# An investigation of optimal feeding ration and effects of probiotic bacteria on the growth of New Zealand Abalone (*Haliotis iris*)

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# *Attestation of Authorship*

I, Te Rerekohu Tuterangiwhiu, declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a University or other institution of higher learning.

Signed…….

Date……8/12/2015.....……

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# ABSTRACT

*Haliotis iris* are long-rearing Pāua that take 4-5 years to reach market size (75mm). A clear gap exists within the aquaculture literature regarding the optimal nutrition in the cultivation of New Zealand *Haliotis iris*. Further, there has been much research across aquatic species to suggest that the use of probiotics can successfully stimulate growth and decrease the time it takes to grow farmed Pāua.

The overall aim of this thesis is to first investigate and identify an optimum feeding ration of the currently used formulated feed, which can improve growth and eliminate wastage of nutrients in *Haliotis iris*. Second, this thesis aims to evaluate the effects of the formulated feed with added probiotic bacteria on the ingestion, digestion, assimilation of Pāua, with the goal to increase growth rates.

There are two growth trials in this study, conducted over 10 weeks, both of which used 160 juvenile abalone (20mm). First, the feeding ration trial compared the growth of Pāua samples from three treatment groups, fed on 3 different rations based on bodyweight of formulated feed (1%, 2%, 5%). Formulated feed rations were calculated by taking a percentage of the mean wet weight of all the Pāua in each feeding treatment.

The second growth trial compares groups of Pāua fed with a probiotic (2%) and non-probiotic (2%) diet at an optimum feeding ration determined from the first trial. Three multi-strain probiotics were used in this study: *Exiguobacterium* sp. strain (JHEb1) and *Vibrio* sp. strain (JH1), and *Enterococscus* sp. strain (JHLDc).

Growth (i.e., shell size, animal wet and dry weights) and survival of abalone were recorded throughout the experiment, with initial measurements at the start of the experiment and every two weeks for a total of 10 weeks. Shell length measurements were obtained by recording the greatest length. Wet weights were measured by lightly drying individual animals using

tissues or hand towels for approximately 30 minutes and weighing them to the nearest 0.001g.

In addition to these morphological and survival parameters, physiological responses that the abalone exhibited under the probiotic treatment were recorded, with a particular interest in the enzyme functionality and the assimilation of nutrients into the body tissues. For this, biochemical analyses were conducted to identify protein, lipid and carbohydrate contents within the tissue of the experimental animals.

The results indicated growth across all treatment groups in both trials as well as a 100% survival rates. For the feeding ration trial the weight gain of the Pāua were higher in the 2% and 5% rations. With regard to shell length, the 1% and 5% groups had greater increase over the 10 week trial. Based on the results of this study, the ideal optimum feeding ration for *Haliotis iris* is a 2% body weight feed ration. A 2% body weight food ration is enough to sustain the nutritional requirements of Pāua without storing an excess of lipids.

The probiotic trial resulted in growth, both weight and shell length, for the probiotic (an average of 8.72g/9.13mm) and non-probiotic treatment (8.91g/11.39mm). However there was no significant difference between the two groups. This suggests abalone that are reared in ideal conditions do not require supplementary probionts to aid their digestive functions. Overall, the conclusions of this research are that probiotics are unlikely to add benefit if used coupled with ideal culturing conditions for the species.

It is hoped that the findings of this work will contribute to efforts to minimize the cultivation period of *Haliotis iris* and will help reduce cultivation costs by making feeding, digestion and assimilation more efficient.

# Chapter 1: Introduction and Literature Review

## 1.1 Background

Abalone belong to the molluscan Family Haliotidae within the Class Gastropoda. The Latin term *Haliotis* refers to the shape of the abalone and likens it to a 'sea ear' (Linnaeus, 1758 from Geiger, 1999). The word abalone is derived from the Spanish name 'Abalón' used to categorise the various species from this family. The abalone family has distinct characteristics that are easily recognisable. The foremost characteristic is an oval rounded shell with a coiling, spiralling arc which flattens out at the base of the shell (Hahn, 1989a). The body of the abalone primarily consists of a large muscular foot that is attached directly to the shell. An abalone's unique identifying trait is the iridescent and colourful underside of its shell that emits a plethora of colours ranging from silver, purple, to green and blue hues (Bevelander, 1988).

In New Zealand, abalone are most commonly known as 'Pāua' which is the Māori term of the indigenous people. 'Pā' which means 'sensitive to touch' or 'strong', and 'ua' meaning 'muscle', giving reference to the characteristics and behaviour of the abalones muscular foot.

### 1.1.2 New Zealand Haliotide

There are three species of abalone which are endemic to New Zealand. The black-footed abalone (*Haliotis iris),* the queen or yellow-footed abalone (*Haliotis australis*) and the virgin or white-footed abalone (*Haliotis virgina huttoni)* (Dutton & Tong, 1981; O'Halloran, 1986). The distribution of all three species of abalone are spread vastly across the coastlines and shallow rocky reefs of all main islands in New Zealand, which also include the Snares and Chatham Islands (Hahn, 1989; Francis & Andrew, 2003). However, Elvy et al. (1994) found abalone populations to be most abundant along the Wairarapa Coast and down towards the South Island.

*Haliotis iris* finds its niche within the rocky reefs of the sub-tidal and sub-littoral shores of the coastline. They settle around boulders and in cracks of rocky substrates that are covered with coralline algae (Francis & Andrew 2003; Tung and Alfaro, 2011*)* which they feed on along with other species of drift macro-algae. Abalone prefer the tempered conditions of inter-tidal zones, with ranges between 8 - 16°C, but can survive in temperatures of up to 21°C (Peter J. Britz ,Thomas Hecht & Stewart Mangold, 1996). They are most common within a depth range of 1 – 15m (Fran, 2003).


**Figure 1: Diagram of the lifecycle of abalone (*Haliotis iris*) and the different phases of their development that highlights the time it takes for abalone to grow from a gametes to a sexually mature adult. This diagram was constructed from the information on the NIWA website *(NIWA, N.D).***

### 1.1.3 Biology

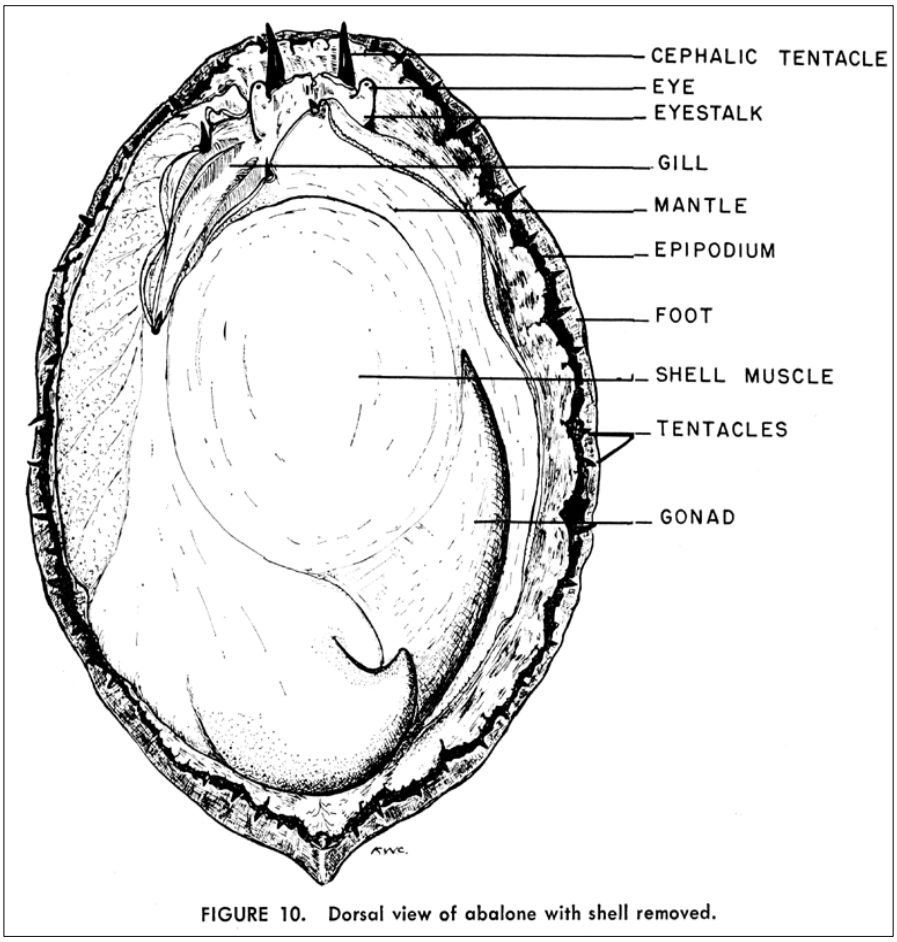


**Figure 2: Illustration of the basic biology of the Haliotis genus. Taken from Cox (1962)**

Abalone are herbivores that feed on algae, such a *Coralline. sp.* and have developed a unique radula which is a ribbon like tooth structure in the mouth that is specifically adapted to rasp and scrape algae from rocks and boulders (Andrew &Francis, 2003). The radula is controlled by hard material called the odontophore and is a pair of cartilaginous-like structures that press the radula down on to algae. Algae are then scraped and broken down to an eatable size before being passed through the mouth and into the oesophagus. (Anderson, 2003).

Figure 2 shows how all of the organs are positioned around a pedal mass, which is the muscular foot. Cox (1962) states that the biological make up of abalone are similar across the

genus of *Haliotis*. The propodial region (front) of the foot muscle is highly prehensile particularly at the front of the foot where the eyes, the sensory antennae and mouth are located. The gullet or oesophagus regulates the amount of food particles that enter into the gut. The sieve protects the gullet by regulating the size of particle that can pass into the gullet.

Respiration is achieved through a gas exchange process around the ctenidia or gills, which are situated on the left side of the body just behind the head, and curve down and around the shell muscle. The respiratory system also circulates water flow into the body, and controls the excretion of waste from the body. Water flow is driven by beating cilia, and is taken in through the sinus, located at the front most hole of the shell. Water enters the mantle cavity, where it passes through the ctenidia where gas exchange occurs. The water flow is then pushed to the back of the mantle cavity where it collects the coronary and digestive waste from the anus and metanephridia openings before expelling the waste water through the three posterior holes of the shell. Throughout this process, the clean water and the waste water output are kept separate from each other.

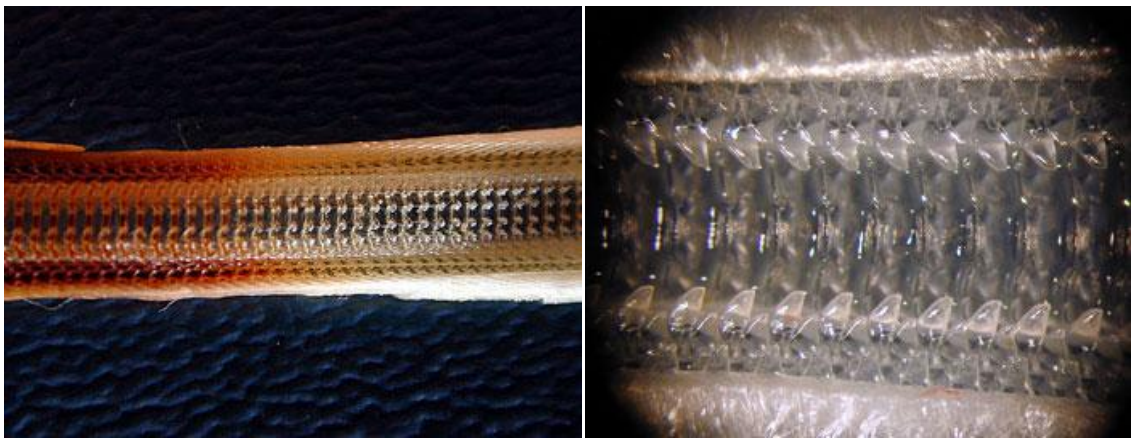

**Figure 3 : Images of the radula of abalone. Taken from Anderson (2003)**

A buccal mass structure contains a range of feeding structures, including the mouth (Figure 3), oesophagus and pharynx follows the same body curvature which link into the visceral mass which contains stomach, gastrointestinal tract, intestines and anus. Surrounding the visceral mass is a mantle, which has the functions of secreting calcium carbonate which forms the abalone shell. The epipodium is a skirt like layer of tissue that surrounds the outer edge of the foot of the abalone, which have small sensory tentacles that aid in the detection of external matter.

### 1.1.4 Digestive System

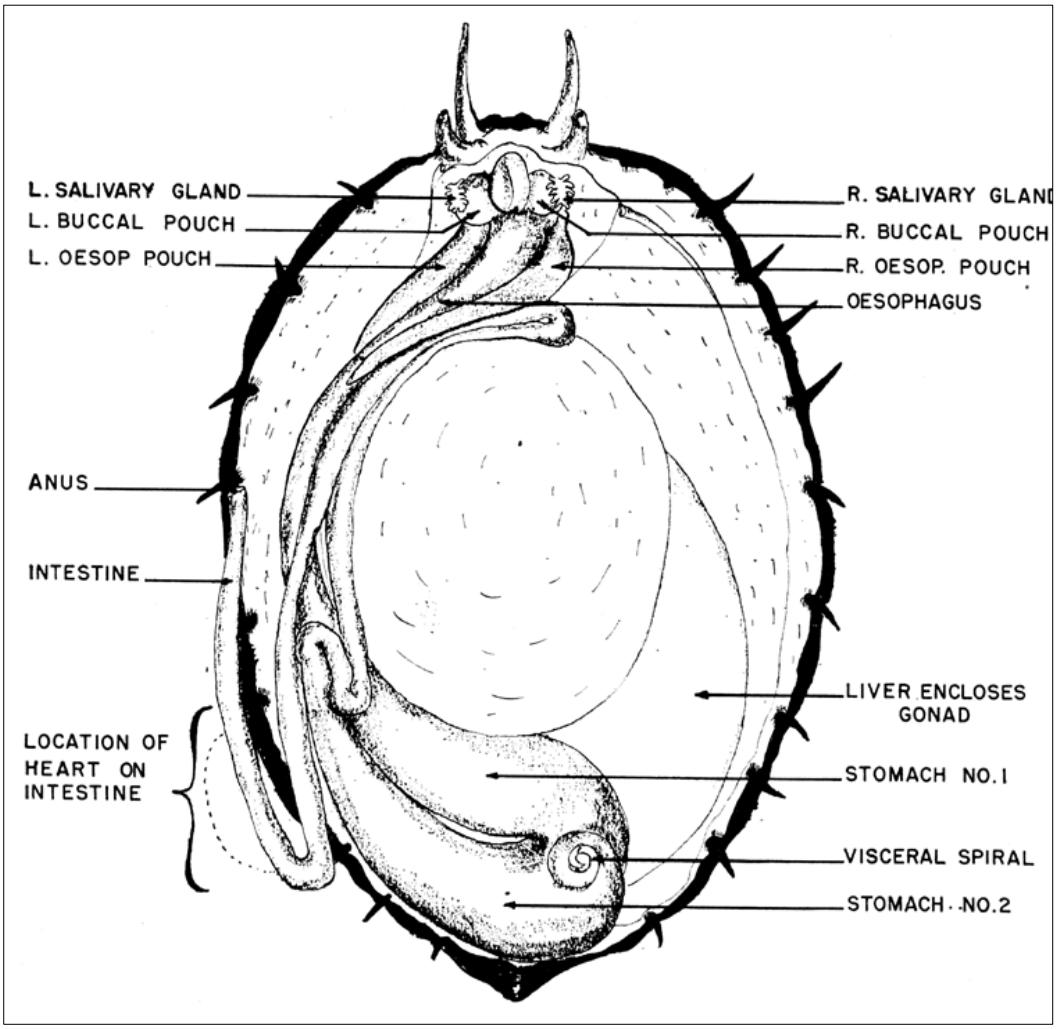


**Figure 4 : Is an illustration of the buccal and visceral mass of the Haliotis genus, taken from Cox (1962)**

The digestive tract (Ffigure 3) is situated on the left side of the body, between the shell muscle and the edge of the epipodium. It consists of the mouth, the esophagus, a three-or four-part stomach and the intestine. The intestine enters and passes through the heart before terminating at the anus.

Garcia-Esquivel and Felbeck (2006) indicate that the digestive system of *Haliotis rufescens* are separated into two distinct regions and are characterized by high levels of specific enzyme activity. The first region encompasses the mouth and intestines, and is characterized by a high level of lipase and aminopeptidase activity. The second region is comprised of the stomach and digestive gland, and is characterized by high amounts of complex carbohydrase enzymes (mainly cellulose and lycosome).

Garcia-Esquivel & Felbeck (2006) suggests that abalone have a very durable digestive system that can adapt quite easily to the food available to them. The enzymes within the digestive system of *H. rufescens* have been shown to consist of two carbohydrase enzymes (cellulose and lysozome) three proteases (trypsin, amino peptidase, and non-specific protease) and a non-specific lipase. Garcia-Esquivel & Felbeck found that abalone have naturally adapted to feed primarily on polysaccharide rich food, but can regulate the level of protease and carbohydrase activity within their gut to utilize the food available. If the digestive system of *Haliotis iris* is similar to that of *Haliotis rufescens* then it will adapt easily to the introduction of new formulated diets that will further enhance the growth rate of abalone.

Viana et al (2006) established how of abalone expended energy on growth and respiration. This was a multi-disciplinary approach to evaluate the metabolism of green abalone as well as trying to establish an *'energy budget'* by using enzyme activity to estimate apparent digestibility. They also compared this with feed intake, caloric estimates, and oxygen consumption to calculate energy assimilation and expenditure estimates. This research

established that a large portion of the energy that is ingested by the digestive system of green abalone (*Haliotis fulgens),* spend at least 10% of the energy acquired from their diet on growth, and between 31-40% is spent on respiration. This information is extremely valuable as it can be related to *Haliotis iris* to establish fundamental energy requirements that are essential for growth of this particular species. This also shows that abalone spend at least 50% of the energy acquired from their diet on maintaining growth and respiration.

### 1.1.5 Fisheries

Abalone are a very significant natural resource, particularly to Māori, and are recognized under Regulation 27 of the Fisheries Act 1996 as being a traditional customary fishery. Management of abalone has been conducted since 1986 under the Quota Management System (QMS) to increase the sustainability on this natural resource (Ministry of Primary Industries, 2014).

The black-footed abalone is the largest abalone species commonly found in New Zealand, and is the only species of abalone currently farmed (Niwa, 2014). This is a relatively new aquaculture species, as it was commercially established in 1986 (New Zealand Abalone Farmers Association, 2008). It is currently one of the more researched and established native aquaculture species on the world market along with Green-lipped mussels. Black-footed abalone have a high market value, as one kilogram of abalone meat can command between $50 -$96 (2013). There is a very high demand for abalone in the Asian market.

Commercial research and statistics estimate Pāua to be worth $60 million dollars in exports (Stats NZ, 2010). In 1991, the industry value was estimated e around $34 million. The value in 1996 rose to $143 million which steadily increased to $260 million in 2002. In September 2008, the asset value of Pāua was $384 million. This contributes 10 percent of the total value of New Zealand's commercial fisheries resources (Statistics NZ, 2009).

Since 2009, the total allowable commercial catch under the Quota Management System is set at 1,058 tonnes. The actual industrial catch for each year since 2009 has decreased from 1, 008 tonne to 926 tonnes reported in 2014 (Ministry of Primary Industries, 2015). These numbers highlight a need for more sustainable strategies to maintain stock populations and fisheries productivity. It also shows a clear need for allowances to be driven by an actual number of stock as opposed to being based on previous year estimates.

### 1.2 Abalone Aquaculture

Exploitation on wild stocks has arisen as a result of demand to capture high revenues and productivity. Driven by high demand, both internationally in the Asian market and locally, the burden on natural stocks and environmental resources has prompted the industry to start looking to aquaculture to alleviate the strain from commercial takes. The majority of abalone exports comes from the natural stock at this stage. Heath and Moss (2009) suggest that there are now 40 farms around the country dedicated to the cultivation and farming of abalone.

Black-footed abalone require up to five years of growth in order to reach market size (125 millimetres). The motivation for further development in the abalone aquaculture industry is to significantly improve this growth rate to make the farming of abalone economically viable. The techniques used in the current day include the use of recirculation systems to provide an optimum living condition, as well as the use of nutritionally formulated diets with boosted amounts of protein and lipids. By adopting these technologies, the New Zealand abalone industry and entities, such as NIWA, have improved the growth by successfully halving growth time to 75 mm after 26 months. Furthermore, there is insufficient knowledge about the effects of growth enhancers, such as probiotics on the digestive system of *Haliotis iris*, and what the correct feeding ration is to apply to a formulated diets that contain probiotic bacteria.

### 1.2.1 Previous studies of abalone growth

Numerous research studies have been conducted in an effort to increase the growth rate of abalone through the development and production of artificial diets (Simpson, 1994; Knauer et al., 1996; Britz and Hecht, 1997). However, there is still very little information available that determines the correct feeding ration (portion size) that will best achieve optimum growth.

### 1.2.2 Feeding rations used in abalone aquaculture

Field research has indicated various feeding rations to use to improve growth. However these have failed to provide a comprehensive rationale as to why they selected the various rations. Through trial and error many have estimated that between 1-6% of the bodyweight of the species is a good ration. However, results are inconclusive or do not provide robust ideas as to how to successfully select the optimum ration. Furthermore, there has been little or no research done on NZ Pāua regarding feeding rations, and more specifically the appropriate amount of food to produce optimum growth.

Cho and Bureau (1998) recognized the importance of uneaten feed as wasteful and an uneconomical loss for aquaculture operations. Their research emphasised feed rations, in particular the use of quantitative analysis through computer software, to analyse ideal rations. Whilst the present study differs in the methods of identifying feeding rations, Cho and Bureau's study highlighted the significance of feeding rations not only to offer benefits to the host organisms but also to alleviate the pressure on industries.

Wang et al (2007) established that the frequency of feeding and ration size are determining factors in regulating the feed intake, growth and waste outputs. Although their study was specific to fish, their application of the feeding ration principle was tested across treatment

groups fed 1,2,3,4 and 5% bodyweight. Their results indicated that the optimum feeding ration was 5% bodyweight.

Puvanendran, Boyce and Brown (2003) suggested that knowing the optimal food ration of cultured fish is a determinant of fish growth. Moreover, knowing the appropriate feeding rations minimises economic loss of uneaten feed, and lowers the risks of water pollution and nutrition lost.

### 1.2.3 The use of Probiotics

### 1.2.4 Definition

The term Probiotics or beneficial bacteria has been widely studied in humans and animals with a shift to the use of probiotics in aquaculture. The most followed definition of probiotics is that by Fuller (1989, p366) as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance'. Additionally, the term probiotic is often used as a synonym for certain types of beneficial members of commensal microbiota. However, in order for microbiota to constitute a probiotic, it must first be isolated as a strains from original commensal microbiota, then characterized for content, stability, the ability to improve the balance of the intestinal microbiota, and the potential health benefit that the stain can offer the host (Sanders, 2008).

### 1.2.5 How probiotics work

Probiotics were primarily used in the aquaculture of molluscs, to manage disease, as bacterial infections are very common illnesses that occurs in intensive culture systems (Kesarcodi-Watson et al, 2008). Bruhn (2006) expressed the idea that probiotic bacteria, as it relates to aquaculture, are live microorganisms which may be administrated for different purposes that offer beneficial effect to their host. These purposes include; disease prevention, water quality

improvement, or as a feed enhancer. All are proposed to promote the health, wellbeing and survival of the farmed host animals, or the environment that the host animals live in.

Irianto & Austin (2002a) reported on the competitive exclusion of pathogenic micro-organisms by probiotic bacteria. These bacteria also assist the metabolism of the host animals by stimulation of the host immunity. Some of the mechanisms that allow the probionts to do this include production of inhibitory compounds, such as bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide, formation of ammonia, diacetyl, and alteration of pH values by organic acids (Verschuere et al., 2000).

One of the most important benefits of probiotic use, is argued by Gómez, et al (2007) as providing a principled alternative to the use of antibiotics in the intensive culture systems, which can inhibit the host animals from developing a tolerance threshold to the antibiotic microorganisms. This was the case for Gómez, et al (2007), by avoiding the use of antibiotics, and using a dry feed containing lactic acid probiotic bacteria (*Carnobacterium divergens*), they were able to improve disease resistance against a pathogenic strain of *Vibrio anguillarum* when they were rearing Atlantic cod fry.

Balcazar, et al (2006) articulates that some probiotics can act as source of nutrients and enzymatic contribution to the digestion of some marine organisms by participating in the digestion processes of host organisms that produce extracellular enzymes, such as proteases, lipases, as well as providing necessary growth factors. Krishnaprakash et al. (2009) reduced the effect of pathogenic organisms in the gut of shrimp, by incorporating probiotic bacteria into the shrimp diet, which further developed a balance in the intestinal microbial community, resulting in enhancement of food absorption and increased activity of digestive enzymes. In fish, it has been reported that Bacteroides and *Clostridium sp*. have contributed to the host's nutrition, especially by supplying fatty acids and vitamins (Sakata, 1990).

Similar observations by Ringo et al (1995) supports this and also argues that microorganisms such as *Agrobacterium sp., Pseudomonas sp, Microbacterium sp, Brevibacterium sp,* and *Staphylococcus sp*. may contribute to nutritional processes in Arctic charr (Salvelinus alpinus L.)

Certain probiotics bacteria have mechanisms that stimulate the host immune response to produce immunostimulants. This has been known to increase macrophage phagocytic activity in red sea bream Pagrus major (Sakai, 1998). This process enhances the defences of the metabolism against pathogens by triggering an increase in phagocytosis, antibodies, chemiluminescent response in the host animal, and by producing superoxide anion (Sakai, 1998).

Balcazar, et al 2006 also reported on how microbionts such as *Bacillus sp*. can improve the water quality of intensive culture systems. This is as a result of introducing probionts that are gram positive and can convert organic matter back to $CO_2$ that gram-negative bacteria. Dalmin et al. (2001) supports this by explaining that during the production cycle gram-positive bacteria can minimize the build-up of dissolved and particulate organic carbon. *Bacillus sp*. has been known to improve water quality, survival and growth rates and increased the health status of juvenile *Penaeus monodon* and reduce the pathogenic Vibrios (Dalmin et al., 2001).

### 1.2.6 Previous research of probiotics in aquaculture

Early aquaculture research has centred on the use of probiotics as a preventative measure against pathogens in aquatic sea-life, as a better alternative to antibiotics. Several studies have sought to isolate probiotic bacterial strains from microbiota in the digestive system, often in the gastrointestinal tract in aquatic species from *Haliotis* and other mollusks, shrimp

and fish (Wang, Li, Lin, 2008; Ninawe and Selvin, 2009; Liu, Chang, Liu and Wang, 2013). The findings from these studies found increased survival rates among abalone and other sea-species, increase in nutrients and enzymatic activity which improve digestion, increased growth and enhanced immune system (Irianto and Austin, 2002; Balcazar, Blas, Ruiz-Zarzu, Cunningham, Verdell, Muizquiz, 2006; Kesarcodi-Watson et al, 2008; Zhao et al, 2011). After outbreaks of disease and increase in pathogens in aquaculture in the 90's, Irianto and Austin (2002) considered the use of probiotics, to combat against *A. salmonicida*, in the digestive tract as an addition to diet in freshwater rainbow trout.

Huddy and Coyne (2015) investigated the use of probiotics in the cultivation of *Haliotis midae* over a 180 day laboratory-based trial. The authors isolated the bacterial strain *Vibrio midae* SY9 and *Vibrio midae* SY9 Mutant (as a stimulant for protein ingestion and absorption) into an artificial feed for three groups including those fed a basal diet. Digestive gut tissue was then analysed and compared to see whether the probiont affected the growth of the abalone. In this instance the authors found larger abalone (shell length of 67mm, 63grams in weight) had a higher growth rate of 33-35% as a result of the treatment. Moreover, a significant finding was the alkaline protease activity was found to be higher in crop/stomach than the intestinal region which suggests the region where amino acids and peptides are absorbed. The *V. midae* SY9 strand of bacteria secreted protease into the intestinal region and resulted in enhanced digestion and contributed to the growth rate.

Other studies have looked at the effect of the adaptation of gut microbiota associated in abalone *Haliotis gigantea*, through the use of *Pediociccus* sp. Ab1 in the hope of improving abalone resistance to pathogens (Iehata, Nakano, Tanaka and Maeda, 2014). The study found the gut biota of probiotic abalone sample is more diverse in comparison to the non-probiotic sample. From Ab1 colonising the gut environment and therefore improving the microbiota environment. The amount of *Vibrio halioticoli*, known to be beneficial for other species of

abalone, was much higher than in non-probiotic group. The authors found that this probiotic increases the abalones defence to pathogens.

As aquaculture research has evolved, more of a focus has been given to the use of probiotics for the purposes of increasing the growth rate of abalone and minimising mortality. Although marine and aquaculture are lucrative industries for various countries, many face similar problems around disease and pathogen outbreaks. Particularly to abalone species is the slow-growth rate, approximately 4-5 years for them to reach market size of 80-100mm.

Macey and Coyne (2005) successfully increased growth and disease resistance in *Haliotis midae* through isolating one SY9 (bacterial) strain and two SS1 and AY1 (yeast) strains and incorporating the probionts into a supplemented feed. Their results indicated an 8% growth in smaller juvenile abalone (20mm) to 33% in larger adult abalone (67mm). Further, the growth was attributed to the probiotic microorganisms in the gut which aided in better digestion and absorption of protein in the digestive tract.

In comparison, another notable study in this field is by ten Doeschate and Coyne (2008) who also investigated the role of probiotics in the growth of South African farmed *Halitotis midae* with three groups who were fed a supplemented feed of probiotics and kelp, antibiotics and kelp and a control group fed a basal diet. The bacterium *Pseudoalteromonas* sp. strain C4 isolated from the gastrointestinal tract. Erasmus et al (1997) previously established the strain to be appropriate for this particular species of farmed abalone who are fed a protein-poor diet of kelp. Whilst the results did not indicate growth as a direct effect of the probiont, it was proposed to be a supplement alongside the kelp which provided additional enzymes and nutrients which could be better ingested by the abalone. Additionally, the antibiotic supplemented feed did not stimulate growth at all among abalone.

A leading study in this area was that investigated by Hadi et al (2014) on New Zealand *Haliotis iris* to increase the survivability and growth through multi-strains of two and three bacterial isolates (*Exiguobacterium* JHEb1, *Vibrio* JH1, *Enterococcus* JHLDc). Three groups of juvenile abalone were fed over a 60 day trial, of which the results indicated both the 2-P and 3-P supplemented fed groups improved in growth (shell length and weight). The 3-P fed group had the most significant growth (19.8% weight, 20.9% shell length). Overall, the authors suggest that the more 'metabolically diverse' the strains of different probiotics used, the more beneficial it is to improving growth of the abalone.

The profiles of the three probionts for the feeding trial are as follow:

*Exiguobacterium* spp.

*Exiguobacterium* are Gram-positive motile microbes that have peritrichous flagella, which possess catalase positive and oxidase negative enzymes. The colonies of this bacteria are circular in shape, raised and smooth, and have an orange colouration. It is both aerobic and anaerobic and has a pH tolerance of 5.5-10.5, and a temperature of 5 - 40°C, and can grow in medium containing salt (NaCl) within the range of 0-5% (Kasana &Yadav, 2007). In relation to aquaculture the genus *Exiguobacterium* have been considered to have the potential to improve the growth and survival rate of brine shrimps by producing beneficial polypeptide antibiotics e.g. *bacitracin, gramicidin S, polymyxin, and tyrotricidin* which defend against a range of against a wide range of bacteria (Sombatjinda et al., 2011).

Phylum *Firmicutes*

Class Bacilli

Order Bacillales

Family Bacillaceae

Genus *Exiguobacterium* (Hadi et al, 2014)

*Vibrio* spp.

*Vibrio* spp. is a Gram-negative, motile strain with a polar flagellum. It is anaerobic and was grown supplemented with NaCl. *Vibrio* spp. They have a transparent colouration, and metabolize glucose by fermentation due to the oxidase-positive, catalase–positive enzymes. Their growth conditions were optimal at 16oC, with a pH of 8 and 2-3% NaCl.

Phylum Proteobacteria

Class Gamma Proteobacteria

Order Vibrionales

Family Vibrionaceae

Genus *Vibrio* (Bergey et al., 1994)

*Enterococcus* spp.

*Enterococcus* is a Gram-positive, lactic acid bacteria. They are non-motile, non-spore-forming, and possess the enzyme catalase-negative, their by-product as a result of fermentation is lactic acid (Ringø &Gatesoupe, 1998).

Phylum: Firmicutes

Class:Cocci

Order:Lactobacillales

Family:Enterococcaceae

Genus: *Enterococcus* (Hadi et al, 2014)

## 1.3 Research Aims

Based on the above literature review, it is clear that a gap exists within the literature regarding first, the optimal nutrition for the New Zealand black-footed abalone. Thus, the overall goal of this thesis is to investigate and evaluate the effects of probiotic bacteria on the ingestion, digestion, assimilation of formulated diets for Pāua, with the goal to increase growth rates. It is hoped that the findings of this work will contribute to efforts to minimize the cultivation period of *Haliotis iris*. It is also envisaged that this contribution will help reduce cultivation costs by making feeding, digestion and assimilation more efficient. Below are the research objectives of this thesis:

Aim One: To identify the optimal feeding ration for Pāua and investigate the physiological effects of different rations on the growth of Pāua.

Aim Two: To identify the effect of added probiotics to optimum feeding rations (from aim 1) to growth of Pāua.

### 1.3.1 Ngā whatu a Tangaroa: Māori Contribution

Pāua have a spiritual and intrinsic meaning to Māori. Traditionally they were a life sustaining resource for Māori tribes particularly those who resided close to coastal areas. Today they are still a delicacy for Māori and New Zealanders alike. Whilst they have been primarily used as a food source, Pāua were also valuable resources used in Māori artistry and ground within Māori spirituality. Within *whare tupuna* or ancestral meeting house of a *Marae* there are posts carved as figures of ancestors and gods from Māori genealogy, history and mythology. The final step upon the completion of the carving of the figures, the eyes were adorned with Pāua shells to give them life. This process is sacred to many tribes of New Zealand and is known as *tiwhaia*.

Pāua was an important aspect of trading between coastal and inland tribes and was used in exchange for goods and services. Traditionally, in the the Northland (Te Tai Tokerau) region, the harvesting period of Pāua and other inshore shellfish stocks were highlighted by the *maramataka* or lunar cycle and the blue moon. The traditional protocols that were set in place to manage and moderate the harvest of seafood such as Pāua were dependant on the seasons. For example, the right time to harvest shellfish (e.g., Pāua and kina) was known by the bloom of the *pohutukawa* tree. In the months of Autumn and Winter when the trees had lost their flowers this signalled the end of the harvesting season.

Iwi groups such as Te Tai Tokerau and Taranaki have a large area of 'rohe-*moana'* which falls under the Quota Management System. They also rely heavily on the profits of their fisheries assets to support the commercial and economic interests of their respected iwi.

Article 2 of the Treaty of Waitangi

'Ko te Kuini o Ingarani ka wakarite ka wakaae ki nga Rangatira ki nga hapu - ki nga tangata katoa o Nu Tirani te tino rangatiratanga o o ratou wenua o ratou kainga me o ratou taonga katoa'

Her Majesty the Queen of England confirms and guarantees to the Chiefs and Tribes of New Zealand and to the respective families and individuals thereof the full exclusive and undisturbed possession of their Lands and Estates Forests Fisheries and other properties which they may collectively or individually possess so long as it is their wish and desire to retain the same in their possession

The fundamental principle of Article 2 is to give Maori the right to manage their affairs, lands and resources under their own authority, customs and lore. Although the wording of the treaty between the English and Maori versions has been problematic, it is this core principle which

has been recognised by the Crown and Parliament and codified into laws such as the Maori Fisheries Act 2004. Under the Fisheries Act 1996, section 2B(1)(b) allocates 20 percent of the quota shares to Te Ohu Kaimoana Trustee Limited, agents of Maori iwi groups in New Zealand. The recognition of customary titles and ownership allows Maori to exercise Kaitiakitanga or 'guardianship' over their resources and pātaka kai or 'customary harvesting site'. It is this underlying responsibility that iwi and hapū have as Kaitiaki to manage, replenish and sustain the resources from depletion. Technology such as aquaculture of native species (such as Pāua) provide avenues to create nurseries (Kōhanga) and farms of additional resource without depleting customary and natural stock, thus, expanding the potential for the Kaitiakitanga of their marine resource.

### 1.4 Thesis Overview

Chapter One has provided the background, research aims and review of the relevant literature on aquaculture research with regard to feeding rations, probiotics and the effects on marine species including *Haliotis iris*. Therefore, the structure for the remaining thesis chapters are outlined below:

Chapter Two provides the Methods and Materials used for both trials. First, it looks at a comparison of food rations to distinguish which feeding ration is the optimum amount that produces the best growth. Second, this chapter outlines the probiotic growth trial which compares probiotic feed to non-probiotic feed by feeding abalone a ration at 2% of their body weight. It also outlines the preparation and cultivation of the probiotic feeds as well as the water system used to grow the abalone.

Chapter Three delivers the results of both trials and discusses theses in regard to the test and analyses used to understand the data in relation.

Chapter Four provides the final discussion of the findings particularly from Chapter Two and Three. The culmination of all previous chapters are summarised into overall findings and contributions from which the limitations are also derived. Lastly, scientific and commercial contributions are discussed post-investigation and lead to recommendations and future avenues of research.

# Chapter Two: Methods and Materials

## 2.1 Introduction

Shortening the growth rate of farmed Pāua is the primary concern of this New Zealand aquaculture industry. The development of improved culture techniques and technologies such as re-circulation water systems and balanced formulated diets have enabled Niwa and East Land Aquaculture to produce high growth rates, with Pāua taking 26 months to reach a market size of 75 mm (NIWA, 2006) which is considerably faster than in the wild. Currently, research is focusing more intensively into the aspects of optimal nutrients and how to capitalise of these attributes using probiotic micro-organisms that have the potential to improve Pāua growth by manipulating the mechanisms within the gastrointestinal tract, to increase nutrient readiness and absorption, to increase the amount of digestive enzymes in the gut (Macey & Coyne 2005; Doeschate &.Coyne, 2008; Hadi, 2012). The present study followed previous research (Hadi et al, 2014) and continued to expand probiotic and the nutritional aspect of New Zealand Pāua farming by exploring the use of a feeding ration to develop a feeding structure that delivers a more efficient growth rate.

There were two growth trials in this study, one looked at comparing the growth of Pāua samples, fed on 3 different rations based on bodyweight of formulated feed (1%, 2%, 5%), and the second was a comparison of a probiotic (2%) and non-probiotic (2%) on an optimum ration feed as a result of the first trial. The experiments of this study were designed to measure the growth dynamics of different samples of Pāua which give logical indicators that determine the overall aims of the study.

The tests that analysed the growth of the Pāua samples independently examine specific aspects of the growth of Pāua that pertain to muscular foot growth as it is a primary focus for the aquaculture industry. These measurable components are made up of wet weight, shell length to give an overall indication of growth. The biochemical analyses examined the

amount of protein, lipids and carbohydrates and tissue of the Pāua. This provided a chemical indication of the amount of cellular growth as protein and amino acids are the building blocks of cellular and muscular growth and repair.

## 2.2 Feeding Ration Test

A feeding trial was conducted with different feeding rations of formulated probiotic diets over a 10 week period, at the Aquaculture Laboratory, AUT, Auckland. Abalone (size 20mm – 30mm) were randomly separated and equally distributed into 40 separate tanks which were supported by an open water flow system that have maintain the water parameters ideal for Pāua (stated below). The rations of feed for the Pāua in this experiment were considered and assessed using different percentages of the mean wet weight of the abalone in each treatment. The rations are 1%, 2%, and 5% bodyweight.

The Pāua were acclimatized for three weeks to the lab conditions and the open flow system at AUT. This allowed the animals to distress from the transportation from the Pāua Farm and also enabled the Pāua to regain an appetite.

Once the animals had settled into their experimental tanks, and prior to the start of the experiment they were starved for three days to clear the digestive system of all previous food and fecal, and to build an appetite to ensure that the animals would eat in the initial stages of the feeding Trial.

Prior to the start of the trial, initial indication data were collected to form a baseline for the experiment. This consisted of the testing measurements that were to be used in the experiment to measure the growth of the Pāua. These measures included shell length (mm), wet weight (0.00g), and a flip test (time in seconds) to gauge and measure responsiveness and overall health and wellbeing, overall mortality was recorded.

Formulated feed rations were calculated by taking a percentage of the mean wet weight of all the Pāua in each feeding treatment. Each ration was weighted prior to feeding each day. Feeding was conducted every day at dusk or around 1800hrs, as abalone are known to feed around dusk (Macey & Coyne, 2005).

The feeding ration values were derived from the literature (Chapter 1) and commercial recommendations (such as NIWA) for feeding abalone. The literature provided no optimal feeding ration that best promoted growth. Moreover, feeding rations ranged from 0.2% (Sales and Britz, 2001), 2.2% (Riche et al, 1995) to 5% (Ganmanee et al, 2010). Hadi et al (2014) is closely followed as it is a recent study conducted on *Haliotis iris*, here the researchers used 1.1% body weight ration to test the effects of probiotics. From the overall findings of Chapter 1, the feeding rations used in this study are 1%, 2% and 5%. Feed rations at 3 and 4% were not used due to the size and scale of the project. Moreover, previous studies have indicated that 1, 2 and 5% are the more common rations in studies of abalone aquaculture. It was the intention of the researcher to isolate a range of ration across the spread of 1, 2 and 5% that would provide optimal benefits to New Zealand abalone.

**2.3 Probiotic versus Non-Probiotic**

A feeding trial was conducted with different feeding rations of formulated probiotic diets over a 10 week period, at the Aquaculture Laboratory, AUT, Auckland. Four Abalone (size 20mm – 30mm per tank) each were randomly separated and equally distributed into 40 separate tanks which were supported by an open water flow system that have maintain the water parameters ideal for Pāua (stated below). The rations of feed for the Pāua in this experiment were considered and assessed using different body weight percentages. The

rations were 2% probiotic diet, and 2% non-probiotic diet (Control) in bodyweight . The purpose of this was to compare the function of the probiotic to the non-probiotic diet.

The Pāua were acclimatized for three week to the foreign lab conditions and the open flow system at AUT. This allowed the animals to distress from the transportation from the Pāua farm and also enabled the Pāua to regain an appetite.

Once the animals had settled into their experimental tanks, and prior to the start of the experiment they were starved for three days to clear the digestive system of all previous food and faecal, and to build an appetite to ensure that the animals would eat in the initial stages of the feeding trial.

Prior to the start of the trial, initial indication data were collected to form a baseline for the experiment. This consisted of the testing measurements that will be used in the experiment to measure the growth of the Pāua. These measures include shell length (mm), wet weight (0.00g), and a flip test (time in seconds) to gauge and measure responsiveness and overall health and wellbeing, overall mortality was recorded.

Formulated feed rations were calculated by taking a percentage of the mean wet weight of all the Pāua in each feeding treatment. Each ration was weighted prior to feeding each day. Feeding was conducted every day at dusk or around 1800hrs, as abalone are known to feed around dusk (Macey & Coyne, 2005).

In much of the research around growth trials in different species of abalone, a wide range of feeding rations were used to stimulate abalone growth. In most cases the feeding rations of abalone many studies calculated the feeding ration based on a percentage of body weight. This range fell between 0.2% body weight (Sales and Britz, 2001) to 1.1 % (Hadi, 2012), 2% (Riche, White and Brown, 1995; Coote, Hone, Van Barneveld and Maguire, 2000) and 3% (Searle, Roberts, Lokman, 2006) to 5% (Ganmee, Sirirustananun, Jarayabhand, 2010;Tung,

2011). For the purposes of this study, the rations 1, 2 and 5% were selected in order to isolate a range of food portion that promoted the optimum growth of *Haliotis iris*.

Growth (i.e., shell size, animal wet and dry weights) and survival was recorded throughout the experiment, with initial measurements (start of the experiment) and every two weeks. Shell length measurements were obtained by measuring the greatest length. Wet weights were measured by lightly drying individual animals using tissues or hand towels for approximately 30 minutes and weighing them to the nearest 0.00g.

In addition to morphological and survival parameters, the aim was to examine the physiological responses that the abalone exhibit under the probiotic treatment, with a particular interest in the enzyme functionality and the assimilation of nutrients into the body tissue. For this, biochemical analyses were conducted to identify protein, lipid and carbohydrate content within the tissue of the abalone. This provided an indication of how efficient the digestive system converts the nutrients in the food to proteins, lipids and carbohydrates that are stored in the body tissue of the abalone.

Additional measurements were also applied to all of the animals better analyze and gain a broader understanding of growth of the animals. These included measuring ‘intake’ by recording the dry weight of the feed before and after feeding time. The Pāua were also dried and the amount of moisture that was extracted was determined, this was also done to acquire Pāua dry weight. Microbiological and chemical analysis of the tissue were conducted to acquire a percentage of the protein, lipids and carbohydrate composition of the Pāua body tissue. This was used to extrapolate the growth trends of the Pāua that are fed each of the different feeding rations.

## 2.4 Methods and Materials

### 2.4.1 Abalone Samples

Juvenile abalone (20 – 30 mm shell length) were obtained from OceaNZ Blue Ltd., Ruakaka, New Zealand. This size class range was selected for the feeding trial since it represents a fast-growing period in the life cycle of abalone and is of commercial importance (Preece and Mladenov, 1999). The juvenile abalone were acquired from OceaNZ Blue Ltd, Ruakaka (NZ). The size class range was selected for the feeding trial as it represents a fast-growing period in the life cycle. 160 animals were individually tagged with a random number for identification, and placed in a common tank to be acclimatised for a period of three weeks. During this time, the animals were fed a formulated feed. After the acclimatisation period, the animals were starved for 3 days prior to the start of the experiment. This starvation period ensured that all animals started the feeding trial with empty stomachs.

Two separate experiments were conducted to investigate nutritional aspects of juvenile abalone. The first experiment was designed to identify the best feeding ration for abalone. Four abalone were randomly placed in each of 10 tanks (40 abalone total) for each of 3 feeding rations (1, 2, and 5% of body weight formulated feed; ten tanks per treatment). The second feeding experiment included a probiotic and non-probiotic diet of 2%. For this experiment, a different set of abalone (4 per tank) were placed in another 10 tanks (40 abalone in total).

### 2.4.2 Dietary components of commercial feed

The commercial feed that was used in the feeding trials was AbMax 16, which is manufactured by E. N. Hutchinson, Ltd., Auckland, New Zealand. The ingredients in this feed include wheat flour, fish meal, seaweed powder, defatted soy flour, and a range of vitamins and minerals.

The composition of the commercial formulated abalone diet is as follow:

- Wheat Flour 35%,
- Fish Meal (Milled NZ) 20%,
- Defatted Soy Flour & Isolate 13%,
- Seaweed Powder, Tapioca Starch, Propylene Glycol, Propylene Glycol, Sugar, Yeast – Saccaromyces, Wheat Gluten, Fish Oil (mixed), Phosphoric Acid (85%),
- Carrageenan, Lecithin Powder, Dicalcium Phosphate, Potassium Sorbate, Vitamin Mineral Mix (Abalone), Betaine, Stay-C (25%), and Vitamin E. Some components percentages are not given due to trade secrets.

The unaltered commercial feed was used in both experiments to form the probiotic diet, and was serve as the control in experiment 2. The treatment included the addition of different probiotic complexes added to the feed. Proximate analyses (i.e., protein, carbohydrates, and lipid) were conducted on AbMax to determine its general composition.

### 2.4.2 Probiont cultivation

The three probiotic strains that were used in this project, *Exiguobacterium* sp. strain (JHEb1) and *Vibrio* sp. strain (JH1), and *Enterococscus* sp. strain (JHLDc) were obtained from Hadi (2014). Pure cultures of these bacteria were retrieved from storage at -80°C by thawing at room temperature and then transferring into Marine Agar, MacConkey Agar, and MRS Agar, respectively. The cultures were incubated at 22°C until colonies were visible.

### 2.4.3 Preparation of Probiotic Feed

The probiotic strains were propagated in enriched media prior to addition to the feed. *Exiguobacterium* (JHEb1) was propagated in Marine broth supplemented with 1% of yeast extract. The culture was incubated at 36°C for 48 hours. Vibrio JH1 was grown in Marine

broth supplemented with 0.5% of glucose at 36°C for 48 hours. *Enterococcus* JHLDc was propagated anaerobically in MRS broth containing 2% NaCl, and was incubated at 36°C for 48 hour.

Hadi (2014) determined that the most effective and efficient method for applying the probionts to the feed of the abalone was in the form of a spray. By applying broth cultures of the probiont isolates directly to the feed pellets of commercial feed was most effective and maintained the health, mobilization and populations of bacteria cultures, particularly when added to the experimental tanks. Other methods such as producing food cakes that contained immobilized probionts did not maintain the structure as they deteriorated when they were added to water, and were not as effective in transporting and mobilizing the probionts into the gut of the abalone.

- Three to five colonies of *Exiguobacterium* JHba1 cultured in Marine Agar were inoculated into 50 ml of modified Marine Broth containing an additional 1% of yeast extract.
- Three to five colonies of *Vibrio* JH1 cultured in Marine Agar were transferred to 50 ml of modified Marine Broth containing 1% yeast extract and 0.5% glucose.
- Three to five colonies of *Enterococcus* JHLDc from MRS agar were transferred to MRS broth containing 2% NaCl.

Viable counts of probiotic were conducted weekly by taking samples from the feed, faeces and the sumps of each water system. Ten-fold dilutions of the sample were prepared and aliquots from each dilution were plated on Marine Agar by spread plating. The plates were incubated for a 48 hour period at $20^0$C. This monitoring was done to ensure that the bacteria in the feed was at a working range and the sump water was not contaminated. This enumeration was necessary to determine if a viable concentration

between $10^6$ CFU /g to $10^8$ CFU/ g was maintained in the feed (Gatesoupe, 1999; Macey &Coyne, 2006b; ten Doeschate &Coyne, 2008).

To collect the cells from propagation, the cultures were transferred into sterile (50 ml) centrifuge tubes with caps to maximize hygiene and minimize contamination. Centrifugation was conducted for 10 minute periods at 36°C. After centrifugation, probiotic biomass from each culture settled at the bottom of the conical shaped centrifuge tube, this biomass was collected and re-suspended in (25ml) sterile marine broth. These three bacterial cultures were then carefully and gently mixed together into clean sterile beaker. 100 grams of abalone food pellets were spread out on a sterile tray (sprayed with 70% ethanol and dried immediately). In order to formulate the probiotic supplemented feed, the probiotic mixture was sprayed over the abalone food pellets using a sterile syringe. The pellets were then mixed manually, using sterilized gloves with 70% ethanol to thoroughly coat the pellets with the bacterial mixture. Pellets were then left in the laminar flow cabinet at room temperature for 2- 4 hours or until dry (Lauzon et al., 2010). Once pellets were dry they were then placed in a zip lock plastic bag and stored in the refrigerator at under 4°C until the feed was needed for feeding.

### 2.4.4 Growth Measurements

Growth (i.e., shell size, animal wet and dry weights) and survival of abalone were recorded throughout the experiment, with initial measurements at the start of the experiment and every two weeks for a total of weeks. Shell length measurements were obtained by measuring the greatest length. Wet weights were measured by lightly drying individual animals using tissues or hand towels for approximately 30 minutes and weighing them to the nearest 0.001g.

In addition to morphological and survival parameters, physiological responses that the abalone exhibit under the probiotic treatment were recorded, with a particular interest in the enzyme functionality and the assimilation of nutrients into the body tissue. For this, biochemical analyses were conducted to identify protein, lipid and carbohydrate contents within the tissue of the experimental animals. This is to help give an indication of how efficient the digestive system converts the nutrients in the food to proteins, lipids and carbohydrates that are stored in the body tissue of the animals.

### 2.4.5 Food Intake

Through the duration of this project, the food intake for abalone samples was monitored. This was determined using the method (below) acquired from Ganmanee et al (2010). Monitoring the food intake of the animals and correlating this with the mass gained (in weight gained) during the trial gives a good indication of feeding efficiency. The experimental diet was offered daily (1800 h) at different rations (different percentage of their body mass) of their biomass. After 12 h (0600 h), uneaten food was collected, dried at 60°C for 24hrs, and weighed. Intake was calculated with the following equation:

$$\boldsymbol{I} = (\boldsymbol{W} - \boldsymbol{DWF}) / \boldsymbol{N}$$

'I' is the feed intake, 'W' is the mean dry weight of food at the onset of the test, 'DWF' is the mean dry weight of food at the end of the test less the correction for stability from controls, and N is the number of animals in the unit. (Ganmanee et al. 2010)

The feed conversion efficiency (FCE) was be calculated using total wet weight gain per abalone and the corresponding feed intake is as follows:

$$\boldsymbol{FCE = 100 \times [\text{wet weight gain (g)}] / [\text{feed intake (g)}]}$$

### 2.4.6 Flip Test

At the end of the experiment, a flip test was performed on each animal to identify their activity level. Tung (2011) adapted the procedures for this test from Searle (2004) who originally developed this test for approximating the health of H. iris at the conclusion of the experiments. The animals from each container were placed upside down on a wet, flat surface. Then, the number of individuals that overturned to their normal position was recorded at 1 minute intervals until all the individuals had flipped to their normal position.

### 2.4.6 Moisture test and drying of samples

A moisture test following De Knegt and Brink (1998), was used to extract the excess water from the body tissue of the sample animals. The moisture content of the samples were determined by calculating the wet weight difference between before and after drying the animal tissue. For this process, individual samples were de-shelled and placed into small individual plates that had been pre-weighed. The weight of each sample was taken prior to placing the samples in the oven at 100 degrees for three hours or until dry. When there was no sign of moisture in the tissue, samples were taken out of the oven and weighed immediately to minimize the absorption of moisture from the air. Total moisture was then determined using the following formula.

$$\text{Total moisture \%} = \frac{\text{M (initial)} - \text{M (dried)}}{\text{M (initial)}} \times 100$$

### 2.4.7 Biochemical analyses

Following the moisture test, the remaining dry tissue samples from the different feeding treatments were pooled together and ground into a powder form with a coffee grinder. In order to prepare for biochemical sample, each group of samples contained the remnants of all of the soft tissue components of the abalone in each feeding treatment. This was done to acquire an overall estimate of protein lipid and carbohydrate content within the tissue of each sample to compare biochemical composition.

### 2.4.8 Bicinchoninic acid assay (BCA Assay)

A BCA protein assay kit from Thermo Scientific New Zealand Limited was used to determine protein concentrations in the samples of abalone tissue and commercially formulated feed. The BCA protein assay is a detergent compatible formulation based on the bicochoninic acid for the colorimetric detection and quantitation of total protein. This method combines the reduction of $Cu^{+2}$ to $Cu^{+1}$ by protein in an alkaline medium with a highly sensitive and selective colorimetric detection of the cuprous cation using a unique reagent containing bicinchoninic acid. The purple coloured reaction product of this assay of the chelation of two molecules of BCA with one cuprous ion. This water soluble complex exhibits a strong absorbance which is read at 562nm. This is nearly linear with the increasing protein concentration over a broad working range (20-2000µg/mL). In this process the peptide bonds in protein reduce $Cu^{+2}$ ions from the copper sulphate to $Cu^{+1}$ and is proportional to the amount of protein present in the solution. Two molecules of bicinchoninic acid chelate with each $Cu^{+1}$ ion forming a purple coloured product which strongly absorbs light at a wave length of 562nm.

### 2.4.9 Working reagents

The protocol for this assay began with preparing a set of known protein standards to form the working range of which can be compared with the unknown protein concentrations of the samples. This was done using the Albumin Standards provided in the kit. The standards were diluted using sodium hydroxide (NaOH) at volumes shown in the table below.

**Table 1:** Preparation of Diluted Albumin (BSA) Standards (taken from Thermo Scientific Assay Kit)

| Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20-2,000µg/mL) | | | |
|---|---|---|---|
| **Vial** | **Volume of Diluent (µL)** | **Volume and Source of BSA (µL)** | **Final BSA Concentration (µg/mL)** |
| A | 0 | 300 of Stock | 2000 |
| B | 125 | 375 of Stock | 1500 |
| C | 325 | 325 of Stock | 1000 |
| D | 175 | 175 of vial B dilution | 750 |
| E | 325 | 325 of vial C dilution | 500 |
| F | 325 | 325 of vial E dilution | 250 |
| G | 325 | 325 of vial F dilution | 125 |
| H | 400 | 100 of vial G dilution | 25 |
| I | 400 | 0 | 0 = Blank |
| Dilution Scheme for Enhanced Test Tube Protocol (Working Range = 5–250µg/mL) | | | |
| **Vial** | **Volume of Diluent (µL)** | **Volume and Source of BSA (µL)** | **Final BSA Concentration (µg/mL)** |
| A | 700 | 100 of Stock | 250 |
| B | 400 | 400 of vial A dilution | 125 |
| C | 450 | 300 of vial B dilution | 50 |
| D | 400 | 400 of vial C dilution | 25 |
| E | 400 | 100 of vial D dilution | 5 |
| F | 400 | 0 | 0 = Blank |

In order to prepare the working reagent for this assay the following formula was used

Total volume of working reagent required =

(# standards + # unknowns) x (# replicates) x (volume of working reagent per sample)

### 2.4.10 Preparation of samples and BCA protocol

In order to process the tissue samples and feed samples using the BCA protocol, the samples first had to be homogenized into liquid and diluted into readable amounts. Samples were prepared by placing 100µg of sample into 15ml sterile centrifuge tubes. Samples were measured using an analytical balance and were measured to the nearest 0.001µg.

Approximately 4ml of NaOH was added to the centrifuge tubes to rehydrate the samples. Samples were then placed on the laboratory shaker machine and shaken to combine mixture for 10 minutes. After they were incubated at 56 degrees for 30 minutes. Samples were centrifuged for 10 minutes at 2000rpm set at 36°C. 150µL of each sample solution was placed into the multiplate followed by 150µL of the working reagent. Each sample including the feed was replicated four times. The multiplate was then incubated at 37 degrees Celsius for two hours. The spectophotometer was set at a wavelength of 562nm and samples were read to determine protein concentrations. After which the protein concentrations were then converted to a ratio of protein per ml.

### 2.4.11 Lipid analysis

The lipid contents were determined following a method extraction modified from Bligh and Dyer (1959) and adopted from Hadi (2014). This method was used to determine the total lipid composition in the feed as well as the abalone body tissue. Five replicates of ground tissue samples from each treatment were prepared into powder form from the biochemical analysis. Approximately 0.5 grams of powder from each sample were put into centrifuge tubes. The content of each tube was weighed using an analytical balance and was measured to the nearest 0.0001 gram. Each sample was then rehydrated with 2ml of deionized water. A solvent mixture was made using a ratio of two parts methanol to one part chloroform. Approximately 3 ml of this mixture was added to the centrifuge tubes and shaken well for 5 minutes on a laboratory machine shaker to acquire a well homogenized mixture. An additional 1 ml of chloroform was added to each tube and mixed in by shaking for 10 minutes. Samples were then placed in a centrifuge and set for 2000 RPM. Following gentrification, samples showed a clear separation between the water content inside the tube and the chloroform layer containing the lipids were separated by the protein disc of the

sample within the centrifuge tubes. The lower layer of chloroform containing the lipids was extracted from the centrifuge tube using a Pasteur Pipette and was transferred into clean glass test tubes. These test tubes had been pre-weighed and they were re-weighed to take into account the chloroform mixture. The evaporation process commenced by placing the test tubes, containing the lipid samples of all feeding treatments, into a water bath heated at 36°C and exposing the chloroform mixture to nitrogen gas to evaporate the chloroform. This process left behind a thick lipid droplet which was then reweighed. The total lipid percent was then calculated using the following formula:

$$\text{Total lipid \%} = \frac{\text{Lipid extract (g)}}{\text{Lipid in chloroform solvent (g)}} \times 100$$

#### 2.4.11 Carbohydrate analysis

This analysis has been adopted from Porter and Earl (1990) and Hadi (2014) to determine the total carbohydrate content of the feed as well as the abalone tissue samples by taking into consideration the sum percentage of protein, lipids and moisture analyses. This formula calculates the sum percentage of carbohydrates by subtracting the sub percentage of protein lipids and moisture from a percentage of 100. The formula for carbohydrate is as follows:

$$\text{Total Carbohydrate \%} = 100 - [\%\ \text{protein} + \%\ \text{lipids} + \%\ \text{moisture}]$$

## 2.5 Aquaculture System design

This experiment was supported by a semi-closed circulation water system, that sourced fresh seawater was collected from the Okahu Bay Marina. This consisted of two 500L sump tanks inside a semi insulated room that are plumbed to an external 1500L sump. The two small

sumps supply water to individual semi closed re-circulatory systems. Each system had its own 155 Watt pump that had a max flow of 11300L/Hour that circulated water through the system and into a bio-filter and protein skimmer before flowing back into the sump.

Abalone are sensitive to high oxygen demand and require a minimum of 100% dissolved oxygen or over 4 mg/L (Ching et al, 2004; Tung 2011; Hadi, 2012). Thus, the holding tanks had a constant supply of air linked to an air stone to increase the dissolved oxygen level.

Naturally occurring sea conditions help and stop the Pāua from being stressed as it removes decaying rotting food of faecal matter that attract decomposing bacteria that consume a lot of oxygen and produce ammonia when the break down matter. Safe level of Nitrate and Nitrite for abalone in an aquaculture setting must remain under 0.5 mg/L (Hadi, 2012). To replicate this process in the aquaculture system a Bio-filter was used to control ammonia levels.

Constantly cool temperature is maintained naturally from water coming from the deep up into the intertidal and coastlines area. For this reason a water cooler was used to stabilise the water temperature, as Pāua are thermo-tolerant and their optimum growth rate is most supported at a temperature range 8-21°C (Tung and Alfaro, 2011).

Pāua are subject to normal day and night periods so the photo period in the wet lab was adapted to (period of time in contact with light) a twelves hour light to dark cycle, with day light starting at 6am through to 6pm..

A sequence of small holding tanks were built which held the Pāua. Each tank had a capacity of 4 litres and had a relief valve to control the overspill and redirect water back into the outflow and waste water. The holding containers also had a dark coloured lid which had a hole drilled through at the top to supply each tank with a constant supply of fresh running water ( flow rate was 335ml per minute), as well as a aeration hose with an oxygen stone to supply a constant supply of oxygen (103% oxygen saturation) to the animals. This was to

emulate the continuous flow of water in the real environment that water supply was set at a controlled temperature and also had set parameters around pH (pH must remain between 7.9-8.3) level and salinity (must remain between 32-35 ppt) (Wright, 2011).

Figure 5: Pāua holding tanks

The waste management within the tanks were managed manually, the faeces and uneaten food within each tank was siphoned out daily and the waste matter was filtered from the water then removed, the water was then returned into the sum. The system was designed with a 335ml/L flow rate into the small sample tanks to keep water movement constant.

The working range of the water circulatory system had a pH level of 7.63 - 8.01 with a salinity level of 33.6-35.6ppl (parts per litre). The flow rate was 335ml per minute and had a dissolved oxygen level of 8.28mg per litre which equated to 103%. The temperature fluctuated between 15.3 – 17.1°C.

NIWA have reported since 2003, as a result of adopting recirculation systems, Pāua farms such as Eastland Aquaculture and Oceans Blue Limited have successfully produced Pāua that grow up to 75 mm in 26 months. This gives a growth rate of around 2.8 mm per month and 34.6 mm per year. From this development it was recognized that recirculation systems provided more growth benefits for New Zealand Pāua farming. As such, the system used in this study was derived from concepts within the NIWA studies. Studies have shown that in

many species of Abalone including Pāua, a high dissolved oxygen level is needed to promote stable growth as abalone have a higher need for oxygen in order to survive, such as that found rocky inshore waters. As they metabolise oxygen, they release carbon-dioxide which is released into sea water and forms acids, which lowers the pH levels in the water. This can stress abalone, in order to offset this, the biomedia used was choral which offset the acidity within the water and stabilized the pH levels.

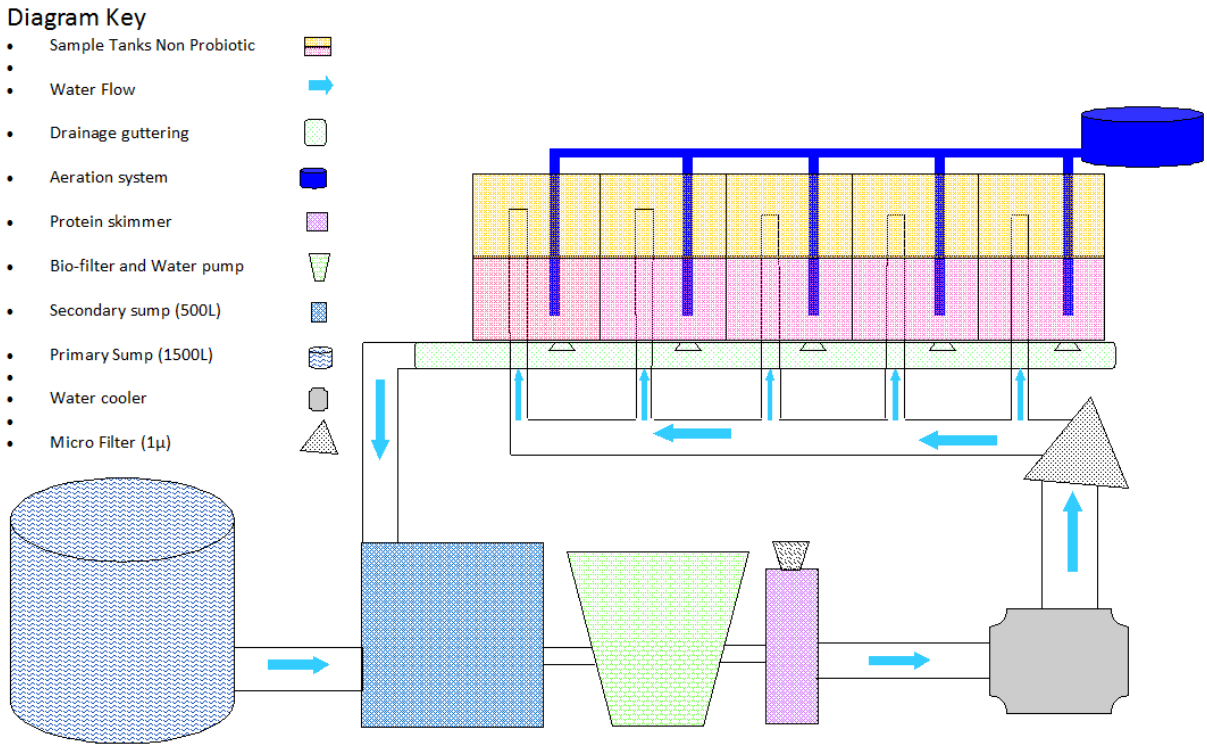


**Figure 6: Non-probiotic Water system above shows the components of the semi-closed circuit, non-probiotic water system.**

This distributes stored sea water into ten 4 litre holding tanks. Each holding tank houses 4 Pāua each, to ensure an equal distribution of feed and to eliminate bias within the feeding trial.

This seawater was then filtered through a 0.2µ prior to filling the sump and was then pumped into the 500L holding tank (secondary sump) which supplied the water to the bio-filter and

the water pump. The bio-filter contained bio-media made up of coral that harboured nitro-bacter and nitro-spira bacteria which consumed and managed ammonia and nitrite levels within the water supply. The water flowed through the bio-filter and was channelled through the protein skimmer. The protein skimmer forces water into different cyclindrical chambers that generates lots of bubbles which are forced through the water. The dissolved proteins and other organic contaminants in the water are turned into foam and are removed. This process also oxygenated the water, before challenging the water through the plumbing and into the water cooler. The wet lab where the experiment was based was not a fully insulated and enclosed environment and was subject to temperature fluctuations, so a water cooler was used to ensure that the water temperature remained between 15 – 17°C. The water was then directed through a 10 inch Standard House water filter at a size of 1 micron filtration to further clean and remove any loose matter before flowing into the sample Pāua tanks. From this point water was dispersed through the plumbing into small hoses which ran through a hole in the top of the lid of the different Pāua tanks (4 litre capacity) at a rate of approximately 335ml/minute, taking just under 12 minutes to fill to capacity. Each tank had an airline that also ran through this hole and was connected to a small air stone to maximise oxygenation. The tanks had an overflow nozzle at the top to ensure that the waste water was directed into the drainage guttering. There is one main distinction between the systems that housed the Pāua that were fed the probiotic diet, and those Pāua that were not. Uneaten food and faeces were carefully removed from the holding tanks each morning. They were remove using a pipette so as to separate them and both the food and the faeces were filtrated through 250 micron filter paper, then placed in a small plastic container for drying.

The system that supports the non-probiotic fed Pāua was plumbed so that the waste guttering would direct the excess water from all of the small Pāua tanks back into the water supply that would recirculate into the 500L sump. This was to conserve water efficiency and

sustainability, as there was no direct threats of recirculating the water. However, the system that housed the Pāua fed the probiotic diet was plumbed so any overflowing water would be directed from the animal tanks out in to the waste water drain. The water from these sample tanks may have contained remnants of the probiotic bacteria, so was not to be reintroduced back in to the water system for fear of the unknown effect that a contaminated water supply could have on the experiment.

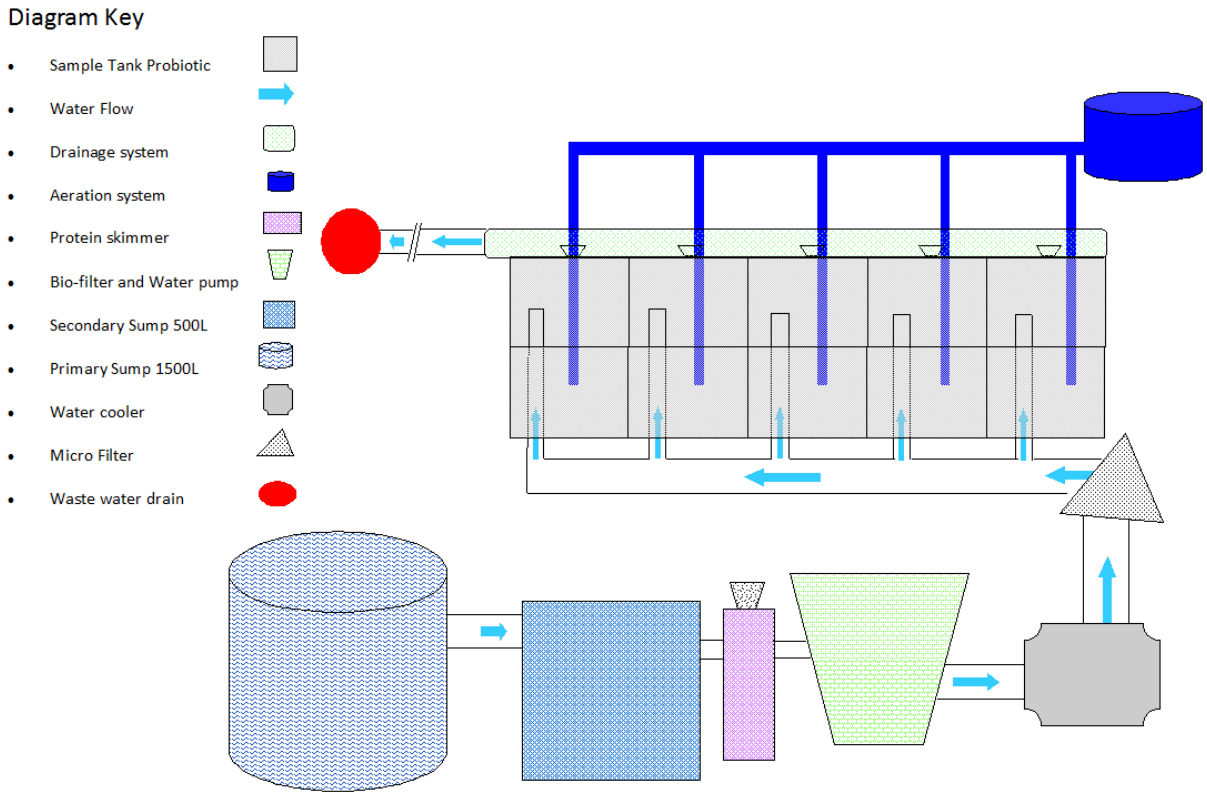


Figure 7: Probiotic Water system above shows the components of the semi-closed circuit.

**As the figure 7 shows, water from the holding tanks was caught in a guttering then directed into the waste water drain. This was to eliminate the problem of bacteria leaching in to the water system and contaminating the water supply. As a result of this, this culture system demanded over twice the amount of water per week to keep it running and would require around 1000 litres per week on average.**

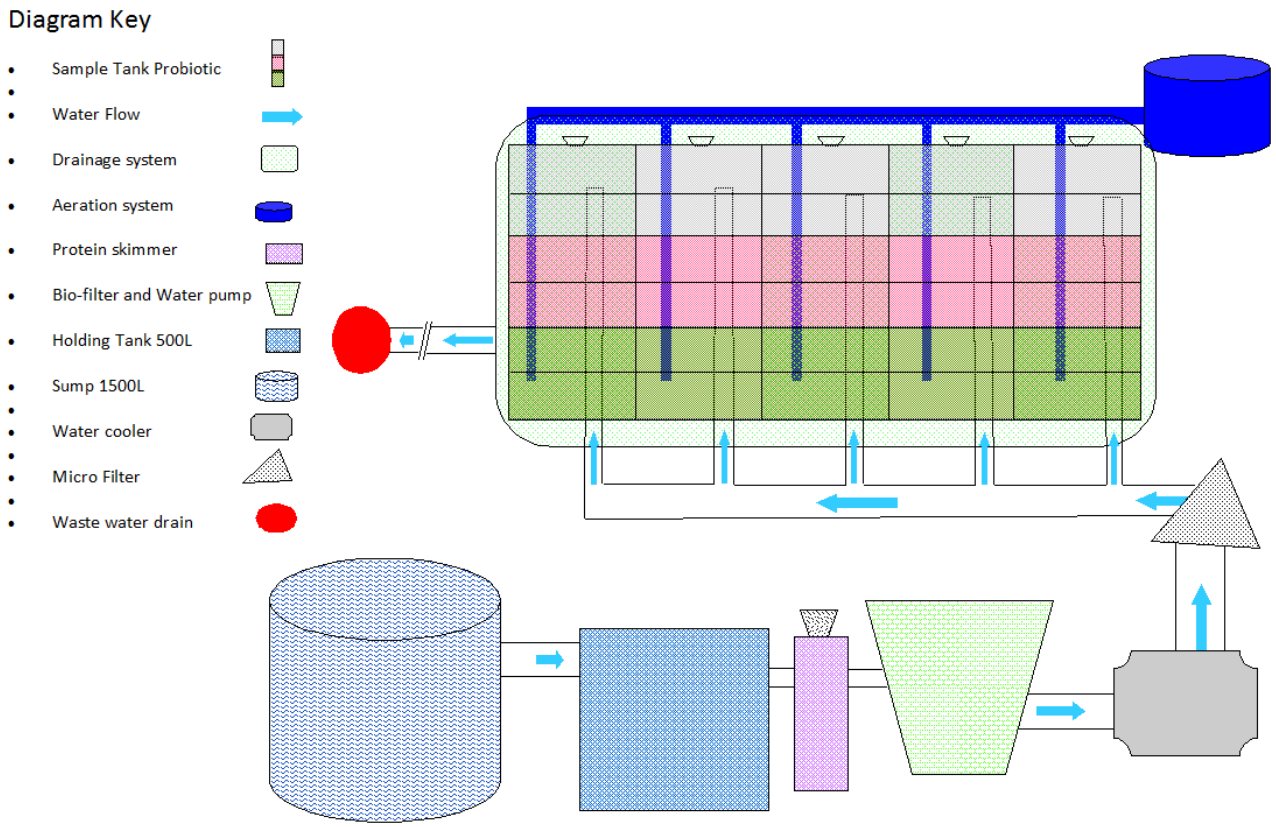


**Figure 8: probiotic Water system above shows the components of the semi-closed circuit, probiotic water system for the food ration test.**

# Chapter Three: Results

## 3.1 Feeding Ration Test Findings

### 3.1.2 Growth Trial Results

The feeding ration results showed some differences among treatments (Figure 1). Abalone grew in all three feeding treatments and those in treatments 2% and 5% increased growth in a linear progression from the beginning of the trial to week 10. Animals in treatment 1% started at a mean of 35.03mm and grew steadily until week 6 (38.33mm) where the growth plateaued until week 10 (41.12mm). At the beginning of week 0-1, animals in treatment 2% had a mean shell length of 34.25mm and those in treatment 5% were 34.55mm. At the end of week 10, animals in treatment 2% concluded with 33.48mm and 5% at 42.96mm.

Figure 2 shows that animals in the 2% treatment had the largest margin of growth, producing 9.13±0.94mm. Animals in the 5% treatment produced 8.14±0.84mm of shell length growth, and those in the 1% treatment produced the least optimum growth, only growing 6.09±0.61mm.

A one-way ANOVA test with shell length as the factor showed there was significant differences ($F=_{2, 117} = 6.10$; p-value $<0.05$) between the three treatment groups (Tukeys post-hoc test p-value = 0.003).

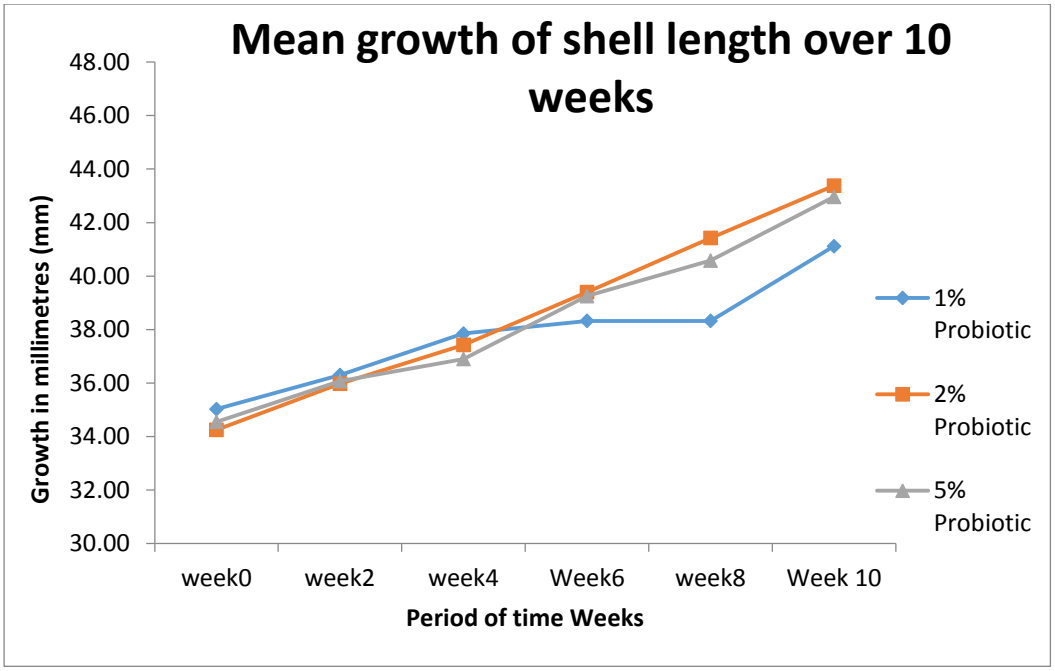


Figure 9: displays the mean shell length growth of all the three probiotic feeding treatments (T1%, T2%, T5%) as they progressed through the 10 week feeding trial.

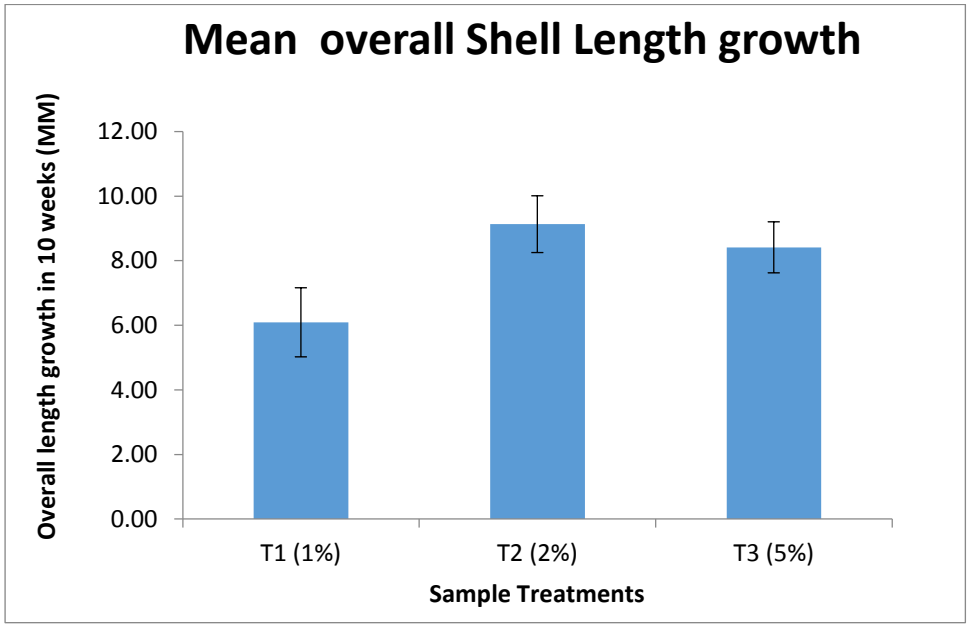


**Figure 10: shows the overall mean shell length growth (in Millimetres) exhibited by the individual treatment (T1%,T2%, T5%) and compares them to isolate which one produces the most optimal results.**

### 3.1.3 Weight gain

The mean total weight gain for the three treatment groups increased throughout the 10 week trial and is plotted below on Figures 3 and 4.

Abalone in treatment 1% began at a mean weight of 7.02grams and steadily increased until a plateau at week 6 (11.193g) and week 8 (11.64g) before drastically increasing to 15.033grams at week 10. Treatment 2% showed a similar pattern of growth compared to 1% , starting at 6.85 grams in weeks 0-1, marginal growth between weeks 2 (8.033g) to week four (8.68g) and then a constant increase (15.57g) in week 10. Treatment 5% 6.69grams in week 0-1 with steady growth to 13.168grams in week 10. The overall growth (Figure 4) of treatment 1 was 8.017±0.636g, treatment 2% showed the greatest increase overall 8.724±0.617g. Treatment 5% showed the least amount of growth 6.475±0.29g.

A single factor ANOVA test revealed there was significant differences between the food rations and weight ($F=2, 117 = 21.12$, Tukey test $p = <0.05$).

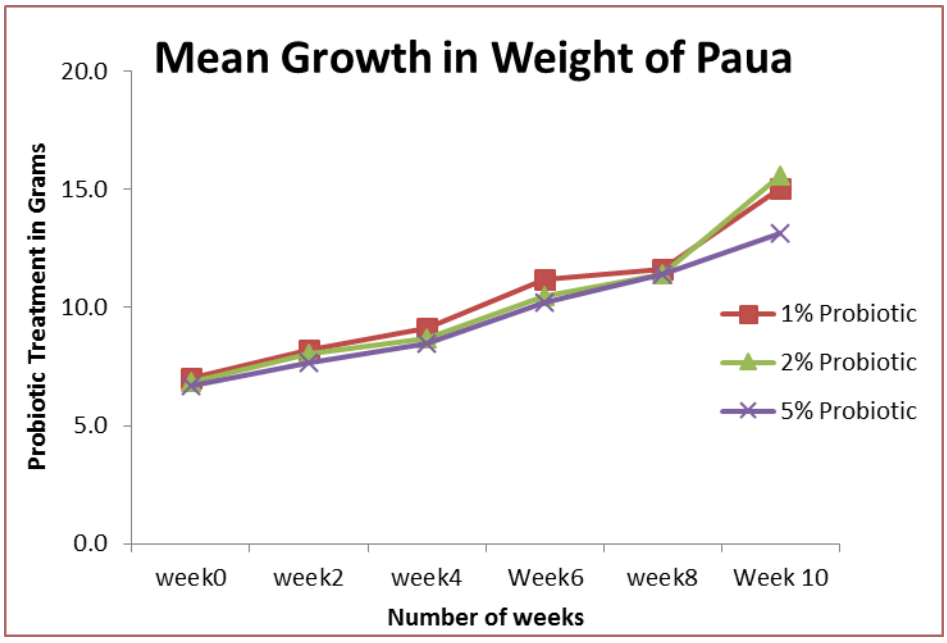


**Figure 11: displays the mean wet weight (g) growth of all the three probiotic feeding treatments (T1%, T2%, T5%) as they progressed through the 10 week feeding trial.**

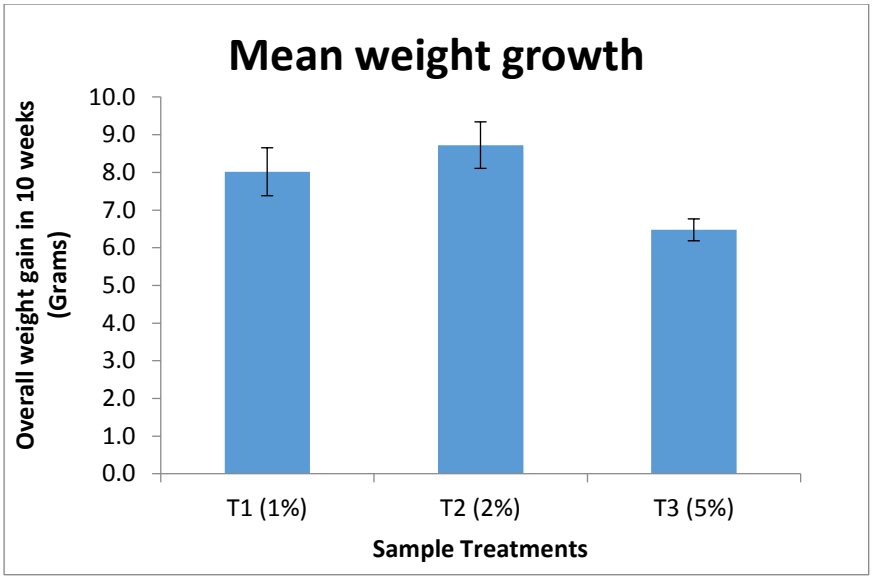


**Figure 12: shows the overall mean wet weight gain growth (g) exhibited by the individual treatments (T1%, T2%, T5%) and compares them to isolate which one produces the most optimal results**

### 3.1.4 Intake

Figure 5 shows the pattern of consumption of the three samples of Pāua as they progressed through the 10 week trial. The 1% and 2% treatment both show a fluctuating, but slightly increasing food intake from week 1-10 starting at 0.06g (T1%), 0.130g (T2%) steadily increasing to 0.108g (T1%), 0.206g (T2%) of food consumed in week 10. However, the 5% treatment had a fluctuating food intake of 0.231g and drastically decreased over a 6 week period to 0.086g, before spiking to 0.408g in week 8, then decreasing in week 10 to 0.298g.

Figure 6 shows the mean overall intake of all three treatments, T1% had a mean intake rate of 0.087±0.016g. T2% had a mean intake of 0.154±0.036g and T5% had a mean intake of 0.218±0.096g.

Figure 7 shows the mean weight of food ration per week for each of the feeding treatments and shows a gradual increase in weight in correlation with the mean weight gain of the different animals, as the feeding ration for each treatment is calculated as a percentage of the overall weight of the Pāua (1%, 2%, 5% of the total body weight).

A one-way ANOVA test resulted in significant differences among treatments ($F=2, 15=4.59$; Tukey test $p = <0.05$).

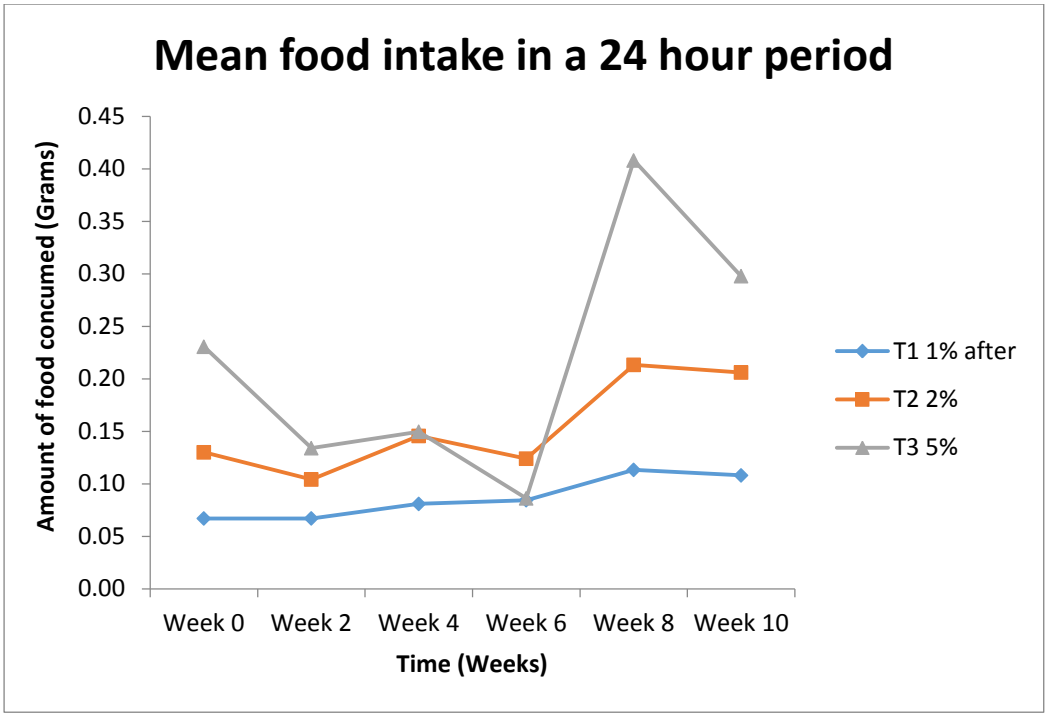


**Figure 13: displays the mean Intake rate or amount of food consumed by Pāua in the three feeding treatments (T1%, T2%, T5%) in a 24 hour period as they progressed through the 10 weeks.**

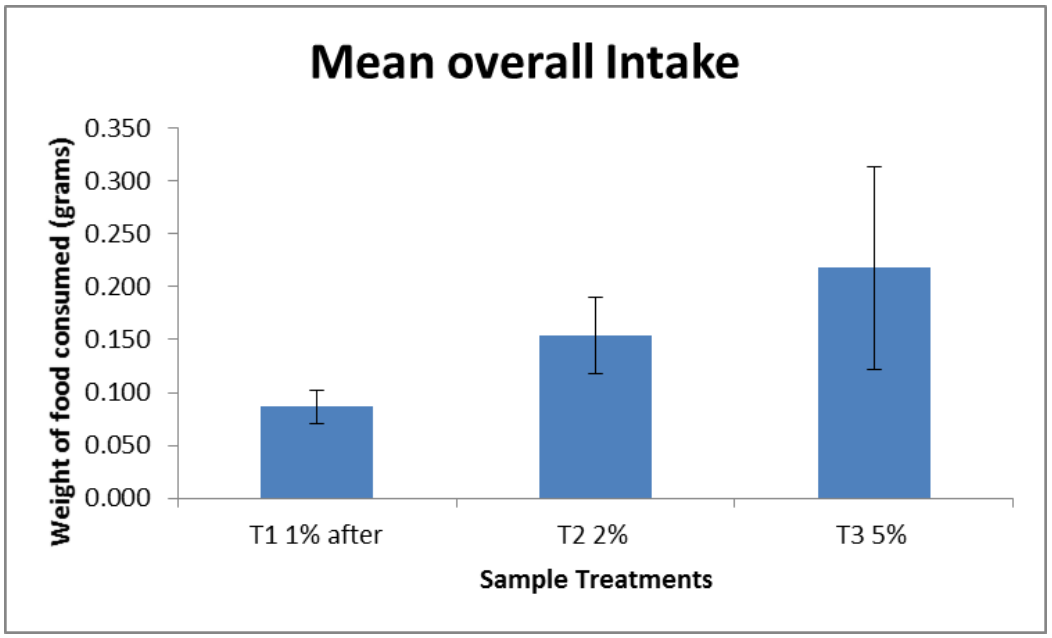


**Figure 14: shows the overall mean Food Intake exhibited by the individual treatments (T1%,T2%, T5%) and compares them to isolate which one produces the most optimal feeding rate within a 24 hour period**

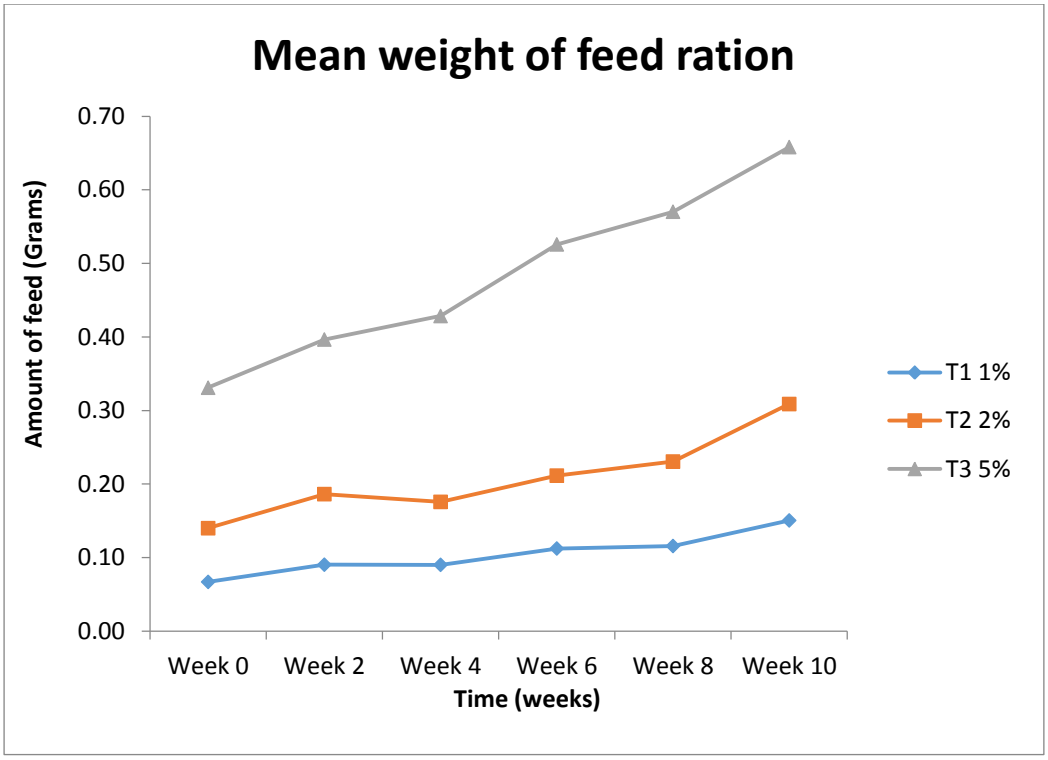


**Figure 15: displays the mean daily food ration for Pāua in the three feeding treatments (T1%, T2%, T5%) as they progressed through the 10 weeks.**

### 3.1.5 Flip Test

Animals in T1% had a mean flip time of 10.32±2.03s (seconds) at week 0 and improved speed 10.03±3.20s in week 10 shown in Figure 8. T2% had a flip test of 10.96±3.21s in week 0, and slowed to 11.15±2.35s in week 10. T5% had an initial flip time of 14.38±5.36s in week 0 and showed a decrease in flip time by week 10, 13.70±4.46s.

The flip test results showed that there was no significant difference between the treatment groups and time it took to flip back to their starting position as a result of a one-way ANOVA test. ($F=_{2,57}= 1.27$, $p = 0.287$).

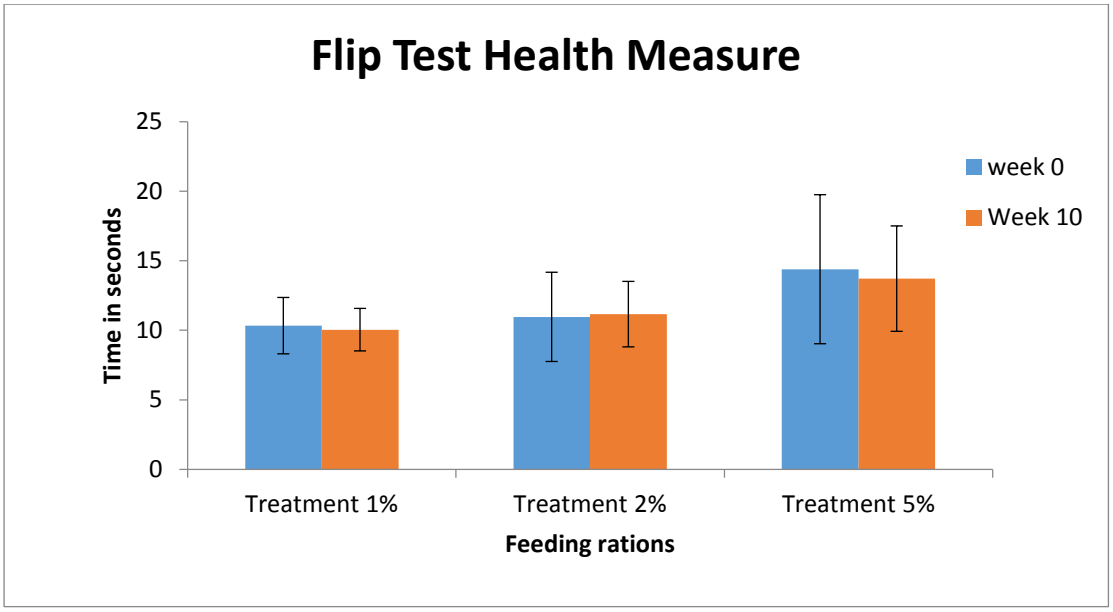


**Figure 16: Shows the mean flipping time exhibited by the Pāua in the three different treatments and compares their responsiveness between week 0 and week 10.**

## 3.2 Bio-Chemical Analysis

### 3.2.1 Moisture test

The amount of moisture in the Pāua tissue at the conclusion of the 10 week trial is shown in Figure 9. T1% had the lowest amount of moisture recorded (53.27±10.57%), T2% contained the most moisture at 69.23±6.08%. T5% contained 63.48±5.68%.

A single factor ANOVA test showed no statistical difference (significant p-value <0.05) between the treatment groups for moisture ($F=_{2, 57} = 23.10$, $p = 4.47$).

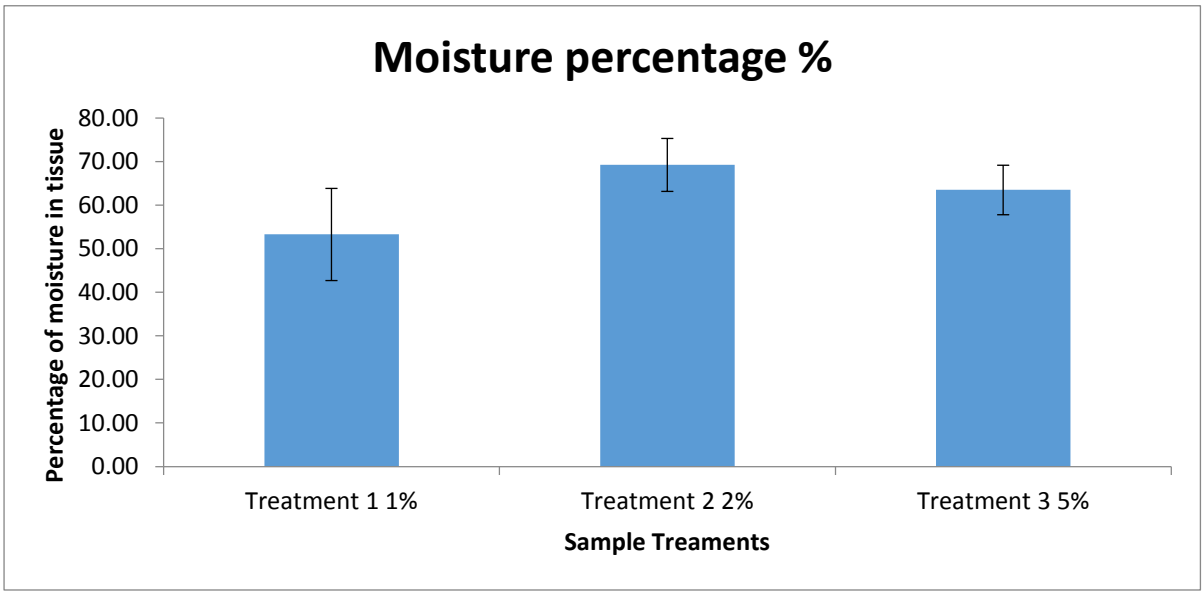


**Figure 17: conveys the mean percentage of moisture removed from the tissue of the Pāua of the three different treatments**

### 3.2.2 Dry Weight

After removing the moisture from the tissue samples, the dry weight was established T1% had a mean dry weight of 2.42grams±0.72, T2% had a mean weight of 2.63grams±0.78, T5% had the heaviest dry weight 2.72grams±0.63 (Figure 10).

The ANOVA test resulted in no significant difference $F(2, 57 = 0.182, p = 0.833 > 0.05)$.

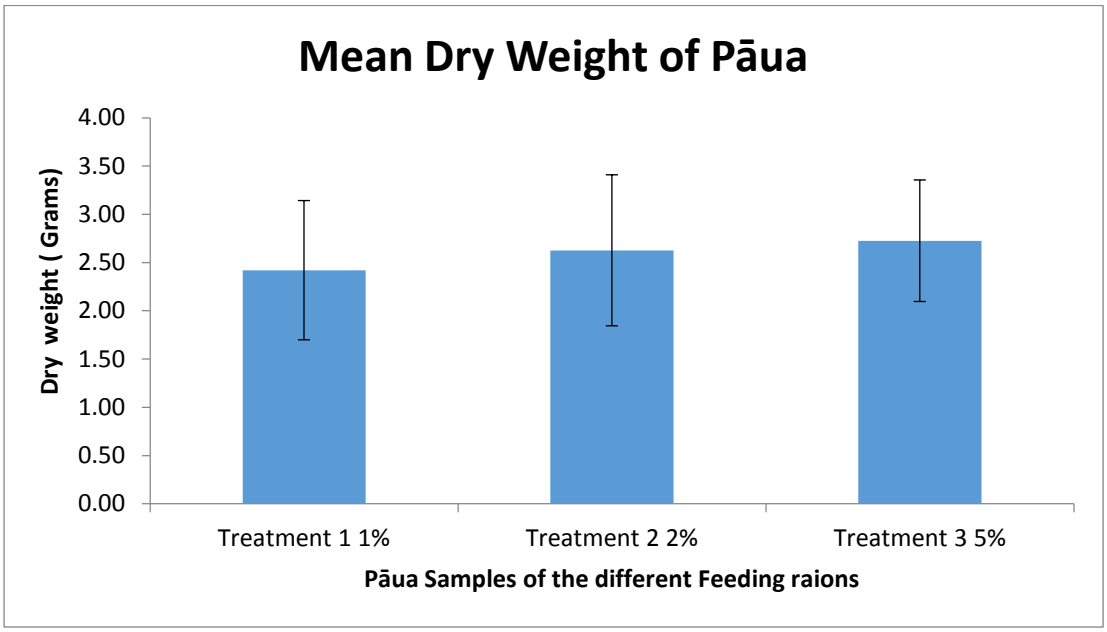


**Figure 18: shows the mean dry weight of Pāua tissue in the three respective treatments after having the moisture removed**

### 3.2.3 Lipid Analysis

The results of the lipid extraction analysis shown in Figure 11 were similar to those of the food intake where an increase in feeding rations among the groups accounted for higher amounts of lipid content (Mean±SR T1% = 0.0775±0.008g, T2% = 0.105±0.033g, T5% = 0.139±0.041g). This is confirmed by the lipid content within the formulated feed which consisted of 0.25 ±0.131g (per gram of tissue).

The single factor ANOVA showed no significant difference F(3,12=3.302, p=0.058 >0.05)

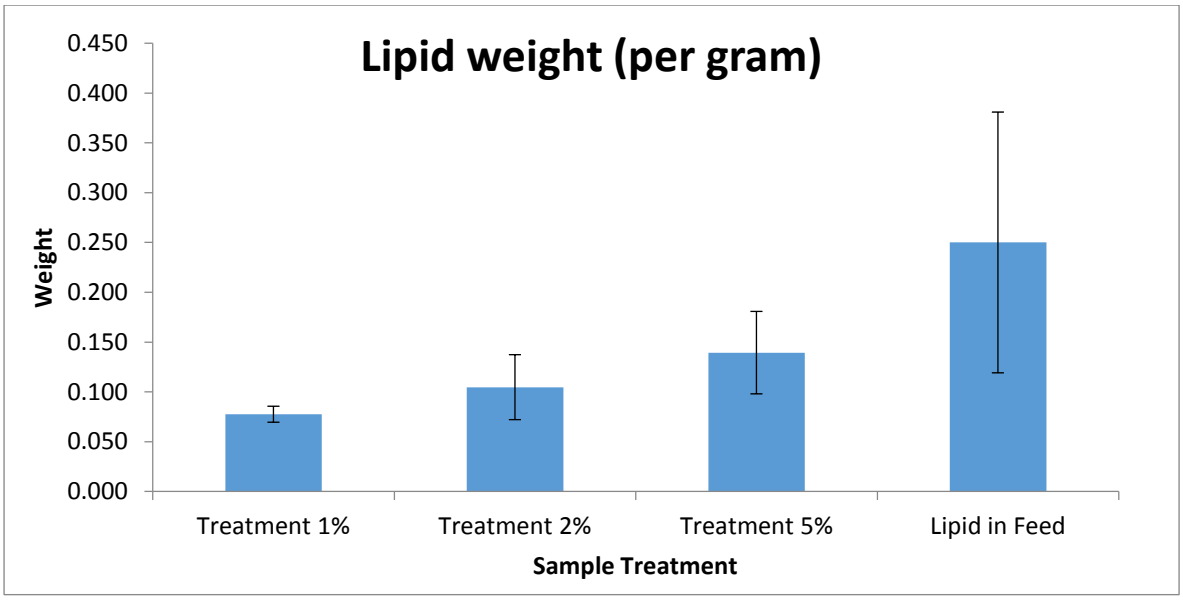


**Figure 19: shows the mean lipid weight per gram of Pāua tissue from each respective treatment, as well as the lipid content of the food.**

### 3.2.4 BCA Protein Analysis

The results of the BCA protein analysis produced protein estimates for T1% that was 1,380µg/100mg of tissue sample which was the highest of the protein readings. T2% 1263.33µg/100mg, T5% consisted of 1330µg/100mg and the formulated feed itself contained a mean of 996.67µg/100mg (Figure 12).

A one-way ANOVA showed no significant difference between the groups and protein amounts $F(3,16=0.549$, $p=0.655$ ($>0.05$).

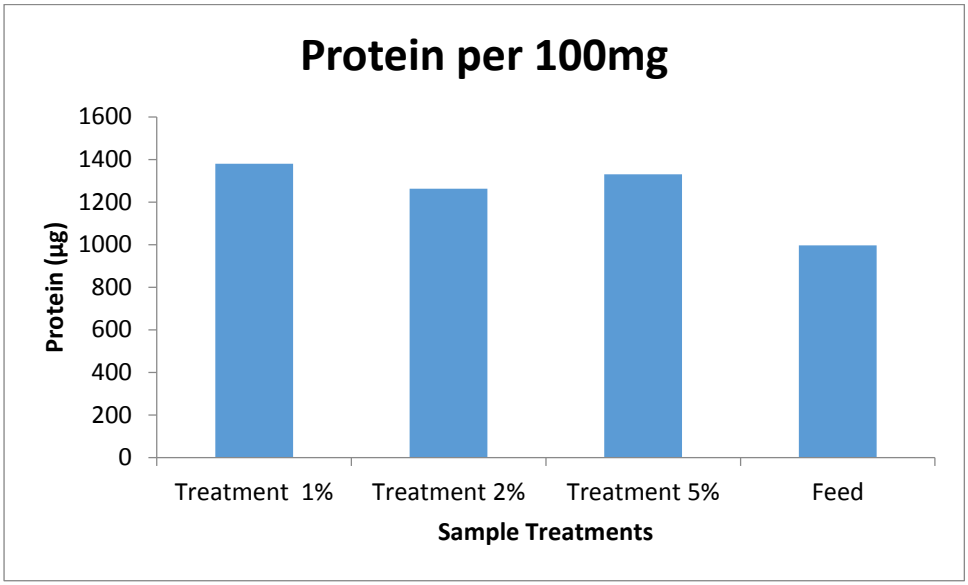


**Figure 20: shows the mean protein weight per 100 milligrams of Pāua tissue from each respective treatment, as well as the protein content of the food. These amounts were calculated from the spectophotometre reading of the BCA protein assay.**

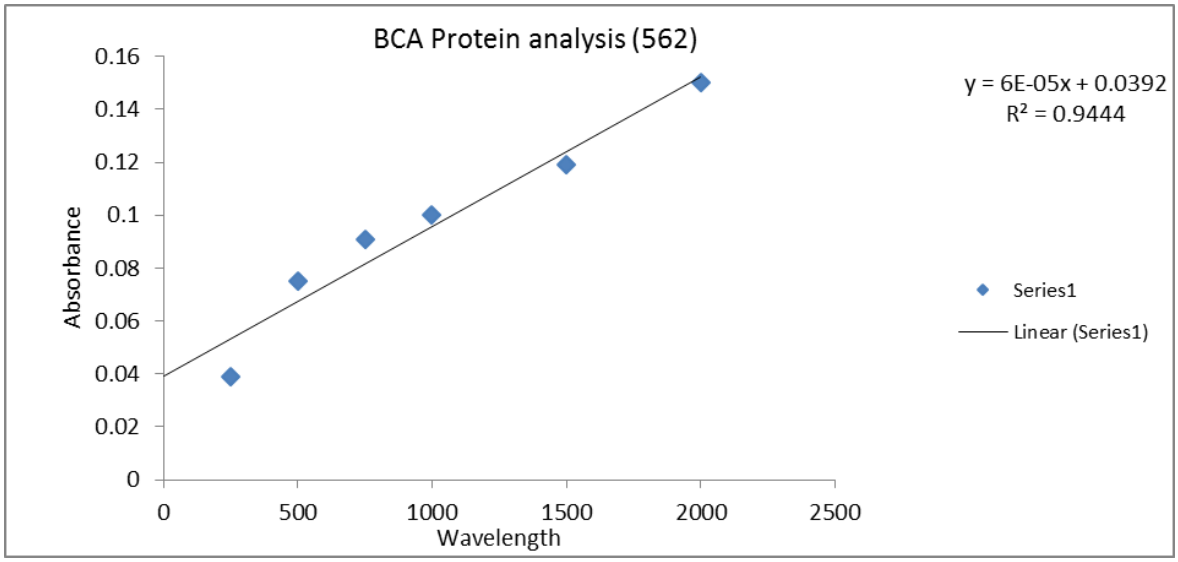


Figure 21: shows the linear progression of protein from the known protein standards, used to identify the unknown protein content of the Pāua tissue samples.

### 3.2.5 Carbohydrates Analysis

As stated in the previous chapter, the carbohydrate analysis was calculated by the sum of percentages of protein, lipids and moisture and the results are shown in Figures 14 - 16. The range of carbohydrates in the Pāua tissue is between 20-40% with 91% of carbohydrates making up the food pellets (Figure 17). Of the three treatment groups, T1% retained the most carbohydrates (41%) and protein (6%).

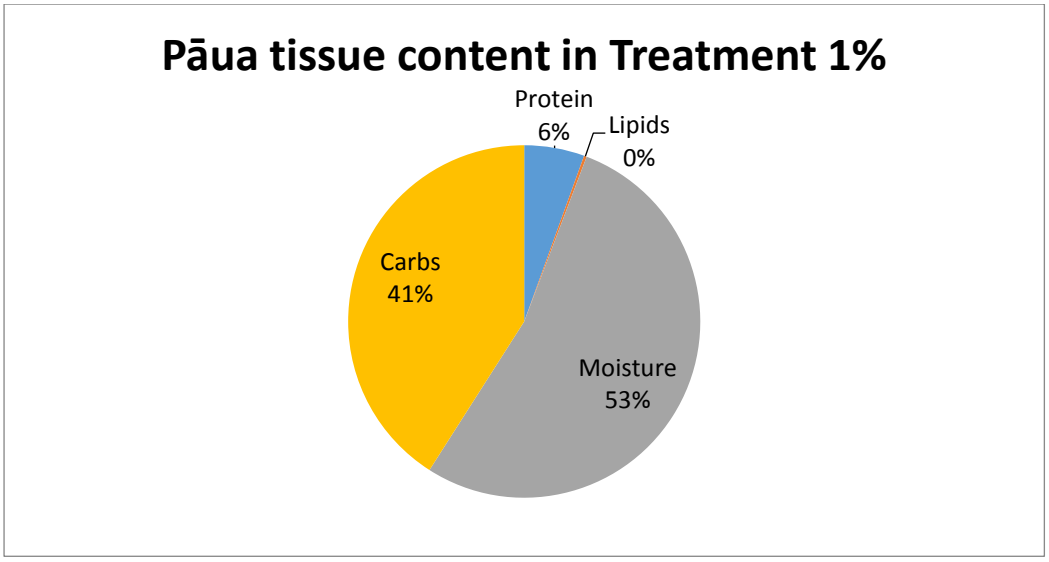


**Figure 22: Shows the combine content of the Pāua in treatment 1% which include moisture, lipids, protein and to highlight the carbohydrate content of tissue content**

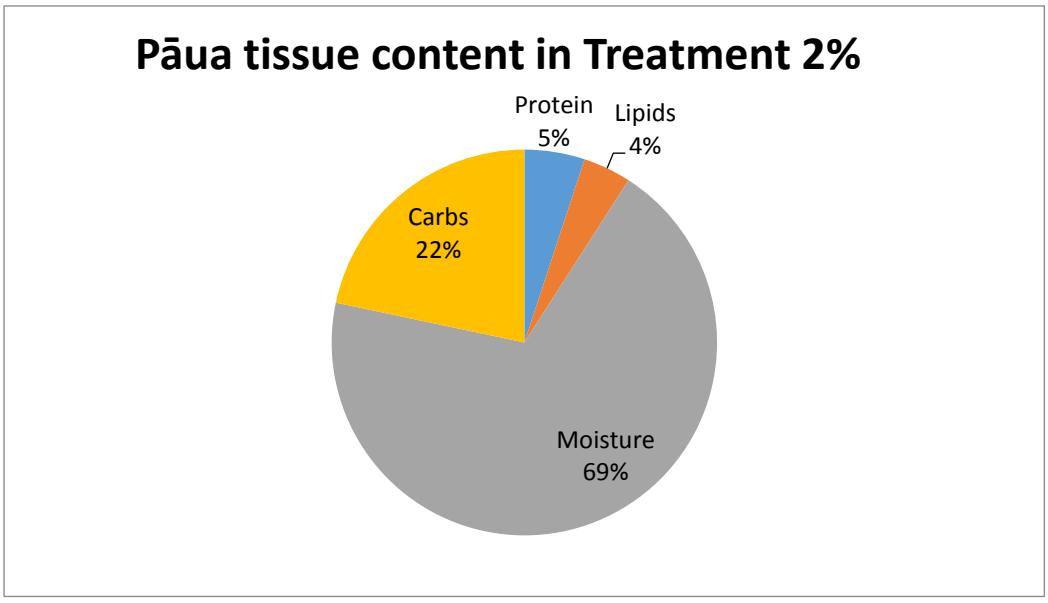


**Figure 23: Shows the combine content of the Pāua in treatment 2% which include moisture, lipids, protein and to highlight the carbohydrate content of tissue content**

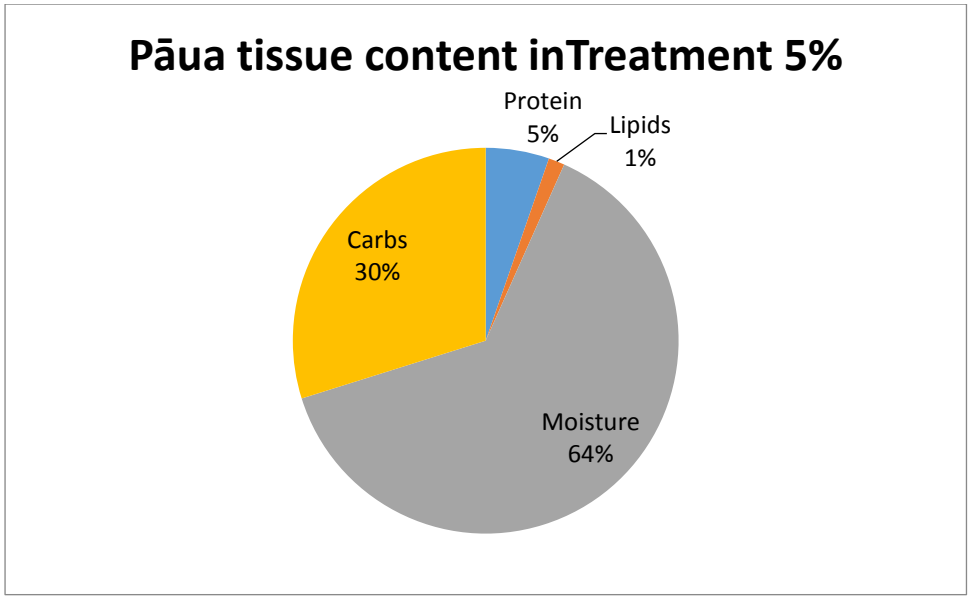


**Figure 24: Shows the combine content of the Pāua in treatment 5% which include moisture, lipids, protein and to highlight the carbohydrate content of tissue content**

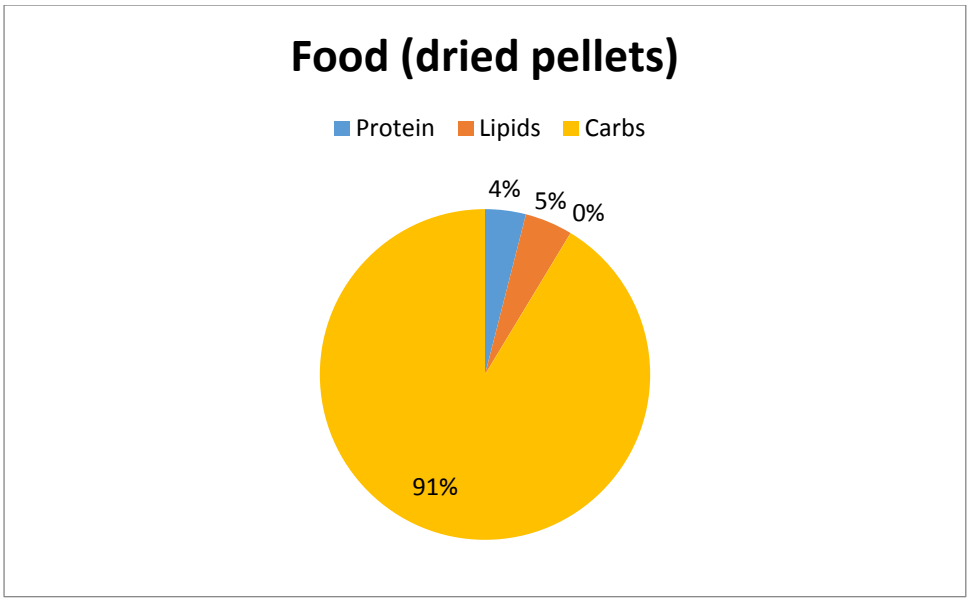


**Figure 25: Shows the combined content of the formulated food pellets which includes moisture, lipids, protein and to highlight the carbohydrate content of food content**

### 3.3 Mortality of Abalone

Forty Pāua were in each treatment for the duration of the 10 week trial. There was 0% mortality for all treatments across the total 160 Pāua (Figure 18).

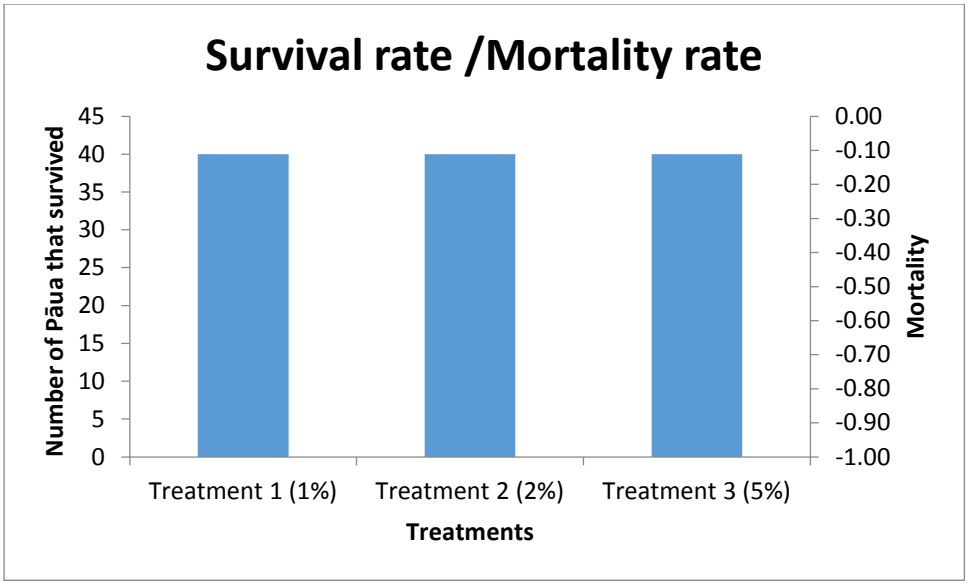


**Figure 26: shows the survival rate compared to the mortality rate over the course of the 10 week trial**

## 3.4 Probiotic vs Non-Probiotic

### 3.4.1 Shell Length

The shell length of the Pāua increased for both T2% probiotic and T2% control group over the 10 week trial. Interestingly, T2% control started at a mean growth of 35.08mm at week 0 and gradually increased every fortnight until week 10 where growth steadied (46.47mm). T2% probiotic showed similar patterns of progression starting week 0 with a mean shell length of 34.25mm to 43.38mm in week 10 (Figure 19).

Figure 20 shows the overall growth gain in both treatments where the 2% non-probiotic grew 11.39±0.84. The 2% probiotic treatment was less effective growing 9.13±0.088.

A one-way ANOVA indicated statistical significance between the probiotic and control groups for shell length ($F_{1,78} = 21.70$, $p=0.00$). This was confirmed by the Tukey Post-hoc test ($p=<0.05$).

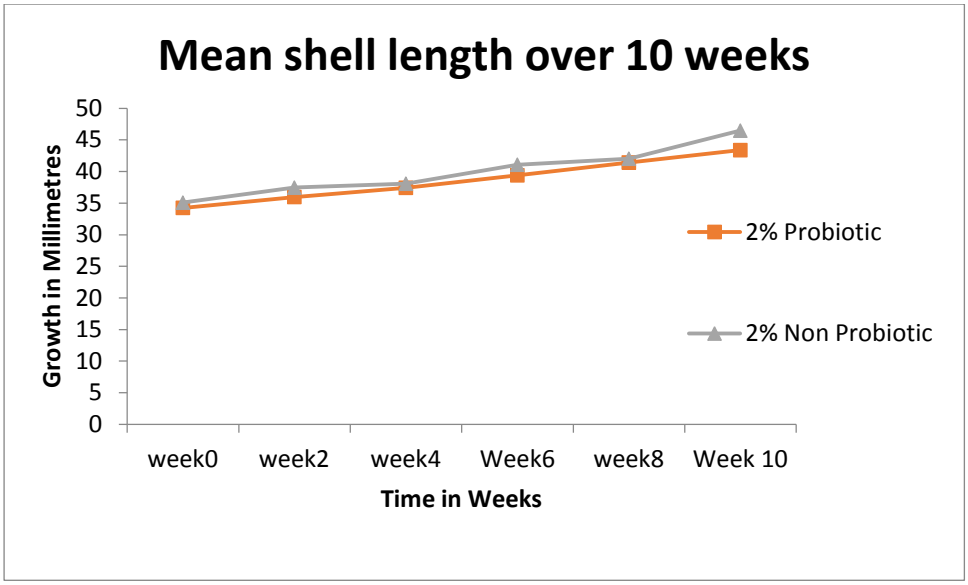


**Figure 27: displays the mean shell length growth of probiotic and non probiotic feeding treatments as they progressed through the 10 week feeding trial. The progressional data was recorded and is displayed fortnightly.**

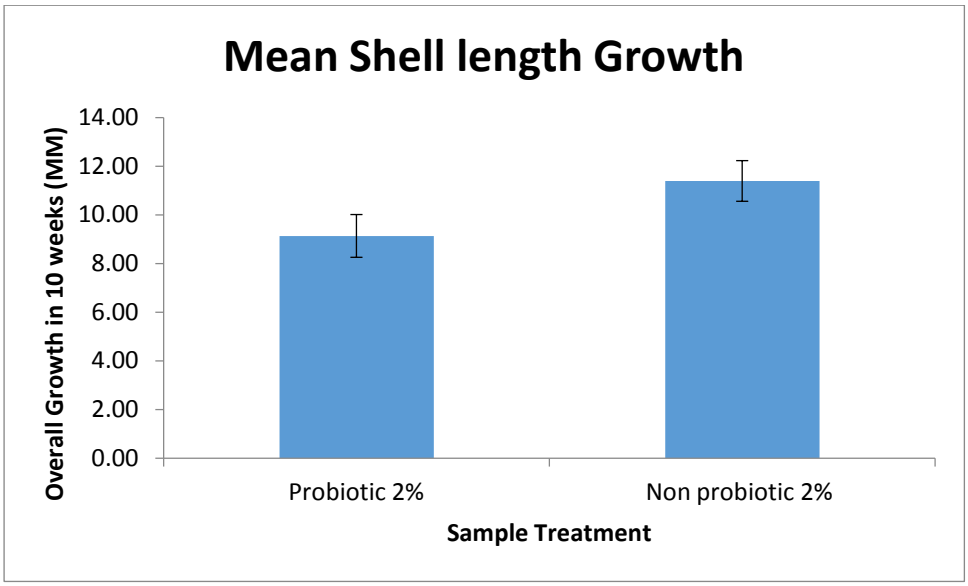


**Figure 28: shows the overall mean shell length growth (in mm) exhibited by the individual treatment (Probiotic 2% and Non-Probiotic 2%)**

### 3.4.2 Weight Gain

As expected, and in agreement with the intake results, Figure 21 shows both groups of Pāua steadily increased in weight from week 1-10. The wet weight growth of T2% probiotic diet was similar linear progression to that of abalone in the control over the course of 10 weeks. T2% Control started at a mean weight of 6.91g at week 0 and gradually increased every fortnight until week 10 where growth steadied (15.82g). T2% showed similar patterns of progression starting week 0 with a mean wet weight of 6.85g to 15.57g in week 10.

Figure 22 shows the overall growth gain in both treatments where the 2% non-probiotic grew 8.91g ±0.49. The 2% probiotic treatment was less effective growing 8.72g ±0.0.62

Furthermore, a one-way ANOVA showed no statistical significance $F(1,78=0.360, p=0.549)$ between the probiotic treatment and control group for weight.

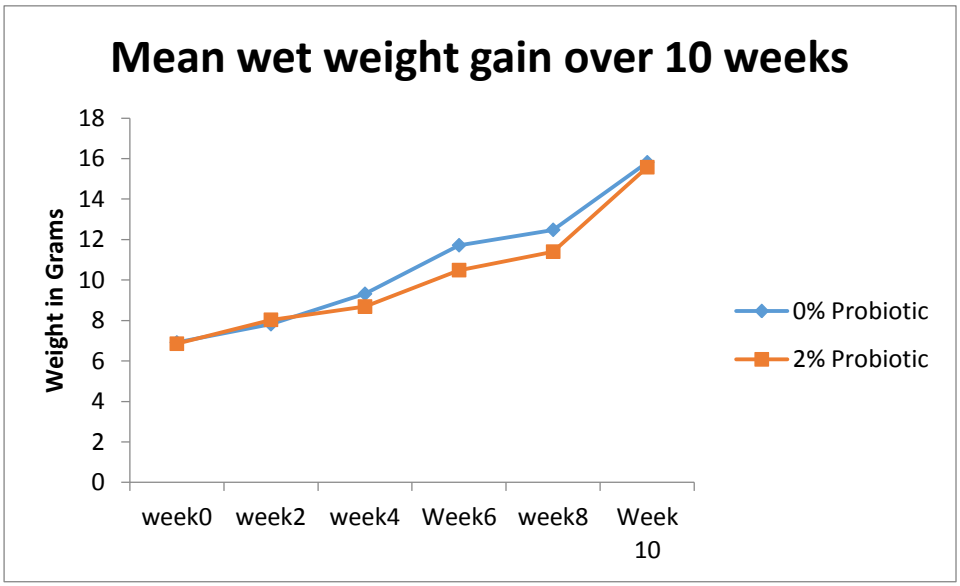


**Figure 29: displays the mean wet weight growth of probiotic and non probiotic feeding treatments as they progressed through the 10 week feeding trial. The progression data was recorded and is displayed fortnightly.**

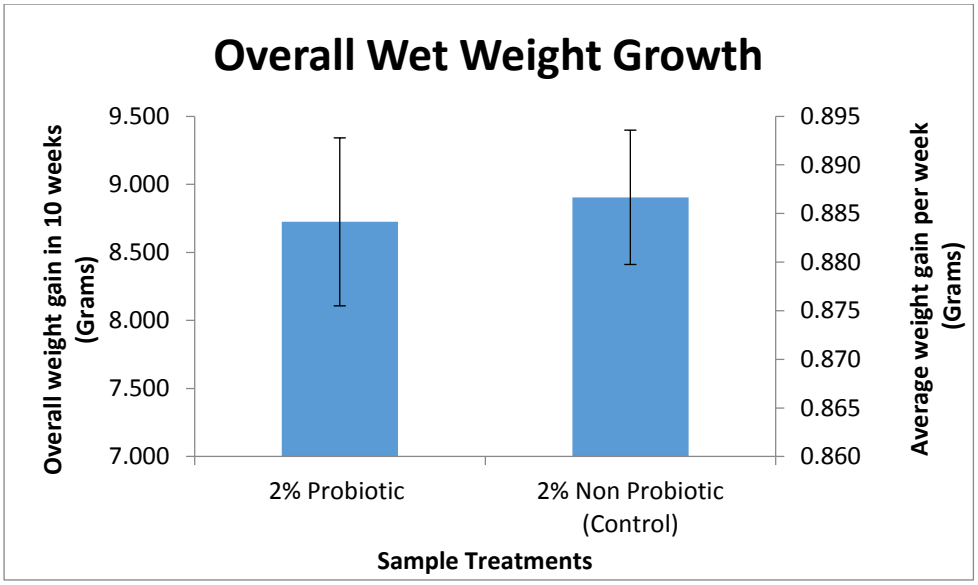


**Figure 30: shows the overall mean wet weight growth (in grams) exhibited by the individual treatment (Probiotic 2% and Non Probiotic 2%).**

### 3.4.3 Intake

Figure 23 shows the food intake for both juvenile Pāua groups was consistent with the overall growth of T2% and T2% Control. Figure 24 shows the mean overall intake of both, T2% control had a mean intake rate of 0.171±0.029g. T2% probiotic had a mean intake of 0.154±0.036g. T2% Control started with a mean food intake of 0.135g and increased to 0.218g by the end of the trial. T2% began with a mean of 0.130g and increased food consumed to 0.218g.

A one-way ANOVA test indicated there were no statistic difference between the probiotic and control diet and intake ($F(1,10)=1.532$, $p=0.997 >0.05$).

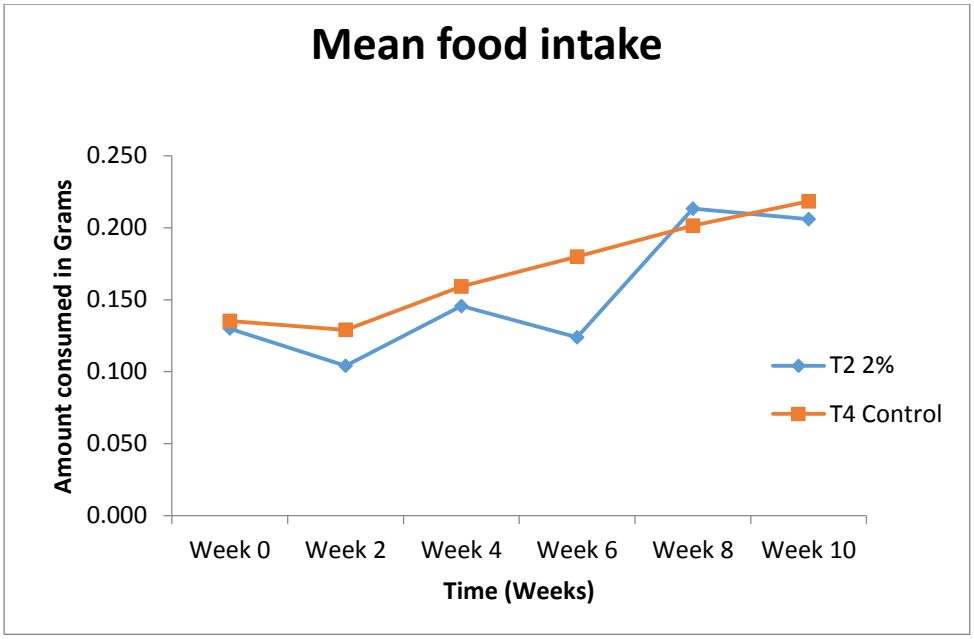


**Figure 31: displays the mean Intake rate or amount of food consumed by Pāua in the 2% Probiotic and 2% Non Probiotic in a 24 hour period as they progressed through the 10 weeks.**

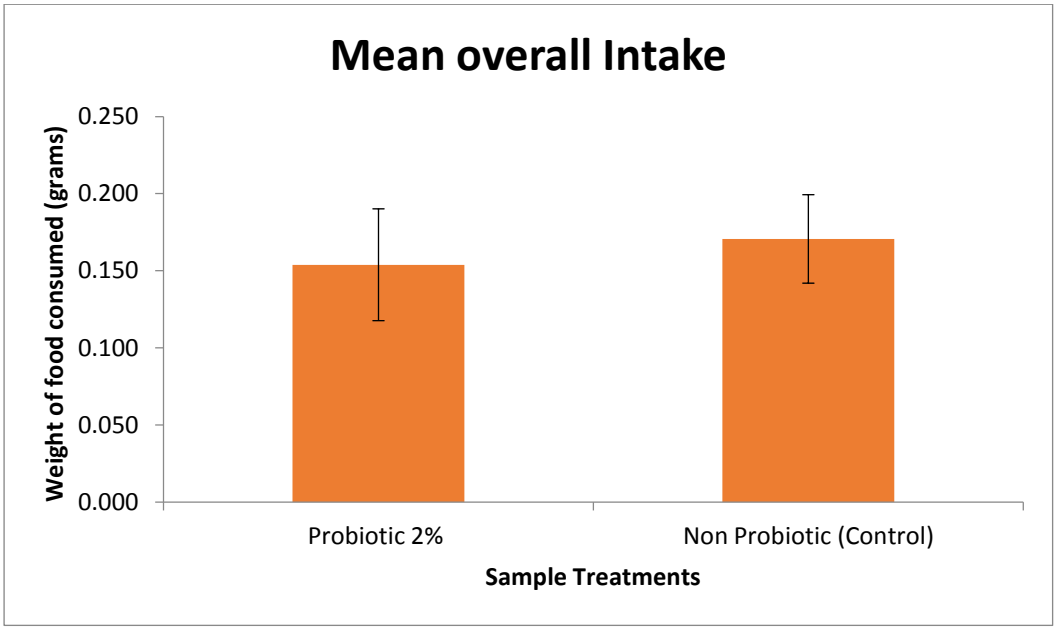


**Figure 32: shows the overall mean Food Intake exhibited by the individual treatments 2% Probiotic and 2% Non Probiotic and compares them to isolate which one produces the most optimal feeding rate within a 24 hour period.**

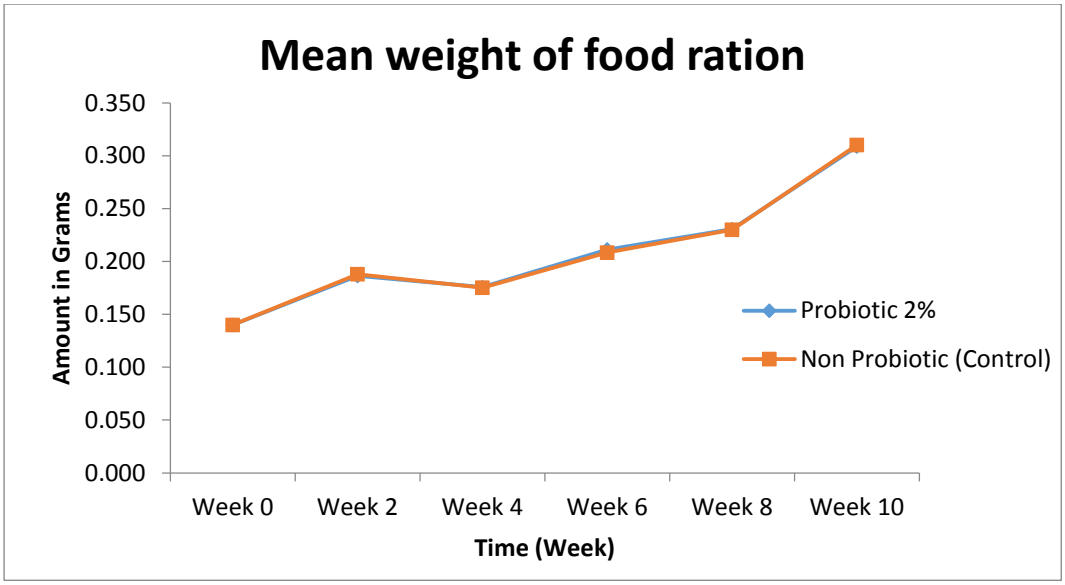


**Figure 33: displays the mean daily food ration for Pāua in the three feeding treatments (T1%, T2%, T5%) as they progressed through the 10 weeks.**

### 3.4.4 Flip Test

The flip test results indicated both groups were marginally faster by the end of the trial (T2% Control, week 0 9.67s±2.92; week 10 9.30s±1.99, T2% Probiotic, week 0 10.96±3.21; week 10 11.15 ±2.35) (Figure 26).

A one-way ANOVA test indicated no significant differences between the groups $F(1,38=0.335, p=0.565, >0.05)$.

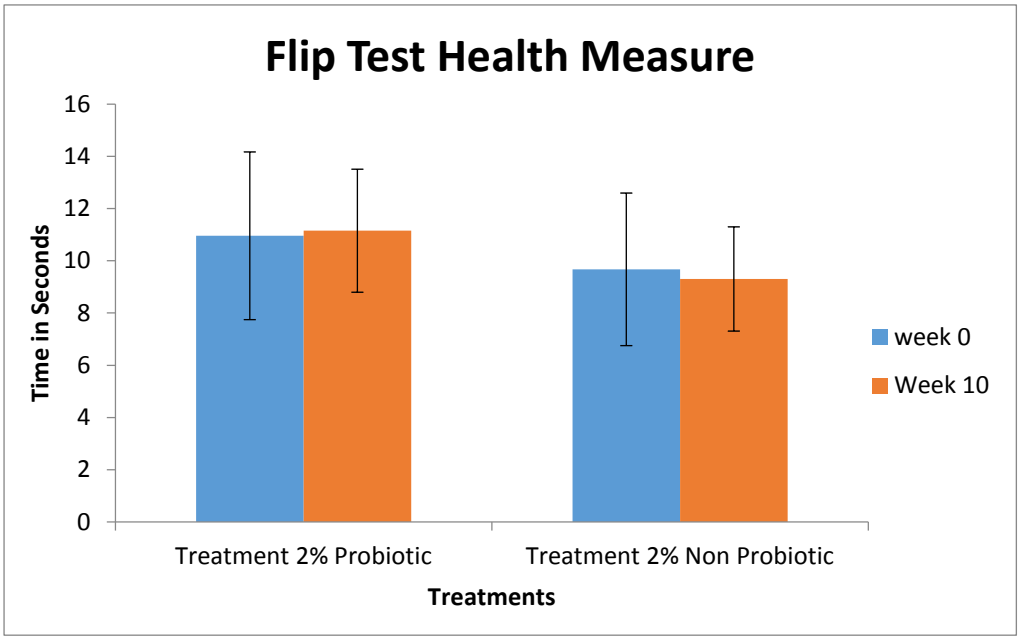


**Figure 34: Shows the mean flipping time exhibited by the Pāua in the Probiotic 2% and Non Probiotic, and compares their responsiveness between week 0 and week 10**

## 3.5 Micro Biological Analysis

### 3.5.1 Moisture

At the conclusion of the 10 week trial, the amount of moisture recorded was T2% probiotic had a moisture content (66.86±8.11%), T2% Control contained the most moisture at 69.23±6.08%.

A single factor ANOVA showed a significant difference ($F=1,38=11.514$, $p=0.01$) between the two groups and level of moisture.

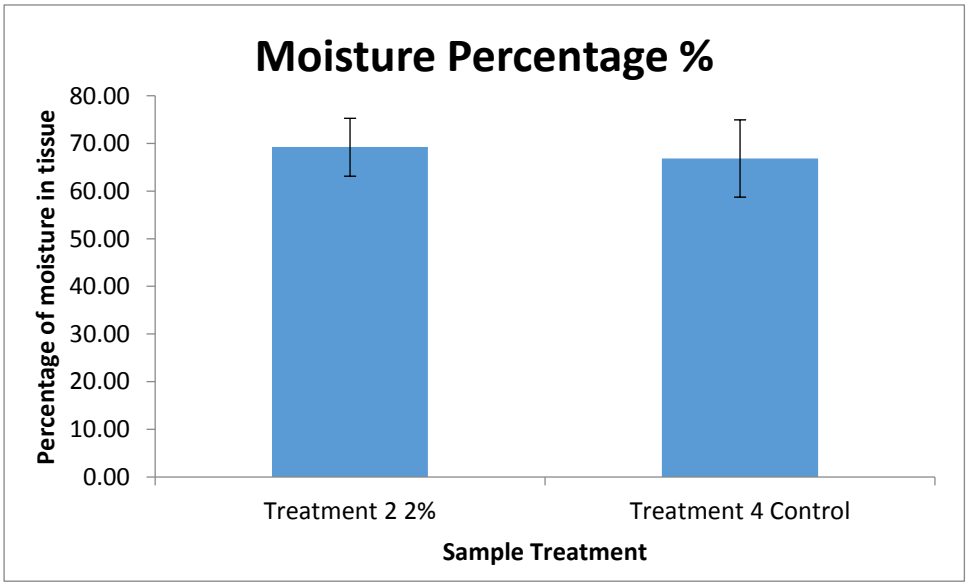


**Figure 35: conveys the mean percentage of moisture removed from the tissue of the Pāua of the 2% Non-probiotic and 2% Probiotic treatments.**

### 3.5.2 Dry Weight

After removing the moisture from the tissue samples, the dry weight was recorded for abalone in the T2% probiotic and control. The results indicate a mean dry weight of 2.63±0.78g for T2% probiotic and T2% non-probiotic had a mean weight of 2.77±0.97g.

The ANOVA test indicated no significant difference between the probiotic and non-probiotic groups ($F=1,38=0.051$, $p=0.821$).

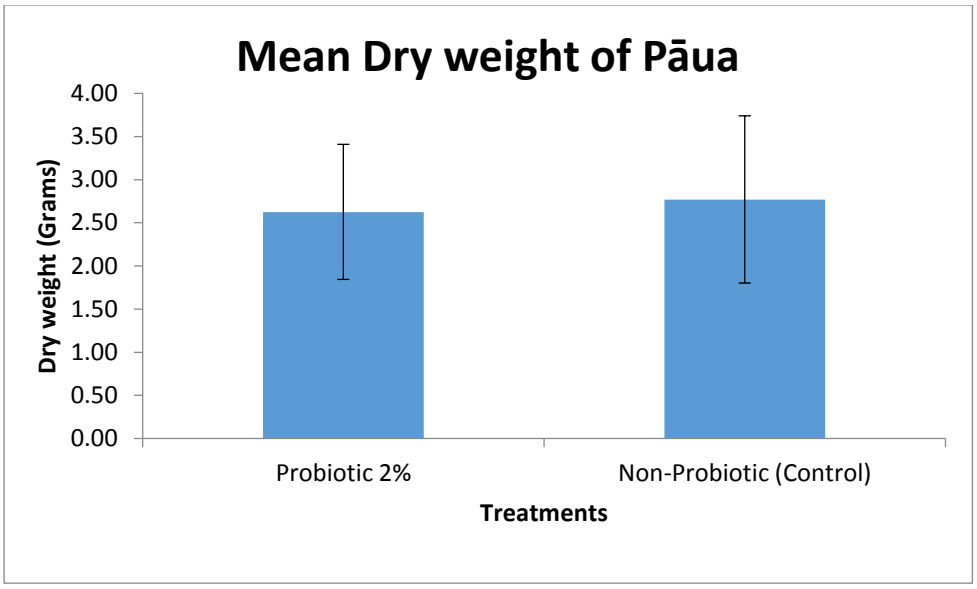


**Figure 36: shows the mean dry weight of Pāua tissue in the 2% Probiotic and Non-Probiotic treatments after having the moisture removed**

### 3.5.3 Lipid Analysis

The results of the lipid extraction analysis (shown in Figure 29) reveal T2% probiotic had a mean content of 0.105±0.003g per gram of sample tissue. T2% non-probiotic had a higher lipid content 0.299±0.09g.

The lipid content within the formulated feed consists of 0.250±0.13g (per gram of tissue). The single factor ANOVA indicated no significant differences between probiotic and non-probiotic groups and lipid content ($F=2,9=3.319$, $p=0.08$).

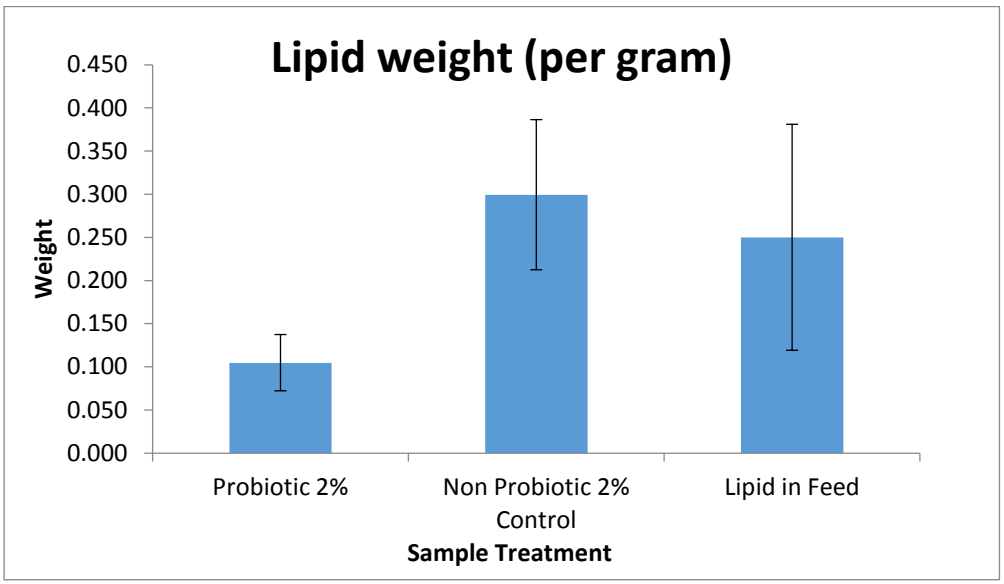


Figure 37: shows the mean lipid weight per gram of Pāua tissue from the 2% probiotic and non probiotic treatment, and the lipid content of the food.

### 3.5.4 BCA Protein Analysis

The BCA protein analysis shown in figure 30 produced protein estimates as expected with a higher protein count as bodyweight treatment increased. The formulated feed contained a mean of 996.67µg/100mg with the higher protein count found within the T5% (1330.33µg/100mg) and lower in the T2% (1263.33µg/100mg).

A one-way ANOVA with BCA protein as the fixed factor showed no statistical difference ($F=1,8=0.032$, $p=0.086$).

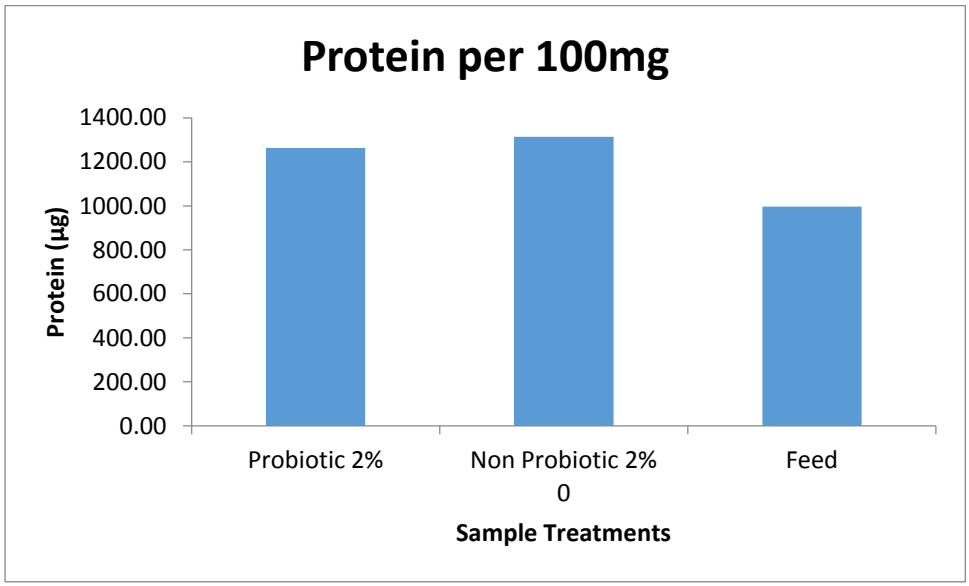


**Figure 38: shows the mean protein weight per 100mg of Pāua tissue from non-probiotic and probiotic treatments, as well as the protein content of the food. These amounts were calculated from the spectophotometre reading of the BCA protein assay.**

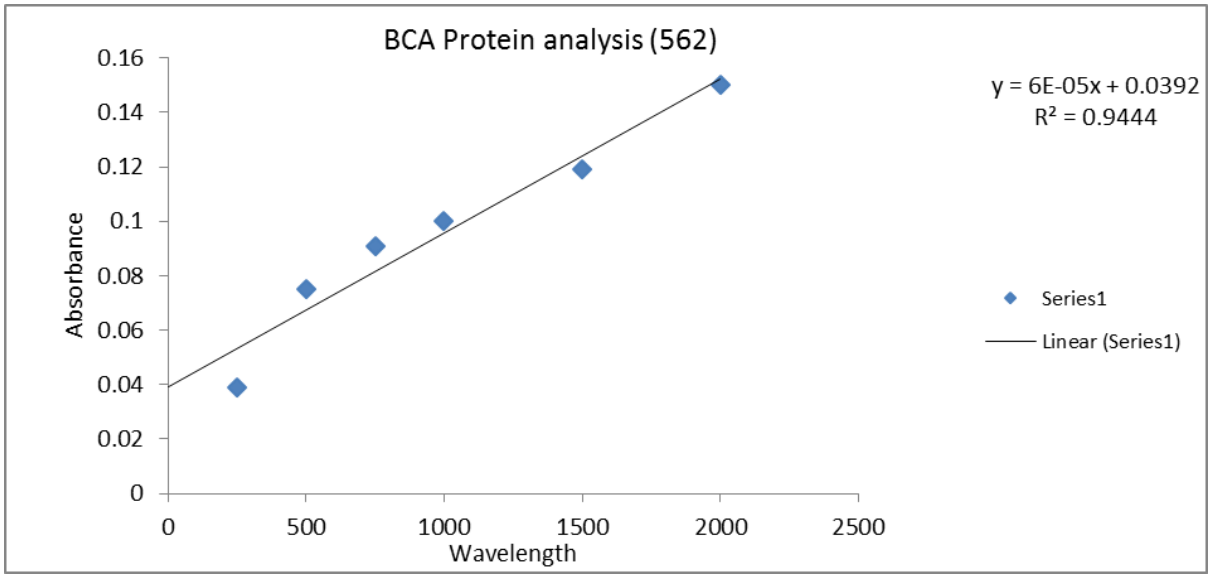


**Figure 39: shows the linear progression of protein from the known protein standards, used to identify the unknown protein content of the Pāua tissue samples.**

### 3.5.5 Carbohydrates

The carbohydrate results indicated both the probiotic (22%) and control (23%) group had a small difference in percentage of carbohydrates. Similarly, both groups had the same protein content and a slight difference in lipids. The results are portrayed below in Figures 32 -34.

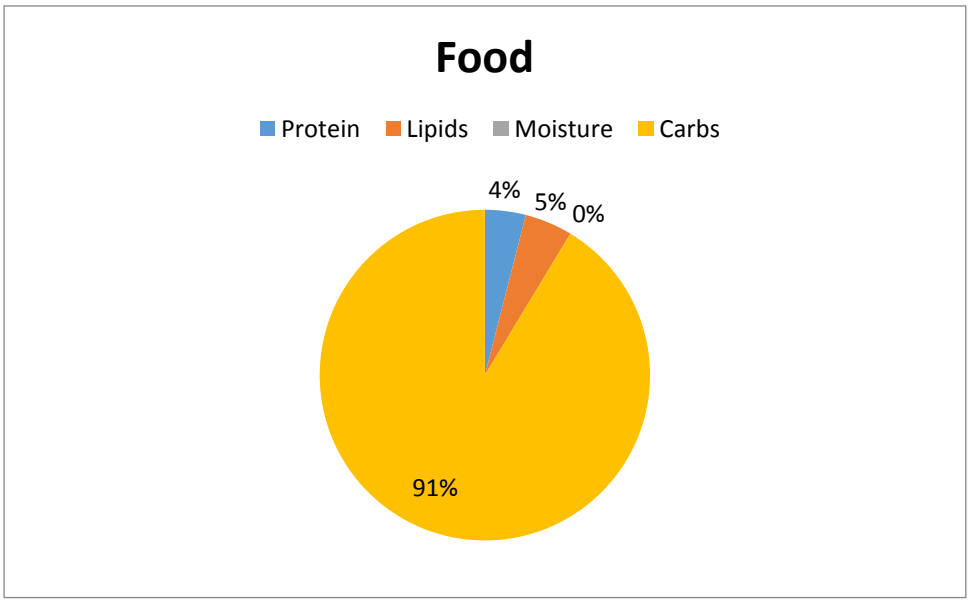


**Figure 40: Shows the combine content of the Pāua in the formulated feed pellets which include moisture, lipids, protein and to highlight the carbohydrate content of tissue content**

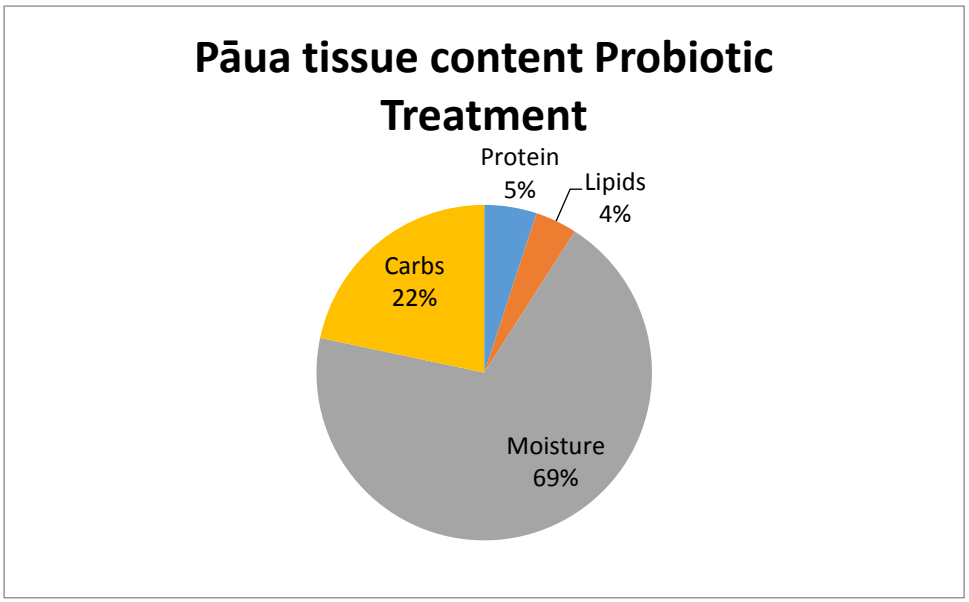


**Figure 41: Shows the combine content of the Pāua in probiotic 2% which includes moisture, lipids, protein and to highlight the carbohydrate content of tissue content**

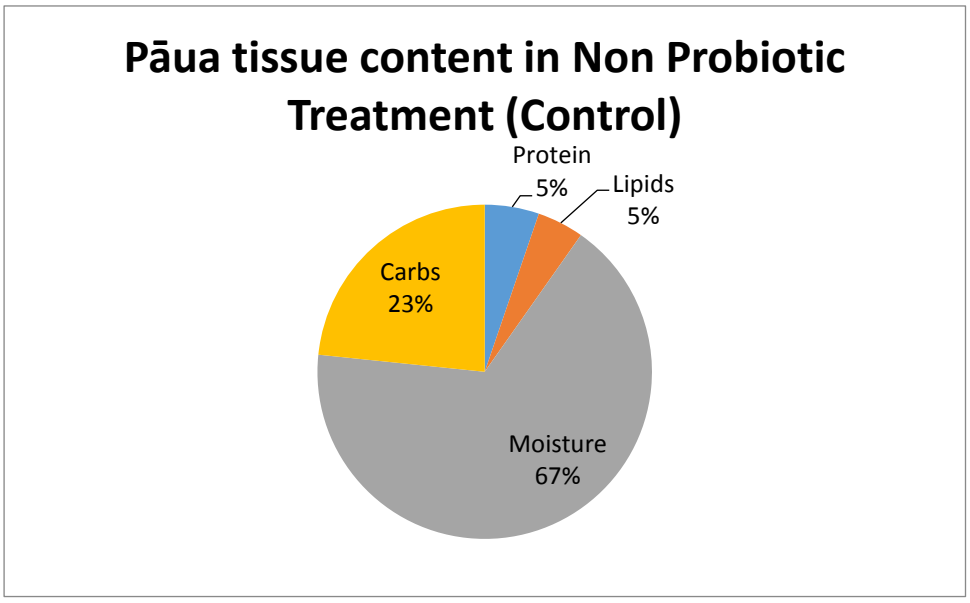


**Figure 42: Shows the combined content of the Pāua in 2% non-probiotic treatment which include moisture, lipids, protein and to highlight the carbohydrate content of tissue content**

## 3.6 Mortality of Abalone

There was a zero percent mortality rate among the Pāua within both the probiotic and non-probiotic treatments.

## 3.7 Bacterial count

The bacterial count in the bacterial count within the feed are within the acceptable range of $10^6$ CFU /g to $10^8$ CFU/g (Table 1). The comparison of the tank water between the Probiotic and Non Probiotic tanks supply showed that bacteria did not leach into the water from the feed, which affirmed the security of the water circulatory systems.

Table 1 show the CFU count of the pro biotic feed and tank water

| CFU Bacterial Count | Feed Bacterial count | Non Probiotic Tank | Probiotic Tank |
|---|---|---|---|
| per/1ml | 2.18 X $10^7$ | 3.56 X $10^4$ | 4.47 X$10^4$ |
| Mean | 21775000.00 | 35600.00 | 44700.00 |
| stdev | 3803452.38 | 1646.55 | 3198.96 |
| Ci | 1666905.87 | 721.62 | 1401.98 |

# Chapter Four: Final Discussions

## 4.1 Interpretation of Results and Findings

A clear gap exists within the aquaculture literature regarding the optimal nutrition for the New Zealand *Haliotis iris*. Thus, the overall goal of this thesis is to investigate and evaluate the effects of probiotic bacteria on the ingestion, digestion, assimilation of formulated diets for Pāua, with the goal to increase growth rates. It is hoped that the findings of this work will contribute to efforts to minimize the cultivation period of *Haliotis iris* and will help reduce cultivation costs by making feeding, digestion and assimilation more efficient.

### 4.1.1 Key Findings of Aim One

Over the 70-day trial, three treatment groups of 1, 2 and 5% of body-weight feeding rations were trialled in a lab-based experiment. Chapter one highlighted the lack of pertinent research in this area, with most studies specific to the study of fish and feeding rations. Although the underlying principles for finding an optimum feeding ration, regardless of species, are the same. The results of aim one indicate that using various feeding rations in aquaculture trials and operations can reveal significant information that can positively or negatively affect the cultivation of aquatic species. Previous research that was conducted mainly on fish revealed the levels of growth, digestibility and utilization of food across the different feeding rations.

The feeding trial confirms as overall body weight increased, as well as the amount of feed ration, so too did the amount of protein consumed. Rowland et al (2005) established similar findings in their study of freshwater silver perch fish, as feed conversion ratio and feeding ration and frequency increased, so did the growth and conversion rate.

The present study data showed growth across all three feeding rations. However, the weight gain of the Pāua were higher in the 2% and 5% rations. With regard to shell length, the 1% and 5% groups increased over the 10 week trial.

The 5% treatment group had the biggest intake of food and highest consumption rate, approximately 0.154g per day. However, this proved to be too excessive as it resulted in wasted food as consumption fluctuated, with 0.218g of the (average) 0.485g being consumed within weeks 2-6 and spiking until week 10. Moreover, the growth data indicates the weight gain of the 5% was significantly less than that in the 1% and 2%.

In contrast, the 1% treatment group data revealed the food intake and ration fed were identical indicating the abalone in this group ate the entire ration (to satiety) and there was no excess. In comparison to the other two treatments this would seem the most efficient. However, when applied with the growth data, this treatment did not produce the best growth (average weight 8.017g).

The 2% treatment group was also efficient with a similar rate for food intake and ration fed. Although it was not always consistent, in week 2 there was a small wastage with a lower food intake. However, in weeks 6, 8, 10 the food consumed indicated the threshold for amount of food that could be eaten without waste. Likewise, when compared with growth the 2% feeding ration produced optimal growth with minimal food wastage.

Based on the results of this study, the ideal optimum feeding ration for *Haliotis iris* is a 2% body weight feed ration. A 2% body weight food ration is enough to sustain the nutritional requirements of Pāua without storing an excess of lipids. *Haliotis* are mollusks which are slow-metabolising animals, and so a change in metabolism can arise as a result of excessive food.

Gomez-Montes et al (2002) stated that abalone consume food to satisfy an energy requirement of 59-67 calories per gram. This reinforces the concept that abalone are not excessive eaters and they do not consume food for long term storage of nutrients.

Previous research suggests there is an increase in dietary lipids inhibits efficiency of metabolism digestion in abalone, low levels of lipases in the digestive gland indicates a limited ability to digest lipids (Green et al, 2011). Moreover, dietary lipid levels of 3-6% reduced energy digestion of nitrogen and amino acids in juveniles (Van Barneveld et al, 1998). This may be attributed to an observed decrease in the condition of soft tissue (Green et al, 2011).

The trial revealed if the abalone were fed too high an amount of lipids, their bodies were unable to metabolise it. Furthermore, past research on various species of *Haliotis* and the role of digestive enzymes observed that abalone had low levels of lipases (Garcia-Esquivel and Felback, 2006) and further, that high levels of lipids may negatively impact the growth of abalone (Thongrod et al, 2003; Viera et al, 2011). The data demonstrated low levels of lipid content in the tissue of abalone across all three feeding ration groups, between 0.24-5%.

Trejchel et al (2013) identified an optimum feeding ration of 2% for juvenile burbot and confirmed by the 100% survival rate and lack of cannibalistic behaviour (known to this species of fish) thereby reinforcing the optimum conditions. The present study increased survival across all three ration groups (with no mortalities) which reinforced healthy animals and optimum conditions, where the food rations were a main determinant.

### 4.1.2 Key Findings of Aim Two

Verschuere et al (2000) posed the question as to whether probiotics were capable of being manipulated. The probiotic trial resulted in growth, both weight and shell length, for the probiotic (an average of 8.72g/9.13mm) and non-probiotic treatment (8.91g/11.39mm). However, as stated in the previous chapter, there was no significant difference between the T2% probiotic and the T2% Control, therefore suggesting probiotics do not have an effect on growth over and above normal conditions. The study by Hadi et al (2014) indicated

successful growth in weight (19.8%; 0.43g) and shell length (20.9%; 4.03mm) for the 3-P multi-strain group, above that of the control group (0.37g/3.33mm). Whilst this is an opposite result to the present study, it is important to note a few factors. Both studies were laboratory-based done over 60-day and 70-day trials and both used probiotic supplemented feed consisting of *Exiguobacterium JHEb1*, *Vibrio JH1* and *Enterococcus JHLDc*. The present study showed significantly higher improvements of growth in weight and shell length with a multi-strain probiotic, supplemented feed of 2% body weight. Whilst the previous study used a 1.1% total body weight ration. In light of the present study, this may suggest that 1.1% is not an adequate feeding ration with probiotic as abalone are possibly underfed and under-utilise the nutrients. Huddy and Coyne (2015) identified the extracellular protease *VmproA* is produced by *Vibrio midae* (probiotic from *Haliotis midae*) in the crop/stomach and intestines of the digestive tract this type of abalone. The *vibrio sp*. used is a similar species (differs by sub-species *Haliotis iris*) used to that in the present study (*Vibrio JH1*) and improved growth and digestibility of protein by increasing the amount of protease activity within the crop gut (Huddy and Coyne, 2015). Growth in the present study may be partially attributed to the *vibrio* strain for these reasons.

Verschuere et al (2000) specified ‘a candidate probiotic should be supplied on a regular basis or be able to colonize and persist in the host or in its ambient environment’ (p667). The non-significant growth results between T2% Probiotic and T2% Control indicate the probiotic is not effective. This may be attributed to the crop/stomach already being colonized with the sufficient bacteria to efficiently digest and ingest nutrients as the probionts in the probiotic compound already having colonized the digestive tract. Hadi et al (2014) identified the current strains as probionts that exist naturally within the digestive system of Pāua.

Probiotics for the present study were fed daily yet still did not result in significantly different growth to the control group. This suggests the probionts may already exist or there is no need for them. Furthermore this may help to understand why the results of Hadi et al (2014) where probiotics were significantly different for growth, are possibly due to the environment of the present study which placed less stress on the animals than the previous study. This suggests abalone that are reared in ideal conditions do not require supplementary probionts to aid their digestive functions. Stocking density (4 Pāua per tank) and feeding rations, dissolved oxygen level (103% maintained throughout) attributed to a higher growth rate. Viera et al (2011) proposed that an increased weight gain and increased protein levels can be linked to culture conditions of an integrated cultural system being of high economic significance for improving production.

The flip test results indicated the Pāua were faster and more responsive in the time it took to flip to their normal position at the end of the trial. No stress on the Pāua is confirmed by the flip test results indicating they were in good health and wellbeing.

The water system and husbandry protocols reflected natural living conditions and resulted in growth across all treatments and zero mortality. Due to the results of the probiotic trial proving non-statistically significant, this thesis concludes that probiotics are unlikely to be successful if used coupled with ideal conditions for the species.

### 4.1.3 Importance of Findings

Over both trials there was a zero percent mortality rate among the animals which may be attributed to the water system design that adhered closely to natural living conditions as opposed to farm conditions. The significance of this paper is not to denounce the use of probiotics in the cultivation of farmed abalone. Rather to suggest situations where it is more beneficial to use them than not. It offers a novel perspective that living conditions of the species such as *haliotis iris* may be a determinant in how well they grow or thrive within the trial.

## 4.2 Scientific Contribution

This thesis adds to the underdeveloped literature around *Haliotis iris* and aquaculture, particularly as it relates to optimal feeding rations and the use of probiotics to further improve growth. By identifying an optimal feeding ration the economic circumstances around food efficiency benefit by limiting food waste. Moreover, the knowledge as it relates to the appropriateness of the use of probiotics and the conditions they can be used, is significant for the cultivation of aquaculture species. These provide tools in further improving feeding efficiency, digestibility and assimilation, and strengthening immune responses, thus further contributing to improving the overall growth rate and survivability of abalone. The ability to explore aquaculture pathways such as new feeding techniques will provide opportunities for the commercial fishing and farming industry to develop tools and techniques such as ‘enhancement programs’ that can increase the sustainability of the abalone fishery within New Zealand. This is an opportunity for the commercial marine industry to increase economic revenue as well as actively acknowledging their roles and responsibilities to provide precautionary steps in the sustainable conservation of marine resources, fisheries, and the Marine Environment for future generations.

### 4.3 Maori Contribution

Pāua are a cultural and ecological keystone species to Māori, in particular to all iwi and hapū who are coastal (McCarthy et al, 2014). Pāua have a very prestigious role in maintaining cultural integrity of Māori not only as a food and economic source of wealth, but also as an artistic and spiritual representation of Māori cultural identity. McCarthy et al (2014) reported that there are many coastal communities who have seen the decline in fisheries stocks such as Pāua which drastically affects their capacity to sustain , manage, to manaaki and tiaki their mahinga kai.

Manaakitanga and Kaitiakitanga of resources such as Pāua requires an extensive knowledge of Pāua in order to be effective as a sustaining a resource for generations to come. At present there is little literature known in Aotearoa that relates to Pāua biology, ecology and aquaculture, that has been developed as a resource to aid Kaitiakitanga and Manaakitanga practices.

'Manaaki' is a compound word which explains the process nurturing caring or rearing a particulat subject. It is defined by the two terms of 'mana' (in this context means life power or energy) and 'akiaki' (encourage or enhance) which means to 'enhance' the 'life energy ', of a particular subject (Marsden & Royal, 2003). The term Kaitiakitanga or more specifically to 'tiaki' is also a compound of 'ti' (tikanga or practices or protocols) and akiaki which translate to the tikanga or actions and protocols put in place in order to enhance the life energy of an entity (Marsden & Royal, 2003). The principal findings of this thesis provides knowledge on the grown of Pāua, specifically as it relates to aquaculture, nevertheless, it is information that can aid the management of Pāua in general. This is essential background knowledge to have in order to provide effective and efficient Kaitiakitanga and Manaakitanga practices.

Aquaculture is a developing industry in Aotearoa that has the potential to extend customary and commercial fisheries capacity and longevity for generation to come. The method and finding of this study can highlight new avenues for aquaculture for organizations such as Rūnanga-a-iwi, and Māori owned organizations like Aotearoa Fisheries and Oceanz Blue in Bream Bay to enable a more holistic approach to how aquaculture can be used. This research also provides valuable insight into Pāua growth and aquaculture provides hapu and iwi with tools which can help produce customary resources such as Pāua by understanding the role that the biology, digestive system, probiotic treatments, husbandry knowledge, nutritional requirements can all play in improving the growth rate and survivability of Pāua.

Additionally this thesis gives valuable knowledge reviews regarding Pāua biology, life cycle, nutrition, husbandry conditions that can be very effective in produce positive enhancement strategies to customary resources such as Pāua, particularly in the enforcement of Regulation 27 of the Fisheries Act 1996. This knowledge will be critical in enable hapū and iwi organizations such as Runanga to incorporate a more holistic knowledge base of Pāua ecology and biology, nutrition and growth into their strategic management of customary Pāua resources, particularly as they relate to Pataka-kai. Aquaculture provides benefit for resource development by creating an alternative stock population and alleviating pressure from commercial recreational and customary harvesting requirements both in the natural environment and in intensive culture.

These fisheries management tools can also be coupled with mechanisms such as taiapure, wahi tapu and rahui, Knowledge relating to the improvement of ecological conditions, nutrition benefits, growth rates , health parameters and living conditions can be used to help iwi better support rejuvenation and sustainable environmental kaupapa such as taiapure and rahui in order to accurately administer appropriate and effective interventions to revitalize depleted stocks of Pataka kai, mahinga kai, rohe moana.

Stock enhancement programs such as that done in Marlborough by Roberts et al (2007) saw the release of hatchery reared Pāua seedlings into the isolated reefs in an effort of replenishing and supplementing naturally recruiting Pāua stocks. This is the type of opportunity that would be able to utilize knowledge of Pāua growth and development as a restorative practice of customary stock. The results showed that at a price of NZ$0.32 per 10 mm SL seed, the survival rate was at a range of 1.3% - 18.6% to harvest. There are obvious risks in survival, however, but aquaculture provides benefit for resource development by creating an alternative stock population and alleviating pressure from commercial recreational and customary harvesting

This Pāua stock enhancement technique, together with the knowledge learnt in this thesis would prove to be an extremely valuable resource in replenishing pataka kai and controlled areas such as rāhui and taiapure. This study suggested that the opportunity of reseeding is will be economically viable if sites and habitat are carefully selected, and there is sufficient resource to cover food requirements. This would be suitable for iwi and hapu in areas such as Te Tai Tokerau as aquaculture development in Northland and particularly the cultivation of black foot abalone (common Pāua), particularly on the eastern coastline, is very viable both for food production and pearl production (Jeffs, 2003, NIWA).

### 4.4 Limitations

Due to the nature of the thesis format and the limited scope of the research, the author acknowledges that ideally longer studies will be more beneficial to comprehensively understand determinants of growth, especially for long-rearing animals like Pāua. The present study was 70 days long, others were 60 days (Hadi et al, 2014) and 168 days respectively (Macey and Coyne, 2002).

Also, should resource and time constraints not be an issue, a wider use of different control groups at different ratios for the probiotic fed groups as the current design of the trials did not allow for an extensive review and analysis of the probiotic at different rations. A lack of resources and facility capabilities meant that the digestive tract could not be evaluated to identify the effect the probiotics had on the naturally occurring enzymes. Should this study be undertaken again this would be the ideal way to analyse the abalone.

Juvenile abalone (20mm) were used in this study however research has indicated that adult abalone (60-70mm length; 60-65grams in weight) have a 33% higher growth rate than juvenile abalone (Huddy and Coyne, 2015).

### 4.5 Future research Avenues and Recommendations

1) Conduct the trial over a longer period of time, preferably 6 months to a year to obtain an accurate record of Pāua feeding patterns, growth spurts and consumption rates. Also to align with the 4-5 year growth of abalone to market size. Also to use different size ranges of abalone that include both juvenile and adults.

2) The food consumption pattern of the 5% treatment group had major fluctuations due to lipid levels slowing metabolism. A longer study would allow to understand whether the feeding rate fluctuation and growth is as a result of the lipase activity and limitations. This could be tested through an in vivo enzyme assay to record the patterns of digestion, food consumption and growth.

3) To conduct in vivo enzyme analysis in the digestive tract to analyse the activity of the protease and lipase and digestibility frequencies of New Zealand Pāua in comparison to other abalone species. Additionally to get a more in depth understanding of how the probiotics affect the activity of these digestive enzymes.

4) To compare the growth of Pāua in the present study with a trial that is supported by a fully closed recirculation water system with an additional AV filter. The husbandry protocols of this system was very time-consuming as water had to be sourced weekly. The benefit of having a water system that is more self-sufficient or has its own water supply would make this trial more economical and user friendly.

5) Undertake more research relating to how the probiotics affect abalone that are of different health levels. This would require conducting a probiotic trial on abalone that are healthy and in ideal conditions and compare to abalone who may have had exposure to pathogens or have diseases; as well as healthy abalone who are in unhealthy conditions such as water with low dissolved oxygen. This would give a good indication of how probiotics react and adapt in various situations and to different health conditions. This may reaffirm the findings of this study and others where probiotics produced different results based on different conditions.

6) Different conditions-such as when abalone are infected with pathogens in order to test for their resistance against pathogen and disease outbreaks. Goosen et al (2014) proposed probiotics which hydrolysed proteins can enhance cellular immune function by stimulating and significantly increasing phagocytic activity. This increases immune-stimulation and haemocytes activity can aid in the assisting towards pathogens.

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