certain pond-season combinations. In particular, in summer, sediment C/N ratios in ponds 1, 2, and 6 were significantly higher than those in ponds 3, 4 and 5. During the winter season, the sediment C/N ratios exhibited statistically significant differences between pond 3 and pond 5, whereas no significant distinctions were observed between the remaining ponds during this season.

Table 4.2 Sediment carbon/nitrogen ratio (mean ± S.E., n = 4) in marron ponds during four seasons

Pond	Spring	Summer	Autumn	Winter
1	8.42 ± 0.07^{de}	10.01 ± 0.05^{g}	8.23 ± 0.24^{cde}	7.15 ± 0.16^{ab}
2	8.25 ± 0.11^{cde}	9.72 ± 0.26^{g}	8.11 ± 0.09^{cb}	7 ± 0.08^{ab}
3	8.36 ± 0.05^{de}	8.79 ± 0.11^{def}	8.89 ± 0.13^{ef}	7.25 ± 0.12^{b}
4	8.89 ± 0.02^{ef}	8.74 ± 0.16^{def}	6.98 ± 0.12^{ab}	6.85 ± 0.19^{ab}
5	8.72 ± 0.12^{def}	8.48 ± 0.02^{de}	6.78 ± 0.11^{ab}	6.47 ± 0.11^{a}
6	8.56 ± 0.09^{def}	9.28 ± 0.07^{fg}	7.52 ± 0.14^{bc}	6.9 ± 0.29^{ab}

Different alphabetical superscripts show significant differences among pond-season combinations ($p<0.05$).

The pooled data showed that C/N ratio in marron pond sediments varied significantly according to seasons ($p<0.05$). The highest and lowest value of sediment C/N ratios were recorded in summer and winter, respectively. In particular, the average sediment C/N ratio

of six ponds was 8.56 in spring, and then increased to 9.27 in summer, followed by decreases to 7.52 in autumn, and to 6.9 in winter (Figure 4.1).

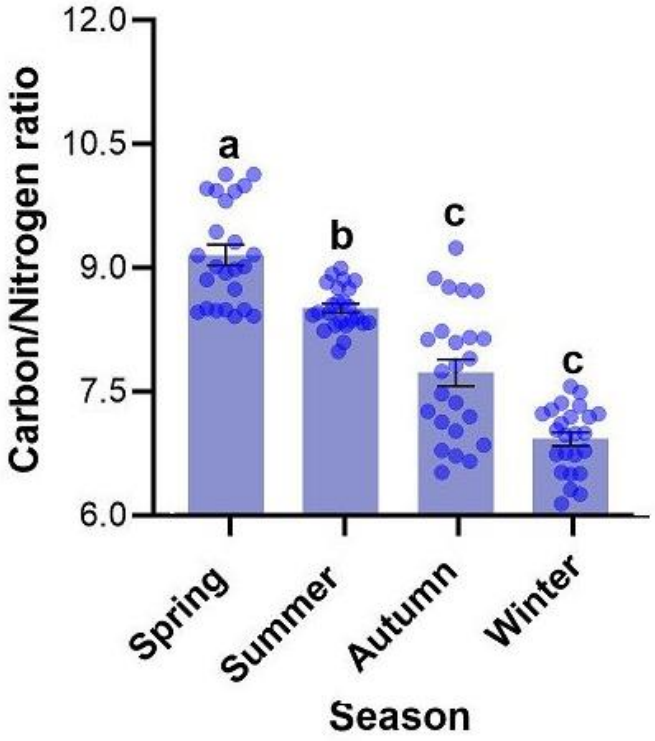


Figure 4.1 Sediment carbon/nitrogen ratio (mean ± S.E., n = 24) in marron ponds in different seasons. Different letters on bars show significant differences ($p<0.05$).

4.3.3 Characterization of bacterial communities in marron pond sediment

4.3.3.1 Sequence statistics

After processing of reads and quality filtering, a total of 247,665 clean PE reads, and an average of 10,319.3 ± 2106.6 reads were obtained, which were classified into 2813 OTUs. Filtering of singleton, low-abundance, and unclassified OTUs resulted in 2004 clean OTUs for further analysis. These OTUs were classified into 10 phyla and 48 unique classified genera. Out of the 2004 clean OTUs, *Aeromonas* (282 OTUs, 14.1%), *Cyanobium* PCC (237 OTUs, 11.8%), *Dechloromonas* (165 OTUs, 8.2%), *Flavobacterium* (182 OTUs, 9.1%), *hgcl-clade* (216 OTUs, 10.8%), and *Polynucleobacter* (157 OTUs, 7.8%) comprised of 61.8% total classified OTUs in 24 sediment samples. An average good's

coverage index value of 0.996±0.001 indicated that each sample was sequenced at enough depth and saturation level to capture most of the bacterial diversity.

4.3.3.2 Alpha-beta diversity and shared and unique OTUs

The alpha diversity measurement showed higher diversity and Shannon evenness in the spring samples, compared to autumn and winter (Figure 4.2A). Beta-ordination in the form of Principal Coordinate Analysis (PCoA) displayed significant and distinct clustering of samples, collected from marron ponds in four different seasons. However, the separation was more significant between summer and winter, followed by spring-winter and autumn-winter samples while no variations were observed in community separation between summer and spring (Table 4.3). In addition, the distance-based separation of microbial community was more significant in terms of rare taxa (unweighted) in the summer and winter seasons (Table 4.3, Figure 4.2B). Among 2813 OTUs obtained, only 11 were shared across the seasons. Spring and summer shared 1231 OTUs signifying the similarity of taxa between these two seasons. Despite having a less diverse bacterial community compared to spring, autumn displayed the highest unshared OTUs among all seasons (Figure 4.3).

Table 4.3 Seasonal variations of microbial communities (Beta-dispersion)

Unweighted		**Weighted**	
Seasons	**Beta-dispersion**	**Seasons**	**Beta-dispersion**
Spring-Summer	p >0.05	Spring-Summer	p >0.05
Spring-Autumn	p = 0.00121	Spring-Autumn	p = 0.001621
Spring-Winter	p = 0.00012	Spring-Winter	p = 0.000159
Summer-Autumn	p = 0.00804	Summer-Autumn	p = 0.001038
Summer-Winter	p = 0.00008	Summer-Winter	p = 0.000121
Autumn-Winter	p = 0.02836	Autumn-Winter	p = 0.047943

Significant difference at p-value<0.05.

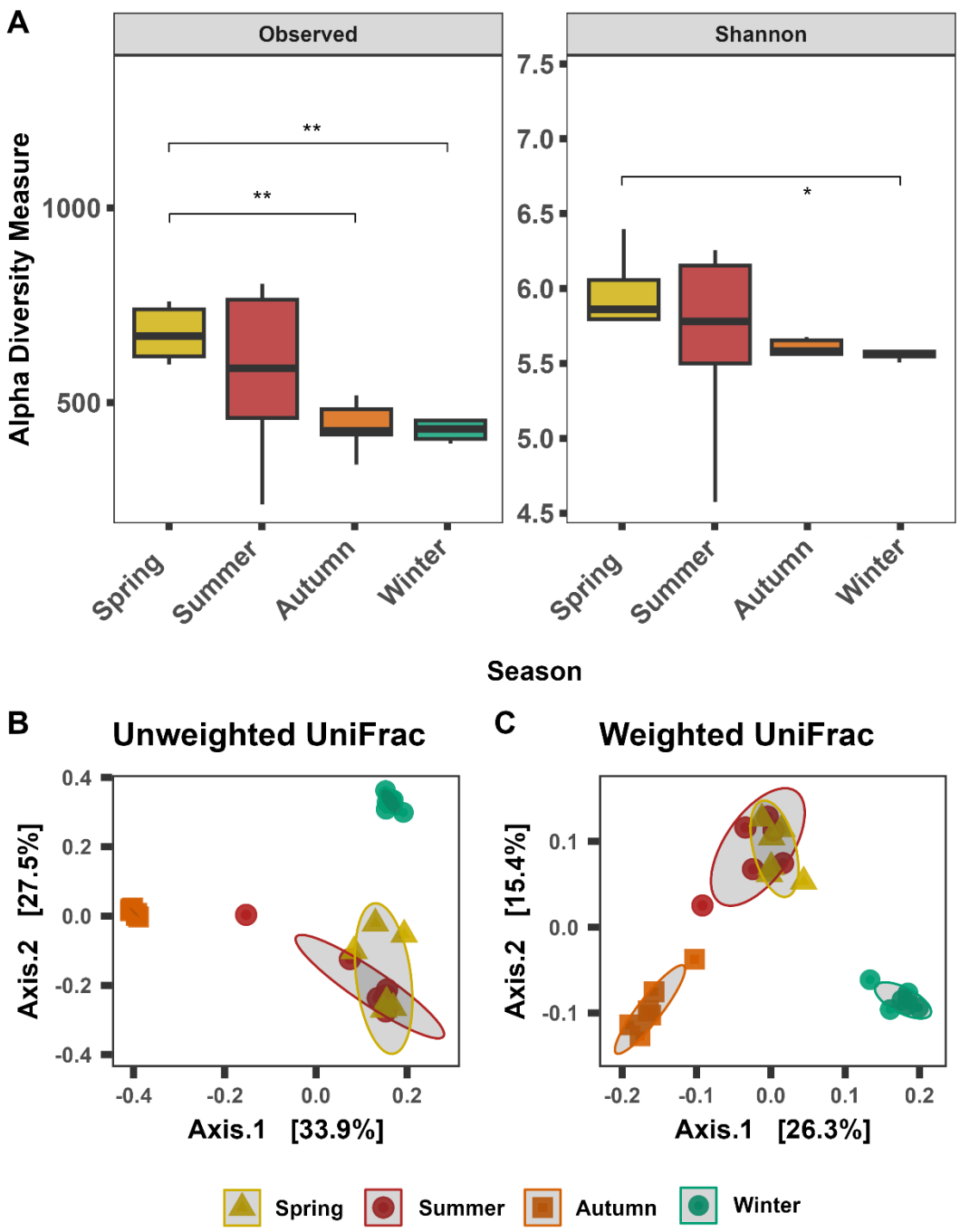


Figure 4.2 Alpha-beta diversity of sediment microbial communities in four different seasons. (A) Observed species and Shannon index were considered for alpha diversity measurements. (B) Beta-diversity measurements were performed as weighted and unweighted UniFrac distance metric and visualized as PCoA plot. * and ** represent significant differences at α-level of 0.05 and 0.01.

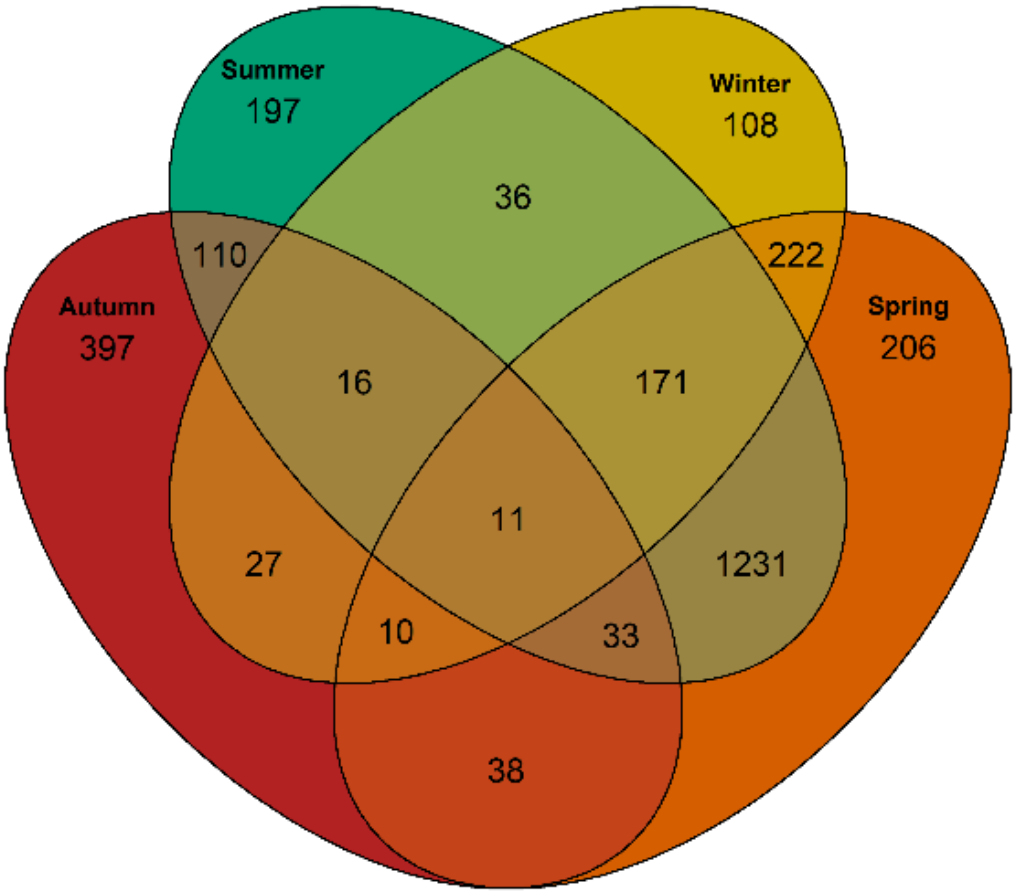


Figure 4.3 Number of shared and unique OTUs in marron ponds for different seasons.

4.3.3.3 Seasonal variation in the bacterial community

Overall, Proteobacteria (41.8%) dominated the bacterial abundance at the phylum level. In addition, the relative abundance of Cyanobacteria (28.6%), Bacteroidetes (19.3%) and Actinobacteria (6.8%) were also higher in the marron pond in all four seasons. In autumn, 99% of the classified reads were assigned to Proteobacteria (53%) and Bacteroidetes (46%). While Proteobacteria abundance did not change significantly amongst seasons, Bacteroidetes in spring, summer and winter dropped to 18%, 10%, and 2%, respectively. An overwhelming abundance of Cyanobacteria was observed in the winter (53%), summer (38%) and spring (23%) samples (Figure 4.4).

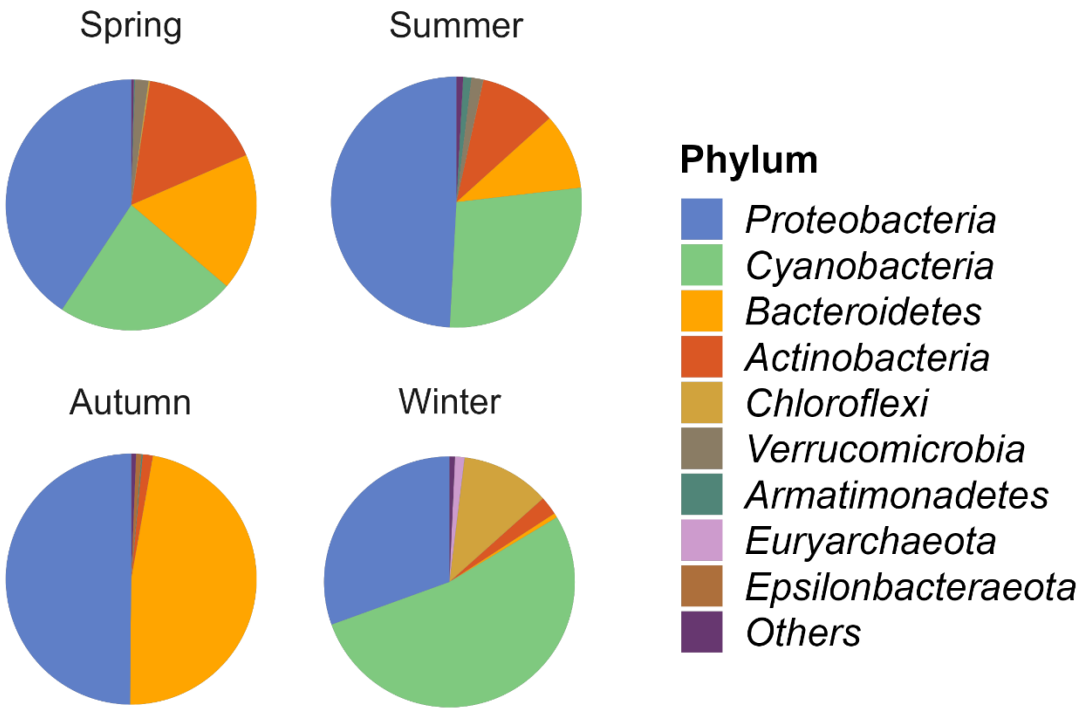


Figure 4.4 Pie chart representing the relative abundance of bacteria at phylum level in four different seasons. Phyla comprised of >1% read abundance are presented here.

At genus level, most of the reads (68%) in the winter samples remained unclassified, and the number was also found high for the spring (32%) and summer (28%) samples. In aggregate, 76% of the reads generated in the autumn samples were classified to *Flavobacterium* (42%) and *Aeromonas* (34%), and the abundance was significantly higher than any other seasons. Winter samples had significantly higher abundance of Cyanobacteria and Chloroflexi than autumn samples. Bacteriodetes abundance was found higher in autumn than any other seasons (Figure 4.5). At genus level, spring favoured the growth of *Polynuceobacter* (18%), hgcl-clade (15%) and *Epipyxis* (9%) wherein the first two had significantly higher abundance, compared to autumn. *Aeromonas* (24.8%) and *Flavobacterium* (45.2%) had significantly higher abundance in autumn and *Dechloromonas* (22%) in winter, compared to their counterparts. *Cyanobium* PCC (24%) dominated (P=0.0012) microbial communities in summer (Figure 4.6).

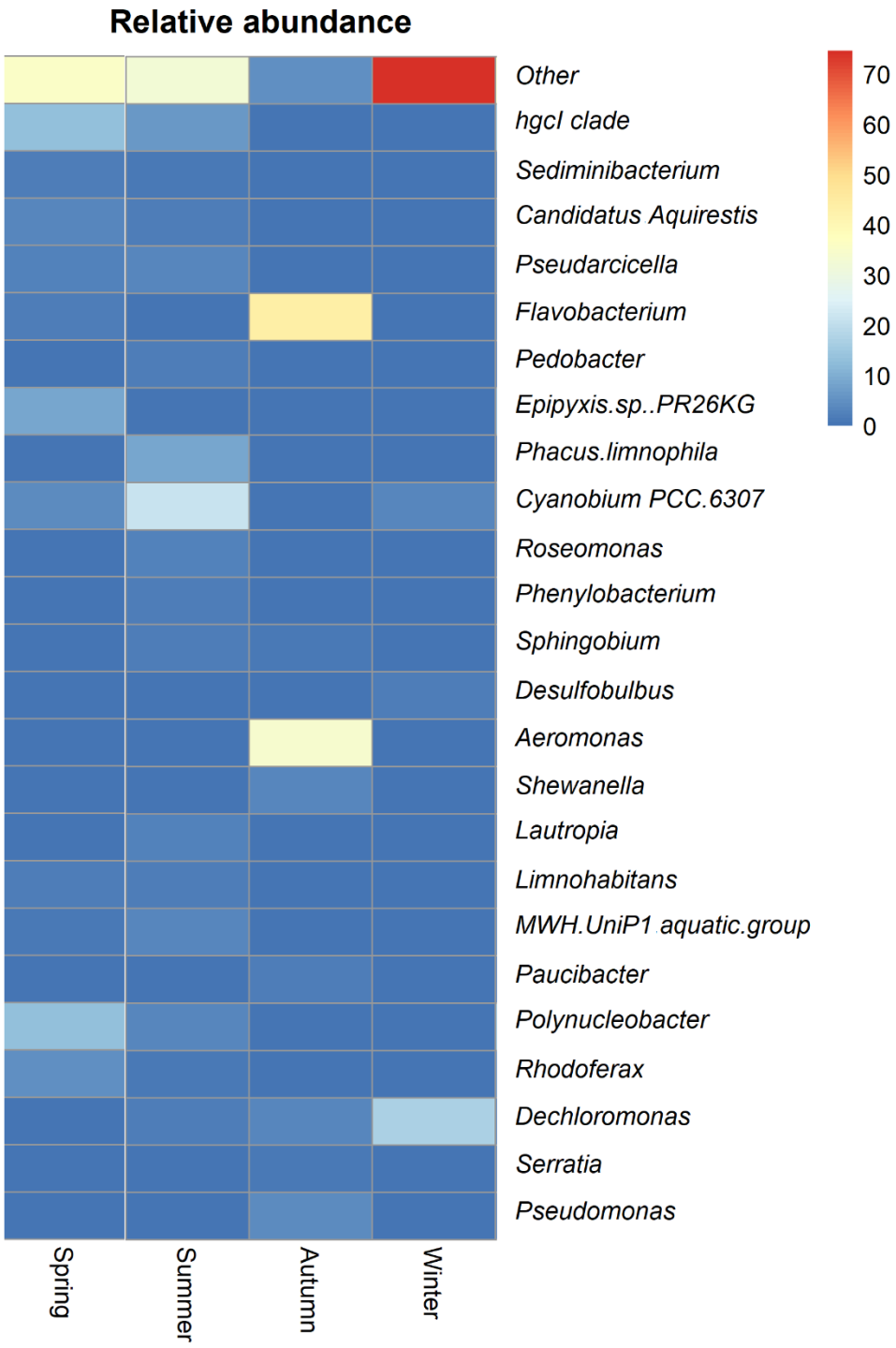


Figure 4.5 Relative abundance of bacteria at genus level in four seasons in the pond sediments. Genera comprised of >1% read abundance are presented here. Low abundant (<1%), uncultured and unclassified bacteria are presented as "other" in the heatmap.

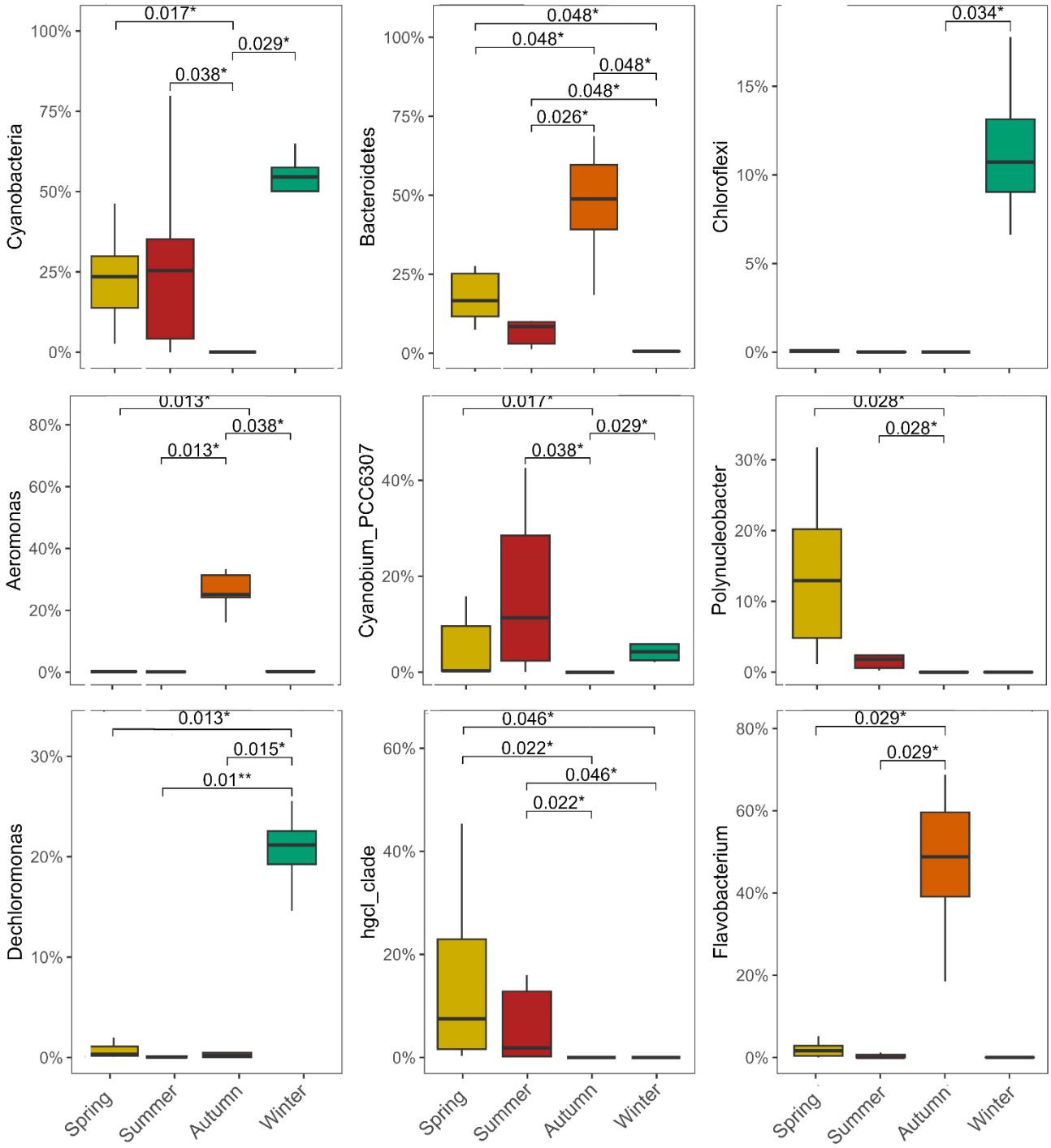
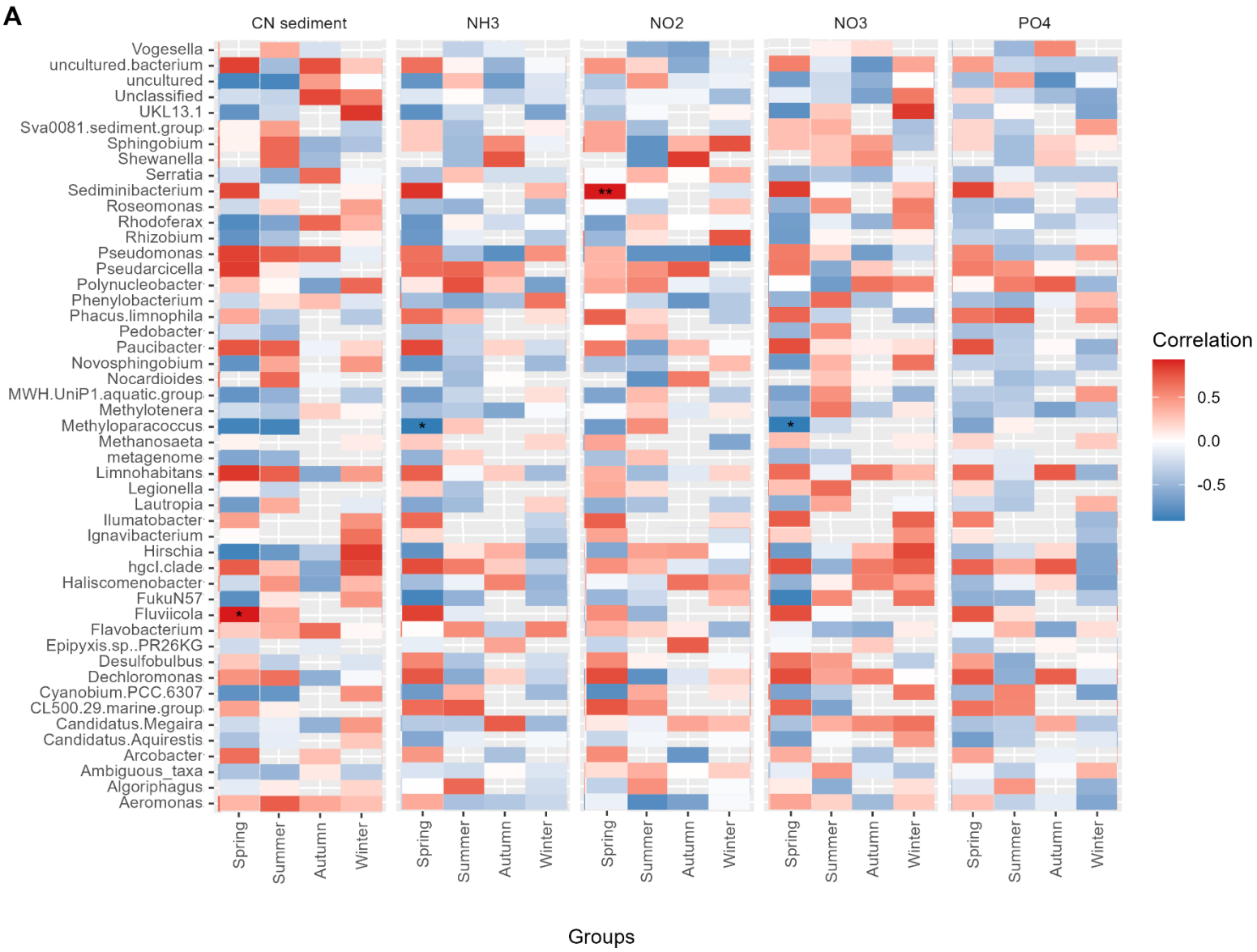


Figure 4.6 Differential abundance of bacteria at phylum (A) and genus (B) level in four different seasons in the pond sediments. Phyla and genera with >1% read abundance were considered for differential abundance analysis. *Significant at α-level of 0.05. **Significant at α-level of 0.01.

4.3.4 Correlation between sediment microbial community and environmental factors

The abundance of *Shewanella* positively correlated with DO in autumn while a positive correlation was observed between *Pseudomonas* and zooplankton abundance in winter. In spring, increasing nitrite in the pond water and the ratio of carbon/nitrogen in the sediment favoured the abundance of *Fluviicola* and *Sediminibacterium*, respectively. However, *Methyloparacoccus* was dominant in this season and negatively correlated with ammonia and nitrate in the pond water (Figure 4.7A-B).



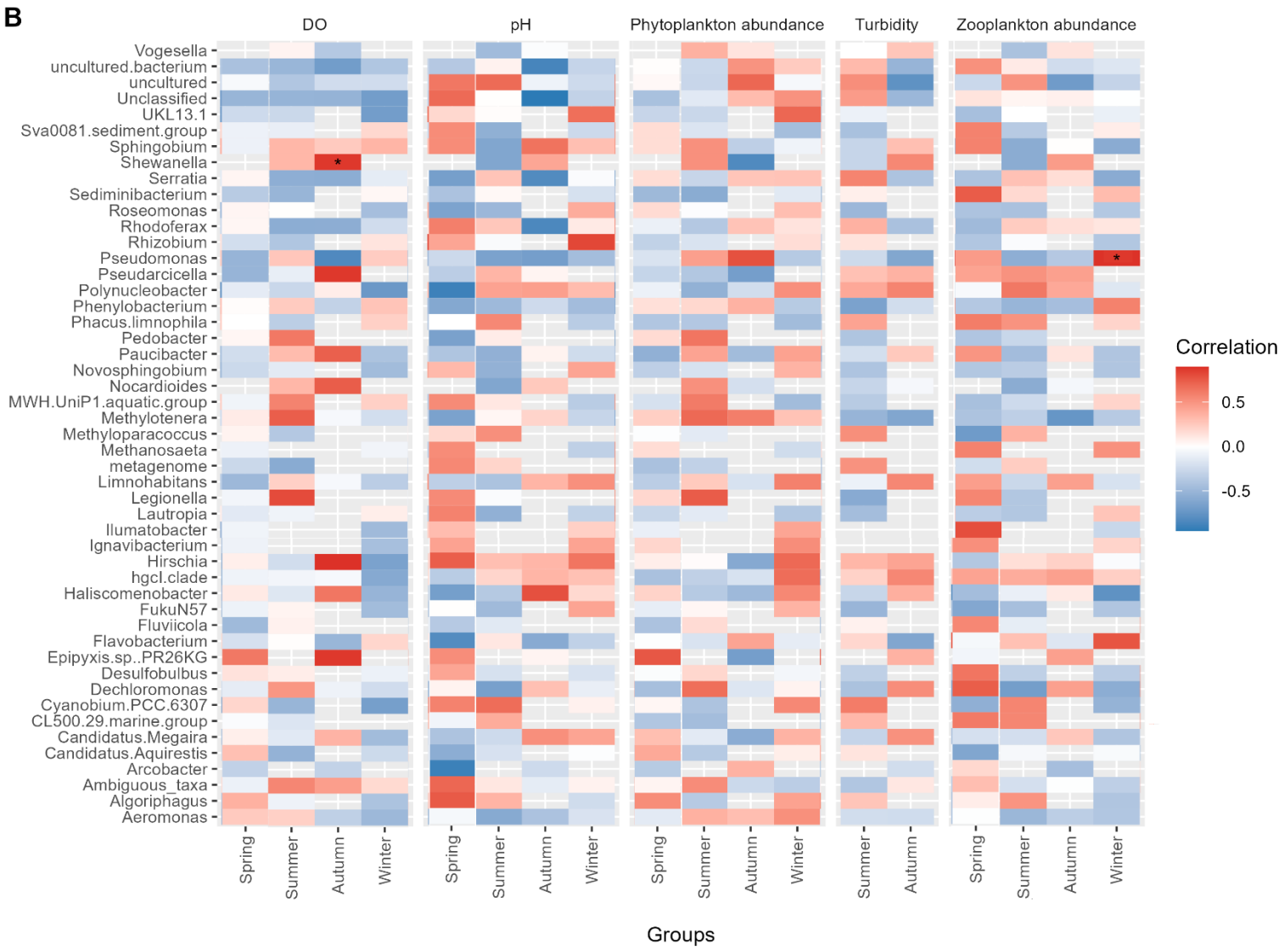


Figure 4.7 Pearson correlation between bacterial genera and abiotic and biotic factors in marron ponds in four seasons. *Alpha level of 0.05. **Alpha level of 0.01

4.4 Discussion

4.4.1 Abiotic and biotic environmental factors

In the present study, water quality and sediment characteristics co-varied in aquaculture ponds, and seasonal variations in similar systems were also observed by Tulsankar et al. (2021b). All measured water quality parameters were within the optimum range for marron

aquaculture, as described by Cole et al. (2019) which was conducted on a comparable marron farm within the same area.

In pond water, while dissolved oxygen can be enhanced by providing aeration, the temperature varies along with the ambient environment while pH depends mainly on pond soil characteristics. Therefore, water temperature and pH are difficult to adjust in pond water and can change significantly during the seasons which are also observed in the present study. Of the nutrient inputs, a small portion was assimilated by farmed animals, and the rest either dissolved in water or settled in the pond bottom where a high percentage of unused nitrogen was found (Avnimelech and Ritvo, 2003, Thakur and Lin, 2003). In contrast, our present study revealed low accumulated concentrations of nitrogenous wastes in the marron pond ecosystem's water. However, a significant accumulation of nitrate concentration was observed in autumn and winter, potentially explaining the low carbon/nitrogen ratio in pond sediment during these two seasons. Water pH value is the indicator for the build-up level of sediment (Delgado et al., 2003). pH values in the present study ranged from 7 to 8 during the four seasons, suggesting that the build-up of sediment at the pond bottom during one year of culture was not significant (Delgado et al., 2003). The increase of pH and plankton abundance co-occurred, attributing to the control of pH by phytoplankton species through photosynthesis (Hammer et al., 2019). This study recorded high abundances of phytoplankton and zooplankton in spring and summer, as a consequence of higher temperatures in these two seasons relative to other seasons, which is in agreement with many previous studies (Guerrero-Galván et al., 1998, Cole et al., 2019, Tulsankar et al., 2021b). However, plankton biomass was relatively poor in the current study compared to another report on the same parameter measured in different but comparable ponds by Tulsankar et al. (2021b), indicating that the current marron ponds were relatively low in nutrients causing lower natural productivity.

Sediment plays an important role in the productivity of an aquaculture system. As unassimilated nutrients end up settling on the pond bottom, the top sediment layer can contain several times more nutrients than the water column (Yang et al., 2017, Avnimelech

and Ritvo, 2003). However, sediment is also a source of nutrients, acting as a buffer in water nutrient concentration (Smith et al., 2006, Avnimelech et al., 1999). The increase in sediment C/N in summer in the present study may be attributed to the accumulation of organic inputs including organic fertilizers and formulated feeds after a few months of marron stocking. High temperature and increased sunlight are optimal conditions for plankton growth which raises the carbon content in the culture system (Dauta et al., 1990, Darvehei et al., 2018). Moreover, temperature is vital to the denitrification process. High temperatures (>25ºC) can enhance nitrate removal out of the culture systems (Qu et al., 2022, Liao et al., 2018). These processes result in increasing carbon and reducing nitrogen accumulation in summer which probably leads to the rise of C/N in marron pond sediments. Although sufficient organic carbon is vital for microbial activity and benthic organism growth in the pond bottom (Tepe and Boyd, 2002, Thunjai et al., 2004), overloaded organic matter can result in high biological oxygen demand and anaerobic state which cause water quality problems and are harmful for cultured animals (Hargreaves and Tucker, 2004, Groffman et al., 2006, Huang et al., 2018). C/N ratio in marron pond sediments is similar to other culture systems, and is within the normal range (6.47 to 10.01) for aquaculture ponds (Munsiri et al., 1995). We also found that sediment C/N ratio reduced in winter and this trend was consistent for all sampling ponds. This can be explained by the high accumulation of organic matter inputs along with animal deaths and waste products present in the final stages of the rearing process.

4.4.2 Microbiota in marron pond sediment

Previous studies (Ning et al., 2022, Liu et al., 2020b) indicated that similar bacteria communities could present in different aquaculture models. However, the presence of different groups of microorganisms in terms of abundance and diversity reflects the culture environment quality and cultured animal health (Dimitroglou et al., 2011, Qin et al., 2016, Huang et al., 2018). This study found that the phylum Proteobacteria including three genera (*Aeromonas*, *Polynucleobacter,* and *Dechloromonas*), and the phylum Bacteroides represented by the *Flavobacterium* genus were the most abundant in the sediment of

marron semi-intensive culture ponds. These observations are consistent with previous findings on white leg shrimp culture systems (Xu et al., 2022, Zeng et al., 2021). Another abundant genus *Cyanobium* belonging to the phylum Cyanobacteria found in the present study was also detected in abundance in rice-crayfish co-culture pond sediments (Ning et al., 2022). The genus *Cyanobium* is recognised as an integral primary producer in aquatic environments (Jezberová and Komárková, 2007), therefore has the potential to increase primary productivity and food resources for marron.

These genera can both negatively and positively influence farmed animals. For example, the genera *Flavobacterium* and *Aeromonas* contain several species such as *F. psychrophilum* (Nematollahi et al., 2003), *F. columnare* (Dong et al., 2015), *A. hydrophila* (Declercq et al., 2013), and *A. veronii* (Hoai et al., 2019, Han et al., 2021) which are pathogens and cause diseases in salmonid fish such as coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*O. mykiss*), catfish (*Pangasianodon hypophthalmus*) and koi carp (*Cyprinus carpio var. koi*). On the contrary, some *Flavobacterium* species are not considered primary pathogens but play vital role in mineralising organic matter in pond ecosystems (Bernardet and Bowman, 2006), and *A. media* (strain A199) can act as probiotics and inhibit pathogenic agents such as *Vibrio tubiashii* and *Saprolegnia sp.* (Gibson et al., 1998, Lategan and Gibson, 2003). The community structure and ecological roles of microbiota in pond sediment could be influenced by various factors, and seasonal variation has been identified as one of the most important parameters (Wolinska et al., 2022, Fan et al., 2016, Zhang et al., 2016). In this study, all identified bacteria were most abundant in spring and summer, likely due to optimum environmental conditions such as temperature, pH, and nutrients promoting microbial diversity in the sediment. It is important to note that Proteobacteria and Bacteroides, the most abundant phyla in this study, are heterotrophic that strongly depend on the availability of nutrients in the culture system during spring and summer. Likewise, Cyanobacteria are known autotrophs and they were less abundant in winter compared to other seasons has been identified by Li et al. (2019b). Additionally, carbon recycling bacteria declined significantly in winter, and it was

predicted that carbon production during this period could be utilised in later seasons such as spring and summer, contributing to bacterial abundance in these seasons (Wilhelm et al., 2014).

4.4.3 Environmental factors shaped sediment microbiota

Environmental factors in aquaculture systems can affect the structure and function of the microflora in water column as well as bottom sediment. Although correlations between environmental factors and microbes in water of various aquaculture systems have been widely documented (Alfiansah et al., 2018, Sun et al., 2021), including in marron tank culture system (Nguyen et al., 2021), the correlation between environmental factors and sediment microbes has received less attention. In our study, *Shewanella* positively correlated with dissolved oxygen in autumn which is in agreement with previous research results that showed an increase in dissolved oxygen levels could enhance *Shewanella* abundance (Niu et al., 2023). Besides dissolved oxygen, zooplankton abundance could also coincide with the growth of bacterial communities (Tang et al., 2010). Our study found that zooplankton abundance dramatically reduced in winter compared to summer, which coincides with lower abundance of *Pseudomonas* in winter. *Pseudomonas* could be less abundant or disappear in winter, which agrees with previous studies (Sultana et al., 2021).

In spring, several prevalent bacterial groups had significant correlations with water column nutrients such as ammonia, nitrite, nitrate and with the carbon/nitrogen ratio in the pond sediment. For example, *Fluviicola* were positively associated with temperature and negatively correlated with total nitrogen in the water column (Yue et al., 2021). This is in accordance with the finding of our study where carbon/nitrogen ratio increased in the sediment during spring, potentially promoting the development of this bacterial genus. Our study found that *Sediminibacterium*, a genus belongs to Bacteroidetes phylum, is predominant in spring, and their growth positively correlated with water nitrite. This correlation suggests that *Sediminibacterium* could play a vital role in the processing or utilization of nitrite as a form of nitrogen which was identified by Hu et al. (2023). In a previous study, it was found that *Methyloparacoccus* use methanol and methane as a source

of carbon and energy to grow (Hoefman et al., 2014). However, another research suggested that this genus may have autotrophic metabolism, allowing it to not only thrive in environments with depleted carbon, but also negatively respond to changes in chemical oxygen demand and total nitrogen (Wang et al., 2020b). This bacteria genus was found to be abundant in both water and sediment in seasons with high temperatures such as summer and autumn (Albakistani et al., 2022). In our study, we observed a significant increase in the abundance of *Methyloparacoccus* while the nitrate concentration in the water decreased, likely due to a change in temperature from winter to spring. This could explain the negative correlation between *Methyloparacoccus* and nitrate.

4.5 Conclusion

Seasons significantly impacted both abiotic and biotic environmental factors in marron ponds. Comparing summer to other seasons, highest temperature, pH level, and plankton abundance in water and C/N ratio in sediment were recorded. In contrast, water dissolved oxygen concentration was highest in winter. *Aeromonas*, *Dechloromonas*, and *Polynucleobacter* of Proteobacteria, *Cyanobium PCC* of Cyanobacteria, and *Flavobacterium* of Bacteroidetes were the prominent bacterial genera in the pond sediment; however, their abundance varied according to seasons. In a specific season, other genera such as *Shewanella*, *Pseudomonas*, *Sediminibacterium*, *Methyloparacoccus* and *Fluviicola* significantly correlated with dissolved oxygen, zooplankton abundance, nitrite, ammonia and nitrate in water, and C/N ratio in sediment, respectively. These findings suggested that environmental conditions have a significant impact on some particular bacteria that may be essential for increasing water and sediment quality and regulating the semi-intensive growth system. To understand the relationship between the water, sediments, and marron health, more research is required.

CHAPTER 5 (Experiment 2) Effect of two diets on sediment characteristics and productivity of marron cultured in semi-intensive earthen ponds

Abstract

Limited research has examined the effects of commercial diets on the properties of the water, sediment, and microbial communities in marron (*Cherex cainii*) aquaculture ponds. The current study examined the impact of two commercially available diets on pond water quality, sediment properties, and marron productivity for one year, in two commercial ponds in Western Australia. The first commercial diet, the premium marron pellets (PMP) had a carbon to nitrogen (C/N) ratio of 12.07, and the second commercial diet called a Solair feed (SPD) had a C/N ratio of 14.52 with supplemented probiotics. Water and sediment samples were taken every two months during the final six months of the culture period. The C/N ratio in the sediment and selected biotic and abiotic water parameters were measured, and the bacterial communities in the sediment and water were characterised. The health parameters of marron were comparable between the two ponds. The results showed no significant variations in water quality or sediment C/N ratio between the two treatments. However, PMP showed significantly reduced bacterial alpha diversities in water and sediment. Proteobacteria, Actinobacteria, Bacteroidetes, and Cyanobacteria were the four most prevalent phyla in the marron pond rearing environment, independent of the type of diet used. Additionally, sediment was also the habitat for the prevalence of Firmicutes. *Limnohabitans* spp was one of the prevalent genus groups in water, and its abundance in PMP was significantly lower than in SPD. The other major bacterial taxa, including *Aeromonas*, *Vibrio*, *hgcl clade*, *Aquabacterium*, and *Vogesella*, were more prevalent in PMP. *Limnobacter* was prevalent in sediment in SPD at a rate of 19%, while in PMP, this bacterial group was just 0.1%. On the other hand, PMP accelerated the development of the *hgcl clade*, *Aquabacterium*, *Aeromonas*, *Vogesella*, and *Vibrio*. The marron receiving PMP resulted in higher production and positively skewed size distribution.

5.1 Introduction

Decapod crustaceans, including marine shrimp, freshwater prawn, crabs, lobsters, and crayfish, have increasingly become important protein sources for human demand. Also, these decapods have various direct and indirect benefits for humans in both health and economy. Recently, high demand and economic value of cultured decapods in global markets have been reported (FAO, 2022). Among them, marron (*Cherax cainii*) is one of the decapod species, with high market value, cultured mostly in Australia, but also exported to a number of countries (Lawrence, 2007, Nunes et al., 2017, McClain, 2020). Although marron farming has been practiced for decades, there are still various challenges to improve the production of this industry (Alonso, 2010, Luckens et al., 2015). Developing a low protein diet with high nutrient digestibility can be seen as a proper measurement that could reduce waste loads but possibly affect health and production of marron. In the aquatic industry, water quality deterioration and effluent discharge have become an increasing concern (Ahmad et al., 2019, Boyd and Tucker, 2019). Therefore, it is necessary to develop commercial diets that can both sustain the environment and improve marron performance.

To date, it has been widely accepted that probiotics play a significant role in aquaculture as dietary probiotics can improve animal immune status and growth performance (Nimrat et al., 2013, El-Saadony et al., 2021). A dietary probiotic supplement can also act as an eco-friendly method for stress and disease control for sustainable aquaculture (Wang et al., 2019, Duan et al., 2017). Previous studies have demonstrated the beneficial effects of dietary probiotics in pond conditions on growth performance of some crustacean species such as tiger shrimp (*Penaeus monodon*) (Dalmin et al., 2001), and white leg shrimp (*Litopenaeus vannamei*) (Zheng et al., 2017). The probiotic approach has been utilised in marron aquaculture both in vitro and vivo scales that showed promising outcomes, especially in indoor systems with controlled environments (Foysal et al., 2019d, Ambas et al., 2013).

Compared to indoor, outdoor aquaculture systems are more complex, and nutrient inputs along with many other factors, including in situ nutrients of the bottom soil, can contribute

to the target yield. The cultured species partially use nutrients entering the pond, most of which are suspended in water and accumulated in the pond sediments. While diet efficiency was assessed through animal production, the impact on the pond sediment was not considered. The present study determined the performance of two marron ponds fed two different commercial diets (one diet was supplemented with probiotics) by evaluating nutrients as well as microbial communities in water and sediment, marron physiological parameters, and marron production.

5.2 Materials and methods

5.2.1 Experimental design

A feeding trial was conducted in ponds located at Blue Ridge marron farm (34°12′22″ S, 116°01′01″ E). Two adjacent rectangular ponds of identical dimensions, each measuring 1000 m^2 and possessing similar structural features, were used for the trial. Pond characteristics were documented previously in Chapter 4. Juvenile marrons obtained on-site from the same broodstock pond were manually graded, counted, and weighed prior being stocked into two ponds. The stocking densities were at three juveniles per square meter for both ponds. Two diets, namely premium marron pellets (PMP) and a Solair feed (SPD), were used as two diet treatments. The proximate composition of these diets was presented in Table 3.1 in Chapter 3. From the commencement of the trial until the day of harvest, an equivalent quantity of each diet (2.5 kg) was used to feed marron in each corresponding pond by hand, once daily at 03:00 pm.

5.2.2 Sample collection

Samples of water, sediment, and marron were collected three times in the last six months of the growing period with the interval of every two months in the middle of March, May and July 2018.

5.2.3 Water quality

Parameters include temperature, pH, dissolved oxygen (DO), and water transparency were measured at four corners of each pond while water samples were collected for laboratory

analysis of ammonia, nitrite, nitrate, phosphate, phytoplankton abundance, and zooplankton abundance (see section 3.1 of Chapter 3).

5.2.4 Sediment collection and analysis of carbon/nitrogen (C/N) ratio

The methods of pond sediment collection and C/N ratio analysis were described in sections 3.2 and 3.3 of Chapter 3.

5.2.5 Microbial ecology in pond water and sediment

The procedures outlined in section 3.4 of Chapter 3 were followed for DNA extraction, PCR amplification and sequence data processing of water and sediment samples.

5.2.6 Marron proximate composition and health indices

At each sampling time, of each diet treatment, three marron were collected to analyse haemolymph and moisture indices, following the process described in sections 3.6 and 3.7 of Chapter 3.

5.2.7 Marron harvest

At the end of the culture period, pond water was drained totally, and marron was collected manually. The number of marron in each diet treatment was counted to calculate the survival rate, as described in section 3.8 of Chapter 3.

Marron at harvest was graded by size, ranging from the smallest size of ≤ 70g to the largest size of ≥ 500g. The final weight of marron at harvest (kg) was calculated by totalling the biomass of all sizes.

5.2.8 Statistical analysis

The dataset was assessed to ensure conformity with the normality and homogeneity assumptions outlined in Section 3.9 of Chapter 3. Subsequently, a two-way ANOVA was employed to examine the interaction between diet types and sampling months. Furthermore, independent sample t-tests were conducted to compare all parameters across the two diet treatments, while variations in parameters among the three sampling months

were assessed using one-way ANOVA followed by post-hoc tests. The p-value of less than 0.05 was considered statistically significant.

5.3. Results

5.3.1 Pond water quality and sediment C/N ratio supplied with two different diets

As shown in Table 5.1 below, no significant differences were observed in abiotic factors (temperature, pH, dissolved oxygen, water transparency, ammonia, nitrite, nitrate and phosphate) and biotic factors (phytoplankton and zooplankton abundances) between two treatments. Regarding to sediment C/N ratio, it was higher in SPD compared to PMP, but not statistically significant ($p>0.05$).

Table 5.1 Water parameters and sediment C/N ratio in marron ponds supplied with two different diets. Data represented as mean ± S.E., n = 12

Parameters	**PMP**	**SPD**
Temperature (°C)	18.88 ± 1.57	18.65 ± 1.57
pH	8.06 ± 0.24	8.09 ± 0.24
Dissolved oxygen (mg/L)	7.15 ± 0.13	7.14 ± 0.18
Water transparency (cm)	66.25 ± 3.99	65.00 ± 3.89
Ammonia (mg/L)	0.06 ± 0.01	0.05 ± 0.01
Nitrite (mg/L)	0.01 ± 0.00	0.01 ± 0.00
Nitrate (mg/L)	0.25 ± 0.01	0.30 ± 0.02
Phosphate (mg/L)	0.38 ± 0.01	0.35 ± 0.03
Phytoplankton abundance ($x10^3$ cells/L)	751.04 ± 70.97	826.04 ± 42.12
Zooplankton abundance (individuals/L)	60.00 ± 9.15	68.33 ± 4.49
Sediment C/N ratio	7.86 ± 0.19	8.27 ± 0.15

Abbreviations: PMP – premium marron pellets; SPD – Solair feed.

5.3.2 C/N ratio in marron pond sediment at different sampling times

In this study, there was no interaction between diets and sampling months ($F=1.631$, $p>0.05$). The results for sediment C/N ratio in specific sampling times are presented in

Figure 5.1. Statistical differences were not found with the samplings in March and May. However, a significantly lower value of sediment C/N ratio was observed in PMP when compared to SPD in July ($p<0.05$).

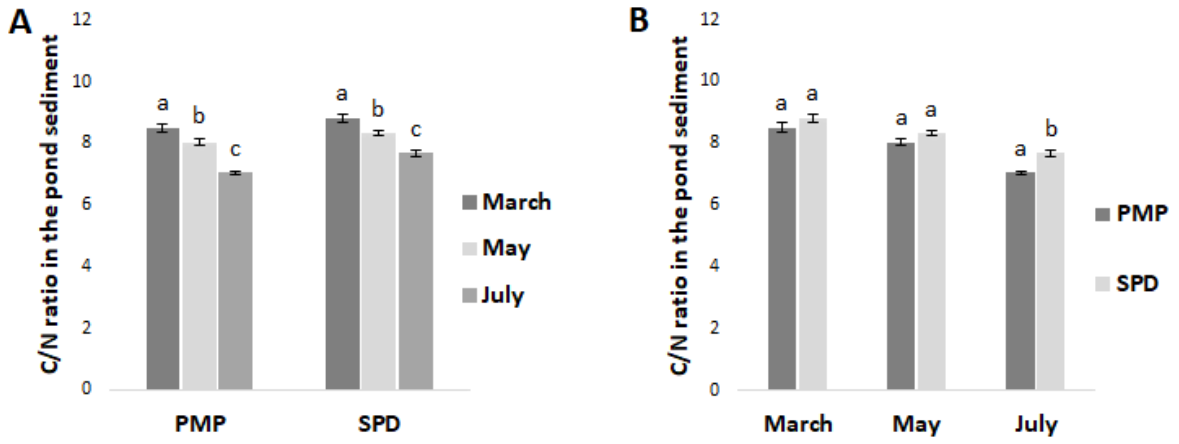


Figure 5.1 Sediment C/N ratio in marron pond sediment. Data represented as mean ± S.E., n = 4. Letters on bars represent the significant differences between sampling months within each diet (A) and between two diets within each sampling month (B) ($p<0.05$). Abbreviations: PMP – premium marron pellets; SPD – Solair feed.

5.3.3 Characterization of bacterial communities in water and sediment of marron ponds provided with two different diets

5.3.3.1 Sequence statistics

A total of 71,776 high-quality reads were obtained from 06 pond water and 06 pond sediment samples. After trimming and filtering, 62,308 reads were retained wherein 98% were classified into phylum level. In total, 258 OTUs were assigned to 14 phyla and 158 genera.

5.3.3.2 Bacteria diversity

The PMP treatment demonstrated lower bacteria alpha diversities in water and sediment when compared to SPD. In water samples, observed species and Chao1 diversity indices of PMP were significantly ($p<0.05$) lower than that in SPD. Similarly, in sediment samples,

apart from the significant differences between observed species and Chao1 diversity indices, the Shannon indices was also found to be augmented in SPD (Table 5.2).

Table 5.2 Diversity indices of bacteria (mean ± S.E.) in marron pond water and sediment

	Parameters (n=3)	**PMP**	**SPD**
Water	Observed species	49.3±1.5[a]	91.7±0.4[b]
	Shannon	2.7±0.3	2.8±0.2
	Simpson	0.88±0.4	0.86±0.3
	Chao1	52.7±2.9[a]	112.9±5.7[b]
Sediment	Observed species	56.7±0.6[a]	103.7±1.3[b]
	Shannon	2.9±0.3[a]	3.2±0.2[b]
	Simpson	0.92±0.1	0.92±0.2
	Chao1	59.3±2.4[a]	143.7±112.8[b]

In the same row, different superscripts represent the significant difference between two diet treatments ($p<0.05$). Abbreviations: PMP – premium marron pellets; SPD – Solair feed.

5.3.3.3 Bacterial community composition

Of the top dominant phyla, Figure 5.2A shows that Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria were four phyla abundant in marron pond environment, regardless of diet types. Proteobacteria was the most abundant phylum in both ponds, accounting for more than 60% of the total sequences. In PMP treatment, Actinobacteria was the second dominant phyla (16.1%), followed by Cyanobacteria (15.7%) while Bacteroidetes (18.5%) and Actinobacteria (8.4%) came second and third, respectively in SPD environment.

Figure 5.2B-C describe the differences in relative abundance of dominant phyla in water and sediment habitats between two tested treatments. The dominant phyla in the two-pond water of PMP (wPMP) and water of SPD (wSPD) were Proteobacteria (66.4%, 73.7%), Actinobacteria (14.2%, 5.1%), Bacteroidetes (0.2%, 17.5%), and Cyanobacteria (17%, 2.5%), respectively. More than 97% of all sequences from water samples belonged to these

four phyla. Sediment habitats of diet treatments were also dominated by these four phyla including Proteobacteria (62.1%, 61.8%), Actinobacteria (17.9%, 11.7%), Bacteroidetes (1.9%, 19.4%), and Cyanobacteria (14.3%, 2.6%), which accounted for 96.2% and 95.5% of the microbial sequences in pond sediment of PMP (sPMP) and sediment of SPD (sSPD), respectively. Sediment was also the habitat for the prevalence of Firmicutes. High percentages of this phylum were recorded in the sediments of both diet treatments.

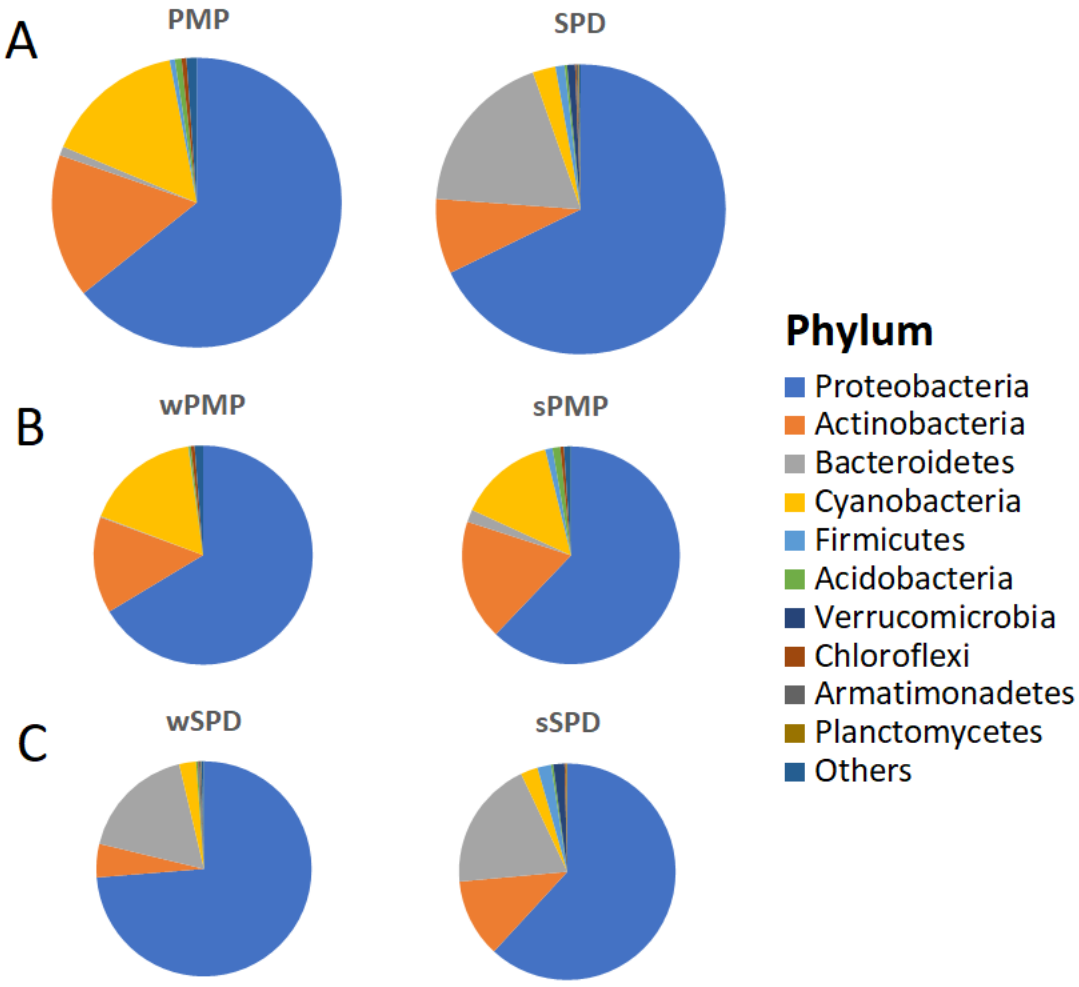


Figure 5.2 Pie charts show percentages of the top dominant phyla in marron ponds treated with different diets. A-microbiota at phylum level in marron rearing environment (pooled data). B-microbiota at phylum level in PMP water (wPMP) and sediment (wPMP). C-microbiota at phylum level in SPD water (wSPD) and sediment (sSPD).

The average relative abundances of significantly different genus taxa in water and sediment samples are shown in Table 5.3. In water, *Limnohabitans* was the highest-dominated genus among six groups, accounting for 33.3% in the SPD while it was only 6.8% in PMP ($p<0.001$). In contrast, PMP significantly enriched *Aquabacterium* (21.4%) compared to about 8% in SPD. The other four dominant bacteria groups including *Aeromonas*, *hgcl clade*, *Vibrio*, and *Vogesella* were significantly higher in PMP. In sediment habitat, the prevalence of *Limnobacter* (19%) was observed in SPD whereas this bacteria group accounted for 0.1% in PMP. However, PMP promoted the development of *hgcl clade*, *Aquabacterium*, and *Aeromonas* which were significantly higher than those in SPD ($p<0.001$). Similar to water habitat, sediments of PMP also contained higher levels of *Vogesella* and *Vibrio* than those bacteria groups in SPD.

Table 5.3 The average relative abundance (mean ± S.E., n=3) of significantly different genus taxa in water and sediment samples

	Genus	**PMP**	**SPD**
Water	*Limnohabitans*	6.80 ± 0.69 [a]	33.3 ± 1.62 [b]
	Aquabacterium	21.40 ± 1.39 [a]	8.10 ± 0.92 [b]
	Aeromonas	9.20 ± 0.81 [a]	3.00 ± 0.46 [b]
	Hgcl clade	8.50 ± 0.75 [a]	2.00 ± 0.35 [b]
	Vibrio	6.60 ± 0.64 [a]	1.40 ± 0.17 [b]
	Vogesella	8.20 ± 0.69 [a]	6.60 ± 0.69 [b]
Sediment	*Limnobacter*	0.10 ± 0.00 [a]	19.00 ± 1.62 [b]
	Aquabacterium	13.00 ± 1.04 [a]	4.00 ± 0.46 [b]
	Aeromonas	11.60 ± 0.92 [a]	1.90 ± 0.23 [b]
	Hgcl clade	14.00 ± 1.27 [a]	0.10 ± 0.00 [b]
	Vibrio	5.60 ± 0.40 [a]	0.10 ± 0.00 [b]
	Vogesella	7.70 ± 0.52 [a]	0.10 ± 0.00 [b]

In the same row, different superscripts represent the significant difference between two diet treatments ($p<0.05$). Abbreviations: PMP – premium marron pellets; SPD – Solair feed.

5.3.4 Marron performances

5.3.4.1 Moisture indices, proximate compositions and immunological parameters of marron

Differences in moisture indices and proximate composition of marron in ponds were not significant. Similar results were also found in most immunological parameters of marron except for granular cell percentage. Marron of PMP had significantly lower granular cell percentage in their haemolymph compared to those in marron of SPD.

Table 5.4 Moisture indices, immunological parameters and proximate composition (mean ± S.E., n = 9) of marron in ponds provided with different diets.

Parameters	**PMP**	**SPD**
HM (%)	70.1 ± 1.03	72.05 ± 0.97
TM (%)	74.54 ± 0.70	74.74 ± 0.31
Tiw	25.38 ± 0.55	26.08 ± 0.26
Tid	6.43 ± 0.08	6.59 ± 0.10
Hiw	5.36 ± 0.26	5.87 ± 0.21
Hid	1.58 ± 0.03	1.63 ± 0.02
THC (x10^6 cells/mL)	1.64 ± 0.02	1.63 ± 0.03
Granular cells (%)	25.28 ± 0.51 [a]	28.56 ± 0.86 [b]
Semi-granular cells (%)	10.17 ± 1.16	8.44 ± 1.11
Hyaline cells (%)	64.56 ± 1.51	63.00 ± 1.07
Protein (%)	82.12 ± 0.22	82.56 ± 0.33
Energy (KJ/g)	20.49 ± 0.11	20.60 ± 0.12
Protein/Energy ratio	4.01 ± 0.02	4.01 ± 0.03
Ash (%)	7.13 ± 0.18	6.97 ± 0.12

Different superscripts in the same row show significant differences ($p<0.05$). Abbreviations: PMP – premium marron pellets; SPD – Solair feed; HM - Hepatopancreas moisture, TM - Tail muscle moisture, Tiw - Wet tail muscle to body ratio, Tid - Dry tail muscle to body ratio, Hiw - Wet hepatosomatic index, Hid - Dry hepatosomatic index, THC - Total haemocyte counts.

5.3.4.2 Marron production

Marron productions of two treatments were summarised in Table 5.5 and Figure 5.3. Similar marron survival was obtained in PMP (60.6%) and SPD (59.5%). However, PMP had 26.4 kg more marron than SPD (Table 5.5). Marron weight distribution varied between two treatments (Figure 5.3). In PMP, marron had a final weight range between 70-400 g with one peak at 151-200g while SPD obtained two peaks at 101-150 g and 201-250 g. In addition, there were fewer marron in the size groups of less than 150 g and more marron in the size ranges of larger than 250 g in PMP compared to SPD. In addition, there was one marron greater than 400 g in PMP while no marron of that size was found in SPD.

Table 5.5 Marron production in ponds provided with two different diets.

Parameter	**PMP**	**SPD**
Number of marron at stocking (individuals)	3000	3000
Total marron weight at stocking (kg)	30.36	30
Number of marron at harvest (individuals)	1818	1769
Survival (%)	60.6	59.5
Total feed offered (kg)	1080	1080
Total marron biomass at harvest (kg)	297.63	271.23

Abbreviations: PMP – premium marron pellets; SPD – Solair feed.

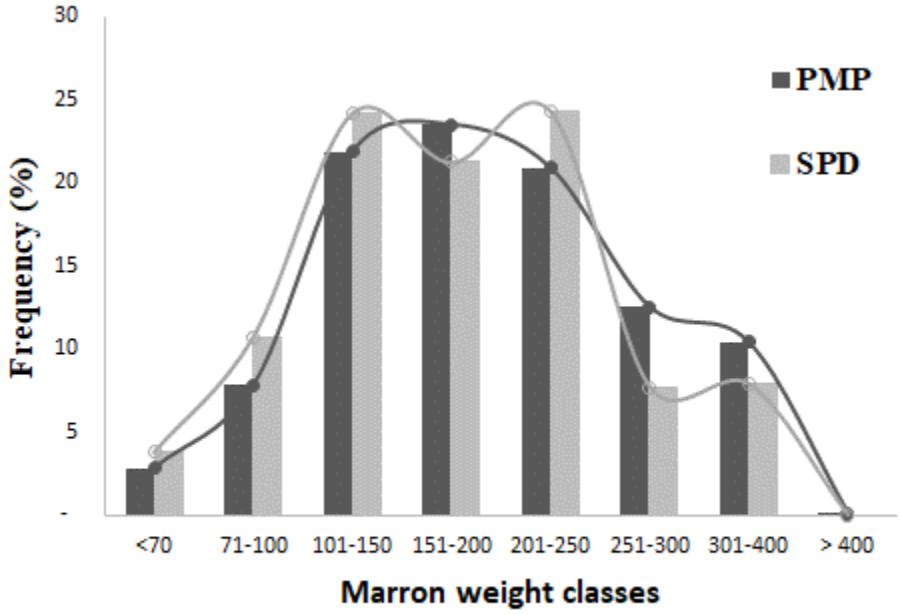


Figure 5.3 Marron weight distribution (%) of ponds provided with two different diets, PMP – premium marron pellets and SPD – Solair feed.

5.4. Discussion

5.4.1 No significant difference in water quality and sediment C/N ratio

The sediment-water interface serves as a link between the water column above and the sediments at the bottom of aquatic systems. The conditions of sediment-water interface can considerably affect growth and health of decapod crustaceans because the animals spend most of their time in this area. Poor quality of this area could lead to negative impacts on the cultural environment and cause a reduction in animal production due to metabolism impairment and disease outbreaks (Avnimelech and Ritvo, 2003). For instance, overfeeding resulted in increasing nutrient accumulation in the pond bottom and caused eutrophication and oxygen deficits (White, 2013, Talbot and Hole, 1994). High concentrations of nitrogenous compounds such as nitrite at the end of the culture period may result in immune ability depression and affect animal growth performance (Tseng and Chen, 2004). Therefore, maintaining an adequate quality of the sediment-water interface

area is the vital management bringing successful pond cultivation (Avnimelech and Ritvo, 2003).

High protein levels in feed can deteriorate water quality (Wang et al., 2012, Bechara et al., 2005). This was attributed to a high release (75%) of feed nitrogen into the water (Avnimelech and Ritvo, 2003). In the current trial, ranges of selected water variables were in acceptable reference values for marron culture (Morrissy, 1990, Cole et al., 2019). A 1.5% difference in the protein levels (20.85% vs. 19.41%) of the two test diets may not be large enough to make a dissimilarity in pond water quality in our study.

Pond sediment is the sink of nutrients and organic matter from the water column (Avnimelech and Ritvo, 2003). High percentages of carbon and nitrogen of the input (water, feed, fertilisers) accumulated in the pond bottom were documented by several researchers (Jackson et al., 2003, Yang et al., 2022, Sahu et al., 2015). In the present study, differences in feed protein levels and C/N ratios significantly impact sediment nutrients at harvest but not in other time points. In previous studies, Hari et al. (2004) and Asaduzzaman et al. (2008) found that increasing the C/N input significantly reduced total nitrogen in sediment. It is important to note that the pond sediment-water interface is not only influenced by the quantity and quality of deliberate inputs but also by the variation of environmental factors and the activity of microbial communities (Huang et al., 2018, Zhao et al., 2018). Therefore, a combination of different variables may contribute to the water and sediment characteristics.

5.4.2 Probiotic diet significantly affected water and sediment microbiota

The interaction between microbial communities in the culture environment (water and sediment) and the health status of cultured animals was concerned (Huang et al., 2018, Rajeev et al., 2021). Understanding the role of microbiota is necessary to better manage animal health in aquaculture (Wei et al., 2016, De Schryver and Vadstein, 2014). Previous studies recognized the importance of microbial relationships between water and sediment

as well as compared the distribution of microorganisms between these two habitats in various aquaculture systems (Li et al., 2018, Sun et al., 2020).

Using 16S rRNA gene sequencing technology to identify, classify and quantify microbial populations of different habitats (water, intestine, sediment) was performed in indoor marron tanks (Foysal et al., 2022b, Foysal et al., 2022a, Nguyen et al., 2021). However, studies have yet to be performed to compare the microbiota of water and sediment habitats in marron pond cultures using this technology. Similar bacterial compositions at the phylum level between these two habitats in the current trial aligns with previous studies on white leg shrimp (Hou et al., 2018), and crucian carp (*Carassius auratus*) (Li et al., 2017). Notably, along with the top four dominant phyla, including Proteobacteria, Actinobacteria, Bacteroidetes, and Cyanobacteria, the phylum Firmicutes is also prevalent in pond sediments, and their abundance brings benefits to aquatic organisms (Xia et al., 2019, Xia et al., 2023, Sun et al., 2020).

Regarding the effects of feed types on environment microbiota, different diets were proven to impact bacterial abundance in water (Qin et al., 2016). Similarly, the two tested diets resulted in differences in the relative abundance of bacteria in the water and sediment of marron ponds. There are several possible reasons for these differences. It could be due to different C/N ratios of the diets. Diverse C/N ratios could influence microbial growth and reflect the type of microorganisms developed in the culture systems (Xu et al., 2022, Abakari et al., 2022, Ghonimy et al., 2023). Prior research has shown that rising the C/N ratio of inputs can lower inorganic nitrogen levels and change the bacterial community in rearing water (Panigrahi et al., 2018, Zhu et al., 2021), which could also be the case in the present study. Furthermore, Ambas et al. (2019) demonstrated that dietary probiotics could enhance bacterial abundance in the marron intestine. Cole et al. (2019) also found a higher abundance of heterotrophic bacteria in water of marron pond due to the addition of probiotic substrates. SPD in our trial was supplemented with probiotics and may also have similar effects on water and sediment of marron ponds. Bacterial diversity plays a fundamental role in maintaining ecosystem stability. High bacterial diversity can enhance

the resilience of ecosystems against disease outbreaks and provide a natural defence against pathogens. Fewer bacterial groups were found in the gut and rearing water of diseased white leg shrimp than healthy ones (Wang et al., 2014, Kurniawinata et al., 2022). In the current study, SPD promoted higher bacterial diversities in pond water and sediment, possibly due to the effect of the beneficial bacteria added to this diet.

Our study also found that the two tested diets differently promoted the growth of specific bacterial genera in marron pond sediments. While the pond sediment supplied with PMP favoured the genera *Aquabacterium* and *Polynucleobacter*, SPD predominated with *Aeromonas, Cyanobium,* and *Candidatus Bacilloplasma* groups. This result may be the root of differences in the diet formulations. With nitrate serving as an electron acceptor, the genera *Aquabacterium* and *Polynucleobacter* were able to effectively oxidise Fe(II) in high ammonia-nitrogen environments (Zhang et al., 2016, Hoetzinger et al., 2017), explaining the abundance of these genera in pond provided with PMP which contains a higher protein level compared to SPD in the present study.

The genus *Aeromonas* consists of some species, such as *A. hydrophyla*, *A. salmonicida*, and *A. veronii* that are recognized as pathogens for fish (Tyagi et al., 2022, Anjur et al., 2021), but it was also determined as the core bacteria in marron gut and abundant in the sediment of marron culture tank and did not negatively affect marron health (Foysal et al., 2022a). Based on previous reports (Janda and Abbott, 2010, Chaix et al., 2017), *Aeromonas* grew better in habitats that were rich in nutrients. A high C/N input should inhibit the growth of *Aeromonas* (Foysal et al., 2022a), which was contradicted by the results of the present study where the high C/N diet was used and high C/N ratio retention in pond sediment. A limitation of this study was that we did not analyze the microbiota at the beginning of the feeding trial, so it is not known whether the difference in microbiota between treatments was due to different diets or to a combination of other factors in the pond. Ultimately, farmers and researchers should manage to achieve beneficial microbial communities for the culture systems, as a healthy microbiome can be vital to successful and sustainable aquaculture (Rajeev et al., 2021).

5.4.3 The effect of diets on marron production

The formulated feed used for aquatic animals is often customized to have high protein and carbohydrate content to compensate for nutrient leaching. Previous studies have proposed the formulated feeds with 25-26% crude protein, 8-9% lipid, and 3-5% ash for marron cultured in ponds (Fotedar, 2004, Ambas et al., 2019) while diets with 29-30% crude protein, 7% lipid, 4-10% ash for marron raised in tanks (Nguyen et al., 2021, Saputra and Fotedar, 2021). The two diets tested in the present study have lower protein contents, while energy, lipid, and carbohydrate contents are comparable to those in previous studies. The feed C/N ratio was usually around 7-10 for most aquatic farmed animals (Avnimelech, 2007), but not documented in the literature for any marron culture systems. In the present study, tested diets have relatively high C/N ratios, attributing by low percentages of protein.

The two diets showed no effect on moisture indices of marron in the present study. This finding agreed with the past study that also obtained data on marron at market sizes (Bryant and Papas, 2007). Results conducted with *Clostridium butyricum* as dietary probiotic supplements illustrated a positive influence on protein content in marron tail muscle (Foysal et al., 2019d). This parameter is higher than in the present study, possibly due to different culture environments (indoor tanks vs. commercial ponds). However, there is an agreement on the effect of probiotic diets on improving the innate immune parameters of marron between previous studies (Foysal et al., 2019d, Ambas et al., 2019) and the present study, attributing to a higher proportion of granular cells in marron haemolymph fed probiotic supplemented diet. An elevation in granular cell percentage was noted among healthy marron, as observed in the study by Tulsankar et al. (2022a), which may be linked to their consumption of live plankton. Moreover, the present study suggests that this increase could potentially be influenced by the positive effects of probiotic dietary intake. The immune systems of marron are relatively sensitive to probiotics; therefore, the utilization of probiotics to improve marron health condition should be considered.

Regarding marron production parameters, the two diets used in the present study resulted in similar marron survival which was around 60%, lower than that in the study by Ambas et al. (2019) (66-74%), but much higher than that reported by Fotedar (2004), where marron survival was less than 40%, all cultured in earthen pond conditions. The diet supplemented with probiotic *Bacillus mycoides* improved marron survival and production (Ambas et al., 2019). However, the probiotic diet tested in our study did not bring higher marron production. Marron pond production in our study was 271-297g/m^2, higher than that documented by Ambas et al. (2019), which was 215-258 g/m^2.

Due to size-related marron prices, marron weight distribution at harvest is contributing to the bottom-line. There is no sale of smaller than 120 g marron into the domestic market (Lawrence et al., 2006). It is crucial to comprehend how diets have affected this size distribution. When comparing the economic benefit of marron fed two tested diets, the results indicated that PMP was more beneficial than SPD because marron fed PMP have larger sizes which can bring more income for growers, therefore higher economic efficiency. These findings indicated that probiotic diet does not always improve marron pond production, but other diet indices such as protein sources and C/N ratio may contribute to increase marron production.

5.5 Conclusion

In conclusion, water quality parameters showed no significant differences among test diets. Significant difference of sediment C/N ratio between two treatments was recorded at harvest time only. SPD showed significantly increased bacterial alpha diversities in water and sediment where *Aquabacterium, Aeromonas, hgcl clade, Vibrio*, and *Vogesella* were less prevalent than the other diet treatment. However, a positively skewed pattern of size distribution and increased production were the outcomes of the marron pond that received PMP.

CHAPTER 6 (Experiment 3) The impacts of two dietary protein sources on the rearing environment and performance of marron (*Cherax cainii*) in laboratory conditions

Chapter 6.1 was conducted to investigate the effect of dietary protein sources on the water quality parameters and the water microbiome. This study found positive correlations between some selected water quality parameters and microbial genera. These results directed us to do further analysis, as explained in chapter 6.2, on the influence of dietary protein sources on the sediment carbon/nitrogen ratio and microbiota, as well as marron growth and health performance.

CHAPTER 6.1 The effect of two dietary protein sources on water quality and the aquatic microbial communities in marron (*Cherax cainii*) culture

(Published in Microbial Ecology: https://doi.org/10.1007/s00248-021-01681-3)

Abstract

Feeding freshwater crayfish species with different diets not only affects the water quality but also induces the abundance of various microbial communities in their digestive tracts. In this context, very limited research has been undertaken to understand the impacts of various protein incorporated aqua-diets on the characteristics of water and its microbial communities. In this study, we have critically analysed the water quality parameters including pH, dissolved oxygen, nitrate, nitrite, ammonia, phosphorus, as well as bacterial communities under marron (*Cherax cainii*) aquaculture, fed fishmeal (FM) and poultry-by-product meal (PBM) based diets for 60 days. The results unveiled that over the time, feeding has significant impacts on organic waste accumulation, especially ammonia, nitrate, nitrite and phosphate while no effects were observed on pH and dissolved oxygen. Analysis of 16S rRNA sequence data of water sample indicated significant ($P<0.05$) shift of microbial abundance in post-fed FM and PBM water with the evidence of microbial transmission from the gut of marron. Post-fed marron resulted in a significant correlation of *Hafnia*, *Enterobacter*, *Candidatus* Bacilloplasma, *Aquitella* with the quality and microbial population of water. The results of this study generated valuable knowledge database of microbes-water relationship for better health management practices and production of marron aquaculture fed with FM and PBM diets in under restricted feeding regime with the feeding ratios provided.

6.1.(1) Introduction

Transmission of pathogenic bacteria including *Vibrio*, *Aeromonas*, *Streptococcus* and *Enterococcus* from water into fish and crustaceans (Foysal et al., 2019a, Oidtmann et al., 2017) emphasise the importance of studying water microbiota in aquaculture. Therefore, alongside the host, the study of water microbial community is equally important to investigate host-microbe interaction in aquatic environment in order to maintain health and immune status of the aqua-cultured species. These microbial interactions are critical for an effective establishment and maintenance of a healthy microbial population and are frequently associated with pathogenic or beneficial effects on the cultured species (Braga et al., 2016). Further, the use of different dietary feed ingredients in aquaculture resulting in the shift of the microbial communities in the rearing water is inevitable (Bentzon-Tilia et al., 2016). Though the impact of different dietary protein supplementations on the gut microbiota and health status of several aquatic animals has been investigated (Foysal et al., 2019b, Miao et al., 2018, Siddik et al., 2020, Gupta et al., 2020), the effects of feeding different protein sources on water microbiota has been paid no attention to. In addition, the health status of aquatic species is highly correlated to the composition and functions of microbial communities inside and outside of the animal's digestion system (Adamovsky et al., 2018, Hillman et al., 2017).

Addition of aqua-diets into water accelerates the colonization of microbes in the water (Elsaidy et al., 2015, Ibekwe et al., 2017). The uneaten feedstuffs containing trace elements, vitamins and minerals are used by the resident microbes as nutrient sources and stimulate their growth in water (Cardona et al., 2016, Crab et al., 2012). Some of these microbes not only play a crucial role in removal of organic wastes or facilitate in remediation of water but also generate microbial proteins that are used as feed for aquatic species (Delamare-Debouteville et al., 2019, Bentzon-Tilia et al., 2016). Augmentation of phyla Bacteroidetes, Firmicutes, Tenericutes and species of *Bacillus, Citrobacter, Pseudomonas, Serratia* can naturally remediate aquaculture water by detoxification of aquatic bio-toxin and decomposition of organic waste (Zhu et al., 2017, Foysal et al.,

2020c). Similarly, advantages of protein supplements on water quality, growth performance and immune status of fish and crayfish have been investigated extensively in previous studies (Asaduzzaman et al., 2008, Azim and Little, 2008, Hari et al., 2004, Hari et al., 2006, Saputra et al., 2019). However, there is no information pertaining to the impacts of different protein diets on microbial communities and quality of water under crayfish aquaculture in general, and marron (*Cherax cainii*) aquaculture in particular.

Marron (*Cherax cainii*) is one of the economically important and popular freshwater crayfishes native to the southern part of Western Australia (WA) (Morrissy, 1990). Due to an omnivorous feeding habit, the protein requirement for marron is moderate (approximately 30%) (Ambas et al., 2017). Fish meal (FM) is one of the major sources of dietary protein for cultured aquatic animals, however, dwindling trend in FM production over the past few decades has propelled the researchers to invent cheaper and sustainable alternatives to FM for different aquatic species without compromising their growth (Wu et al., 2018). Poultry by product meal (PBM) is one of those promising alternative ingredients that demonstrated positive effect on the growth rate, digestibility, and immune status of fish and crayfish by replacing FM in aquaculture diets (Yang et al., 2006, Bransden et al., 2001, Saoud et al., 2008).

In post-genomic era, high throughput sequencing (HTS) and downstream bioinformatics have provided comprehensive tools with diverse packages and pipelines to impart deeper insight into microbial communities and their role in specific environment (Liang et al., 2019, Foysal et al., 2020e). The combinatorial approaches have been applied to enable in-depth analysis of feeding effects on health and immunity of fish and crayfish (Foysal et al., 2019b, Foysal et al., 2020a). These approaches are limited to gut microbiota, innate immune response and disease resistance, however, none of the studies reported the dietary effects on microbiome community in the culture water of aquatic species till to date. On the above backdrops, the present study aimed to investigate the shift of microbial communities, using high throughput sequencing and bioinformatics tools, and their role in

water quality management post dietary feeding of marron with two different protein diets FM and PBM.

6.2.(1) Materials and Methods

6.2.1(1) Experiment set-up and sampling

A total of 24 marron with average weight of 71.0 ± 0.8 g were procured from Blue Ridge marron farm, Manjimup, Western Australia and transported alive to Curtin Aquatic Research Laboratories (CARL). Marron were then distributed into six different tanks with a density of four marron per tank, each of 200 L capacity, filled with 150 L freshwater. Each marron was reared in a specially prepared plastic mesh cage as described previously by Ambas et al. (2013). Marron in the tanks were acclimatised for 7 days, and during this period they were fed only commercial diet – premium marron pellets (as described in section 3.5 of Chapter 3). Post acclimation, marron were randomly assigned into two different treatment groups, namely FM pellet fed group and PBM pellet fed group and trial was conducted for 60 days. The ingredients of feed were purchased from Glenn Forrest, Perth, Australia, and after formulation, final diets were produced by the same company. The feed ingredients with proximate composition of the final diets are presented in Table 3.2 described earlier by Foysal et al. (2019b) (see section 3.5 of Chapter 3). Marron were fed everyday evening at 3% of the body weight, during acclimation and dietary trial (Saputra et al., 2019). No daily water exchange was conducted. However, every week, approximately 30% of water from every culture tank with faecal waste was transferred to another tank, and after careful removing of waste, the water then was transferred back into the respective tank. Samples from water microbiota were collected at first week on days 1, 3, 5 and last week on days 56, 58, 60, from FM and PBM fed marron tank water and labelled as FMASE, Fishmeal treated water at the start of experiment; FMATE, Fishmeal treated water at the end of experiment; PBMASE, Poultry-by-product meal treated water at the start of experiment; PBMATE, Poultry-by-product meal treated water at the end of experiment, to analyse the changes in bacterial communities (Bavithra et al., 2020). A pool was created by mixing and homogenization of water samples from three tanks for each of

the treatment groups and respective day. From each tank, 200 ml of water sample was collected for DNA extraction. The samples were centrifuged in refrigerated centrifuge at 10,000 × g for 10 min. to form desirable amount of pellet for DNA extraction.

6.2.2(1) Water quality analysis

Water quality parameters including temperature, pH, dissolved oxygen, ammonia, nitrite, nitrate, and phosphate were measured at the first week and the eighth week of the trial following the methods as described in section 3.1 of Chapter 3.

6.2.3(1) Microbial ecology in tank water

The methodologies outlined within section 3.4 of Chapter 3 were followed to processes of DNA extraction, PCR amplification, and sequence data.

6.2.4(1) Data analysis

The alpha diversity was calculated based on observed species, Shannon and Simpson indices. Non-parametric statistical test of the distance metric was done using ANOSIM (1000 permutations). Feeding effect on water was analyzed based on Bray-Curtis dissimilarity using non-metric multidimensional scaling (NMDS) test. Significant bacteria at genus level from differential abundance was identified using Linear Discriminant Analysis (LEfSe) at 0.05 level of significance (Segata et al., 2011). Correlation between water quality parameters and bacterial abundance was performed in microbiomeseq (https://github.com/umerrijaz/microbiomeSeq). One-way analysis of variance (ANOVA) with Tukey's HSD was used to calculate any significant differences ($p<0.05$) between treatment groups in R-Studio.

6.3(1) Results

6.3.1(1) Water quality

No dietary treatment and sampling time had any influence on water temperature, pH and dissolved oxygen. Within a sampling period, nitrogen metabolites and phosphate were not different ($p >0.05$) when marron were fed different diets, whereas concentrations of these

parameters in 8th week were significantly higher ($p<0.05$) than 1st week within each dietary treatment (Table 6.1(1)).

Table 6.1(1) Water quality parameters (mean ± S.E.) in the trial with FM and PBM

Parameters (n=3)	Diet	Sampling Time	
		1st week	8th week
Temperature (°C)	FM	20 ± 0.03	20 ± 0.09
	PBM	20 ± 0.06	20 ± 0.12
pH	FM	7.6 ± 0.06	7.8 ± 0.09
	PBM	7.6 ± 0.06	7.7 ± 0.12
DO (mg/L)	FM	7.5 ± 0.07	7.3 ± 0.06
	PBM	7.5 ± 0.05	7.4 ± 0.08
Nitrate (mg/L)	FM	1.1 ± 0.04^{a}	1.8 ± 0.05^{b}
	PBM	1.0 ± 0.01^{a}	1.6 ± 0.05^{b}
Nitrite (mg/L)	FM	0.12 ± 0.009^{a}	0.19 ± 0.012^{b}
	PBM	0.14 ± 0.012^{a}	0.18 ± 0.015^{b}
Ammonia (mg/L)	FM	0.15 ± 0.018^{a}	0.24 ± 0.015^{b}
	PBM	0.15 ± 0.012^{a}	0.24 ± 0.015^{b}
Phosphate (mg/L)	FM	0.92 ± 0.03^{a}	1.3 ± 0.03^{b}
	PBM	0.91 ± 0.04^{a}	1.2 ± 0.03^{b}

Different alphabetical superscripts (a, b) in the same row (comparisons among sampling time by paired t-tests at $p<0.05$) and independent t-tests showed no significant difference between tested diets for all water quality parameters $p>0.05$. Abbreviations: FM, fishmeal diet; PBM, poultry-by-product meal diet.

6.3.2(1) Sequence statistics and alpha diversity measurements

After quality trimming, a total of 494,948 reads were extracted from 12 samples before and after feeding trial. Taxonomic classification yielded 911 OTUs, 18 phyla, 26 classes, 63 orders, 101 families and 141 genera. The rarefaction curve indicates that samples were sequenced at high depth and near about saturation to capture enough diversity for all samples (Figure 6.1(1)A). The good's coverage index values ranging from 0.968 for sample PBMATE to 0.998 for PBMASE was employed that indicates samples were sequenced around highest saturation level. After trial, significant increase of major alpha-diversity indices including richness (observed species), and Chao1 was observed in

PBMATE tank water, compared to other groups. The results also showed that feeding the aquatic species with PBM diet increase Shannon diversity in water after two months (PBMATE), in relation to the first week of the trial (PBMASE). However, at the start of the experiment, alpha-diversity indices showed no differences between FMASE and PBMASE (Figure 6.1(1)B-E).

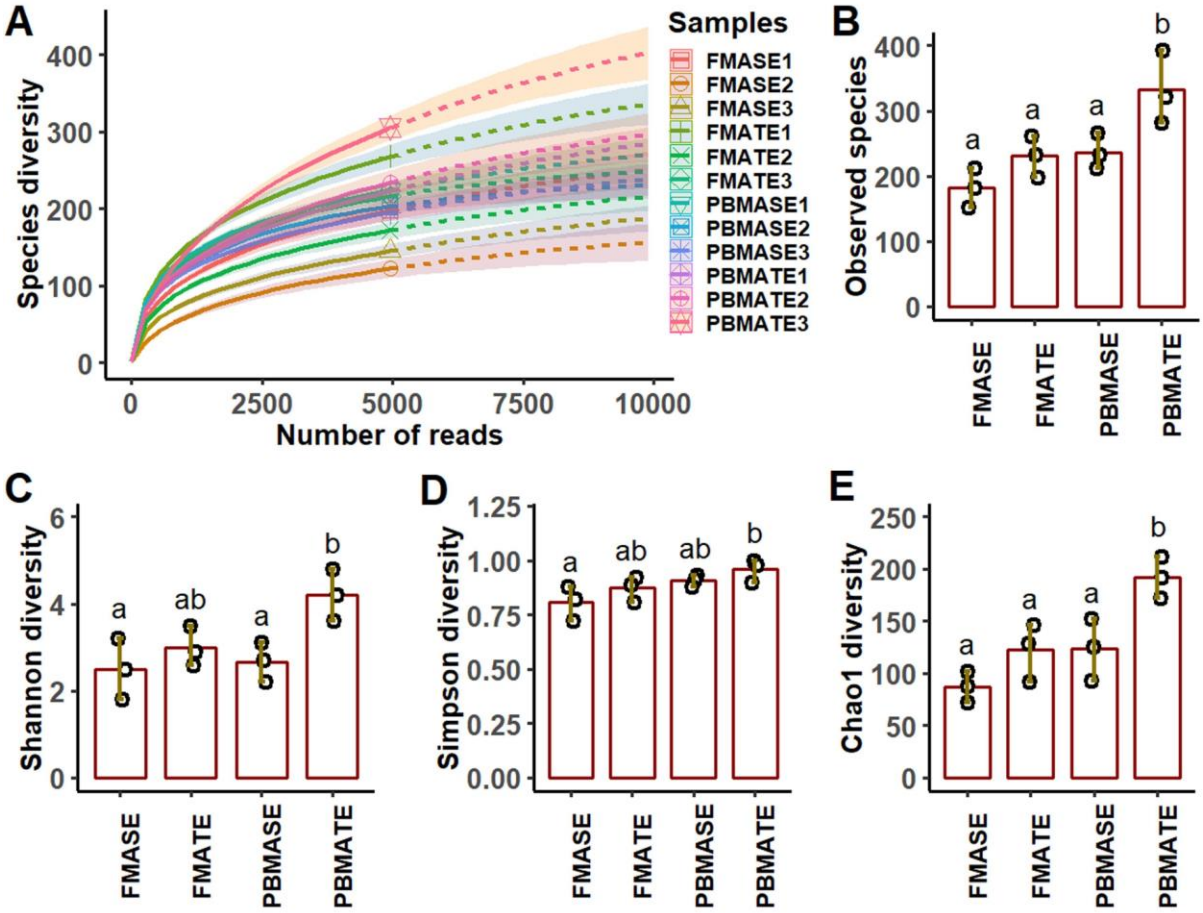


Figure 6.1(1) Alpha-diversity measurements of present study samples. (A) Rarefaction depth showing saturation level of the sequences; (B) Observed species; (C) Shannon diversity; (D) Simpson diversity; (E) Chao1 diversity. Abbreviations: FMASE, Fishmeal water at the start of experiment; FMATE, Fishmeal water at the end of experiment; PBMASE, Poultry-by-product water at the start of experiment; PBMATE, Poultry-by-product water at the end of experiment.

6.3.3(1) Beta diversity and microbial community

Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity showed differential clustering of samples based on treatment groups (Figure 6.2(1)A). The R–value (0.70) and P-value (<0.05) revealed significant correlation between the water microbial population at the start and end of trial and protein feeds. Feeding with both FM and PBM enriched microbial community in water with more unshared genera at the end of experiment. A total of 38 genera were found common in water at the start and end of trial for all four groups (FMASE, FMATE, PBMASE and PBMATE). Alongside shared, 30 and 27 unshared genera were identified in FMATE and PBMATE groups, respectively (Figure 6.2(1)B). At phylum level, Proteobacteria comprised of 63.8-98.2% of bacteria in all groups, followed by Bacteroidetes (1.03-34.5%) and Tenericutes (1.2-12.7%). At genus level, *Aeromonas*, *Candidatus* Bacilloplasma, *Hafnia Obesumbacterium* and *Serratia* were identified from all samples (FMASE, FMATE, PBMASE and PBMATE). In addition, compared to first week, PBM feeding (PBMATE) significantly reduced the *Aeromonas* abundance in water by 24.6% while both diets increased the OTUs for *Vibrio* after trial (FMATE and PBMATE) by 2.2% and 6.4%, respectively (Figure 6.3(1)).

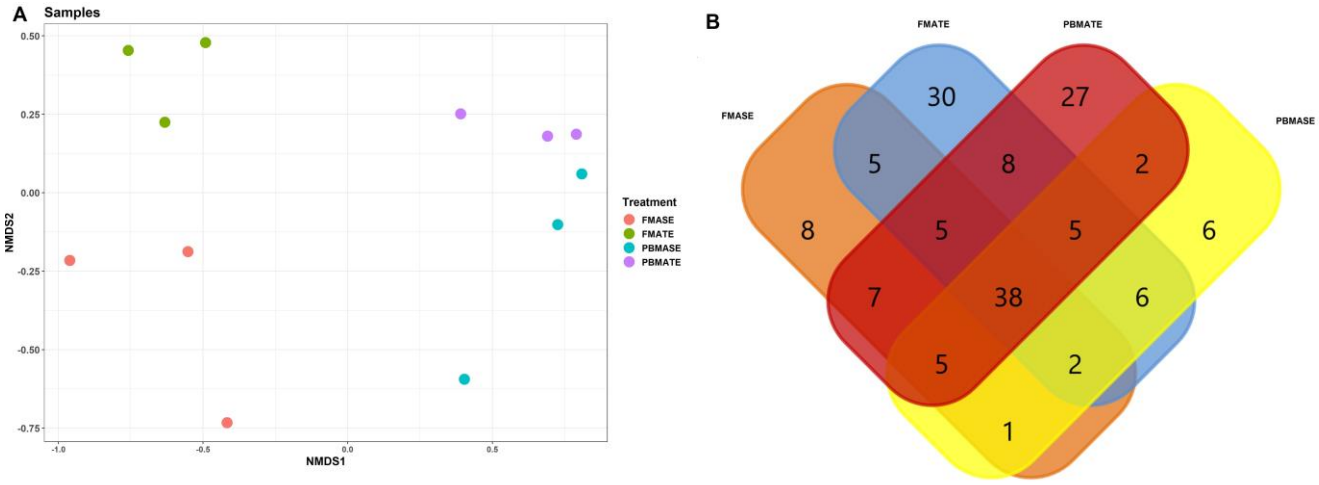


Figure 6.2(1) (A) Beta-ordination plot in terms of NMDS showing clustering of samples. (B) Venn diagram contrasting shared and unique genera in four different groups.

Abbreviations: FMASE, Fishmeal water at the start of experiment; FMATE, Fishmeal water at the end of experiment; PBMASE, Poultry-by-product water at the start of experiment; PBMATE, Poultry-by-product water at the end of experiment.

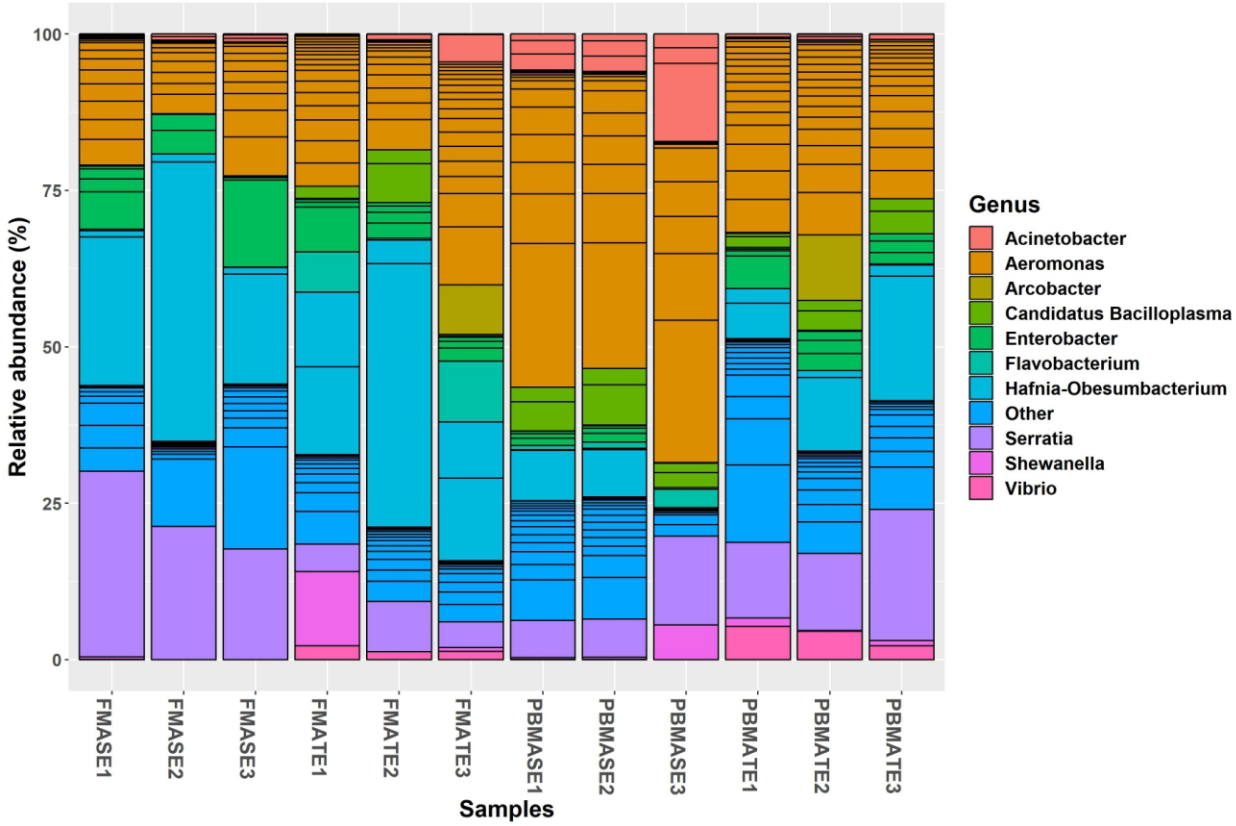


Figure 6.3(1) Relative abundance (%) of bacterial OTUs at genus level. Genera comprised of >1% of OTUs in all 12 samples are presented here. Abbreviations: FMASE, Fishmeal water at the start of experiment; FMATE, Fishmeal water at the end of experiment; PBMASE, Poultry-by-product water at the start of experiment; PBMATE, Poultry-by-product water at the end of experiment.

6.3.4(1) Differentially abundant bacteria and their role

Application of Linear Discriminant Analysis revealed four bacterial genera to be expressed differentially. *Aeromonas* and *Enterobacter* were found significantly enriched in PBM served tank's water after trial while *Candidatus*Bacilloplasma and *Hafnia* were significant abundant bacteria in FMASE group, respectively (Figure 6.4(1)). Correlation analysis

between most abundant genera (≥1%) and water quality parameters showed significant positive association of *Candidatus* Bacilloplasma, *Hafnia* and *Enterobacter* with the concentration of ammonia, nitrate and nitrite in both treatments while *Aquitella* had significant positive correlation with phosphate for only PBM treated water. *Arcobacter*, *Megasphaera* and *Negativibacillus* were negatively correlated (p>0.05) with ammonia, nitrate and nitrite (Figure 6.5(1)).

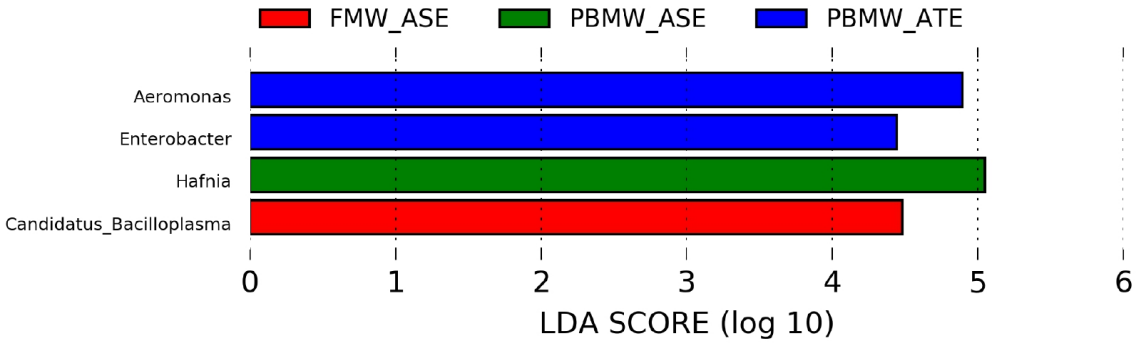


Figure 6.4(1) Significantly abundant bacterial OTUs at genus level. Abbreviations: FMASE, Fishmeal water at the start of experiment; FMATE, Fishmeal water at the end of experiment; PBMASE, Poultry-by-product water at the start of experiment; PBMATE, Poultry-by-product water at the end of experiment.

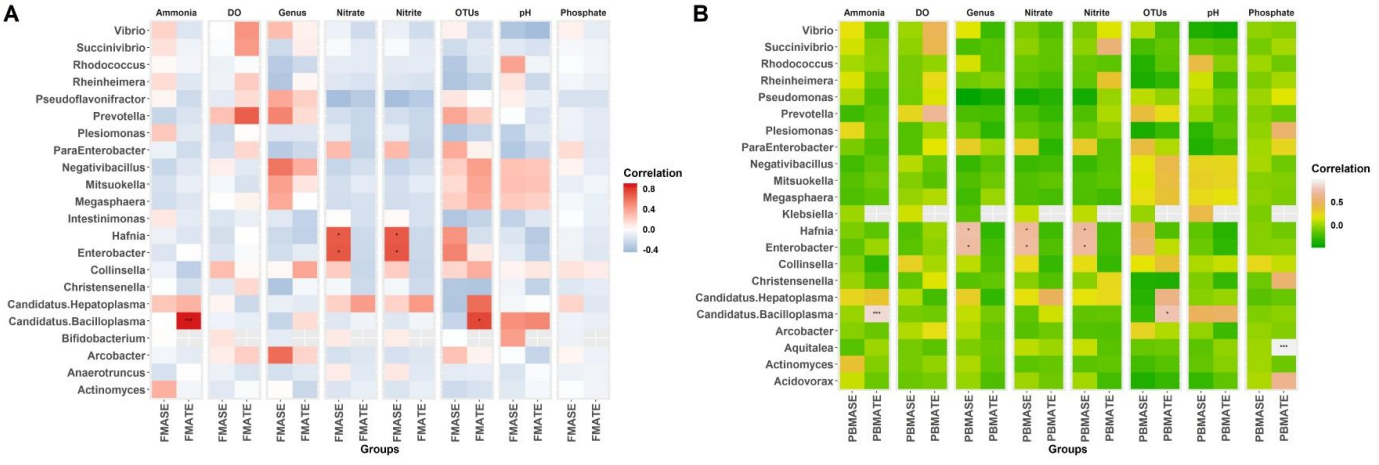


Figure 6.5(1) "Pearson" correlation between bacterial genera and water quality parameters. Abbreviations: FMASE, Fishmeal water at the start of experiment; FMATE,

Fishmeal water at the end of experiment; PBMASE, Poultry-by-product water at the start of experiment; PBMATE, Poultry-by-product water at the end of experiment. *Alpha level of 0.05. ***Alpha level of 0.001.

6.4(1) Discussion

High quality protein feed ingredients such as FM and PBM in aquaculture feed formulations may improve the efficiency of nitrogen assimilation and digestion depending on the host species and their life stages (Begum et al., 1994, Lazzari and Baldisserotto, 2018). These high-quality protein diets usually have 10:1, carbon (C) and nitrogen (N) ratio that may slow down the rate of bacterial waste decomposition, resulting into the accumulation of inorganic nitrogen in the culture system (Azim et al., 2008). Further, a large portion of nitrogen and phosphorus in the feed is not converted to shrimp biomass, but is released into the environment (Jackson et al., 2003, Páez-Osuna and Ruiz-Fernández, 2005, Xia et al., 2004). This can be correlated with our finding of increased nitrogen and phosphorous metabolites in the rearing water of marron fed FM and PBM.

Researchers (Gallagher and Degani, 1988, Emre et al., 2003, de Yta et al., 2012, Fasakin et al., 2005, García-Pérez et al., 2018) in the past either did not compare or find any difference in water quality parameters when FM was replaced by PBM (Webster et al., 2000, Waldemar and Allen, 2012, Rawles et al., 2011, Riche, 2015). Our research confirms no differences in water quality with marron under laboratory conditions, which might have contributed to the advancement/refinement of PBM quality over time as PBM could replace up to 100% of FM without compromising growth performance and feed utilization in red drum (*Sciaenops ocellatus*), sunshine bass (*Moronechrysops*× *M. saxatilis*), gibel carp (*Carassius auratus*), humpback grouper (*Cromileptes altivelis*) (Webster et al., 2000, Yang et al., 2006, Kureshy et al., 2000, Shapawi et al., 2007). Despite the vital importance of physicochemical properties of water in aquaculture, the qualitative microbiological assessment of water is often under-appreciated specially in freshwater aquaculture, posing higher risks to cultured organism than marine aquaculture (Ibrahim et al., 2014). Moreover, the effects of protein based aqua-diets on quality and microbial network of water have not

been studied yet, which impose a great gap in the immune and health status of cultured aquatic organisms.

In limited water exchange aquaculture systems, waste from uneaten feed and faeces are assimilated and mineralized into other metabolites by dense microbial communities (Ebeling et al., 2006, Avnimelech, 2006) and thus positively influence their growth and regeneration (Harder et al., 2019, Pathma and Sakthivel, 2012, Rastogi et al., 2020). No water exchange was applied in the current experiment, therefore the microbial communities in tank water were primarily generated from the uneaten feed and marron's faeces. In this study, with the aid of high throughput sequencing and comprehensive bioinformatics, bacterial species and their correlation with water quality were identified and compared between different diets. Though the concentration of harmful inorganic nutrients including nitrate, nitrite, ammonia and phosphate in water were significantly higher at the end of the trial, yet the ranges were far from the lethal value for marron aquaculture conditions (Nugroho and Fotedar, 2013a). The relative abundance at phylum level had not deviated significantly due to Proteobacterial dominance in all groups whereas at genus level, differential abundance showed that PBM incorporated diet accelerated the growth of *Aeromonas* and *Enterobacter* spp. The relative and differential data also revealed that most of the bacterial colonization occurred in the first week of the feeding, due to the rapid utilization of inorganic nutrients by the heterotrophs such as, Proteobacteria. This group of bacteria is known to play a crucial role in organic waste decomposition from aquatic environment (Alfiansah et al., 2018, Allen et al., 2004, Emerenciano et al., 2012). Consistent to the results of our earlier study (Foysal et al., 2019b) in marron gut, present investigation also reported higher abundance of *Hafnia* and *Aeromonas* in FM and PBM treated water. In addition, the metabolic features of bacterial communities in the marron gut revealed increased activities for *Vibrio*, *Aeromonas*, *Pseudomonas* and *Escherichia coli* after feeding with FM and PBM incorporated diets (Foysal et al., 2019b). Therefore, the enhanced growth and functional activities of heterotrophic bacteria in the gut can be correlated with the microbial communities in water, mediated through uneaten feed and

faecal release by marron into water. To corroborate our findings, earlier study from our laboratory reported a similarity of microbial communities in terms of relative abundance between gut microbiota of marron fed above two different protein diets (FM and PBM) (Foysal et al., 2019b) and water microbiota in the present study.

Present study identified significantly higher alpha diversity of observed species at the end of the feeding trial. Higher unshared genera at the end of trial revealed that different protein based diets could increase bacterial diversity in water under marron aquaculture, which is in accordance to the results of (Qin et al., 2016). In addition to classified OTUs, the numbers of unclassified and uncultured OTUs that have been grouped as others were detected from all samples but high numbers at the end of trial revealed greater abundance of unknown microbial lineages in the water that needs further characterization and their role should be investigated further in-depth.

Environmental factors shape the structure and function of microbial communities in aquaculture water (Allison and Martiny, 2008). Previous studies reported a significant correlation of microbial growth with total nitrogen (Xiong et al., 2014a), nitrate and nitrite (Noorak et al., 2018), total phosphate (Zhang et al., 2014a), and feed supplements (Qin et al., 2016). Current study demonstrated a shift of bacterial abundance and species richness in water at the end of trial wherein the growth of some genera had significant correlation with inorganic nutrients and microbial interaction in water. For instances, the genus *Candidatus* Bacilloplasma, a lineage of bacteria that belongs to class *Mollicutes*, specially found in crustacean gut, and mostly regarded as beneficial bacteria group in the gut for its role in digestion, and host-microbe interaction was shown to have correlation with nutrients and microbial interaction (Meziti et al., 2010, Leclercq et al., 2014). In the present study, growth of *Candidatus* Bacilloplasma was influenced significantly by ammonia and other bacterial lineages in the water at the end of the trial (Figure 6.5(1)). In previous studies, *Candidatus* Bacilloplasma has been identified as a core hub in microbial interaction network in the gut of Pacific white-leg shrimp (*Litopenaeus vannamei*) and marron (Chen et al., 2017b, Foysal et al., 2019b). The transmission of these bacteria from gut into water

was possibly linked to faecal discharge by marron, and its influential role in microbial interaction network. The positive correlation with OTUs can be defined, however, the association with ammonia utilization remains to be discovered. *Aeromonas* spp. and *Enterobacter* spp. are naturally occurring opportunistic bacteria, ubiquitous to aquatic ecosystems and commonly reported from the gut flora of healthy aquatic animals whereas some of the species including *A. hydrophilla*, *A. sobria*, *A. veronii*, and *E. cloacae* are reported to cause pathogenicity to both fish and crayfish (Foysal et al., 2019c, Thillai Sekar et al., 2008, Gołaś et al., 2019). A study characterized high percentage of *Enterobacter* (24%) from the freshwater and their growth was significantly correlated with the availability of protein sources, especially nitrogen, as they are known to fix atmospheric nitrogen (Surendraraj et al., 2009, Jahangiri and Esteban, 2018). Similarly, *Aquitalea* species characterized from lake water and their growth reported to be linked with the depth of water and concentration of total and soluble phosphorus (Lau et al., 2006, Pontes et al., 2009). However, in this case the correlation between dietary protein sources, phosphorus and growth of *Aquitalea* warrants further investigations.

6.5(1) Conclusion

In totality, dietary FM for marron culture can be replaced by PBM without any changes in the selected water quality parameters and heterotrophic bacterial diversity in water under restricted feeding regime with the feeding ratios provided. This study also reported some unknown microbial lineages that warrants further in-depth investigation to characterize those and decipher their role in the water.

CHAPTER 6.2 Carbon/Nitrogen ratio in the sediment drives microbial diversity and composition under marron (*Cherax cainii*) aquaculture: A study with two different protein diets

Abstract

A 60-day feeding trial was carried out to evaluate the effects of fishmeal (FM), and poultry by-product meal diets (PBM), on the sediment carbon/nitrogen (C/N) ratio, microbial communities and growth and health status of 1^{+} marron (*Cherax cainii*). Both diets were isonitrogenous having a crude protein level of approximately 30%. The diets were randomly assigned to triplicated tanks of four marrons housed individually in a plastic cage in every tank. During the trial, tank sediment samples were taken weekly to measure the C/N ratio, whereas microbial communities were analyzed in weeks one and eight. Marron growth and health indices were analyzed at the conclusion of the trial. The results showed that C/N ratios in the tank sediments fluctuated during the culture period and were independent of marron diets. Regardless of diets, the most dominant genera in the tank sediments were *Flavobacterium*, *Aeromonas,* and *Hafnia-Obesumbacterium*. There was a significant rise in *Flavobacterium* in both dietary treatments at week eight compared to week one. *Aeromonas* abundance in the tank sediments with FM diet was significantly lower than in PBM diet at week one, while an opposite result was observed for *Hafnia-Obesumbacterium*. However, at week eight, the abundance of these three genera was similar between the two dietary treatments. None of the diets significantly influenced the growth and health indices except higher protease activity in marron hepatopancreas fed FM diet. The study's findings propose further investigations into the inclusion of exogenous proteases in PBM diets. Additionally, it is recommended to characterize microbial functions to enhance comprehension of how this terrestrial animal protein source could enhance the efficacy of feed in marron aquaculture.

6.1(2) Introduction

The efficacy of aquaculture diets relies on the fishmeal (FM) protein due to its rich nutritional content (Tacon and Metian, 2015, Boyd et al., 2007). However, FM is a relatively expensive and limited resource, which triggers the search for more sustainable and nutritionally beneficial alternatives (FAO, 2018). Among potential protein candidates to replace FM, poultry-by-product meal (PBM) presents a high protein content with a favourable composition of essential amino acids (Hernandez et al., 2010). These characteristics, together with its comparable price to FM and wide availability (Vikman et al., 2017), make PBM an ideal meal for replacing FM in aquaculture feeds. Various aquatic species have been successfully cultured using PBM as a substitute source of protein and the replacement proportion is species-specific. For example, the optimal inclusion level of PBM was 30.75% for the culture of juvenile cobia (*Rachycentron canadum*) (Zhou et al., 2011). PBM-based diet was given to crucian carp (*Carassius auratus gibelioto*), partly and totally replacing FM without significant reductions in fish performance in the study of Yang et al. (2004). The growth rate of marron was not negatively impacted by the complete substitution of PBM for FM in their diet (Foysal et al., 2019b). In contrast, past studies reported that health parameters of marron fed PBM have received better outcomes relative to FM. For example, in the study of Saputra et al. (2019), total haemocyte count and the microvillus length of the intestine tract of marron were increased by PBM diet. Previous studies also found that PBM either being fermented, or combined with black soldier fly meal could significantly improve immune responses and digestive enzymatic activities of cultured marron (Siddik et al., 2020, Foysal et al., 2019b).

In aqua-cultured marron, the microbial communities considerably contribute to growth and health of the animals (Foysal et al., 2019b). Some studies have been done on the effects of PBM on water and gut microbiota while no research has been done to investigate the impacts of dietary PBM on sediment characteristics in marron culture systems. Nguyen et al. (2021) suggested that FM and PBM diets both have significant influences on nitrogen and phosphate accumulation in the water column. The authors also found that similar

predominating bacterial taxa such as *Aeromonas, Hafnia Obesumbacterium, Candidatus Bacilloplasma, and Serratia* were in the water column regardless of dietary protein sources (Nguyen et al., 2021). In the intestine of marron, the abundance of *Aeromonas* was reduced while that of *Lactobacillus* was significantly upregulated when marron fed fermented PBM (Siddik et al., 2020). Many strains of *Aeromonas* are pathogenic bacteria that cause disease in white leg shrimp (*Litopenaeus vannamei*) (Zhou et al., 2019b) and a wide range of fish species (Ringo et al., 2007) meanwhile *Lactobacillus* group acted as probiotics and were responsible for better nutrient absorption and utilization (Siddik et al., 2020). A later study by Foysal et al. (2022a) documented the higher diversity of bacterial communities in both water and gut habitats when the crayfish was fed with PBM, further suggesting the potential positive effects of this protein source for the marron farming industry.

On the one hand, nutrient availability can be a critical factor causing considerable variations in the compositions and functions of bacterial communities (Jia et al., 2020). Total nitrogen and organic matter are the main elements restricting bacterial growth as well as bacterial abundance and activity (Jia et al., 2020). Furthermore, significant correlations between nutrients and microbiota were found in the rearing water (Nguyen et al., 2021). Huang et al. (2018) found that the changes in nitrogenous compounds in water can induce the alterations of microbial communities in sediment. As sediment is an organic matter sink of the water column, it represents not only a strong reservoir of nutrients but also a source of microbial communities between water and sediments (Xiong et al., 2015, Del'Duca et al., 2015). Although most dominant bacterial taxa were shared between the rearing environment and the animal guts, the sediments and animal guts had a similar profile of microbial communities (Wang et al., 2014, Huang et al., 2018). Therefore, it is critical to understand which microbial group not only is present in the gut but also in the surrounding environment, enhancing the health of cultured animals and preventing disease outbreaks.

Overloaded nutrient accumulation in the aquaculture systems can deteriorate the culture environment through the predominance of pathogenic bacterial species (Wang et al., 2021). In a previous study by Foysal et al. (2022a) the nutrient accumulations and microbial

composition in the sediments of marron culture systems were studied. Those studies were conducted on juvenile marrons fed an FM-based diet. Therefore, further investigation is necessary to compare the nutrients and the microbiota in marron tank sediments provided with a PBM-based diet as more sustainable than FM. In this laboratory study, sediment samples from marron tanks fed FM and PBM diets were collected to compare C/N ratios and microbial communities and evaluate the replacement effects on the growth and health of farmed marron.

6.2(2) Materials and Methods

6.2.1(2) Experiment design

The experiment was set up as a part of another trial described in Chapter 6.1. There were two treatments, each with three culture tanks, namely fishmeal-based diet (FM) and poultry-by-product meal-based diet (PBM). The husbandry of marron was described previously in section 6.1.1 of Chapter 6.1.

6.2.2(2) Marron growth and health performances

The measurements of marron proximate composition, growth, and health performances followed the procedures and calculations outlined in sections 3.6 to 3.8 of the general methodology (Chapter 3).

6.2.3(2) Sediment sampling and analyses

6.2.3.1(2) Sediment sampling for analysing carbon to nitrogen ratio

On a weekly basis, sediment samples were collected by siphoning, kept in the freezer as described previously in section 3.2 of Chapter 3. The carbon and nitrogen concentrations in these sediment samples were then analysed according to the procedure described in section 3.3 of Chapter 3.

6.2.3.2(2) Sediment sampling for analysing microbiota

Sediment samples were collected at the start of the experiment on days 1, 3, 5 and at the end of experiment on days 56, 58, 60 to analyse the changes in bacterial communities. The method of sampling tank sediments was mentioned in section 3.2 of Chapter 3.

Three sediment samples of the same treatment collected on the same day were pooled together by mixing thoroughly. By doing this, three sediment samples respective to sampling days of each treatment were created. After that, each sample was centrifuged for 10 minutes at 10,000 × g in a refrigerated centrifuge to discard the supernatant and retain the sediment pellet for DNA extraction.

DNA extraction, PCR amplification, amplicon sequencing, and data processing were conducted followed the protocols as described in sections 3.4.1 and 3.4.2 of Chapter 3.

6.2.4(2) Statistical analysis

The dataset was checked to verify its normality and homogeneity assumptions as detailed in Section 3.9 of Chapter 3. Following this, a two-way ANOVA was utilized to explore the interaction between diet types and sampling weeks. Then, independent sample t-tests were performed to compare all parameters between the two diet treatments, while variations in parameters across the eight sampling weeks were evaluated through one-way ANOVA, followed by post-hoc tests. A significance level of $p<0.05$ was applied to determine statistical significance.

6.3(2) Results

6.3.1(2) Marron growth and health indices

During 60 days of the experiment, no marron deaths were recorded in both treatments. None of the growth parameters, survival and moisture indices of marron were influenced by experimental diets, according to the independent t tests ($p>0.05$) (Table 6.1(2)).

Table 6.1(2) Marron survival, growth and health indices (mean ± S.E., n=3) at the end of the feeding trial

Parameters	**FM**	**PBM**
Survival (%)	100 ± 0.00	100 ± 0.00
Initial weight (g)	70.73 ± 0.83	70.68 ± 0.24
Final weight (g)	76.36 ± 0.7	76.37 ± 0.17
Weight gain (%)	7.99 ± 0.27	8.06 ± 0.16
Specific growth rate (%)	0.13 ± 0.00	0.13 ± 0.00
Protein (%)	82.68 ± 0.73	82.18 ± 0.58
Energy (KJ/g)	69.93 ± 1.14	70.68 ± 0.82
HM (%)	75.63 ± 0.78	74.66 ± 0.67
TM (%)	29.09 ± 1.15	28.49 ± 0.45
Tiw	7.08 ± 0.24	7.23 ± 0.29
Tid	5.74 ± 0.33	5.78 ± 0.28
Hiw	1.72 ± 0.06	1.7 ± 0.09
Hid	69.93 ± 1.14	70.68 ± 0.82
Total haemocyte count ($x10^6$ cells/mL)	2.33 ± 0.03	2.26 ± 0.02
Granular cells (%)	30.17 ± 0.44	31.5 ± 0.87
Semi-granular cells (%)	9.17 ± 2.33	11 ± 2.93
Hyaline cells (%)	60.67 ± 2.46	57.5 ± 2.75

Abbreviations: FM - fish meal-based diet; PBM - poultry-by-product meal-based diet; HM, hepatopancreas moisture content; TM, tail muscle moisture content; Tiw, tail muscle wet weight indice; Tid, tail muscle dry weight indice; Hiw, hepatopancreas wet weight indice; Hid, hepatopancreas dry weight indice.

Protease activity in hepatopancreas was significantly higher in marron fed with the FM-based diet compared to those fed with the PBM-based diet ($p<0.05$, Figure 6.1(2)A). However, no such similar results were recorded for lysozyme activity in marron haemolymph ($p>0.05$, Figure 6.1(2)B).

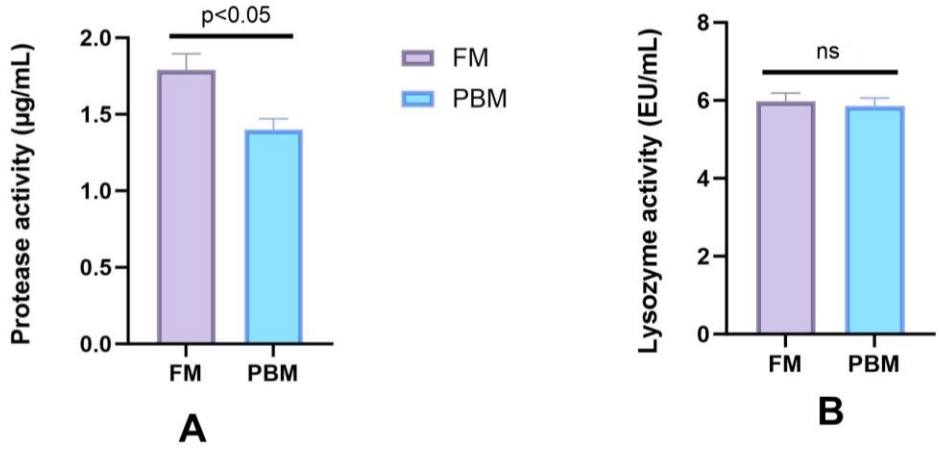


Figure 6.1(2) Protease (A) and lysozyme (B) activities in marron fed different diets. *Abbreviations: FM, fishmeal-based diet; PBM, poultry-by-product meal-based diet; ns, not significant.*

6.3.2(2) Sediment characteristics

6.3.2.1(2) C/N in marron tank sediment

This study found no interaction between diet types and sampling weeks ($F = 0.758$, $p>0.05$). The C/N values in tank sediments, as presented in Table 6.2(2), were found to be independent of dietary treatments. Within the same treatment, sediment C/N decreased gradually over time to reach the lowest points after one-month of the feeding trial in both treatments. Although the ratios increased steadily in the following weeks, they were still significantly lower in the closing week compared to the commencement, regardless of dietary protein treatments.

Table 6.2(2) Changes in carbon/nitrogen ratio (mean ± S.E., n=3) in the tank sediment in different treatments over the trial.

Week	FM	PBM
1	$_{a}$ 8.36 ± 0.02	$_{a}$ 8.60 ± 0.09
2	$_{b}$ 7.32 ± 0.11	$_{b}$ 7.39 ± 0.11
3	$_{bc}$ 7.00 ± 0.04	$_{bc}$ 7.17 ± 0.27
4	$_{c}$ 6.40 ± 0.03	$_{c}$ 6.47 ± 0.03
5	$_{cd}$ 6.50 ± 0.07	$_{cd}$ 6.42 ± 0.21
6	$_{cd}$ 6.73 ± 0.19	$_{bc}$ 6.69 ± 0.15
7	$_{cd}$ 6.69 ± 0.11	$_{bc}$ 6.74 ± 0.25
8	$_{cd}$ 6.61 ± 0.14	$_{bc}$ 7.11 ± 0.20

Different subscript letters of the same column indicate statistically different ($p<0.05$). Abbreviations: FM - fish meal-based diet; PBM - poultry-by-product meal-based diet.

6.3.2.2(2) Sequence statistics and sediment alpha-beta diversity

The obtained OTUs were classified into 10 phyla and 72 genera. Alpha diversity measurements showed significantly higher observed species at the end trial data for both FM and PBM diets, compared to the starting time-point. No changes ($p>0.05$) in Shannon and Simpson diversity matrices were observed between the start and end-point data (Table 6.3(2)).

Table 6.3(2) Alpha diversity (mean ± S.E.) of sediment microbial communities

Parameters (n=3)	**Diet**	**Sampling time**	
		1st week	8th week
Observed	FM	43.3 ± 7.3^{a}	55.7 ± 15.7^{b}
	PBM	47.3 ± 5.3^{a}	65.7 ± 12.3^{b}
Shannon	FM	2.1 ± 0.4	2.4 ± 0.7
	PBM	2.4 ± 0.5	2.7 ± 0.8
Simpson	FM	0.91 ± 0.2	0.94 ± 0.2
	PBM	0.92 ± 0.2	0.94 ± 0.3

Different superscripts of the same row indicate statistically different ($p<0.05$). Abbreviations: FM - fish meal-based diet; PBM - poultry-by-product meal-based diet.

6.3.2.3(2) Sediment microbial composition and correlations with C/N ratios

Aeromonas and *Hafnia* were found in higher abundance compared to other genera in all samples (Figure 6.2(2)).

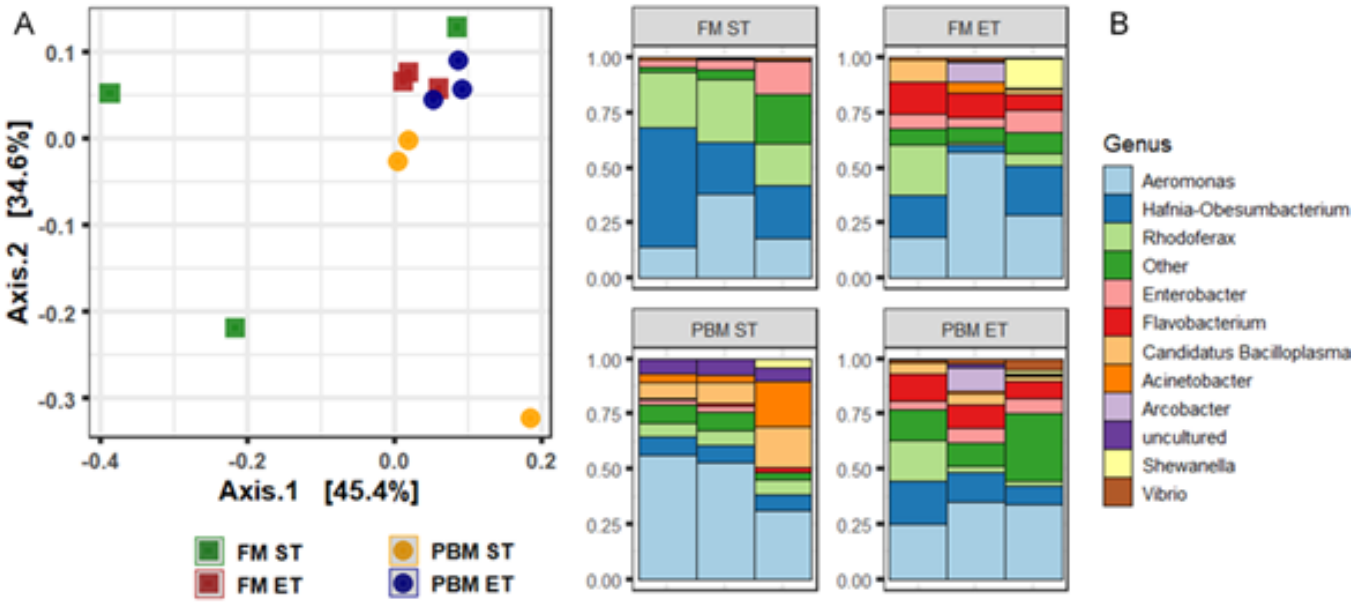


Figure 6.2(2) (A) Beta-ordination plot showing clustering of bacterial OTUs. (B) Microbial composition at genus level in two different diet groups. Abbreviations: FM ST and FM ET sediment microbial communities with fishmeal diet at the start and the end of experiment, respectively; PBM ST and PBM ET sediment in poultry-by-product treatment at the start and the end of experiment, respectively.

At week one of the trial, *Flavobacterium* abundance showed a similarity between the two diet treatments, while the abundance of *Aeromonas* was lower in FM compared to PBM. Conversely, *Hafnia* presented a higher abundance in FM as opposed to PBM. However, at week eight, no significant differences in the abundances of these bacteria genera between FM and PBM were recorded (Table 6.4(2)).

Flavobacterium had a significantly higher abundance at the end for both FM and PBM treatments than at the start of the experiment. PBM reduced *Aeromonas* prevalence while FM significantly decreased *Hafnia* abundance in the tank sediment at the end of the feeding trial (Table 6.4(2)).

Table 6.4(2) Significantly abundant microbial communities in the sediment in the 1st and 8th week of the trial

Genera (n=3)	**Diet**	**Sampling time**	
		1st week	**8th week**
Flavobacterium	FM	$_1$ 0.3 ± 0.1^a	$_1$ 2.8 ± 0.2^b
	PBM	$_1$ 0.2 ± 0.1^a	$_1$ 2.6 ± 0.3^b
Aeromonas	FM	$_1$ 3.4 ± 0.4^a	$_1$ 4.2 ± 0.5^a
	PBM	$_2$ 5.4 ± 0.6^a	$_1$ 4.2 ± 0.8^b
Hafnia-Obesumbacterium	FM	$_1$ 3.2 ± 0.7^a	$_1$ 2.4 ± 0.8^b
	PBM	$_2$1.8 ± 0.4^a	$_1$ 2.1 ± 0.2^a

The different superscripts of the same row and different subscripts of the same column within each genus indicate significant differences ($p<0.05$). Abbreviations: FM - fish meal-based diet; PBM - poultry-by-product meal-based diet.

The correlation data of top abundant genera and environmental variables showed a positive Pearson correlation between *Candidatus Bacilloplasma* and C/N ratio in the sediment at the end of the trial (Figure 6.3(2)).

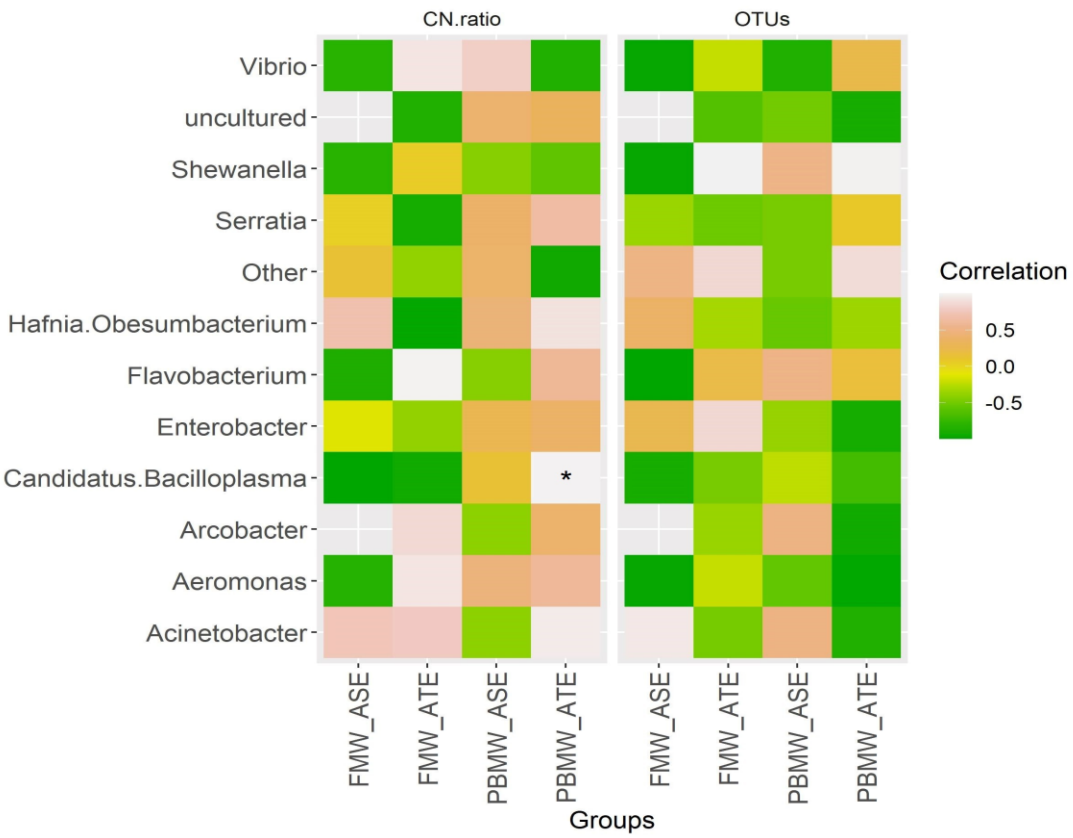


Figure 6.3(2) Pearson correlation between bacterial genera with C/N ratio and OTUs in tank sediment. FMW_ASE and FMW_ATE indicate sediment samples of fish meal diet treatment at the start and end of experiment; PBMW_ASE and PBMW_ATE indicate sediment samples of poultry-by-product meal diet treatment at the start and end of experiment. *Alpha level of 0.05.

6.4(2) Discussion

In light of this study's findings, it was determined that the C/N ratio in sediment was not influenced by the dietary protein sources used. Interestingly, the C/N ratio in sediment decreased while bacterial diversity increased at the closing phase of the experiment compared to the commencement, regardless of the protein source present in the feed. Proteins from by-products of terrestrial animals are high in quantity and quality, especially when locally available, free of anti-nutritional factors, and comparable price compared to fish meal (El-Sayed, 1999). Among these animal protein sources, PBM was reported as a good candidate in diets for several freshwater species (Cruz-Suárez et al., 2007, Saoud et

al., 2008, Yang et al., 2004). Sathishkumar et al. (2021) documented that a diet with a combination of 66.67% high-quality bio-processed PBM and 33.33% FM was superior to a diet with 100% FM as protein sources in terms of growth rate and nutrient utilization of tilapia. However, similar effects of FM and PBM-based diets on marron growth were documented (Saputra et al., 2019, Foysal et al., 2019b, Siddik et al., 2020). Consistent with these findings, our study confirmed the same effects of the two diets on marron survival and growth performance. It has been demonstrated that the nutrient balance in diets but not the protein sources can affect an animal proximate composition (Sathishkumar et al., 2021). Likewise, the comparable nutrient composition of the two diets used in the present study did not alter protein and energy levels in the marron tail muscle. These findings are in line with other studies on marron (Foysal et al., 2019b, Siddik et al., 2020). On the aspects of marron moisture and immunity indices, no effects of dietary protein sources are obtained in this trial, which agree with another study on the same species (Foysal et al., 2019b). In contrast, a positive effect of PBM diet on the exposure susceptibility of marron at high temperature (30°C) was found Saputra et al. (2019). Different outcomes between studies could also be explained by differences in experimental animal size, quality of ingredients, composition and preparation of the diet (Dawson et al., 2018, Cheng et al., 2002).

A good quality dietary protein source is vital as it can improve animal growth and health (Cruz-Suárez et al., 2007, Bureau et al., 1999). Lack of essential amino acid, for example methionine in PBM diet (Li and Wu, 2020) can be responsible for lower protease activity in our research, hence dietary supplementation of methionine to PBM diet is recommended (Li et al., 2021c, Ren et al., 2018). Dietary protein quality can influence protease activity both in digestive tract (Lee et al., 1984) and hepatopancreas (Ezquerra et al., 1997) of white leg shrimp. Therefore, feed quality evaluation is necessary to better understand the correlation of feed quality with digestive enzyme activities in marron.

Despite the efficiency of PBM on animal health and production, no research reported its influence on sediment characteristics of the culture system in terms of nutrient accumulation and microbial community. Therefore, the information on these aspects

obtained from the present study can add novel findings that may have an integral contribution to our current knowledge. This study found that dietary protein had no significant influence ($p>0.05$) on C/N ratio in tank sediment. A previous study in tilapia found that compared to FM, PBM-based diet did not affect the feed conversion and feed efficiency ratios (Sathishkumar et al., 2021). The non-significant differences in nutrient retention in animals (Sathishkumar et al., 2021) coupled with the water quality treated with different dietary protein sources (Nguyen et al., 2021) could explain the similarity in C/N ratios in the tank sediments in the present study. Sathishkumar et al. (2021) also found that in aquatic animals, protein content has a positive correlation with nutrient utilization while protein sources are not as important as protein contents. Thus, when choosing a protein source, its amino acid profile should be one of the vital criteria.

Sediment C/N ratio experienced a reduced trend during the experimental period, indicating that feeding practice significantly affects C/N ratio in sediments. Carbon and nitrogen loads in indoor aquaculture systems may vary depending on the culture management practices, but carbon accumulation in the sediments is often lower than nitrogen. Less than 20% of total carbon input stayed in the tank sediments when the experiment ended (Tinh et al., 2021), and CO_2 emission significantly contributed to the carbon loss (Tinh et al., 2021, Hu et al., 2014). In contrast, at the termination of the culture period, up to 71% of total nitrogen output was within the sediments (Hari et al., 2006, Khoi and Fotedar, 2010). Sediment is the ultimate sink of particulate organic and suspended matters from the water column due to gravities and sedimentation processes (Avnimelech et al., 1999). Therefore, high inorganic nitrogen compounds accumulated in the water column of marron tanks at the end of the experimental period (Nguyen et al., 2021) might also be the reason for the increased total nitrogen levels in the tank bottom, which reduced the sediment C/N ratio.

In the present study, feeding marron with PBM-based diet generated some rare bacteria species without affecting Shannon diversity in tank sediments, a trend that was also observed in marron culture water (Nguyen et al., 2021). This result indicates that feeding different diets does not affect the relative abundance of top-abundant taxa in the sediment.

Higher abundance for *Aeromonas* in the sediment for all groups proved the dominancy of this genus in the aquaculture rearing environment alike other previous studies (Zhao et al., 2018, Foysal et al., 2022a, Huang et al., 2022a). This bacteria group is a potential pathogen for many aquatic species (Bruhn et al., 2005, Dong et al., 2017, Pang et al., 2015) but possibly its number is below the threshold point for disease establishment in the present study. Previous reports (Zhang et al., 2019, Chen et al., 2014) also indicated *Aeromonas* could be involved in nutrient cycling processes. We predicted that the nutrients and wastes generated from feeding FM and PBM-based diets lead to higher bacterial diversity in the sediment at the end of the trial. *Flavobacterium*, an opportunistic pathogen for aquaculture animals, also utilized the nutrient sediment contents and enhanced its abundance in the final stage of the trial (Itoi et al., 2007, Itoi et al., 2006). Surprisingly, *Hafnia* which was reported as probiotic agents in several studies (Didinen et al., 2016, Legrand et al., 2020) showed growth at the end of the trial with PBM. This indicates that dietary PBM was beneficial to the microbial ecology which in turn could possibly have a positive long-term effect on marron health. *C. bacilloplasma* is one of the core gut microbiota of marron and other crustaceans, positively correlated with health and immune index of host species (Boopathi et al., 2023, Foysal et al., 2022a). Therefore, the release of faecal materials from animals may link to the presence of *C. bacilloplasma* in the sediment. In this study, only *C. bacilloplasma* abundance was positively correlated to sediment C/N ratio for both diets as time progressed. So, augmenting this bacterial abundance is crucial in the retention of C/N ratio in the sediment over the time.

6.5(2) Conclusion

In conclusion, FM and PBM-based diets had similar effects on marron growth rate and health indices in the present study. However, PBM-based diet had a substantial effect causing a reduced protease activity in the marron hepatopancreas as well as shifting microbial composition in the tank sediments. Further research is needed to elucidate if the impacts of PBM on protease activity and sediment microbial communities have a biologically significant impact on marron's health and growth performance.

CHAPTER 7 (Experiment 4) Carbon supplementation in rearing water: Effect on sediment characteristics, growth, and health of cultured marron (*Cherax cainii*) under laboratory conditions

(Published in Scientific Reports: https://doi.org/10.1038/s41598-024-51585-8)

Abstract

Carbon sources are considered as critical input for the health and immunity of aquatic animals. The present study investigated the impact of different carbon sources on water quality parameters, carbon to nitrogen (C/N) ratio and microbial community in sediments, and health responses of marron (*Cherax cainii*) under laboratory conditions. Following one week of acclimation, 120 marron were randomly assigned to 12 experimental tanks. There were four treatments including one untreated control and three groups with carbon addition to maintain a C/N ratio of 12 maintained in culture water. Carbon supplementation groups included corn flour (CBC12), molasses (MBC12) and wheat flour (WBC12). At the end of the 60-day trial, MBC12 resulted in the highest sediment C/N ratio, followed by CBC12. Weight gain and specific growth rate were higher in MBC12, compared to control. The protease activity in marron hepatopancreas, total haemocyte count and lysozyme activity in haemolymph were highest in MBC12. Analysis of 16S rRNA sequence data of tank sediments revealed increased bacterial alpha diversity in MBC12 and WBC12. Proteobacteria was the most abundant phylum in MBC12 (88.6%), followed by control (82.4%) and CBC12 (72.8%). *Sphingobium* and *Novosphingobium* were the most abundant genera in control and MBC12 groups, respectively. Higher *Aeromonas* abundance in CBC12 and *Flavobacterium* in WBC12 were observed. Overall results indicated that MBC12 led to improved water quality, retaining high C/N ratio and enriched the bacterial populations in sediments resulting in improved growth and immune performance of marron.

7.1 Introduction

Marron (*Cherax cainii*) is an important farmed freshwater crayfish in Western Australia (Holdich, 1993). It has a significant potential to expand and improve its farming productivity (Fotedar, 1998) due to its large size, mobility and omnivore (Reynolds et al., 2013). Numerous studies on marron (Foysal et al., 2022b, Tulsankar et al., 2022b) have been conducted and further research is in progress (Achmad et al., 2023, Cole et al., 2023) with the aim to enhance marron productivity through feeding of formulated diets and improving the management practices. Like other decapods, the natural habitat of marron is the sediment-water interface which is the boundary between the bottom and overlying water column. The detritus/sediment is recognised as a vital *in-situ* food source for marron nutrition (Tulsankar et al., 2022a). However, research efforts to decode the sediment characteristics in marron aquaculture is still in infancy stage, and its effects on marron's growth and health parameters are obscure.

In aquaculture systems, a large amount of undigested feed limits the conversion of carbon (C) and nitrogen (N) into final biomass (David et al., 2021). For instance, a maximum of 16% (C) and 37% (N) were found to be converted into the flesh of freshwater prawn (*Macrobrachium rosenbergii*) (Sahu et al., 2013), tiger shrimp (*Penaeus monodon*) (Thakur and Lin, 2003, Jackson et al., 2003) and white leg shrimp (*Litopenaeus vannamei*) (David et al., 2021) farmed in earthen ponds. The retained percentage could be even lower to 10% (C) and 6% (N) due to low shrimp survival in integrated rice-shrimp culture systems (Dien et al., 2018). A relatively high percentage of unused N settles as sediments in ponds (Jackson et al., 2003) or in tanks (Tinh et al., 2021). From the total inputs, up to 53% of the N can be deposited as tank sediments (Thakur and Lin, 2003, Tinh et al., 2021). Hence, it is absolutely imperative to optimise feed utilisation in order to reduce the amount of undigested feed and the deposition of N wastes in sediments.

Carbon to nitrogen (C/N) ratio is an integral element that plays a vital role in converting N wastes into bacterial biomass (Avnimelech, 1999, Bossier and Ekasari, 2017, Crab et al., 2012) and thereby promoting circular growth of blue economy. Analysis of the C/N ratio

in sediment is vital for gaining insight into the source of essential elements such as C and N in the aquaculture pond environment (Avnimelech, 1999, Wudtisin and Boyd, 2006). Therefore, managing the C/N ratio and understanding their interaction with microbial communities in sediment is necessary for water quality control and animal performance in aquaculture ecosystems. By adding external carbon sources into aquaculture rearing systems, the C/N ratio can be manipulated for enhanced nitrogen uptake by heterotrophic bacteria leading to decreased ammonium concentration and increased microbial biomass (Abakari et al., 2021b). Carbohydrates such as corn and wheat flour are not only essential ingredients in the diets of decapod species (Fotedar, 1998, Sang and Fotedar, 2010, Saputra et al., 2019) but are in vogue as exogenous carbon sources in aquaculture rearing systems. Supplementation of corn starch and wheat flour are reported to enhance water quality, heterotrophic bacterial biomass, growth and survival of various cultured shrimp species such as pink shrimp (*Farfantepenaeus brasiliensis*) (Emerenciano et al., 2012), freshwater prawn (Hosain et al., 2021a) and white leg shrimp (Tinh et al., 2021). Unlike corn and wheat, molasses are not the prevalent ingredient in crustacean diets including marron, but has been widely used in aquaculture of tiger shrimp (Kumar et al., 2014) and white leg shrimp (Samocha et al., 2007) as an external carbon source. Compared to other exogenous carbon sources, molasses have been validated as superior carbon source in removing ammonia and improving the growth of white leg shrimp (Serra et al., 2015, Khanjani et al., 2017). Therefore, molasses has the indisputable potential for their use as additional carbon sources in marron aquaculture.

Previous studies on red claw (*Cherax quadricarinatus*) (Azhar et al., 2020) and red swamp (*Procambarus clarkii*) (Li et al., 2021a, Li et al., 2019a, Li et al., 2023) reported beneficial effects of carbohydrate supplementation. For example, molasses supplementation improved water quality and feed utilization efficiency of red claw (Azhar et al., 2020). Adding external carbon sources (glucose and wheat bran) enhanced the growth performance and proximate composition of red swamp (Li et al., 2019a). Additionally, another study found that narrow-clawed crayfish (*Astacus leptodactylus*), as indicated by

their normal haemolymph indices, can adapt well to high stocking densities in the carbon supplementation system (Doğukan et al., 2021). However, similar studies are lacking for marron, necessitating further investigation to elucidate the potential advantages of carbon supplementation on the growth and overall health of marron. It is hypothesized that carbohydrate supplementation might play a vital role in stimulating the assimilation of nitrogenous wastes either suspended in the water column or sediments, thereby improving water quality as well as augmenting physiological and immunological performances of marron. The present experiment aims to assess the effect of three carbohydrate sources, corn flour, wheat flour, and molasses, on the water quality characteristics, C/N ratio and microbial communities of the tank sediments in relation to the growth and health responses of marron under laboratory conditions.

7.2 Materials and Methods

7.2.1 Preparation of microbial inoculum water for carbon-added treatments

Microbial inoculum water was prepared following the procedure provided by Ahmad et al. (2019) with some modifications. Three indoor plastic tanks were filled with 150 L freshwater. Each tank contained 3000 g of bottom soil from the commercial Blue Ridge Marron farm, Manjimup, Western Australia (34°12′22″ S, 116°01′01″ E), 1.5 g ammonium sulphate $(NH)_4SO_4$, and 60 g selected carbon sources being either corn flour, molasses or wheat flour. These tanks were kept in a laboratory with controlled temperature (22°C), wherein a photoperiod of 12-hour light and 12-hour dark was provided with optimum aeration for 24 h to stimulate the growth of heterotrophic microbial biomass.

7.2.2 Experimental design

A total of 120 marron with an initial body weight of 11.67 ± 0.11 g (mean ± S.E.) was purchased from Blue Ridge Marron farm. The marron were acclimated in experimental tanks for one week before being weighed at the start of the experiment, which lasted for 60 days. There were one clear water treatment (control) and the supplementation of corn flour, molasses and wheat flour as treatments of carbohydrate sources to maintain a C/N of 12 in

the culture water, namely CBC12, MBC12, and WBC12, respectively. A complete randomized design with three replications per treatment was employed. Experimental tanks with 300 L water capacity were filled with 250 L freshwater for the control tanks and 200 L for carbon-treated tanks, and stocked with ten marron per tank housed individually in black plastic mesh cages. Throughout the experiment, a 12-h cycle of light and dark was maintained. Fishmeal based diet was formulated for marron containing 29.93% crude protein following the standard protocol as described in our previous study (Foysal et al., 2019b). Marron were fed once daily in the evening, and the feeding rate was set at 3% of the total stocked biomass during the acclimation and also during the experimental period. Uneaten feed and faeces were removed out from the tanks every morning to prevent the deterioration of water quality.

The prepared inoculum was used to inoculate the experimental tanks once at the beginning of the experiment, 50 L of microbial inoculum water per tank, to provide a known community profile of the microbial source to imitate the microbial community in pond culture. Then to maintain the C/N ratio of 12 in the rearing water, method of Perez-Fuentes et al. (2016) was followed by daily adding the carbon source into the experimental tanks after feeding marron with a formulated feed. The amount of carbohydrate used per treatment was determined by the content of protein (%) in the formulated feed and the amount of feed supplied during the experiment, not considering the amount of carbon contained in the feed. Assuming that protein is 16% nitrogen and that marron excrete 65% of protein as nitrogen. In 1000 g of marron feed (29.93% crude protein), there is 47.89 g nitrogen. Of the nitrogen used, 31.13 g nitrogen is excreted into water by marron. Corn flour, molasses, and wheat flour contained 39.55%, 40.38%, and 39.53% carbon, respectively. Therefore, to maintain a C/N ratio of 12 in the rearing water, 944.37 g corn flour, 924.96 g molasses, and 944.85 g wheat flour are required for 1000 g of feed supplied. Based on this calculation, the amount of corn flour, molasses, and wheat flour required per treatment per day was 9.78 g, 9.74 g, and 9.77 g, respectively. During the experiment, no water exchange was done in all treatments. However, every week, 30% of the water from

each culture tank containing sediments was transferred to another empty tank, and the water was then carefully returned to the original tank after the sediments were collected.

7.2.3 Data collection

7.2.3.1 Water quality

The experiment was carried out in a wet laboratory room at a constant temperature of 22°C. Air diffusers and air pumps were used to continuously aerate the tanks. Daily measurements were made of water temperature, pH and dissolved oxygen (DO). Other water parameters including ammonia, nitrate, nitrite, and phosphate were measured weekly. These measurements were followed the procedures outlined in section 3.1.1 of Chapter 3.

7.2.3.2 Sampling of sediments

Sediment sampling for analysing C/N ratio

On weeks 2, 4, 6 and 8 of the experiment, sediment samples were collected for analysing the C/N ratio. Tank sediments were collected as mentioned above and described in section 3.2 of Chapter 3. Assessment of carbon/nitrogen ratio in tank sediments was followed the procedure as stated in section 3.3 of Chapter 3.

Sediment sampling for analysing microbiota

On the final week of the experiment (days 52, 54, and 56), sediment samples were collected as described previously for analysing the microbial community. A pool was produced by thoroughly mixing the sediment samples from three replicated tanks within each treatment on a respective day. After pooling, three sediment samples of each treatment were created. These samples were then centrifuged and the obtained sedimentary pellets were used for microbiome analysis.

Microbial composition in tank sediments was assessed according to the protocols as described in section 3.4 of Chapter 3.

7.2.3.3 Calculations of marron growth and health

Sections 3.6 to 3.8 of the general methodology (Chapter 3) contained the processes and calculations used for measuring marron growth and health performances.

7.3 Results

7.3.1 Water quality

Water temperature did not differ significantly between treatments during the trial. Overall, pH, dissolved oxygen, and nitrogenous compound levels were significantly higher in control than in carbon-added treatments (Table 7.1). No significant differences between the three carbon-treated groups were observed in all tested water quality parameters, except for a lower pH level in WBC12 than in the other two carbon-added treatments. Phosphate level in MBC12 was significantly lower than in the control and WBC12; however, it did not differ from CBC12.

Table 7.1 Water quality (mean ± S.E.) in different treatments over a 60-day trial.

Parameters (n=3)	Control	CBC12	MBC12	WBC12
Temp. (°C)	21.61 ± 0.01 ^{a}	21.55 ± 0.02 ^{a}	21.59 ± 0.05 ^{a}	21.41 ± 0.1 ^{a}
pH	7.74 ± 0.01 ^{a}	7.46 ± 0.00 ^{b}	7.44 ± 0.02 ^{b}	7.37 ± 0.00 ^{c}
DO (mg/L)	8.24 ± 0.01 ^{a}	7.57 ± 0.02 ^{b}	7.57 ± 0.01 ^{b}	7.58 ± 0.04 ^{b}
Nitrate (mg/L)	0.93 ± 0.01 ^{a}	0.76 ± 0.02 ^{b}	0.74 ± 0.03 ^{b}	0.77 ± 0.01 ^{b}
Nitrite (mg/L)	0.10 ± 0.00 ^{a}	0.08 ± 0.00 ^{b}	0.07 ± 0.00 ^{b}	0.07 ± 0.00 ^{b}
Ammonia (mg/L)	0.05 ± 0.00 ^{a}	0.04 ± 0.00 ^{b}	0.04 ± 0.00 ^{b}	0.04 ± 0.00 ^{b}
Phosphate (mg/L)	0.32 ± 0.00 ^{a}	0.3 ± 0.01 ^{ab}	0.28 ± 0.00 ^{b}	0.31 ± 0.01 ^{a}

Within the same row, data having different superscript alphabet (a, b, c) are significantly different ($p<0.05$). Abbreviations: Temp. – temperature; DO – dissolved oxygen.

7.3.2 Sediment C/N ratio

Adding different carbon sources in the culture system significantly affected C/N ratio in the tank sediments from week four onwards (Figure 7.1). The sediment C/N ratio was significantly higher in MBC12 relative to the control at weeks four, six, and eight of the trial. In the fourth and sixth weeks, there were significant increases in sediment C/N ratio

of MBC12. In the eighth week, MBC12 still had the highest sediment C/N ratio (8.12 ± 0.10), followed by CBC12 (8.08 ± 0.08) and WBC12 (7.78 ± 0.08), and the lowest C/N ratio was observed in control (6.36 ± 0.02).

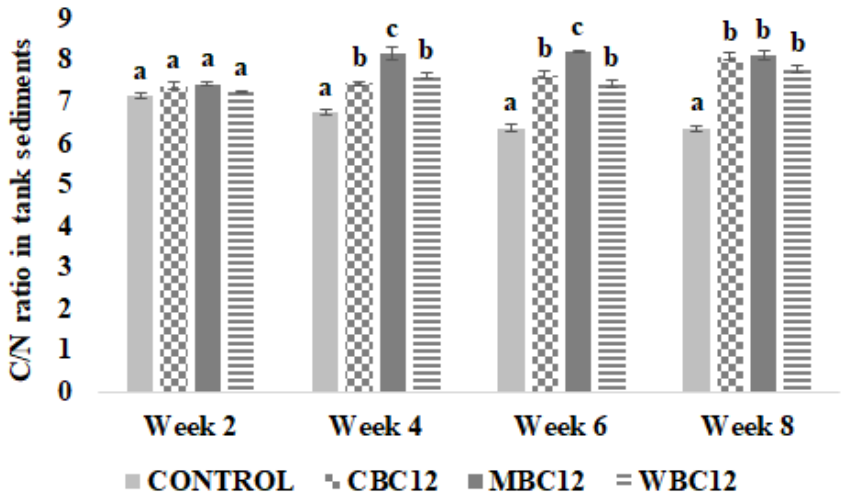


Figure 7.1 Changes in carbon/nitrogen in the tank's sediment in different treatments at four sampling points. The alphabet letters (a, b, c) on the top of each bars indicate statistical difference between treatments ($p<0.05$).

7.3.3 Bacterial load and composition in tank's sediments

7.3.3.1 Sequence statistics and alpha-beta diversities

A total of 639,788 quality reads were obtained from 12 samples after trimming, ranging from 47,490 to 127,236 reads. Reads were assigned to 1300 OTUs, 8 phyla, 56 families, and 129 genera. The rarefaction depth curve (Figure 7.2A) and Good's coverage indices (0.992-0.997) indicated that each sample was sequenced at enough depth to capture maximum diversity. The alpha diversity measurements showed that MBC12 and WBC12 had a positive influence on bacterial diversity, compared to the control (Figure 7.2B), however WBC12 generated the highest unique OTUs in the sediments (Figure 7.2C). The clustering of bacterial OTUs for four different groups was found distinct in the Beta-ordination principal coordinate analysis (PCoA) where PERMANOVA R and P-value of

weighted uniFrac metric revealed significantly different of bacterial composition in different groups (Figure 7.2D).

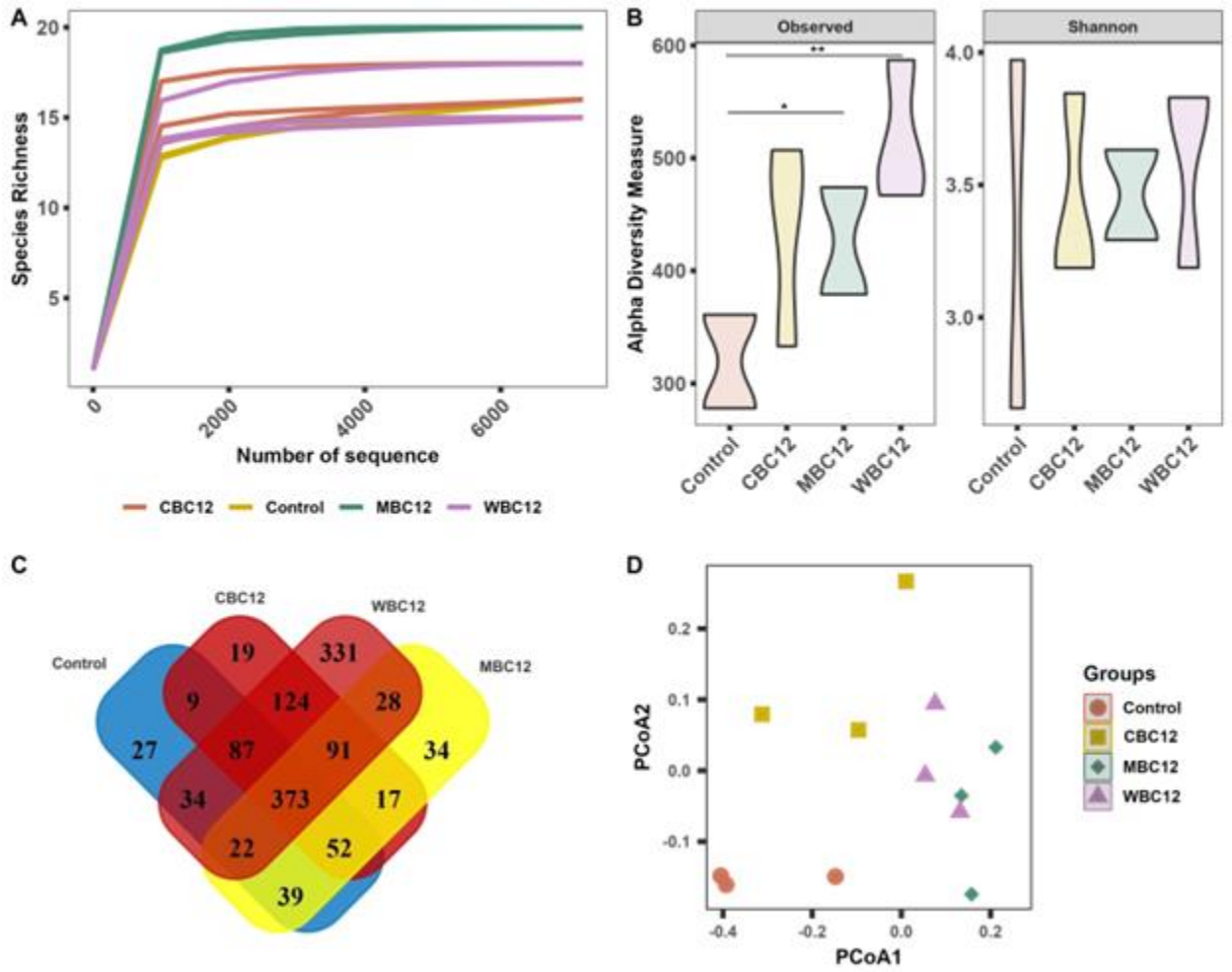


Figure 7.2 Alpha-beta diversity measurements of bacterial diversity in sediment samples collected from tanks treated with three different carbon sources relative to one control group. (A) Rarefaction curve showing the depth and saturation level of 16S rRNA sequence.

7.3.3.2 Microbial composition

At the phylum level, Proteobacteria was the most abundant bacteria in the control (82.4%), CBC12 (72.8%), and MBC12 (88.6%) while Bacteroidetes (56.2%) dominated microbial communities in WBC12 sediments. At the genus level, *Sphingobium* and

Novosphingobium comprised 62% and 48% of the read abundance in the control and MBC12, respectively, whereas, in CBC12 and WBC12, *Aeromonas* and *Flavobacterium* accounted for 82.2% of the read abundance (Figure 7.3A-B).

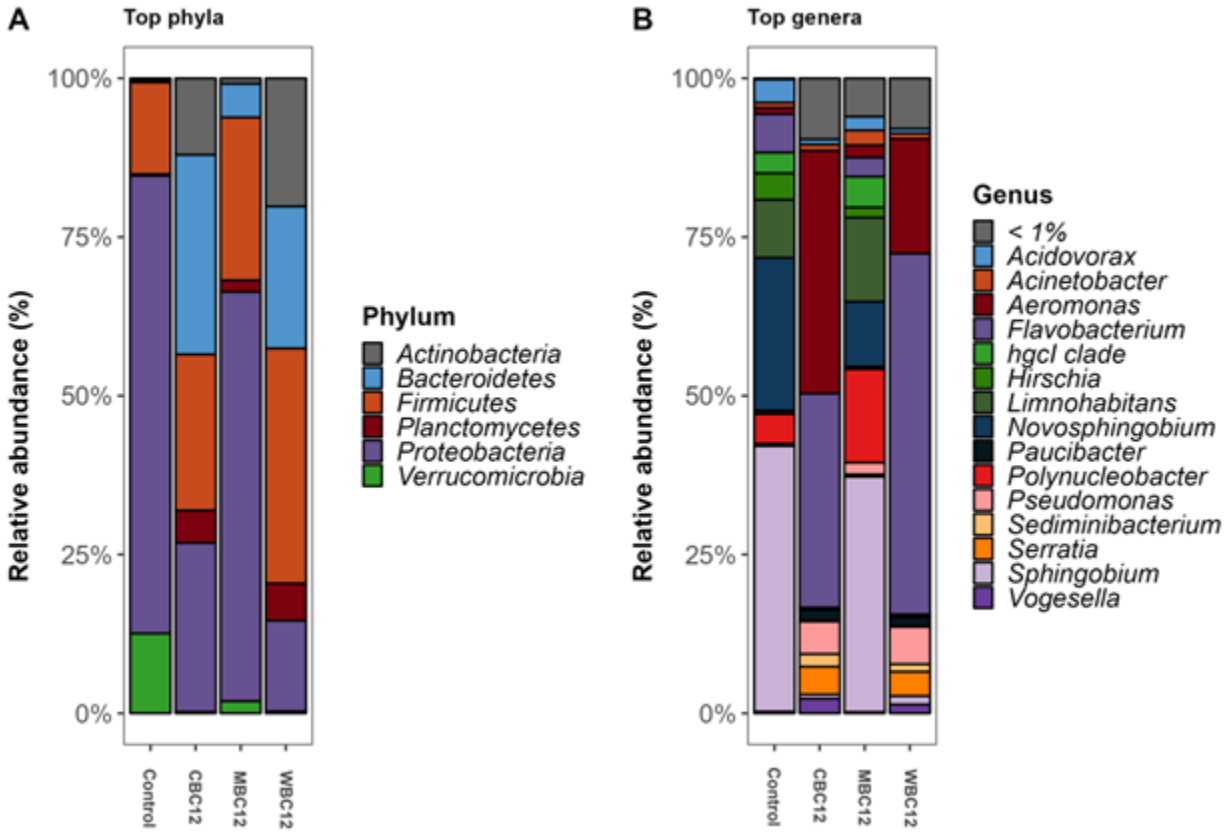


Figure 7.3 Relative abundance of bacteria at (A) phylum and (B) genus level. Only OTUs representing at least 1% of the total reads are shown.

Alongside these four bacteria groups, *Acinetobacter* in the control and MBC12, *Dechloromonas* in CBC12 and WBC12, *Hirschia* in control, and *Serratia* in CBC12 and WBC12 had more than 1% read abundance in the sediment bacterial communities. Relative to the control group, eight bacteria groups at the genus level had significantly different reared read abundance in three carbon-treated groups. The abundance of *Massilia* and *Paucibacter* was high in CBC12 while MBC12 favoured the growth of *hgcl clade*, *Paraperlucidibaca,* and *Sphingobium* whereas WBC12 promoted the abundance of *Aeromonas*, *Deefgea* and *Nocardioides* (Figure 7.4A). PICRUSt2 data showed that CBC12

and MBC12 enriched pathways for carbohydrate, protein and amino acid metabolism. In addition, MBC12 enhanced activities for the biosynthesis and metabolism of amino acids. WBC12 only enriched beta-alanine metabolism compared to others. Control samples on the other hand mostly involved in amino acid metabolism (Figure 7.4B).

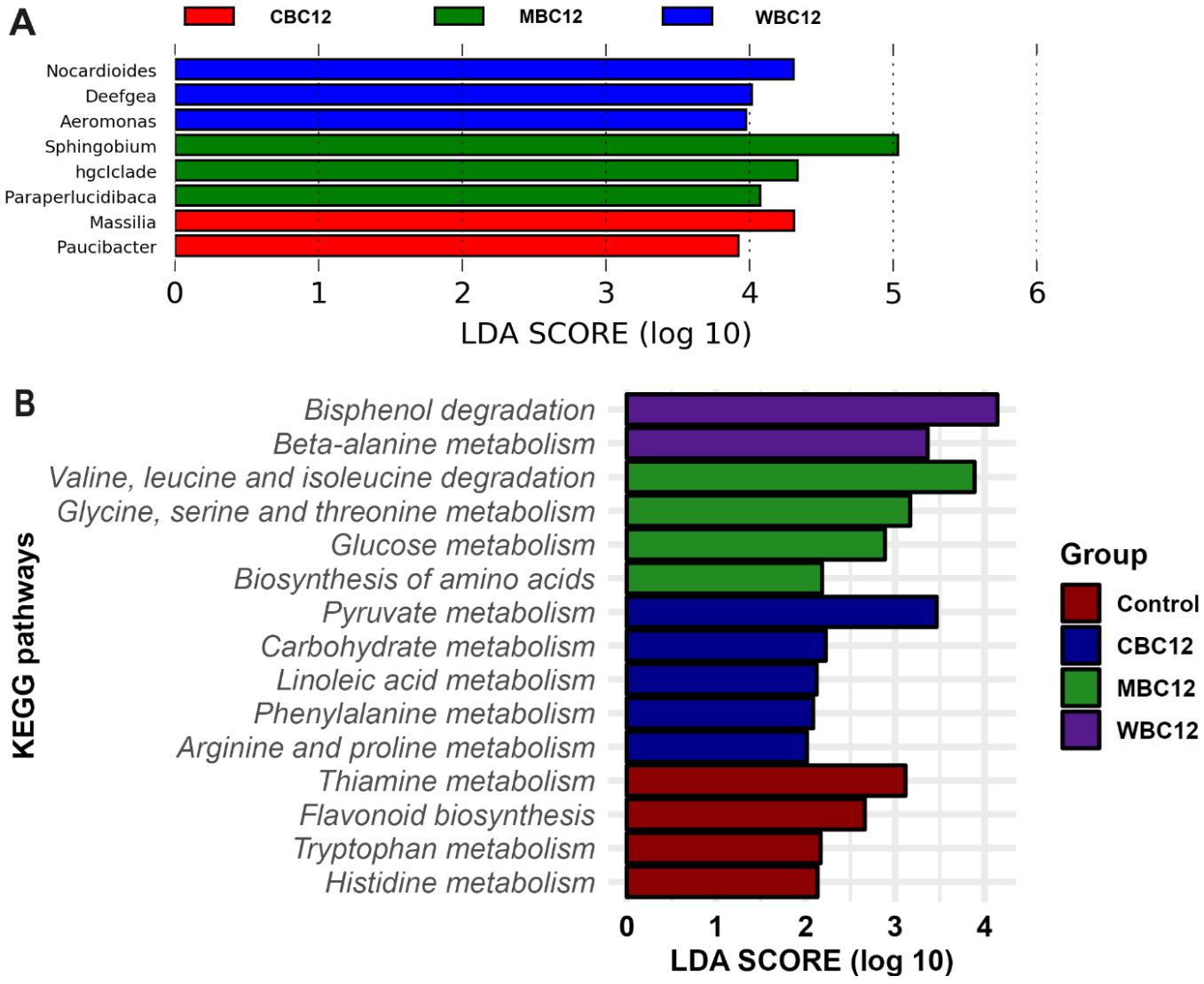


Figure 7.4 Significantly abundant genera (A) and metabolic pathways (B) in three different treatment groups with Linear Discriminant Analysis (LDA). No genus had differential abundance in the control group with LDA value 2.0 and 0.05 level of significance.

7.3.3.3 Sediment C/N ratio-taxa correlations

Among the correlations between sediment C/N ratio and the relative abundance of most abundant genera, sediment C/N ratio was found to have significant correlations with *Acinetobacter* and *Acidovorax*. C/N ratio was positively and negatively correlated to *Acinetobacter* and *Acidovorax*, respectively (Figure 7.5).

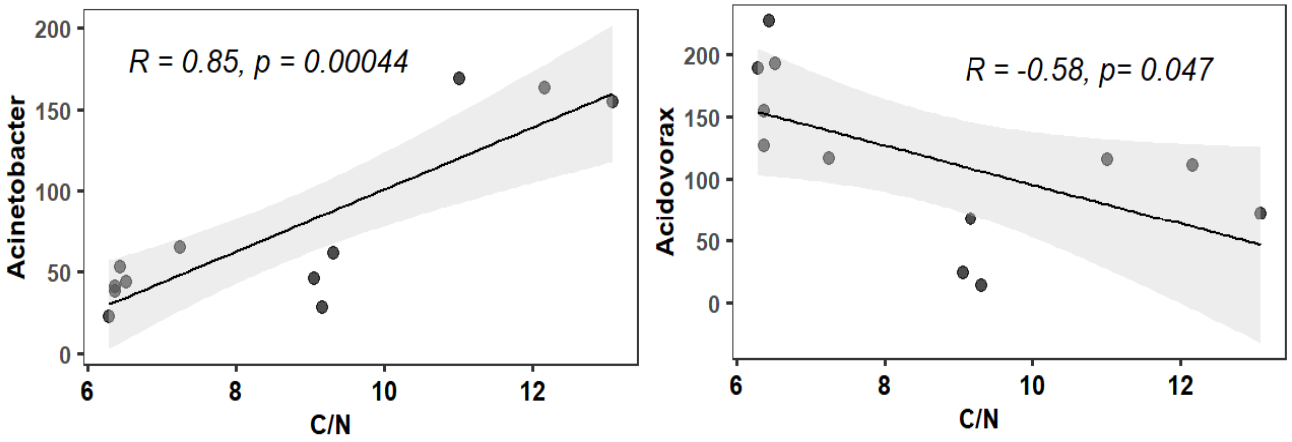


Figure 7.5 Pearson correlations between the most abundant bacterial taxa and sediment C/N ratio. Significant level at $p<0.05$.

7.3.4 Marron growth performance

The supplementation of different carbon sources into the culture system led to significant changes in WG and SGR of marron; however, it did not significantly affect the survival rate at the end of the trial (Table 7.2).

Table 7.2 Marron growth (mean ± S.E.) performance parameters.

Parameters	Control	CBC12	MBC12	WBC12
Initial weight (g)	11.98 ± 0.09 [a]	11.5 ± 0.1 [a]	11.7 ± 0.2 [a]	11.49 ± 0.05 [a]
Final weight (g)	14.47 ± 0.06 [a]	14.49 ± 0.06 [a]	16.15 ± 0.17 [b]	15.55 ± 0.5 [ab]
Survival (%)	90 ± 0.00 [a]	96.67 ± 3.33 [a]	93.33 ± 3.33 [a]	90 ± 5.77 [a]
Weight gain (%)	21.27 ± 1.16 [a]	26.38 ± 1.15 [ab]	32.23 ± 2.71 [b]	28.05 ± 0.58 [ab]
SGR (%/day)	0.34 ± 0.02 [a]	0.42 ± 0.02 [a]	0.5 ± 0.04 [b]	0.44 ± 0.01 [ab]
MWI (%)	22 ± 1.07 [a]	26.53 ± 0.26 [bc]	29.36 ± 0.85 [c]	23.77 ± 0.5 [ab]
MI (days)	32.44 ± 1.28 [a]	31.42 ± 1.23 [a]	32.67 ± 1.5 [a]	31.02 ± 1.65 [a]
Protease (μg/mL)	1.05 ± 0.08 [a]	0.99 ± 0.05 [a]	1.65 ± 0.05 [b]	1.34 ± 0.04 [c]

Rows having different superscript are statistically different ($p<0.05$). Abbreviations: SGR – specific growth rate, MWI – moult weight increment, MI - Moult interval.

The highest and lowest growth performances, as measured by WG and SGR were observed in MBC12 and control group, respectively. Similarly, MWI was significantly higher in MBC12 than in the control. No significant differences were observed in moult intervals between treatments. The highest protease activity in the hepatopancreas of marron was also observed in the MBC12 (1.65 ± 0.05 μg/mL), followed by WBC12 (1.34 ± 0.04 μg/mL). In contrast, the lowest protease activity among the treatments was observed in CBC12 (0.99 ± 0.05 μg/mL), which was statistically similar to the control (1.05 ± 0.08 μg/mL).

7.3.5 Marron organosomatic indices

Organosomatic indices of marron are presented in Figure 7.6. No significant changes in TM (%), Tid, and Hid among the different carbon-treated groups were observed in marron by the end of the experiment. HM (%) in the control was significantly lower than those in all carbon treatments. In MBC12, Tiw was significantly higher than in WBC12; however, there was no significant difference in this parameter between MBC12 and the other two treatments. Hiw was significantly higher in CBC12 than in the control but did not differ statistically from other carbon-treated groups.

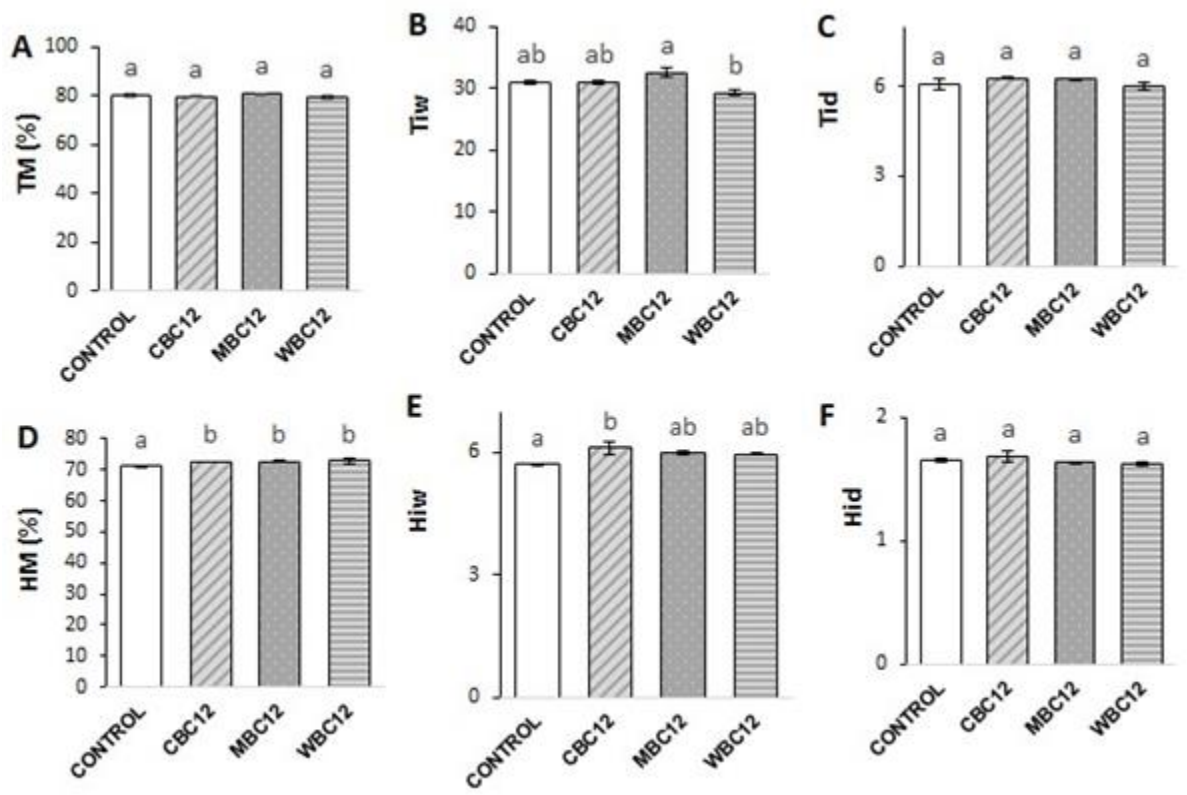


Figure 7.6 Moisture measures (mean ± S.E.) of marron treated with different carbon sources. A. Tail muscle moisture content (TM%), B. Dry tail muscle index (Tid), C. Wet tail muscle index (Tiw), D. Hepatopancreas moisture content (HM%), E. Wet hepatosomatic index (Hiw), F. Dry hepatosomatic index (Hid). Different superscript letters (a, b) on the top of the bar represents significant difference at $p<0.05$.

7.3.6 Marron immunological parameters

Figure 7.7 shows that by the end of the experiment, THC in the marron's haemolymph was significantly higher in MBC12 relative to the control; however, it did not differ from other carbon-treated groups. There was no significant difference in the proportions of granular, semi-granular, and hyaline cells between all groups. However, the highest lysozyme activity was obtained with MBC12, and the differences were significant compared to all other treatments, including the control.

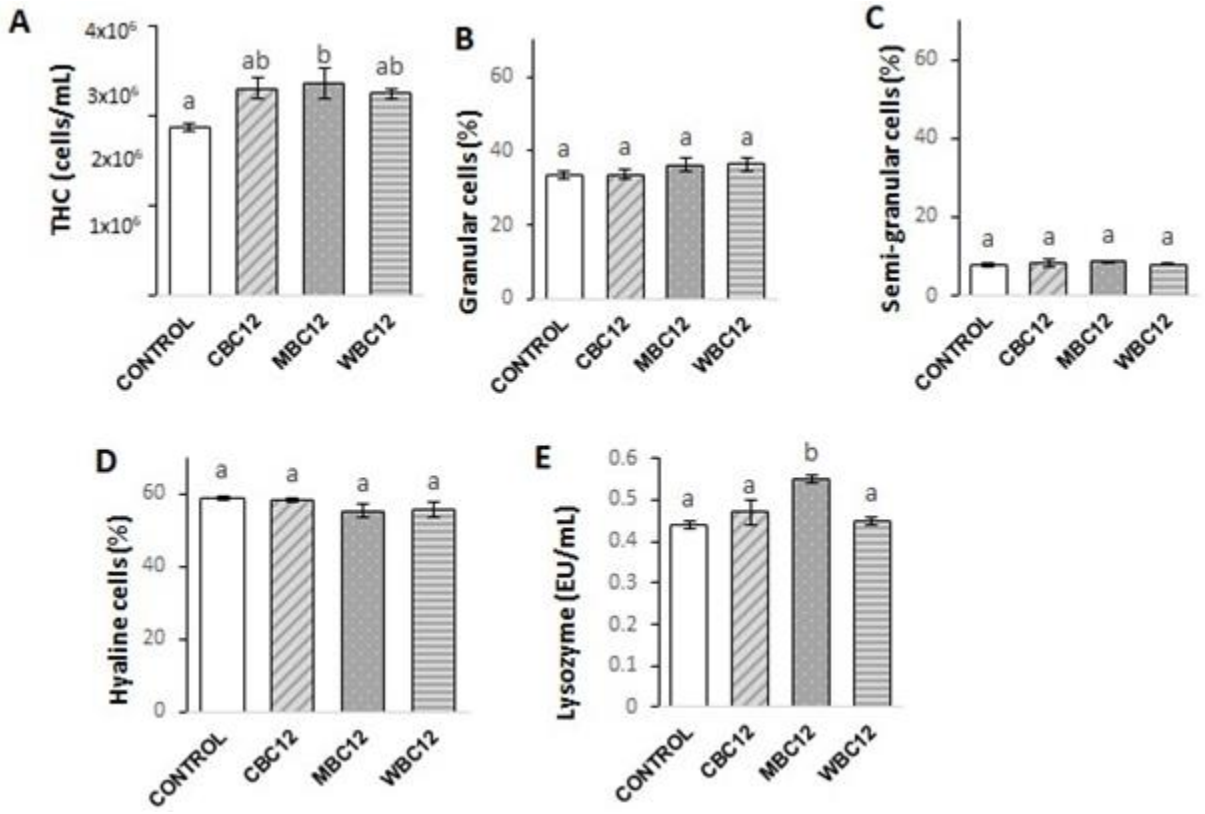


Figure 7.7 Immune responses of marron in different groups treated with different carbohydrate sources. Different superscript letters (a, b, c) on the top of the bar represents significant difference at $p<0.05$. THC – Total haemocyte count.

7.4 Discussion

Addition of an external carbon source is absolutely necessary to retain an optimal C/N ratio for promoting the growth of beneficial heterotrophic microorganisms in aquaculture water (Avnimelech, 1999, Abakari et al., 2021b). Moreover, using a suitable external carbon source is vital to enhance the production of the targeted species cultured in this system (El-Sayed, 2021). The present study demonstrated that adding external carbon sources to marron culture led to beneficial effects on water quality, sediment bacteria, growth performance and the health status of marron. Previous reports (Crab et al., 2012, Ebeling et al., 2006) demonstrated that water quality could be improved since heterotrophic bacteria can metabolize wastes in carbon-added aquaculture systems. Consistent with studies on other decapod species (Rajkumar et al., 2016, Tinh et al., 2021), the present research

showed significantly reduction in concentration of nitrogen and phosphorus metabolites following carbon addition in the culture tanks. All carbohydrate sources can affect the immobilization of nitrogenous compounds (Lima et al., 2018, Mansour and Esteban, 2017). That could be the reason why various forms of added carbon investigated in the present study were effectively associated with reduced level of toxic nitrogenous wastes. Our results are in agreement with the prior studies on Nile tilapia (*Oreochromis niloticus*) (Luo et al., 2014) and white-leg shrimp (Luis-Villaseñor et al., 2015) that reported lower phosphorus levels in carbon-treated systems. In our study, among the three carbon sources, molasses treatment was found to be more effective in reducing phosphate concentrations in marron culture. It is likely that molasses are made up of simple sugars and thereby readily and rapidly used by microbes (Serra et al., 2015, Wei et al., 2020), which leads to phosphate reduction (Correia et al., 2014, Miao et al., 2017a).

However, the inclusion of external carbon into marron culture resulted in declining dissolved oxygen and pH in the water. Similar to our results, low dissolved oxygen and pH values were reported in water of pink shrimp culture when sugarcane molasses and wheat bran were added as external carbon sources (Emerenciano et al., 2012). Reduced dissolved oxygen and pH might be correlated with the increased respiration rates of heterotrophic microbial flora that enhanced the carbon dioxide concentration in limited water exchange systems (Tacon et al., 2002); therefore, applying sodium bicarbonate ($NaHCO_3$) is necessary when water pH drops below 7.0 (Rajkumar et al., 2016).

Sediments in aquaculture systems receive most of the total carbon and nitrogen inputs from exogenous sources (Tinh et al., 2021). Adding different carbon sources into the culture systems resulted in different amounts of carbon and nitrogen accumulating (Tinh et al., 2021). In the present study, the sediment C/N ratio declined in control over time while it increased in carbon-added treatments. The lowest C/N ratios were recorded in the control over the entire trial. These results imply that nitrogenous wastes accumulated more in the sediment of the control but have been efficiently decomposed by bacteria in treatments that received carbon supplementation. The highest C/N ratio retained in the sediments of the

molasses-treated group could be due to the greater solubility of molasses in the tank water (Serra et al., 2015). Molasses produce higher levels of dissolved organic carbon compared to the complex carbon sources of corn and wheat flour, which often require additional time for microbial degradation prior to the carbon source being utilisable by microorganisms (Serra et al., 2015). In addition, high C/N ratio could benefit the diversity and community structure of microbial communities in tank sediments (Foysal et al., 2022b).

In the present study, analysis of 16S rRNA sequence data exhibited that adding carbon sources significantly increased bacterial diversity in the sediments. The augmented bacteria in the sediments with wheat flour are reported to play diverse roles, including degradation of complex organic compounds such as atrazine, isoprene by the *Nocardioides*, hydrolytic *Deefgea* with unknown soil function, and one of the most diverse environmental bacteria *Aeromonas* that is mostly pathogenic to the aquatic species (Gibson et al., 2020, Piutti et al., 2003). On the other hand, increased bacteria abundance with molasses supplementation are primarily involved in the degradation of contaminated soil by *Sphingobium*, organic waste decomposition by *hgcl-clade* (Ghylin et al., 2014), and seawater bacteria *Paraperlucidibaca* (Oh et al., 2011). The augmented genera in wheat flour and molasses attributed to better water quality and improved health status of marron.

In this study, PICRUSt2 has been employed for the metagenome prediction from the bacterial 16S dataset. Due to the low resolution of Illumina amplicon data, the phylogenetic classification has been restricted to the genus level only, and therefore, extraction of metabolic information from short-reads is difficult and not precisely accurate. However, PICRUSt2 has a co-efficiency of more than 80 for metagenome prediction for environmental samples (Douglas et al., 2020). Alongside the building blocks of protein, amino acids are the primary sources of energy for the gut and hepatopancreas of crustaceans (Li et al., 2021d). Carbohydrate is essential, but the requirement for aquatic animals, including crustaceans, is very low (Wang et al., 2016), yet enrichment of glucose and carbohydrate metabolism by CBC12 and MBC12 is an indication of balanced carbohydrate, protein and amino acids biosynthesis and metabolism. These findings also

support our previous studies with marron and different fed supplementations wherein elevated protein and amino acids activity positively linked to better immune and gut health of marron (Foysal et al., 2019b, Foysal et al., 2022a). No toxic activity or biofilm formation signifying no negative impacts of carbon supplementation on gut health and metabolism and the possibility of CBC12 and MBC12 to be used in marron aquaculture.

One of the most significant findings of this study is the correlation between bacterial communities and C/N ratio in the sediments. An increased C/N ratio is linked to better water quality features in aquaculture by removing toxic nitrogenous compounds such as ammonia (da Silva et al., 2013). We found that *Acinetobacter* positively correlated to C/N ratio in the sediments. This is the bacterial group that can increase C/N ratio through the evolution of CO_2 (Hoyle et al., 1995). Hence, an increase in relative abundance for *Acinetobacter* in the molasses supplemented group might be associated with a higher C/N ratio in the sediment. No parallel reports are available on the negative correlation between *Acidovorax* and C/N ratio in the sediment to further substantiate the findings. Therefore, future research should be focussed on microbial community network and sediment C/N ratio to better understand taxa-environmental correlation under marron aquaculture. However, despite having higher bacterial diversity in the sediment of both wheat flour and molasses added groups, the overall data largely suggest beneficial effects of molasses in terms of beneficial bacteria, and C/N ratio retention.

The addition of external carbon sources significantly benefited the growth performance of various fish (Ahmad et al., 2019, Perez-Fuentes et al., 2016) and shrimp species (Rajkumar et al., 2016, Tinh et al., 2021). In the present study, all three exogenous carbon sources included in the marron culture did not significantly affect marron survival, marron weight gain; however specific growth rate, and moult weight increment were improved. The survival rate of the marron in this study was comparable to those reported in a previous study (Tulsankar et al., 2022a), which may be due to the same approach of housing the marron individually to prevent cannibalism. Moreover, heterotrophic bacteria presented in the carbon-enriched treatments could assimilate nitrogenous waste and convert it into

protein-rich microbial biomass, thereby supplying the nutrients for the growth of aquatic organisms (Khanjani et al., 2017, Wei et al., 2020). Furthermore, decapod growth is restricted by the ability of their digestive systems to break down and absorb specific nutrients (Houlihan et al., 1988). Red swamp crayfish raised in the culture system with external carbon addition obtained higher hepatopancreatic pepsin activities (Li et al., 2019a). Results of the current study reveal that molasses treatment increased protease activity in marron hepatopancreas, resulting in the highest moult weight increment and specific growth rate of marron which is in line with the results documented in other studies (Khanjani et al., 2017, Serra et al., 2015). The lower specific growth rate of marron in the current study is in contrast to the findings of Tulsankar et al. (2022a), which might be associated due to a larger size of marron used as experimental animal.

Moisture and immunological parameters have been widely used as robust indicators of marron health conditions (Fotedar, 1998). High dry tail muscle and hepatopancreatic indices indicate greater energy storage in marron flesh and hepatopancreas, respectively (Fotedar, 1998). In our study, carbon supplementation had significant impacts on some moisture contents of marron such as hepatopancreas moisture, wet hepatosomatic and wet tail muscle indexes. In contrast to our results, hepatopancreas index and meat yield were unchanged in red swamp crayfish cultured in the system with carbon addition relative to the control (Li et al., 2019a). The different crayfish species and carbon sources used in these two studies could be responsible for the different outcomes. El-Sayed (2021) reported that some probiotic agents presented in carbon-added systems can augment the immune system in shrimp. In the present study, molasses addition improved the immunological parameters, including THC and lysozyme activity. High THC and lysozyme activity are associated with better marron health conditions (Jussila, 1997a). As an external carbon source, molasses has proven its effectiveness in strengthening the immune system of various farmed animals. For example, Zhao et al. (2016a) reported significantly higher THC in the haemolymph of the shrimp treated with molasses relative to shrimps reared with other carbon sources. Similar results were confirmed by farming white-leg shrimp

with molasses supplementation (Khanjani et al., 2017, Serra et al., 2015). The findings of similar proportions of differential haemocyte cells in marron reared in carbon-enriched environments are reported for the first time in the scientific literature. These blood cells function in wound healing and phagocytosis; thereby, their fluctuations in the differential haemocyte profile suggest a response to foreign invaders (Aladaileh et al., 2007, Ruddell, 1971). Further research is needed to clarify roles of haemocyte cells in marron health when the animals are cultured in carbon-supplemented systems.

7.5 Conclusion

In conclusion, adding carbon sources to the marron culture system led to improved performance of marron which are reflected on the water quality improvement, and high C/N ratio retention in the sediments. Among all the carbon sources tested, the molasses treated group remarkably fostered the diversity of bacterial populations in tank sediments. These conditions further contributed to improved growth and health performances of marron. Future research efforts on the optimisation levels of molasses will provide insightful understanding for better management and profitability of commercial marron farming and for promotion of sustainable blue economy.

CHAPTER 8 (Experiment 5) Supplementation of molasses increases bacterial diversity in the sediments and growth of juvenile marron (*Cherax cainii*) cultured under laboratory conditions

Abstract

Adding exogenous molasses to maintain a high C/N ratio in aquaculture systems has been reported as a practical approach to improve water quality and enhance the growth of target species. This study investigated the effects of two levels of molasses supplementation on water quality, sediment characteristics and growth performances of 3.16 ± 0.21 g marron (*Cherax cainii*) in 300 L circular tanks. Three treatments were in quadruplicates, including one untreated control and two groups with different quantities of molasses added into culture water to maintain a C/N ratio of 12 (MBC12) and 15 (MBC15). Marron were individually reared in custom-designed cages at a density of 10 marron per tank. Once a day, during the 60-day trial, formulated pellets were provided to the marron at 3% body weight, followed by adding molasses with the quantities according to treatments. The results showed that MBC12 and MBC15 led to significant decreases in ammonia and nitrite in water and increases in C/N ratio in sediment compared to the control. MBC12 and MBC15 both enhanced marron growth performances relative to the control group. However, MBC12 had the highest sediment microbial diversity and the lowest relative abundance of potentially pathogenic bacteria such as *Vibrio*. These findings suggest that MBC12 had particularly beneficial impacts on marron ecosystem.

8.1 Introduction

As aquaculture keeps on expanding to meet the rising demand for seafood due to an increasing world population, increasing productivity with water saving protocols (Boyd et al., 2020) is required. As freshwater is a limited resource, especially in Australia (Lake and Bond, 2007, Naylor et al., 2009), thereby restricted or no water exchange practices need to be implemented. The biofloc aquaculture systems (BFT) is able to meet these criteria (Ahmad et al., 2017, Abakari et al., 2021a) where external carbon sources are added to increase the carbon-to-nitrogen (C/N) ratio in the water (Avnimelech, 1999). An increase in water C/N ratio can stimulate the activities of heterotrophic microorganisms which can convert nitrogenous wastes into microbial biomass. This benefits the system by maintaining suitable water quality and providing an additional food source for the growth of various cultured species (Wei et al., 2016, Panigrahi et al., 2018). Many studies have documented that increasing the water C/N ratio can result in an improvement of feed utilisation, innate immunity (Panigrahi et al., 2018, Miao et al., 2017a, Miao et al., 2017b, Panigrahi et al., 2019), and health of hepatopancreas and digestive tracts in crustaceans (Tong et al., 2020, Xu and Pan, 2013).

Among crustacean species, marron (*Cherax cainii*) holds significance as a key aquaculture species in Australia (Beatty et al., 2019). The increasing market demand for this species (Alonso, 2010) has resulted in substantial research to improve their growth performance (Foysal et al., 2019b, Nguyen et al., 2021, Tulsankar, 2021). In marron aquaculture, very little research on the role of C/N ratio is available. In contrast, studies on controlling water C/N ratio in aquaculture systems by supplementing different quantities of exogenous carbohydrate sources were conducted on various decapod species (see review by Ahmad et al. (2017); Minaz and Kubilay (2021); Dauda (2020)). Based on the outcomes of our preliminary trial (Chapter 7) on the effects of various external carbohydrate sources (corn flour, wheat flour and molasses) on marron performances, molasses was found to be a suitable candidate for marron culture in a limited water exchange system. Therefore, the present study was conducted to assess the impact of supplementing two levels of C/N ratio

by the addition of molasses on water quality, sediment characteristics, and growth performances of marron.

8.2 Materials and Methods

8.2.1 Preparation of microbial inoculum water for marron culture system

Microbial inoculum preparation was followed a previous study by Ahmad et al. (2019) with some modifications. Two indoor plastic tanks filled with 200 L freshwater were set under light 24 hours/day. Each tank contained 4000 g soil from the marron pond, 2 g ammonium sulphate and 80 g molasses. The suspensions were kept under optimum aeration for 24 hours at a controlled laboratory temperature (22°C) to grow heterotrophic microbial biomass.

8.2.2 Experimental design

A hundred twenty marron 0^+ (3.16 ± 0.21 g) was obtained from Blue Ridge Marron farm (34°12′22″ S, 116°01′01″ E). Marron were introduced into experimental tanks a week prior to the commencement of the 60-day experiment for acclimatization. Each tank, with a water capacity of 300 L, accommodated ten marron, each housed in custom-made black plastic mesh cages. The tanks followed a completely randomized design with three treatments (control, MBC12, and MBC15), each with four replications. While the control was not added with any exogenous carbon sources, molasses was supplemented to MBC12 and MBC15 treatments to maintain C/N ratio in marron culture water of 12 and 15, respectively. The photoperiod of the experimental room was held a 12-hour light and 12-hour dark cycle. On day 1, each experimental tank was filled with 200 L of freshwater and 50 L of water from the prepared microbial inoculum tanks. A fishmeal-based diet (29.93% crude protein) prepared as described in section 3.5 of Chapter 3 was used to feed marron once a day with a feeding rate of 3% during both acclimatization and experimental periods. Feed and marron wastes were removed from the cages each morning to prevent water quality deterioration and water was not exchanged throughout the experiment. Molasses was added to experimental tanks every day after feeding except for the control tanks. The

amount of added molasses was calculated based on the method of Perez-Fuentes et al. (2016).

8.2.3 Data collection

8.2.3.1 Water quality

The laboratory was set at a controlled 22°C. Continuous aeration, facilitated by an air pump linked to air diffusers, was sustained within the tanks. Daily monitoring of water temperature, pH, and dissolved oxygen levels, while other parameters (phosphate, ammonia, nitrite, and nitrate) were checked weekly according to the protocols outlined in Chapter 3, section 3.1.

8.2.3.2 Sampling and analysing C/N ratio of sediments

Sediment samples were collected for analysis of the C/N ratio every week. Tank sediments were collected using the method previously outlined in section 3.2 of Chapter 3. Determining the carbon/nitrogen ratio within the tank sediments was conducted per the procedure detailed in section 3.3 of Chapter 3.

8.2.3.3 Analysing sediment microbiota

On the final week of the experiment (days 54, 56, 58, and 60), sediment samples were collected for analysis of the microbial community in all treatments. For microbiome analysis, on each sampling day, pools were created by thoroughly mixing the sediment samples from four tanks of the same treatment.

Sediment sampling and microbiome analysis followed the protocols described previously in sections 3.2 and 3.4 of Chapter 3.

8.2.3.4 Marron growth and health analysis

The methodologies outlined in Chapters 3.6 to 3.8 provided the procedures and calculations were used in evaluating the growth and health performances of the marron.

8.3 Results

8.3.1 Water quality

Apart from water temperature ranging from 20.7°C to 22.8°C in all tanks and it did not differ significantly among tanks, other observed water quality parameters varied between control tanks and those supplemented with molasses (Figure 8.1A-D). Data in weeks six and eight showed that significantly lower pH, nitrite and dissolved oxygen values were recorded in MBC12 and MBC15 than in the control. However, the water parameters remained the same between MBC12 and MBC15 over the experimental period.

Significantly lower nitrate levels were recorded in MBC12 and MBC15 groups than the control from week four of the experiment, and the nitrate level in MBC15 was significantly lower than in MBC12 in week eight. Similarly, from week four, phosphate concentrations in MBC15 were lower than in the control, while phosphates in MBC12 were less than the control only in weeks six and eight. Except for week four, similar phosphate concentrations were observed between different molasses treatments (Figure 8.1E-F).

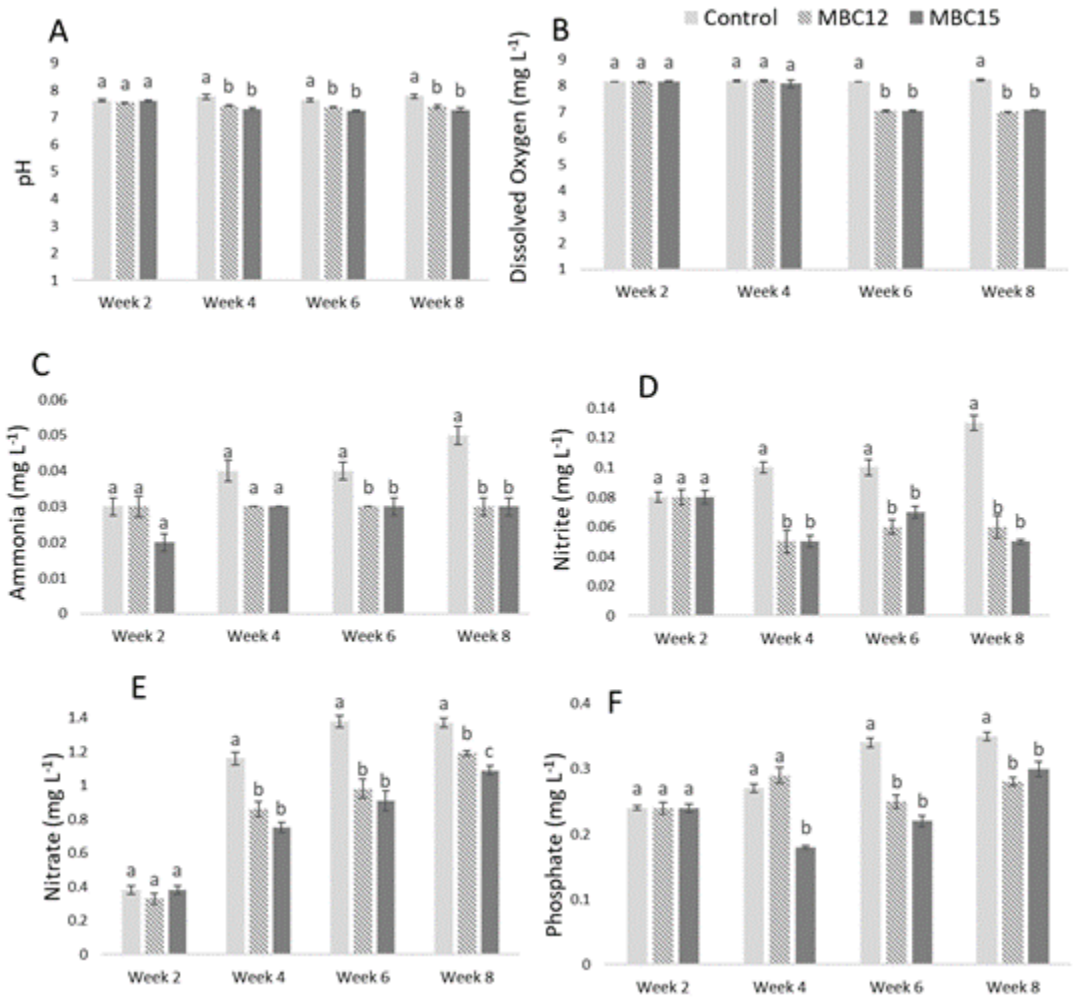


Figure 8.1 Water quality parameters between different treatments. Different superscript alphabets (a, b, c) indicate significantly different between treatments at specific sampling times ($p<0.05$).

8.3.2 Sediment carbon/nitrogen (C/N) ratio

The two-way ANOVA analysis indicated significant interactions between diet treatments and sampling weeks ($F=9.937$, $p<0.05$). The MBC15 had the highest sediment C/N ratios, followed by the MBC12 (Table 8.1). In particular, significantly higher sediment C/N ratios in MBC12 and MBC15 were detected compared to the control from week three onwards. Although there were no differences in sediment C/N ratios between MBC12 and MBC15 at most sampling points, the MBC15 had higher sediment C/N ratios than the MBC12 at

the final sampling time. Sediment C/N ratios showed a declining trend in control, while sediment C/N ratios of MBC12 and MBC15 treatments increased to reach their peaks at week three and remained high for the remainder of the experiment.

Table 8.1 Changes in C/N ratio in the tank sediment in different treatments over the experimental period. Data are presented as mean ± S.E., n=4.

Week	Control	MBC12	MBC15
1	7.64 ± 0.13^{cdefg}	7.77 ± 0.32^{defgh}	7.99 ± 0.13^{fghij}
2	7.55 ± 0.08^{cdef}	7.62 ± 0.05^{cdefg}	7.85 ± 0.08^{efghij}
3	7.34 ± 0.07^{cde}	8.41 ± 0.09^{j}	8.43 ± 0.14^{j}
4	7.24 ± 0.09^{cd}	8.15 ± 0.05^{ghij}	8.31 ± 0.08^{ij}
5	7.16 ± 0.07^{bc}	8.11 ± 0.05^{ghij}	8.23 ± 0.04^{hij}
6	7.12 ± 0.07^{bc}	8.20 ± 0.05^{hij}	8.12 ± 0.03^{ghij}
7	6.70 ± 0.04^{ab}	8.09 ± 0.07^{fghij}	8.21 ± 0.08^{hij}
8	6.46 ± 0.06^{a}	7.91 ± 0.07^{fghij}	8.42 ± 0.05^{j}

Different superscript letters indicate statistically different ($p<0.05$).

8.3.3 Bacterial load and composition in tank sediment

8.3.3.1 Sequence statistics and alpha-beta diversities

After processing of reads and quality filtering, a total of 586,432 PE reads and an average of 48869 ± 12688 reads were obtained, which were classified into 1359 OTUs. Filtering singleton and low-abundance OTUs and removing non-functional taxa resulted in 953 clean OTUs for further processing. These 953 OTUs were classified into six phyla and 26 genera. Out of 953, *Aeromonas* and *Candidatus Bacilloplasma* comprised of 586 OTUs. An average good's coverage index value of 0.997 indicated that each sample was sequenced at enough depth and saturation level to capture maximum bacterial diversity.

The alpha diversity analysis revealed that MBC12 had the highest bacterial richness in Observed and Fisher measures compared to the control. Further increase of the additional level of molasses, MBC15 treatment significantly decreased the bacterial diversity, Chao1

and ACE indices, relative to the control treatment. No statistical difference was observed for Simpson evenness between control and MBC treatments (Figure 8.2A). A visual representation of beta-diversity regarding PCoA displayed distinct clustering of bacterial OTUs for MBC15 treatment compared to the control and MBC12 groups whereas many overlaps between them were apparent in the plot (Figure 8.2B).

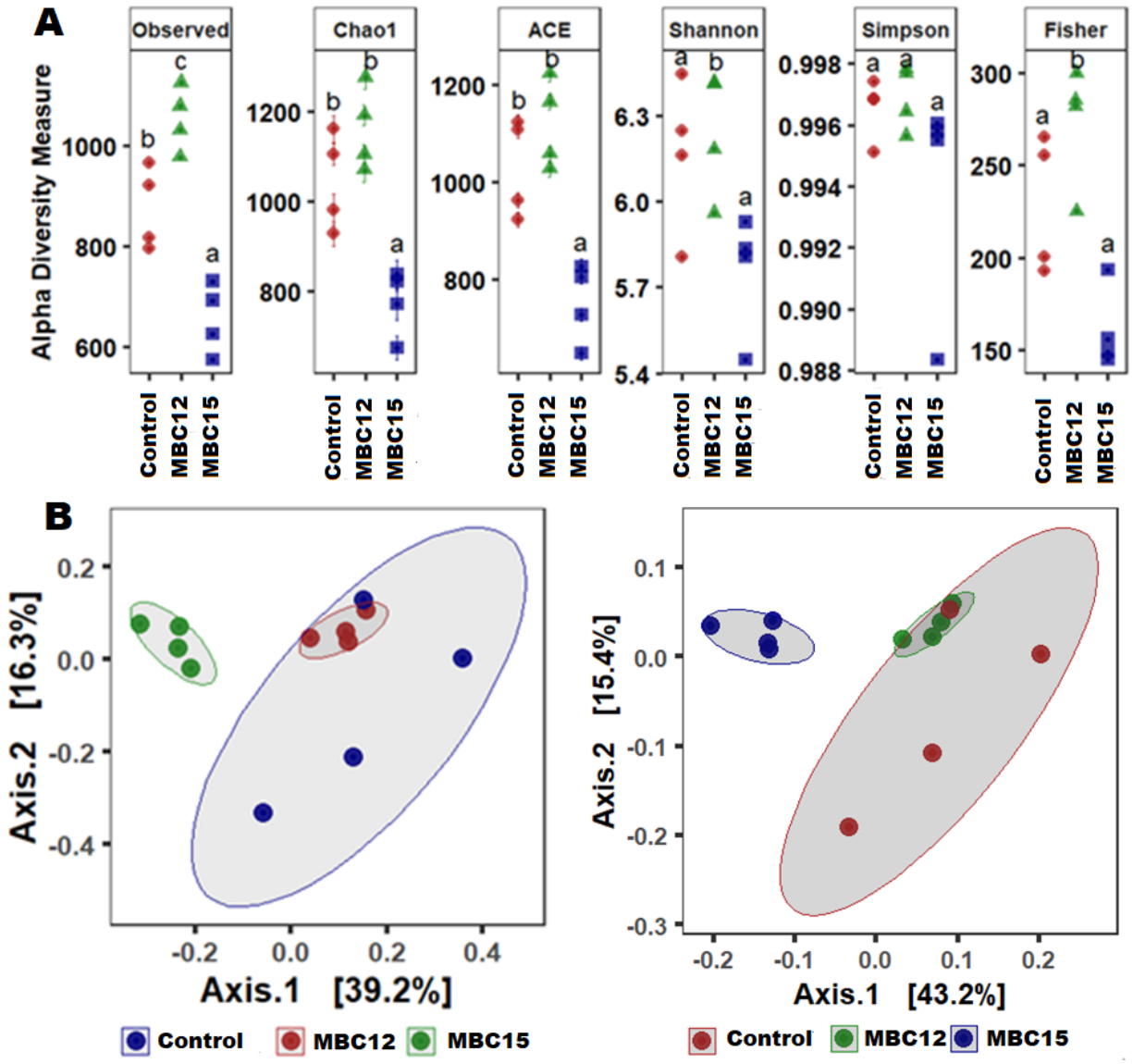


Figure 8.2 Alpha (A) and beta (B) diversity measurements of bacterial communities in sediment samples collected from tanks treated with different levels of exogenous molasses.

8.3.3.2 Microbial composition

Proteobacteria and *Aeromonas* were the most represented phylum and genus in all samples, consisting of 81% and 19% of the OTUs on average. Following Proteobacteria and *Aeromonas*, Tenericutes and *Candidatus Bacilloplasma* were the second most dominated phylum and genus, represented by 18% and 17% of classified OTUs in 12 sediment samples (Figure 8.3). At the genus level, the high relative abundance of *Sphingobium* (12%) was also found across all sediment samples of molasses treatments; however, the control group was further enriched (21%).

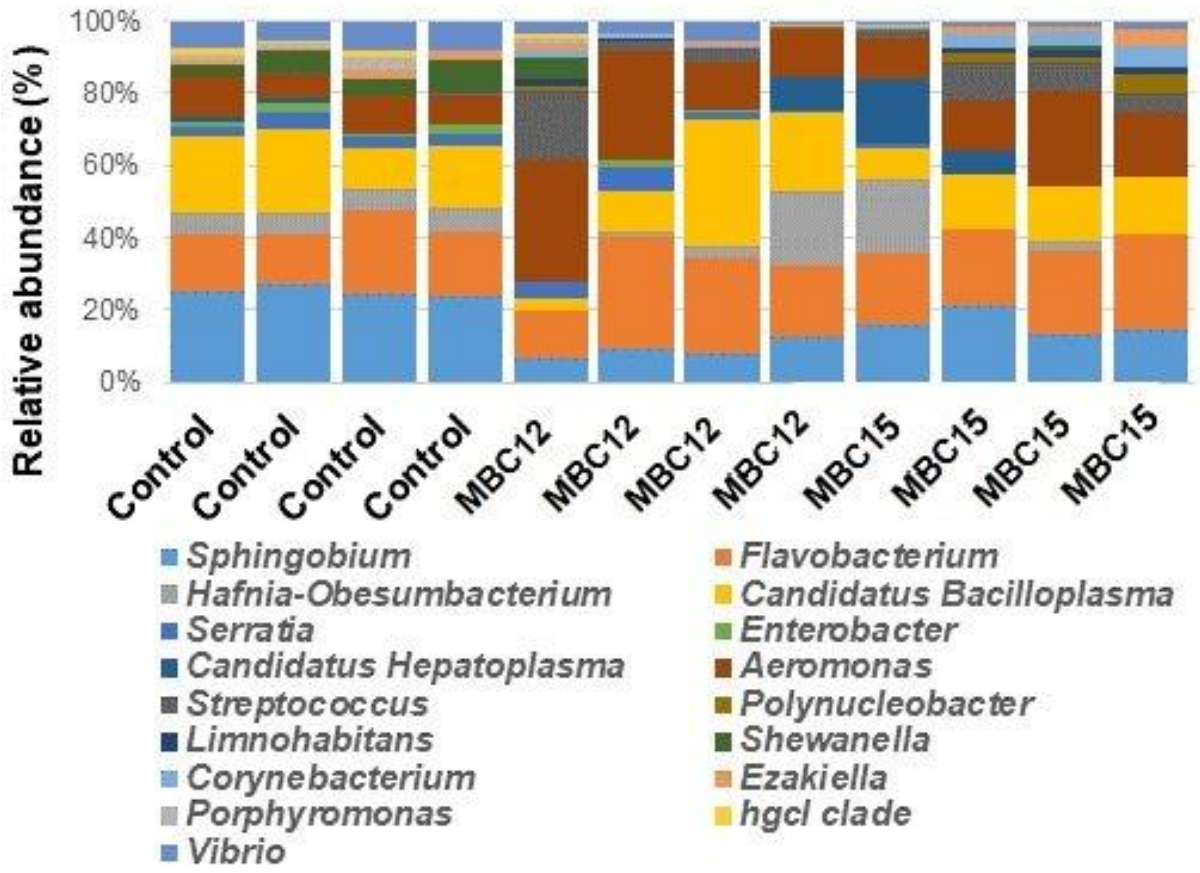


Figure 8.3 Relative abundance of bacteria at the genus level. Only OTUs representing at least 1% of the total reads are shown.

Differential analysis showed prevalent changes in bacterial abundance with MBC15 treatment, wherein treatment significantly reduced the richness of *Serratia*, *Acinetobacter*,

and *Candidatus Bacilloplasma*, and increase the growth of *Flavobacterium* after 60 days, compared to the control treatment. On the other hand, *Vibrio* abundance was significantly lower in the MBC12 group than in the control group (Figure 8.4).

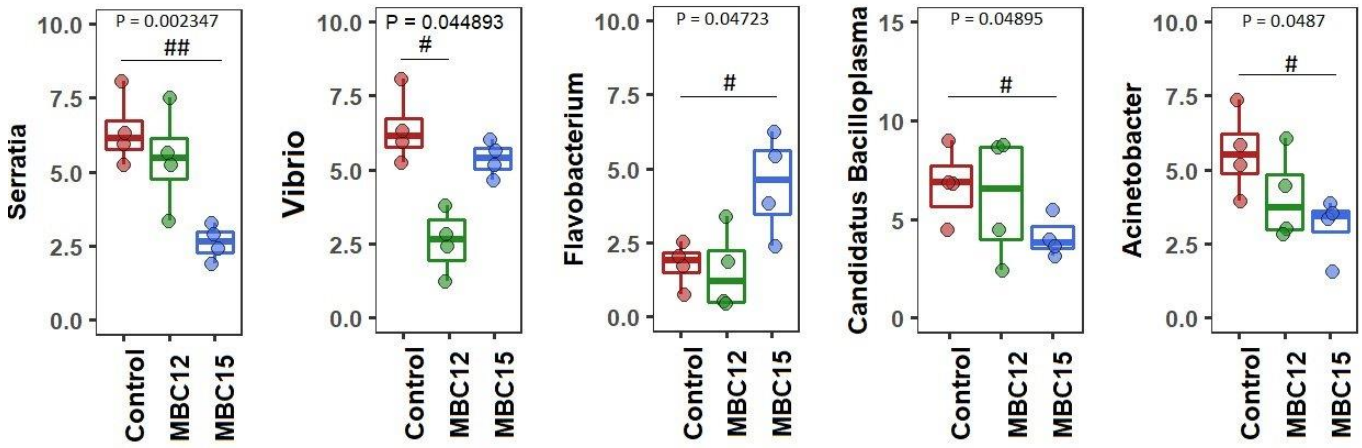


Figure 8.4 Significantly different bacteria at genus level in control and two molasses-treated groups.

8.3.4 Marron growth performance

As shown in Table 8.2, there was no significant difference ($p>0.05$) in marron initial weight between treatments; however, higher marron final weights were recorded due to the molasses supplementations. The survival rate of marron was about 90% in all treatments, and it was not affected by adding molasses. MBC12 and MBC15 increased the same weight gain and specific growth rate of marron.

Molasses supplementation did not affect the moulting interval of marron. However, marron inter-moult increments and protease activity in MBC12 and MBC15 were higher than in the control, and there were no significant differences between these two molasses-treated groups.

Table 8.2 Marron survival and growth performance parameters (mean ± S.E., n=4) in different treatments.

Parameters	Control	MBC12	MBC15
Initial weight (g)	2.97 ± 0.04 [a]	3.10 ± 0.02 [a]	3.08 ± 0.08 [a]
Final weight (g)	3.61 ± 0.04 [a]	3.90 ± 0.02 [b]	3.87 ± 0.08 [b]
Survival (%)	90.00 ± 0.00 [a]	92.50 ± 2.50 [a]	90.00 ± 4.08 [a]
Weight gain (g; %)			
0 – 30 days	$_1$ 9.66 ± 0.28 [a]	$_1$ 11.58 ± 0.29 [b]	$_1$ 12.07 ± 0.51 [b]
30 – 60 days	$_1$ 10.49 ± 0.23 [a]	$_1$ 12.97 ± 0.60 [b]	$_1$ 12.73 ± 0.30 [b]
0 – 60 days	$_2$ 21.19 ± 0.13 [a]	$_2$ 26.11 ± 0.78 [b]	$_2$ 26.42 ± 0.81 [b]
SGR (g; %/day)			
0 – 30 days	0.31 ± 0.01 [a]	0.36 ± 0.01 [b]	0.38 ± 0.02 [b]
30 – 60 days	0.33 ± 0.01 [a]	0.41 ± 0.02 [b]	0.40 ± 0.01 [b]
0 – 60 days	0.32 ± 0.01 [a]	0.38 ± 0.01 [b]	0.39 ± 0.01 [b]
MI (days)	28.05 ± 0.88 [a]	26.47 ± 0.84 [a]	28.06 ± 0.24 [a]
MWI (%)	20.37 ± 0.02 [a]	26.42 ± 0.56 [b]	26.13 ± 0.33 [b]
Protease (μg/mL)	1.19 ± 0.03 [a]	1.59 ± 0.02 [b]	1.58 ± 0.03 [b]

The different superscript letters (a, b, c) in rows and the different subscript numbers (1, 2) in columns indicate statistically different ($p<0.05$). Abbreviations: SGR – specific growth rate, MI - Moult interval, MWI – moult weight increment.

8.3.5 Marron health indices

Significantly higher moisture contents of marron dry tail muscle index and hepatopancreas moisture content in molasses-treated tanks relative to the control were recorded. Similarly, total haemocyte counts were significantly higher in MBC12 and MBC15 than in the control. However, the difference in marron total haemocyte counts between these two molasses-treated groups was not significant, and no statistical differences were found between treatments in other haemolymph parameters (Table 8.3).

Table 8.3 Organosomatic and immunological measures (mean ± S.E.; n=4) of marron treated with different molasses levels.

Parameters	Control	MBC12	MBC15
TM (%)	81.36 ± 0.32 [a]	80.83 ± 0.34 [a]	80.83 ± 0.54 [a]
Tiw	32.32 ± 0.52 [a]	33.47 ± 0.36 [a]	33.23 ± 0.55 [a]
Tid	6.02 ± 0.04 [a]	6.41 ± 0.06 [b]	6.36 ± 0.08 [b]
HM (%)	70.9 ± 0.31 [a]	73.55 ± 0.18 [b]	73.15 ± 0.73 [b]
Hiw	5.81 ± 0.16 [a]	6.18 ± 0.14 [a]	6.06 ± 0.17 [a]
Hid	1.69 ± 0.04 [a]	1.63 ± 0.05 [a]	1.63 ± 0.07 [a]
THC (million cells/MBC)	1.84 ± 0.02 [a]	2.35 ± 0.04 [b]	2.24 ± 0.07 [b]
GC (%)	30.63 ± 1.64 [a]	35.17 ± 1.79 [a]	33.29 ± 1.01 [a]
Semi-GC (%)	8.08 ± 1.24 [a]	9.58 ± 1.09 [a]	8.29 ± 1.16 [a]
Hyaline cell (%)	61.29 ± 2.86 [a]	55.25 ± 2.36 [a]	58.42 ± 1.94 [a]

Different superscript letters (a, b) in the same row represent significant differences at $p<0.05$. Abbreviations: TM - tail muscle moisture content, Tid - dry tail muscle index, Tiw - wet tail muscle index, HM - hepatopancreas moisture content, Hiw - wet hepatosomatic index, Hid - dry hepatosomatic index, THC – total haemocyte count, GC – granular cells.

8.4 Discussion

8.4.1 Effects of molasses level addition on water quality

The results of the present study show that ammonia and nitrite concentrations in water were reduced significantly due to molasses supplementation. Previous research (Panigrahi et al., 2019, Dauda et al., 2018, Magondu et al., 2013) found a further reduction in ammonia and nitrites with increasing the amounts of carbohydrate addition. However, the present study did not provide evidence that a higher level of molasses supplementation could reduce ammonia and nitrite from marron culture system. In contrast, a further reduction in nitrate, a less toxic form of nitrogen, and phosphate at some points of the experimental period was found. Nitrate and phosphate are important elements for phytoplankton proliferation (Sahu et al., 2012, Achary et al., 2014); therefore, their reduction was possibly related to the

enhanced growth of the plankton in the culture system. High plankton abundance can also result in declined dissolved oxygen concentration when the systems are treated with exogenous carbon (Hosain et al., 2021a) but still in the optimum range for marron culture (Cole et al., 2019). The water quality results also suggested that low-level molasses (MBC12) supplementing is sufficient to convert nitrogenous metabolites into microbial proteins. Increasing molasses levels may not result in any further benefits in marron culture.

8.4.2 Effects of supplementing molasses on sediment characteristics

Sediment C/N ratios remained high during the experiment when molasses were supplemented, which is consistent with the results of our previous study (Chapter 7) when various carbon source treatments (cornflour, molasses, and wheat flour) maintained high C/N ratios in the sediments of the marron tanks. High sediment C/N also indicate a positive relationship between C/N ratios in water and sediment, which agrees with a published study by Han et al. (2022b).

A high C/N ratio is vital for the growth of microbial communities in the culture system. Previous findings (Xu et al., 2016, Khanjani and Sharifinia, 2022, Qiu et al., 2021) suggested that autotrophic bacteria communities predominated in the culture water with low C/N ratios, whereas heterotrophic bacteria prevailed when more carbon was available. In general, sediment has a higher accumulation of uneaten feed and faeces than water, providing abundant carbon and nitrogen sources for bacterial growth. Hostins et al. (2019) reported that providing an appropriate C/N ratio input can reduce pathogens and improve probiotics in white leg shrimp (*Litopenaeus vannamei*) culture system. In the present study, molasses supplementation did not cause an imbalance in the relative abundance of the different bacterial species in the communities. However, bacteria richness in the sediment of the marron tank was highest in the low-level molasses supplementation, indicating that carbon levels influenced the microbial diversity in marron culture systems. Therefore, the quantity of molasses supplementation needs to be considered to promote bacteria diversity.

Proteobacteria was the highest-dominated phylum in the bacterial communities in the present study. At the genus level, the four bacteria groups, including *Aeromonas*, *Candidatus Bacilloplasma*, *Flavobacterium*, and *Sphingobium*, dominated in all treatments. These bacteria groups were also prevalent in marron tank sediments of our unpublished study (Chapter 6) and consistent with the findings of Foysal et al. (2022b), where marron culture systems were supplemented with zeolite.

Our results highlight the lower abundance of *Vibrio* of the family Vibrionaceae in molasses-added marron tanks. A significantly lower abundance of Vibrio was observed in the MBC12 treatment than in the control. The family Vibrionaceae has been declared as a family predominated in intestinal and sediment samples with necrotic disease acute hepatopancreatic disease (Gopal et al., 2005, Walling et al., 2010, Cornejo-Granados et al., 2017). The reduction of *Vibrio* in the present study may be due to the increase of potential probiotic bacterial groups against *Vibrio* in the carbon-added systems (Hostins et al., 2019).

8.4.3 Effects of molasses level addition on marron growth and health performances

This study is the first attempt conducted on marron to evaluate the effects of controlled supplementation of molasses in water to alter the water C/N ratio on marron growth and health. In contrast, several research have been published on other aquaculture species such as tilapia (*Oreochromis niloticus*) (Elayaraja et al., 2020) and white leg shrimp (Ren et al., 2019, Panigrahi et al., 2019). These studies have conclusively demonstrated that the systems with high water C/N ratios can promote the growth performance of cultured animals. In our study, adjusted water C/N to 12 and 15, resulted in higher growth of marron, similar to that previously observed by Pérez-Fuentes et al. (2013) and Hosain et al. (2021b) for freshwater prawn (*Macrobrachium rosenbergii*). The higher growth performance of marron in molasses-supplemented treatments in the current study is also in line with the findings of our earlier investigation conducted on the larger size of marron (Chapter 7). Previous reports (Xu et al., 2016, Khanjani and Sharifinia, 2022, Rajkumar et al., 2016) documented that organic carbon supplementing to the culture water can form flocculate aggregates, which are composed of a wide range of microalgae, zooplankton and bacteria.

These aggregates can contain high crude protein and gross energy (Wei et al., 2016, Rajkumar et al., 2016). Therefore, the positive results in marron growth performance in the present study are possibly due to marron consuming these flocculates as an additional food source. Pérez-Fuentes et al. (2013) and Abu Bakar et al. (2015) reported that flocculates generated in carbon-treated systems frequently contain a variety of plankton species that crustacean species can consume. Recent studies have documented the effectiveness of live plankton in improving the growth and health status of juvenile marron (Tulsankar et al., 2021a, Tulsankar et al., 2022a). Therefore, further studies need to investigate the diversity and abundance of plankton in water and marron gut to clarify the assumption that marron can consume these food sources in carbon-supplemented culture systems.

Previous studies demonstrated that carbohydrates added to the culture system to maintain a C/N ratio at 15 could improve the growth performance of juvenile tiger shrimp (*Penaeus monodon*) (Anand et al., 2013), and African catfish (*Clarias gariepinus*) (Abu Bakar et al., 2015). However, the culture systems of freshwater prawn (Xu et al., 2016, Khanjani and Sharifinia, 2022) and tilapia (Perez-Fuentes et al., 2016) obtained higher animal growth performance at a lower C/N ratio input. Consistent with these published findings, increasing molasses levels did not result in higher marron growth in the present study. Similar marron growth between different molasses treatments, possibly due to feed formulation for marron was already high in carbohydrates (see Table 3.2 in section 3.5 of Chapter 3), so only a small amount of external supplementation was needed to stimulate higher growth. The significantly higher moult increment of marron in treatment with a low molasses level indicated that sufficient nutrition had been provided for marron growth and health in this treatment. According to McLaughlin et al. (1983), the hepatopancreas plays critical roles in decapod crustaceans' digestion, absorption, synthesis, and production of digestive enzymes. These roles could be attributed to higher moisture hepatopancreas content, dry tail muscle index and protease activity in marron supplemented with molasses.

8.5 Conclusion

Adding molasses at two levels can maintain water quality and enhance marron growth performance in a stagnant marron system under laboratory conditions. A low-level molasses input (MBC12) is the most favourable treatment level for marron culture systems with limited water exchange. Supplementing molasses to maintain C/N of 12 can also improve sediment characteristics by retaining high C/N ratios, reducing pathogenic bacteria in the sediment, and enhancing marron growth and health.

CHAPTER 9 General discussion, Conclusions and Recommendations

9.1 Introduction

This chapter discusses the results of all trials, including two field trials and three indoor laboratory-based trials. The correlations between sediment carbon to nitrogen (C/N) ratios and the selected environmental factors in four seasons in marron ponds are also discussed.

The effects of C/N ratio due to formulated feed and carbohydrate supplementation on nutrient wastes in marron tank water are summarized and discussed. The response of different size classes of marron to C/N ratios were also analyzed and discussed, followed by some recommendations.

9.2 The correlation between environmental variables and the C/N ratio of the pond sediments

Sediment plays a significant role in maintaining the stability of the pond bottom, making it one of the essential determinants for the sustainability of aquaculture activities (Fan et al., 2019). The characteristics of the bottom sediment and its effect on water quality in aquaculture ponds have been reported (Boyd and Tucker, 2012, Hasibuan et al., 2023). Nutrients in sediments originate from the settlement of solid materials settled from the water column (Zhang et al., 2014b, Zhang et al., 2015, Meyers, 2003). The field experiments (Experiments 1 & 2), conducted in semi-intensive marron ponds, where feed and other nutrient sources were added to support marron production, found that environmental variables in ponds fluctuated according to seasons (Experiment 1) and also responded to different formulated feeds (Experiment 2). Analyzing the pooled-data of selected rearing environment parameters showed that many factors can potentially contribute to carbon and nitrogen accumulation in the pond sediments. C/N ratio of marron pond sediments significantly correlated with various water parameters, including temperature (+), ammonia (+), plankton abundances (+), dissolved oxygen (-), water transparency (-), nitrite (-), nitrate (-), and phosphate (-) as shown in Table 9.1. Multiple linear regression analysis illustrated that sediment C/N ratio is highly affected by water

parameters in autumn and summer with R-squared values of 0.91 and 0.78, respectively. In addition, temperature, pH, ammonia and nitrite are the four primary determinants, significantly contributing to the change of sediment C/N ratio (Table 9.2).

Temperature is an essential factor in all aquatic ecosystems, and the positive correlation between temperature and carbon and nitrogen of lake sediments has been recognized (Wang et al., 2022, Dong et al., 2012). Other studies also found that temperature significantly regulates carbon mineralization (Gudasz et al., 2010), and the contribution of water temperature to the variation of sediment nitrogen can be up to 60% (Xia et al., 2015). An increase in temperature could lead to accelerated organic matter decomposition due to an enhancement of enzyme activities of certain bacteria groups (Staal et al., 2003, Gray et al., 2019, Tian et al., 2021). The process uses dissolved oxygen and releases nutrients from sediments (Iber and Kasan, 2021, Musa et al., 2023), promoting phytoplankton yields and reducing water transparency (Woznicki et al., 2016). Besides, the C/N ratio significantly regulates denitrification in aquaculture systems (Chen et al., 2018a, Gou et al., 2019, Lu et al., 2022). In the present study, this regulation could explain the negative correlations between sediment C/N ratio and nitrite, nitrate, and phosphate. Similar observations were described by Chen et al. (2018a)) and Gou et al. (2019), who found an increasing sediment C/N ratio co-occurring with reducing nitrite, nitrate, and total phosphorus levels in pond water. Therefore, sediment C/N ratios need to be maintained at the appropriate level to ensure the optimal environment for marron growth, and the adjustment of sediment C/N ratio may depend on carbon and nitrogen inputs.

Table 9.1 Pearson correlation matrix between all selected water quality parameters and sediment C/N ratio in marron ponds

Parameters (n=120)	Temperature	pH	DO	Water trans.	Ammonia	Nitrite	Nitrate	Phosphate	Ph. Ab.	Zoo. Ab.
pH	0.518**									
DO	-0.445**	0.045								
Water trans.	-0.693**	-0.445**	0.376**							
Ammonia	0.305**	0.002	-0.407**	0.135						
Nitrite	-0.480**	0.074	0.530**	0.254**	-0.217*					
Nitrate	-0.417**	0.049	0.767**	0.313**	-0.174	0.586**				
Phosphate	-0.239**	0.045	0.059	-0.001	0.114	0.438**	0.205*			
Ph. Ab.	0.591**	0.545**	-0.193*	-0.818**	-0.309**	-0.106	-0.283**	-0.039		
Zoo. Ab.	0.601**	0.478**	-0.182*	-0.486**	-0.124	-0.145	-0.172	-0.267**	0.604**	
Sedi. CN	0.703**	0.024	-0.666**	-0.506**	0.306**	-0.626**	-0.690**	-0.421**	0.402**	0.413**

*Positive (+) and Negative (−) correlations. * and ** Correlation is significant at p-value <0.05 and <0.01, respectively.*

Abbreviations:DO-Dissolved oxygen; Water trans. – Water transparency; Ph. Ab. - Phytoplankton abundance; Zoo. Ab. - Zooplankton abundance; Sedi. CN - Carbon/nitrogen ratio in the pond sediment.

Table 9.2 Multiple linear regression with sediment C/N as the response and water parameters as predictors in different seasons. R^2 is shown in brackets.

Factor	Autumn (0.913)		Spring (0.781)		Summer (0.777)		Winter (0.561)	
	RS	p-value	RS	p-value	RS	p-value	RS	p-value
Temperature	1.245	**0.002**	0.110	0.648	-0.651	**0.006**	-0.336	0.537
pH	-0.779	**0.000**	-0.496	0.110	-0.583	0.050	0.128	0.614
DO	0.027	0.884	-0.203	0.221	-0.137	0.543	-0.160	0.140
Water trans.	-0.044	**0.002**	2.577	0.439	-0.025	**0.021**	-0.003	0.824
Ammonia	-2.989	0.742	2.239	0.865	38.081	**0.009**	-26.131	0.153
Nitrite	3.322	0.825	1.222	0.485	-122.331	**0.024**	-4.283	0.868
Nitrate	0.167	0.876	0.932	0.397	3.610	0.155	-0.572	0.669
Phosphate	0.192	0.892	0.003	0.064	1.309	0.512	-1.578	0.387
Ph. Ab.	0.000	0.299	-0.001	0.719	0.001	0.234	2.364E-05	0.984
Zoo. Ab.	0.002	0.383	0.110	0.648	0.000	0.929	-6.684E-05	0.991

Abbreviations: RS – Regression slope; DO-Dissolved oxygen; Water trans. - Water transparency; Ph. Ab. - Phytoplankton abundance; Zoo. Ab. - Zooplankton abundance; Sedi. CN - Carbon/nitrogen ratio in the pond sediment. Significance level at p-value<0.05.

9.3 Responses of rearing environment to different ratios of C/N supplementation

9.3.1 Responses of water quality

The uncontrolled addition of nutrient to increase aquaculture productivity can deteriorate water quality (Makori et al., 2017, Bhari and Visvanathan, 2018, Boyd and Tucker, 2012). The negative impacts of accumulation of toxic nitrogenous compounds prompted research towards biofloc technology which can mitigate the drawbacks of intensive culture systems to the environment (Avnimelech, 2009, Boyd and Tucker, 2012). In these biofloc systems, maintaining proper water C/N input between 10 and 20 has been suggested to effectively control ammonia, nitrite and nitrate (Perez-Fuentes et al., 2016, Elayaraja et al., 2020). In a similar vein, the results of our experiments showed that without external carbon supplementation, C/N input of 9 (control treatments) had significantly higher nitrite and nitrate concentrations than those in the carbon-added treatments with C/N inputs of 12 and 15. These results were consistent for the cultures of marron in different age groups (results of experiments 4 & 5).

Comparing the results of treatments that have similar settings from different experiments showed that larger marron (juvenile 1^{+}; 71 ± 0.8g) released more wastes in the forms of ammonia, nitrite, nitrate, and phosphate to the rearing environment than small marron (juvenile 0^{+}; 11.67 ± 0.11g and 3.16 ± 0.21g) (Figure 9.1). In treatments with a C/N input of 12, differences in water quality were observed among the two size classes of juvenile 0^{+}. Specifically, the 11.67 ± 0.11g size class culture showed higher levels of ammonia in weeks 5 and 7, increased nitrite levels in week 8, and decreased nitrate levels in week 4 compared to those in the 3.16 ± 0.21g size class culture (Figure 9.2). These results can be explained by larger marron tend to produce more waste, leading to the potential accumulation of a greater amount of waste in their environment.

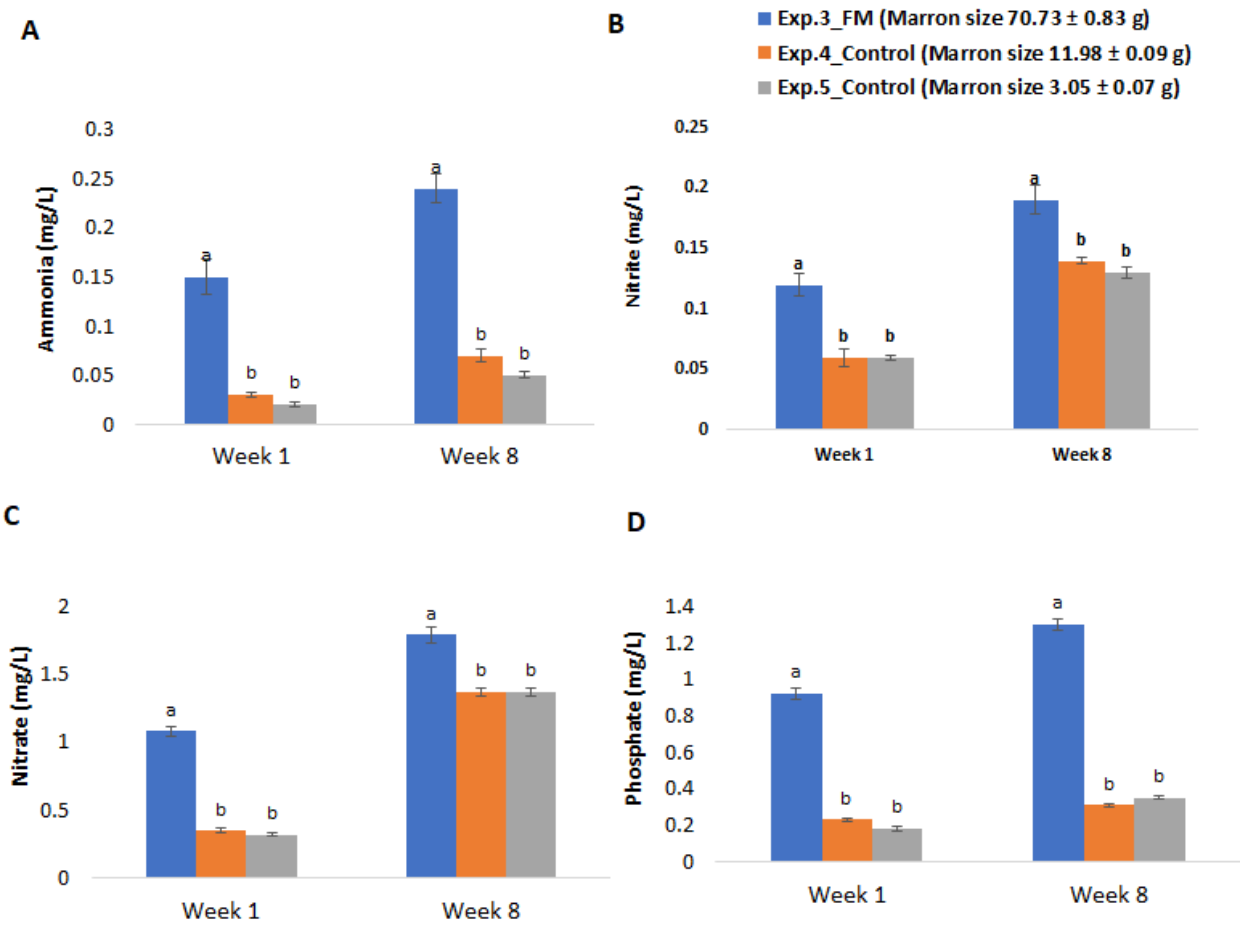


Figure 9.1 Water quality characteristics in the control treatments (C/N input of 9) of the experiments with different marron size classes (compare control treatments of experiments 3 & 4 & 5). Abbreviations Exp.- Experiment; FM – fishmeal. Different superscripts on bars within the same week show significant differences at $p<0.05$.

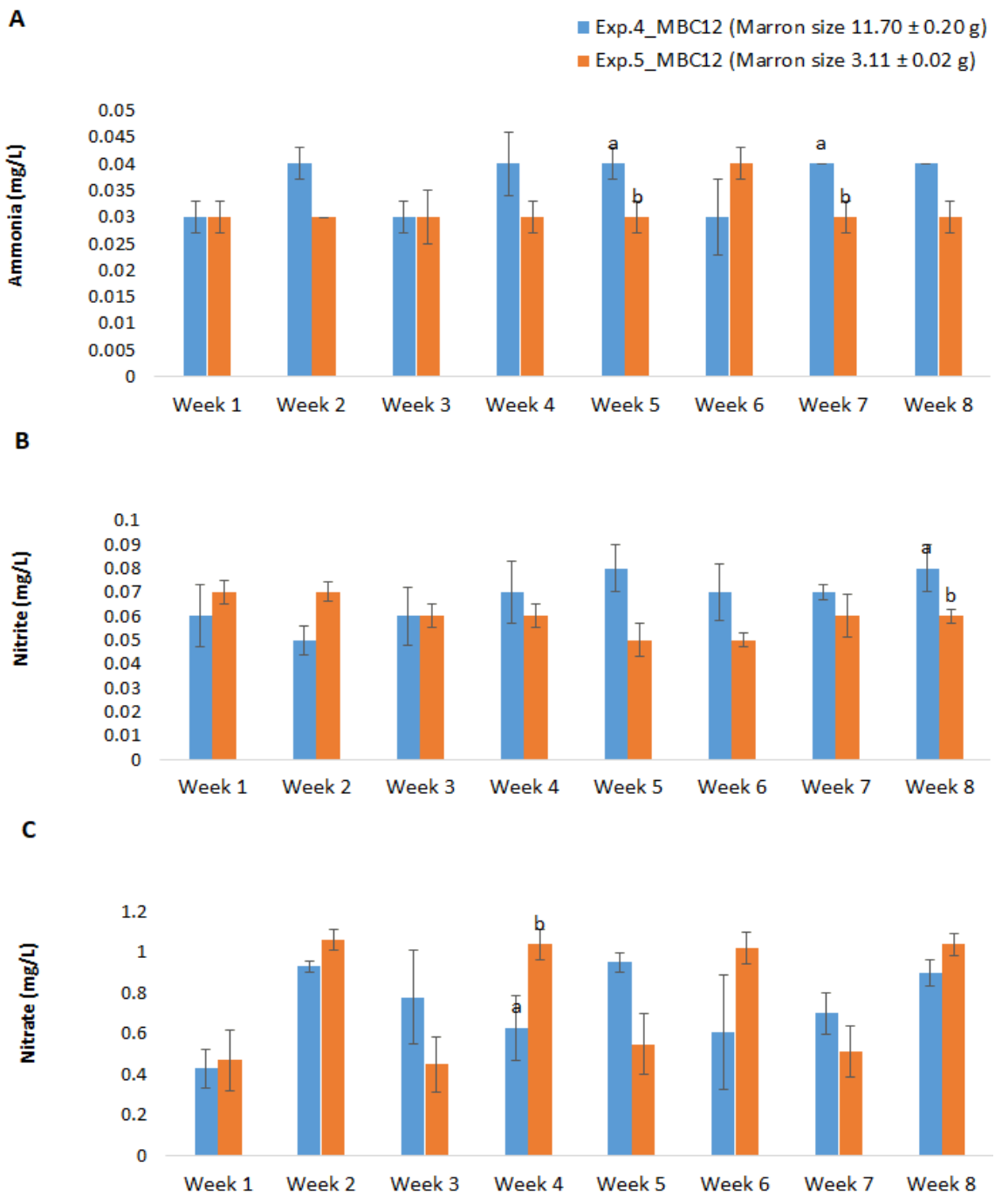


Figure 9.2 Water quality characteristics in the MBC12 treatments (C/N input of 12) of the experiments with different marron size classes (compare MBC12 treatments of experiments 4 & 5). Abbreviations Exp.- Experiment; MBC12 – molasses based carbon supplementation to obtain C/N ratio of 12. Different superscripts on bars within the same week represent significant differences at p-value<0.05.

9.3.2 Carbon supplementation to rearing water increased sediment C/N ratio

The presence of C and N in intensive aquaculture systems are mainly from inputs like formulated feed, and sediment acts as a primary storage of these nutrients (Hopkins et al., 1994, Boyd and Tucker, 2012, Junior et al., 2021, David et al., 2021, David et al., 2017) where up to 66% of unconsumed feed could end up (Hasibuan et al., 2023). Of the total unutilized nutrients, Sahu et al. (2013) demonstrated that organic carbon and nitrogen deposited in sediments of tiger prawn (*Penaeus monodon*) culture systems were 64.8 and 49.1%, respectively. The buildup of nutrient sediment linearly increased with nutrient input (Ioannis et al., 1998, Pouil et al., 2019). Therefore, managing the input is necessary to estimate the waste load of the culture systems (Chatvijitkul et al., 2018). To reduce nutrient waste, various strategies, including enhancing feed quality can be used (White, 2013, Hassan et al., 2021, Nunes et al., 2019). Previous studies have shown conflicting results regarding feed efficiency and nutrient retention when using different diets. While a high dietary protein/carbohydrate ratio increased the feed efficiency of gibel carp (*Carassius gibelio*) (Zhao et al., 2016b), a similar diet reduced nutrient retention of gilthead seabream (*Sparus aurata*) (Basto-Silva et al., 2022). Choosing the right carbohydrate type to formulate feed can also contribute to the nutrient utilization and the economic efficiency of the cultivation system (Basto-Silva et al., 2022).

The productivity of the aquaculture system can decrease due to nutrient entrapment in the sediments. Therefore, removal of the pond sediments after the harvest or regular tank siphoning is a solution to renew the bottom environment quality of these culture systems (Boyd and Teichert-Coddington, 1994, Thunjai et al., 2004, Shafi et al., 2021, Wudtisin and Boyd, 2006). However, this process is always costly and time-consuming (Jasmin et al., 2020). Besides that, the bottoms of these culture systems cannot be completely returned to their previous state by sediment removal. Moreover, the removed sediment needs to be handled properly to not negatively affect the receiving areas (Yuvanatemiya and Boyd, 2006). Adding exogenous carbon sources is another solution that can mitigate the drawbacks of sediment wastes by making use of these trapping nutrients (Avnimelech, 2009, Liu et al., 2019, Delgado et al., 2020, Ahmad et al., 2021). By organic carbon

addition, Liu et al. (2019) demonstrated that nitrogen accumulation in the sediment declined from 5.98 g/m^3 to 0.59 g/m^3. Another study by Han et al. (2022a) found a linear relationship between the water and sediment C/N ratios. Han et al. (2022a) also reported that high C/N input causes a decrease in inorganic nitrogen concentrations in the sediment which can explain for high C/N ratio retention in the sediment of marron systems in our study (results of experiments 4 & 5).

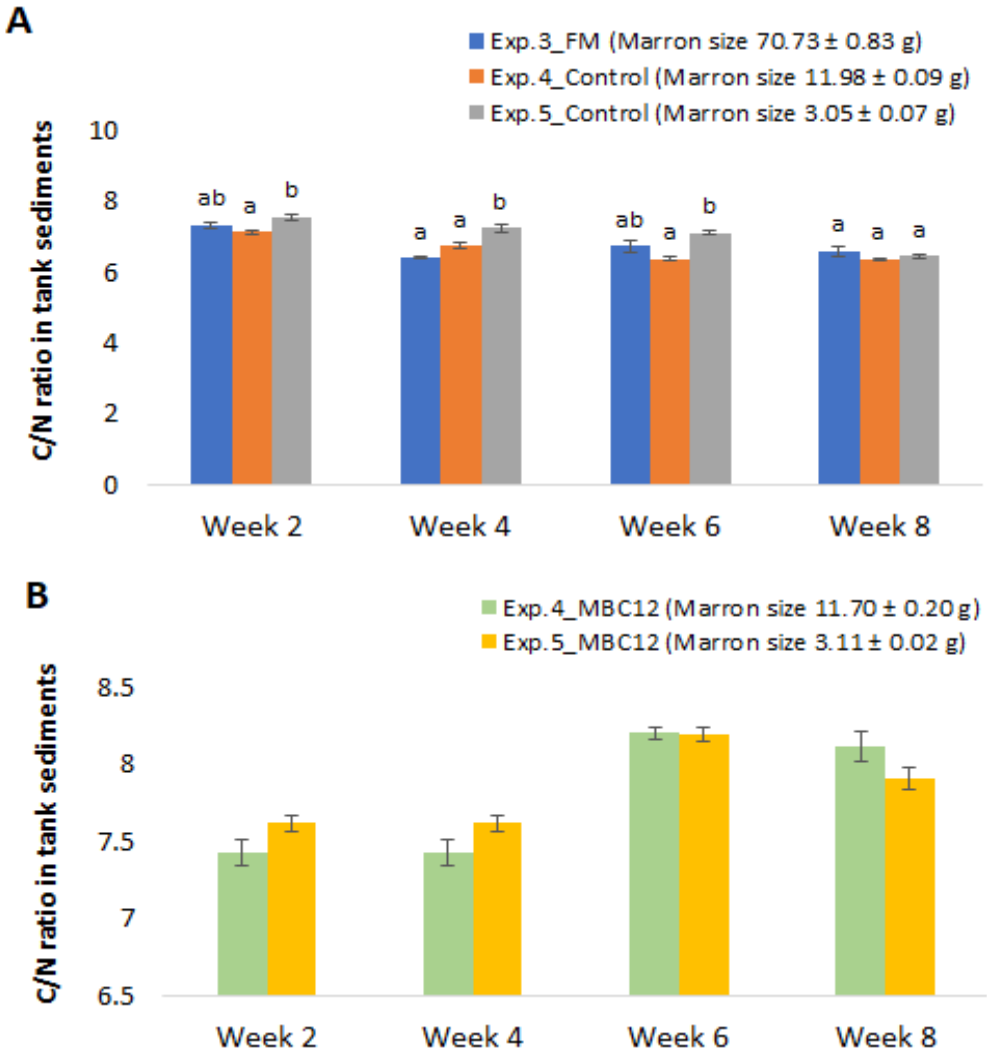


Figure 9.3 Sediment C/N ratio: **A**-in the control and **B** – in the MBC12 treatments of the experiments with different marron size classes. Abbreviations Exp.- Experiment; FM-fishmeal; MBC12 – molasses based carbon supplementation to obtain C/N ratio of 12. Different superscripts on bars within the same week represent significant differences at p-value<0.05.

The current study determined that the sediment C/N ratio is influenced by the size of the marron, with a C/N input of 9 derived from formulated feed sources (Figure 9.3A). Specifically, sediment C/N ratios within the system featuring larger marron sizes (71 ± 0.8g and 11.67 ± 0.11g) exhibited lower values in comparison to those observed in the culture of smaller marron sizes (3.16 ± 0.21g). However, this size dependence was not observed when the trials were terminated (Figure 9.3A). Similarly, in instances where the systems were supplied with molasses (MBC12) as an external carbon source to augment the C/N input to 12, sediment C/N ratios exhibited a consistent uniformity across two 0^{+}marron size classes (11.67 ± 0.11g and 3.16 ± 0.21g) throughout all selected sampling times (Figure 9.3B). These results may be ascribed to the presence of an adequate amount of carbon, which facilitates the biochemical processes required for the conversion of nitrogenous wastes in the culture systems when external carbon is supplemented.

Grebliunas and Perry (2016) showed that denitrification rates trended downward when high concentrations of nitrogenous compounds were presented. Sufficient dissolved organic carbon can satisfy nitrogen recycling (Grebliunas and Perry, 2016). As the C/N ratios grew, the denitrification rate is promoted, and more nitrogen in water and sediment is eliminated by this process (Grebliunas and Perry, 2016, Choi et al., 2008, Stelzer et al., 2014). These findings indicate that high C/N input can significantly contribute in removing nitrogen from the ecosystems, thereby playing an integral role in reducing the impacts of excess nitrogenous wastes in the water column and at the bottom sediments. However, the specific mechanisms can be complex, involving the activity of a wide range of microorganisms as well as depending on various factors such as the type and amount of organic matter, sediment characteristics, and environmental conditions (Wang et al., 2018, Zhu et al., 2018, Han et al., 2022a).

9.3.3 Various factors shifted microbial communities in marron culture systems

The bacteria account for more than 90% (Hahn, 2006) and play a crucial role in nutrient cycling (Abakari et al., 2022, Khanjani et al., 2022, Imaizumi et al., 2022). Bacteria can consume or produce carbon and nitrogen compounds, depending on the specific conditions

of the system (Johnson et al., 2017, Pajares and Ramos, 2019). For example, in a culture system that contains high levels of organic matter, bacteria may consume it and release carbon dioxide and nitrogen compounds as by-products. Conversely, in nitrogen-limited culture systems, bacteria may fix atmospheric nitrogen and convert it into forms that plants or algae can more easily take up. Previous studies established that C/N ratio input and soil condition after each cultivation could alter the structure of the soil microbial community (Boyd and Teichert-Coddington, 1994, Liang et al., 2017, Xue et al., 2018). While autotrophic bacteria predominate in water bodies with low C/N ratios (Liu et al, 2021), heterotrophic bacteria become dominated by high C/N (Zhu et al., 2018, Dilmi et al., 2021). The latter bacteria group proliferates when C/N is seven or more, and their abundance is preferable in aquaculture systems as they can enhance the bioremediation of wastes containing nitrogen through the denitrification process (Choi et al., 2008, Gou et al., 2019, Coyne et al., 2020) which transforms nitrogenous wastes into bacterial biomass as a useful protein source (Beveridge et al., 1989, Dilmi et al., 2021).

In many aquaculture conditions, despite the complexity, the predominant populations of bacteria, such as Proteobacteria, Cyanobacteria, Actinobacteria, and Bacteroidetes, are comparable at the phylum level (Huang et al., 2014, Tang et al., 2014, Zhang et al., 2016, Kim et al., 2021) which is also the case in our marron culture systems. Nevertheless, bacterial communities in the culture environment are sensitive to culture conditions. Many factors, including environmental variables (Experiment 1), dietary probiotics (Experiment 2), dietary protein sources (Experiment 3), and the addition of exogenous carbon (Experiments 4 & 5) significantly influence bacterial structure. These factors affect the nutrient enrichment, particularly the C/N ratio in the system (Liu et al., 2017, Chen et al., 2018a), thereby driving the microbial communities in marron culture, like previous studies on other aquaculture ecosystems (Jia et al., 2023, Addo et al., 2023). The current findings illustrated that Proteobacteria and Bacteroidetes are more prevalent when C/N input is increased, consistent with the study by (Kathia et al., 2018). Addition of organic carbon to enhance C/N can also boost the variety of heterotrophic bacteria (Deng et al., 2018), and

differentiate the abundance of several genera in the present study. The differentiation is probably due to their ability to utilize high C/N to produce energy and replenish nutrients (Zhou et al., 2019a). Increasing C/N ratio to reach more than 15 has brought benefits in higher abundances of probiotic bacteria genera such as *Bacillus, Lactobacillus, Enterococcus, Rhodotorula* (Gutiérrez et al., 2016, Islam et al., 2021). The present study did not have the same positive effect when increasing the C/N input to 15. On the contrary, the C/N input of 15 in our study was not effective in reducing the abundance of *Vibrio*, a bacterial group that causes disease in various aquaculture systems (Defoirdt et al., 2011, Haenen et al., 2014, Novriadi, 2016). In general, understanding the effects of driving factors on microbial communities to create the optimal environment for favouring beneficial bacteria and limiting harmful species to benefit marron production is crucial (Kim et al., 2012, Pang et al., 2022, Wang et al., 2022).

9.4 C/N input of 12 enhanced marron growth and health

Marron requires a balanced and adequate supply of nutrients, including carbon and nitrogen, to support their growth and development (Fotedar, 1998). This can be achieved through proper management practices such as controlling nutrient inputs, managing water quality parameters, and implementing appropriate nutrient cycling and waste management strategies (Avnimelech and Ritvo, 2003, White, 2013). The high water C/N input could generate *in situ* protein, fat, and carbohydrate that accounted for a considerable proportion of nutritional needs for cultured species (Crab et al., 2010, Kuhn et al., 2010, Emerenciano et al., 2012) maintaining proper water C/N can control water quality and enhance the activity of heterotrophic bacteria in marron culture systems, which may indirectly benefit marron growth and health, similar to previous studies on other cultured crustacean species (Guo et al., 2020, Xu et al., 2022).

Marron growers in Western Australia are adding fermented barley straw as an additional carbon source to marron ponds to maintain an equilibrium between nutrients and bacterial loadings. This equilibrium enhanced natural productivity leading to increase marron growth (Tulsankar et al., 2021b). Such practice was also used in our field trials

(Experiments 1 & 2). Promising outcomes in water quality, sediment C/N ratio (Experiment 1) and also marron production cultured in earthen ponds were achieved (Experiment 2). In laboratory experiments (Experiments 4 & 5), supplementing organic carbon to increase the water C/N ratio input had similar positive effects on the growth and health of two size classes of reared marron. By adding exogenous carbon from various sources, marron growth and health were improved. Adding molasses to manipulate water C/N equal to 12 performed the best among all treatments (Experiments 4 & 5). We found similar results in the experimental systems of marron at different size groups, confirming positive effects on marron growth due to the presence of exogenous carbon (Tables 9.3 and 9.4).

Marron growth rates in our study were comparable to previous studies where supplemental immune-stimulants (Sang et al., 2009, Sang and Fotedar, 2010) or trace elements (Tulsankar et al., 2022a) were used. In our studies, culture systems with proper C/N inputs created a suitable environment for plankton to proliferate as an in-situ food source for marron growth. Previous studies found that the culture systems with high water C/N were also enriched with amino acids (Emerenciano et al., 2014), fatty acids (Anand et al., 2014) and micronutrients including vitamins and minerals (Fimbres-Acedo et al., 2020, Flefil et al., 2022). Our study showed that considering the body size, the moulting interval was shortened while enhancing the marron's innate immune system and hepatopancreatic enzyme activity in the high C/N input treatments. These results can also explain the high survival and growth rate of marron in our experiments. Ahmad et al. (2017) and Correia et al. (2014) reported another benefit of C/N adjustment systems is that feed use can be reduced.

Table 9.3 Marron growth performance (mean ± S.E.) from treatments with different C/N inputs and marron size classes.

A	C/N input = 9		
	Exp.3_FM (n=3)	**Exp.4_Control (n=3)**	**Exp.5_Control (n=4)**
Marron size class (g)	70.73 ± 0.83 [a]	11.98 ± 0.09 [b]	3.05 ± 0.07 [c]
Survival (%)	100 ± 0.00 [a]	90 ± 0.00 [b]	90 ± 0.00 [b]
SGR (%/day)	0.13 ± 0.01 [a]	0.34 ± 0.02 [b]	0.32 ± 0.00 [b]
WG (%)	7.99 ± 0.27 [a]	21.27 ± 1.16 [b]	21.19 ± 0.13 [b]
MWI (%)	NA	22.00 ± 1.07 [b]	20.37 ± 0.02 [c]
MI (days)	NA	32.44 ± 1.28 [b]	28.05 ± 0.88 [c]
Protease activity (ug/mL)	1.79 ± 0.11 [a]	1.05 ± 0.08 [b]	1.19 ± 0.03 [b]

B	C/N input = 12	
	Exp.4_MBC12 (n=3)	**Exp.5_MBC12 (n=4)**
Marron size class (g)	11.70 ± 0.20 [a]	3.11 ± 0.02 [b]
Survival (%)	93.33 ± 3.33 [a]	92.5 ± 2.50 [a]
SGR (%/day)	0.50 ± 0.04 [a]	0.38 ± 0.01 [b]
WG (%)	32.23 ± 2.71 [a]	26.17 ± 0.83 [b]
MWI (%)	29.36 ± 0.85 [a]	26.42 ± 0.56 [b]
MI (days)	32.67 ± 1.50 [a]	26.47 ± 0.84 [b]
Protease activity (ug/mL)	1.65 ± 0.05 [a]	1.59 ± 0.02 [a]

Superscript letters in the same row show significant differences in marron growth performance between treatments at p-value<0.05. Abbreviations: Exp. - Experiment; FM - fishmeal; MBC12 – molasses based carbon supplementation to obtain C/N ratio of 12; SGR – specific growth rate; WG – weight gain; MWI – moult weight increment; MI – moult interval.

Table 9.4 Marron health indices (mean ± S.E.) from treatments with different C/N inputs and marron size classes.

A	C/N input = 9		
	Exp3_FM (n=3)	**Exp4_Control (n=3)**	**Exp5_Control (n=4)**
Marron size class (g)	76.36 ± 0.70 [a]	14.47 ± 0.06 [b]	4.15 ± 0.36 [c]
THC (x10^6 cells/mL)	2.33 ± 0.03 [a]	1.87 ± 0.04 [b]	1.84 ± 0.02 [b]
Granular cells (%)	30.17 ± 0.44 [a]	33.33 ± 0.88 [a]	30.63 ± 1.64 [a]
TM (%)	75.63 ± 0.78 [a]	80.24 ± 0.55 [b]	81.36 ± 0.32 [b]
HM (%)	69.93 ± 1.14	71.06 ± 0.28	70.90 ± 0.31
Tid (%)	7.08 ± 0.24 [a]	6.09 ± 0.18 [b]	6.02 ± 0.04 [b]
Hid (%)	1.72 ± 0.06 [a]	1.65 ± 0.01 [a]	1.69 ± 0.04 [a]

B	C/N input = 12	
	Exp.4_MBC12 (n=3)	**Exp.5_MBC12 (n=4)**
Marron size class (g)	16.15 ± 0.17 [a]	3.88 ± 0.03 [b]
THC (x10^6 cells/mL)	2.37 ± 0.17 [a]	2.35 ± 0.04 [a]
Granular cells (%)	36.17 ± 1.74 [a]	35.17 ± 1.79 [a]
TM (%)	80.78 ± 0.38 [a]	80.83 ± 0.34 [a]
HM (%)	72.73 ± 0.10 [a]	73.55 ± 0.18 [a]
Tid (%)	6.24 ± 0.02 [a]	6.41 ± 0.06 [a]
Hid (%)	1.63 ± 0.01 [a]	1.63 ± 0.05 [a]

Superscript letters in the same row show significant differences in marron health indices between treatments at p-value<0.05. Abbreviations: Exp. - Experiment; FM - fishmeal; MBC12 – molasses based carbon supplementation to obtain C/N ratio of 12; THC – total haemocyte count; TM – tail muscle moisture content; HM – hepatopancreas moisture; Tid - dry tail muscle index; Hid - dry hepatosomatic index.

9.5 Conclusions

1. The seasons significantly influenced the biotic and abiotic variables in commercial marron ponds.
2. There were significant correlations between sediment C/N ratios and various water quality parameters in semi-intensive marron ponds.
3. The research effectively characterized the resident microbial communities in marron pond sediments across seasonal cycles, identifying the environmental factors responsible for driving changes in these microbial communities. These factors will need to be considered in future research on marron.
4. Significant impacts on both water and sediment microbiota were observed due to the use of commercial diets with varying C/N ratio inputs, as well as the inclusion of probiotics.
5. Marron size distribution at harvest was influenced by different commercial diets used in pond farming conditions.
6. In our laboratory experiments, marron are isolated in cages, which eliminates agonistic interactions, thereby enhancing their survival and growth. Future studies should involve marron being freely housed in tanks.
7. Laboratory experiments indicated distinctions in the abundance of certain bacterial genera in response to dietary protein sources derived from FM and PBM.
8. Only a positive correlation between *Candidatus Bacilloplasma* and sediment C/N ratio was found at the end of the trial when marron was fed with the PBM diet.
9. Among dietary protein sources with a C/N ratio of 9, no significant impact on growth and health indices was observed, except for an increase in protease activity in marron hepatopancreas when fed a FM-based diet.
10. The addition of carbohydrates to enhance the C/N ratio input resulted in improved water quality, maintenance of a high C/N ratio, and enrichment of bacterial populations in sediments. Molasses emerged as the superior external carbon source for marron culture in the 11g size class, outperforming corn flour and wheat flour.

11. For marron in the 3g size class, increasing the C/N ratio input to 15 by augmenting molasses led to significant reductions in nitrogenous wastes in water and elevations in the C/N ratio in tank sediments.

12. While a C/N input of 12 effectively reduced the presence of *Vibrio*, a further increase in C/N input did not produce the same effect, and growth and health responses of marron were comparable between the two treatments.

9.6 Limitations and Recommendations

1. The research primarily concentrated on water quality parameters and sediment C/N ratios, with no quantification of C and N concentrations in the water of marron culture systems. Consequently, the correlation between C/N ratios in water and sediment could not be established.

2. In the context of pond culture systems, sediment sampling should be conducted at more frequent intervals and over multiple consecutive years to confirm seasonal variations in sediment characteristics. Additionally, sediments from marron ponds that host broodstocks warrant examination to facilitate comparisons with findings from juvenile ponds.

3. While plankton abundance in marron ponds was assessed, an evaluation of the benthos community, a crucial component of sediment quality in both pond and tank cultures, was absent.

4. The study primarily targeted protease activity in marron hepatopancreas. Future investigations should consider the inclusion of additional digestive enzymes, such as amylase and lipase.

5. Experiments involving carbon supplementation were solely conducted on 0+ marron. Further studies should explore 1+ marron.

6. The research did not quantify the bioflocs formed in the supplemented-carbon systems or their nutritional compositions, which are critical for assessing their contributions to marron growth and overall health.

7. Feeding rate reduction in marron culture systems with additional carbon sources could be the next research direction for the marron farming industry to reduce the feed costs.

8. Furthermore, assessing marron texture and flavour in biofloc culture systems is crucial for quality assurance and meeting consumer expectations.
9. It is recommended to have pond replications, if possible for future studies. Additionally, conducting a similar feeding trial in the laboratory would help confirm the results.

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APPENDIX

Appendix 1

(List of publications during PhD study)

1. FOYSAL, M. J., NGUYEN, T. T. T., CHAKLADER, M. R., SIDDIK, M. A., TAY, C.-Y., FOTEDAR, R. & GUPTA, S. K. 2019. Marked variations in gut microbiota and some innate immune responses of fresh water crayfish, marron (*Cherax cainii*, Austin 2002) fed dietary supplementation of *Clostridium butyricum*. *PeerJ*, 7, e7553.
2. FOYSAL, M. J., NGUYEN, T. T. T., SIALUMANO, M., PHIRI, S., CHAKLADER, M. R., FOTEDAR, R., GAGNON, M. M. & TAY, A. 2022. Zeolite mediated processing of nitrogenous waste in the rearing environment influences gut and sediment microbial community in freshwater crayfish (*Cherax cainii*) culture. *Chemosphere*, 298, 134276.
3. NGUYEN, T. T. T., FOYSAL, M. J., FOTEDAR, R., GUPTA, S. K., SIDDIK, M. A. B. & TAY, C. Y. 2021. The effect of two dietary protein sources on water quality and the aquatic microbial communities in marron *(Cherax cainii*) culture. *Microbial Ecology*, 82, 299-308.
4. NGUYEN, T. T. T., FOYSAL, M. J., GUPTA, S. K., TAY, A., FOTEDAR, R. & GAGNON, M. M. 2024. Effects of carbon source addition in rearing water on sediment characteristics, growth and health of cultured marron (*Cherax cainii*). *Scientific Reports*, 14(1), 1349.

Appendix 2

(Author contributions in published article)

	Concepts	Funding	Methods	Data curation	Investigation	Software	Formal analysis	Validation	Writing: Draft	Writing: Reviewing	Resources	Supervision	Total % contribution
Chapter 6.1 (Paper 1)- Title: The effect of two dietary protein sources on water quality and the aquatic microbial communities in marron (*Cherax cainii*) culture. *Microbial Ecology*, 82, 299-308.													
First Author Thi Thu Thuy Nguyen	√	√	√	√	√	√	√		√				82.5%
Co-Author 1 Md Javed Foysal			√	√			√		√				5 %
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 2 Ravi Fotedar		√						√		√	√	√	5 %
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 3 Sanjay Kumar Gupta							√		√				2.5%
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 4 Muhammad M.A.B. Siddik							√		√				2.5%
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 5 Chin-Yen Tay										√	√		2.5%
I acknowledge that these represents my contribution to the above research output. Signed:													
Total %													100

Appendix 3

(Author contributions in published article)

	Concepts	Funding	Methods	Data curation	Investigation	Software	Formal analysis	Validation	Writing: Draft	Writing: Reviewing	Resources	Supervision	Total % contribution
Chapter 7 (Paper 2)- Title: Effects of carbon source addition in rearing water on sediment characteristics, growth and health of cultured marron (*Cherax cainii*). Published in Scientific Reports.													
First Author Thi Thu Thuy Nguyen	√	√	√	√	√	√	√		√				82.5%
Co-Author 1 Md Javed Foysal			√	√			√		√				5 %
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 2 Sanjay Kumar Gupta			√						√				2.5 %
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 3 Alfred Tay			√								√		2.5%
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 4 Ravi Fotedar		√						√		√	√	√	5%
I acknowledge that these represents my contribution to the above research output. Signed:													
Co-Author 5 Marthe Monique Gagnon			√	√						√	√	√	2.5%
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												Total %	100

Appendix 4

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SPRINGER NATURE

The Effect of Two Dietary Protein Sources on Water Quality and the Aquatic Microbial Communities in Marron (Cherax cainii) Culture

Author: Thi Thu Thuy Nguyen et al
Publication: Microbial Ecology
Publisher: Springer Nature
Date: Jan 11, 2021

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Appendix 5

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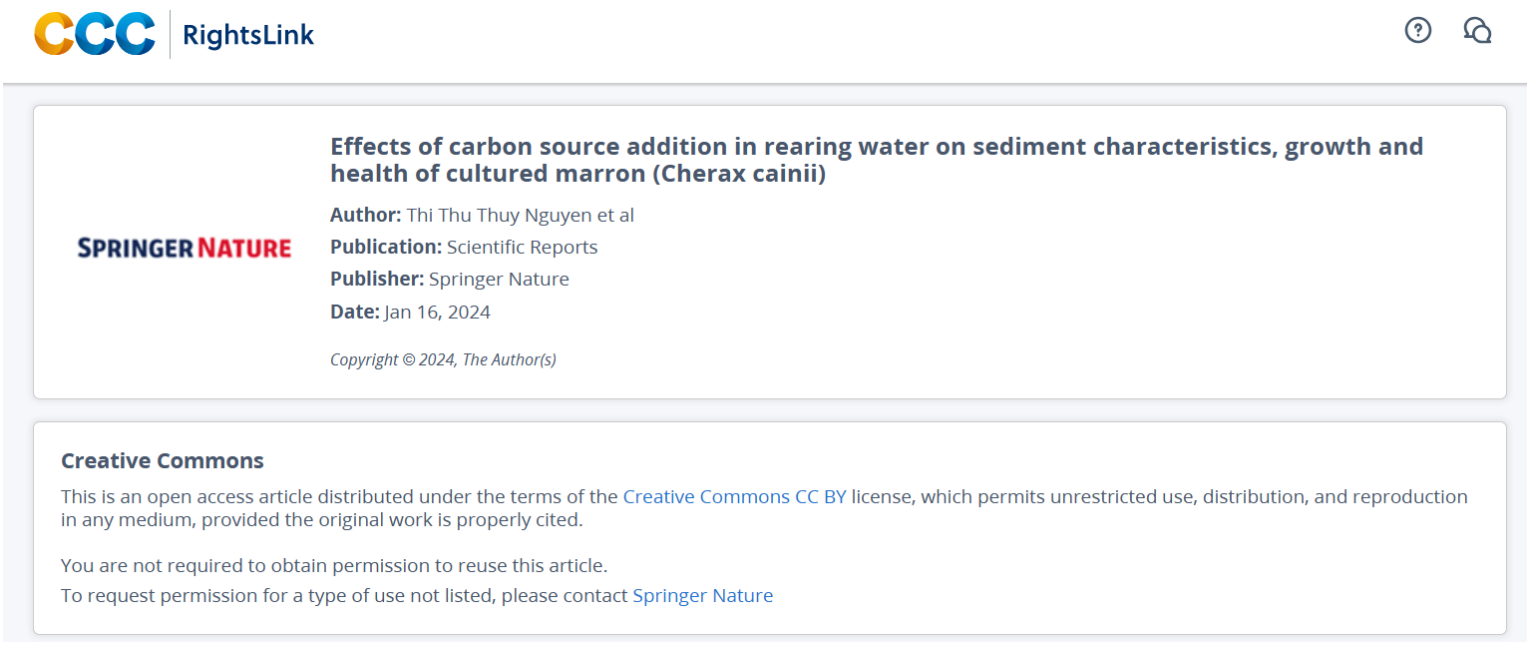

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SPRINGER NATURE

Effects of carbon source addition in rearing water on sediment characteristics, growth and health of cultured marron (Cherax cainii)

Author: Thi Thu Thuy Nguyen et al

Publication: Scientific Reports

Publisher: Springer Nature

Date: Jan 16, 2024

Appendix 6

(Data storage and availability)

All digital data have been kept in a personal computer and backed up into a USB hard drive. The amplicon sequence data of some chapters have been deposited in the National Centre for Biotechnology Information (NCBI) and are available under the BioProject with the accession numbers presented below:

Chapters	Accession numbers
4	PRJNA1015109
6.1	PRJNA613507
6.2	PRJNA1025831
7	PRJNA1015270