School of Molecular and Life Sciences

An Assessment of Digestibility and Water Stability of a Poultry By-Product Meal (PBM) based diet for Marron (*Cherax cainii*)

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This thesis is presented for the Degree of
Master of Philosophy
of
Curtin University

May 2021

Declaration

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

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

Abstract

Over the last 2 decades fish meal (FM) production has been steadily declining due to a decrease in wild fish stocks and as a consequence feed sustainability has been brought to the forefront of the aquaculture industry. The decrease in FM production coincides with the rapid expansion of the industry and with it an increase in diversity and volume of farm fed aquatic species. Currently, FM is the preferred protein in aquatic diets due to its “ideal” amino acid (AA) balance. However, the expansion of aquaculture globally has driven the demand for FM and fish oil products up and with no additional raw material expected from wild fish stocks the increasing demand for FM will need to be met by alternatives. Poultry by-product meal (PBM) has effectively replaced FM in various aquatic species diets (i.e *Litopenaeus vannamei, Cherax quadricarnatus, Penaeus monodon, Salmo salar* L., *Tilapia zilli*) and has an AA profile similar to FM making it an ideal candidate for substitution.

In this study, PBM was sourced from Talloman (Hazelmere, WA) as a protein alternative in crustacean diets. PBM from Talloman is marketed as >60% crude protein. Experiment 1 defined product “quality” as a product that consistently meets proximate composition standards, particle size distribution and milling standards as defined by manufacturing specifications. Proximate analysis and particle distribution analysis was conducted on random samples collected from Talloman over a 3 month period. Results found PBM to meet specifications consistently in all proximate specifications (crude protein, moisture, crude lipids and ash) and had a particle distribution similar to FM. The product is marketed advertised as a “free-flowing” powder and the majority (~70%) of feed particles were ≤250µm. This has positive flow on effects in terms of manufacturing capabilities as smaller more uniform feed particles aid in mixing and homogenizing of compound feeds so that each individual pellet reflects the entire feed composition aiding in nutrient delivery. Investigations in the potential of PBM is warranted as poultry production in Australia is estimated to continue rising over the next 5 years and this increase is reflected in projections for PBM production at Talloman. The sustainable supply of raw poultry by-products and consistency of the product is an added advantage as a protein source in aquatic diets.

In experiment 2 an experimental diet was formulated to utilising PBM as the primary protein source, at a crude protein level of 34%. PBM-based feed was made using an electronic meat mincer and dried at 60°C; a commercial marron feed (Specialty Feeds, WA) was purchased as a control diet. Marron were fed for 8 weeks twice per

day until satiated. Immune parameters of total and differential haemocyte counts were taken as an indicator for health. No significant difference between the PBM and control diets was observed in either growth or health of crayfish. *In-vitro* digestibility values obtained using commercial enzymes (pepsin, α-amylase and cellulase) were similar to *in-vivo* apparent digestibility values. In the livestock industry *in-vitro* digestibility feed evaluation is accurate, rapid and repeatable providing the industry with a useful and inexpensive tool to test the quality of newly formulated feeds. This study provided evidence for the application of livestock industry feed evaluation methods to be utilised for aquatic feed evaluation and demonstrated no adverse effects on growth or health of marron fed diets utilising PBM.

There is limited information available on the performance of PBM based aquatic diets in regards to water stability. However, it is important to identify interactions between the protein source and manufacturing processes used to produce a water stable feed targeted at aquatic animals. Experiment 3 presented evidence that there are interactions between drying temperature and feed shape with water stability and crude protein retention in feeds formulated using PBM. Physical instability (breakdown) and nutrient leaching in aquatic feeds can impact on feed utilisation depending on the target species. Crustaceans such as prawns, crayfish and crabs manipulate their feed using their appendages – this action can increase the physical disintegration of the feed. Therefore, these species require feed to be stable in water for a longer period of time than fish which are generally instant feeders. In the absence of a commercial extruding technology, drying temperature and feed shape are two factors that can be evaluated under lab conditions. This information would be useful to producers who make feed rather than purchase commercial products. This study compared 3 feed shapes (pellet, wafer and mash) and 3 drying temperatures (50, 60 and 70°C). The study investigated the effects of these factors on water stability and protein leaching of feeds utilising PBM as the major protein source. It was found that crude protein leaching was higher in marine water (33ppt) than freshwater (0ppt). This was attributed to a change in protein solubility due to the presence of chloride ions and low concentrations of other salts. Results showed feeds in the ‘wafer’ shape, dried at 60°C were most stable indicated by minimal physical disintegration and maximum protein retention. This alternative feed shape was more water stable and retained more protein than pelleted feeds (made using a meat mincer). This new shape could be applied in other experimental feed studies where sophisticated feed manufacturing machinery is unavailable. By improving the pellet stability of feed, the time the animal has to consume the feed is increased, which in turn improves feed

conversion ratio and growth performance of the animal and also can help to more accurately evaluate feed formulations. These 3 studies have revealed the valuable and applicable properties (quality, application and manufacturing capability) of PBM as an alternative protein in crustacean diets.

Acknowledgements

The work described here would not have been possible without the guidance and contributions of my supervisors, Dr. Sue Low and Dr. Ben Saunders. The work has been funded by Curtin University and supported by Talloman, specifically, Ben Barron and Andy Bennett. Rowan Kleindienst has been of great assistance in providing technical advice and support. I am very grateful for the help of numerous volunteers, in particular Tahlia Hendry, for assisting me with my field work and lab work on countless late nights. I would also like to thank my family and Robert Worst whose continued support has made this possible.

Table of Contents

List of Figures

List of Tables

List of Equations

List of Abbreviations

AA	Amino Acid
ADC	Apparent Digestibility Coefficient
AFMA	Australian Fisheries Management Authority
ANF	Antinutritional Factor
CP	Crude Protein
DH	Degree of Hydrolysis
DM	Dry Matter
DHC	Differential Haemocyte Count
ECOP	Environmental Codes of Practice
EAA	Essential Amino Acid
FAO	Food and Agriculture Organization
FCR	Feed conversion Ratio
FM	Fish Meal
MEMP	Management and Environmental Monitoring Plan
MSY	Maximum Sustainable Yield
MT	Million tonnes
NPF	Northern Prawn Fishery
PBM	Poultry By-product Meal
PL	Post-larvae
THC	Total Haemocyte Count

Chapter 1 – Introduction

1.1 Contribution of Aquaculture to World Fish Production

Aquaculture is defined by the Food and Agriculture Organization (FAO) as the farming of aquatic organisms in both coastal and inland areas (FAO 2019b). It involves interventions in the rearing process to enhance production such as selective breeding, modified growing environments and specialized feeding regimes. Some farmed examples include fish, crustaceans, molluscs, aquatic plants and algae. Farming systems can be extensive, semi-intensive or intensive, depending on the level of input/output per farming area and the stocking density. *Extensive* farming systems are defined as having no external nutrient input and aquatic species are dependent on naturally available food; *semi-intensive* farming uses a supplementary nutrient diet input as well as naturally available food and *intensive* farming requires complete nutrient diet input with growth entirely dependent upon external nutrient sources (Tacon and De Silva 1997; Naylor et al. 2000). Marine culture is practiced in the sea, also known as mariculture, coastal aquaculture is practiced in coastal ponds or gated lagoons and inland aquaculture is land-based and can be both saline and freshwater (FAO 2018).

Over the last three decades aquaculture's contribution to total world fish production has been increasing rapidly (figure 1.1) and in 2016 was estimated to have a first-sale value of 340 billion (AUD) (at 110.2 MT). Figure 1.2 shows the contribution from each aquatic species group with finfish contributing more than half (54.1 MT) of the total production, followed by molluscs (17.1 MT) and then crustaceans (7.9 MT). The category "Other" includes aquatic animals such as turtles, sea cucumbers, sea urchins, frogs and edible jellyfish (FAO 2018). Although world aquaculture's annual growth has slowed since the 1980s and 90s (10.8% and 9.5%, respectively), the sector remains to be one of the fastest growing major food sectors (Naylor et al. 2009; Deutsch et al. 2007).

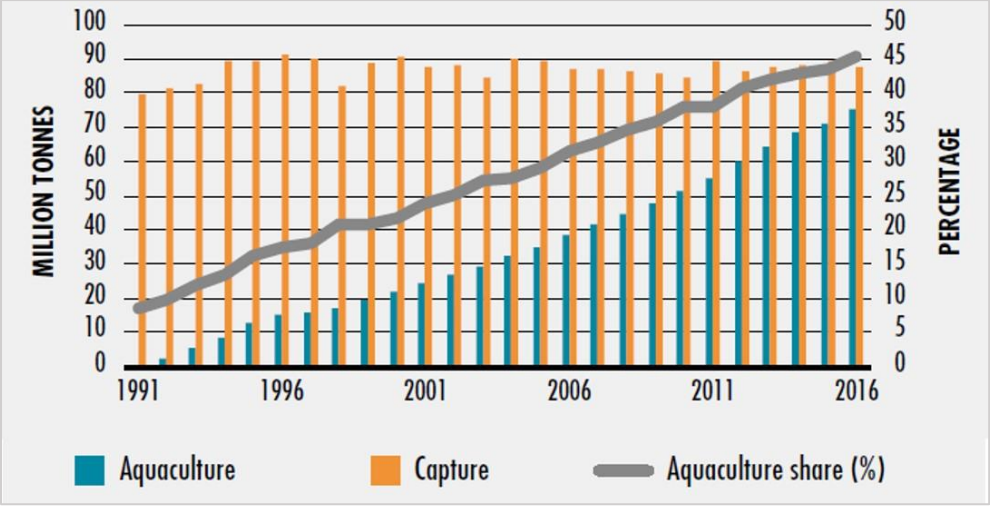

Figure 1.1 – Aquaculture contribution to total fish production (excluding aquatic plants) (FAO 2018).

The majority of the crustacean and mollusc production is cultured in marine and coastal environments, where cultured species are farmed either at sea or in human-made structures adjacent to the sea. Shelled molluscs make up 58.8% (16.9 MT) of marine and coastal aquaculture and crustaceans 39.9% (4.8 MT) (FAO 2018) globally. A diversification of the inland aquaculture mainly in crustaceans such as prawns, crayfish and crabs has been mirrored by the lessening dominance of the sector by finfish production (FAO 2018).

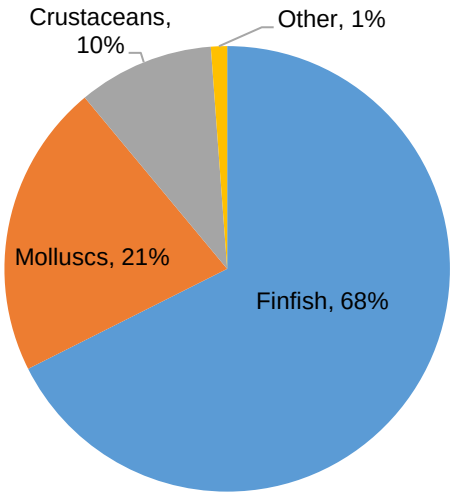

Figure 1.2 – Contribution of each aquatic group in 2016 of all farmed food fish production in the world (FAO 2018).

The overall increase in aquatic farming has had a trickledown effect on the diversity of farmed species. Globally the number of farmed species has increased by 26.7% in 2006 from 472 species to 598 in 2018 (FAO 2018). Even though species diversity continues to increase, aquaculture production by volume is dominated by a small number of “staple” species (table 1.1). Globally, finfish production is dominated by

carp species, crustaceans by Whiteleg Shrimp (Litopenaeus vannamei) with 53% of the total crustacean production and oysters contributing the most to the mollusc group. Finfish species are the most diverse group and rely on 27 species while crustaceans, molluscs and other animals are *much less diverse (6, 9 and 4 major species, respectively)*.

Table 1.1– Top 5 species (by volume) from each aquatic group farmed in 2014 and 2016 (thousand, tonnes) (FAO 2018).

Species	**(thousand tonnes)**		
	2014	2016	% of total, 2016
Finfish			
Grass carp (*Ctenophayngodan idellus)*	5, 539	6, 068	11
Silver Carp (*Hypophthalmichthys molitrix)*	4, 968	5, 301	10
Common Carp *(Cyprinus carpio)*	4, 161	4, 557	8
Nile tilapia *(Oreochromis niloticus)*	3, 677	4, 200	8
Crustaceans			
Whiteleg shrimp (*Litopenaeus vannamei*)	3, 697	4, 156	53
Red swamp crawfish *(Procambarus clarkia)*	721	920	12
Chinese mitten crab (*Eriocheir sinensis*)	797	812	10
Giant tiger prawn *(Penaeus monodon)*	705	701	9
Molluscs			
Cupped oysters nei *(Crassostrea spp.)*	4, 374	4, 864	28
Japnese carpet shell (*Ruditapes philippinarum)*	4, 014	4, 229	25
Scallop nei *(Pectinidae)*	1, 650	1, 861	11
Marine molluscs nei *(Mollusca)*	1, 135	1, 154	7

1.2 Farmed Aquaculture

The growth in farmed aquatic species has seen an increase in the number of both fed and non-fed species, although growth has been faster in fed species (figure 1.3). Fed species can be defined as species that are fed using purchased resources in the form of compound, farm-made or live feed in semi-intensive and intensive culture systems.

Non-fed species are species that are farmed and sustained with naturally occurring feed resources (i.e filter feeding oysters) (Fry et al. 2018; Tacon and De Silva 1997). Fed aquatic animals raised on commercial or farm-made feed reach harvest weight faster than non-fed animals where no feed is admnisterd to animals (Hasan et al. 2007; Fry et al. 2016). Fed aquaculture makes up approximately 70% of aquatic-based animal production (Hua et al. 2019).

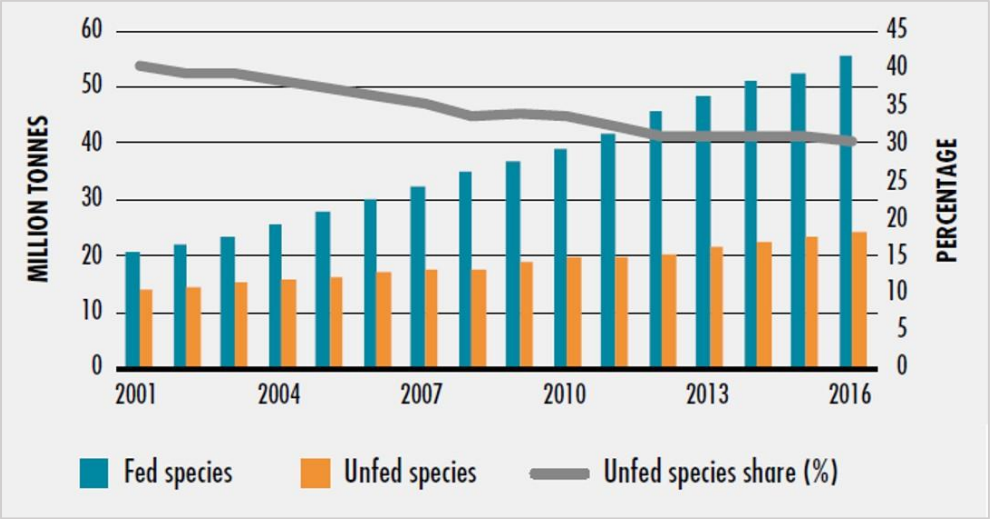

Figure 1.3 – Global Fed and Non-Fed Food Fish Aquaculture Production (FAO 2018).

In 2008, 46.1% of the total global production of farmed aquatic animals and plants was dependent on the supply of externally provided fresh, farm-made or commercial pellet feeds i.e fed production systems (figure 1.4) (Tacon, Hasan and Metian 2011). Of the fed species, carps and other cryprinids (9 species) accounted for 67% of fed freshwater fish, marine prawns accounted for 93% of fed marine crustaceans and salmon for 48% of the fed diadromous fish. Marine fish and freshwater crustaceans were not dominated heavily by one species. The expansion in the farmed fish sector has caused a sharp rise in demand for products in particular fish meal and fish oil for use in feed products. Fish meal is considered an ideal protein for aquatic feeds as it has an ideal proportion of essential amino acids that closely corresponds with the requirements of aquatic animals (Nguyen 2017). The majority of these products are obtained from the wild. Capture fisheries whose catch is not used for direct human consumption are utilized in the “industrial sector” for reduction into fish meal and fish oil products (FMFOP) for other human food supply systems (poultry, pigs, aquaculture etc.), adding to the pressure on wild fish stocks (Péron et al. 2010). The sustainability of these fisheries has come into question and whether they can meet the demands of the growing aquaculture industry.

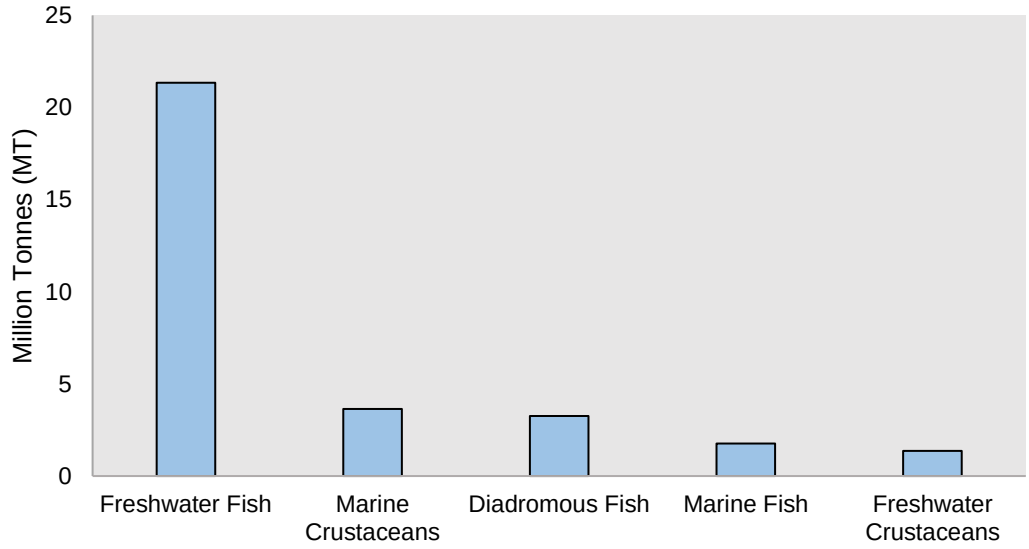

Figure 1.4 – Global volume of farmed fed species groups in 2008 (Taco, Hasan and Metian 2011).

Aquaculture is forecasted to overtake capture fisheries as the primary source of fish and seafood worldwide by 2020 (OECD/FAO 2019). To feed the growing volume of fed farmed fish, wild fish, capture has gradually shifted from large high value species to smaller less-valuable species (Naylor et al. 2000). For example, anchoveta, Chilean jack mackerel, Atlantic herring, chub mackerel, Japanese anchovy, round sardinella and European anchovy are all small pelagic fishes that are used in fish meal production for use in animal feeds including aquatic diets (Naylor et al. 2000). Compound feeds that contain fish meal are used predominantly used to feed carnivorous finfish such as salmon (*Salmo solar)* and marine prawns such as Whiteleg Shrimp (*Litopenaeus vannamei).* Carnivorous finfish and marine prawns need a supply of essential amino acids (particularly lysine and methionine); these amino acids are deficient in plant proteins but found in animal proteins such as fish meal (De Silva and Anderson 1998). However, compound feeds targeted at herbivorous and omnivorous finfish can also contain low to moderate amounts of animal protein (Naylor et al. 2000). Generally, herbivorous and omnivorous finfish are able to utilize plant-base proteins obtained from crops and other agricultural by-products and are considered net protein producers, producing more protein in the body biomass than was consumed (Fry et al. 2016). Carnivorous species are moving slowly towards becoming net protein producers by replacing the fish meal and fish oil with non-marine ingredients. For example, in 1990 90% of ingredients in Norwegian salmon feed were of marine origin, however, by 2013 this proportion was reduced to 30% with inclusion rates of fish meal of 18% and fish oil 11%. That same year it took 0.7 kg of marine protein to produce 1 kg of salmon protein making Norwegian salmon a net protein producer (Ytrestøyl et al. 2015).

The diverse production systems used in aquaculture give rise to a paradox where aquaculture is a possible solution to the development of sustainable fish resources and also a contributing factor to the depletion of wild fish stocks (Naylor et al. 2000). These systems may also have a negative impact on the environment. For example, farming of prawns may cause environmental damage through waste discharge, disease spread and habitat destruction and is heavily reliant on capture fisheries for feed (Naylor et al. 1998). In contrast, farming of herbivorous species such as carp or filter feeders (e.g. molluscs) and some carnivorous species do not rely solely on capture fisheries for feed and therefore have a net contribution to fish production, (Naylor et al. 2000; Ytrestøyl et al. 2015). Of the 2012 total catch, 86% was directly consumed by humans. The remaining 14% (21.7mt) was used for non-food products, mainly in the manufacture of fish meal and fish oil (FAO 2015). The demand for fish meal is rising at 5% per year and feed is the industry's largest production cost (Naylor et al. 2000; Cruz-Suárez et al. 2007). If the aquaculture sector is to sustain its current growth and to alleviate pressures on wild fisheries, utilization of current sources needs to be improved as well as sourcing suitable fish meal alternatives such as poultry by-product meal, soybean meal, insects or algae (OECD/FAO 2019; Tacon and Metian, 2008).

1.3 The State of Crustacean Aquaculture

1.3.1 Global

The main crustacean species farmed globally are marine prawns (*L. vannamei* and *P. monodon*) 60% of global crustacean production, freshwater prawns (*Machrobrachium nipponense* and *Macrobrachium rosenbergii*) 5%, crabs (*Eriocheir sinensis*) 8% and crayfish (*Procambarus clarkii*) 18%. Between 2013 and 2017 the global crustacean trade saw a rise, where crustacean imports increased in the US, the EU, and China. The main suppliers being India, Vietnam, Mexico, and Ecuador due to higher more efficient production in these areas (Byrne 2019). Although produced in modest numbers crayfish are an ecologically and commercially important animal that are consumed as a luxury food around the world, they also hold customary value in some countries such as Sweden and Finland (Seemann et al. 2015; Harlioglu and Farhadi 2017; Crandall and Buhay 2008). Crayfish farming is carried out by China, USA and Europe farming *Procambarus clarkii* as the main species and Australia farming *Cherax caiini, C. quadricarinatus and C. destructor* (Harlioglu and Farhadi 2017).

1.3.2 Australian Seafood/Fishing Industry

In Australia, combined aquaculture and wild caught seafood production increased by 4% between 2007 and 2017. Over this time period, production of aquaculture products grew by 53% (93,968 tonnes) (Mobsby 2018). Aquaculture products accounted for 44% of the total production value of Australian produced seafood ($1.3billion). In the same period the value of wild-caught fisheries declined by 0.4% worth $1.7 billion (AUD) (Mobsby 2018). The increase in contribution from aquaculture to seafood supply in Australia is consistent with the global trend of meeting increasing demand for seafood from aquaculture.

Although only a minor contributor to global fish production (0.16%), exported Australian products are high unit value products. Australian products are highly sort after by multiple countries such as Japan, Hong Kong and Vietnam (Whittle et al. 2015; Mobsby and Koduah 2017). Additionally, seafood consumption in Australia has increased annually by 1.1% from 2006 to 2016 (Mobsby and Koduah 2017). Local production of seafood has remained steady over the same period, producing approximately 110,000 tonnes annually and the increase in demand has been met by imported products mainly tuna, frozen fish, frozen prawns and preserved and prepared prawns (Mobsby and Koduah 2017).

1.3.3 Australian Crayfish

There are three main species of crayfish farmed in Australia; *Cherax cainii* (a.k.a smooth marron) and *Cherax tenuimanus* (a.k.a hairy marron) both are known as ‘marron’ and *Cherax destructor* known as ‘yabby’. In the year of 2017-18, 65.8 tonnes of marron was produced in Australia with a value of $3million (AUD) and 51.5 tonnes of yabby produced with a value of $1.4million (AUD). A significant increase in production value from the previous year – 19% increase in marron production and 75% increase in yabby production (Steven et al. 2020). The Australian freshwater crayfish industry has had a slow growth and therefore is much smaller in size and volume. In Western Australia (WA) until the 1960s marron were grown on farms that also produced cattle or sheep. Export of marron overseas was prohibited until 1980 when legislation was passed to allow sale of marron globally (Fotedar et al. 2015). In 1995 and 1998 changes to legislation were made to make marron farming more attractive. As of 2015 there were 188 licensed marron growers in WA, table 1.2 shows production of marron in the late 2000’s and number of licensed growers. Marron are now exported to the EU and Asia (Fotedar et al. 2015).

Table 1.2 – Production of marron in Western Australia and number of licensed marron growers (Fotedar et al. 2015, pg. 16).

Year	Marron Production (tonnes)	# of Marron Growers
2009	52	183
2010	52	188
2011	51	184
2012	52	175

Although still in its infancy, marron farming is Western Australia's largest aquaculture sector, this sector contributes to the diversity of Western Australian aquaculture as well as diversifies and supplements income of farmers leaving them more robust to economic volatility (Department of Fisheries in WA 2015).

1.4 Sustainable Practices

The main pieces of legislation that regulate aquaculture in Australia are the *Fish Resources Management Act 1994* and the *Fish Resources Management Regulations 1995.* The *Fish Resources Management Act 1994* states that all commercial aquaculture activities in Australia require a license (Government of Western Australia 2012). With the license application a management and environmental monitoring plan (MEMP) is required to manage any potential environmental impacts (Department of Fisheries 2013). In Western Australia, MEMPs are developed with guidance from Environmental Codes of Practice (ECOP) developed by the Aquaculture Council of Western Australia. There are 7 ECOPs outlining codes for the 7 major aquaculture sectors in Western Australia; abalone, pearls, land-based finfish, marine finfish, marron, mussel and oyster and prawn farming (ACWA 2019). ECOPs provide guidelines on facility operations and risk management; minimization of environmental impacts; water quality and waste management. For example, if feed ingredients come from overseas, they must undergo quarantine testing, a requirement of the Australian Quarantine and Inspection Service to mitigate biosecurity risks. In addition, licensees must notify the Department of Fisheries of the presence or suspicion of any notifiable disease to protect WA's ecosystems and policy guidelines outlined by the Department of Fisheries must be followed to minimize disease spread during translocation of animals between regions (ACWA 2013). This legislation aims to develop and manage the industry in a sustainable way and to share and conserve the nation's aquatic resources and their habitats for the benefit of present and future generations.

1.5 The Fish Meal Crisis

Fish meal (FM) is one of the major sources of protein used in fish feeds not only for its desirable nutritional and palatability characteristics (protein content and amino acid content) (Cruz-Suárez et al. 2007) but also as a source for nucleotides, essential fatty acids, phospholipids, minerals and fat and water soluble vitamins (Tacon et al. 2009). The majority of FM is sourced from small pelagic fisheries comprised of sardine, anchovy, capelin and menhaden fish species as whole fish (Barlow 2003b). The sourcing of raw materials for feed from wild capture fisheries has negatively impacted the fisheries and stocks have been declining due to fishing pressures (FAO 2018). It is estimated that approximately 10% of the fish biomass caught from wild fisheries is destined to be used as feed to high-value farmed species bringing in to question the sustainability of the commodity (Hua et al. 2019; FAO 2018). Currently only 33% of the world's FM production comes from fish by-products (fish offal) (Jackson and Newton 2016) (figure 1.5). In 1994 global FM production was at its peak yielding 30 MT (wet weight) and since then has exhibited a fluctuating but overall declining trend (Barlow 2002). However, due to improved supply conditions in Peru, the main supplier of fish meal and oil products, an increase in trade has been observed in the last 4 years (Byrne 2019). Hua et al. (2019) estimate an additional 37.4 million tons of aquafeeds will be required by 2025 with the increasing demand from farmed seafood, therefore making the use of forage fish to meet this demand unsustainable. Thus, with decreasing quantities of raw material expected from wild caught fisheries the increasing demand for FM products will rely more heavily on the use of alternative proteins such as by-products (Delgado 2003; Kobayashi et al. 2015; FAO 2018). Future projections estimate FM production to be 19% higher in 2030 than in 2016, where 54% of the growth is derived from fish by-products (FAO 2018). As well as the use of fishery and aquaculture by-products, Hua et al. (2019) highlights the potential use of food waste through biotransformation and/or bioconversion of raw waste materials.

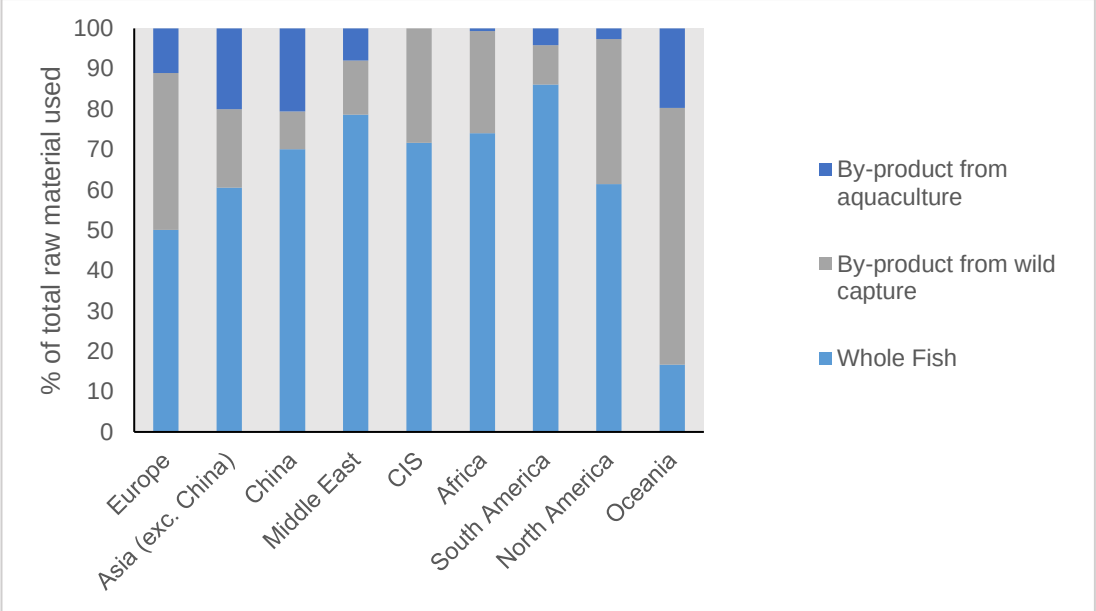

Figure 1.5 – Source of raw materials used for fish meal production as of 2016 (Institute of Aquaculture, University of Sterling and Marine Ingredients Organisation (IFFO) in Seafish, 2016).

Possible consequences of increased use of fish by-products include lowered protein, increased ash (due to inclusion of fish frames) and inconsistency in comparison to fish meal derived from whole fish and therefore will need to be accounted for during the processing of fish by-products (FAO 2018). Fish by-products, especially those that contain viscera, deteriorate rapidly and therefore need to be preserved or processed as soon as possible after being produced (Olsen et al. 2014). Even at low storage temperatures the lipids in fish by-products are susceptible to rapid degradation and oxidation (Rustad et al. 2011). At sea by-products are generally discarded due to lack of space; however, if caught close enough to shore or if resources permit (on-board processing facilities) by-products can be brought to shore to be processed fresh or stored (Rustad et al. 2011; Ramirez 2013; Olsen et al. 2014). The key factor to utilization of by-products is retaining the quality of raw materials or ideally processing by-products immediately after collection (Rustad et al. 2011). In this sense aquaculture by-products have an advantage over by-products produced at sea. By-products that are produced on land can be quickly transferred to processing facilities (Rustad et al. 2011). The addition of onboard technologies for handling by-products for example, refrigeration can also add value to fishing fleets (Falch et al. 2007).

Consequently, the rapid expansion in volume and diversity of farmed fish, crustaceans, molluscs and other aquatic animals has increased the demand for FM

and fish oil (Kobayashi et al. 2015; Rana et al. 2009). Yield from small pelagic fisheries that produce a major portion FM and fish oil products to feed farmed fish have been declining due to decreasing catch volumes (Lazzarotto et al. 2018). The main issues faced by the aquaculture industry arise from (i) the lack of sustainability of feed inputs which are derived from wild capture fisheries, (ii) the increasing reliance on fish by-products to supplement this decline and, in turn, (iii) the inherent variability from use of such materials due to lack of processing facilities on board fishing vessels that see potentially valuable raw materials discarded at sea. In order to sustain the growing aquaculture sector sourcing a sustainable protein alternative has become an industry priority. The complex nature of fish nutrition in regards to nutritional requirements, physical stability of feeds and quality of both raw and compound feeds must all be taken into account when assessing the viability of a replacement product.

1.7 Potential Fish Meal Replacement Protein Sources

In the last two decades inclusion rates of FM and fish oil have steadily decreased and feed prices although still increasing, are no longer as volatile as they would be if previous formulations were still sold today (Turchini et al. 2019; Tacon et al. 2011; Tacon and Metian 2015). This trend has mainly been influenced by decreasing availability of marine resources and the need for more sustainable feed inputs. Modern aquafeeds are a sophisticated mix of raw ingredients made up of commodity meals, oils, pigments, minerals and concentrates that are intended to facilitate efficient production of aquaculture species (Hua et al. 2019; Turchini et al. 2019). Turchini et al. (2019) highlights the importance of nutrient-based formulations and advocates that nutritionist's role is to identify a combination of ingredients that satisfy the target species' dietary requirements and tolerances and that can be manufactured to optimal pellet specifications. While FM and fish oil can greatly simplify this formulation, they can be replaced by other ingredients. Nutrient-based formulation requires very detailed nutrient data sets. However, what is often overlooked as an important insight to diet formulation is data on digestibility, palatability, utilization and functionality (on at least one representative species) of ingredients. Products need to also be judged on how they complement other raw materials and not just on their similarity to marine-derived ingredients (Turchini et al. 2019).

1.7.1 Plant Protein Meals as Potential Replacement for Fish Meal

Many FM replacement studies have been conducted using plant alternatives such as soy bean and canola as well as other plant processing by-products such as sunflower oil press cake (table 1.3). Plant protein meals such as soybean meal or canola meal have been recognized as possible substitutes due to having sufficient supply, low

price and relatively good amino acid balance (Gatlin et al. 2007; Biswas et al. 2017). For some finfish diets, FM has been completely replaced using plant protein meals such as rapeseed protein concentrate (Slawski et al. 2012; Kissil et al. 2000) or canola meal (Burel et al. 2000). Pakravan et al. (2016) achieved complete FM replacement in *Litopenaeus vannamei* using *Spirulina platensis* (marine microalgae).

However, limitations to using plant protein meals as FM replacements include poor palatability, limiting essential amino acids (i.e methionine and lysine) and presence of antinutritional factors (Gatlin III et al. 2007). Huang et al. (2018) evaluated the amino acid profiles of 4 diets that utilised common plant protein meals (soybean meal, cottonseed meal, rapeseed meal, and peanut meal) by replacing 25% of FM in *Litopenaeus vannamei* diets (diets were isonitrogenous). All 4 diets that contained plant protein meals had lower methionine levels than diets containing FM and prawns fed rapeseed meal and peanut meal had significantly lower survival rates. Forster and Dominy (2006) demonstrated that lack of methionine supplementation in diets that contained soybean meal significantly lowered prawn growth rate; optimal methionine supplementation was equivalent to 1.5% of total amino acids.

Plant protein meals may contain undesirable components known as antinutrional factors (ANF). ANF's can be defined as "substances which by themselves, or through their metabolic products arising in living systems, interfere with food utilisation and affect the health and production of animals" (Francis et al. 2001. pp. 199). For example, soybean meal, rapeseed meal and sunflower oil cake contain protease inhibitors, tannins and lectins which affect protein utilisation and digestion (Francis et al. 2001). ANF's need to be removed or deactivated prior to being incorporated into aquatic diets; methods of removal include fermentation which can predigest ANF's and improve digestibility (Refstie et al. 1999; Park et al. 2003) or removal through heat treatment (van der Poel and Blonk 1990).

Furthermore, a study testing the effects of various dietary protein meals on the performance of freshwater crayfish *(Cherax tenuimanus)* found that the inclusion of lupin protein significantly lowered the protein levels in the hepatopancreas (digestive gland) when compared to those fed on animal protein, from 27% CP in the heptopancreas of those fed with lupin protein to 30% CP in the heptopancreas of those fed with fish protein (Fotedar 2004). The digestive gland is where hemocyanin, a protein contained in haemolymph is created. Increased dietary protein has resulted in increased concentrations of protein in crayfish haemolymph suggesting better physiological condition in lobsters (Martin and Hose 1995; Castell and Budson 1974;

Fotedar 2004). Haemolymph is comprised of various cells (i.e granulated, hyaline) that are involved in crustacean immunity and defence and are used an indicator of health (Bachere 2000; Cheng et al. 2004; Yang et al. 2000).

Table 1.3 – Plant products used in aquatic feed studies to replace fish meal. Percent of FM replaced by plant protein is the maximum amount able to be replaced without adverse effects on performance.

Plant Product	Species	Amount of FM replaced (%)	Specific Growth Rate (%/day)	Reference
Soy Bean Meal	Red Sea Bream *(Pagrus major)*	10	2.30	Biswas et al. 2007
	Korean Rockfish *(Sebastes schlegeli)*	20	3.01	Lim et al. 2004
	Spotted-nose snapper *(Lutjanus guttatus)*	20	1.27	Silva-Carillo et al. 2012
	Pacific Bluefin Tuna *(Thunnus orientalis)*	10	24.6	Biswas et al. 2011
Canola Meal	Angel Fish *(Pterophyllum scalare)*	24	1.50	Erdogan and Olmez 2010
	Yellow Croaker (*Pseudosciaena crocea*)	26	3.60	Zhang et al. 2008
Rapeseed Protein concentrate	Rainbow trout (*Oncorhynchus mykiss*)	100	1.1	Slawski et al 2012
	Gilthead sea bream *(Sparus aurata)*	100	1.92	Kissil et al. 2000
Spirulina platensis (Marine microalgae)	Pacific White Shrimp *Litopenaeus vannamei*	100	1.71	Pakravan et al. 2016
Sunflower Oil Cake	Black Tiger Prawn. (*Penaeus monodon*)	20	1.76	Dayal et al. 2011

In conclusion, while there have been promising results in FM replacement with plant protein meals such as soy bean meal and spirulina in some aquatic diets, lower amounts of lysine and methionine in these products require feeds to be supplemented with individual amino acids, (Furuya et al. 2004). Further research on the effects of utilising plant protein meals in crayfish diets may be required to ensure no adverse health effects would arise from using these protein meals. Partial replacement of FM using plant protein meals has been shown to be effective and is valuable in reducing FM amounts in aquatic diets. However, complete replacement is likely to require supplementation of limiting amino acids and additional processing costs.

1.7.2 Animal By-Product Meals as a Fish meal Alternative

To replace FM in aquatic diets without adversely affecting growth performance the replacement protein needs to provide the optimum content and ratio of amino acids needed for optimal growth (Miles and Chapman 2007). Currently, FM is the

considered the first ideal protein feedstuff as it has an amino acids profile very similar to the target amino acid content of the farmed product. Nutritionists formulate feeds to match the profile of this product (Trushenski et al. 2006). In addition, there is a qualitative difference in essential fatty acid (EFA) requirements between freshwater, diadromous and marine fish species (Oliva-Teles et al. 2015). It has been found that EFA requirements for marine fish are only met with long-chain polyunsaturated fatty acids (LC-PUFAs; namely 20;5n-3 and/or 22:6n-3) (Oliva-Teles et al. 2015; Sargent et al. 2002). Fish oil is a rich source of these LC-PUFA's therefore, making it essential to cover EFA requirements in addition to amino acids and proteins (Oliva-Teles et al. 2015; Turchini et al. 2009). For several decades terrestrial animal by-products have been used in aquatic feeds. During the 1930's to 1970's salmon and trout were fed on semi-moist "meat meal mixtures" made up of unprocessed slaughterhouse by-products such as beef, pork or horse liver and spleen; these products were deemed as cost effective sources of protein (Bureau 2006). Stickney (2000) outlines the types of animal by-products that have been utilised in aquatic diets in the past such as poultry by-product meal and meat and bone meal (table 1.4). Table 1.5 shows studies that have utilised these products in experimental feeding trials of different aquatic species.

The use of animal by-products to completely replace FM in aquatic feeds has had varying success. Lysine deficiencies have been demonstrated as a limiting amino acid in using feather meals. For example, FM could only be replaced up to 15% before negatively affecting growth of rainbow trout (*Oncorhynchus mykiss)* (Bureau et al. 2000). Feather meal has been reported to contain lower amounts of essential amino acids such as lysine, methionine and histidine when compared to FM (Hertampf and Peidad-Pascual 2000). Higher replacement levels have been achieved using poultry by-product meal or meat and bone meal in freshwater prawn (*Macrobrachium nipponense*) diets (50% FM replacement for both meals) (Yang et al. 2004). Conversely, the inclusion of meat and bone meal in marine fish diets has been observed to negatively affect fish performance when included at levels greater than 18% (Kikuchi et al. 1997) and >24% (Bureau et al. 2000).

Table 1.4 – Animal By-products used in aqua-diets (Stickney 2000).

Product	Processing Procedure	Crude Protein Content	Suitability in Aqua-Diets
Fish meal	Prepared from dried ground tissues or whole marine fish or from fish waste.	~55% to 75%	Amino acid profile and apparent digestibility highly suitable
Prawn meal and crab meal	Prepared from the waste of prawn processing.	~32%	Good source of *n-3* fatty acids.
Meat and bone meal	The rendered product of beef/pork/sheep processing	~45 to 50%	Lower lysine than FM and high ash content.
Blood meal	Prepared from fresh animal blood.	~80 to 85%	Good lysine source, less methionine than FM.
Poultry by-product meal	Prepared from the heads, feet, underdeveloped eggs and visceral organs (does not contain feathers) of poultry.	~58%	Often used as a partial replacement in aqua-diets.
Poultry feather meal, hydrolyzed	Prepared by the high-pressure treatment of clean, undecomposed feathers from poultry.	~85% protein	High protein, can have a low apparent digestibility.

Table 1.5 – Animal by-products tested in aquatic species diets to replace fish meal. Percent of FM replaced by animal protein is the maximum amount able to be replaced without adverse effects on performance.

Animal By-Product	Species	Amount of FM replaced (%)	Crude Protein (%)	Specific Growth Rate (%/day)	Author(s)
Meat and bone meal (MBM)	Fresh water Prawn (*Macrobrachium nipponense)*	50	38	0.74	Yang et al. 2004
	Gilthead seabream *(Sparus aurata)*	50	44	1.94	Moutinha et al. 2016
	Rainbow trout *(Oncorhynchus mykiss)*	24	48	2.46	Bureau et al. 2000
Poultry by-product meal (PBM)	Fresh water Prawn (*Macrobrachium nipponense)*	50	38	0.63	Yang et al. 2004
	Crayfish *(Pacifastacus leniusculus* Dana, Astacidae)	45	50	4.37	Fuertes et al. 2013
	Whiteleg Shrimp *(Litopenaeus vannamei)*	80	35	1.22	Cruz-Suárez et al. 2007
	Cobia *(Rachycentron canadum)*	30	45	4.59	Zhou et al. 2011
	Black sea bream *(Acanthopagrus schlegelii)*	16	42	2.9	Gao et al. 2013
Blood meal	Hybrid catfish *(Clarias gariepinus x Heterobranchus bidosarlis)*	10	-	1.58	Aliu and Dako 2018
Poultry feather meal blended with poultry by-product meal	Giant Croaker *(Nibea japonica)*	40	47	3.18	Wu et al. 2018
Poultry feather meal blended with meat and bone meal	Nile tilapia *(Oreochromis niloticus)*	66	25	5.9	Bishop et al. 1995
Poultry feather meal	African catfish *(Clarias gariepinus)*	20	40	7.79	Chor et al. 2013
	Rainbow trout *(Oncorhynchus mykiss)*	15	55	1.73	Bureau et al. 2000

Limitations of utilizing Poultry By-Product Meal (PBM)

Poultry by-product meal (PBM) is obtained from rendering poultry offal (heads, feet, underdeveloped eggs, carcasses and visceral organs, excluding feathers) produced as a waste product from chicken processing and is not used for human consumption (Cruz-Suárez et al. 2007; Stickney 2000). Like most FM alternative growth studies PBM limitations arise in the form of limiting or variable amino acids and/or ratios and crude protein content. For example, methionine levels have been shown to be a limiting factor when included in aquafeeds for salmonids (Dong et al. 1993) and cobia (*Rachycentron canadum*) (Zhou et al. 2011). Nengas et al. (1991) explained in a study of gilthead seabream (*Sparus aurata* L.) that the first limiting essential AA when utilising PBM was methionine and that the presence of cystine can reduce the amount of methionine necessary to maximise growth, highlighting the importance of AA ratios.

Variation in crude protein and amino acid content of both FM and PBM from different origins is highlighted in table 1.6. The table shows FM used in 7 studies had a range of 40-75% crude protein content and PBM used in 6 studies had a range of 47-72% crude protein content. However, table 1.6 shows the large variation in methionine levels of FM from different sources, Peruvian FM has 1.72% methionine and Spanish FM has 4.24% methionine. Likewise, the levels of methionine in PBM products also can have a large range and depending on source can have increased levels of methionine when compared to FM. For example, when comparing methionine levels of PBM from Spain (2.04%) to FM from Peru (1.72%) or China (1.59%). This inconsistency in product can be attributed to a number of factors such as whether raw materials are by-products (fish offal) or whole fish or a combination and the differing processing techniques. Excessive heating can denature proteins and lower the overall crude protein levels and digestibility of amino acids (Hamilton 2004). Wang (1996) investigated the effect of processing temperature on amino acid digestibility and found that lysine and methionine digestibility was increased when raw poultry materials were processed between 100-130°C when compared to higher temperatures (>140°C). Lysine digestibility increased from 82.3% to 84.6% and methionine increased from 88.4% to 90.2%.

Additionally, the “freshness” or the time between slaughter and raw material processing directly affects the quality of the product. Biogenic amines are derived from the decarboxylation of amino acids and are non-volatile low molecular-weight nitrogenous organic bases (Feddern et al. 2019). Higher amounts of toxic amines can be found in food products or feedstuffs (such as fish or meat products) due to poor

quality raw materials, long times between slaughter and processing and microbial contamination and inappropriate storage conditions (Feddern et al. 2019). Biogenic amines are resistant to heat applied during processing and therefore have been considered good indicators of raw-material quality and processing conditions (Feddern et al. 2019). Pike (1991) demonstrated that as storage time of raw fish material increased total volatile nitrogen content and biogenic amines levels increased. Salmon fed on FM made of “fresh” (processed within 12 hours of catching) raw materials had significantly better feed conversion ratios (FCR = 0.89) than those fed with “moderately fresh” FM (processed within 48 hours), FCR = 0.95 and those fed “stale” (processed after 7 days), FCR = 1.47. In regards to PBM, the transport, handling and processing of poultry by-products within Australia is governed by standard operating procedures (within each processing plant) and federal legislation to help manage the risks associated with animal feed manufacture (PISC 2007). The primary objective of the protocols is to provide safe rendered products by ensuring hygienic rendering of biological material from poultry farms. These standards ensure that spoilage of raw material is minimized and helps to improve the quality in terms of consistency of the meal by standardizing operations throughout the country.

Table 1.6 – List of essential and non-essential amino acids found in Fish Meal and Poultry By-product meals from different origins.

Protein Source	FM	FM	FM	FM	FM	FM	FM	Flash Dried PBM	PBM	PBM	PBM	SBM+PBM (co-extruded)	PBM
Origin Country	Lima, Peru	Shandong, China	Burgos, Spain	Tasmania, Australia	NIL	Shandong, China	Beren, Norway	Kentucky, USA	Hong Kong China	Burgos, Spain	Shandong, China	Missouri, USA	North Carolina, USA
Crude Protein (%)	67.8	64.2	67.9	74.9	40.95	61.6	68.85	72.2	67.2	60.1	47.8	53.1	62.17
Essential Amino Acids													
Arginine	3.45	3.96	9.78	4.10	5.30	3.72	4.24	5.60	4.11	9.24	2.65	3.72	4.47
Histidine	1.74	1.82	1.29	1.30	2.20	1.72	1.65	1.36	1.76	1.00	0.81	1.29	1.39
Isoleucine	2.59	2.74	3.51	2.90	2.70	2.58	3.16	2.84	2.75	2.93	1.53	2.32	1.97
Leucine	4.67	5.07	4.54	5.60	4.30	4.70	5.39	-	4.97	4.47	2.75	-	4.00
Lysine	4.76	5.28	6.11	5.50	5.20	4.33	5.75	4.53	4.53	3.94	2.65	3.20	3.42
Methionine	1.72	1.82	4.24	2.10	2.40	1.59	2.15	0.91	1.63	2.04	0.51	0.89	1.13
Phenylalanine	2.62	2.74	2.91	3.00	3.70	2.58	2.95	2.99	2.76	2.11	1.63	2.61	2.20
Threonine	-	2.94	3.73	3.20	3.50	2.72	2.95	3.27	-	2.90	1.63	2.15	2.95
Tryptophan	-	-	0.17	3.30	0.78	0.75	0.79	0.57	-	0.11	-	0.68	0.30
Valine	-	3.35	2.47	-	0.50	3.16	3.52	3.53	-	3.42	1.83	2.58	2.29

Non-essential Amino Acids													
Alanine		-	4.32	-	4.60	4.12	-	-	-	3.79	-	-	-
Aspartic Acid	-	-	6.11	-	10.69	5.33	-	-	-	4.96	-	-	-
Cysteine	-	-	0.38	-	1.19	0.62	-	0.66	-	0.63	0.36	0.79	-
Glutamic Acid	-	-	8.54	-	18.09	8.36	-	-	-	5.51	-	-	-
Glycine	-	-	0.82	-	5.59	4.75	-	8.12	-	0.66	-	-	-
Met + Cys	2.36	0.58	-	-	-	-	-	-	2.38	-	-	-	-
Proline	3.05	2.13	2.53	-	5.86	3.79	-	-	4.76	4.81	-	-	-
Serine	2.35	-	3.83	-	3.78	24.30	-	3.20	2.71	4.48	-	-	-
Tyrosine	2.06	-	2.01	-	2.53	1.80	-	2.53	1.96	1.54	0.24	-	-
Species	*L. vannamei*	*L. vannamei*	*P. leniusculus*	Salmonid	*P. monodon*	Paralichthys olivaceus	*L. vannamei*	*L. vannamei*	*L. vannamei*	*P. leniusculus*	*L. vannamei*	*L. vannamei*	*L. vannamei*
Author(s)	Luo et al. 2012	Ye et al. 2011	Fuertes et al. 2013	Glencross et al. 2010	Dayal et al. 2011	Deng et al. 2006	Cheng et al. 2002	Davis and Arnold 2000	Luo et al. 2012	Fuertes et al. 2013	Ye et al. 2011	Davis and Arnold 2000	Cheng et al. 2002

Currently processing by-products only provide a small portion of raw materials that produce FM (figure 1.5). As wild stock numbers deplete these proportions will change over time and an increasing amount of by-products from both aquaculture and wild capture will be used as raw material inputs to meet growing aquaculture demands (FAO 2018). Variability in quality is particularly evident in FM (table 1.7); given the seasonality of wild stocks coupled with increasing demand, fisheries are in constant decline and this decline is likely to affect the nutritional consistency of FM. Resources have recently been invested in PBM as a protein alternative with some success but like FM, quality and consistency of PBM has emerged as an obstacle (Dozier 2003).

Advantages of utilizing Poultry By-Product Meal (PBM)

Historically animal by-product protein meals have exhibited high variability with increased levels of ash and low digestibility but with modern rendering facilities, high slaughter volumes and increased automation these problems have been minimised to achieve a more consistent product (Cruz-Suárez 2007). Additionally, PBM is sourced from a controlled, commercialised and growing poultry industry with a consistent supply of raw materials it has an advantage over FM as a more available, cheaper and sustainable resource (Abdul-Halim et al. 2014; Galkanda-Arachchige et al. 2020). Quality control systems ensure that processing and conditioning can be controlled thus reducing product variability. Therefore, by-products like PBM could have significant applications in aquatic diets making it a logical candidate for protein substitution. Due to its high protein content, essential fatty acids, vitamins, minerals, palatability and protein quality PBM has emerged as a protein alternative with significant potential (Galkanda-Arachchige et al. 2020; Cruz-Suárez 2007)

PBM has been able to replace FM in crustacean diets at levels ranging from 30 to 100% (Yang et al. 2004; Fuertes et al. 2013; Cruz-Suárez et al. 2007; Saputra et al. 2019). In crayfish species *(Cherax* spp.) FM has been completely replaced by both plant and animal protein meals (table 1.7). PBM has completely replaced FM in red claw crayfish (*Cherax quadricarnitus)* diets (Saoud et al. 2008; Garza de Yta et al. 2012) and in marron (*Cherax cainii*) diets (Saputra et al. 2018). These studies found that replacing 100% of FM with PBM did not affect survival or growth of these species. Furthermore, Saputra et al. (2018) demonstrated that in *Cherax cainii* crayfish health was improved (as indicated by increased total haemocyte count) when fed diets containing PBM (crude protein 30%) compared to those fed diets containing FM. These marron were also less susceptible to stresses caused by exposure to increased temperatures outside of their optimal range. PBM has been demonstrated as a good protein alternative candidate in *Cherax* spp based on the growth and health

data of the presented studies. Data collection that assesses the manufacturing capability of PBM in aquatic feed in terms of water stability is the logical progression in its application to crustacean feed.

Table 1.7 - Fish meal replacement studies on *Cherax* spp. Percent of FM replaced is the maximum amount able to be replaced without adverse effects on performance.

Alternative	Species	Amount of FM Replaced (%)	CP of diet (%)	SGR (%/day)	Reference
Plant Alternatives					
Combination of soybean meal and brewer's grains with yeast	Red claw crayfish (*Cherax quadricarnitus)*	100	40	6.72	Muzinic et al. 2004
Soybean meal	Australian freshwater crayfish (*Cherax destructor)*	20	30	3.21	Jones et al. 1996
Soybean meal, distillers' dried grains with solubles and milo	Red claw crayfish (*Cherax quadricarnitus)*	100	28	2.46	Thompson et al. 2006
Soybean meal and prawn protein hydrolysate	Red claw crayfish (*Cherax quadricarnitus)*	5	35	3.29	Arredondo-Figueroa et al. 2013
Animal By-Product Alternatives					
Poultry by-product meal	Red claw crayfish (*Cherax quadricarnitus)*	100	26	5.60	Saoud et al. 2008
Poultry by-product meal	Red claw crayfish (*Cherax quadricarnitus)*	100	35	6.12	Garza de Yta et al. 2012
Poultry by-product meal	Marron (*Cherax cainii*)	100	30	0.25	Saputra et al. 2019

Poultry By-Product Meal Production Projections

One of the main advantages of using PBM in aquatic diets is supply. Poultry meat production in Australia has been steadily increasing over the last decade and is projected to continue increasing over the next 5 years as a result of increasing consumption (ABARES 2019). This increasing trend in poultry production and consumption is reflected in the steady increase in production volume of PBM at Talloman (figure 1.6). The steady increase in PBM at Talloman is attributed to a

relatively stable growth in human population (forecast 1.6%) (ABS 2019), increase in poultry meat consumption per capita and increase in local capacity. WA poultry producers are aiming to meet more the state's demand with locally grown and slaughtered product (B.Barron 2011, personal communication 10 July).

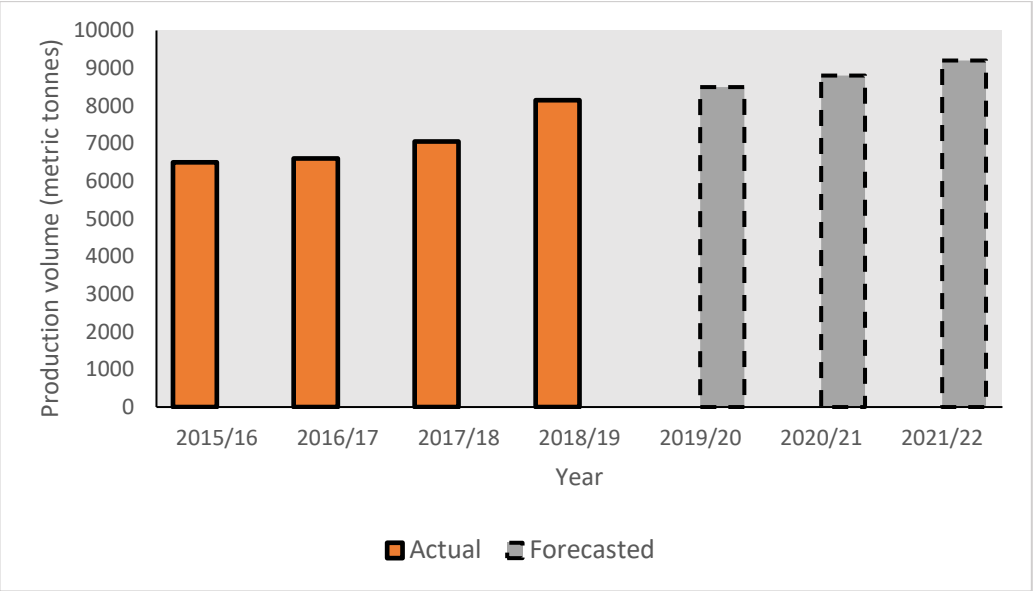

Figure 1.6 – Production volume (metric tonnes) of Poultry By-Product Meal at Talloman (B.Barron 2011, personal communication 10 July).

To conclude, the steady production of poultry and in turn of PBM is a significant advantage over FM as the main protein source in aquatic diets. Raw material inputs are sourced locally and sustainably and are therefore impacted less by seasonal availability and environmental issues. In comparison to plant protein meals, the amino acid profile of PBM is relatively similar to FM. However, supplementation of some amino acids may be required depending on source of the protein meal and/or fed species requirement. Investigations into finding protein alternatives have been many, however, for application of this research at a commercial scale, the quality of the substitute needs to be assessed to ensure its longevity and consistency. Due to the variability between input products as seen in table 1.6 the consistency of nutrient composition over time should be assessed to ensure the product is reliable long term.

Chapter 2 – Literature Review

2.1 Protein Requirements of Crustaceans

2.1.1 Proteins and Amino Acids

The basic unit of all proteins are amino acids (AA) and come in two forms; essential amino acids (EAA) and non-essential amino acids (Halver 1989). EAAs cannot be synthesized by the animal and therefore need to be provided in the diet (Li et al. 2009); non-essential AAs can be synthesized by the animal (table 2.1). Due to difficulties such as moulting and feeding habits crustacean nutrition has progressed slower than that of fish (Mente 2006; D'Abramo et al. 1997). Compared to finfish, optimal dietary protein requirements for crustaceans varies from 30 to 57% for various prawn species (Cuzon et al. 2004; Kureshy et al. 2002; D'Abramo et al. 1997) and 27 -35% for crayfish species (table 2.2). Currently, FM is the main protein source in aquatic diets and is considered to have the "ideal" AA balance (Boyd 2015). This is based on the premise that, if the AA profile of the formulated feed and the whole-body composition of the target animal are the same, protein utilization and growth are theoretically maximized (Trushenski et al. 2006).

Table 2.1 – Essential and non-essential amino acids for aquatic animals, adapted from Li et al. (2009).

ESSENTIAL AA	NON-ESSENTIAL AA
Arginine	Alanine
Histidine	Asparagine
Isoleucine	Aspartate
Leucine	Glutamate
Lysine	Glycine
Methionine	Serine
Phenylalanine	Tyrosine
Threonine	
Tryptophan	Cysteine*
Valine	Hydroxyproline*
	Proline*
	Taurine*
	Glutamine*
Non-essential AA's denoted with a '*' indicate AA's that must be provided by the diet where rates of utilization are greater than rates of synthesis.	

2.1.2 Crustacean Protein Requirements

The protein requirements of crustaceans vary depending on species and have been attributed to species, size, availability of natural food sources, feeding rate, culture intensity and water quality (Kureshy and Davis 2002; Jones and Ruscoe 2000; Mente 2006). Protein requirements of crustaceans have not been well defined (Alam et al. 2000). Protein is often considered as a single dietary component. However, studies have brought to light the negative effects of AA imbalances on growth and performance of fed species (Almquist 1953; Wilson and Halver 1986; Harper and Elvehjem 1955; Scott 1998; Richard et al. 2011). Of the 10 EAAs (table 2.1), lysine

and methionine have been identified as the first limiting AAs in fish diets (Craig 2017) and prawn diets (Millamena et al. 1998; Nunes et al. 2014). Millamena et al. (1998) reported a significant reduction in growth rate and reduced survival in prawns (*Penaeus monodon* Fabricius) fed a diet of 1.18% lysine when compared to a supplemented diet of 2.02% lysine thus defining the lysine requirement of juvenile *P.monodon* as 2.08% of the diet. Millamena et al. (1996) highlighted the breakpoint in which weight gain of the juvenile *P. monodon* begins to decrease as methionine concentration increases, defining the optimal methionine level as 2.4% of protein. Methionine supplementation has also been found to be essential in the moulting process (Floreto et al. 2000). Moreover, amino acid imbalances have also given rise to not only growth deficiencies, reductions in feed consumption and lowered survival rate but also in the form of defects such as bilateral cataracts in Atlantic salmon (*Salmo salar*) (Scott 1998).

Table 2.2 shows a wide range of optimum crude protein levels in diets fed to crustaceans' species and table 2.3 demonstrates the wide range in EAA content (prawn tail muscle) from different species of prawns. Feed is formulated to the AA requirements of the species at different life stages by attempting to match the AA profile of the feed to the AA profile of the target animal (Trushenski et al. 2006). By formulating diets based on an ideal protein (or AA balance), protein can be used more efficiently (Miles and Chapman 2007). However, there is apparent variation in AA requirements of crustaceans and Millamena et al. (1998) demonstrates that this dissimilarity occurs even within penaeid species, which is also reflected by the large range in crude protein content of commercially available feeds (table 2.2).

Table 2.2 – Growth studies of various commercially farmed crustaceans.

Species	Initial Weight (g)	Specific Growth Rate (%/day)	DietCP (% DM)	Protein Source	Reference
Whiteleg Shrimp *(Litopenaeus vannamei)*	1.02	3.63	35	53% Anchovy FM 32% prawn meal 15% squid meal	Lim and Dominy 1990
	0.37	5.59	32	Menhaden Fish meal	Davis and Arnold 2000
	2.6	1.46	37	Fish meal	Pakravan et al. 2016
Black Tiger Prawn *(Penaeus monodon)*	3.11	2.10	44	Fish meal	Glencross et al. 1999
	0.21	4.41	37	Fish meal	Luo et al. 2012
	0.21	3.41	37	Purified	Millamena et al. 1996
Kuruma Prawn *(Penaeus japonicas)*	0.19	2.42	40	Fish meal	Bulbul et al. 2013
	0.55	0.96	45	Soy Bean Meal	Teshima et al. 1992
Signal Crayfish *(Pacifastacus leniusculus)*	0.49	3.60	50	Fish meal	Fuertes et al. 2013
Marron *(Cherax cainii)*	3.29	1.35	27	Fish meal	Nugroho and Fotedar 2013
Freshwater crayfish (*Cherax quadricarinatus*)	1.08	3.55	37	Fish meal	Cortes-Jacinto et al. 2003
	4.81	1.23	17	-	Jones and Ruscoe (2000)
Freshwater River Prawn *(Macrobrachium rosenbergii)*	0.145	1.05	35	Anchovy meal, Prawn head meal	*Misra et al. 2002)
	0.149	1.12	35	Anchovy meal, Prawn head meal	
Freshwater Prawn *(Machrobrachium nipponense)*	0.36	0.73	38	White fish meal	Yang et al. (2004)

'*' – Misra et al. (2002) values were determined using one formulation manufactured two different ways.

Table 2.3 - Essential Amino Acid content obtained from prawn tail muscle.

Amino Acid	(%)			
Arginine	3.49	4.27	7.35	9.06
Histidine	0.67	0.67	1.63	2.90
Isoleucine	2.41	2.58	2.72	5.86
Leucine	3.15	2.97	5.32	7.79
Lysine	0.63	0.65	6.10	8.40
Methionine	1.30	1.40	2.34	3.68
Phenylalanine	1.97	2.28	2.62	5.55
Threonine	1.13	1.21	2.72	3.79
Tryptophan	-	-	2.38	4.06
Valine	1.08	1.16	2.66	5.46
Species	*L. vannamei*	*P. monodon*	*M. rosenbergii*	*P. japonicas**
Reference	Sriket et al. 2007	Sriket et al. 2007	Farmanfarmaian and Lauterio 1980	Teshima et al. 1986

* *P. japonicas* obtained from prawn larvae

Requirement data is available for some EAAs for crustaceans due to extensive works by Millamena et al. (1996 and 1998) and D'Abramo et al. (1997). However, not all AA requirements have been quantified due to the time-consuming and expensive nature of AA determination analysis (Alam et al. 2000). In the search for a protein alternative in aquatic diets potential substitutes should ideally match the AA profile of whole-body composition of the fed animal (table 2.3) or when this information is lacking the AA profile of the current ideal protein, FM (Trushenski et al. 2006).

2.1.3 The Importance of Bioavailability

Bioavailability is “the degree to which an ingested nutrient in a particular source is absorbed in a form that can be utilized in metabolism by an animal” (Ammerman et al. 1995, pg. 1). The availability of AA’s varies between protein source, the processing treatment in which that protein product has undergone and the interaction of that protein product with other constituents in the diet (Lall and Anderson 2005). There is no direct measure of AA bioavailability and traditionally estimates have been obtained using slope-ratio assays which tend to underestimate bioavailability due to the estimation reflecting the animals’ metabolic cost induced by feeding a particular ingredient (Stein et al. 2007). The assay also assumes that the animals’ response to graded AA intake levels is linear and not influenced by dietary nutrient balance (Stein et al. 2007).

Other methods such as AA digestibility can be argued as a more appropriate method to estimate bioavailability. Determining AA digestibility of diets is generally derived from measuring the difference between the amount of AAs consumed and the amount of AAs excreted as faeces (Masumoto et al. 1996; Anderson et al. 1995). However, the current methodologies to collect faecal matter from fish and crustaceans are a challenge due to leaching of nutrients from faeces to the water column if faeces are not collected immediately after excretion. Concurrently, pellet stability and leaching of crystalline amino acids of poorly bound diets have also been a challenge as reported by Meyers and Zein-Eldin (1972) and Goldblatt et al. (1980). Storebakken et al. (1998) evaluated three methods of faecal collection (stripping, sieving and dissection) and found that all three methods gave significantly different results. It was found that sieving produced significantly higher values than the other two treatments for apparent digestibility coefficients of organic matter and nitrogen. It was calculated that 42% of the variation in apparent digestibility coefficients was attributed to faecal collection method. Crystalline amino acids which are added to formulations are known to leach into the water before feed is consumed which is a significant obstacle when assessing AA requirements for slow-eating aquatic eaters such as prawns and crayfish (Alam et al. 2000). Lall and Anderson (2005) outlined the essential prerequisites for ideal AA estimation: 1) the same basal diet, sex and strain of animals, and the same test period in all studies; 2) true digestibility values of AAs in the basal diet; 3) clear-cut graded response of the limiting AA under investigation; and 4) proper statistical methods with consistent and appropriate curve fitting.

In order to formulate protein-efficient diets understanding both AA requirements and their availability within a feed and to the animal is required. By adjusting the

combination of protein alternatives and supplementation of insufficient EAAs in the free form, a significant improvement can be made in the utilization of alternative protein sources (Lall and Anderson 2005). While macronutrients such as proteins, carbohydrates and fats have a high bioavailability with approximately 90% being utilized by the body, availability of amino acids can vary greatly. For maximised bioavailability, AAs need to be supplied in the correct proportions (Schönfeldt et al. 2016).

In conclusion, methionine, arginine and lysine have been identified as limiting AAs essential for optimal growth and survival in crustaceans. These EAAs should be carefully considered when formulating a complete diet and also when developing methods to evaluate the effectiveness of a formulation. Formulations should take into account the "ideal protein" concept to improve feed efficiency as potential FM replacement proteins need to be matched closely to published AA requirement data with emphasis on known limiting essential amino acids.

2.2 Feed Quality

Feed "quality" can be broken into two components; digestibility and physical stability. Understanding how feed is digested by an animal and the main metabolic pathways occurring during digestion is very important (France et al. 2000). It is a complex process in which feed provides an animal with the required energy and nutrients for maintenance and growth (Moyano et al 2015). The physical stability of feed is the method of nutrient delivery which requires feed to be robust enough to withstand transportation and delivery to the animal without reducing availability of nutrients (digestibility) due to heavy compaction. Physical stability is particularly important when feeding aquatic animals as these feeds are required to stay intact while immersed in water. Crustaceans, in particular require specialized feed that sinks and can withstand manipulation from their appendages, an additional factor to consider when manufacturing feed (Dominy et al. 2004; Jussila and Evans 1991). When selecting diet ingredients both of these components must be taken in consideration to manufacture an effective feed.

Feed Digestibility (*In-vitro* vs *in-vivo*)

The main method for determining digestibility of aquatic feed is through *in-vivo* apparent digestbility trials by using an inert marker/substance (mainly chromic oxide) that becomes concentrated as feed is digested by the animal; the concentration of marker found in the feed and faeces is used to estimate dry matter digestibility. Use of an inert marker to determine digestibility relies on the effective collection and

analysis of faeces (Jones and De Silva 1997). Faecal collection must be rapid with minimal residence time in the water to minimize leaching of compounds. It must also ensure only faecal matter is analysed and uneaten feed is separated. Jones and De Silva (1997) demonstrated faecal collection method (single vs pooled) to have strong influence on digestibility estimations of (*Cherax destructor*) and found that limitations of using chromic oxide (Cr_2O_2) could be minimized if the majority of deposited faecal matter was collected. Similarly, Shipton and Britz (2001) using abalone (*Haliotis midae)* demonstrated a significant correlation between faecal chromic oxide and faecal protein (r^2= 0.89) at a 0.5% inclusion of chromic oxide. The marker did not interfere with digestive processes, moved through the digestive tract at a similar rate to the protein and produced replicable and reliable results.

One of the main obstacles associated with using digestibility markers such as chromic oxide or yttrium oxide is the differential movement of the marker to other nutrients through the digestive tract. However, until the rate of movement is accurately quantified it is likely that these methods will remain preferential (Hatlen et al. 2015; Jones and De Silva 1997; Luo et al. 2012; Nieto-Lopez et al. 2011; Burel et al. 2000; Cuzon et al. 1994; Akiyama et al. 1989; Catacutan 1991). For small or juvenile species, such as penaeid prawns, faecal collection is time consuming as waste is only produced in very small volumes and faecal collection must span over extended periods of time. Therefore, development of *in-vitro* digestibility assays would greatly benefit the evalution of feed quality to improve aquatic feed efficiency and especially crustacean feed efficiency.

Methods for *in-vitro* digestibility assays for aquatic species and diets has been extensively covered in a review by Moyano et al. (2015). In contrast to terrestrial methods, aquaculture applications focus on protein hydrolysis of protein-rich feeds rather than carbohydrate hydrolysis. *In-vitro* trials have been utilised by the livestock industry to determine dry matter digestibility by simulating digestion using enzymes. Enzymes such as; pepsin, amylase and cellulase are used to determine dry matter digestibility which is then adjusted to predict *in-vivo* apparent digestibility values using a linear regression based on samples of known *in-vivo* apparent digestibility values (AFIA 2014). This method is less expensive than *in-vivo* methods and can provide accurate and rapid results. However, *in-vitro* methods used for aquatic diets show poor correlation between *in-vitro* and *in-vivo* values (Nengas et al 1995; Gomes et al. 1998). This may be due to the simplicity of closed system digestion simulations which may be too simple and do not capture the entirety of the digestive process. Strong correlations between the methodologies must be established before *in-vitro* assays

can be used to accurately predict *in vivo* digestibility values (Moyano et al. 2015). In the Moyano et al. (2015) review a total of 9 papers focus on crustaceans and all of them are marine prawns. Only 2 out of the 9 papers compare the use of terrestrial animal enzymes and prawn heptopancreatic (digestive gland) digestive enzymes from the prawn itself. Terrestrial animal enzymes are commercially available and could be utilised for the aquacuclture industry if correlations can be made rather then extracting enzymes from the target animal.

Ezquerra et al. (1997) used trypsin, chymotrypsin, peptidases, pronase, casein and azocasein obtained from terrestrial animal sources and showed a positive correlation between degree of hydrolysis (DH) and apparent digestibility coefficiens (ADC) values of *Litopenaeus vannamei* feeds (r^2=0.71). A positive correlation was also observed when using hepatopancreas (digestive gland) enzymes (r^2= 0.77). The degree of hydrolysis is the percentage of peptide bonds that are hydrolyzed during digestion (Dimes and Haard 1994). This study provides grounds for further investigation of the use of commercial enzymes utilised by the livestock industry and their suitability to aquatic feed studies for rapid feed evaluation. Table 2.4 highlights the variability when comparing *in-vitro* and *in-vivo* digestibility values. The regression analysis completed by Ezquerra et al. (1997) provides compelling evidence for the application of commercial enzymes (trypsin, chymotrypsin and peptidase) in aquatic feed evaluation methods.

Table 2.4 – *In-vitro* and *in-vivo* protein digestibility values of aquatic feed ingredients/diets, *in-vitro* methods use various terrestrial animal enzymes.

Ingredients evaluated	Protein (%)	*In-vitro* Digestibility (%)	*In-vivo* Digestibility (%)	Species	Author(s)
Mackerel meal	68.7	97.1	69.5	*Litopenaeus vannamei*	Lazo et al. (1998)
Anchovy meal	64.0	96.3	80.5		
Menhaden meal (premium)	61.0	96.0	-		
Menhaden meal (regular)	60.9	95.5	-		
		Degree of hydrolysis (%)			
Anchovy meal	60.2	30.3 (80.1)*	83.6	*Litopenaeus vannamei*	Ezquerra et al. (1997)
Tuna Waste meal	61.3	19.0 (63.1)*	63.6		
Menhaden fish meal	63.8	24.2 (70.9)*	67.1		
Soybean protein	49.5	30.8 (80.9)*	90.9		
Fish hydrolysate Diet	39.0	6.1	85.2	*Litopenaeus vannamei*	Córdova-Murueta et al. (2002)
Krill hydrolysate Diet	42.2	4.8	91.8		
Squid meal Diet	44.4	6.0	75.1		

'*' – Values contained in brackets were the calculated in-vitro protein digestibility value using regression analysis obtained in Ezquerra et al. (1997), r^2 = 0.71

In summary, a standard method for evaluating aquatic feeds has yet to be developed. Current *in-vivo* apparent digestibility methods to evaluate aquatic feed digestibility involve the use of inert markers such as chromic oxide, the main obstacle of using this method is the need for rapid and accurate faecal collection. Residence time of faeces in the water columns should be minimized as it allows for leaching of nutrients and of the marker itself which effects quantification. Despite these minor difficulties, strong correlations have been established and the technique is still the preferred *in-vivo* assay (Shipton and Britz 2001; Hatlen et al. 2015; Jones and De Silva 1997; Luo et al. 2012; Nieto-Lopez et al. 2011; Burel et al. 2000; Cuzon et al. 1994; Akiyama et al. 1989; Catacutan 1991). In attempt to move away from time consuming *in-vivo* assays *in-vitro* methodologies have been investigated as a more resourceful and rapid feed evaluation tool. Currently, no standard *in-vitro* assay has been established to evaluate aquatic feed however, there is strong evidence to suggest that commercially available terrestrial enzymes can be used to predict aquatic feed digestibility similar to those methods used in the livestock sector (Ezquerra et al. 1997; Moyano et al. 2015). By using digestive enzymes that are already commercially available methodologies from the livestock sector can be adapted to aquatic feeds without the need to develop new enzymes. To improve feed efficiency in the

aquaculture industry a standardised tool for rapid and reliable feed evaluation is required. Therefore, further research comparing *in-vivo* values and *in-vitro* assays to accurately estimate aquatic feed digestibility is needed.

2.3 Aquatic Feed Pellet Stability

The most important difference between aquatic feeds and terrestrial feeds is the requirement of aquatic feeds to remain intact while immersed in water, this is known as physical or water stability. Igwhela (2013) defines high pellet water stability as "the retention of pellet physical integrity with minimal disintegration and nutrient leaching while in the water until consumed by the animal". Aquatic animals require feed to remain stable (resistant to physical breakdown) and intact while immersed in water for a long enough period of time to be consumed. Without an appropriate method of nutrient delivery, focusing on meeting the nutritional requirements of species is of less importance.

Degree of water stability, whether feed sinks or floats, its size, its texture and its shape are all important pellet physical qualities. The standards for these physical qualities are dependent on species (Meyers and Sein-Eldin 1972). An Australian survey conducted in 1995 (Gleeson and Evans n.d.) asked producers to rank physical charateristics of feeds for a range of species including; silver perch *(Bidyanus bidyanus)*, barramundi (*Lates calcarifer*), prawns (*Penaues monodon*) and Atlantic Salmon (*Salmo salar*) and international (European and American) farms growing Atlantic Salmon *(Salmo salar)* and channel catfish *(Ictalurus punctatus).*The physical characteristics ranked were; density/sinking rate, stability (dry/wet), mechanical strength, size, texture, shape and gelatinization. Results showed that regardless of species, density/sinking rate and stability ranked 1 and 2, respectively as the most important with gelatinization the least important. The lower ranking of characteristics of texture, shape and gelatinization may have been due to lack of understanding of the role of other parameters (Gleeson and Evans n.d.).

Fish consume feed almost instantaneously when it is presented; whereas crustaceans are benthic feeds who forage and manipulate feed therefore, stability is of even greater importance in crustacean culture (Dominy et al. 2004; Jussila and Evans 1991; Meyers & Zein-Eldin 1972; Volpe et al. 2012). Unstable (i.e., rapid disintegration of pellets) feeds are known to have negative environmental impacts such as lowering water quality through fouling, emphasizing the importance of pellet stability regardless of species (Saalah et al. 2010). Feeds can be classified as either

wet, moist or dry depending on their moisture content (Hardy 2002). Generally, wet feeds contain 50 – 70% moisture, moist feeds contain 35-40% moisture and dry feeds contain less than 10% moisture (Jobling et al. 2001). Commercial aquaculture farms tend to use dry feeds as they have a longer shelf life and are available in a variety of different forms (compressed, expanded and extruded) depending on what the farmed animal requires (i.e sinking/floating).

2.3.1 Pellet Types

Commercial aquatic feeds are generally manufactured in floating/buoyant form through extrusion or as sinking pellets through steam/pressure pelleting (Craig et al. 2017). Extrusion systems apply energy in the form of heat, pressure and steam into either a preconditioner or an extruder barrel that uses a screw to move materials through the barrel. Wet barrel extruders are jacketed for steam injection and aid in controlling the cooking process (Obaldo et al. 2000). Currently, these systems are the primary continuous cooking apparatus utilised for aquatic feed manufacture (Rokeyet al. 2010; Obaldo et al. 2000). For example, steam/pressure pellets are manufactured by injecting steam at a predetermined temperature to feed mash which is put through a steam pellet press; pellets are then dried to a desired moisture content (<10%) (Booth et al. 2002). Steam/pressure pelleting produces feeds that are suitable for crustacean culture as these pellets tend to be denser allowing the pellet to sink to the bottom into the crustacean feeding zone.

There are three main types of pellets; expanded, extruded and compressed. *Expanded* pellets are made using high amounts of pressure within an angular expander coupled with various steam injection points. Temperatures within the chamber can exceed 120°C. The sudden drop in pressure as pellets leave the die hole cause large vacuoles to occur which enable pellets to trap air allowing them to float on the surface of the water (Misra et al. 2002; Kop and Korkut 2017). *Extruded* pellets use the same techniques as expansion processing, with greater ranges of temperatures, pressures and moisture content. Extrusion processing can reach temperatures between 80 - 200°C and the moisture content can be increased to a range of 20-30% using steam injection. Extrusion technology has the versatility to manufacture pellets with different densities and buoyancy profiles by altering the temperature and pressure profiles within the extruder (Bandyopadhyay and Rout 2001). This is a significant advantage as it enables manufactured feeds to be tailored according to the feeding behaviour each individual species and is widely used in the aquaculture industry. Table 2.5 summarizes aquatic feed studies investigating the

effect of extrusion parameters on species performance. *Compressed* pellets produce a dense pellet that sinks rapidly, suitable for crustacean feeds. Pellet compaction is the process of forcing feed mash through a die opening with the aim of producing a complete pellet that can withstand rigorous transport and handling (Jobling et al. 2001). The mash is passed through a die and warm air is forced over the pellets to dry the pellets and reduce the moisture content. All three pellet types utilize heat, pressure and mechanical shear in various forms to enhance pellet water stability through starch gelatinzation and compaction.

2.3.2 Factors that affect Pellet Stability

The pelleting process cohesively combines ingredient particles by mechanical action using heat, pressure and moisture (Muramatsu et al. 2015). The effectiveness of the pelleting process is assessed using the pellet durability index (PDI). The PDI indicates the percentage pellet that remains intact after exposure to mechanical forces such as loading and transportation (Muramatsu et al. 2015). Pellet water stability is a measure of resistance to disintegration and dissolution. Although several methods have been used to evaluate pellet water stability a standard method has yet to be established. The majority studies that assess water stability immerse pellets in static water (Meyers et al. 1972; Obaldo and Tacon 2001; Obaldo et al. 2002; Igwhela 2013; Volpe et al. 2012; Solomon et al. 2011; Jussila and Evans 1998; Fagbenro and Jauncey 1995). Other methods include using mechanical agitation (Obaldo et al. 2002; Heinen 1981) or water flow through systems (Ruscoe et al. 2005; Cheng et al. 2005; Goldblatt et al. 1980). Obaldo et al. (2002) compared the static water, vertical shaking (5.5cm stroke distance at 30cpm) and horizontal shaking (300 cpm) methods and found that all three methods provided reproducible results and recommended either shaking methods to assess water stability of prawn feeds as it simulates actual culture conditions. When assessing pellet water stability, it is important to match the test conditions to the conditions of the target culture system.

There are a number of factors that affect the physical and chemical stability of a pellet; nutritional composition, particle size of raw materials, conditioning (heat processing), moisture content, shape and drying temperature (Thomas et al. 1996; Thomas et al. 1997; Muramatsu et al. 2015). The influence of these factors and their interactions have become increasingly important in the context of producing aquatic feeds. As water stability of feeds is one of the most important characteristics the effects of particle size, conditioning (heat processing and moisture content), fat inclusion, physical shape and drying temperature will be discussed.

Particle Size

In general, the finer the particle size of the ingredients, the more physically stable the pellet as the pellets increase in density with decreasing particle size (Lim and Cuzon 1994). Smaller particle sizes are also easier to mix to achieve homogeneity and each pellet can more accurately reflect the original formulation (Thek & Obernberger 2010, p. 47). The recommended particle size distribution of ingredients targeted for prawn feeds is 95% <250µm (Bortone and Kipfer 2016). In general, grinding improves the mixing properties of ingredients, increases bulk density and improves steam penetration into the feed mixture (Jobling et al. 2001).

Obaldo et al. (1999) investigated the effect of ingredient particle size (fish meal, wheat, soy bean meal and prawn meal) on physical pellet stability and the physiological performance of *L.vannamei*. As particle size decreased pellet stability, durability and starch gelatinization increased. The improvement was attributed to a reduction in air space between particles which increased the contact surface area between particles to allow for greater bonding and compaction. The compaction improved the density of pellets and reduced the number of “break points” that can facilitate leaching. As particles became larger, water stability decreased in a linear fashion (r=0.91). Similarly, degree of starch gelatinization improved with decreasing particle size (r=0.95), where starch increased in surface area exposed to heat and steam, the catalysts for gelatinization. Similar results were observed in trials by Garber et al. (1997), Desrumaux et al. (1998) and Rolfe et al. (2000). In terms of performance, *L. vannamei* grew significantly faster when fed diets made up of smaller particle sizes (124µm) when compared to prawns fed diets made up of larger particle sizes (603µm).

Palaniswamy and Ali (1991) examined the effect of the particle sizes of *Penaeus indicus* diets made with casein, prawn waste and FM. Reduction in particle size from 500µm to 212µm increased water stability of pellets (P>0.05). However, for pellets with particle sizes less than 212µm (100µm and 50µm) water stability decreased. Digestibility also declined with descending order of particle sizes of casein based diets; in contrast the digestibility of prawn waste based diets increased with decreasing particle size. All diets were 40% CP and increased digestibility was apparent across all feeds containing prawn waste. Reasons for the increased digestibility of prawn waste based diets was unclear but may reflect pellet hardness.

Feeding trials with *Palaemon serratus* and *Palaemon platycero* demonstrated that feed particle size must be <5µm in diameter to pass through the posterior chamber

of the proventriculus (Forster and Gabbott (1971). The larger feed particles had to be reduced in size to pass through the filter in the posterior chamber of the proventriculus and into the alimentary canal (Vonk 1960 cited in Forster 1972, p. 213). Hunt et al. (1992) also demonstrated that macrophagous feeding where the animal (*Penaeus merguiensis*) is able to reduce the size of larger pieces of feed through mandible action was preferred. This suggests that larger pellets made up of smaller particles may improve crustacean feed ingestion. However, it has been calculated that up 50% of feed can be wasted due to external maceration and manipulation of feed from spiny lobsters (*Jasus edwardsii* Hutton, 1987); waste could be reduced by 19% by providing lobsters with optimal feed size (5x5mm pellets to 34-45mm CL size lobster, 7x7mm pellets to 60-70mm CL size) (Sheppard et al. 2002). Hunt et al. (1992) also demonstrated that feed texture influenced the ingestion of particles of different sizes. Particles fed as alginate microcapsules between 8-20µm in diameter produced the same feeding sequence (mouthpart movement) in *P. merguiensis* as much larger (>5mm^2) particles in the form of scallop, fish or prawn flesh. It is suggested that the soft texture of the latter makes it easier to ingest allowing for larger particles to be fed to the animal.

Smaller particles mix more uniformly increasing the homogeneity of ingredients including micro-nutrients within each pellet (Hunt et al. 1992; Thek & Obernberger 2010). Grinding or sieving of raw materials to 200µm was found to yield the best growth and performance results in prawn diets and particle sizes smaller than 200µm showed a decrease in water stability and digestibility (Palaniswamy and Ali 1991). Palaniswamy and Ali (1991) suggested reasons for this reduction in water stability and digestibility of feeds to be attributed to the hardness in pellet brought on by the ability of smaller particles to be compacted heavily and the increased surface area facilitating large amounts of dry matter to be lost when placed in water. Thus, setting limits on ingredient particle size for optimum output may be an option.

Heat Processing and moisture content

The manufacturing processes for crustacean and fish feeds are the same, that is, grinding, mixing, extrusion and drying (Cuzon et al. 1994). Lim and Cuzon (1994) argued that the steam compaction pelletizing method was the most popular, however two decades later extrusion technology has several advantages over steam pelleting making it commercially popular but also more expensive (Bandyopadhyay and Rout 2001; Hardy and Barrows 2002). The temperatures and pressures applied in

extrusion processing have a dramatic effect on starch chemistry when compared to milder processes of steam pelleting (Glencross et al. 2011).

Starch Gelatinization

Starch gelatinization plays two important roles in aquatic diets, (1) to spare proteins and lipids from catabolism by acting as an inexpensive source of energy, (2) improving water stability of pellets as gelatinized starch molecules improve particle cohesion (Kanmani et al. 2018). Starch gelatinization is greater in extruded feeds due to the increased temperatures and pressures (Misra et al. 2002; Kraugerud et al. 2013; Cai and Diosady 1993). Starch acts as an adhesive or binding agent (Thomas et al. 1998) by gelatinising at temperatures above 80°C. Crystalline structures are broken causing amylose in the starch granule to leach outand form a gel thus increasing the viscosity. Physical quality is improved due to increased binding between feed particles (Svihus et al. 2005; Han and Hamaker 2001). Larger starch granules are easier to gelatinize and therefore have a stronger influence on the physical properties which may potentially increase the physical quality of pellets (Chiotelli and Le Meste 2002; Svihus et al 2005). Additionally, the process of gelatinization breaks bonds making starch molecules more easily accessible to enzymatic degradation leading to improved digestibility (Stone et al. 2003; Svihus et al. 2005). The degree of starch gelatinization can be manipulated by manipulating screw speed, barrel temperature and feeding rate (Domenech et al. 2013). (Misra et al. 2002; Kraugerud et al. 2013; Cai and Diosady 1993; Gropper et al. 2002). Extrusion is considered to result in starch gelatinization and therefore produces a more stable feed, which is required in both floating and sinking extruded feeds (Barrows 2007; Welker et al. 2018).

Misra et al. (2002) compared the pellet quality and growth performance of freshwater prawns (*Macrobrachium rosenbergii*) for extruded pellets and steamed pellets. Extruded pellets had greater water stability, staying stable for up to 4hrs in water, whereas steam pellets disintegrated within 10min of immersion. *M.rosenbergii* fed extruded diets had significantly better feed conversion ratio (FCR= 1.94) than those fed steam pellets (FCR= 2.16). The higher water stability of extruded pellets may have delayed gastric emptying of the prawn, similar to observations in trout *(Salmo gairdneri* R.) (Hilton et al. 1981). The delayed passage of feed through the digestive tract may have lowered feed intake but increased digestibility by increasing exposure to digestive enzymes, therefore improving feed efficiency. The increased water stability was due to greater starch gelatinzation through high temperatures, pressures

and shear (Bandyopadhyay and Rout 2001; Kraugerud et al. 2011). Table 2.5 shows outcomes of studies investigating the effect of extrusion conditions on water stability and performance.

Table 2.5 – Studies on the effect of extrusion processes on pellet quality.

Author	Process	Species	Result
Welker et al. 2018	Extrusion vs Expansion Pelleting	*Oncorhynchus mykiss*	Extruded pellets better quality. Improves faecal collection and reduces effluence.
Liam et al. 2014	Steam Processing	*Clarias gariepinus*	Improved water stability when steamed at 80°C for 40mins.
Yoshitomi 2004	Extrusion Processing Conditions	N/A	Expansion rate increased to >160% at 145°C and was negatively correlated with bulk density.
Misra et al. 2002	Extrusion vs Steam Pelleting	*Macrobrachium rosenbergii*	Extruded pellets had better water stability than steamed pellets but performance showed no difference.
Bandyopadhyay and Rout 2001	Extrusion from a low-cost single extruder	*Penaeus monodon*	Optimum parameters: Moisture content: 30-35% Speed: 92-94rpm Barrel Temp: 68-94°C
Rolfe et al. 2000	Extrusion Processing Conditions	Catfish	Starch gelatinization was maximized at: Moisture: 25% Particle size: 700µm Speed: 400rpm
Obaldo et al. 2000	Wet Extrusion Conditions	*Penaeus vannamei*	Shear had the greatest effect on starch gelatinization. Optimum parameters for pellet quality: Moisture: 20% Temp: 90°C
Rout and Bandyopadhyay 1999	Extrusion vs Meat mincer	*Penaeus monodon*	Extruded pellets had better water stability. Optimal parameters: Particle size: 300µm Temp: 94°C Moisture: 35%

Individual ingredient manufacturing performance is important to consider when formulating aquatic feeds that are required to remain stable while immersed in water. Principles of conditioning and heat treatment can be applied to rudimentary lab equipment such as meat mincers that are often used in aquatic feed evaluation studies.

Lipid Inclusion

Physical stability of pellets is reduced when fats present in feed formulations lubricate feed particles; the lubrication of particles lowers the compression in the die as feed mash passes through (Lim and Cuzon 1994). (Murmatsu et al. 2015; Lim and Cuzon 1994). Pellet quality is significantly reduced with fat inclusion at levels greater than 35 g/kg producing significantly more fines. The hydrophobic nature of fats reduces the binding capacities of feed ingredients as feeds are bound using water soluble components (Thomas et al. 1998). Lipids coat carbohydrates and inhibit gelatinization effectively lowering the binding capacity of these constituents (Eliasson et al. 1981). At levels greater than 15 g/kg, a significant decline in pellet durability index (PDI) (Muramatsu et al. 2013) has been found. Similar results were demonstrated by Mortiz et al. (2003) showing that as fat addition varied from 36 g/kg to 50 g/kg, PDI decreased from 75% to 54%. The conclusions drawn from these studies suggested that the increased fat levels increased the flow rate of the mash through the extruder and reduced frictional heat. A decrease in exposure time to thermal treatment created by friction due to the increased flow rate would have reduced the starch gelatinization (Moritz et al. 2003). Fats such as fish oil, vegetable oil or other fats added to formulations to boost fat content are known as mixer added fats (MAF) and can reduce pellet quality as the mash slips through the die and reduces pressure and compaction (Thomas et al. 1998). Wamsley and Mortiz (2013) investigated two manufacturing techniques that included either 1% or 3% addition of MAF's and results showed that 3% MAF addition reduced pellet quality by approximately 5%.

PBM generally has a greater crude fat level than FM, ranging from 11 – 19% (table 2.6). Cheng et al. (2002) replaced FM using 2 types of PBM- regular (crude fat 11.16% DM) and defatted (crude fat 3.5% DM); in defatted PBM diets, fat was replaced using fish oil as a MAF. Although water stability was not assessed, there was no significant difference in prawn growth between the diets. The influence of crude fat content in PBM on pellet water stability has yet to be investigated and compared to FM based aquatic diets.

Table 2.6 – Crude lipid content of fish meal and poultry by-product meals used in aquatic feed experiments.

FM	PBM	Reference
11.92	12.21	Luo et al. 2012
9.5	17.2	Ye et al. 2011
8.87	19.01	Fuertes et al. 2013
13.4	11.16	Cheng et al. 2002

Physical Form of pellets

Studies investigating this characteristic have focused on the effects of pellet shape and size on growth response and feeding behaviour of aquatic species (table 2.7). Salmon *(Salmo salar)* have been observed to detect longer pellets (≥ 10mm) over smaller pellets (≤ 8mm) as well as pellets with a greater diameter (8-10mm). However, although longer pellets were detected the fastest once grasped these longer pellets were more likely to be rejected over shorter pellets, suggesting that this species has a preference in pellet size (Smith et al. 1995). Similarly, Obaldo and Masuda (2006) highlighted the need to further investigate the relationship between prawn feeding behaviour and pellet size. Aggressive prawn behaviour was observed more frequently in tanks provided with larger pellets (3.0mm) than those fed smaller pellets (0.7 and 0.2mm). Prawns fed larger pellets were often seen to monopolize feed which may cause size variation and increased mortality of smaller prawns.

A correlation between water stability and pellet size (r=0.97) in *L. vannamei* diets was demonstrated by Obaldo and Tacon (2001); as pellet size decreased from 3mm to 0.7mm, water stability decreased from 82.5% to 76.3%. The correlation can be explained by the method of size reduction, pellets were crumbled to produce smaller pellets which in turn caused stress fractures that allowed water to penetrate the pellet faster and reduce pellet water stability. Futhermore, as pellet size was reduced the surface area to volume ratio increased making the pellets more susceptible to disintegration and nutrient leaching. Prawns of different size classes also showed a preference for pellet size. 1g and 13g prawns gained more weight when fed 3mm pellets whereas 7g prawns gained the most weight when fed 2.2mm feed which demonstrates a need for optimum diet size for prawn size (age) or optimal prawn size to feed size ratio. Prawns have shown a preference to larger feed particles that they can hold and break into smaller pieces upon consumption (Hunt et al. 1992; Igwhela et al. 2013).

There is limited information on the feeding behaviour of prawns when fed diets of different shapes. The feeding behaviour of crustaceans exacerbates DM loss and

nutrient leaching to the environment as prawns prefer to forage and to break large feed particles into smaller pieces prior to ingestion (Hunt et al. 1992; Igwhela et al. 2013). The behaviour is distinctive and unique; prawns will cradle pellets with the maxillipeds, and begin to tear and crush the end of a pellet with the mandibles (Obaldo and Masuda 2006). During consumption prawns use their appendages to masticate their food outside the buccal cavity before ingestion, this can create fines which are swept away by exhalent gill currents, making it unavailable to the animal and producing waste (Forster 1972).It is possible that feed form (shape and size) is likely to have a significant impact on pellet water stability and crustacean feed intake.

Huu and Lan (2015) investigated the effect of noodle (1.5mm or 3mm diameter) and disk shaped (3, 5 and 7mm diameter) feeds on crayfish (*Panulirus ornatus*). Noodle shaped pellets (1.5mm) supported better crayfish survival (78% survival) than the other treatments (69% survival). However, the overall poor survival rate of all treatments when compared to those fed on the control diet of trash fish indicated a possibly deficient diet formulation.

Table 2.7 – Studies investigating the effect of pellet physical form on species performance.

Species	Form	Result	Author
Spiny Lobster (*Panulirus ornatus*)	Noodle (length = 1.5 and 3mm) Disk (diameter = 3, 5 and 7mm)	1.5mm noodle pellets showed improved crayfish survival.	Huu and Lan 2018
Whiteleg Shrimp (*Litopenaeus vannamei*)	0.7, 1.2, 1.7, 2.2, 2.6 and 3.0mm crumble	1g prawns grew best when fed 3mm crumble. 7g prawns grew best when fed 2.2mm crumble.	Obaldo and Tacon 2001
Atlantic Salmon (*Salmo salar*)	Cylinder Shape at varying diameters and lengths D: 5, 6 8 and 10mm L: 6, 13 and 20mm	Salmon preferred shorter pellets >13mm	Smith et al. 1995
Atlantic Salmon (*Salmo salar*)	Round D:2.6mm Long thin L: 10mm Long flat L: 100m D:2mm	Salmon preferred long thin pellets over long flat and round pellets.	Stradmeyer et al. 1988
Juvenile Atlantic Salmon (*Salmo salar*)	Semi-moist Dry Starter diets	Salmon grew fastest when fed semi-moist diets.	Lemm 1983

There is limited information available on the impacts of pellet shape and size on pellet water stability. Of the information available, all diets tested are FM based. Fish species in particular salmon prefer cylinder shaped feeds that are >13mm (Stradmeyer et al. 1988; Smith et al. 1995). Crustacean species, in particular penaeid prawns prefer large feeds made of finely ground particles (>500 - 212µm) that are water stable (Obaldo and Tacon 2001; Huu and Lan 2018; Palaniswamy and Ali 1991). Although crustaceans have shown a preference for larger feed broken down to smaller pieces upon ingestion; an optimal prawn size to feed size ratio has not been identified (Obaldo and Tacon (2001). The information presented above provides grounds for investigation into the effect of physical form of pellets made from protein alternatives on pellet water stability as different feed ingredients are likely to possess varying manufacturing capabilities. This information would be especially beneficial to crustacean culture due to their benthic feeding behaviour as feeds must undergo substantial manipulation before ingestion.

Drying Temperature

Drying temperature refers to the temperature in which pellets are dried at to remove excess moisture for safe and long-term storage. Feed with a high moisture content is not suitable for storage and transportation, therefore feed is dried to improve shelf life (Chevanan et al. 2009; Thomas et al. 1996; Wang et al. 2012). Over-drying of pellets can result in wasted energy consumption (economic loss) and more directly can cause cracks in the pellet surface to form which in turn affects their durability (Thomas et al. 1997; Lambert et al. 2018). Lambert et al. (2018) highlight the lack of information available on pellet the drying process despite its large economic importance in respect to energy saving and feed shelf life (Pacheco et al. 2011; Houben et al. 2011). As feeds exit the extruder pellet temperatures can be up to 90°C and up to 19% moisture, air must be passed over the pellets to cool and dry them to <10% moisture for storage and handling (Houben et al. 2011; Jobling et al. 2001; California Pellet Mill Co. 2019). There is little information on the effect of drying temperature on pellet stability in terms of either leaching rate (crude protein loss) or water stability (dry matter loss). The majority of information available on the effect of thermal treatment and effects on pellet stability is focused on manufacturing processes such as thermal pre-treatment (i.e steam injection), barrel temperature, screw speed and die pressure/temperature. Flores and Martinez (1993) found that drying temperature and thermal pretreatment strongly influenced pellet water stability. Pellets that were dried at 80°C without steam treatment had greater water stability than pellets that had been

dried at the same temperature with steam treatment and pellets dried at 60°C with and without steam treatment. The study concluded that drying temperature was a critical parameter that affects the stability of pelleted feeds (Flores and Martinez, 1993).

There is very little information that investigates the water stability of feeds that contain PBM. The impacts of drying temperature on PBM based feeds have yet to be investigated. This information is important in order to effectively utilize PBM in aquatic diets. A better understanding of the functional properties and manufacturing capabilities of PBM would provide insight in how to efficiently use this protein in an aquatic feed.

In conclusion, the rapid growth of the aquaculture industry has not been able to be matched by FM production and the pressure to produce FM to supply fish and crustacean farms has caused a decline in wild fish stocks. This has driven the industry to source more sustainable feed inputs. The complicated nature of fish and crustacean nutrition is reflected in the wide range of AA and nutrient requirements. While some information is available on AA requirements of aquatic species there are gaps and limitations due to the expensive and time-consuming nature of determining AA requirements. Inexpensive sources of crude protein are continuing to play a vital role in the industry. Crustaceans compound the issue due to their feeding behaviour adding a further complication to sourcing a suitable alternative. The manufacturing capabilities of a protein alternative must also be taken into account when assessing its viability as a substitute.

PBM has been demonstrated as a promising alternative in aquatic feed studies (table 1.6). However, there are still gaps in knowledge in regards to its suitability as a protein alternative. In the past, by-products have had a tendency to display increased proximate composition variation, effecting the quality of the protein. This in turn can have flow on effects to the consistency of produced feed. However, automation of technology has helped to decrease this variation. In this study a local source of PBM will be investigated as a FM replacement and tested for variation in proximate composition over a 3 month period. Further evaluation of the product using *in-vitro* digestibility methods utilised by the livestock industry will be investigated and compared to *in-vivo* apparent digestibility values to provide information to further the effort to standardise aquatic feed evaluation methods.

PBM has successfully replaced 100% of FM in freshwater crayfish diets (table 1.8) making it a suitable candidate for further investigation into how the product effects water stability in aquatic feeds, an important component of aquatic feed manufacture. There is limited information on the effects of PBM on pellet stability and this will be investigated by manipulating feed shape and drying temperature. These parameters have been chosen as they are easily manipulated under laboratory conditions where extrusion equipment may be unavailable. Where sophisticated machinery is unavailable researchers should aim to mimic commercial standards as closely as possible to more accurately investigate protein alternatives to have prospective commercial application. Figure 2.1 illustrates the rationale of this study and outlines the specific objectives of each chapter. The combined chapters aim to optimise pellet stability of compound feeds using PBM as a protein alternative in crustacean diets and compare *in-vitro* and *in-vivo* digestibility methods to test for quality of aquatic feeds.

2.4 Research Questions

Is the poultry by-product meal produced at Talloman, WA a consistent and quality product that can be used in crustacean diets as a protein alternative?

How does poultry by-product meal perform in terms of water stability when made into aquatic feeds of different shapes and dried at different temperatures?

2.5 Aims and Objectives

Aim – Optimise pellet stability of compound feeds using poultry by-product meal as a fish meal replacement in crustacean diets and compare *in-vitro* and *in-vivo* digestibility methods to test for quality.

Objective 1 – Investigate the quality and consistency of locally sourced poultry by-product meal to be used in compound feeds.

Objective 2 – Investigate the digestibility of poultry by-product meal in *Cherax cainii* diets using *in-vitro* and *in-vivo* digestibility methods and examine the viability of utilizing *in-vitro* methods as a test for quality in aquatic diets.

Objective 3 – Identify the most suitable feed form and drying temperature of manufactured feed utilizing poultry by-product meal to minimize leaching of protein and disintegration.

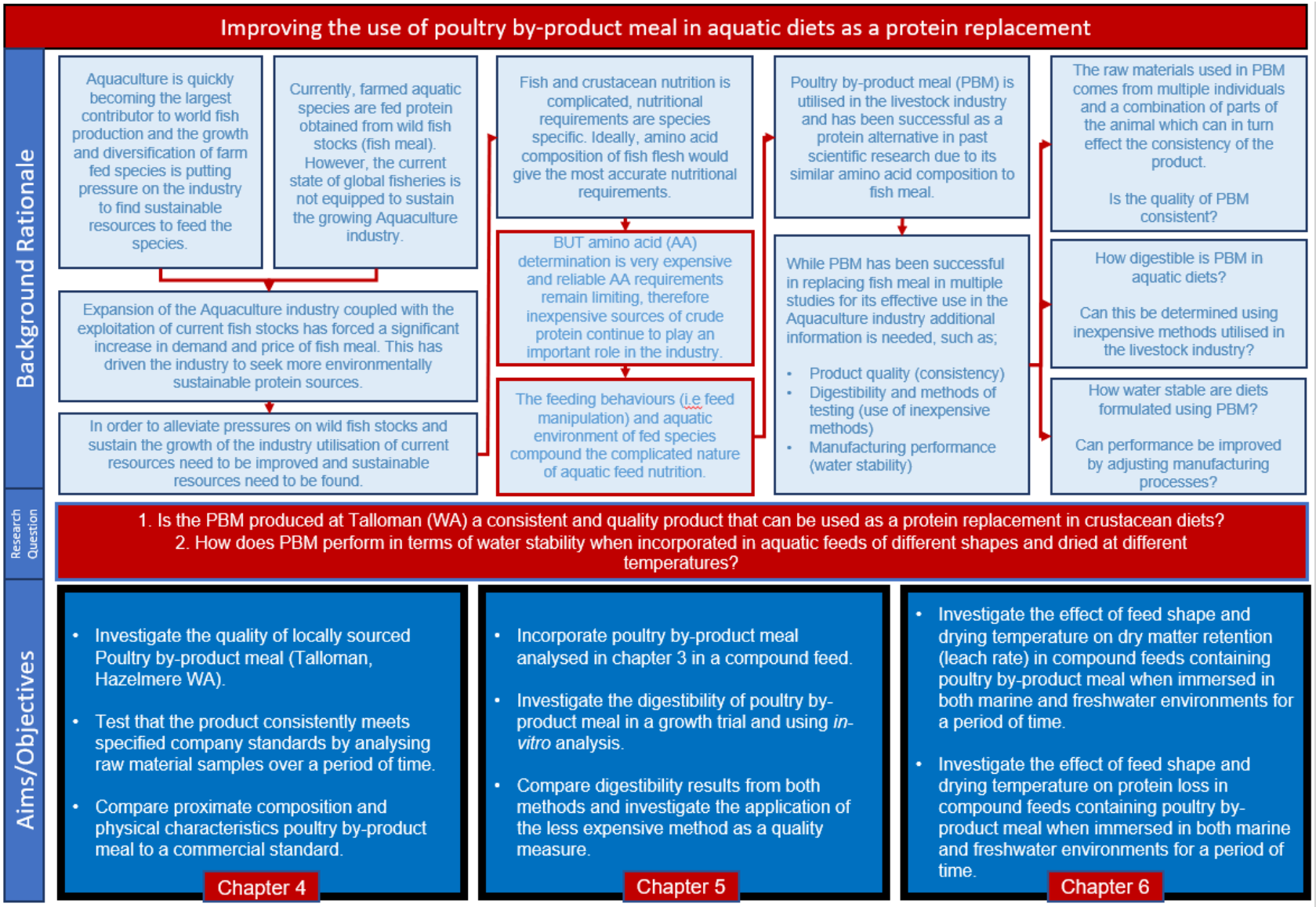

Figure 2.1 – Flow diagram of research rationale.

Chapter 3 – Poultry by-product meal background, proximate and digestibility analysis methods.

3.1 Poultry By-Product Meal from Talloman

Background

Talloman is an Australian company part of the larger Craig Mostyn group that specializes in processing material in a range of processing plants including material such as fat, bone, blood, feather and poultry by-products to produce a range of products such as meat and bone meal, poultry by-product meal, feathermeal, bloodmeal and poultry oil. The following information about the origins of the poultry by-product meal produced at Talloman was obtained during an interview with Ben Barron, the production manager of the company on the 25th February 2016. The poultry by-product meal described in this section is used as the FM substitute throughout the three experimental chapters.

Poultry By-product Meal Production

Raw poultry by-product material arrives daily between 5am and 7.30am from Western Australian poultry producers, namely Inghams, Steggles, Mount Barker and other smaller independent companies. Purpose built trucks are loaded with by-products as they are produced and delivered to Talloman. Once raw material arrives at the processing plant it is dumped into bins (feathers and kill products are already separated at external companies) at the start of the production line, all raw material is required to be processed within a 15 hour window of receiving.

A metal screw transports raw material through a metal detector to remove any metal and then into a pre-cooker. The pre-cooker heats the offal up to 98°C and ensures the temperature is consistent throughout the mixture, this is the first stage of sterilization. The offal is then transported to strainers to drain out the separated oil and the remaining product is then transported to a dryer (114°C to 130°C) for 2 to 2.5 hours. Offal is then pressed (approximately 90°C) to remove any remaining oil and producing a dry cake. The dry cake transported to a mill and processed through a hammer mill. The milled material is passed through a 3mm shaker screen to achieve a uniform meal is. The product is specified as a “free flowing powder”. Table 3.1 shows the minimum, maximum and target proximate composition of each batch of poultry by-product meal produced.

Table 3.1 - Proximate Composition Specifications of Poultry By-Product Meal produced at Talloman, taken from Talloman Protein Conversion Analysis Report (February, 2016).

	Minimum (%)	***Maximum (%)***	***Target (%)***
Protein	60	-	68
Moisture	3	6	5
Fat	11	16	14
Ash	-	16	13
Pepsin (DMD)	86	-	80

The majority of the rendering process is automated however, the company uses trained technicians at each stage of the process to quality check the material to achieve the desired texture, temperature, composition etc. Each batch of PBM is tested to ensure all sold batches adhere to the standards displayed in Table 3.1.

3.2 Moisture/Dry Matter Content

Dry matter was determined using method 1.3R of the Australian Fodder Industry Association (AFIA) Laboratory Methods Manual (Version 8, 2014). Dry matter of dry ground samples was determined by calculating the loss of moisture in samples dried at 105°C for 24 hours.

Porcelain crucibles (30 mL) were oven dried at 105°C for 24hrs and the dried crucibles weight was recorded (4dp). 2g samples of feed were weighed (4dp) and placed into cooled crucibles, recording the total weight (crucible + air dried feed sample) and then placed back into the drying oven (105°C) until samples reached a constant weight (approximately 24 hours). Moisture and dry matter content was calculated using the equations below. All weights were grams.

Equation 3.1 – Percent Dry Matter

$$\%\ \text{Dry matter} = \left(\frac{((\text{oven dried cruc.} + \text{air dried sample}) - \text{oven dried cruc.})}{\text{air dried sample}}\right) \times 100$$

Equation 3.2 - Percent Moisture Content

$$\text{Moisture Content}\ (\%) = 100 - \%\ \text{Dry matter}$$

3.3 Ash

Ash was determined using method 1.10R of the Australian Fodder Industry Association (AFIA) Laboratory Methods Manual (Version 8, 2014). Ash content of dry ground samples was determined by ashing samples in a furnace at 600°C and weighing the remaining inorganic residue.

Prior to analysis 30mL porcelain crucibles were oven dried at 105°C for 24 hours. 2g samples were weighed (4dp) into the crucibles placed into a 600°C muffle furnace, this temperature was held for 2hours (samples formed a uniform grey/white powder) and then the muffle furnace was turned off and left closed overnight to cool. Samples were then removed from the muffle furnace while still warm and placed in a desiccator to cool completely. The total weight (oven dried crucible + ash) of the crucibles was recorded to 4dp and percent ash was calculated using the equation below.

Equation 3.3 - Percent Ash Content.

$$\text{Ash Content (\%)} = \frac{\text{Weight of remaining sample}}{\text{Weight of original sample}} \times 100$$

3.4 Crude Protein

Crude protein was determined using an automated Kjeltec 8200 Distillation Unit (Figure 3.1) and Tecator Digestive system with reference to AOAC Official Method 2001.11 (2005). Total nitrogen was analysed and crude protein was calculated using the standard Kjeldahl factor for protein (6.25) (AOAC 954.01, 2005). The Kjeldahl method consists of 3 parts; 1) Digestion – Decomposition of nitrogen in organic matter by boiling a sample in concentrated sulfuric acid resulting in an ammonium sulfate solution, 2) Distillation – converting NH_4^+ to NH_3 in the sulfate solution by using sodium hydroxide (NaOH) and boiling and condensing the gas in a receiving solution such as boric acid to form ammonium borate and 3) Titration – quantifying the amount of ammonia in the receiving solution.

0.3g (4dp) of dry ground sample was weighed into digestion tubes and 12mL 98% sulfuric acid and 2 Kjeltec digestion tablets (containing 3.5g potassium and 3.5g selenium) were added to the tubes; tubes were heated at 420°C using a block Tecator Digestor for 2 hours or until a pale straw colour was achieved. Digestion tubes were then removed from the block and left to cool for 25 minutes. Digested samples were distilled in the Kjeltec Auto Analyser where 30mL sodium hydroxide was added to the digested sample and the sample was boiled using steam to condense NH_3 gas into a 4% boric acid receiving solution in a 250mL conical flask. The final distillate was titrated using 0.1M Hydrochloric acid (HCl) as a titrant. The final volume of HCl required to change the indicator was recorded. Total nitrogen and crude protein was calculated using the equations below.

Equation 3.4 - Total Nitrogen (%)

$$\text{Nitrogen (\%)} = \frac{14.01 \times \text{M} \times (\text{mL titrate} - \text{mL titrate blank})}{\text{weight dry ground sample (mg)}}$$

Where;

$14.01 =$ the atomic weight of nitrogen

$\text{M} =$ molarity of the HCl

Equation 3.5 - Percent Crude Protein

$$\text{Crude Protein (\%)} = \text{Nitrogen} \times 6.25$$

Figure 3.1 – Foss Kjeltec 8200 Distillation Unit, taken from Foss Brochure (2018)

3.5 Crude Lipid Analysis

Crude Lipid analysis was conducted using a Buchi E-816 Extraction Unit (Figure 3.2) with reference to AOAC Official Method 920.39 (2005).

Glass beakers compatible with the E-816 Extraction unit were washed and oven dried at 105°C for 24 hours. Dried crucibles were placed in a desiccator to cool completely and then weighed to 4dp. Ground samples (2g) were weighed (4dp) into cellulose extraction thimbles (Whatman 33mmx94mm) and inserted into the extraction chambers. The dried glass beakers were fitted in place below the extraction chambers and 100mL of petroleum ether was added to the extraction chamber immersing the bottom of the thimbles that contained dry ground sample in solvent. The solvent refluxed from the extraction chambers into the beakers until 15 to 18 cycles had been completed (approximately 2 hours). The sample was rinsed with fresh solvent. The remainder of the solvent was evaporated and the beakers containing the lipid (fat) extract were left in a fume hood to cool and evaporate residual solvent before being

placed into an oven at 105°C for 24 hours. Crude fat was calculated using the equation below.

Equation 3.6 - Percent Crude Fat.

$$\text{Crude Fat } (\%) = \left(\frac{((\text{oven dried cruc.} + \text{air dried sample}) - \text{oven dried cruc.})}{\text{air dried sample}}\right) \times 100$$

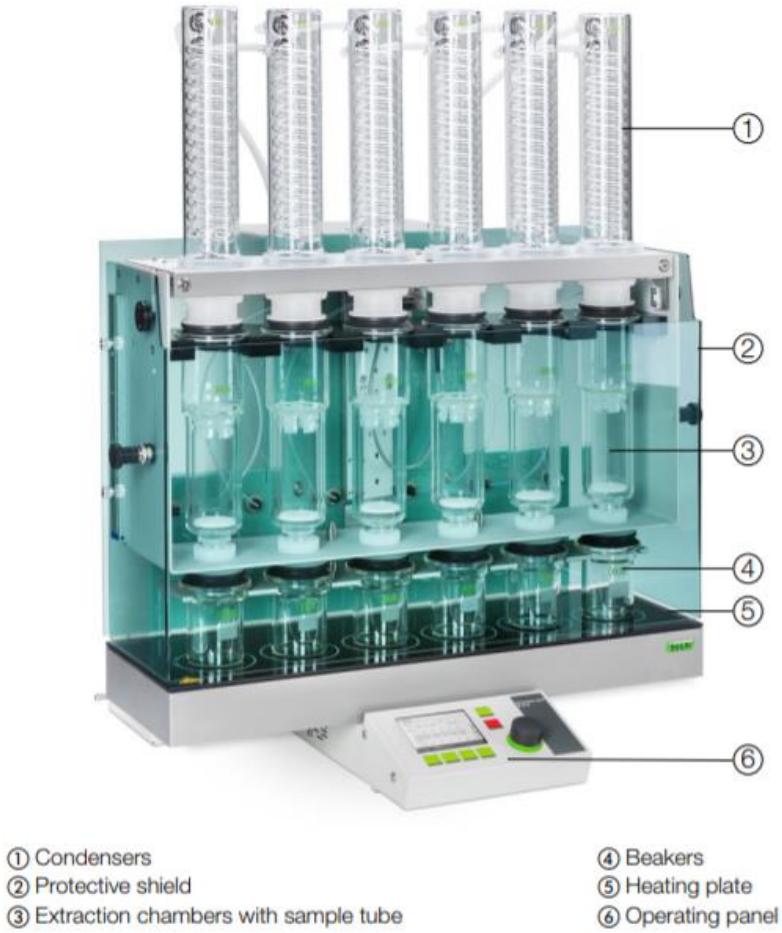

Figure 3.2 - Buchi Extraction Unit E-816 ECE, taken from Operation Manual (Version A, 2015).

3.6 Dry and Organic Matter Digestibility Determination

To determine dry and organic matter digestibility methods were taken from the AFIA Laboratory Methods Manual (Method 1.78R, 2014), the pepsin-cellulase digestibility methods were used. Digestibility was determined over four days. Dry ground samples were incubated with acidified pepsin (0.3% w/v in 0.125M HCl) and incubated in a water bath at 40°C. After 24 hours the sample was heated to 80°C and 2 4mL of thermostable α-amylase (2% v/v) was added and incubated for 45 minutes. The waterbath temperature was lowered to 40°C and pH was adjusted to 4.6 using 0.8mL sodium carbonate solution (1M); the samples were incubated a final time at 40°C with a 10mL buffered cellulose solution (12.5g Yakult Cellulase, Onozuka FA in 20%

sodium acetate buffer). The residue was used to calculate the dry matter and organic matter digestibility.

30mL sintered glass crucibles (porosity 1) were oven dried at 105°C for 24 hours and weighed to 4dp. Digested samples were cooled to room temperature and then filtered through the crucibles under vacuum using deionised water to wash any residue left in the tubes. Crucibles were oven dried at 105°C for 24 hours. Crucibles were cooled in a desiccator and weighed to 4dp. Dry matter digestibility was calculated using the equation below.

Equation 3.7 - Pepsin Cellulase Dry Matter Digestibility.

$$\text{Pepsin Cellulase Dry Matter Digestibility (\%)} = \frac{[\mathrm{DM_S} - ((\mathrm{W_{R(sample)}} - \mathrm{W_C}) - (\mathrm{W_{R(blank)}} - \mathrm{W_C}))] \times 100}{\mathrm{DM_S}}$$

Where $\mathrm{DM_s} = \mathrm{W_s}$ x dry matter (g)

$\mathrm{W_s}$ = Sample weight (g)

$\mathrm{W_{R(sample)}}$ = Weight of sample residue plus crucible (g)

$\mathrm{W_{R(blank)}}$ = Weight of blank residue plus crucible (g)

$\mathrm{W_C}$ = Weight of crucible (g)

After oven dried crucibles were weighed for dry matter crucibles were then placed in a muffle furnace and ashed at 600°C for 2hrs and then left to cool overnight in the furnace with door closed. Samples were removed from the furnace while still warm and placed in a desiccator to cool completely and then weighed to 4dp. Organic matter digestibility was calculated using the equation below.

Equation 3.8 – Pepsin - Cellulase Organic Matter Digestibility.

$$\text{Pepsin Cellulase Organic Matter Digestibility (\%)} = \frac{[\mathrm{OM_S} - ((\mathrm{W_{R(sample)}} - \mathrm{W_C}) - (\mathrm{W_{R(blank)}} - \mathrm{W_C}))] \times 100}{\mathrm{DM_S}}$$

Where $\mathrm{OM_s} = \mathrm{DM_S}$ x Ash (g)

$\mathrm{W_s}$ = Sample weight (g)

$\mathrm{W_{R(sample)}}$ = Weight of sample residue plus crucible (g)

$\mathrm{W_{R(blank)}}$ = Weight of blank residue plus crucible (g)

$\mathrm{W_C}$ = Weight of crucible (g)

3.7 Chromium Determination

Chromium determination was analysed by external laboratories using company methods MET1BTICP and MET1SFICP (Chem Centre, WA, Bentley) with reference to SW-846 Method 3052: Microwave Assisted Acid Digestion of Siliceous and Organically Based Matrices (US EPA 1996) and ISO 12740: Lead sulphide concentrates (ISO 1998). 0.2g (4dp) of dry ground samples were weighed into Teflon tubes with nitric acid, hydrochloric acid and hydrogen peroxide. The tubes were then sealed and placed into a high power microwave and digested at 170 - 220°C. Tubes were then cooled and a green residue was noticed at the bottom of the tubes therefore the next step of measuring the absorbance through spectrophotometry could not be followed. Therefore, additional steps were taken as follows.

The acid digested solutions were made up to 25mL and filtered through N0. 540 Whatman filter papers and the filtrate retained. Filter papers were placed in zirconium crucibles, dried and then ashed at 500°C holding this temperature for 2 hours. Crucibles were cooled in a desiccator and 2g of sodium peroxide (Na_2O_2) and four pellets of sodium hydroxide (NaOH) were added to the crucibles. The crucibles were then heated to 600°C holding this temperature for 2 hours. Crucibles were cooled in a desiccator and water was added to the crucibles. The water was added to the filtrate obtained from the acid digestion, crucibles were rinsed with water several times and finally with diluted hydrochloric acid with all rinsing water added to the filtrate. The combined solution was made up to 250mL in a volumetric flask and percent chromium was determined by inductively coupled plasma-atomic emission spectrometry (ICPAES). Apparent DM digestibility was calculated using the following equation:

Equation 3.9 – Apparent Dry Matter (DM) Digestbility.

$$\text{Apparent DM Digestibility (\%)} = 100 \times \frac{\left(\frac{1}{Cr2O2}\right)Diet - \left(\frac{1}{Cr2O2}\right)Faeces}{\left(\frac{1}{Cr2O2}\right)Diet}$$

Inductively coupled plasma-atomic emission spectrometry (ICPAES).

Atomic emission spectrometry is used for the qualitative and quantitative determination of chemical elements. The principle of ICPAES is “the absorption or emission of light of certain wavelengths after the atomization and excitation of the sample” (Ulrich 2003, pg. 5441). The intensity of the emission before and after the absorption is proportional to the concentration of the sample. Suggested wavelengths for chromium determination are 205.560, 267.716 and 276.653 from the AOAC Official Method 2017.02 (2018).

Chapter 4 - Assessment of the quality of locally sourced Poultry By-product meal (PBM) using proximate analysis, *in-vitro* digestibility and particle size distribution.

4.1 Introduction

PBM exists in two grades; feed grade and pet-food grade and over the last two decades there has been an increase in demand for high quality and protein rich PBM in the pet food industry. The pet industry is willing to pay a premium for PBM that has a defined composition which is marketed as “pet-food grade” and thus has driven efforts in the rendering industry to improve its processing technology to minimize variation and increase protein retention for this market. As a result, feed grade PBM has been widely suggested to have great variation. Dozier et al. (2003) investigated the variation between the two grades of PBM and found that feed grade PBM had greater CP variability (49.3 – 63.7%) then pet-food grade (63.0% - 69.3) and greater lipid variation, feed grade 10.5 – 24.5% and pet-food grade 2.2 – 7.2%. The variation in CP and lipid levels of feed grade PBM was suggested to be due to residues and sludge included in the rendering processes. To be suitable for use in aquatic diets that require a precisce balance of nutrients for optimal growth, high quality PBM needs to be produced consistently with minimal variability to proximate composition.

Studies investigating the use of PBM as complete or partial replacement for FM have shown postive growth results when used in crustacean feeds (table 1.6 and 1.8). The positive response from the crustaceans to the inclusion of PBM was attributed to the high quality of the raw materials and processing technology used to produce the meal. AA analysis showed lysine and methionine content to be similar to that of FM, 3.97% and 1.24% respectively (Cruz-Suárez et al. 2007). Poultry by-products are sourced from the commercial sector where the product is produced from the waste resulting from producing chicken for human consumption. The transport, handling and processing of poultry by-products within Australia is governed by standard operating procedures and federal legislation to help manage the quality and food safety risks associated with rendered products (PISC 2007). The standards ensure that spoilage of raw material is minimized and helps to improve the quality in terms of consistency of the meal by standardizing operations throughout the country.

Feed ingredients can be evaluated for quality both physically and/or chemically. The physical evaluation of feed provides information such as weight, colour, smell and hardness. Particle size is an important physical characteristic that can affect both physical stability of pellets as well as digestibility of feed. Small particles are retained

in the digestive tract, increasing the exposure time to digestive enzymes which improves digestibility (Wade et al. 2018). Similarly, the increased surface area to volume ratio of smaller particles allows greater access to digestive enzymes (Palaniswamy and Ali 1991; Rezaeipour et al. 2014). Smaller more uniform particles are more easily compacted which allows for greater bonding and compaction which in turn provides greater stability within the pellet (Obalado et al.1999). The opposite, larger particles, allow for increased gap size between particles which can exacerbate leaching of nutrients when immersed in water and therefore ideally all feed ingredients are milled to a specified size prior to pelleting to maximize physical stability and in turn physical quality.

Chemical (proximate) analysis of feed quantifies the organic and inorganic components of the feed. Organic components consist of mainly proteins, carbohydrates, vitamins and fats and inorganic components (known as ash) consist of the mineral elements. This quantification of components is known as proximate analysis (McDonald et al. 2010). Proximate analysis partitions feed components into 6 categories (water/moisture, crude protein, ether extract, crude fibre, nitrogen free-extract and ash) based on the chemical properties of the compounds (Hardy 2002). These methods have been standardised by the Association of Official Analytical Chemists (AOAC 2005) and are used to define feed ingredients for feed formulation, for quality control to measure nutrient loss during manufacture and as quality control of the formulated end product (Hardy 2002).

A “quality protein” in this chapter is defined as a product that consistently meets proximate composition standards as defined by manufacturing specifications. Producing a consistent product improves the overall quality of a feed ingredient by providing a consistent source of protein. Inclusion of ingredients of consistent quality and nutritive value can reduce variations in growth and yield from the enterprise. The first step to FM replacement in aquatic diets is sourcing a reliable and sustainable substitute. **This chapter investigated the variability of poultry by-product meal from a Western Australian rendering company (Talloman, Hazelmere, WA) to assess the “quality” of the product over a 3 month period, using particle size, *in-vitro* digestibility and proximate analysis as a measure.**

4.2 Methods and Materials

PBM samples were collected from Talloman from February 2016 to April 2016 (total of 28 samples). Samples were collected on randomly selected dates during the 3

month period. Each sampling day, a 500g sample of PBM was taken from the whole batch of PBM that had been processed and milled that day. Samples were kept in ziplock bags with minimal air inside. As a comparison one batch of commercial FM (Specialty Feeds, Glenforest, WA) was analysed for proximate composition in triplicate for crude protein, moisture, ash, dry mater and organic matter digestibility.

Proximate composition (crude protein, moisture and ash) was determined using the methods stated in chapter 3 and all 28 samples were tested in duplicate. To analyze crude fat only 6 samples were analyzed, 2 randomly chosen samples from each month of the 3 month period. Crude fat was analysed using the methods stated in chapter 3 (3.5). Dry and organic matter digestibility was analysed on 4 random samples of PBM from the 28 samples taken over the 3 month period. Digestibility was analysed using the method stated in section 3.6.

4.2.1 Particle Distribution

One random sample of each protein (PBM and FM) was chosen and tested in quadruplicate 20g samples. Samples were sieved for 5mins through 2000µm, 500µm, 250µm, 125µm and 63µm sieves, distribution of each particle size fraction was obtained using the below equation (equation 4.1);

Equation 4.1 – Percent of total particle fraction.

$$\text{Particle Fraction (\%)} = \frac{\text{weight of sample in fraction (g)}}{\text{total weight of sieved sample (g)}} \times 100$$

4.2.2 Statistical Analysis

All statistical analyses was carried out using the SPSS program (IBM SPSS Statistics 25) for Windows (v. 10.0). Data was checked for normality using the Shapiro-Wilk test of normality. Data were expressed as the mean ± SD of duplicate or triplicate samples. The means within each treatment and among treatments were compared using ANOVA followed by Tukey's test of multiple comparison with a 95% significance level.

4.3 Results

On average over the three-month test period crude protein, moisture and ash of PBM samples all remained within the labelled specifications (table 4.1). However, the crude protein low range of 63% was below the specified target during the end March. Table 4.1 shows relatively consistent product averages and ranges with minimal variation

in all of the tested parameters. Talloman PBM is advertised at 65% protein (minimum 60%) which coincides with the results found in this study.

Table 4.1 - Proximate analysis and dry matter (DM) and organic matter (OM) digestibility of PBM from Talloman over a 3month period and of commercial FM.

	% Crude Protein	% Crude Fat	% Moisture	% Ash	% DM Digestibility	% OM Digestibility
RANGE	63 - 66	12 - 13	3 - 4	13 - 16	73 – 77	70 - 75
AVERAGE	65.65 ±0.23	12.47 ±0.07	3.94 ±0.07	13.89± 0.12	76.05±0.32	73.65±0.35
REQUIREMENT	60 (min)	16 (max)	6 (max)	16 (max)	Pepsin Digestibility (%) 80 (min)	
FISHMEAL	58.32 ±0.63	-	5.54 ±0.05	22.63 ±0.13	80.17±0.25	76.92±0.26

Figure 4.1 shows the particle distribution of both proteins (FM and PBM), both have similar distributions. The dominating particle size for both meals is 125 - <250µm, followed by 63 - <125µm and then 250 - <500µm. The largest and smallest fraction sizes contained <1% of the total sieved sample. Statistically, PBM has a significantly higher proportion of particles than FM in the larger fraction sizes (250 - <500µm and >2000µm) (table 4.2). Figures 4.2 show the contents in both meals at different fraction sizes. Parts of bones, feathers and other debris were visually observed in the largest fraction size of PBM and mainly scales and fish bones in the larger FM fractions. At 250 - <500µm (figure 4.3) it is evident that FM contains various cotton-like particles and is less consistent than its counterpart, this fraction is where the majority of the scales and fish bones collect in FM samples. The smaller fraction sizes (C, D and E) display homogenous and cohesive meal in both PBM and FM.

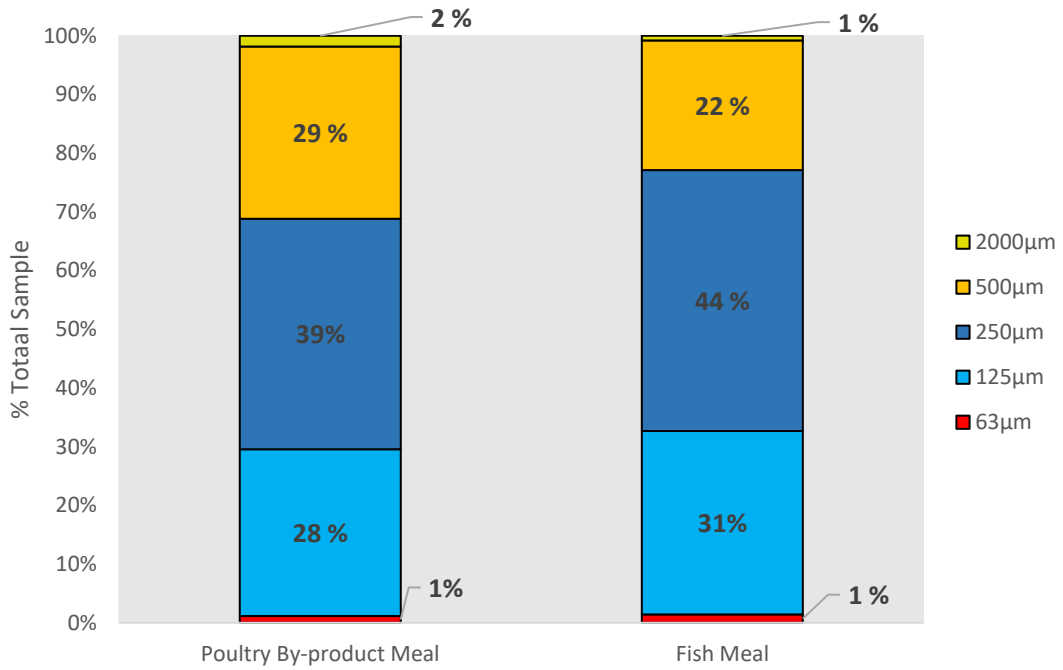

Figure 4.1 - Particle size distribution of 20g feed samples sieved into 2000µm, 500µm, 250µm, 125µm and 63µm fractions.

Table 4.2 – P values comparing particle size proportion of PBM and FM. Significant difference in **bold.**

	% of Total		
Fraction (µm)	**PBM**	**FM**	**P-Value**
<63	1.12±0.23	1.32±0.07	0.50
63 - <125	28.16±4.72	30.34±1.49	0.72
125 - <250	38.86±4.17	44.21±0.95	0.33
250 - <500	29.04±1.03	22.20±0.69	**0.00**
>2000	1.79±0.14	0.81±0.08	**0.00**

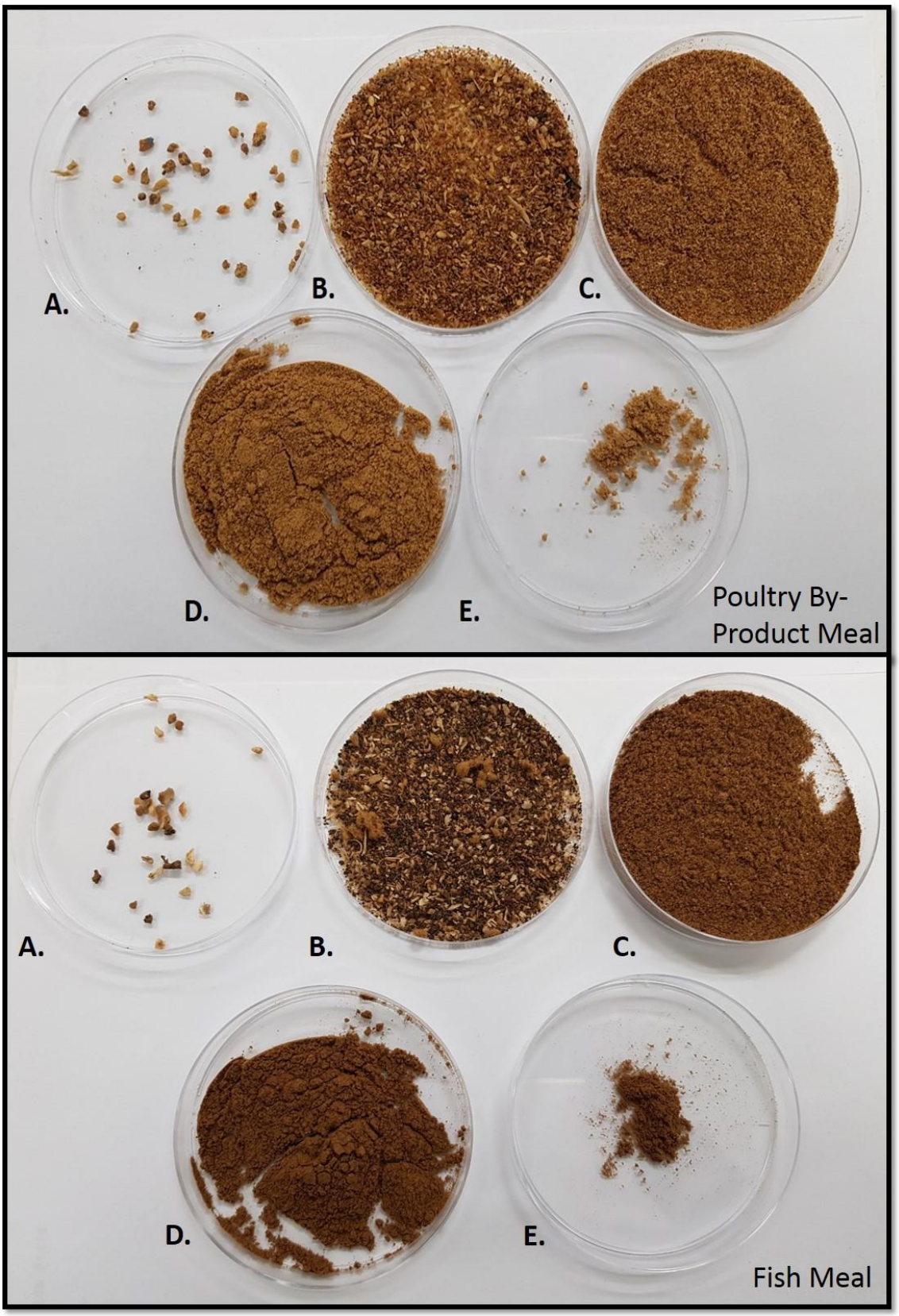

Figure 4.2 - Particle size distribution of 20g Poultry by-product meal and Fish Meal samples sieved into >2000µm (A), 250 - <500µm (B), 125 - <250µm (C), 63 - <125µm (D) and <63µm (E) fractions.

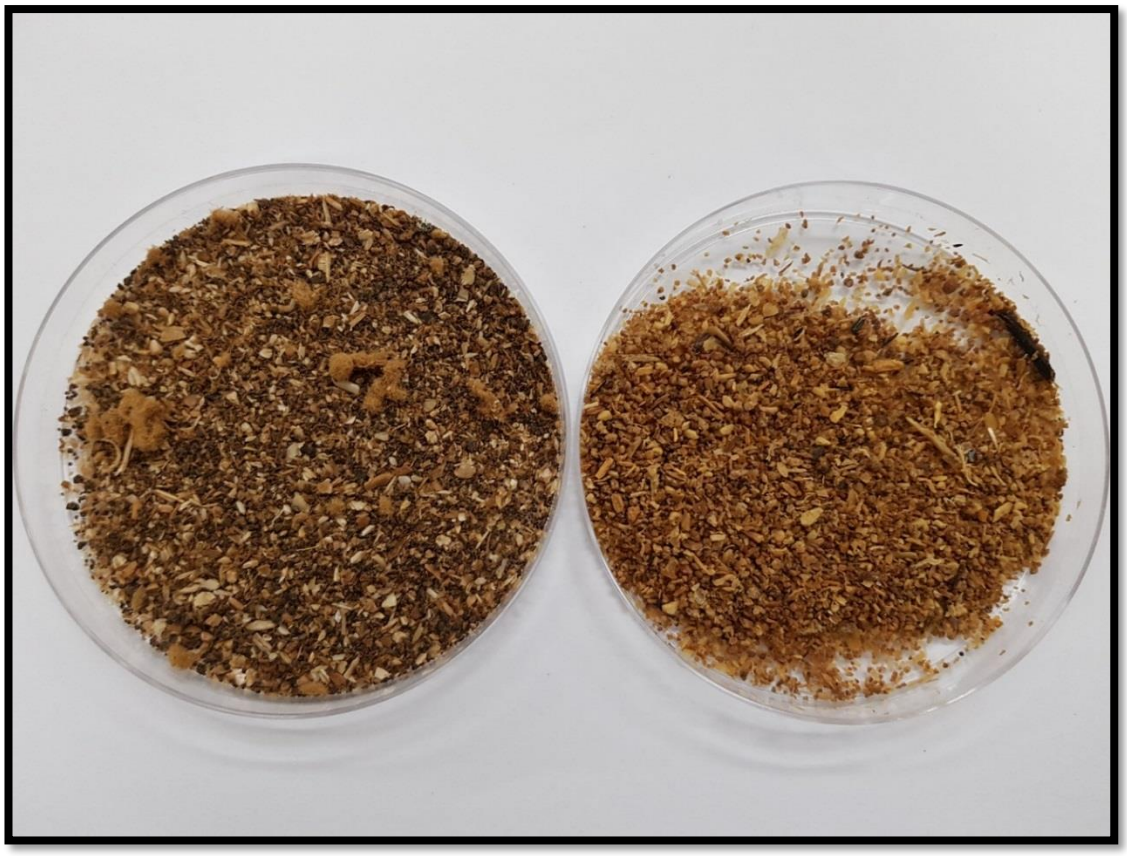

Figure 4.3 – 250 - <500µm sieved sample of Fish Meal (Left) and Poultry by-product meal (Right).

4.4 Discussion

The results obtained from the 3 month temporal study of PBM from Talloman (Hazelmere, WA) demonstrated a consistent product that met manufacturing specifications of crude protein, crude fat, ash and moisture content. Dry and organic matter digestibility values did not meet the 80% digestibility minimum requirement. However, this may be due to differences in test methods or pepsin source and activity and sample number. The PBM ash content had a range of 13 – 16% which may have affected the *in-vitro* digestibility values. Ash content in meals indicate the presence of indigestible material such as bone and has been used as an indicator of poor protein quality (Johnson and Parsons 1997).

The average crude protein level was well above the minimum requirement of 60%. While this can be beneficial as aquatic animals often require protein rich feeds, nutrients can be wasted in the form of fecal output and leaching if the protein level in feed is greater than the animal's requirement resulting in economic loss (Salaah et al. 2010). This must be accounted for during diet formulation to minimize negative effects. A balance between energy and nutrients in a diet must be met to maximize its utilization by the animal (Cho and Bureau 1997). Food wastage and water fouling

due to the leaching of protein in faeces or uneaten feed to the water column is a substantial environmental obstacle in aquaculture (Salaah et al. 2010).

Crude fat and moisture content of PBM remained within a very narrow range over the 3 month period adhering closely to the specifications. Fats contain the most energy per unit than any other biological compound and can be used to offset the cost of proteins (Craig and Helfrich 2017; Halver 1989). The rendering process removes fat from the product; the fat is used to produce poultry oil which is another by-product of use to the stockfeed industry (Woordard et al. 2005). However, fat content in raw ingredients can have an effect on the physical stability of pellets. Lipids lubricate individual particles and lower the compression in the die, this lowered compression decreases the pressure at the die head and pellets are unable to be compacted densely which in turn produces a less physically stable pellet (Lim and Cuzon 1994). This can have a major influence on the effectiveness of an aquatic feed as stability in water is one of the major hurdles faced when manufacturing aquatic diets. Nevertheless, the fat contained within the cells of materials does not have the same effect as when oil or other die lubricating materials (i.e mixer added fats (MAFs)) are added to the formulation (Thomas et al. 1998). Therefore, the fat contained within PBM is unlikely to adversely affect its pelletability.

When compared to FM the particle size distribution of PBM was very similar. The dominating particle size in both meals was between 125 - <250µm and is supported by Obaldo et al. (1999) to be more beneficial in regards to pellet stability. Obaldo et al. (1999) demonstrated that as feed particle size was reduced from 603µm to 69µm, pellet durability, water stability and starch gelatinization increased (Desrumaux et al. 1998; Garber et al. 1997; Rolfe et al. 2000). Feed stability allows for nutrients to stay available to animals while in the water column for longer periods of time which is beneficial for benthic feeders such as crustaceans. Fraction sizes <500µm displayed a uniform and homogenous meal in both FM and PBM (figures 4.3 and 4.4), this is a desirable characteristic when manufacturing feed as smaller and more uniform particles are able to be bound more tightly. The additional compaction in the pellet die increases density and in turn there are fewer breakpoint fissures which improves pellet durability (Obaldo et al. 1999).

Furthermore, it is beneficial to finely grind material to not only aid in physical stability but also aid in feed availability and digestibility. For example, Palaniswamy and Ali (1991) found that feed sieved to 212µm exhibited the highest digestibility (94.23%)

while feeds with particles below this fraction (less than 100µm) had a decreased digestibility when fed to prawns (*Penaeus indicus*). This was attributed to the smaller particles being able to be compacted too heavily, the resulting pellets being very dense and hard, making it difficult for prawns to ingest.This pattern has been supported by Zhu et al. (2001) who found that feed ingredient particle size impacted feed conversion ratio of rainbow trout (*Oncoorhynchus mykiss* Walbaum) (3mm diameter FCR = 1.070 and 0.6mm diamter – 0.6mm FCR = 1.022) and when fed pellets made of smaller particles fish exhibited greater weight gain (4.6%). The majority of the PBM and FM particles were sieved to 250µm similar to Palaniswamy and Ali (1999) study, this indicates that PBM behaves similarly to FM and may be readily incorporated into aquatic diets with favourable particle size characteristics that improves feed physical stability and digestibility.

In conclusion, while protein quality is determined by AA levels and availability. When equipment is not available to measure AA levels, proximate analysis, digestibility and particle size can be used as indicators of quality and consistency. These results show that PBM is a consistent and quality product that behaves similarly to FM in terms of particle size and distribution. Over the 3 month test period PBM adhered to labelled specifications closely, indicating consistency in manufacture and supply. At fraction sizes <500µm PBM exhibited a uniform and homogenous meal free of bones, feathers and other debris that would otherwise hinder pelleting functionality. It would be beneficial for manufacturers to mill PBM at fraction sizes ≤500µm to gain an advantage over FM to improve the utilization of PBM in aquatic diets.

Chapter 5 – Dry Matter Digestibility of diets for marron *(Cherax cainii)* containing PBM

5.1 Introduction

The suitability of a compound feed can be determined by analysing the growth and health response of the target animal and by measuring how much of the feed is digested by using *in-vivo* and/or *in-vitro* methods. Aquatic animal culture mainly uses *in-vivo* apparent digestibility methods as *in-vitro* methods have yet to be standardized. However, *in vitro* methods are commonly used throughout the terrestrial animal farming industry using methods adopted from Tilly and Terry (1963). Methods used to determine aquatic diet digestibility have been adapted from terrestrial methods and focus on protein digestibility rather than carbohydrates. A review by Moyano et al. (2015) extensively covers the existing papers on aquatic animal *in-vitro* digestibility models and concludes that there is comparatively less information available for aquatic nutrition than terrestrial animal nutrition. Crustacean studies have focused on simulating the digestion of protein-rich feed ingredients in penaeid prawn diets, using various different methods such as the pH-stat method, by measuring proteolytic activity and *in-vitro* evaluation using both terrestrial and aquatic animal digestive enzymes (Ezquerra et al. 1997; Moyano et al. 2015; Lemos et al. 2000; Córdova-Murueta & García-Carreño 2002) and crayfish diets using both terrestrial and its own digestive enzymes (Perera et al. 2010). *In-vitro* assays aim to estimate feed digestibility using methods that can be routinely utilized by a laboratory and that are cheaper and faster than *in-vivo* apparent digestibility methods which can be costly and time consuming (Anderson et al. 1993; Fox and Lawrence 2009). In order for *in-vitro* methods to be adopted by the aquatic industry reliable data from *in-vivo* studies are required.

One of the main *in-vivo* methods used to determine apparent digestibility of aquatic feeds uses inert markers incorporated into feeds; the proportion of the marker found in the feed and faeces is used to calculate the digestibility of the diet. This method is widely used throughout the animal production sector and has been utilised in a number of digestibility studies involving chickens (Short et al. 1996) and pigs (Jagger et al. 1992) using titanium dioxide as the inert marker and in fish and crustacean studies using chromic oxide (Opstvedt et al. 2003; Sørensen et al. 2002; Dayal et al. 2011). Limitations of these methodologies exist such as, leaching of the inert marker from pellets prior to ingestion and nutrient leaching from faeces while in the water column. Leaching of protein and other molecules can result in inaccuracies in the

results. Divakaran et al. (2002) reviewed the methodology for chromic oxide determination, demonstrating ash samples oxidized by percholoric acid and quantified by DPC colorimetry to give the most accurate results. While alternative digestibility methods using different trivalent metal oxides such as yttrium have been investigated, accurate determination of these metal oxides in feed and faecal samples has yet to be established. Therefore, the use chromic oxide as an inert marker remains the main method of apparent digestibility determination of aquatic feeds. Using these values correlations can be made to help to improve and standardise *in-vitro* methods to accurately estimate digestibility.

In an attempt to standardise an *in-vitro* methodology, digestibility studies that use enzymes extracted from the digestive glands of the aquatic target animal have been used. Limitations to using such enzymes include, lower protein hydrolysis values (enzyme activity) using extracted enzymes when crustaceans had not been fed on experimental diets, this suggests the need for adaptation of gut enzymes to the diet to be tested (Divakaran et al. 2004). Therefore, the use of standard commercially available enzymes rather than those obtained from digestive glands could yield more consistent results. Although, the use of gut enzymes from the target animal has exhibited promising results (Ezquerra et al. 1997; Fox and Lawrence 2009; Ezquerra et al. 1998; Lemos et al. 2000) these enzymes are not yet commercially available. Enzymes derived from terrestrial animals such as pigs, sheep or even enzymes derived from bacteria are currently commercially available and used to reliably and accurately estimate the apparent digestibility of forages and compound feeds for cows, sheep and goats (Moyano et al. 2015). The aquaculture industry could benefit from the standardization of such methods much like that of the livestock sector, however, correlations between *in-vitro* and *in-vivo* nutritive values must be established in order for *in-vitro* assays to accurately estimate digestive values. Only then can routine laboratory tools be developed for evaluating aquatic feed stuffs and take the place of time-consuming and costly *in-vivo* procedures.

Furthermore, in comparison to fish, crustacean *in-vitro* digestibility models are even fewer. Of the 65 papers covered by Moyano et al. (2015) only 10 of the papers compare both *in-vitro* and *in-vivo* nutritive values in crustacean diets and no papers investigate freshwater crustacean diets. **This study aims to determine the digestibility of feed utilizing PBM as a partial protein replacement in freshwater crayfish/marron (*Cherax cainii*) diets. *In-vivo* and *in-vitro* values will be**

compared to assess the viability of routine *in-vitro* methods utilised in livestock studies to be applied to aquatic animal feed evaluation.

5.2 Materials and Methods

5.2.1 Feed preparation

Experimental Feed

The composition of the experimental diet is displayed in table 5.1. Dry ingredients were mixed together by first combining all smaller quantities (wheat flour, calcium carbonate, vitamin premix, cholesterol and chromium oxide) and larger quantities (PBM, wheat, soy bean meal) separately into bowls and mixing thoroughly for 5mins each. All ingredients were combined using a large plastic bag and shaken for 10mins to ensure the mixture was homogenous. Wet ingredients were combined and mixed thoroughly, lecithin which was in tablet form was dissolved in 100mL warm water and combined with wet mixture. Wet and dry ingredients were then combined in a large mixing tub with a wide bottom to allow 'crumbing' of wet and dry ingredients together to ensure dough was homogenous. Once both mixtures were thought to be well homogenised warm water was added to the mixture until a dough-like consistency was achieved and kneaded for 5mins. Pellets were made using a single screw meat mincer (Reber #12 Electric Meat Mincer) using a 4mm die. Dough was fed through the mincer at a steady rate with pressure only being applied to start extrusion and to finish. This ensured spaghetti-like strands were not compressed heavily. Spaghetti-like strands were then separated and spread over two aluminum drying trays and dried at 60°C. After drying for 24 hours feed was left to cool for 20mins at room temperature and broken into smaller pieces and stored in zip lock bags in a cool dry storage room.

Control Feed

Commercial standard marron feed was purchased from Speciality Feeds (Glenforest, Hazelmere, WA), "Vegetarian Marron Pellets". Pellets were ground to a loose flowing powder using a coffee grinder (Sunbeam). The powder was then weighed and 0.5% chromium oxide was added to the powder in a large plastic bag, the dry mix was shaken for 10mins to ensure mixture was homogenous. Warm water was added to the mixture until a dough-like consistency was achieved and kneaded for 5mins. Pellets were made using a single screw meat mincer (Reber #12 Electric Meat Mincer) using a 4mm die. Dough was fed through mincer at a steady rate with pressure only being applied to start extrusion and to finish to ensure spaghetti-like strands were not compressed heavily. Spaghetti-like strands were then separated

and spread over two aluminium drying trays and dried at 60°C. After drying for 24 hours feed was left to cool for 20mins at room temperature and broken into smaller pieces and stored in zip lock bags in a cool dry storage room.

Proximate Analysis

Proximate analysis (dry matter, crude protein, crude lipid and ash) was conducted on the control feed and the experimental feed. Proximate analysis methods were followed according to chapter 3. Feed analysis of dry matter, crude protein and ash were carried out in triplicate, and crude fat analysis was carried out in duplicate.

5.2.2 Experimental Design

24 farm bred marron (*Cherax cainii*) were ordered from Forest Fresh Marron (Pemberton, WA). The initial weight of individuals was 101.76g ± 4.34g SE. Prior to the feeding trial all marron were fasted for 24 hours to purge any leftover feed in their system. Marron were randomly assigned into 8 tanks (100L) using a random number generator. Each 100L tank had 3 holding chambers (20L bucket with drilled holes to allow for water flow, labelled A, B or C) to isolate marron from each other (figure 5.1). The tanks were then randomly assigned to either an experimental feed treatment or control feed treatment (4 control replicate tanks and 4 experimental replicate tanks), there was no significant difference in size between treatments (control – 103.99g ± 0.42g SE and experimental – 99.71g ± 0.38g SE).

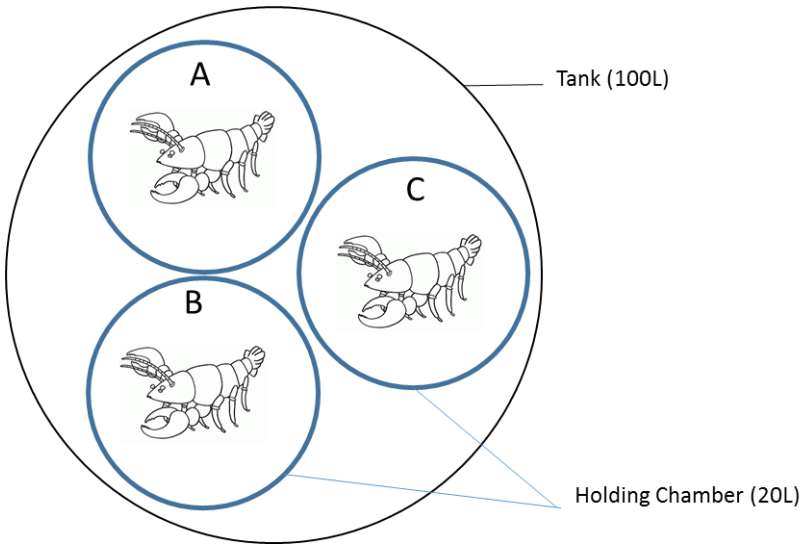

Figure 5.1 – Tank set up and holding chamber configuration, holding chambers were made from 20L buckets with a singular ring of holes drilled around the bucket. Buckets shared tank water.

Feeding rate was determined according to Jussila and Evans (1998), and animals were fed at approximately 1% body weight per day. Uneaten feed and faeces were siphoned out of each holding chamber every morning before feeding. Collected

faeces were separated from uneaten feed manually by immersing material in approximately 50mL of freshwater and removing faecal “strands” using forceps as faecal matter would sink to the bottom edges of the buckets. Faeces were then dried at 60°C in a 20mL porcelain crucibles and frozen for chromic oxide determination at the end of the feeding trial. The total length of the trial lasted 8 weeks with growth parameters (weight, carapace length, tail length and total length) and health parameters (total and differential haemocyte counts) taken at the start and end of the trial. Apparent DM digestibility was calculated using the methods detailed in chapter 3.

5.2.3 Total and Differential Haemocyte Count (THC and DHC)

At the end of the 8 week trial, haemolymph was withdrawn from individual marron at the base of the fifth thoracic leg (figure 5.2) using a 25 gauge needle coated with anticoagulant (1% glutaraldehyde and 0.2M sodium cacodylate) and dispensed into a 0.5mL Eppendorf tube and inverted several times to mix with the anticoagulant.

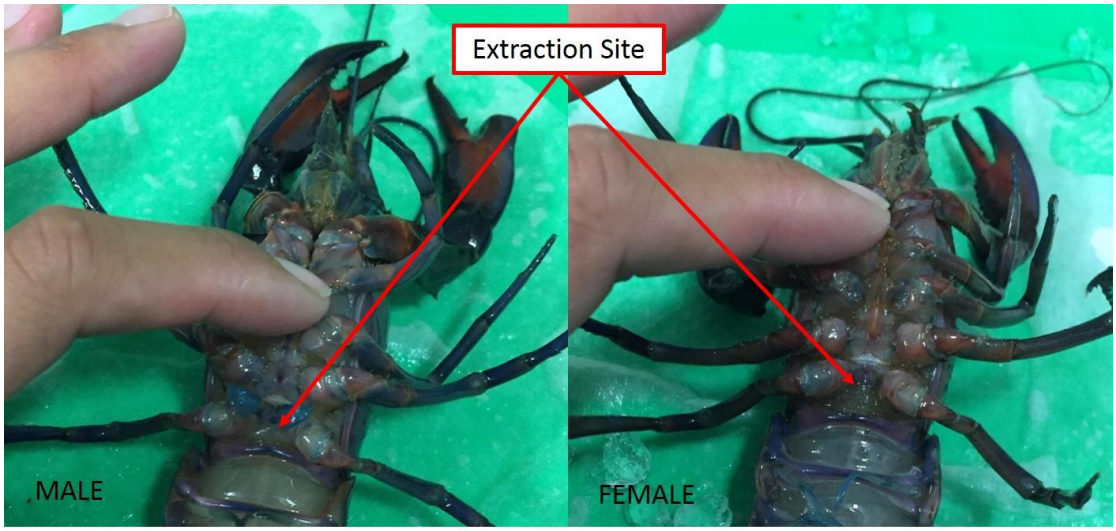

Figure 5.2 - Male vs Female marron (*Cherax cainii*) site of haemolymph extraction.

THCs were counted using a haemocytometer (Weber England, Improved Neubauer B.S 748) under 100x magnification, the average of both grids was used in the following calculation.

Equation 5.1 - Total Haemocyte Count (cells/mL)

$$THC = \frac{cells\ counted \times 1000}{volume\ of\ grid\ 0.1mm^3}$$

Images were taken of haemocytes on the haemocytometer grid immediately after extraction (5.3). A minimum of 200 haemocytes were counted and the percentage of granulated, semi-granulated and hyaline cells was calculated using the below equation.

Equation 5.2 - Differential Haemocyte Count (cell/mL)

$$DHC = \frac{Number\ of\ different\ haemocyte\ cell\ type}{Total\ haemocytes\ counted} \times 100$$

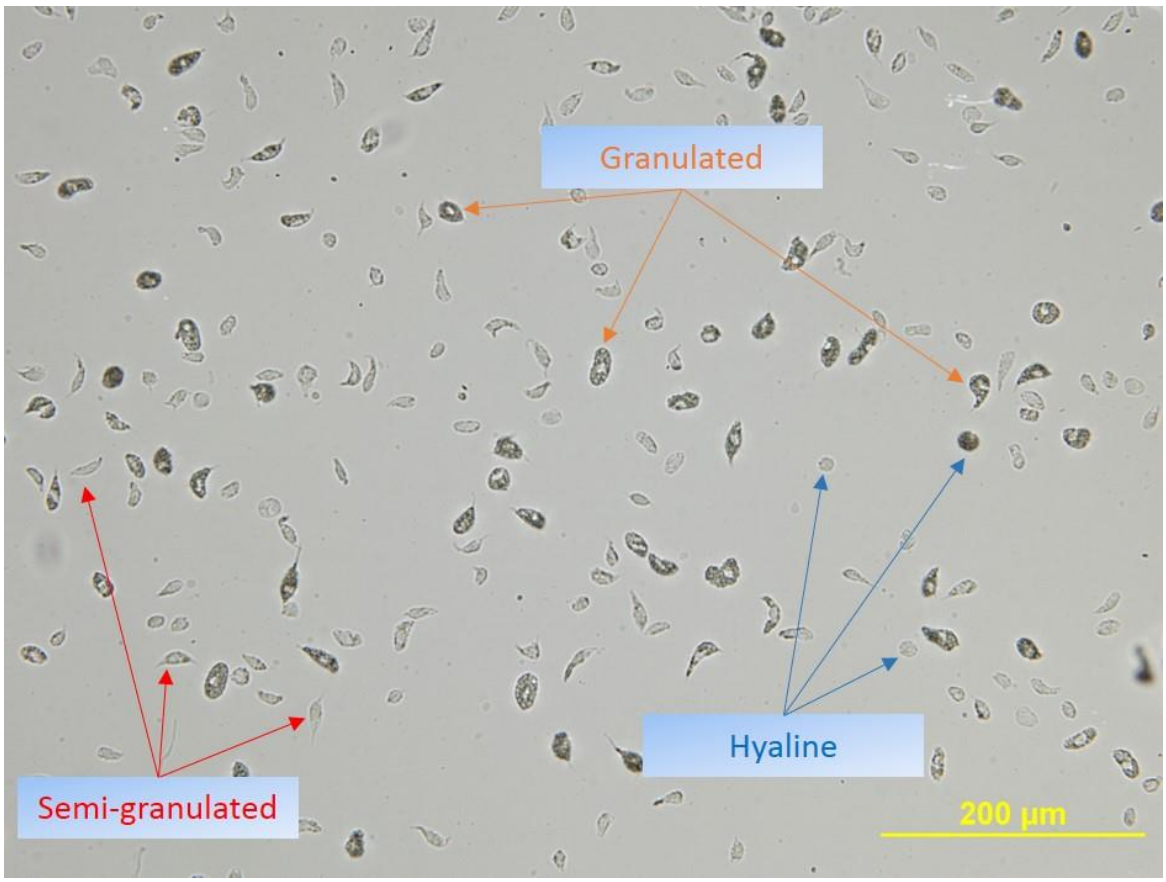

Figure 5.3 - Marron (*Cherax cainii*) haemolymph in 1% glutaraldehyde in 0.2M sodium cacodylate buffer. 100x (Dec, 2018)

5.2.4 Tail Flesh Preparation

At the conclusion of the 8 week feeding trial marron were frozen to euthanize. Frozen marron were taken and put in a refrigerator to thaw for 24 hours. Tail flesh was removed by twisting tail clockwise and firmly holding the carapace, the shell was removed with aid of scissors and wet weight of the tail flesh was recorded (4dp). Flesh was then placed into porcelain crucibles (100mL) that had been previously dried at 105°C for 24 hours and weighed (4dp). Crucibles with the tail flesh were then placed in an oven and dried at 60°C for 7 days to ensure all moisture was removed. Moisture content was calculated according to the equations below (equation 5.3 and 5.4).

Equation 5.3 – Percent dry matter of marron tail flesh.

$$\%\ \text{Dry matter} = \left(\frac{((\text{oven dried cruc.} + \text{wet weight of tail flesh}) - \text{oven dried cruc.})}{\text{wet weight of tail flesh}}\right) \times 100$$

Equation 5.4 – Percent moisture content of marron tail flesh.

$$\text{Moisture Content}\ (\%) = 100 - \%\ \text{Dry matter}$$

Once moisture content of the tail flesh was recorded the dried flesh was ground using a coffee grinder (Sunbeam) into a free flowing powder. Tail flesh powder was then used for proximate composition analysis according to the methods in chapter 3.

5.2.5 Statistical Analysis

All statistical analyses was carried out using the SPSS program (IBM SPSS Statistics 25) for Windows (v. 10.0). Data were expressed as the mean ± SE of triplicate samples. The means within each treatment and among treatments were compared using ANOVA followed by Tukey's test of multiple comparison with a 95% significance level.

5.3 Results

The formulation of the experimental and control diets can be seen in table 5.1. Dry matter, ash and crude lipid of the diets was similar, crude protein of the standard marron diet (22% CP) was lower in protein than the experimental diet (34% CP). The experimental diet replaced whole lupin, lupin kernel, wheat flour and barley with PBM to form a complete diet for marron at 34% CP.

Table 5.1 - Formulation of control and experimental marron diet with 0.5% Cr_2O_3.

Ingredient	g/kg	
	Control	**Experimental**
Poultry By Product Meal	-	338
Lupin Whole (milled)	106	-
Lupin Kernel	70	-
Wheat Flour	27	494
Barley	16	-
Wheat Starch	-	20
Soybean Meal	-	102
Calcium Carbonate	*	6
Vitamin Premix	*	6
Fish Oil	*	32
Cholesterol	-	2
Chromium Oxide	5	5
Dry Matter	92.03±0.30	92.60±0.03
Crude Protein	22.14±0.40	34.70±0.04
Crude Lipid	6.01±0.71	7.72±0.07
Ash	6.50±0.16	6.60±0.14

"*" – Ingredients were included at least cost ration.

Table 5.2 shows the proximate composition of marron tail flesh after the 8 week feeding trial and the final dry weight of the tail. Dry matter, crude lipid, ash, crude protein and tail dry weight between the two treatments did not differ significantly.

Table 5.2 - Proximate Composition of marron tail flesh after 8 week feeding trial.

(%)	Control	Experimental
Moisture	79.38±0.35	80.95±0.79
Crude Protein	75.96±1.35	73.43±1.75
Crude Lipid	1.64±0.15	1.71±0.04
Ash	5.40±0.38	6.54±0.15
Tail Dry Weight (g)	6.01±3.00	5.69±2.84

No significant difference in weight gain was observed between treatments (figure 5.4). Immune competence assays exhibited no significant difference in differential or total haemocyte counts between the two groups (P>0.05) (figure 5.5).

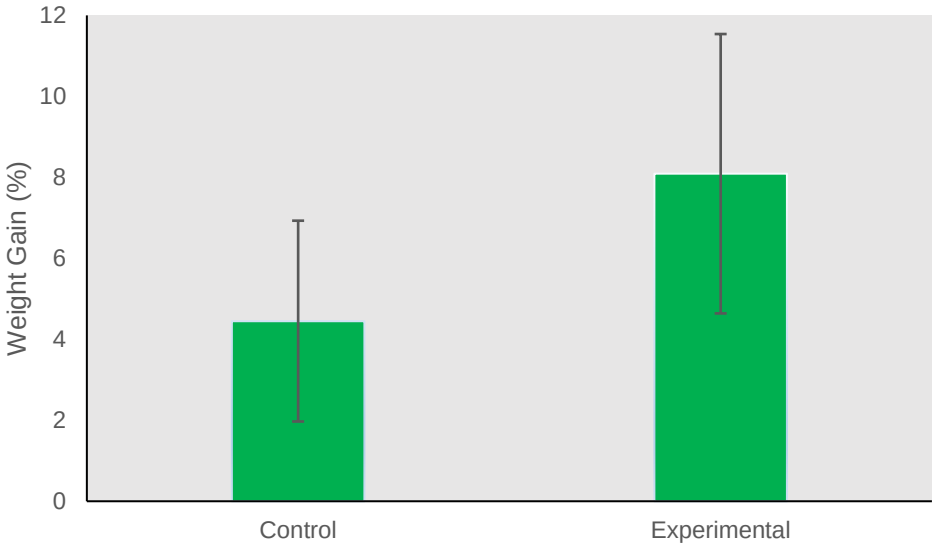

Figure 5.4 - Percent weight gain from initial liveweight of marron fed compound feeds, experimental feed using PBM as a protein replacement.

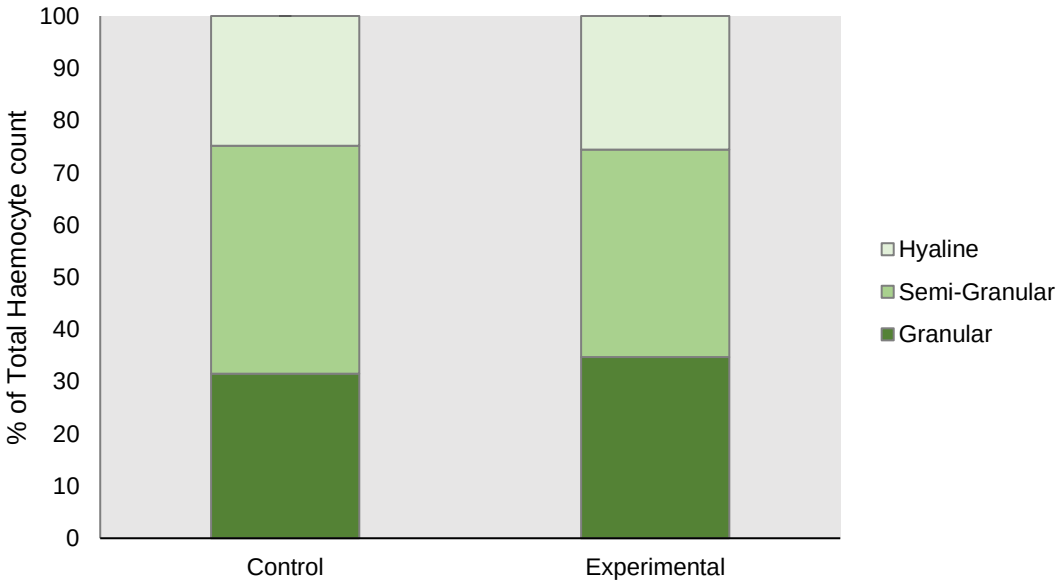

Figure 5.5 - Haemocyte counts of marron fed experimental compound feeds using PBM as a protein replacement.

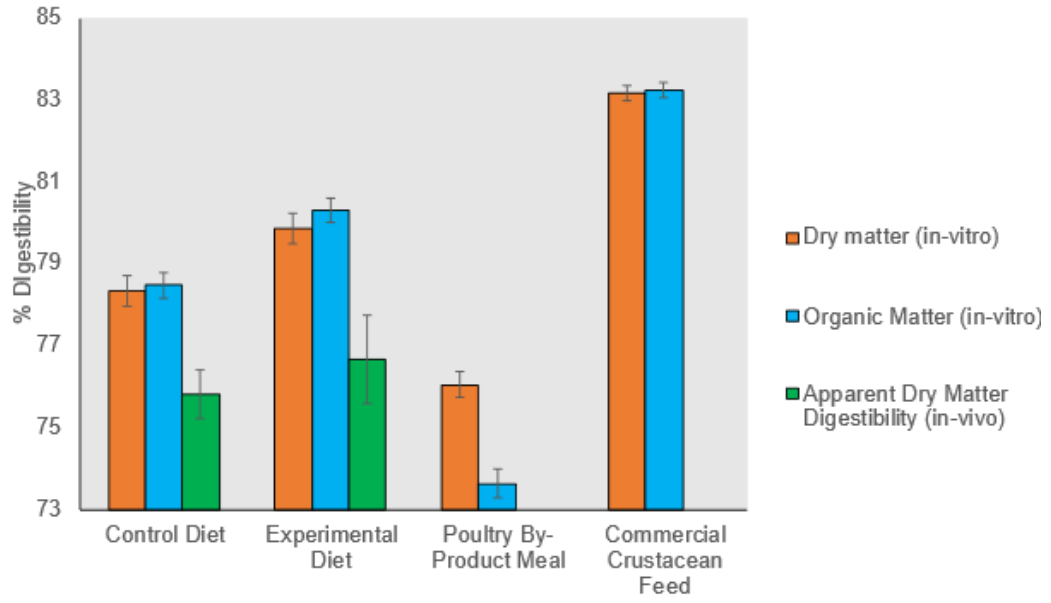

Figure 5.6 –Pepsin cellulase dry matter and organic matter digestibility (*in-vitro*) and apparent dry matter (*in-vivo)* digestibility values of single proteins and compound feeds fed to marron.

No significant differences were found when comparing digestibility values of the three test methods within each diet/test ingredient and no significant difference was found when comparing digestibility methods among all diets/test ingredients (P= 0.652). Apparent digestibility of dry matter did not differ from *in-vitro* values. Due to the low volume of faecal samples, apparent digestibility of individual nutrients was unable to be analysed. PBM in its raw form and in compound feed (experimental diet) had similar digestibility values for all three treatments (P= 0.960). However, in raw form PBM had significantly lower organic matter digestibility (P= 0.000) than all other diets/ingredients.

5.4 Discussion

Replacing protein using PBM in a marron diet did not affect marron (*Cherax cainii*) growth performance or health over the 8 week trial period. The experimental diet contained 33.8% PBM and 10.2% soybean meal with an overall CP content of 34%. The control diet contained lupin as the main protein source with an overall CP content of 22.1%. Marron growth performance was similar with no significant difference in weight gain after the trial period between the two groups. Both diets were able to sustain marron for the trial period and can be considered complete. No moulting was observed during the trial period therefore growth data was inconclusive. However, tail flesh dry weight was used as an indicator of nutritional condition The concept of nutritional condition assumes “a positive relationship between body energy reserves and fitness” (Fitzgibbon et al. 2015, pg. 265). For crustaceans this can be defined as

the extent to which reserves have accumulated to allow normal physiological function and growth (Moore et al. 2000; Fitzgibbon et al. 2015). Crustacean life stages, in particular ecdysis (i.e moulting) give rise to periods of sequentional accumulation and depletion of internal metabolic reserves, natural fluctuations of nutritional condition (Fitzgibbon et al. 2015).The tail flesh dry weights represent accumulation of protein into muscle and indicates relative muscle deposition, results showed comparable tail dry weights between the two groups.

In this study, dietary protein between groups differed in source and crude protein content, however, this level of dietary protein did not appear to affect crayfish health, or growth negatively. The “ideal protein concept” refers to the idea that whole body or tail muscle amino acid profiles can be used as reference dietary profiles (Trushenski et al. 2006; Reed and D’Abramo 1989). Amino acid profiles of marron tail flesh and diets were unable to be analysed due to lack of equipment and funds, however, the proximate composition of the tail flesh of fed marron did not differ significantly between treatments therefore, providing supporting evidence for the use of PBM as a non-marine protein alternative in marron diets. A longer growth trial testing isonitrogenous diets is required for more conclusive evidence on marron growth performance of the protein where marron are able to moult at least twice.

Health parameters (total and differential haemocyte counts) showed no adverse health effects from the use of PBM as no significant difference was observed between groups. Haemocytes play an important role in crustacean defense with the three main types of haemocyte cell found in crustaceans being hyaline cells which are involved with coagulation and/or clotting (Omori et al 1989), semi-granular and granular which are involved in phagocytosis of pathogens and other bacteria (Gargioni and Barracco, 1998; Yang et al. 2003).Total number of haemocytes and proportion of haemocyte types have been used as an indication of health in crustaceans (Fotedar et al. 2001; Sang et al. 2009; Bachere 2000; Cheng et al. 2004; Yang et al. 2003). THC’s and DHC’s from the present study were at similar levels to those expected for healthy marron obtained from Nugroho and Fotedar (2013) and Sang and Fotedar (2010). Both studies examined the immunological response of crayfish from the *Cherax* family.

Digestibility of a feed can be increased with extensive thermal and mechanical treatment, like those used in commercial feed production. The processes have been observed to break down complicated molecular structures making them more easily

digestible. Energy in the form of heat and pressure have the ability to break native bonds in molecules (i.e proteins and carbohydrates) making them more easily accessible to enzymatic degradation and in turn can improve the digestibility of feeds (Stone et al. 2003; Svihus et al. 2005; Wade et al. 2018). In contrast, the experimental diet used in this study was handmade in a lab using rudimentary equipment and therefore was not subjected to intensive conditioning. The lab made feeds in the present study were dried at 60°C which is within the range to break down the quaternary and tertiary structures of protein molecules (Camire 1991). However, DM digestibility values of handmade feeds in the current study were not observed to have significantly higher DM digestibility values when compared to the raw ingredient, therefore it is unlikely that DM digestibility was altered due to manufacturing processes. Although handmade feeds were subjected to thermal treatment within the range of denaturing this exposure only occurred at the end of the manufacturing process (drying) for a maximum of 24hrs which may not have been sufficient exposure to thermal treatment to alter protein digestibility.

Compared to fish, the crustacean digestive process is much simpler. To simulate *in-vitro* digestion of crustaceans' researchers have adopted the pH-stat method. This method reproduces one step enzyme hydrolysis which may be more appropriate for crustaceans as it mimics the intestinal stage of digestion using enzymes derived from terrestrial animals such as procine trypsin, bovine chymotrypsin and pronase (Shipton and Britz 2002; Ezquerra et al. 1997). Whereas, more complex systems (two step configurations) with semi permeable membranes may be more suitable for fish species (Moyano et al. 2015). Research on crustacean enzymes has shown that marine decapod crustaceans synthesize a range of proteolytic enzymes in the digestive gland such as endopeptidases (trypsin and chymotrypsin) and exopeptidases (carboxypeptidases and aminopeptidases) which differ from the mammal enzymes used in this study (Rossano et al. 2011). The use of mammal or bacterial enzymes in digestibility assays is beneficial to show differences in susceptibility to hydrolysis under controlled conditions (Lazo et al. 1998; García-Carreño et al. 1997; Córdova-Murueta and García-Carreño 2002)

The enzyme to substrate ratio is also an influential factor in obtaining accurate digestibility values and Moyano et al. (2015) explains that these ratios tend to be chosen to obtain clearly measurable effect and that there is lack of explanation for the rationale behind these ratios. Standardised enzyme-substrate ratios that exist in the digestive tracts of different aquatic species have not been established and

therefore current *in-vitro* values may not accurately reflect the *in-vivo* process. The standardised substrate-enzyme ratio in terrestrial and human digestibility studies are the foundation of accurate results (Kitessa et al. 1999; Mabjeesh et al. 2000; Hur et al. 2011; Guerra et al. 2012; Moyano et al. 2015). Operating conditions of *in-vitro* assays should also be based around the physiological parameters existing in the selected species, therefore should closely reflect that of the target aquatic species.

In conclusion, practical application of *in-vitro* digestibility methods would require a standard operating procedure that is straightforward and reliable to reduce human error and inter-laboratory differences. Greater inter-laboratory collaboration would benefit the industry by establishing a standard protocol and help to reduce the number of *in-vivo* assays needed to evaluate raw constituents and compound feeds. This would result in savings in both cost and time. In this study, no significant difference was observed between apparent digestibility values and pepsin cellulase dry and organic matter digestibility values in marron fed on diets utilizing PBM as the primary protein. This provides supporting evidence for the use of this method to estimate digestibility values. However, more data is needed to develop accurate correlations between apparent digestibility values and *in-vitro* values for its further application.

Chapter 6 – Effect of feed shape and drying temperature on pellet stability using PBM as an alternative protein in crustacean diets

6.1 Introduction

The performance of aquatic feed is judged on three main factors: (1) nutritional quality, (2) pellet stability and (3) palatability (Yoshitomi 2004). Pellet water stability can be defined as "the retention of pellet physical integrity with minimal disintegration and nutrient leaching while in the water until consumed by the animal" (Obaldo et al. 2002, pg. 369). The physical integrity of aquatic feeds play a vital role in farmed fish production, feed must stay intact with minimal leaching to ensure delivery of nutrients to the animal and to minimise water pollution (Halver 1989; Ali 1988). The required degree of stability is dependent on the species and its feeding behaviour. Most finfish species such as trout and catfish are 'instant feeders' (Chen & Jenn 1992) and therefore feed is only required to remain stable in water for a few minutes. In contrast, crustaceans such as prawns/crayfish are benthic feeders and use appendages to manipulate their food and therefore require a feed that sinks and remains stable for multiple hours (Dominy et al. 2004; Jussila and Evans 1991). The feeding behaviour of crustaceans allows for greater dry matter loss and nutrient leaching to the medium as crustaceans prefer to forage and to break large feed particles into smaller pieces prior to ingestion (Hunt et al. 1992; Igwhela et al. 2013). During consumption crustaceans use their appendages to masticate their food outside the buccal cavity before ingestion, if feed is not sufficiently water stable it can create fines which are swept away by exhalent gill currents, becoming unavailable to the animal and producing waste (Forster 1972). Highly water-stable feeds minimise water pollution and improve the feed conversion ratio through a reduction of nutrient loss (Meyers et al. 1972). The functional properties of each individual component included in a compound feed as well as the choice of equipment, processing conditions and system variables are all factors that affect the physical quality of a feed (Thomas et al. 1997). Feed formulation is a compromise between meeting the nutritional requirements of an animal and developing a product that is physically stable, can be mass produced, transported and stored (shelf-life). Therefore, it can be argued that pelleting functionality is as important as nutritional composition in feed formulation (Halver 1989).

The growing demand and rising prices of fish meal (FM) due to unsustainable pressure on fish wild stocks have made this protein an unfeasible ingredient at the current inclusion rates (Fuertes et al. 2013; Naylor et al. 2009; Tacon & Metian 2008). As a result, research has been focused on finding a suitable alternative. PBM is a

sustainable, readily available and cheaper animal protein alternative that has had success in partial or complete FM replacement in aquatic feeds (table 1.7 and 1.8). Studies of PBM as a replacement protein have been focused mainly on biological performance of cultured species and there is limited research on its manufacturing capabilities in aquatic diets and the effects of pellet shape on water stability. On the contrary, there have been multiple studies on the impact of production processes on pellet water stability comprised of other constituents such as FM, where parameters such as heat, pressure, moisture, particle size and pellet size are adjusted throughout the production stages to improve pelleting characteristics and consequently the biological performance of animals (Barrows et al. 2007; Booth et al. 2002; Bowzer et al. 2016; Yoshitomi 2004). Huu and Lan (2015) briefly investigated the effect of two pellet shapes; noodle and disc on spiny lobster (*Panulirus ornatus*) growth and survival. However, no water stability data was collected and diet was nutritionally deficient giving inconclusive results.

Information on the physical performance of protein alternatives in feeds is lacking. Under lab conditions where feed manufacturing machinery is limited, two easily manipulated processes are drying temperature and feed shape. **This chapter aims to examine the effect of drying temperature and feed shape on the water stability of crustacean feed using PBM as a protein alternative. It was hypothesized that altering the shape and drying temperature of pellets made with PBM will affect pellet water stability.**

6.2 Methods and Materials

Diets utilizing PBM as the primary source of protein were tested to investigated the effects on pellet stability. Crustacean feed was formed into three different pellet shapes (wafer, mash and pellet) (figure 6.1) and dried at 3 temperature treatments (50, 60 and 70°C) for 24 hours. The composition of each experimental diet is listed in table 6.1 on a percentage dry matter basis. Shapes were developed from pilot feed studies with *Cherax cainii*. Observations of crayfish consuming handmade and hand cut wafer like biscuits suggested that crayfish consume the handmade wafer shaped feed more readily than pellets. Mash shaped feed was a derivation of this shape with added protrusions to increase surface area to aid in feed manipulation by crayfish.

6.2.1 Feed Preparation

Dry ingredients were mixed together by first combining all small quantities (calcium carbonate, salt, vitamin premix, casein and cholesterol) and large quantities (PBM,

wheat, wheat starch, and soybean meal) separately using mixing bowls and mixing by hand thoroughly for 5mins each. All dry ingredients were then combined using a large plastic bag and shaken by hand for 10mins to ensure mixture was homogenous. Wet ingredients were combined and mixed, lecithin which was in tablet form was dissolved in warm water and combined with wet mixture. Wet and dry ingredients were then combined in a large mixing tub with a wide bottom to allow 'crumbing' of wet and dry ingredients together to ensure dough was homogenous. Once both mixtures were thought to be well homogenised warm water was added to the mixture until a dough-like consistency was achieved and kneaded by hand for 5mins. The dough was then weighed and divided into 3 equal portions. Shapes were decided from preliminary observations from a separate study using hand made feeds where crayfish appeared to prefer holding feed using their appendages and breaking larger pieces into smaller pieces.

Wafer (W) pellets were made by rolling and compressing dough onto an aluminium tray using a rolling pin until approximately 0.5cm thick. Tray dimensions were 30x25cm, once rolled out the dough was cut into 1.5cm x 10cm strips and dried at different temperature treatments.

Mash (M) was formed by dividing dough into approximately 2g pieces and pressing between forefinger and thumb to create oddly shaped clumps with 2 to 3 protrusions on each clump. Clumps were laid out on an aluminium drying tray to dry at different temperature treatments.

Pellets (P) were made using a conventional single screw meat mincer (Reber #12 Electric Meat Mincer) using a 4mm die. Dough was fed through mincer at a steady rate with pressure only being applied to start extrusion and to finish to ensure spaghetti-like strands were not compressed heavily. Spaghetti-like strands were then separated and spread over two aluminium drying trays and dried at different temperature treatments.

After drying for 24 hours feed was left to cool for 20mins at room temperature before being broken into smaller pieces and stored in zip lock bags in a cool dry storage room.

Figure 6.1 – Crustacean feed manufactured into 3 experimental shapes using Poultry by-product meal as a Fish meal replacement.

Table 6.1 - Formulation of experimental crustacean diet.

Ingredient	g/kg
Poultry By Product Meal	480
Wheat	233
Wheat Starch	188
Soybean Meal	50
Calcium Carbonate	2
Salt (NaCl)	2
Vitamin Premix	1
Casein	2
Fish Oil	30
Olive Oil	10
Lecithin	1
Cholesterol	1
TOTAL	1000

6.2.2 Determination of Water Stability of Pellets

The method used to determine water stability of feed was adapted from Igwhela, Ahmad and Abol-Munafi (2013). Leach rate (LR) refers to the physical disintegration of feed (loss of mass to the aqueous medium) while the percent crude protein (CP) of each feed was the amount of protein remaining in the feed after the designated immersion time.

5g samples of each of the 9 experimental diets were weighed (4dp) and immersed in either 0ppt deoinised water or 33ppt saline solution, 1000mL. Feed was immersed in six time treatments of 1hour intervals up to a maximum 6hr immersion period.

Immersed samples were then filtered through glass fibre filter paper (GF-C 7.0cm, GE Whatman) placed inside a Buchner funnel attached to a 1.0L vacuum flask. Samples were filtered completely and filter paper and left over feed was transferred into pre-dried (105°C for 24 hours) and weighed (4dp) porcelain crucibles (30mL). Crucibles containing feed and filter paper were dried at 105°C for 24 hours, cooled in a desiccator and then weighed (4dp). Percentage dry weight loss and leaching rate was obtained using equations 6.1 and 6.2. All diets were tested in 2 solutions to simulate marine (33ppt) and freshwater (0ppt) environments and time treatments (1, 2, 3, 4, 5, 6hrs) were tested in triplicate. Figure 6.2 illustrates the experimental design.

Equation 6.1 – Percent Dry Matter Loss.

$$\text{Dry Matter Loss } (\%) = \frac{\boldsymbol{Initial\ Sample\ Weight - Remaining\ Solid}}{\boldsymbol{Initial\ Sample\ Weight}} \times \mathbf{100}$$

Equation 6.2 – Leach Rate.

$$\text{Leach Rate } (\%) = \frac{\boldsymbol{A}\times(\mathbf{1}-\boldsymbol{r})-\boldsymbol{R}}{\boldsymbol{A}\times(\mathbf{1}-\boldsymbol{r})} \times \mathbf{100},$$

Where $\boldsymbol{A}$ =weight of pellets before immersion;

$\boldsymbol{r}$ =moisture content of pellets and

$\boldsymbol{R}$ =Dry weight of the remaining solid

6.2.3 Feed Analysis

The remaining crude protein after immersion was determined for every treatment. Once dry matter loss and leach rate had been calculated the dried feed samples were removed from filter papers and feed was ground to a free-flowing powder using a coffee grinder (Sunbeam). Crude protein was analysed following procedures presented in chapter 3 (equation 3.4 and 3.5). Proximate analysis was conducted on the 9 experimental diets according to the methods outlined in chapter 3. Remaining crude protein of immersed feed was obtained using the same methods, crude protein was determined for each time treatment replicate.

6.2.4 Statistical analysis

All statistical analyses was carried out using the Primer-e with PERMANOVA+ (version 7). Data were expressed as the mean ± SD of triplicate samples. Data was checked for normality using the Shapiro-Wilk test of normality. The means within each treatment and among treatments were compared using PERMANOVA followed by pairwise comparisons with a 95% significance level.

Experimental Design

Figure 6.2 illustrates the experimental design, handmade feeds were dried in 3 shapes; wafer, mash and pellet; at 3 temperatures; 50, 60 and 70°C and then immersed in 2 different aqueous solutions; 0ppt and 33ppt. Feed was immersed in 6 time treatments of 1hour intervals up to a maximum 6hr immersion period, triplicate samples were tested in each time treatment. Crude protein retention and leach rate was calculated for each sample.

All data were assessed for normality using the Shapiro-Wilk test for normality and Levene's test for homogeneity of variances. Once normality and homogeneity of variances was confirmed data were tested using a three factor ANOVA (Factors: Feed Shape, Drying Time, Immersion Time, Figure 6.2) followed by post-hoc pairwise testing to identify differences among time treatments. Statistical analysis was performed using SPSS (IBM SPSS Statistics 25) for parametric assumptions and Primer 7 for multi factor analysis (PRIMER 7).

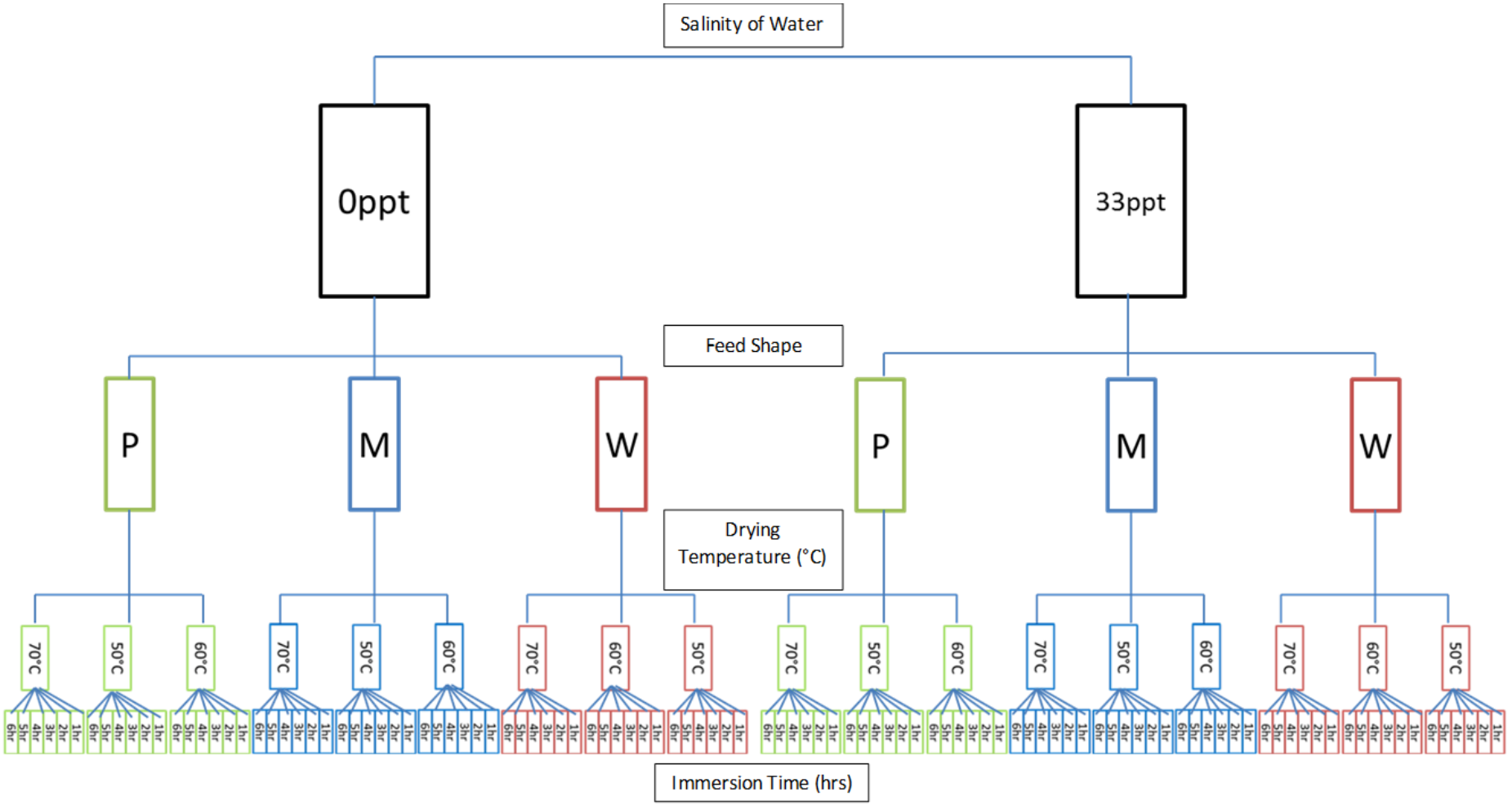

Figure 6.2 - Experimental design of water stability testing on 9 experimental diet, where P= Pellet, M=Mash and W=Wafer to depict the shape of the feed. Each immersion time was tested in triplicate (not pictured).

6.3 Results

The proximate composition of the 9 diet variations is shown in table 6.2. The intended crude protein level was 37% however, the final product of each of the diets ranged from 34 to 36.8%. Crude lipid values were the highest on average in feeds dried at 70°C with exception to mash shaped feed dried at 50°C which had an increased crude lipid value of 14.07%. Dry matter and ash values among diets were similar.

Table 6.2 - Proximate Composition feeds dried at 3 different temperatures and in 3 feed shapes.

Drying Temperature	Feed Shape	Dry Matter (%)	Ash (%)	Crude Protein (%)	Crude Lipid (%)
50°C	Wafer	92.54	8.77	36.08	9.52
	Mash	92.68	8.21	36.80	14.07
	Pellet	92.54	7.96	34.39	9.91
60°C	Wafer	92.51	8.45	36.19	9.41
	Mash	93.12	8.11	36.55	9.60
	Pellet	93.50	7.79	34.29	10.36
70°C	Wafer	93.93	7.68	36.21	13.68
	Mash	93.97	7.83	35.94	12.29
	Pellet	93.30	8.28	35.53	11.42

6.3.1 Freshwater (0ppt)

LR was affected by drying temperature for all shape treatments and increased with time (table 6.3 and figures 6.3). Feed dried at 60°C performed the best with the lowest LR among temperature treatments while feed dried at 70°C performed poorly, with the highest LR. Wafer shaped feed had the lowest LR in all temperature treatments (fig 6.3), followed by mash and then by pellet shaped feed in the 50°C and 60°C treatments. When feed was dried at 70°C feed tended to leach faster and no significant difference was found between mash or pellet shaped feed whereas wafer shaped feed performed significantly better than the other shapes after 4 hours of immersion (table 6.3).

Drying temperature had an effect on CP after immersion in freshwater (figure 6.3). CP was lowest when feed was dried at 60°C and highest when dried at 50°C. At 50°C drying temperature the feed shape that performed the best in terms of CP retention was wafer, retaining significantly more CP throughout the 6hour immersion period, mash and pellet shaped feed did not significantly differ after 1hour of immersion in

freshwater (figure 6.4). At 60°C drying temperature, in the first 2 hours of immersion CP was retained most by wafer shaped feed, after this time shape did not have an effect on CP until at 6 hours where pellet shaped feed had significantly less CP then the other two shapes (figure 6.4). At 70°C drying temperature mash shaped feed retained the most CP staying relatively stable throughout the 6hour period, whereas pellet and wafer shapes declined in CP.

Table 6.3 – Results of a multi factor ANOVA on leach rate and crude protein retention of feeds immersed in freshwater (0ppt) and marine water (33ppt). Feed shape and drying temperature are fixed factors. Significant differences at α=0.05 are shown in **bold.**

Factors	df	Sum Sq	Pseudo-F	P
Freshwater (0ppt) Leach Rate				
Feed Shape	2	265.39	97.59	0.0001
Drying Temp (°C)	2	94.755	34.843	0.0001
Immersion Time (hrs)	5	422.26	62.109	0.0001
Feed Shape x Drying Temp (°C)	4	45.778	8.4166	**0.0001**
Feed Shape x Immersion Time (hrs)	10	55.187	4.0587	**0.0001**
Drying Temp (°C) x Immersion Time (hrs)	10	7.4508	0.54796	0.8586
Feed Shape x Drying Temp (°C) x Immersion Time (hrs)	20	32.322	1.1885	0.2759
Residual	108	146.85		
Total	161	1070		
Freshwater (0ppt) Crude Protein				
Feed Shape	2	32.776	44.715	0.0001
Drying Temp (°C)	2	34.769	47.435	0.0001
Immersion Time (hrs)	5	7.8154	4.2649	0.0012
Feed Shape x Drying Temp (°C)	4	46.343	31.612	**0.0001**
Feed Shape x Immersion Time (hrs)	10	1.8053	0.49259	0.8942
Drying Temp (°C) x Immersion Time (hrs)	10	5.0523	1.3785	0.2045
Feed Shape x Drying Temp (°C) x Immersion Time (hrs)	20	9.7615	1.3317	0.1773
Residual	108	39.582		
Total	161	177.9		
Marine Water (33ppt) Leach Rate				
Feed Shape	2	113.84	114.67	0.0001
Drying Temp (°C)	2	10.514	10.59	0.0001
Immersion Time (hrs)	5	182.96	73.719	0.0001
Feed Shape x Drying Temp (°C)	4	13.825	6.9629	**0.0001**
Feed Shape x Immersion Time (hrs)	10	15.602	3.1433	**0.0015**
Drying Temp (°C) x Immersion Time (hrs)	10	15.064	3.0348	**0.0026**
Feed Shape x Drying Temp (°C) x Immersion Time (hrs)	20	9.4885	0.95579	0.5258
Residual	107	53.111		
Total	160	412.32		

Factors	df	Sum Sq	Pseudo-F	P
Marine Water (33ppt) Crude Protein				
Feed Shape	2	77.044	80.582	0.0001
Drying Temp (°C)	2	88.851	92.931	0.0001
Immersion Time (hrs)	5	28.892	12.088	0.0001
Feed Shape x Drying Temp (°C)	4	98.95	51.747	**0.0001**
Feed Shape x Immersion Time (hrs)	10	7.0311	1.4708	0.1596
Drying Temp (°C) x Immersion Time (hrs)	10	10.053	2.103	**0.0268**
Feed Shape x Drying Temp (°C) x Immersion Time (hrs)	20	9.8613	1.0314	0.4382
Residual	107	51.151		
Total	160	375		

Df - degrees of freedom; Sum Sq - sum of squares; Pseudo-F - F value by permutation, P-values based on 9999 permutations.

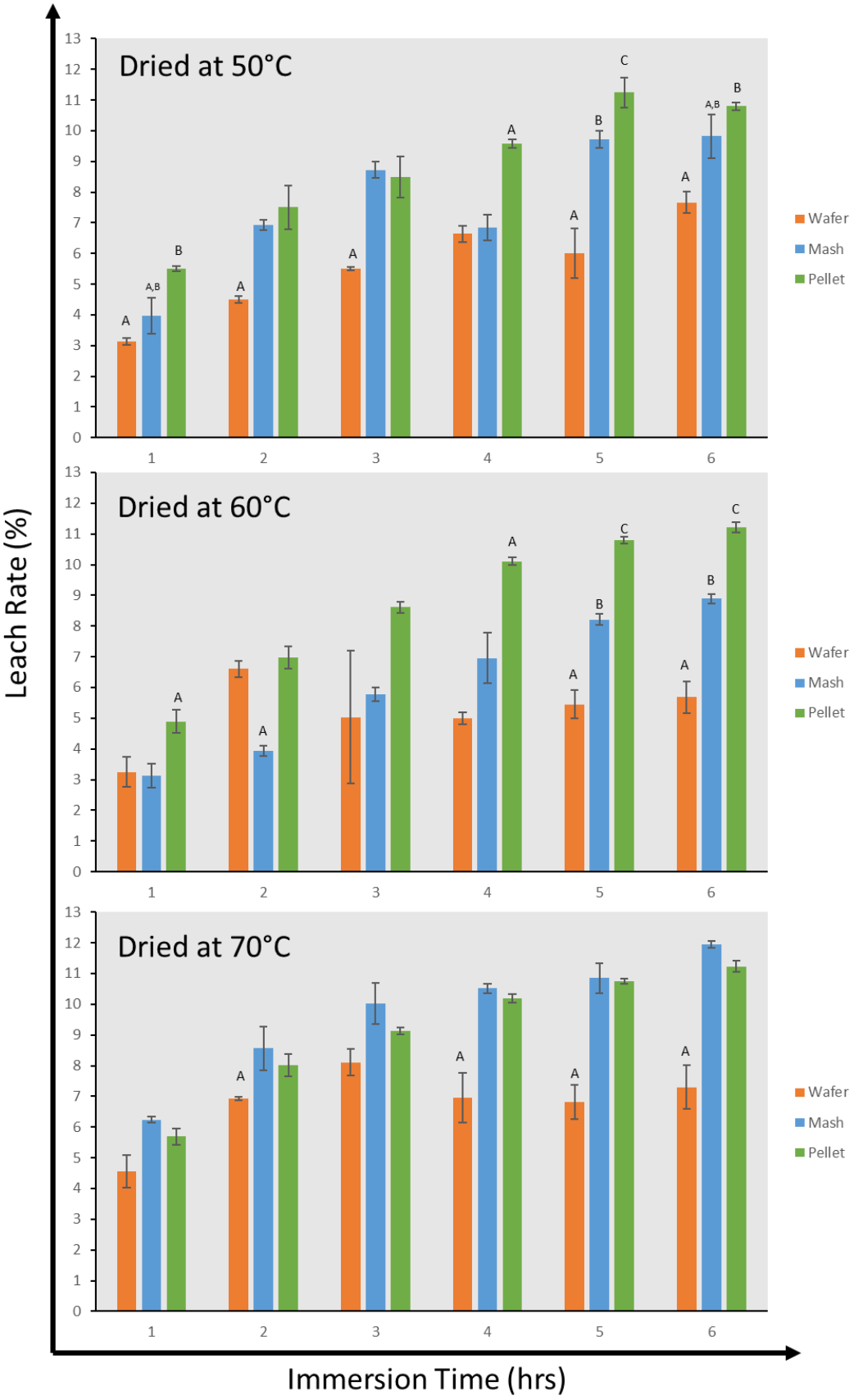

Figure 6.3 – **Leach rate** of 9 experimental diets immersed in **0ppt freshwater** over a 6hour period. Significant difference within each time treatment is indicated by "A, B or C". Bars with a different letter above indicate significant difference between shapes within the time hourly treatment.

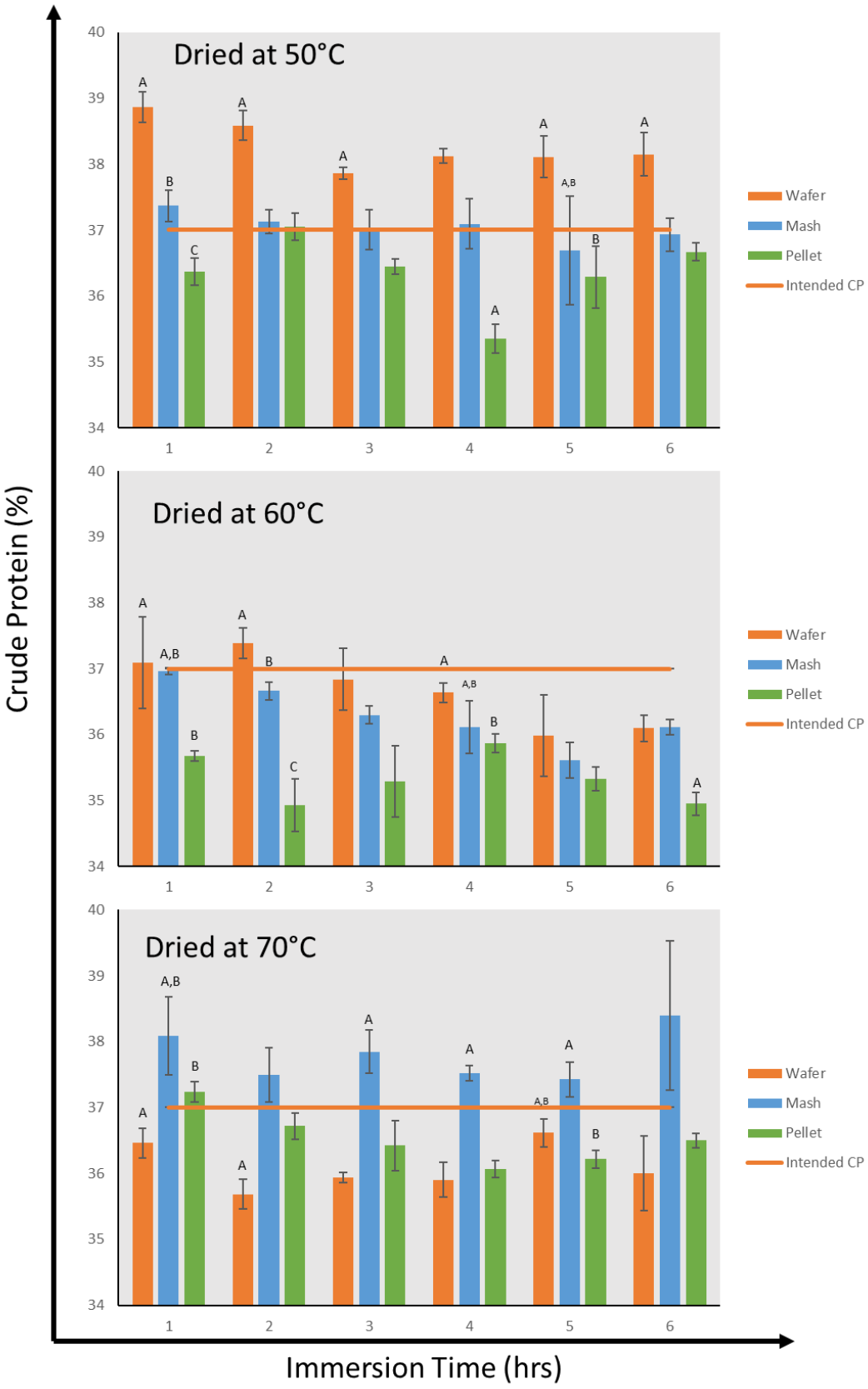

Figure 6.4 - **Crude protein** of 9 experimental diets immersed in **0ppt freshwater** over a 6hour period. Significant difference within each time treatment is indicated by "A, B or C". Bars with a different letter above indicate significant difference between shapes within the time hourly treatment.

6.3.2 Marine Water (33ppt)

Similar to the freshwater results, the LR of feed immersed in marine water increased over time at all temperature treatments and feed shapes (figure 6.5 and table 6.3). However, in marine environments feed leached less on average over 6 hours than feed immersed in freshwater. In the first 1 to 2 hours of immersion in marine water all feeds lost <5% dry matter, after 2 hours the LR of feed dried at 50°C began to increase, with pellet performing the worst, followed by mash and then wafer (figure 6.5). Feed dried at 60°C performed the best with wafer shaped feed losing <3% dry matter over the entire 6hour immersion period and pellet shaped feed had a significantly higher LR for the entire immersion period then both mash and wafer shaped feed (table 6.3).

On average feed immersed in marine water lost more CP then feed immersed in freshwater. The pellet shaped feed had the lowest CP of all shapes however, mash shaped feed had the highest CP (figure 6.6). Feed dried at 60°C performed the best in terms of percent CP retained after immersion, similar to the LR of feed dried at the same temp. Over the 6hour immersion period feed dried at 60°C lost little CP. In the first 1 to 2 hours of immersion there was no significant difference between feed shapes, but after 2hrs pellet shaped feed had significantly less CP (table 6.3). At a 50°C drying temperature pellet shaped feed retained a significantly lower percent CP then the other two feed shapes, however, this level stayed relatively stable in all time treatments. In feed dried at 70°C wafer shaped feed retained significantly less CP compared to other shapes, but the CP stayed relatively stable overtime (figure 6.6). A decrease in CP over time was observed in mash and pellet shaped feed with mash shaped feed retaining significantly more CP in the first and fourth hour of immersion.

In summary, feed immersed in freshwater lost significantly more dry matter and CP than feed immersed in marine water.

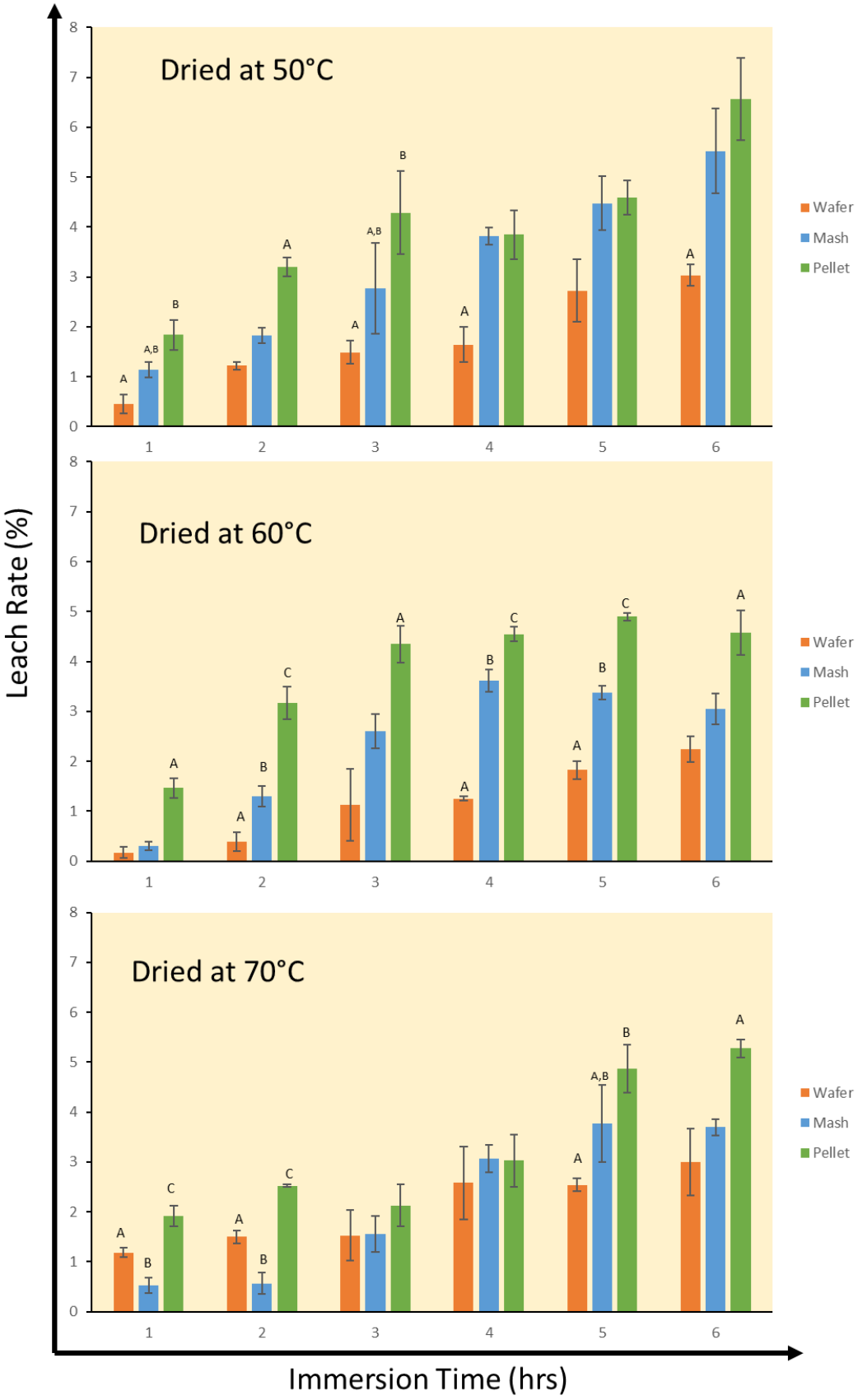

Figure 6.5 - **Leach rate** of 9 experimental diets immersed in **33ppt marine** water over a 6hour period. Significant difference within each time treatment is indicated by "A, B or C". Bars with a different letter above indicate significant difference between shapes within the time hourly treatment.

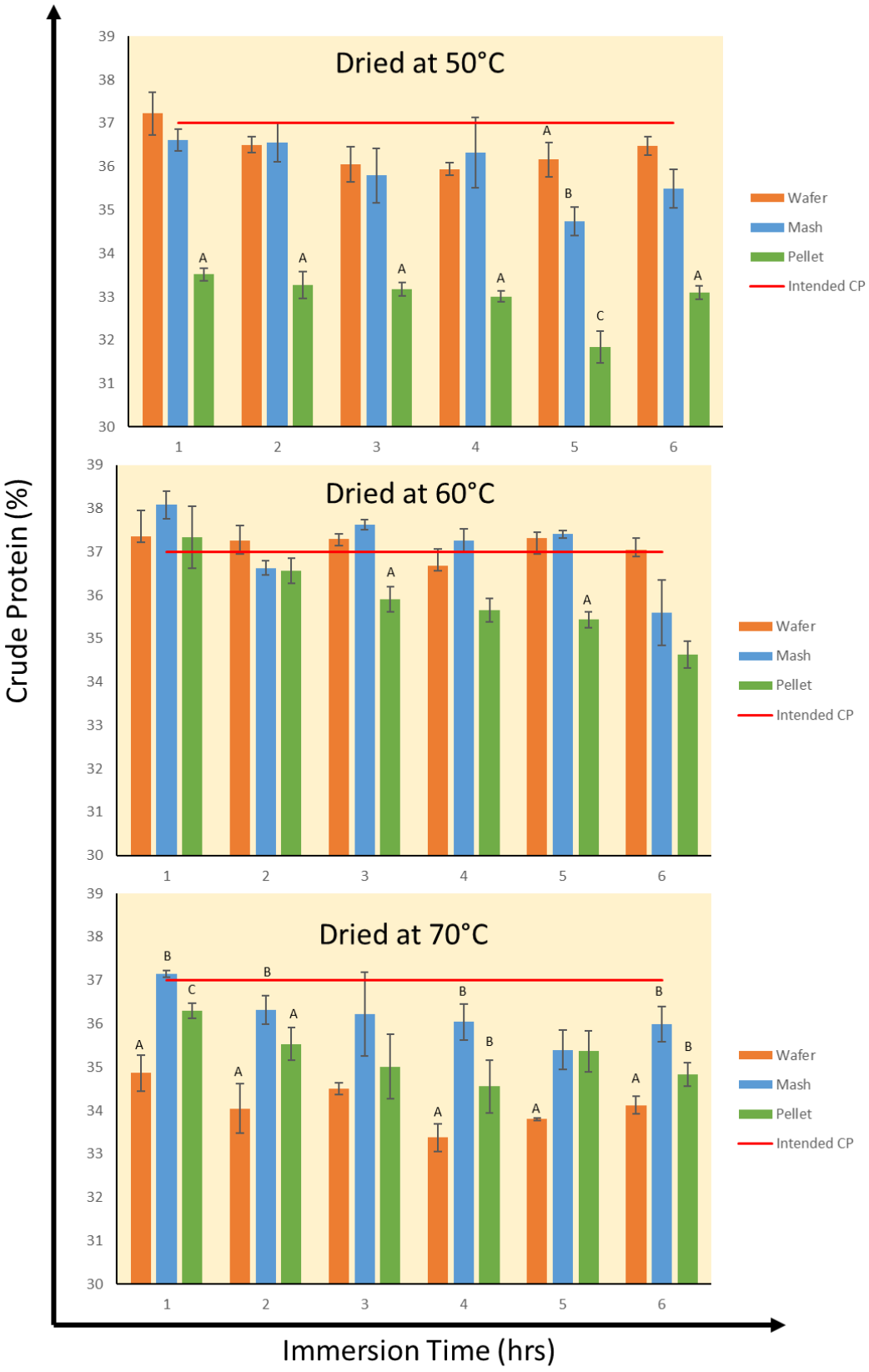

Figure 6.6 - **Crude protein** of 9 experimental diets immersed in **33ppt marine water** over a 6hour period. Significant difference within each time treatment is indicated by “A, B or C”. Bars with a different letter above indicate significant difference between shapes within the time hourly treatment.

6.4 Discussion

Pellet shaped feed lost the most dry matter (DM) over the 6hour time period and leached more CP than wafer and mashed shaped feed. This poorer performance may be attributed to stress fractures in individual pellets caused by crumbling after drying. Crumbling of large pieces into smaller pieces has been seen to cause stress fractures in pellets making feed more susceptible to disintegration and nutrient leaching when placed in water (Obaldo & Tacon, 2001). The breaking of the spaghetti-like strands after drying is likely to have caused some fractures in individual pellets jeopardizing their stability. Comparatively, mash and wafer shaped pellets underwent some compaction, and required less breaking up before storage and/or feeding. Compaction of pellet shaped feed was minimal occurring only at the die head of the meat mincer. Whereas, mash feed dough was hand-pressed into smaller pieces to the desired size which subsequently required less crumbling after drying and wafer shaped feed was pressed into a pan and had cut lines pressed into the dough prior to drying. Therefore, very little force was needed to break wafers. As a result both mash and wafers were likely to be bound more cohesively and have fewer fractures within their structure. Stress fractures in pellets could have been minimized by cutting strands while dough was wet, prior to drying. Wafer shaped feed performed the best in terms of DM loss which is especially beneficial for crustacean culture where the animals are slower feeders than fish. Jussila and Evans (1998) have observed marron (*Cherax tenuimanus*) handling stable feed up to 24 hours after being fed and ignoring disintegrated pellet parts and therefore improving feed ingestion (Jussila and Evans 1998). Additionally, stable feed helps to minimise water pollution and improve feed efficiency through reduced loss of nutrients (Meyers et al., 1972). Feed that remains intact after a period of time in water remains an available source of food for animals that may have not consumed feed during the feeding period of the farm.

Furthermore, the pelleted feed in this study had a greater surface area to volume ration compared to the mash and wafer shaped feeds, which could have also contributed to the reduced water stability. The surface area to volume ratio of a pellet increases as pellets decrease in size which therefore, exposes more of the pellets outer surface to water making the pellets inducing great water absorption. Similar results were observed by Obaldo and Tacon (2001) where pellets were crumbled from 3.0mm pellets to 0.7mm pellets which saw a reduction from 82.5% to 76.3% water stability. This reduction was also attributed to particle size, Obaldo and Tacon (2001) suggest a reduction in pellet size should be accompanied by a reduction in ingredient particle size. This helps to improve uniformity and nutrient content of each

individual pellet and to the same effect the reduction in particle size exposes greater surface area between particles which improves binding during manufacturing which in turn improves water stability (Obaldo et al. 1998).

Temperature has a significant influence on the microstructure of pellets. When exposed to temperatures of 66.7 to 101.5°C vacuoles begin to appear in the microstructure of pelleted feeds, above these temperatures, between 147.4 and 180°C these vacuoles grow much larger (Yoshitomi 2004). This might explain the patterns observed in the current study where feed dried at the highest temperature (70°C) exhibited the highest leach rate regardless of water salinity (freshwater or marine). Drying at 70°C may have allowed for small vacuoles to develop in feed, resulting in lowered water stability. When feed was dried at 60°C, outside of the range Yoshitomi (2004) tested, water stability appeared to improve. This may be due to smaller or less vacuole formation within the feed. Vacuoles in pellets can exacerbate pellet disintegration where either moisture remains within the pellet within these vacuoles and inhibit effective drying or by producing gaps within the pellet that allows the uptake of water when immersed. Similarly, expansion from heat can cause the evaporation of trapped water and lead to the formation of air pockets in pellets which can also reduce water stability (Misra, Sahu & Jain 2002). These air pockets allow for pellets to float on the surface of the water. While this is usually a requirement for instant feeders which are less impacted by leaching, it is not suitable for benthic feeding crustaceans. Also, additional heat is often generated mechanically from pressure generated through friction at the die which can cause some expansion in pellets. While heat is a catalyst for important binding reactions such as starch gelatinization a balance must be met to minimize expansion to create sinking feed.

Starch gelatinization is main chemical bonding mechanism of pelleted feeds, starches are derived usually from cereal grains such as wheat or corn or tubers such as potato and tapioca (Olkku & Rha 1978). The energy used to gelatinize starches is usually provided by heat, between 60 - 80°C and adequate levels of water (Kraugerud et al. 2011; Romano & Kumar 2019). This variability is largely influenced by the amylose/amylopectin ratios within the starch source (Copeland et al. 2009). These ratios can vary even from the same starch sources (e.g soil and climate conditions) (Park et al. 2007; Romano & Kumar 2019). Additionally, factors such as temperature, pressure and/or shear forces influence the degree of gelatinization and generally elevating these influences tend to increase starch gelatinization (Romano & Kumar 2019). However, it is likely the response observed was influenced by both the differing

degrees of starch gelatinization due to the differing drying temperatures and manufacturing techniques, simultaneously. The use of pre-gelatinized starches have been found to reduce the reliance on high temperatures, pressures or shear forces in order to create stable pellet which could otherwise denature proteins or cause undesired expansion which cause pellets to develop air bubbles and float which is unsuitable for crustacean feeds (Romano & Kumar 2019; Kanmani et al. 2018). Similarly, pre-gelatinized starches have had success when pellets were manufactured using hand pelleters which create substantially less pressure and shear forces and heat is generated from friction or added water (Romano et al 2018).

The presented study showed that different drying temperatures (50 - 70°C) may have had an effect on the starting CP level. It has been suggested that moisture levels within cells protect cellular proteins by preventing excessive thermal levels from being achieved (Camire 1991). For example, during high-moisture (>25% weight basis) extrusion, minimal protein denaturing was observed by Cheftel (1985). Feed dried at 70°C exhibited a lower crude protein level after drying, the increase in drying temperature would have reduced the moisture level at a faster rate in comparison, consequently exposing the proteins to potential denaturation. Feeds dried at lower temperatures (50 and 60°C) began the immersion period at the intended crude protein level and sustained this level over the course of the 6 hour. The reduced temperature during the drying process may have minimized the thermal effects on proteins as higher temperatures tend to denature proteins (Camire 1991). Similarly, exposure to heat has been demonstrated to affect protein solubility. Zayas (1997) showed that heat treatment between 40 - 50°C increased the solubility of proteins and at temperatures higher than 40 - 50°C protein solubility was lowered. The effect of heat on protein solubility has also been demonstrated by deWit and Klarenbeek (1984) and Rangavajhyala et al. (1997). Feed exposed to 60°C temperatures in the present study retained the highest CP level over the 6 hour immersion period in a marine environment and the lowest DM leach rate in both environments. This is likely due to a decrease in protein solubility at the 60°C drying temperature and therefore lowered the loss of protein to the water, similar results were observed at the higher temperature treatments as well. In addition, pellet shaped feed began at CP levels below the intended levels, as pellet size decreases the surface area to volume ratio increased and therefore during the drying process pelleted feed may have been more uniformly exposed to thermal treatment which could have denatured proteins (Obaldo and Tacon 2001).

In terms of environment; freshwater or marine, protein solubility is known to fluctuate due to the presence of chloride ions and therefore should be taken into account when evaluating stability feeds targeted at marine or freshwater species. After binding to positively charged protein group's chloride ions increase protein solubility through electrostatic repulsion. Initially, protein solubility is increased at salt molarities between 0.5-1.0M (29 - 58ppt) in a process termed "salting in", once molarity increases above 1.0M protein solubility begins to decrease (Zayas 1997; Kinsella 1979). The marine environment in this study was within range of the "salting in" process and therefore may explain the low crude protein retention levels of feed immersed in marine water.

In conclusion, drying temperature and feed shape had a marked effect on the water stability and CP retention of feed immersed in aqueous solutions. Feed is influenced differently depending on the aquatic environment. Marine environments are less favourable for CP retention of feed but fair better in terms of DM retention. In contrast, feed in freshwater environments retained the highest percent of protein but lost DM at a greater rate than those immersed in marine water. Pellet shaped feed had the highest CP loss and highest leach rate, which can be attributed to a high surface area to volume ratio and fissures created during manufacture. The results of this study suggest that wafer shaped PBM based feed dried at 60°C to be the most suitable for application to crustacean aquaculture. Wafer shaped feed faired the best in both CP and DM retention, and it was also the easiest and quickest feed to manufacture. Wafer shaped feed has better pellet stability than laboratory pelleted feed. Crustaceans manipulate their feed and the larger shape would allow for the animal to hold the wafer and break into smaller pieces to consume at will, while minimizing leaching (Dominy et al. 2004; Jussila and Evans 1991; Hunt et al. 1992; Igwhela, Ahmad and Abol-Munafi 2013). This study demonstrates that PBM in aquatic feed has exhibited favourable manufacturing capabilities for both marine and freshwater environments. Future studies to investigate the biological performance of crustaceans fed utilizing the novel shape are the next step in its application. A commercial manufacturing processes (such as extrusion) of "wafer" shaped feed would not be difficult as it is similar to products already available on the aquatic feed market (sinking wafers/algae wafers) and in the human food industry (i.e rectangular biscuit).

Chapter 7 – General Discussion

This study investigated the potential of poultry by-product meal (PBM) as a protein replacement in crustacean (freshwater crayfish) diets. The study investigated the quality of PBM, feasibility of including it in an aquatic diet and the effect of manufacturing conditions on the stability of diets containing PBM. Taken together the three experiments highlight that PBM is an appropriate protein in crayfish diets, one of the main advantages of utilising PBM is that it is a practicable source of protein with similar performance to commercially available feed. Optimal manufacturing parameters when formulating with PBM have also been outlined when using rudimentary lab equipment to produce water stable feeds for better feed evaluation (figure 7.1).

7.1 Poultry By-Product Meal Quality

A “quality protein” in this study was defined as a product that consistently meets proximate composition standards and milling standards as defined by the manufacturer specifications (figure 7.1). The tested PBM was a consistent quality protein. In the present study, on average over the 3 month period the tested PBM remained well above the 60% crude protein minimum requirement specification, ranging between 63-66% (dry matter basis). Ingredient consistency is an important factor to consider when formulating compound feeds because it improves quality and a reliable feed that meets specifications improves farm output. In aquatic compound feeds protein is the most expensive feed ingredient and is the driving factor in crustacean growth. Therefore, when formulating aquatic diets finding a consistent protein source to produce high quality feed is very important. By law in Western Australia all manufactured stockfeed sold or supplied is required to be labelled with the minimum protein level, however this does not indicate the actual protein level of the product (Biosecurity and Agriculture Management (Agriculture Standards) Regulations 2013). Generally, the crude protein content of feedstuffs is higher than the labelled minimum requirement in order to meet legislation standards.

Protein measurements were based around the proximate system of measuring total nitrogen (N) (determined by the Kjeldahl method) multiplied by a specific factor (6.25) to estimate protein content. However, total protein content can also be obtained by measuring amino acid content. Although this approach is more expensive than proximate analysis, amino acids are the basic units of proteins and quantifying the amount of each individual amino acid has been considered a more accurate method

of determining total protein (Hall and Schönfeldt 2013). The PBM met specifications, however crude protein levels of >6% above the minimum requirement may result in waste in the form of faeces, leaching and water fouling. As a result of exceeding specifications, the feed formulation may contain protein at higher levels than can be absorbed by the animals which must be accounted for during formulation (i.e reduced inclusion). Similarly, ash content of raw materials is another factor that impacts the properties of a compound feed. In this study the ash content of PBM ranged between 13-16%, when PBM was tested for dry and organic matter digestibility, organic matter digestibility values were 2-3% lower than dry matter digestibility values. Once dry matter digestibility is calculated the residue is heated to 600°C and the leftover inorganic material (ash) is used to calculate the amount of digestible organic material in a feedstuff. Therefore, if organic digestibility is low, it is an indication of high ash content.

Raw poultry offal is processed daily and the final product consists of multiple batches of processed material, the minimal variation in proximate composition over the 3 month period indicated a well-controlled and reliable processing technique. When particle size distribution of PBM was compared to FM it was found that PBM and FM had very similar particle size distributions with less than 2% of particles being larger than 2000 µm and majority of particles remaining under 500 µm. Feed ingredients that have the ability to be sieved to <500 µm are better suited in aquatic feeds as it increases water stability and improves digestion in crustaceans (Palaniswamy and Ali 1999; Desrumaux et al. 1998).

Thus, as an addition to existing literature on the utilization of PBM in aquatic feeds, this chapter has provided compelling evidence for the suitability of using PBM as a replacement protein in aquatic feeds.

7.2 Feed Evaluation

Chapter 5 evaluated PBM as alternative protein in freshwater crayfish *(Cherax cainii)* diets. Feed performance was measured in crayfish growth, health and the digestibility of the feed. Three digestibility evaluation methods were employed; two *in-vitro* methods; dry and organic matter digestibility and one *in-vivo* (apparent dry matter digestibility) method using chromic oxide as a marker. No adverse effects of using PBM in crayfish diets was observed in the present study, although dietary protein of the experimental diet was 12% higher (34% crude protein) than the commercial control diet (22% crude protein). No negative effects from this level of dietary protein

was observed in crayfish growth or immunity therefore it is possible to conclude that PBM at 33.8% dietary CP was adequate to provide the crayfish with nutrient requirements. Previous studies utilizing PBM in crayfish diets observed reduced growth and lowered survival rate when greater than 38.2% dietary PBM was included in diets attributing these effects to lowered methionine levels (Fuertes et al. 2013). Survival and growth in the present study did not appear to be affected by the inclusion of PBM at 35%.

Furthermore, digestibility was also similar between feeds with the *in-vitro* (both dry and organic matter) and *in-vivo* apparent digestibility methods. This shows excellent potential for the application of *in-vitro* methods in evaluating feeds at a basic level. Crustaceans have a simple digestive system and therefore their digestion may be more easily replicable through *in-vitro* methods when compared to the more complex digestive systems of fish. Further testing and inter-laboratory collaboration will provide more evidence to support the relationship between *in-vitro* and *in-vivo* assays and help to standardize a simple method that will minimise operator error and inter-laboratory differences to more accurately estimate the digestibility of crustacean feeds (Moyano et al. 2015). By establishing the base information needed to develop routine *in-vitro* laboratory methods to evaluate aquatic feed, costly and time consuming *in-vivo* assays can be reduced.

This study has demonstrated the value of PBM as replacement protein in crayfish diets. Freshwater crayfish readily digest compound feeds that utilize PBM at a rate similar to that of commercially available feed. The livestock industry has established a toolbox of methods to evaluate feed that is rapid and repeatable. In a novel application to evaluate aquatic diets, it was found that with further investigation a similar system could be established in place of the current expensive *in-vivo* methods.

7.3 Physical Properties

This study presented evidence for interactions between drying temperature and feed shape having an effect on water stability and crude protein retention in feed formulated using PBM. Experimental feed immersed in marine water (33ppt) lost more crude protein than those immersed in freshwater (0ppt). When comparing freshwater and marine environments protein solubility is known to increase due to the presence of chloride ions and low concentrations of other salts. Therefore, environment should be an additional factor to consider when manufacturing feed.

It’s possible that a commercial extruder would produce feed with superior water stability than the feed manufactured during this study. However, in the present study the lab made feed in the wafer shape provided a feed with increased pellet stability with properties that closely reflected commercially made feed. By improving the pellet stability of feed, the time the animal has to consume the feed is increased, which in turn improves feed consumption and possibly growth performance of the animal. Maximising the water stability of lab made feeds also helps to more accurately evaluate the feed. Experimental feeds that have been made by hand or using rudimentary lab methodologies such as cold pelleting through a meat mincer may be inaccurately evaluated. Reduction in nutritional quality due to poor pellet stability may be mistakenly attributed to formulation. In addition, for the feed to be of nutritional benefit to the organism it must first be ingested. For example, when evaluating feed targeted at crustaceans whose feeding behaviour is benthic, the influence of pellet stability is critical and can have a significant impact upon feed performance. Crustaceans such as prawns, crayfish and crabs have a tendency to manipulate their food and therefore require feed with properties such as sinking feed and extended water stability (Dominy et al. 2004; Jussila and Evans 1991; Hunt et al. 1992; Igwhela, Ahmad and Abol-Munafi 2013). Moreover, during consumption prawns use their appendages to masticate their food outside the buccal cavity before ingestion. Therefore, if feed is not sufficiently water stable it can create fines which are swept away by exhalent gill currents, becoming unavailable to the animal and producing waste (Forster 1972). Wafer shaped feed provides the animal with a pellet that is water stable and consumable without the need for sophisticated commercial machinery and consequently a more accurate evaluation of protein alternatives can be conducted.

7.4 Thesis Summary Figure

Taken together the three chapters present a strong argument for the use of PBM as a FM replacement in aquatic feeds. The study presents recommendations and new hypotheses to be tested and future applications. The ideas, conclusions and recommendations are illustrated in figure 7.1 and expounded upon in the following sections.

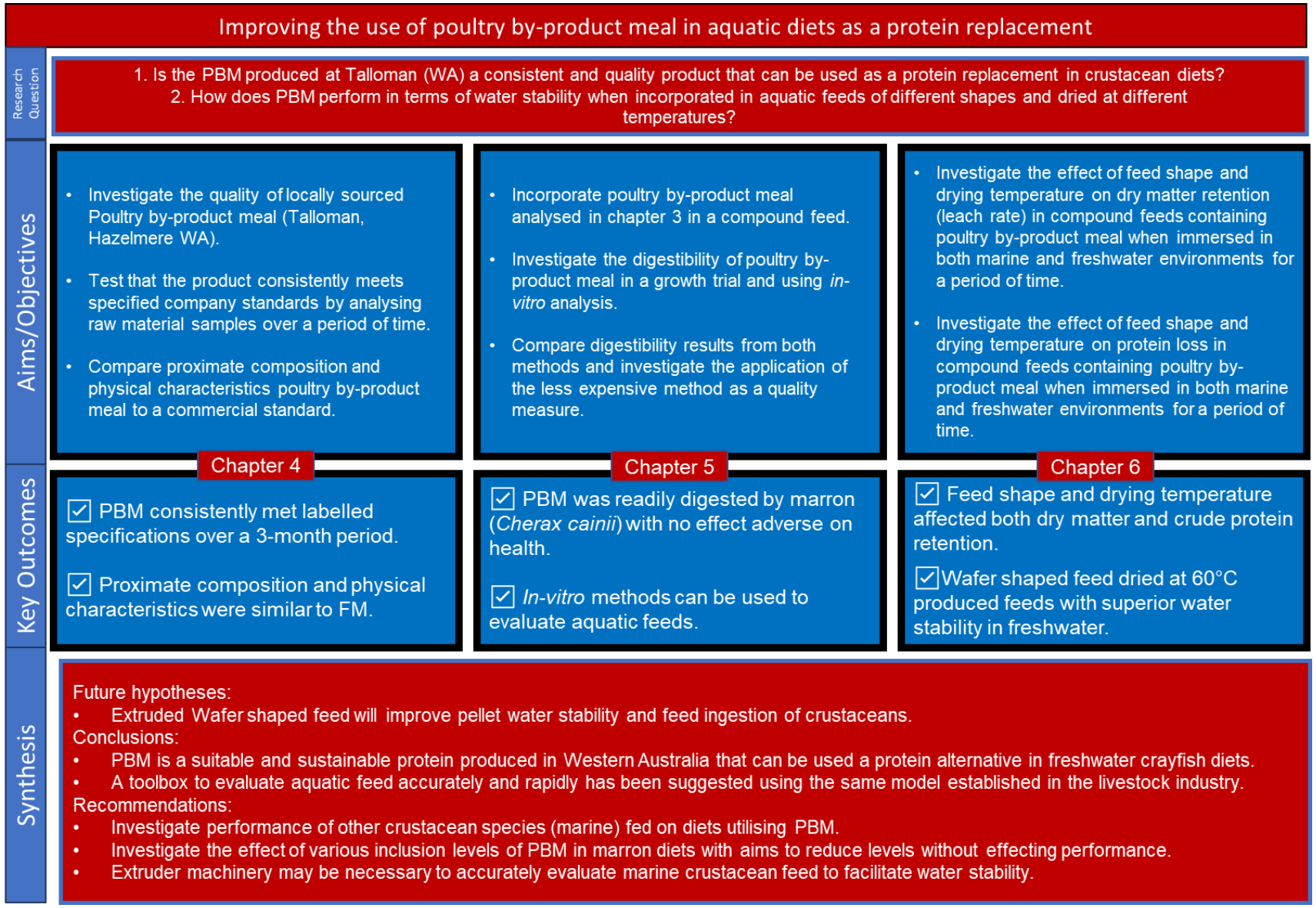

Figure 7.1 – Flow diagram of summary of key outcomes, conclusions and future recommendations.

7.5 Limitations and Future Recommendations

Developing a protein alternative first requires the substitute to be readily available and sourced sustainably, this will ensure that the same difficulty of supply faced with using FM is not repeated. In 2020 the cost of PBM was between $830 - $1220 per metric tonne (B. Barron, personal communication, August 18, 2021), less expensive than FM predicted at $1670 - $2240 per metric tonne in 2020 (Byrne, 2019). PBM is produced from recycled poultry by-products which adds value to the growing Australian poultry industry and also minimises waste. Secondly, preliminary quality testing of the product ensures that it meets labelled specifications (proximate composition) consistently over time and that it has similar manufacturing capabilities to FM. Where possible, AA analysis should be conducted for a more in-depth measure of quality. However, these two initial steps of developing a protein alternative overcome the main issue of supply and investigate quality at the preliminary stages of feed development.

The next step in developing a protein alternative should be in the evaluation of the suitability of the substitute protein. PBM has been successfully used in a number of crustacean and fish diets. However, a standardised method for aquatic feed evaluation has not been established. To a greater extent than the livestock industry aquatic feed must be durable and water stable in order to accurately evaluate feed formulation. The results from the present study showed that handmade feed dried at 60°C and in wafer shape produced feed of greater water stability and superior crude protein retention when immersed in water than feed made using a meat mincer, a commonly used method to evaluate feeds in laboratories. This superior shape should be utilised to evaluate handmade feed under lab conditions. The wafer shape is also commonly seen in commercially produced human foods such as biscuits, confectionary and cereals as well as fish feed and therefore can be commercially applied to crustacean feeds. The water stability of wafer shaped feed in marine environments did not perform as well as wafer shaped feeds in freshwater and therefore use of commercial extruders to produce wafer shaped feed may help to improve water stability of feeds targeted for marine species. As crustaceans prefer to hold large pieces of feed and break it up into smaller pieces to ingest this shape may help to improve feed ingestion and has yet to be tested in a feeding trial. Recommended future studies should investigate the behaviour and feed intake response of crayfish fed wafer shape feed to further its application.

PBM in marine crustacean diets has not been as successful as it has with freshwater crayfish, which may due to the areas they inhabit. Freshwater crayfish are more likely to be exposed to terrestrial animal protein than marine species which may explain why freshwater crayfish respond better to diets utilising protein from terrestrial animals. However, there is no literature available and a comparison between marine and freshwater species fed on these diets would provide greater insight. The experimental diet in this study was 34% CP, which was significantly greater than the control diet, 22% CP. This inclusion level could be lowered substantially in a future study comparing PBM and FM diets of 22% CP, also for a trial period where marron moult at least twice and/or double in weight. However, if this is done health indices should be monitored along with growth response. *In-vivo* apparent digestbility comparisons and correlations to *in-vitro* values are needed for accurate feed evaluation for the industry to move away from time consuming and costly *in-vivo* methods and to model the livestock industry systems. Inter laboratory collaboration is required to create a data pool, increase the amount of samples tested and minimise sampling error to help build correlations between *in-vitro* and *in-vivo* values.

7.6 Aquatic Feed Evaluation Toolbox

A standardised method to evaluate aquatic feed has yet to be established. This study presents a series of steps that can be followed to evaluate the performance of aquatic feed, and an 'aquatic feed evaluation toolbox'. It is suggested that the established principles of feed evaluation from the livestock industry are adopted to develop a standardised method for aquatic feed evaluation. Below are the suggested aquatic feed evaluation tools as outlined in various chapters of the present study.

Feed Water Stability (see chapter 6) – Arguably the most important factor in crustacean feed manufacture. This will quantify dry matter and crude protein that is leaching out of the pellet and becoming unavailable to the animal while immersed in water. Test conditions should be matched as closely as possible to culture systems of the aquatic species.

Wafer Shaped Feed Method (see chapter 6) – In the absence of extruder machinery hand pressed wafer shaped pellets have a higher water stability and better reflect commercial standards when compared to cold pelleted feeds through rudimentary lab equipment. A maximised pellet stability will help to more accurately evaluate feed formulations. Use of binders such as guar gum or alginates to further aid in water

stability is also another option to consider, however, are an added cost with little to no nutritional benefit.

In-vitro digestibility (see chapter 5) – Further investigation is needed before *in-vitro* digestibility methods become established for aquatic species. However, the initial results presented indicate promising applications of the pepsin-cellulase digestibility methods to evaluate crayfish feeds.

Pellet Durability Index (see chapter 2.2) – A tool that can be adapted from the livestock industry. This index will indicate shelf life and storage and handling capabilities of feed.

7.7 Conclusion and Recommendations

In conclusion, this study investigated PBM as a protein replacement in crustacean diets. The study investigated the quality of PBM and found the protein to be of high quality in terms of meeting specifications consistently over time and highlighted the advantage of consistency in supply over FM (figure 7.1). The feasibility of including the protein in an aquatic diet and methods of rapid and replicable feed evaluation were tested on freshwater crayfish (*Cherax cainii*). Use of PBM as the primary protein was achieved with no adverse effects on crayfish growth or health. *In-vitro feed* evaluation methods employed by the livestock industry were tested as a method for aquatic feed evaluation and compared to apparent digestibility values. Results were similar providing compelling evidence for the application of routine *in-vitro* methods to evaluate aquatic feeds. However, further investigation is recommended.

Manipulating the drying temperature and feed shape of compound feeds utilizing PBM highlighted an optimal drying temperature (60°C) and a novel shape (wafer) that can be applied to evaluate feed made in laboratories where sophisticated feed manufacturing machinery is lacking (figure 7.1). Experimental feed dried at 60°C and in wafer shape exhibited improved crude protein retention and water stability that more closely reflected that of commercially manufactured feed.

Specifically I recommend that:

- Poultry by-product meal be used in freshwater crayfish diets as it consistently meets specifications, is a practical source of protein and no adverse health effects were observed in the growth experiment utilising the product

- To produce a water stable feed in the absence of sophisticated extruder machinery, feed should be dried at 60°C and hand pressed into wafer form.

This thesis has reinforced the existing knowledge on utilising poultry by-product meal as a replacement protein in aquatic diets. Using the feed evaluation toolbox presented here, the more sustainable poultry by-product protein source has been shown to be suitable to protein alternative in freshwater crayfish diets.

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