Application of exogenous enzymes in Haliotis midae diets *with soy*bean meal *as fish meal replacement*

By

Christopher Murray de Villiers

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Agriculture (Animal Science)

at

Stellenbosch University

Department of Animal Sciences

Faculty of AgriScience

Supervisor: Lourens De Wet

Co-supervisor: Dr Elsje Pieterse

Date: March 2012

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: March 2012

Abstract

A 240-day growth study was conducted to determine the suitability of soybean meal (SBM) as an alternative protein source to fish meal (FM) in the diet of commercially produced South African abalone (*Haliotis midae)*. The suitability of SBM was determined by a close evaluation of the following key factors: feed water stability, morphological impact on the abalone intestine and the effect on the growth performance of abalone.

The study was comprised of two phases: a fish meal replacement phase (Phase A) and an enzyme treatment phase (Phase B). Diets used in Phase A consisted of a control fish meal diet (Control=22%FM, 0%SBM), a fish meal-soybean meal diet (FMSBM=20%FM, 15%SBM), a soybean meal-low diet (SBMlow=0%FM, 15%SBM) and a soybean meal diet (SBM=0%FM, SBM30%). In Phase B, the FM diet and SBM diet were used as basal diets (FME0 and SBME0). These diets were then treated with three commercial enzyme products, namely, a β- glucanase (FME1 and SBME1), xylanase (FME2 and SBME2) and α-D-galactosidase (FME3 and SBME3). Subsequently, all three enzymes were combined to make two treatments (FME123 and SBME123). With regard to the gut morphology and growth trials, a thirteenth energy enhanced commercial animal protein-free diet (ECO) was used.

In Phase A (fish meal replacement), the findings revealed that water stability did not differ significantly between treatments. In Phase B (enzyme treatment) however, the water stability of β- glucanase treated feeds was significantly lower than that of the control FM diet. It was also observed that in comparison to the control FM diet, soybean meal based diets have a significantly greater effect on intestinal morphology.

With reference to Phase A (fish meal replacement), by the end of the 240 day growth trial period, it was evident that animals fed on the commercial (ECO) diet were significantly heavier than those given the control FM diet. With regard to final length in mm, feed conversion ratio (FCR) and specific growth rate (SGR) for mass and length, no differences between the treatments were noted. It was also found that the condition

of the ECO fed animals was significantly better in comparison to the other treatment fed animals. No significant differences were observed between the FM and three FM-replaced diets however.

With reference to Phase B (enzyme treatment), it was noted that once again, after the 240 day period, abalone fed on the ECO diet were significantly heavier in terms of their final weight when compared to those fed on the other diets. As in Phase A, no differences in FCR and SGR for mass and length were observed. Measurements of the animals' final length (as observed on day 240) revealed that those fed on the ECO diet were significantly longer than those given the FME1, SBME1 and SBME3 diets. At the end of the trial, abalone fed on the ECO diet were also in significantly better condition than those fed on the SBM, FME3 and FME123 diets. In terms of production performance, no significant difference was found between the SBM diets and FM diets and enzyme supplementation did not significantly increase the production performance either. The results of this study therefore show that SBM has great potential to be used as a FM-replacement diet. The improved performance of the ECO diet was expected due to its energy content.

Key Words: extrusion, water stability, gut morphology, growth, feed conversion ratio, specific growth rate, commercial diet

Opsomming

‘n Groeistudie is gedoen met die perlemoen (*Haliotis midae*) oor ’n tydperk van 240 dae om die geskiktheid van sojaboonoliekoek (SBM) as ‘n alternatiewe proteïenbron ter vervanging van vismeel (FM) in die rantsoen te evalueer. Geskiktheid van SBM is getoets aan die hand van waterstabiliteit van voer, morfologie van die spysverteringskanaal en die invloed daarvan op groei van die perlemoen.

Die studie het uit twee fases bestaan naamlik ‘n vismeel (FM) vervangingsfase (Fase A) gevolg deur ‘n ensiem behandelingsfase (Fase B). Die diëte wat gebruik was sluit in ’n Kontrole dieet wat slegs vismeel as proteïenbron bevat (Kontrole = 22%FM, 0%SBM), ‘n 2de dieet wat beide vismeel en sojaboonoliekoekmeel bevat (FMSBM =20%FM, 15% SBM), ‘n 3de dieet wat ‘n lae vlak sojaboonoliekoekmeel bevat (SBMlow =0%FM, 15%SBM) en 4de dieet met ’n hoër sojaboonoliekoek vlak (SBM = 0%FM, 30% SBM).

Die basale diëte van Fase B was dieselfde as die FM en SBM diëte van Fase A (FME0 en SBM0) met die verskil dat dit met kommersiële ensieme behandel is. Die onderskeie behandelings was gedoen met β-glukanase (FME1 en SBME1), xylanase (FME2 en SBME2) en α-D-galactosidase (FME3 en SBME3) asook ‘n kombinasie van die drie ensieme (FME123 en SBME123). ‘n Addisionele behandeling bestaande uit ‘n kommersiële diereproteïenvrye dieet (ECO) is as bygevoeg as kontrole vir die histologie gedeelte van die proef.

Tydens Fase A is gevind dat waterstabiliteit van die onderskeie diëte nie betekenisvol verskil het nie. Tydens Fase B het ensiembehandeling met β-glukanase egter aanleiding gegee tot betekenisvolle laer waterstabiliteit van FME1 en SBME1 diëte in vergelyking met die FM dieet. Histologiese ontledings het getoon dat die SBM diëte ‘n groter negatiewe effek op die morfologie van die spysverteringkanaal gehad het as die kontrole FM dieet.

Fase A het getoon dat die ECO dieet beter groeiresultate opgelewer het as die FM dieet, in terme van liggaamsmassa en kondisiefaktor van die perlemoen. Finale

skulplengte (mm), voeromsetverhouding (VOV) en spesifieke groeitempo (SGT) vir massa en lengte was egter nie betekenisvol verskillend vir enige van die behandelings nie. Geen betekenisvolle verskille is ook gevind tussen die FM en enige van die FM vervangingsdiëte nie.

Resultate vir Fase B het getoon dat diere wat die ECO dieet gevoer is betekenisvol swaarder was as diere wat ander voere gevoer is. Geen betekenisvolle verskille is waargeneem vir VOV en SGT van massa en lengte nie. Finale lengte van die diere wat ECO gevoer is was langer as die van die FME1, SBME1 en SBME3 diëte. Die ECO diere het ook in betekenisvol beter kondisiefaktor vertoon as diere wat SBM, FME3 en FME123 diëte gevoer is. Geen betekenisvolle verskille in produksie parameters is opgemerk tussen die FM en SBM diëte nie en die toevoeging van ensieme het ook nie ‘n betekenisvolle invloed gehad nie. Die gevolgtrekking is dat sojaboonoliekoekmeel suksesvol aangewend kan word vir die vervanging van vismeel in perlemoen diëte.

Sleutel woorde: Ekstrusie, waterstabiliteit, derm-morfologie, groei, voeromsetverhouding, spesifieke groeitempo, kommersiële dieet

ACKNOWLEDGEMENTS

God for salvation and gracious blessings in my life

My supervisors, Lourens De Wet and Dr Elsje Pieterse for their enthusiasm, guidance and support

The Department of Agriculture, Forestry and Fisheries for financial support

THRIP with NutroScience as industrial partner

NutroScience for their provision of ingredients for the experimental diets

Donovan Hawker and Jannie van Aswegen from Novozymes, South Africa for supplying enzymes

Mandi Albas from the Department of Histology, Tygerberg Campus, Stellenbosch University for help with histology

Frik Venter and the staff at Hondeklip Bay for their assistance and support

Desmare Van Zyl, Sanna Van Wyk, Le Daan, Stefan and Amy for assistance in weighing and measuring

Mrs Beverly Ellis and the lab staff at the Department of Animal Science

Paul Reader for supplying reagents

Gail Jordaan for help with statistical analyses

Granny, Dad, Mom, Sara, Amy, Andrew and all cousins and relatives for support and love

To Michelle Tait for help with proof reading and editing

Gys, Greg, Alison, Liz, Warren, Niel, Magdel, John and all other peers who assisted in any way.

LIST OF ABBREVIATIONS

ANF Anti-nutritive factors

ADF Acid detergent fibre

CF Crude Fibre

DGR Daily growth rate

DM Dry matter

FCR Feed conversion ratio

FI Feed intake

FM Fish meal

L_0 Length initially

L_{240} Final length

NDF Neutral detergent fibre

NSP Non-starch polysaccharide

PER Protein efficiency ratio

ROL Rate of loss

SBM Soybean meal

SGR Specific growth rate

SL Shell length

t Time

VFA Volatile fatty acids

W_0 Weight initially

W_{240} Final weight

WG Weight gain

WS Water stability

LYS VAN AFKORTINGS

WS Water stabiliteit

VM Vismeel

SBM Sojaboonmeel

SGT Standaard groietempo

VOV Voeromsettingsverhouding

TABLE OF CONTENTS

List of Tables:

List of Figures:

List of Equations:

Chapter 1

General Introduction

The production of abalone in South Africa has seen a marked increase from less than 100kg in 1996, to over 1000 tons in 2010. Abalone production in South Africa constitutes over 51% of the total aquaculture sector in value, with an estimated market value in excess of two hundred and fifty million rand (Mowlana, 2007; AFASA, 2010).

Due to its good amino acid profile and high digestibility, fish meal has been used as the primary protein source in abalone diets (Fleming *et al*., 1996). An increased demand on limited fish meal resources has resulted in steep increases in the price of this feed. A search for alternative protein sources as a replacement for fish meal is therefore necessary. Soybean meal (SBM) has been the most studied replacement for fish meal in finfish diets (Lin *et al*, 2010). The inclusion of soybean meal in fish diets has, however, been limited due to the presence of various plant based antinutritional factors, non-starch polysaccharides (NSP), oligosaccharides and other antigenic compounds (Lin *et al*, 2010). These antinutritive factors are plants' inherent chemical defence against herbivores. Consequently, they disturb the digestion and/or physiology of these animals. Therefore, significant technological and nutritional challenges are faced when soybean meal is included in the diets of animals (Refstie, 2007).

Enzyme supplementation has also been shown to reduce antinutritive properties contained in soybean meal and other plant-based monogastric animal feed ingredients (Walsh *et al*., 1993). The antinutritive properties found in soybean meal are known to lead to the alteration of gut function as they act as physical barriers to nutrient digestion and absorption (Choct, 1997). Antinutrients in soybean meal are known to cause an inflammatory immune response (enteritis) in the distal gut of salmonoids and other finfish (Sinha *et al*., 2011). The ability of soybean meal based diets to induce enteritis in the gut of other fish species, including abalone, has not yet been reported (Krogdahl *et al*., 2010).

The effect of exogenous enzyme supplementation in monogastric species is well documented, particularly in swine and poultry (Walsh *et al*., 1993; Choct, 1997; Cowieson, 2005; Nadeem *et al*., 2005; Choct, 2006). With regard to the effects of exogenous enzyme supplementation in aquatic animals, however, data remains insufficient. (Choct, 2006). Unique challenges are faced with the inclusion of enzymes in abalone feeds due to unique thermal and physical conditions that occur during the pelleting process (Fleming *et al*.,

1996). A further challenge with regard to effective nutrient delivery is the exposure of pellets to water for extended periods (approximately sixteen hours), as a consequence of the slow feeding behaviour of abalone. Thus, enzyme activity, nutrient leaching, gelatinisation of feed and pellet stability are all important factors that need to be considered in assessing the potential of enzyme supplementation in abalone feeds (Fleming *et al*., 1996; Guzm´an & Viana, 1998).

The aim of the study was to evaluate the effect of enzyme supplementation in diets on feed conversion, growth rate and gut morphology of abalone, as well as to investigate the efficiency of soybean meal as a partial to complete replacement for fish meal. The water stability of feed, specific growth rate (weight and length) and gut morphology were used as references for comparison. Enzyme supplementation was done using three individual enzymes (β-glucanase, α-galactosidase and xylanase) as well as a combination of the three enzymes based on the recommendation by Fourij (2007).

Literature on the histology and physiology of the gut of abalone is limited (Knauer *et al*., 1996; Serviere-Zaragoza *et al*., 1997), and a better understanding of the abalone intestinal tract is necessary to help increase current knowledge and development in abalone nutrition. Although soybean meal seems to be one of the most promising fish meal replacements in monogastric diets (Choct *et al*., 2010; Lim *et al*., 2010), little is known about its impact on gut morphology in abalone, hence the inclusion of investigating digestive tract morphology in this study's analyses.

The effect of fish meal-replacement and enzyme supplementation on water stability was investigated to see whether water stability would be negatively impacted by fish meal-replacement. The effect of these diets on intestinal morphology was also investigated to see how their morphological impacts compared to those of a control fish meal diet. Lastly, a growth trial was conducted to evaluate production performance parameters of fish meal-replaced and enzyme treated diets, when compared to a control fish meal diet. These studies were deemed necessary to create a holistic view of fish meal-replacement and enzyme treatment to assess their potential in abalone diet.

Chapter 2

Literature review

2.1 Current state of nutrition in abalone culture

In 2009, the global abalone harvest was estimated at over 49000 tons, with abalone culture being responsible for 70% of this figure (FAO, 2012). South Africa has become the largest producer of abalone outside of Asia, with the culture of *Haliotis midae* reaching over 1000 tons in 2010 (Troell *et al*., 2006; Mowlana, 2007; AFASA, 2010).

The development of abalone farming as a globally competitive industry has resulted in greater emphasis being placed on feed related research and development and the application of animal feed science principles within this sector (Troell *et al*., 2006). In the 1990's seaweed was still the primary feed source for South African abalone culture. Britz (1995) noted that if abalone culture was to be developed as a sustainable industry, an artificial diet in the form of a pelleted feed would need to be developed. He noted that although kelp and other seaweeds are a cheaper feed source than pelleted feeds, greater economic benefit could be achieved when pelleted feeds were used. This is mainly due to the cost benefits achieved through the use of pelleted feeds, such as lower feed conversion ratios, faster growth and hence shorter production cycles. The practicality of using pelleted feeds and the ease with which they may be managed and stored also give them an advantage over kelp based diets (Britz, 1996). Due to its good amino acid profile and digestibility, fish meal has been used as the primary protein source in abalone diets (Drew *et al*., 2007).

2.2 Protein sources in aqua feeds

2.2.1 Animal Source

Fish meal is widely considered as the primary protein source of commercially produced feeds for carnivorous fish species (Drew *et al*., 2007). This is due to its excellent amino acid profile, high amino acid digestibility, essential fatty acid composition, presence of vitamins and other unidentified growth factors (Dersjant-Li, 2002; Amaya *et al*., 2006). Increased demand for this resource along with a limited supply has led to an increase in fish meal prices (Cook & Gordon, 2010). It is a concern that if the demand for this resource continuous to rise as predicted, it will soon exceed the supply, resulting in extreme economic and ecological pressure (Guzm´an & Viana, 1998; Pratoomyot *et al*., 2010). This

has led to efforts to replace or substitute the use of fishmeal in fish feeds with alternative animal or plant based protein sources (Fleming *et al*., 1996; Dersjant-Li, 2002; Cremer, 2004; Lunger *et al*., 2005; Amaya *et al*., 2006; Drew *et al*., 2007; Pratoomyot *et al*., 2010; Sinha *et al*., 2011).

2.2.2 Plant sources

Certain plant proteins such as soybean meal, sunflower meal, canola, lupines and cottonseed meal appear to translate into good growth and have good apparent protein digestibilities compared to fish meal (Sales & Britz, 2001; Lunger *et al*., 2005; Amaya *et al*., 2006). Soybean meal has a high protein content and relatively well balanced amino acid (AA) profile making it a prime choice for fish meal replacement in finfish diets (Sinha *et al*., 2011).

However, the use of soybean meal in aqua feeds is limited due to the imbalance in amino acids, especially methionine and lysine as well as the many anti-nutritional factors it contains (Pratoomyot *et al*., 2010). Fishmeal, soybean meal and casein are known protein sources in abalone diets, with fishmeal being the main source. The inclusion levels of other sources tend to vary according to commodity prices (Shipton, 1999).

Plant based ingredients generally contain more than one antinutrient factor and thus it is difficult to identify a single factor as the sole cause of an adverse effect observed in an ingredient when fed to fish. Most antinutrients do not lead to mortalities in fish but result in decreased productivity (Francis *et al*., 2001). High inclusion rates of plant proteins have been shown to have a negative effect on intestinal morphology and physiology i.e. inflammation of the intestinal wall, widening and shortening of mucosal folds and disruption of the intestinal membrane (Krogdahl *et al*., 2010) in many finfish species including Atlantic salmon (*Salmo salar*), rainbow trout (*Onchorynchus mykiss*), chinook salmon (*Oncorhynchus tshawytscha*) and carp (*Cyprinus carpio*), with the effect being more pronounced in carnivorous species (Francis *et al*., 2001; Drew *et al*., 2007). Amaya *et al.* (2006) reported that fish meal can be completely replaced in shrimp diets with no adverse effects observed on production (Amaya *et al*., 2006).

The success achieved with fish meal replacement in the feeds of various other aquaculture species has prompted the question as to how abalone will respond to the use of higher levels of plant protein in the diet as an alternative to fish meal. **Error! Reference source not found.** presents a summary of reports on the use of soybean meal as a replacement for fishmeal in the diets of aquatic species.

Table 2.1 Research conducted on soybean meal (SBM) as fishmeal replacement in diets of aquatic species.

Species	SBM as fishmeal replacement (g/100g)	Reference
Tiger puffers	30	Lim *et al*., 2010
Atlantic\Chinook\Coho Salmon	0-20	Francis *et al*., 2001; O'Keef, 2003; Pratoomyot *et al*., 2010
Shrimp	14.5-100	O'Keef, 2003; Amaya *et al*., 2006
Abalone	15-39	Guzm´an & Viana, 1998; Shipton, 1999
Common carp	12-25	O'Keef, 2003
Rainbow trout	12-17	O'Keef, 2003

O'Keef (2003) showed that inclusion of soybean meal in Atlantic salmon, carp and rainbow trout diets reduced inflammation of the intestine and reduced growth. When compared to a fish meal diet, adverse effects were noted around 30, 25 and 17g/100g soybean meal inclusion. Lim et al., (2010) and Francis et al., (2001) validated the findings of O'Keef (2003), observing similar responses. Production performance of tiger puffers decreased when soybean meal was fed in excess of 30g/100g (Pratoomyot et al., 2010). The production performance of abalone, fed partial fish meal replaced diets, were negatively influenced when fed soybean meal at over 40g/100g inclusion (Guzm´an & Viana, 1998; Shipton, 1999).

2.2.2.1 Soybean meal

Soybean meal is the largest global source of vegetable protein and the most abundant legume seed crop (Gatlin *et al*., 2007). Soybeans contain nearly as much carbohydrates as they do protein (Approximatley 35% carbohydrate and 40% protein), yet the anti-nutritive effects of these carbohydrates have often been over looked (Choct *et al*., 2010).

Soybean meal products are considered to be economical and highly nutritious feeds, with a high crude protein content and balanced amino acid profile when compared to other plant proteins. Soybean meal's main advantages include its high yield and high crude protein (CP), along with its stable supply and competitive cost. Disadvantages include its high level of antinutritional factors and relatively low protein efficiency ratio (PER: gain in body mass in grams divided by protein intake in grams) of 1.60, when compared to fish meal which has a PER of 3.1-3.2. This low PER is primarily due to its low methionine content. Supplementation of soybean meal with1000ppm methionine can increase the PER value to a more acceptable 2.55 (Drew *et al*., 2007).

Nutritional deficiencies of soybean meal further extend to below standard levels of 10 essential amino acids; only cysteine is found at a higher level than required in aquafeeds. Crude fat and ash levels are also found at lower levels in solvent extracted soybean meal. These shortages and imbalances can be resolved with the addition of commercial amino acids and fats. Of much greater concern are the high levels of antinutritive carbohydrates. Trypsin inhibitor, lectins, oligosaccharides (sucrose, raffinose and stachyose), soy antigens, β-mannans and sapponins are all antinutritional factors found in soybean meal (Drew *et al*., 2007). Carbohydrates in soybean meal are mainly present in the form of oligosaccharides. Simple oligosaccharide sugars like sucrose are readily digested by aquatic animals while the more complex sugars like raffinose and stachyose are not digested due to the absence of endogenous α-galactosidases (which are necessary to hydrolyze these complex sugars) (Gatlin, 2003). The antinutritive factor found in soy reduces its potential to be a primary fish meal replacer. Primary antinutritional factors that are harmful to fish and found in feed are presented in **Error! Reference source not found.**.

Table 2.2 Heat stable and heat labile secondary compounds found in feed protein sources that are harmful to fish (Adapted from Drew *et al*, 2007).

Ingredient	Crude protein (g/kg)	Protein efficiency ratio	Heat labile secondary compounds	Heat stable secondary compounds
Fishmeal	500-720	3.1-3.7	None	None
Soybean meal	480	1.60	Trypsin inhibitor, lectins	Saponins, non-starch polysaccharides, phytoestrogens, protein antigens
Canola meal	380	3.29	Myrosinase	Glucosinolates, phytate, tannins, sinapine, phenolic compounds, fibre
Maize	-	-	Trypsin inhibitor, lectins	Phytin, arabinoxylans
Wheat	-	-	-	Arabinoxylans and β-glucans

The effects of these antinutritional factors may be due to their direct interactions with epithelial cells of the intestine or may also be caused by the alteration of the bacterial populations in the gastro intestinal tract. In poultry, pigs and certain finfish (atlantic salmon), this has been well documented, yet in many other aquatic species there is little research regarding these effects (Drew *et al*., 2007).

2.2.2.2 Maize

Maize is the most abundantly produced cereal crop and is the most commonly used cereal grain in commercial poultry systems (Cowieson, 2005). Maize is characterised by antinutritional factors like lectins, trypsin inhibitors and arabinoxylan. Lectins and trypsin inhibitors are heat unstable and will be inactivated during the pelleting process, but arabinoxylans and phytin remain a concern. It may be possible to reduce the levels of these antinutritional factors by enzymatic treatment (Drew *et al*., 2007).

2.2.2.3 Kelp

Kelp is the natural feed of abalone. Kelp contains high levels of complex carbohydrates such as cellulose, fucoidan, agarose, alginate and carrageenan, which are digested by the abalone's endogenous carbohydrases. It is known however, that invertebrate herbivores hydrolyse structural carbohydrates less effectively, and thus the contribution of these carbohydrates to the energy portion of the diet is yet to be quantified (Britz, 1996; Sales & Britz, 2001). Restrictions in kelp harvesting, a poor comparable feed conversion ratio (FCR) and growth rates, when compared to formulated feeds, are the major reasons why kelp based diets are barely relevant in the current context (Troell *et al*., 2006).

The feed supplied to the abalone influences endogenous enzyme activity. Abalone fed on artificial diets with protein inclusions of 25% and 38% exhibit higher endogenous cellulase activity (39.8 ± 4.6 and 14.2 ± 0.8 mU mg^{-1} protein, respectively) than those fed on kelp diets (5.5 ± 0.7 mU mg^{-1} protein). Protease activity however, is higher in the case of kelp diets. The abalone used in this particular trial (*Haliotis rufescens)* showed that they possess the ability to increase their endogenous carbohydrase and protease secretions to maximize protein and carbohydrate secretions. Formulated diets can thus be developed to ensure optimal nutrient absorption, unlike kelp (Garcia-Esquivel & Felbeck, 2006).

2.4 Antinutritional factors in abalone diets

The development of artificial feeds for abalone has exposed the animals to nutrients and substrates that are foreign to endogenous physiological structures and secretions (Britz, 1996). Plant based ingredients are known for their abundance of antinutritional factors (Chesson, 1993; Bedford & Schulze, 1998; Francis *et al*., 2001; Dersjant-Li, 2002; Choct, 2006; Ghoush, 2006; Drew *et al*., 2007). Due to their complex gut structure and endogenous secretions, abalone are able to digest more complex carbohydrates than most other aquatic species. The effects of antinutritional factors in abalone are therefore of interest (Sales, 2004), and remain far from being fully understood. Interactions between

the effects of antinutritional factors seem to play an important role and to complicate matters further, the intestinal micro biota may also modify antinutrients and subsequently alter the observed biological effects (Choct & Kocher, 2001; Francis *et al*., 2001).

2.5 Non-starch polysaccharides as antinutrients

The non-starch polysaccharide content of feeds varies between ingredients as well as between crop varieties and geographical location. The main structural features of the non-starch polysaccharides however, are unaffected by these varietal and environmental factors (Choct, 1997).

Historically non-starch polysaccharides were classified according to the method used for their extraction. Initially, the residue remaining after a series of alkali extractions was called cellulose, and the residues solubilised by these alkali extractions were termed hemicellulose. Another classification based on differences in solubility included three categories, namely, crude fibre (CF), acid detergent fibre (ADF) and neutral detergent fibre (NDF). The remaining plant material after alkali and acid extraction yields CF, which includes some insoluble non-starch polysaccharides. The NDF consists of insoluble non-starch polysaccharides and lignin, whilst ADF contains mostly insoluble non-starch polysaccharides with large portions of cellulose and lignin. It must be noted however that this classification lacks biological and chemical precision and the relevance for these methods in monogastric nutrition is questionable due to their inaccuracies (Sinha *et al*., 2011). Modern classification groups non-starch polysaccharides into three main groups, namely, cellulose, non-cellulose and pectic polysaccharides. Arabinoxylans, β-glucans and mannans are in the category of water soluble non-starch polysaccharides seen in Figure 2.1 (Choct, 1997).

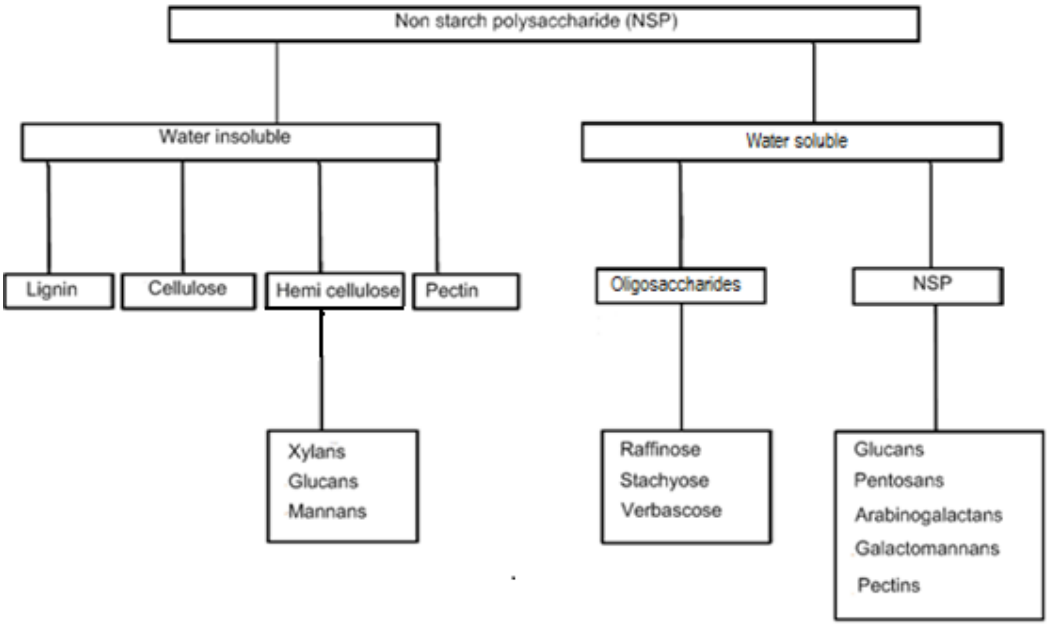


Figure 2.1 Classification of non-starch polysaccharides (Liang, 2000).

2.5.1 Oligosaccharides

Oligosaccharides are found in legumes and cereals and are α- galactosyl derivatives of sucrose (sucrose, raffinose, stacchyose, verbascose and fructose). Raffinose, stacchyose and verbascose are not hydrolysed by endogenous enzymes in monogastrics and therefore offer little if any direct nutritive value. These compounds, for which the structure is presented in **Error! Reference source not found.**.2, are osmotically active as they are water-soluble. They may also cause osmotic diarrhoea, flatulence and may interfere with nutrient digestion (Dersjant-Li, 2002; Krogdahl *et al*., 2010).

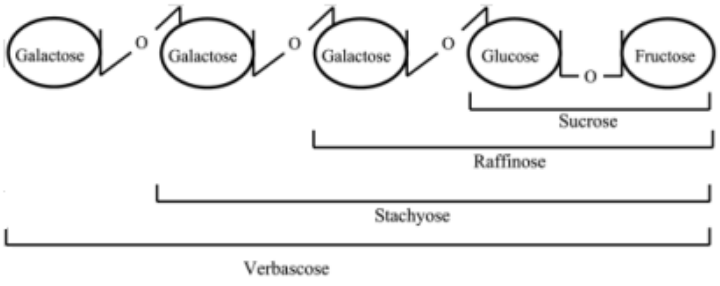


Figure 2.2 Molecular outline of an α-galactosyl homologues (Krogdahl *et al*., 2010).

The role of oligosaccharides in soybean-induced enteritis has been questioned, yet no definitive answer exists to this question (Krogdahl *et al*., 2010). Further negative effects of

oligosaccharides in fish are their ability to bind bile acids, and their ability to obstruct the action of digestive enzymes and movement of substrates in the intestine due to non-starch polysaccharides entrapment of nutrients (Francis *et al*., 2001,Sinha *et al*., 2011).

2.5.2 β - Glucan

β- Glucans are non-starch polysaccharides consisting exclusively of β- (→1-3) and β- (→1-4), glycosidic linkages. The molecular weights and proportion of distribution of these linkages vary considerably however (**Error! Reference source not found.**). These mixed glycosidic linkages render β- glucans more soluble than most non-starch polysaccharides such as cellulose (Walsh *et al.*, 1993). Cellulose and β- glucans are both composed of β-linked glucose units, yet they have very few structural features in common. This is because the β-(→1-3) linkages break the regular structure of the β- (→1-4) linkages, preventing a close packing of the chains and thus resulting in a more soluble molecule (Walsh *et al*., 1993; Choct, 1997).

β- Glucans are abundant in barley (approximatley 3-4 %) and oats and act as antinutritional factors in poultry and pig diets. They are known to increase the viscosity of internal digesta and increase the incidence of sticky droppings in poultry. They are found in the aleuronic layer of the endosperm of barley and oats (Aehle, 2004). The schematic representation of β-glucan is presented in **Error! Reference source not found.**.

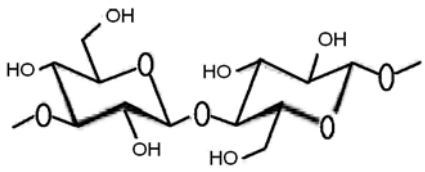


Figure 2.3 Schematic β-glucan structure (Aehle,2004).

2.5.3 Arabinoxylans

The structure of cereal arabinoxylans is comprised of mainly two pentoses, namely, arabinose and xylose (**Error! Reference source not found.**). Most arabinoxylans in cereal grains are insoluble in water since they are anchored to the cell wall by ester-like cross links. However, arabinoxylans not bound to the cell walls can form highly viscous solutions that are capable of absorbing up to 10 times their weight in water (Choct, 2002). In the presence of oxidative agents like peroxidase, arabinoxylans rapidly develop a gel network, caused by the establishment of cross-links. These cross-links result in the formation of a viscous matrix which decreases the transit time of the intestinal digesta.

Apart from covalent cross-links, arabinoxylans can form junctions by bonding between regions of the xylan backbone (Sinha *et al*., 2011).

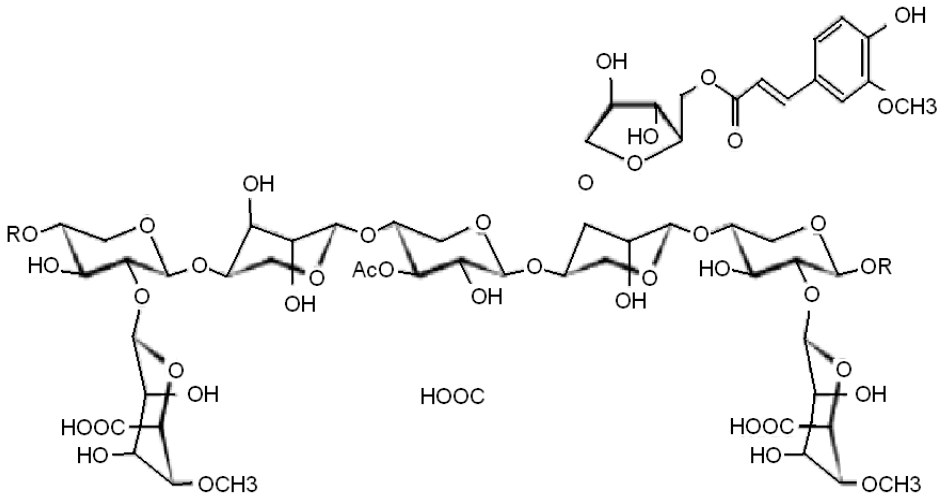


Figure 2.4 Schematic arabinoxylan structure (Aehle,2004).

2.6 Effects of non-starch polysaccharides in monogastrics

Soybean meal, maize, wheat, sunflower meal, canola, lupins and other plant ingredients are known for their non-starch polysaccharide content. The soluble non-starch polysaccharides they contain act as physical barriers to nutrient digestion and absorption (Bedford & Schulze, 1998; Choct, 2002). The combined effects of the non-starch polysaccharides are thought to cause observed effects, not merely singular non-starch polysaccharides' actions (Fourij, 2007). The use of soybean meal in aquaculture feeds has been limited mainly due to the many plant based antinutritional factors (Lim *et al*., 2010) and the level of antinutrients in feed greatly varies between raw materials as seen in **Error! Reference source not found.**.

Enzymes required to hydrolyse antinutritional factors such as β-glucan, arabinoxylans and α-galactosides are low or completely absent in fish. Consequently, non-starch polysaccharides remain undigested and therefore negatively affect animal performance.

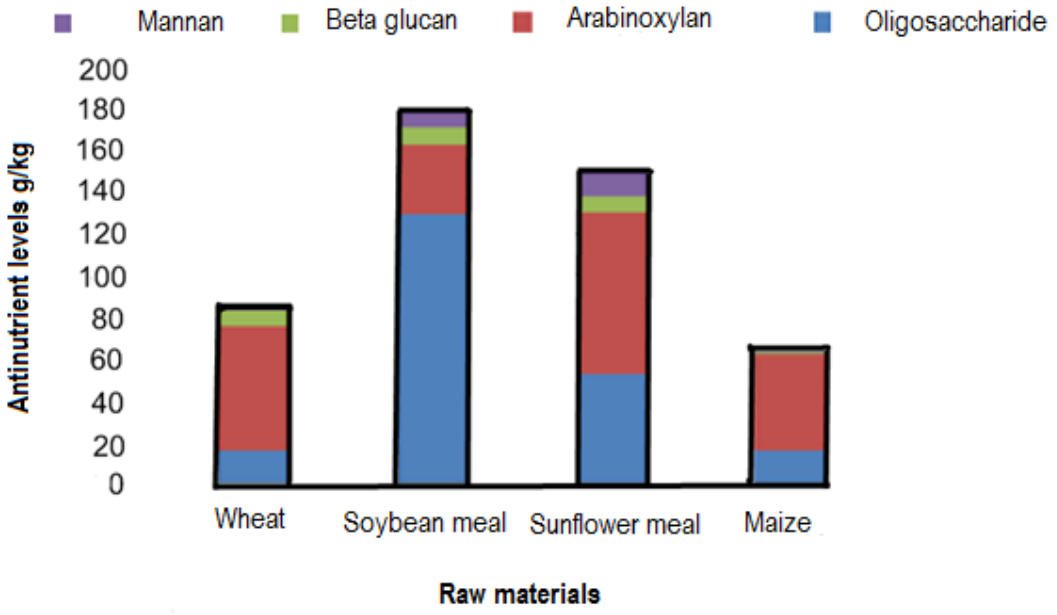


Figure 2.5 Main plant ingredients in commercial Japanese, New Zealand and South African abalone diets as reviewed by Flemming *et al.,* (1996) and main antinutrients present (Knudsen, 1997).

2.6.1 Digesta viscosity

The solubility and molecular weight of non-starch polysaccharides determine their viscosity. Solubility is not specific to the sugar composition but more to the chemical structure and relationship of non-starch polysaccharides with cell wall components (Choct, 1997).

The physical effects of viscosity on the digestion and absorption of nutrients are similar, irrespective of the non-starch polysaccharide source. The binding of non-starch polysaccharides with intestinal brush border increases the thickness of the water layer adjacent to the mucosa, resulting in impaired nutrient digestion and absorption. This increase in endogenous water secretion from the intestines has been suggested as a cause of reduced nutrient digestion. High viscosity increases the time of digesta in the gut, which, in turn, results in an increase in volatile fatty acid (VFA) production. This has drastic effects on the gut ecosystem causing microbial proliferation and a decrease in nutrient digestion and performance in the long run (Williams *et al*., 1997; Kocher *et al*., 2003; Nadeem *et al*., 2005).

Inclusion of soybean non-starch polysaccharides in Atlantic salmon diets caused high viscosity in the intestinal content, translating to reduced AA and lipid digestion. Atlantic cod (*Gadus morhua*) showed signs of decreased absorption of AA, nitrogen (N) and sulphur.

This is thought to be due to the high water-binding capacity of the soybean meal non-starch polysaccharides in the diet. Endogenous and bacterial N secretions are thought to be responsible for the apparent decrease in N utilization (Sinha *et al*., 2011).

2.6.2 Change in gastric emptying and passage rate

Soluble non-starch polysaccharides increase digesta viscosity and decrease rate of passage in monogastrics, whilst insoluble non-starch polysaccharides, like cellulose and hemicellulose, increase this passage rate (Sinha *et al*., 2011).

Inclusion of soluble non-starch polysaccharides in aquafeed diets can reduce the rate of gastric emptying in fish, causing a delay in intestinal absorption of glucose and other nutrients. Significant decreases in the blood cholesterol levels of trout, yellow tail and Atlantic salmon, fed diets with non-starch polysaccharides-rich soybean meal, have been reported by authors (Kaushik *et al*., 1995; Refstie *et al*., 1999). These reduced levels are likely to be caused by the binding and trapping of bile salts in the gut, due to the increased viscosity. This has also been observed in rats fed galactomanans from guar gum (Demigné *et al*., 1998; Sinha *et al*., 2011).

2.6.3 Alteration of gut physiology, gut morphology and gut micro flora

Excessive secretion of bile acids is triggered by high levels of non-starch polysaccharides and as a result, may cause significant bile acid loss to faeces. This, in turn, may result in an increased hepatic synthesis of bile acids from cholesterol to re-establish homeostasis. This will ultimately affect lipid absorption and cholesterol levels in the intestine as non-starch polysaccharides bind with bile salts, lipids and cholesterol, resulting in lower blood cholesterol levels. These changes may have a negative impact on gut physiology,, resulting in poor nutrient assimilation efficiency by the animal (Sinha *et al*., 2011).

It is accepted that non-starch polysaccharides have a marked impact on gut anatomy and gut development. Prolonged consumption of soluble non-starch polysaccharides is associated with an increase in the size and length of digestive organs in pigs (McDonald *et al*., 1999), poultry (Choct, 1997) and fish (Leenhouwers *et al*., 2007) accompanied by a decrease in nutrient digestion. These authors also noted an enlargement in the width of intestinal villi, increased crypt depth of the intestinal crypts found in the jejunum and ileum, and an increased rate of cell proliferation in the large intestine as well (Sinha *et al*., 2011).

The delayed transit time of the digesta can lead to microbial fermentation in the intestine. Fermenting non-starch polysaccharides produce volatile fatty acids (VFA) as end products.

Acetic, butyric and proprionic acids are produced in herbivorous fish (Nile tilapia and African catfish). Levels of these acids differ between species, but in all species, the pH of the gut is lowered which may lead to alteration of the micro flora (Sinha *et al*., 2011).

The ways in which non-starch polysaccharides alter the mucous layers of the gut are not well understood. However, physical scraping and proteolytic breakdown are thought to be the main factors causing the release of mucin into the gut lumen. The current hypothesis therefore stands that the erosion of the gut layer is caused by stretching and abrasion, which occurs as a result of the increased bulk of the digesta. These mechanisms are thought to occur in fish, but no studies have researched this as of yet (Sinha *et al*., 2011).

2.6.4 Enteritis

Enteritis in fish is an inflammatory response characterized by the shortening of the primary and secondary mucosal folds and a widening of the *lamina propria*, which is then infiltrated by a mixed population of inflammatory cells. These inflammatory cells have been identified as lymphocytes, macrophages, eosinophilic and neutrophilic granular cells. This inflammatory response has been intensively documented in salmonid fed diets containing soybean meal. More importantly, salmonids are not the only species to show soybean meal-induced enteritis. These effects have also been documented in other aquatic species. Common carp, tiger puffer, sea bass and trout are amongst other species that have been observed with this condition (Krogdahl *et al*., 2010). There has also been some speculation and documentation of the various roles played by soy proteins, oligosaccharides and sapponins in causing entritus. (Glenncross *et al*., 2007; Krogdahl *et al*., 2010).

Necrotic enteritis is a condition found in poultry, caused by an increase in dietary non-starch polysaccharides. Harmful bacteria like *Clostridium perfringens A, B, C, D and E* proliferate in the favourable conditions created by the non-starch polysaccharides and induce an inflammatory condition (*C. perfringes A* and *C* in particular). Enzyme supplementation has been shown to drastically reduce levels of *Clostridium perfringens*, by reducing non-starch polysaccharide levels (Choct *et al*., 2010).

2.7 Methods of eliminating antinutritive factors

As already mentioned, antinutritional factors consist of heat-stable and heat-labile factors (**Error! Reference source not found.**). Various methods, varying in cost and efficacy, have been and are being developed to remove these factors.

2.7.1 Heat treatments

Certain plant ingredients may contain both heat stable and heat labile antinutrients. Heat-labile secondary compounds are easily destroyed during the heat treatment of processing (Drew *et al*., 2007) and the high temperatures of pelleting and extrusion over 80 °C is capable of destroying or altering the composition of heat-labile antinutritional factors.

Insoluble fibers are redistributed as more soluble fibers by heat and pressure treatment in the extrusion process, rendering carbohydrates more available to fermentation. It is thus evident that improved digestibilities by these heat treated products can be ascribed to the reduced antinutritional factors in the diet and improved fermentation of cell wall products by bacteria in the caeca (poultry) and ileum (pigs) (Karr-Lilienthal *et al*., 2005).

Heat treatment may also have an adverse effect on feed, as extreme temperatures endured during processing are able to alter the chemical nature of proteins and carbohydrates, adversely affecting their nutritional quality (Francis *et al*., 2001).

The heat-stable secondary compounds are more resistant to processing conditions and require special treatments such as aqueous or solvent extraction, fractionation or exogenous enzyme supplementation (Drew *et al*., 2007). Heat-stable antinutritional factors require further treatments to be removed. The most common ones are listed below.

2.7.1.1 Extraction

During manufacturing of soy protein concentrate (SPC), additional ethanol or water washing is done, proving successful in removing non-starch polysaccharides, phytosterols, saponins and enteritis inducing agents. Ethanolic extraction causes an improved feed intake, whilst aqueous extraction has been shown to depress feed intake. This results in an effective product with very low levels of inherent antinutritional factors and non-starch polysaccharides. It is, however, a high-cost product, due to the elaborate treatment methods used. As a result, if soy is to be used as a successful fish meal replacement, eliminating critical antinutritional factors in abalone feeds must be achieved through more cost-effective technologies. (Refstie, 2007).

2.7.2.2 Cultivar

The type of soybean cultivar is a source of variation in the chemical composition, digestibility and availability of soybean meal carbohydrates in monogastrics. The carbohydrate composition may be affected by factors such as the cultivar type, growing conditions, climactic conditions and even fertilizer application. Nutrient values for crude

protein, crude fat and crude fiber have been shown to vary by 9.3%, 10% and 12.8% just between different cultivars and non-starch polysaccharide-levels have also been shown to vary greatly between cultivars (Karr-Lilienthal *et al*., 2005).

2.7.2.3 Enzymes

Exogenous enzyme supplementation to soybean meal is a new area of emphasis. Enzymes could break down portions of the carbohydrates, making them easily available to the animal. Karr-Lilienthal, (2005) concluded his review paper by emphasizing the ability of enzymes to improve dietary soybean meal carbohydrate utilization. He also stressed the need for evaluation of *in vivo* enzyme hydrolysis of non-starch polysaccharides.

Enzymes have been successfully used in reducing non-starch polysaccharide levels in poultry (Bedford & Schulze, 1998) and pigs (Choct, 2006). Enzymes could therefore prove to be a cheaper and more easily implemented technology than washing and soaking in the removal of non-starch polysaccharides in aquaculture feeds.

2.8 Enzymes

2.8.1 Background

Enzymes are proteins that catalyze biological processes. These proteins are of a high molecular weight (10 000-500 000 Daltons) and are sensitive to variations in their physiochemical environment which may lead to modifications in their activity. In contrast to other feed additives, like vitamins and amino acids, enzymes only function through their catalytic action, as opposed to endogenous metabolism. They can catalyze reactions of large quantities of material and substrate in a short time. As an example, 1 mol enzyme can react 1000-10000 times per second with a said substrate. This fast reaction rate is due to the high affinity of the enzyme for its specific substrate (Sabatier & Fish, 1996).

Isolated enzymes were first used in the cheese industry in 1914. In 1926, their protein nature was proven, with large scale commercial production starting in the 1960s. The commercial enzyme industry has seen tremendous growth due to improved technology and an increase in applications (food, feed, cosmetics and other industries). Global demand for enzymes was valued at $2.5 billion in 2004, with an annual growth of 5-10% per annum. Food and animal feeds make up 17% of the total market and are considered as one of the major sectors in this industry (Iyer & Ananthanarayan, 2008).

The use of enzymes in animal feeds has a history of just over 20 years (Choct, 2006). Feed is the single biggest operating cost in intensive agriculture, including aquaculture, and it could therefore be beneficial to utilize the application of enzymes to help improve feed efficiency (Britz, 1996)

There is a significant amount of literature citing the success of enzyme supplementation in animal feeds (Mascarell & Ryan, 1996; Bedford, 2002; Aehle, 2004; Choct, 2006). Despite successful applications in this field however, there are still many challenges regarding enzyme application. Long storage periods, heat processing, pelleting and certain trace mineral interactions can either inactivate or reduce the enzymes activity (Mascarell & Ryan, 1996). Typical fybrolytic feed enzymes are described in **Error! Reference source not found.**3.

Table 2.3 Fibrolytic feed enzymes and their substrates (adapted from Remus, 2009).

Antinutrient	Problem	Level of substrate	Enzyme
Arabinoxylans	Relatively resistant to digestion, reduces nutrient digestion and increases viscosity	Moderate	Xylanase
β-glucans	Soluble form causes extreme viscosity	Moderate to low, not found in maize	β-glucanase
Oligosaccharides	Resistant to digestion	Variable	α-galactosidase
Cellulose	Insoluble and resistant to digestion	High	Cellulase
Starch	Structural resistance, protein binding	High	Amylase

The ability of exogenous enzyme supplementation to increase digestibility, remove anti-nutritional factors and increase availability of feed components has been well documented in monogastric species, such as pigs and poultry (McCleary, 2003). Yet there is a lack of data regarding effects of exogenous enzyme supplementation in aquatic animals. Research in this direction is thus important, as nutritional benefits (as seen in the poultry and pig industries) could be obtained. (Marquadt & Brufay, 1997; Choct, 2006).

Initially, enzymes were exclusively used to increase nutrient digestibility, with the main focus being the removal of anti-nutritive effects of non-starch polysaccharides, such as

arabinoxylans and β-glucans. These non-starch polysaccharides are found in grains like wheat, barley and rye and result in an increased digesta viscosity. During the early 1990s, the value of enzymes was recognised and applied to other nutrients in order to increase the digestibility of more ingredients (Choct, 2006). The use of exogenous enzymes in animal feed is now a global practice. Reasons for their common use include the fact that targeted ingredients are available in greater abundance, manufacture costs are reduced as expensive refined products are not needed and the variation of nutrient quality in ingredients is reduced (Bedford & Schulze, 1998). An increase in dietary antinutritional factors in animal feed has been observed as more plant-based protein sources are used.

The key to addressing these issues is the use of enzymes, as they help in the optimization of feed digestibility and thus absorption of nutrients. Enzymes are often able to convert antinutrients into more digestible forms, thus increasing digestion whilst removing antinutritional functions of ingredients (Bedford; Schulze, 1998). This is done by matching the activity of the enzymes with their suitable substrates (Choct, 2006). The enzymes necessary to break down non-starch polysaccharides like β-glucanase, xylanase and oligosaccharidases are very scarce and mostly absent among the endogenous secretions of most fish species (Sinha *et al*., 2011).

Enzymes have the potential to increase nutrient digestion as well as having a large role in improving gut health (Bedford, 2002). Although commercial exogenous enzymes are mainly used for pigs and poultry, they are potentially applicable in diets of abalone and other fish species (Marquadt & Brufay, 1997; Aehle, 2004). Data available on various abalone species could be comparable to that of pigs and poultry, due to the similar body composition, enzyme activity, ability to tolerate a wide range of feed ingredients and comparable growth performances already observed between species. They also have fairly similar digestion and physiological features to commercially produced monogastric animals. Results seen in pigs and poultry species can, therefore, be expected in abalone to a limited extent (Britz, 1996). Largely contrary to Britz's, (1996) findings, it has been shown that digestibility values of feedstuffs for abalone are not necessarily similar to other aquatic or land-based animals (Sales, 2004).

Enzymatic treatments have been proven effective in modifying the ratio between soluble and insoluble fibers. For example, treatment of cell walls with xylanase increases the level of soluble dietary fibers making them more available to the animal. This is because more

nutrients are available to be degraded as they are released from the viscous matrix (Elleuch *et al*., 2010).

2.8.2 Endogenous abalone enzymes

Herbivorous marine invertebrates, like abalone, possess polysaccharidase enzymes in their digestive fluids (Johnston *et al*., 2005). The endogenous enzymes of abalone include lyase, amylase, cellulase and mannanase which are effective in degrading dextrans, cellulose and β-mannans found in seaweeds (Kumagai & Ojima, 2009). Oligosaccharidases and monosaccharidases are either produced by the animals themselves or are productions of intestinal bacterial fermentation (Erasmus *et al*., 1997). An endogenous β-1-3-glucanase has been identified and was able to hydrolyse laminarian but not xylans and mannans. The ability of endogenous enzymes in abalone to digest terrestrial plant sources is still unknown (Kumagai & Ojima, 2009). The activity of abalone carbohydrases are lower than the enzyme activities found in terrestrial herbivorous species (Garcia-Esquivel & Felbeck, 2006). Micro flora native to abalone intestine have been found to be limited to certain areas of the gut due to pH specificity and are associated with degradation of algal polysaccharides and thus digestion (Harris *et al*., 1998b; Zhang *et al*., 2004).

2.8.3 Methods of non-starch polysaccharide enzyme actions

The mechanisms in which exogenous non-starch polysaccharide-degrading enzymes act in fish are not yet fully understood, although three basic methods have been suggested in literature and are described below.

2.8.3.1 Disruption of cell wall integrity

Cereal and legume cell walls are constructed of cellulose, hemicellulose and arabinoxylan with some β-glucan components. The enzyme activity creates ‘holes’ in the cell walls making hydration possible. Endogenous amylase and pancreatic proteases can then digest the cell content better (Sinha *et al*., 2011).

2.8.3.2 Reduction of digesta viscosity

It has long been accepted that supplementation of non-starch polysaccharide-degrading enzymes are responsible for the reduction of digesta viscosity in monogastrics (Aehle, 2004). Enzymes act by cleaving backbones of polymers rather than side chains, and due to the fact that viscosity is a function of chain length, this process reduces viscosity. A relatively small number of breaks in the chain will greatly reduce or destroy the gel-forming

capacity of the non-starch polysaccharides, thus reducing viscosity (Chesson, 1993). Literature illustrating this effect in fish is lacking (Sinha *et al*., 2011).

2.8.3.3 Stimulation of bacterial population

Non-starch polysaccharide-degrading enzymes in animal feeds cause the breakdown of plant cell wall carbohydrates and reduce chain length, producing small polymers and oligomers. These substrates are broken down further until they become small enough sites for beneficial bacterial proliferation. These beneficial bacteria are responsible for volatile fatty acid production and beneficial gut health. The biggest challenge is the lack of information on specific plant antinutrients and their intricate interactions, as to allow adequate enzyme quantity and substrate specificity supplementation (Sinha *et al*., 2011). Mathlouthi *et al*., (2002) showed that β-glucanase and xylanase supplementation to chicken feed containing high levels of soluble non-starch polysaccharides resulted in improved gut health with an increase in villi length and crypt depth.

2.8.4 Fibrolytic enzymes with potential in abalone feeds

Due to the raw material composition of most commercial abalone feeds in South Africa, the following enzymes show the greatest possibility of matching specificity of their action to predominant non-starch polysaccharides, as seen in Figure 2.5.

2.8.4.1 Xylanase

The mode of action pertaining to the way in which xylanases increase digestibility is not clear. There is a theory suggesting that xylanases release protein and starch molecules that are held within the xylan structure, which are then used by the animal. The other theory is based on the ability of the enzymes to reduce the viscosity in the GIT and that the oligomers produced are less viscous and thus allow improved digestion (Aehle, 2004). Williams, (1997), affirms these two theories but suggests that both are responsible for the enzymatic action and observed effects.

2.8.4.2 β-glucanases

β-glucan consists of linear glucose units linked by β-1,3 and β 1-4 linkages, which are easily cleaved by β-glucanases to produce free glucose and oligosaccharides (Aehele, 2004). The exact mode of action of these enzymes is still unclear although the beneficial results obtained from their use are well documented (Chesson, 1993; Bedford & Schulze, 1998). What is known about their action is that they act on reducing viscosity of β-glucans

and pentosans by cleaving their backbones, which may allow nutrients to be freed from the viscous feed matrix and improve nutrient utilization (Abudabos, 2010).

2.8.4.3 α-galactosidases

These enzymes hydrolyze α-galactosides found in legumes like soybeans. They also neutralize anti-nutritional factors like trypsin inhibitors from soy meal (Avitech Scientific Bulletin, 2002). Animals lack endogenous α-1,6 galactosidase enzyme activity in the mucosa, and thus supplementation is essential for hydrolyzing oligosaccharides. Although these enzymes have been proven to be successful additions to reducing oligosaccharide antinutrients, there is some literature which shows no significant differences in pig and poultry diets (Choct *et al*., 2010).

2.9 Factors influencing enzyme and plant-protein suitability for abalone feeds

2.9.1 Extrusion

Extrusion is a process in which a food material is forced to flow, under one or more of a variety of conditions of mixing and heating, and sheared through a die which is designed to form and/or puff-dry the ingredients (Bhattacharya & Hanna, 1987).

The ingredient composition of diets affects extrusion parameters and should therefore be evaluated. There are some advantages in using plant-protein ingredients in aquafeed extrusion, namely, increased pellet expansion, improved feed binding properties resulting in improved water stability, less plugging of the die by white mineral deposits, greater absorption properties of plant-protein, allowing for increased lipid addition and absorption and lastly, a reduced fish meal dependency which lowers the feed cost, due to the cheaper plant alternatives (Riaz, 2008). Enzymes are heat-labile and fully hydrated enzymes are easily denatured. In a dry state, enzymes are found to be remarkably stable. Enzyme loss during extrusion is therefore dependent on temperature and hydration (Bioferm, 2010).

2.9.2 Gelatinisation

Carnivorous and omnivorous fish are considered to have a limited ability to utilize carbohydrates. Gelatinisation of carbohydrates (a thermal modification of raw starch) is a processing method employed during extrusion and has been reported to improve the nutrient bioavailability of carbohydrates to fish. During this process, the carbohydrate granules are modified in such a manner that their susceptibility to enzymatic action increases, thus allowing for a more complete digestion and utilization of the nutrient

(Sørensen *et al*., 2008). The presence of non-starch polysaccharides impairs the process of starch gelatinisation, mainly due to their water-binding ability and high viscosity. The action non-starch polysaccharides exert on the gelatinisation event is mediated by the reduction in the volume fraction of water in the system, thus restricting water mobility and availability. Non-starch polysaccharides hinder the effect of processing and starch digestibility. A better understanding of specific non-starch polysaccharides and their specific effects of gelatinisation would greatly assist in the utilization of plant carbohydrates in fish nutrition (Sinha *et al*., 2011).

The action of exogenous enzyme supplementation could result in the breakdown of in-feed non-starch polysaccharides and result in improved starch gelatinisation. These effects have been observed as differences in water stability and animal growth. (Sørensen *et al*., 2008).Legumes have also been found not to gelatinise as well as cereal based starch sources, thus legume based diets have often shown poorer pellet quality than cereal based diets. Non-starch polysaccharides may contribute to harder, more durable pellets which are beneficial for handling and distribution (Kraugerud *et al*., 2007). Legumes like soybean meal in particular, are known to improve the durability of feed, as well as improve feed texture; this is thought to be as a result of the adhesiveness of the soy proteins caused by the intermolecular forces of the proteins (Sørensen *et al*., 2008). Although durability is improved by non-starch polysaccharides, their negative effect on water stability and nutrient leaching is of concern.

2.9.3 Heat stability limitations

Uncertainty exists as to how feed processing (pelleting and extrusion) affects enzyme activity. This problem is compounded by the lack of a suitable in-feed enzyme assay procedure. These assay problems have resulted in enzyme activity being assayed *in vivo*, looking at the animal response (Walsh *et al*., 1993). Although thermo tolerant enzymes are being produced, *in vivo* assays and trials showing in-feed activities need to be designed. Interference and interactions of the substrate on the enzyme have been blamed for difficult and inaccurate in-feed assays. High extrusion temperatures and insufficient hydration remain challenges to producers as these factors negatively alter enzyme activity (Bedford, 2008). Under-heating of soybean meal in extrusion results in decreased digestibility and lack of heat-labile antinutrient inactivation, whilst over heating decreases digestibility due to damaging of protein and possible Maillard reaction occurring (Barrows *et al*., 2006).

2.9.4 Water Stability

Pellet water stability refers to the stability of the pellet when immersed in water for a given amount of time. The water stability required can be up to 2-3 days (shrimp), with feed losing over 30% of its dry-matter weight. This stability is reported as the dry weight of the recovered feed after a given period of time (Fleming *et al*., 1996; Friedman, 1996).There is no standard procedure for determination of water stability, whilst the above mentioned principle is agreed upon in literature (Jayaram & Shetty, 1981; Guzm´an & Viana, 1998; Rout & Bandyhophaday, 1998; Tsanigab, 2009).

Water stability is an important aspect of abalone and shrimp feeds. These animals feed slowly and the feed is thus subjected to its aqueous environment for extended periods. It is thus necessary to test water stability in order to determine the ability of the pellet to maintain its physical integrity in the water column (Abin, 2007). Water stability is affected by many factors such as: water temperature, water salinity, water movement, pellet coatings, pellet moisture and immersion time (Mair, 2008). Materials are used in pellet processing and the pellet requires some form of assessment of its physical properties. **Error! Reference source not found.**4 shows the water stability of various test diets in a trial done by Sales & Britz (2003). This largely determines the potential application of the raw materials considered (Glenncross *et al*., 2007). Soybean meal-based diets thus need to be compared to a control fish meal diet.

Table 2.4 Amount of dry matter leached ($g.kg^{-1}$) from test diets with different protein sources and the same basal ingredient formulation after 16 hour water exposure (Adapted from Sales & Britz, 2003).

Test diet	Dry matter leached from feed ($g.\ kg^{-1}$)
Fish meal	16.1
Soya bean meal	54.3
Sunflower meal	17.6
Canola meal	79.0
Peanut meal	43.1
Lupines	120.1
Faba beans	128.3

Water stability incorporates two aspects of nutrient loss, namely, disintegration and leaching. Disintegration is the dry matter loss due to the physical breakdown of the pellet in the aqueous environment, whilst nutrient leaching refers to the loss of soluble nutrients from the feed to the surrounding aqueous environment. During pellet exposure to water,

dry matter is lost to the water, with water soluble nutrients like sugars, starches, salts, amino acids and water-soluble vitamins being mostly affected (Suresh, 2006). Sales and Britz, (2003) observed that, in comparison to legume based diets (faba beans, soybean meal and lupins) fish meal based diets were least affected by nutrient leaching. They concluded that the inclusion of legumes could be problematic because of the high level of leaching associated with these diets as indicated in **Error! Reference source not found.** (Sales & Britz, 2003).

2.10 Abalone morphology

Challenges faced when replacing animal protein sources with plant protein sources are not limited to pellet characteristics and production performance effects (growth in length and mass, specific growth rates, feed conversion rates etc.). The influence of plant-based ingredients on the gastro intestinal tract of animals is also of importance (Sinha *et al*., 2011). This is especially true when plant proteins are used in species where little is known about such physiological effects, as is the case with abalone (Kemp, 2001).

Literature published on abalone gut morphology and histology is scarce and incomprehensive (Campbell, 1965; Bevelander, 1988; Harris *et al*., 1998a; Edwards *et al*., 2003). Currently, there is no published intestinal histological work on the particular species of *H.midae.* Anatomical literature mainly focuses on the digestive gland, diseases and endogenous enzyme activities of juveniles, and lacks histological identification and comparisons (Knauer *et al*., 1996; Erasmus *et al*., 1997; Serviere-Zaragoza *et al*., 1997; Long-Bo *et al*., 2001; Macey & Coyne, 2005; Garcia-Esquivel & Felbeck, 2006; Mouton & Gummow, 2011). Limited data are published on gut pH (Gomez-Pinchetti & Garcia-Reina (1994) and Harris *et al* (1998b)) and much work is still required in the area of documenting and understanding abalone morphology and nutritional interactions (Harris *et al*., 1998a; Harris *et al*., 1998b; Kemp, 2001). The influence of different feed raw materials on gut parameters of the abalone *H. midae* are to date unpublished.

2.10.1 Overview of intestinal regions

The intestinal region of the abalone is long and complex. It originates from the style sac of the stomach and is differentiated into five (I, II, III, IV and V) distinct regions (Bevelander, 1988; Harris *et al*., 1998a), or three less descript regions (Long-Bo *et al*., 2001). Food from the style sac enters region I and exits through region V, which terminates in the anus. The nature of the *Haliotis* spp. digestive tract has been examined by Campbell (1965), with drawings from *Kaliotis cracherocli*, Bevelander (1988), with photographs of *H.tiiberculata*

and Harris *et al.,* (1998) and Kemp (2001), with photographs of *H.laevigata*. A summary of current literature on abalone gut morphology is shown in

5.

Morphology between various regions of the intestinal tract is quite similar (Harris *et al.*, 1998), with the intestine being mainly made up of columnar, ciliated epithelium. Cell height and abundance of cilia are the major morphological differences observed. Region I and region II have large folds and abundant epithelium, whilst region III has smaller cells and sparser epithelium than the former. The epithelial cells of region IV and V are characterised by an increased abundance of mucus cells. Region V possesses the longest cilia of the intestine and is highly folded. Cell height increases nearer to the typhlosole and the typhlosole extends the entire length of the intestine as is common in molluscs (Harris *et al.*, 1998).

2.10.2 Intestinal features

The intestine becomes more complex and folded with age, with intestinal region II only appearing after 158 days (Johnson *et al*., 2005). The oesophagus and intestine III are very similar, but differ with regard to the fact that the oesophagus is more abundant in cilia, as its function is propelling food towards the stomach region, and the intestine may assume a more absorptive role. Region III also has no muscularis present. Phagocytes have been observed near distal epithelial cells in the stomach and intestine III and V, and have thus been attributed with the role of food absorption and waste rejection. Harris and his co-workers contrasted these phagocytes between starved and fed abalone. In starved abalone, their concentrations greatly decreased in the mentioned digestive regions (III and V), and these regions were thus credited as being the most absorptive. Campbell (1965) merely stated that these areas were responsible for consolidation of the faecal string and thought that region V of the intestine played a more prominent role in absorption.

The structural changes observed in the abalone gut could also be influenced by factors such as maturity, water quality and temperature, and thus conclusive data on abalone gut absorption seems to be largely absent. Harris *et al*. (1998) and Kemp (2001) shared the same sentiment and each proceeded to document the morphology of the regions of the digestive tract of the greenlip abalone, *H. laevigata*. Kemp (2001), however, investigated the effect of plant proteins on intestinal characteristics. He noted a change in gut structure when legumes were fed, but mooted further investigation into this topic in his concluding remarks. This currently appears to be the most comprehensive work available on abalone

digestive morphology. Prior to this work, Cambell (1966) and Bevelander (1988) were responsible for producing accounts of abalone physiology. Although these works provided new insights, they were not entirely comprehensive, as varying opinions on the function of the muscularis, the areas of nutrient absorption (stomach, intestine II, III and/or IV) and the function of cells and cilia throughout the intestine are found(

5). A factor which further complicates comparison of gut histology and morphology of abalone is the great variation in isolation and sectioning techniques described in literature (Campbell, 1965; Bevelander, 1988; Harris *et al*., 1998a; Joly, 2011).

Table 2.5 A review of current knowledge on abalone digestive system.

Author, species and focus	GIT Region						
			Absorption Intestine (post-digestive region)				
	Anterior, mid and posterior oesophagus	Crop and stomach	I	II	III	IV	V
Crofts (1939) H.Turbulacta Organogenesis	Juvenile torsion						
Campbell (1965) H.cracherodii, Structure and function	A: Cillia M: Cillia pigmented and columnar P: Epithelial, mucous and secretory	S: Tall and narrow cells	Similar to III and IV	Longest	Scattered epithelia	Mucous cells	Cillary current
Bevelander (1988) H. Rufescenes, Gross and fine structure	Tall, ciliated, pigmented	C:Tall, ciliated, folded S: Shorter than crop	Tall, columnar	Shorter, columnar		Mucous and cilia	4-6 pleats
Harris *et al.,* (1998) H lavigata Characterization of digestive tract	A: Mucous, ciliated and secretory cells, no muscularis M: Columnar secretory P: Cillia on floor	S: Similar to crop	More cuboidal than crop	More mucous than III			
Long-Bo *et al*., (2001)			Three regions anterior, middle and posterior Mucous cells and absorption in posterior region				
Edwards *et al.,* (2002) H.rubra, Gut muscle movement		No peristalsis				No peristalsis	

Britz (1996) stated that the digestive capabilities of abalone and monogastric species are comparable. However, vast differences in gut function, motility and absorption exist between molluscs and monogastric species (Edwards *et al*., 2003). When looking at the histology in the gastro-intestinal tract (GIT) of poultry and abalone, it is evident that

differences in digestive capacity exist, which may drastically alter nutrient uptake. Abalone have complex intestines, with five specific regions. Muscular movement of food through the intestine is minimal and cilia is the main force of feed movement as opposed to peristalsis and contractions in monogastric animals (Edwards *et al*., 2003).

Gut micro flora is known to contribute towards the nutritional value of the food. The extent of this contribution remains unknown however. The micro flora interaction with the intestinal epithelium differs from that of terrestrial herbivores and interaction is thought to occur more with bacteria in the lumen than with intestinal epithelial cells as in livestock (Harris *et al*., 1998a). The difference in micro flora action raises the question of whether enteritis, or a form of gastric inflammation, would occur or be induced by dietary soybean meal in the absorptive areas (II, III, IV and V) of the abalone. Endogenous enzyme secretions in abalone include protease, lipase and complex cellulose activities, thus endogenous cellulosic secretions could assist abalone in hydrolysing complex soybean meal carbohydrates without decreased production performance or detrimental morphological effects (Fleming *et al*., 1996; Knauer *et al*., 1996; Macey & Coyne, 2005).

2.11 Conclusion

Abalone farming is a significant contributor to the South African aquaculture sector, worth more than R250 million. The abalone farming industry is characterized by a fast rate of growth of over 10% per annum, with increased production mainly dependant on the use of artificial feeds.

The option to reduce production costs and increase sustainability through the partial or complete replacement of fish meal with soybean meal is a possibility that needs to be thoroughly explored. The harmful effects of non-starch polysaccharides found in soybean meal and other plant based ingredients is well documented. Enzymes have been successfully used in the pig and poultry industries to reduce the harmful effects of antinutritive factors (Sinha *et al*., 2011), which justifies an assessment with regard to abalone feeds. It must be kept in mind though that abalone digestive morphology differs from monogastrics, and thus the possibility that enzyme supplementation will be beneficial in their production should not be assumed. Pellet water stability, the morphological impacts of such diets as well as production performance need to be critically assessed in order to determine the suitability of fish meal replacement and enzyme supplementation in commercial abalone diets.

References

Abin, J. 2007. Effects of the digestion of carbohydrates in soya on poultry performance. Doctoral dissertation, Cargill.

Abudabos, A. 2010. Enzyme supplementation of corn-soybean meal diets improves performance in broiler chicken. International Journal of Poultry Science. 9(3): 292-297.

Aehle, W. 2004. Second completely revised edition; enzymes in industry production and application. Aehle W., Ed. Whiley-VCH Verlag GmbH & Co.

Aquaculture Farmers Association South Africa, 2010. Abalone Sector Report, 1-1.

Amaya, E. A., Davis D. A. & Rouse, D. B. 2006. Replacement of fish meal in practical diets for the pacific white shrimp (*Litopenaeus vannamei*) reared under pond conditions. Aquaculture. 262: 393-401.

Avitech Scientific Bulletin. 2002. Rationale for enzyme supplementation.

Barrows, F. T., Stone D. A. J. & Hardy, R. W. 2006. The effects of extrusion conditions on the nutritional value of soybean meal for rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 265: 244-252.

Bedford, M. R. 2002. The Role of carbohydrases in feedstuff digestion In: Poultry feedstuffs supply, composition and nutritive value. Editors. McNab, J. M., and Boorman, K. N. CABI Publishing, pp. 319-331.

Bedford, M. 2008. Next generation thermo-tolerant enzymes for reducing feeding costs. Annual ASA-IM SEA feed technology and nutrition workshop, Singapore.

Bedford, M. R. & Schulze, H. 1998. Exogenous enzymes for pigs and poultry. Nutrition Research Reviews. (11): 91-114.

Bevelander, G. 1988. Abalone, gross and fine structure. First edition. The Boxwood Press. 183 Ocean View Boulevard, Pacific Grove, California 93950.

Bhattacharya, M. & Hanna, M. A. 1987. Influence of process and product variables on extrusion energy and pressure requirements. Journal of Food Engineering 6(2): 153-163.

Bioferm. 2010. Questions concerning enzymes. 2011(July, 21).

Britz, P. J., 1996. The nutritional requirements of *Haliotis midae* and development of a practical diet for abalone aquaculture. Doctoral dissertation, Rhodes University.

Campbell, J. L. 1965. The structure and function of the alimentary canal of the black abalone, *Haliotis cracherodii* leach. Transactions of the American Microscopial Society. 84(3): 376-395.

Chamberline, G. W. 2004. Re-emergence of extruded shrimp feed. Aqua Feeds; Formulation and Beyond. 1(2): 18-20.

Chesson., A. 1993. Feed enzymes. Animal Feed Science and Technology 45, (1993): 65-79.

Choct, M. 1997. Feed non-starch polysaccharides: Chemical structures and nutritional significance. Feed Milling International. (June): 13-26.

Choct, M. & A. Kocher. 2001. Non-starch carbohydrates: Digestion and its secondary effects in monogastrics. Doctoral dissertation, Department of Animal Science, University of New England.

Choct, M. 2002. Non-Starch Polysaccharides: Effect on Nutritive Value In: Poultry Feedstuffs Supply, Composition and Nutritive Value. Eds. McNab, J. M. & Boorman,K. N. CABI Publishing, pp. 221-231.

Choct, M. 2006. Enzymes for the feed industry; past, present and future. World's Poultry Science Journal. 62: 5-16.

Choct, M., Dersjant-Li Y., McLeish J. & Peisker M. 2010. Soy oligosaccharides and soluble non-starch polysaccharides: A review of digestion, nutritive and anti-nutritive effects in pigs and poultry. Journal of Animal Science. 23(10): 1386-1398.

Cook, P. A. & Gordon H. R. 2010. World abalone supply, markets and pricing. Journal of Shellfish Research. 29(3): 569-571.

Cowieson, A. J. 2005. Factors that affect the nutritional value of maize for broilers. Animal Feed Science Technology. 119(3-4): 293-305.

Cremer, M. C. 2004. Use and future prospects for soy in aquaculture feeds. Doctoral dissertation, American Soybean Association.

Demigné, C., Levrat M., Behr S. R., Moundras C. & Rémésy C. 1998. Cholesterol-lowering action of guar gum in the rat: Changes in bile acids and sterols excretion and in enterohepatic cycling of bile acids. Nutritional Research. 18(7): 1215-1225.

Denstadli, V., Hillestad M., Verlhac V., Klausen M. & Øverland, M. 2011. Enzyme pretreatment of fibrous ingredients for carnivorous fish: Effects on nutrient utilisation and technical feed quality in rainbow trout (*Oncorhynchus mykiss*). Aquaculture. In Press, Corrected Proof.

Dersjant-Li, Y. 2002. The use of soy protein in aquaculture feeds. Advances in aquaculture nutrition, Cancun, Mexico.

Drew, M. D., Borgeson., T. L. & Thiessen D. L. 2007. A review of processing of feed ingredients to enhance diet digestibility in finfish. Animal Feed Science and Technology. 138: 118-136.

Edwards, S., Geraghty D. & Ralph J. 2003. Pharmacological and histological assessment of gut muscle movement in Blacklip abalone, *Haliotis rubra* (leach). Aquaculture Research. 34(5): 383-388.

Elleuch, M., Bedigian D., Roiseux O., Besbes S., Blecker C. & Attia H. 2010. Dietary fibre and fibre-rich by-products of food processing: Characterisation, technological functionality and commercial applications: A review. Food Chemistry.

Erasmus, J. H., Cook P. A. & Coyne V. E. 1997. The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. Aquaculture. 155(1-4): 377-386.

Food and Agricultural Association, 2012. Fishery and aquaculture statistics, http://www.fao.org/fishery/statistics/en, January (2012).

Fleming, A. E., Bameveld R. J. V. & Hone P. W. 1996. The development of artificial diets for abalone: A review and future directions. Aquaculture. 140: 5-53.

Fourij, J. L. 2007. The effect of multiple-enzyme combinations in maize-soya diets for broiler chickens. Doctoral dissertation, Stellenbosch University.

Francis, G., Makkar H. P. S. & Becker K. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture. (199): 197-227.

Friedman, M. 1996. Nutritional value of proteins from different food sources. A review. Journal of Agriculture and Food Chemistry. (44): 6-29.

Garcia-Esquivel, Z. & Felbeck H. 2006. Activity of digestive enzymes along the gut of juvenile red abalone, *Haliotis rufescens*, fed natural and balanced diets. Aquaculture. 261(2): 615-625.

Gatlin III, D. M. 2003. Nutrition and Fish Health In: Fish Nutrition (Third Edition). Eds. John E. Halver, and Ronald W. Hardy Academic Press, San Diego. pp. 671-702.

Gatlin, D. M., Barrows F. T., Brown P., Dabrowski K., Gaylord T. G., Hardy R. W., Herman E., Hu G., Krogdahl Å., Nelson R., Overturf K., Rust M., Sealey W., Skonberg D., J Souza E., Stone D., Wilson R. & Wurtele E. 2007. Expanding the utilization of sustainable plant products in aquaculture feeds: A review. Aquaculture Research. 38(6): 551-579.

Ghoush, K. 2006. Application of enzymes in aquaculture feeds. Aqua Feeds: Formulation & Beyond. 3(4): 7-10. Retrieved 13 November 2010,

Glenncross, B., Carter, C. & Refstie S. 2007. Harvesting the benefits of grain aquaculture feeds. Soybean in aquaculture feeds, Freemantle, Western Australia.

Gomez-Pinchetti, J. L. & Garcia-Reina G. 1994. Acid deoxyribonuclease activity in crude extracts from marine phycophages used for seaweed protoplast isolation. Scientia Marina. 58(3): 133-236.

Guzm´an, J. M. & Viana M. T. 1998. Growth of abalone haliotis fulgens fed diets with and without fish meal, compared to a commercial diet. Aquaculture. 165: 321-331.

Harris, J. O., Burke C. M. & Maguire G. B. 1998a. Characterization of the digestive tract of greenlip abalone, *Haliotis laevigata*. Morphology and histology. Journal of Shellfish Research. 17(4): 979-988.

Harris, J. O., Burke C. M. & Maguire G. B. 1998b. Characterization of the digestive tract of greenlip abalone, *Haliotis laevigata*. Microenvironment and bacterial flora. Journal of Shellfish Research. 17(4): 989-994.

Iyer, P. V. & Ananthanarayan. 2008. Enzyme stability and stabilization—Aqueous and non-aqueous environment. Process Biochemistry. 43: 1019-1032.

Jayaram, M. G. & Shetty H. P. C. 1981. Formulation, processing and water stability of two new fish feeds. Aquaculture. (23): 355-359.

Johnston, D., Moltschaniwskyj N. & Wells J. 2005. Development of the radula and digestive system of juvenile blacklip abalone (*Haliotis rubra*): Potential factors responsible for variable weaning success on artificial diets. Aquaculture. 250(1-2): 341-355.

Joly, J. P. 2011. Molluscs Processing for Diagnosis by Histology In: Ifremer. 3rd ed. European Union Reference Laboratory for Mollusc Diseases, France. pp. 1-7

Karr-Lilienthal, L. K., Kadzere C. T., Grieshop C. M. & Fahey J., G.C. 2005. Chemical and nutritional properties of soybean carbohydrates as related to non-ruminants: A review. Livestock Production Science. 97(1): 1-12.

Kaushik, S. J., Cravedi J. P., Lalles J. P., Sumpter J., Fauconneau B. & Laroche M. 1995. Partial or total replacement of fish meal by soybean protein on growth, protein utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 133(3-4): 257-274.

Kemp, R. 2001. The effect of diet on intestinal structure and function in the abalone (*Haliotis laevagata)*. Doctoral dissertation, University of Adelaide.

Knauer, J., Britz P. J. & Hecht T. 1996. Comparative growth performance and digestive enzyme activity of juvenile South African abalone, *Halioits midae*, fed on diatoms and a practical diet. Aquaculture. (140): 75-85.

Knudsen, K. E. B. 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. Animal Feed Science. 67: 319-338.

Kocher, A., Choct M., Ross G., Broz J. & Chung T. K. 2003. Effects of enzyme combinations on apparent metabolizable energy corn-soybean meal-based diets for broilers. Poultry Science Association. 12: 275-283.

Kraugerud, O. F., Penn M., Storebakken T., Refstie S., Krogdahl A. & Svihus B. 2007. Nutrient digestibilities and gut function in Atlantic salmon (*Salmo salar*) fed diets with cellulose or non-starch polysaccharides from soy. Aquaculture. 273: 96-107.

Krogdahl, A., Penn M., Thorsen, J., Refstie, S., and Bakke A. 2010. Important antinutrients in plant feedstuffs for aquaculture: An update on recent findings regarding responses in salmonids. Aquaculture Research. 41: 331-344.

Kumagai, Y. & Ojima T. 2009. Enzymatic properties and the primary structure of a β-1,3-glucanase from the digestive fluid of the pacific abalone haliotis discus hannai. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 154(1): 113-120.

Leenhouwers, J. I., ter Veld M., Verreth J. A. J. & Schrama J. W. 2007. Digesta characteristics and performance of african catfish (*Clarias gariepinus*) fed cereal grains that differ in viscosity. Aquaculture. 264(1-4): 330-341.

Li, P., Mai K., Trushenski J. & Wu G. 2009. New developments in fish amino acid nutrition: Towards functional and environmentally oriented aquaculture feeds. Amino Acids. (37): 43-53.

Liang, D. 2000. Effect of enzyme supplementation on the nutritive value of canola meal for broiler chickens. Doctoral dissertation, University of Manitoba.

Lim, S., Kim S. ., Ko G., Song J., Oh D., Kim J., Kim J. & Lee K. 2010. Fish meal replacement by soybean meal in diets for tiger puffer, *Takifugu rubripes*. Aquaculture. 290 (2): 120-122.

Long-Bo, C. U. I., Chuan-Lin L. I. U., Xun L. I. U. & Yao-Hua L. U. 2001. Structure and function of mucous epithelium of the intestine in *Haliotis discus hanna*i. Acta Zoological Sinica. 47(3): 324-338.

Lunger, A. N., Craig S. R. & McLean E. 2005. Replacement of fish meal in cobia (R*achycentron canadum*) diets using an organically certified protein. Aquaculture. 257: 393-399.

Macey, B. M. & Coyne V. E. 2005. Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment. Aquaculture. 245(1-4): 249-261.

Mair, C. 2008. Improving shrimp feed production with a forming extruder. Feed Technology Update. 3(3): 4-9.

Marquadt, R. R. & J. Brufay. 1997. Futuer of feed enzymes: Overview and perspectives. Zaragoza : CIHEAM-IAMZ, 1997. p. 181-187 : 18 ref. south european feed manufacturers conference, 1996/05/09-11, Reus, Spain.

Mascarell, J. & M. Ryan. 1996. Technical aspects of enzyme utilization: Dry vs liquid enzymes. South european feed manufacturers conference, Spain.

Mathlouthi, N., J. P. Lalles, P. Lepercq, C. Juste & M. Larbier. 2002. Xylanase and ß-glucanase supplementation improve conjugated bile acid fraction in intestinal contents and increase villus size of small intestine wall in broiler chickens fed a rye-based diet. Journal of Animal Science. (80): 2773-2779.

McCleary, B. V. 2003. Dietary fibre analysis. Nutrition Society. 62: 3-9.

McDonald, D. E., Pethick D. W., Pluske J. R. & Hampson D. J. 1999. Adverse effects of soluble non-starch polysaccharide (guar gum) on piglet growth and experimental colibacillosis immediately after weaning. Research Veterinary Science. 67(3): 245-250.

Mouton, A. & Gummow B. 2011. The occurrence of gut associated parasites in the South African abalone, *Haliotis midae*, in Western Cape aquaculture facilities. Aquaculture. 313(1-4): 1-6.

Mowlana, R. 2007. Marine aquaculture in South Africa. Department Environmental Affairs and Tourism. (1): 1-16.

Nadeem, M. A., Anjum M. I., Khan A. G. & Azim A. 2005. Effect of dietary supplementation of non-starch polysaccharide degrading enzymes on growth performance of broiler chicks. Pakistan Vetenary Journal. 25(4): 22-35.

O'Keef, T. 2003. Plant protein ingredients for aquaculture feeds: Use considerations & quality standards. Doctoral dissertation, American Soybean Association.

Pratoomyot, J., Bendiksen E. A., Bell J. G. & Tocher D. R. 2010. Effects of increasing replacement of dietary fishmeal with plant protein sources on growth performance and body lipid composition of Atlantic salmon (S*almo salar L*.). Aquaculture. (305): 124-132.

Refstie, S. 2007. Soybean in aquaculture feeds. Harvesting the benefits of grain in aquaculture feeds, Freemantle, Western Australia.

Refstie, S., Svihus B., Shearer K. D. & Storebakken T. 1999. Nutrient digestibility in Atlantic salmon and broiler chickens related to viscosity and non-starch polysaccharide content in different soybean products. Animal Feed Science and Technology. 79(4): 331-345.

Riaz, N. R. 2008. Extrusion of Aquaculture feeds Formulated with Alternative Protein Sources In: Alternative Protein Sources in Aquaculture feeds. Eds. Li, C., Webster,C. D., and Cheng-Sheng,L. 1st ed. The Haworth Press, USA, New York. pp. 523-545.

Rout, R. K. & Bandyhophaday S. 1998. A comparative study of shrimp feed pellets processed through cooking extruder and meat mincer. Aquaculture. (19): 71-79.

Sabatier, A. M. & N. M. Fish. 1996. Method of analysis for feed enzymes: Methodological problems. Improving nutrient utilization by ingredient and dietary modification.

Sales, J. 2004. Aqua feeds: Formulation & beyond, volume 1 issue 3 2004. Aqua Feeds: Formulation and Beyond. 1(3): 23-26.

Sales, J. & Britz P. J. 2003. Apparent and true availability of amino acids from common feed ingredients for south african abalone (*Haliotis midae L*.). Aquaculture Nutrition. 9: 55-64. from the Blackwell Publishing database.

Sales, J. & Britz P. J. 2001. Research on abalone (*Haliotis midae L*.) cultivation in South Africa. Aquaculture Research. 32: 863-874.

Serviere-Zaragoza, E., Toro M. A. N. & Garcia-Carrefio F. L. 1997. Protein-hydrolyzing enzymes in the digestive systems of the adult Mexican blue abalone, *Haliotis fulgens* (gastropoda). Aquaculture. (157): 325-336.

Shipton, T. A. 1999. The protein requirements of the south african abalone, *Haliotis midae*. Doctoral dissertation, Rhodes University Editor.

Sinha, A. K., Kumar V., Makkar H. P. S., De Boeck G. & Becker K. 2011. Non-starch polysaccharides and their role in fish nutrition – A review. Food Chemistry 127(4): 1409-1426.

Sørensen, M., Stjepanovic N., Romarheima O. H., Krekling T. & Storebakken T. 2008. Soybean meal improves the physical quality of extruded fish feed. Animal Feed Science and Technology. 149: 149-161.

Suresh, A. V. 2006. Improving nutrient delivery in aqua feeds: Implications for nutritionists and formulators. Feed Technology Update. 1(4): 1-32.

Tacon, A. G. J. 1997. Review of the state of world aquaculture. 2011(12 July): 163.

Troell, M., Robertson-Andersson D., Anderson R. J., Bolton J. J., Maneveldt G., Halling C. & Probyn T. 2006a. Abalone farming in south africa: An overview with perspectives on kelp resources, abalone feed, potential for on-farm seaweed production and socio-economic importance. Aquaculture. (278): 266-281.

Tsanigab, S. M. 2009. Evaluating namibian macrophytic algae as a dietary source for South African abalone. Doctoral dissertation, Stellenbosch University, Department of Food Science.

Walsh, G. A., Power R. F. & Headon D. R. 1993. Enzymes in the animal-feed industry. TIBTECH. 11: 424-429.

Williams, P. E. V., P. A. Geraert & G. Annison. 1997. Factors affecting non-starch polysaccharide digestibility in poultry. South European Feed Manufacturers Conference, Reus, Spain.

Zhang, Z., Yu G., Guan H., Zhao X., Du Y. & Jiang X. 2004. Preparation and structure elucidation of alginate oligosaccharides degraded by alginate lyase from vibro sp. 510. Carbohydrate Research. 339(8): 1475-1481.

Chapter 3

Effect of extrusion temperature on water stability of enzyme supplemented abalone feed

3.1 Abstract

A study was conducted to evaluate the effect of five extrusion temperatures (57.34 ± 1.31 °C, 61.88 ± 2.33°C, 65.38 ± 1.26°C, 68.20 ± 1.42°C, 68.88 ± 1.28°C) on the water stability (WS) of abalone feed. The best result was obtained at 68.88 ± 1.28°C, yielding an extruded feed with significantly better water stability than at any of the other temperatures. Results from this study indicate that water stability is significantly affected by extrusion temperature, which may compromise the potential inclusion of enzymes in abalone feed due to their thermo-instability.

Key words: gelatinisation, pellet stability, *Haliotis* midae, soybean meal

3.2 Introduction

Temperatures during conventional extrusion and drying of aquaculture feed range from 80-130°C (Jayaram & Shetty, 1981; Inborr & Bedford, 1994; Barrows *et al*., 2006; Sørensen *et al*., 2008; Kraugerud & Svihus, 2010). Enzymes tend to denature when exposed to high temperatures i.e. during extrusion. This may limit the application of heat labile feed enzymes in aquaculture feeds due to a complete loss of activity, as the optimum activities of enzymes range between 40-70°C (Walsh *et al*., 1993). The use of cold extrusion processes may be a solution to this problem, providing processing temperatures appropriate for incorporating enzymes into aquaculture feeds.

Physical pellet quality is determined by the amount of gelatinisation of the starch component of the feed (Chamberline, 2004). The importance of good gelatinisation is not only beneficial for pellet quality and water stability but also for digestion in aquatic species, since most carnivorous aquatics have a limited ability to utilize raw carbohydrates (Sinha *et al*., 2011).

Farmed abalone are generally fed daily rations from 16:00, and only start feeding after dark. During summer, feeding may take place as late as 21:00. (Frik Venter, personal communication). The water stability of the feed is therefore important in ensuring that optimal quantities of dry matter (DM) and nutrients are available for consumption.

The aim of the trial was to evaluate the efficacy of reduced extrusion temperatures on the water stability of the feed. Testing the effect of extrusion temperature on enzyme activity was not possible in this study due to lack of funding for this area.

3.3 Materials and methods

3.3.1 Experimental procedure

The experiment was conducted at the Welgevallen experimental unit of Stellenbosch University's Feed Technology Group. A commercial abalone feed (Aqua Nutro abalone grower phase one, NutroScience (Pty) Ltd, Malmesbury) was extruded at five different temperatures: 57.34 ± 1.31°C, 61.88 ± 2.33°C, 65.38 ± 1.26°C, 68.20 ± 1.42°C and68.88 ± 1.28°C. These different temperatures were achieved by varying the temperature of the mixing water used in the extrusion process (2˚C (T_1), 28˚C (T_2), 50˚C (T_3), 75˚C (T_4) and 80˚C (T_5)). Mixing water was added at a rate of 240ml per kg of feed.

Temperatures were measured throughout the mixing and manufacturing process by a commercial hand-held infrared thermometer (Thermo laser TMTL 500. Panchshil Bearing Company) and measurements were replicated five times during the extrusion of the 10kg batches of feed. After extrusion (post-extrusion phase) an enzyme conditioning phase was simulated by a pre-drying resting period of 45 minutes. Thereafter, feeds were dried in a preheated convection oven for 12 hours at 70°C. **Error! Reference source not found.**, shows the production process used in the production of feed.

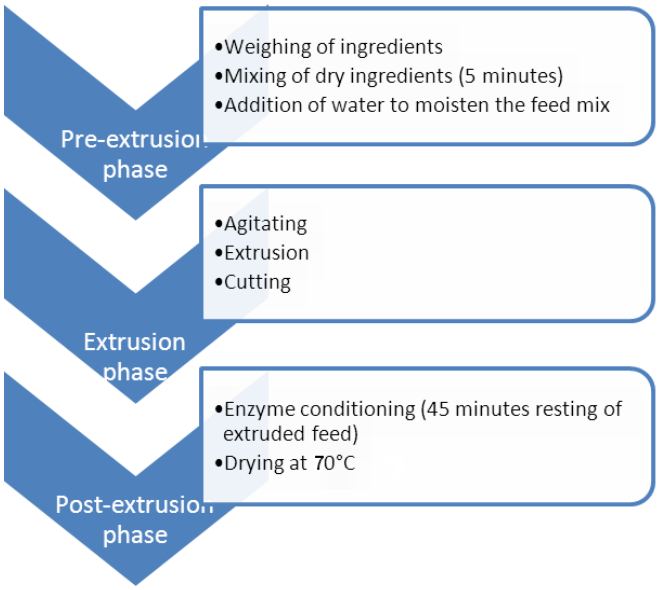


Figure 3.1 Flow chart of abalone feed manufacturing process.

Post-extrusion temperatures were measured at the point where feed left the die-head and were used as the extrusion temperatures. After the oven drying phase, feeds were then left to cool to room temperature after which five 20g samples of each treatment were placed in mesh aeration canisters for the testing of water stability.

The water stability trial was conducted as prescribed by Viljoen (2007), adhering to the methods of Obaldo *et al*., (2002) and Bautista-Teruel *et al*., (2003). Following the feed manufacturing phase, treatment samples of 20g each were weighed on a three decimal UWE HGS-300 scale and placed into closable stainless steel mesh (1mm gauze size) canisters with a 100mm diameter. The canisters were then submersed in an aerated water bath and each treatment was replicated five times (aeration is achieved at an equal rate throughout a uniform system with nozzles placed beneath each canister). Treatments were allocated to three time exposures of four, eight and sixteen hours respectively, after which the respective canisters were removed from the bath and transferred to a drying oven, set at 70°C and dried for twelve hours. Following drying, canisters were weighed again and the relative water stability was calculated as the percentage difference of weight lost from pre- to post-water exposure, as shown below in Equation 3.1.

Equation 3.1 Formula for the calculation of water stability

$$\boldsymbol{Water\ stability\ (WS)} = \left(\frac{\boldsymbol{Final\ feed\ weight\ (g) - \ Initial\ feed\ weight\ (g)}}{\boldsymbol{Initial\ feed\ weight\ (g)}}\right) \times \mathbf{100}$$

3.3.2 Statistical analysis

Regression analyses were carried out on the individual time blocks using PROC REG. Thereafter, a comparison of the slopes between treatments was executed using PROC GLM. A one way ANOVA was also performed in order to determine differences at each time point. All analyses were done using SAS for Windows version 9.1.3.

3.4 Results and discussion

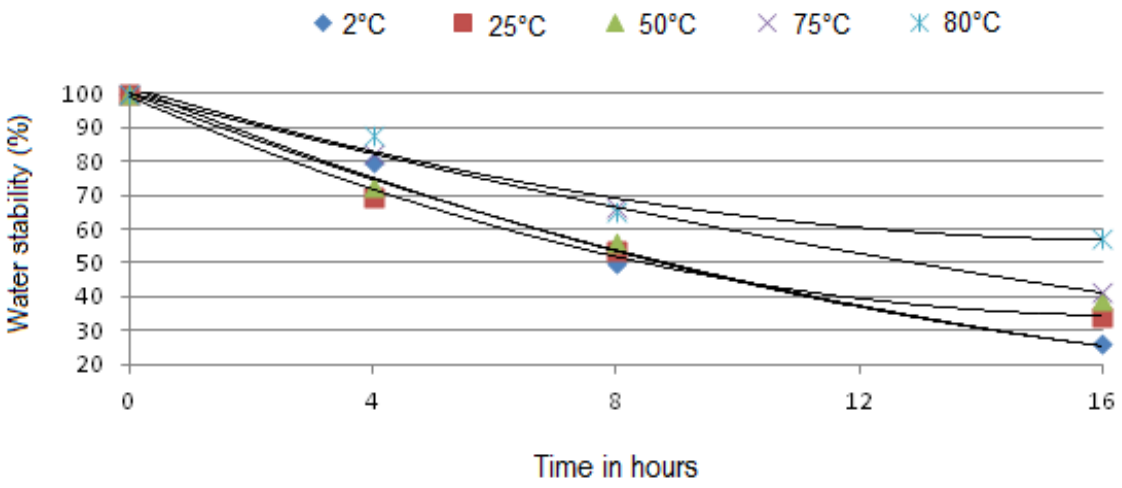
The average temperatures of the mixing water, the post extrusion temperatures and the post 60 minute drying temperatures are shown in **Error! Reference source not found.**3.1.

Table 3.1 Temperature of feed through processing phases.

Treatment	Water temperature (°C)	Dry feed	Pre-extrusion phase	Post-extrusion phase (TE)	Oven drying phase
		Duration of phase			
		0 minutes	15 minutes	45 minutes	60 minutes
T_1	2	26.60 ± 1.41	23.06 ± 0.30^{e}	57.34 ± 1.31^{d}	60.98 ± 1.44^{d}
T_2	25	26.60 ± 1.41	28.14 ± 0.28^{d}	61.88 ± 2.33^{c}	68.12 ± 0.93^{c}
T_3	50	26.60 ± 1.41	33.06 ± 0.82^{c}	65.38 ± 1.26^{b}	66.08 ± 1.94^{bc}
T_4	75	26.60 ± 1.41	41.40 ± 0.92^{b}	68.20 ± 1.42^{ab}	69.2 ± 1.46^{ab}
T_5	80	26.60 ± 1.41	42.96 ± 0.72^{a}	68.88 ± 1.28^{a}	71.14 ± 0.39^{a}
P-value		-	0.0001	0.0001	0.0001

$^{a-b}$ Means with a common superscript are not significantly different (P>0.05); n=5

Results for water stability are shown in **Error! Not a valid bookmark self-reference.**, while the regressions describing the rate of change in dry matter loss are shown in **Error! Reference source not found.** and



3.1.

Table 3.2 Average water stability (%) of treatments over 16 hour water exposure (expressed on an as-is basis).

	Time (hours)

Treatment	Temperature	0	4	8	16
T_1	2	100.00 ± 0	78.96 ± 1.56^{b}	49.84 ± 2.67^{b}	26.05 ± 3.42^{c}
T_2	28	100.00 ± 0	78.26 ± 2.12^{b}	53.77 ± 1.84^{b}	34.14 ± 5.19^{b}
T_3	50	100.00 ± 0	80.55 ± 2.21^{b}	55.99 ± 3.88^{b}	39.22 ± 1.37^{b}
T_4	75	100.00 ± 0	82.31 ± 2.31^{b}	66.31 ± 2.57^{a}	41.16 ± 2.82^{b}
T_5	80	100.00 ± 0	87.84 ± 2.10^{a}	65.37 ± 3.49^{a}	57.31 ± 5.54^{a}
	P-value	0.00	0.0001	0.0001	0.0001

$^{a-b}$ Means with a common superscript are not significantly different (P>0.05); n=5

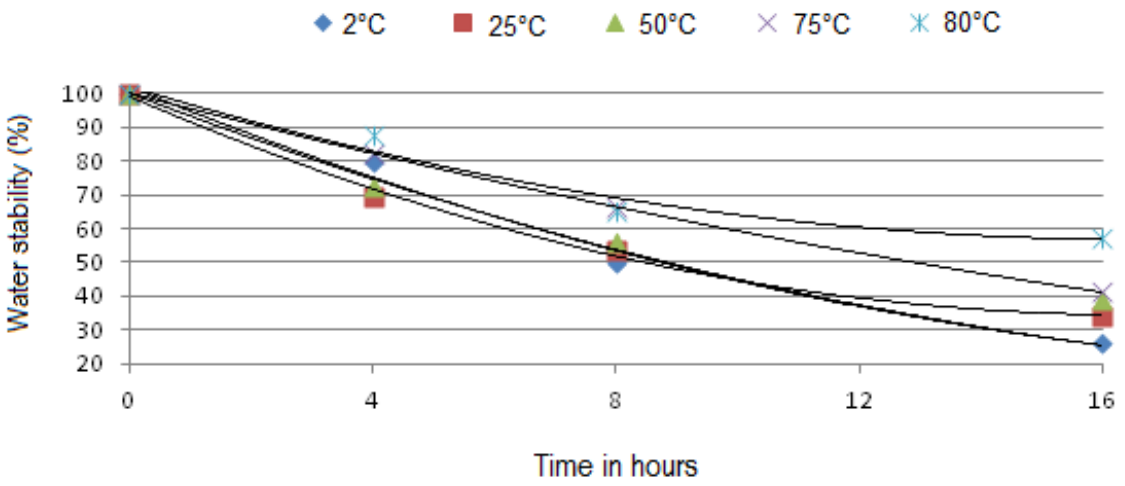


Figure 3.2 Regressions of water stability over time, data points indicate water temperature.

All treatment temperatures differed significantly (P<0.05) from each other in the pre-extrusion phase (PT). In the post- extrusion and oven drying phases T_5 and T_4 did not differ significantly (P>0.05). For the pre-, post-extrusion and oven drying phase, T_1 resulted in significantly cooler (P<0.05) temperatures in comparison to the other treatments.

Table 3.3 Regression equations and R-square values for WS over time.

Treatment	Regression equation	R^2	Significance*
T_1	$y = 0.14x^2 – 6.89x + 100$	0.99	C
T_2	$y = 0.24x^2 -7.98x + 100$	0.99	Bc

T_3	$y = 0.23x^2 – 7.46x + 100$	0.99	B
T_4	$y= 0.065x^2 – 7.72x + 100$	1.00	B
T_5	$y= 0.14x^2 – 4.94x + 100$	0.96	A

*Slopes with different letters, differ significantly (P<0.05)

y= WS %; x= time in hours

At four hours, the T_5 treatment demonstrated significantly (P<0.05) better water stability in comparison to the other treatments, which did not differ significantly (P>0.05) from each other. At eight hours, the T_5 and T_4 treatments did not differ statistically from each other, although they were significantly (P<0.05) more water stable than all other treatments, which did not differ significantly from each other. At sixteen hours, T_5 was again significantly more water stable (P<0.05) than all the other treatments, while T_1 was significantly (P<0.05) less water stable than the remaining treatments.

The best performing feed, T_5, differed in dry matter loss from the worst performing T_1. For water stability, T_5 and T_1 differed by 10.11% over four hours, 43.22% over eight hours and 54.44% over sixteen hours. All other treatments (T1, T_2, T_3 and T_4) lost dry matter at a rate significantly faster than T_5. These findings are substantiated by the findings of Chamberlin (2004) whose results speculated that gelatinisation of feed begins at a minimum temperature range of 55-85°C, with gelatinisation (and thus water stability) improving as the temperature increases (as was seen in this study). A lack of pellet gelatinisation reduces the ability of starch to support pellet integrity in the water column, thus resulting in dry matter loss in feeds with inferior gelatinisation, as was observed. It can thus be concluded that the high levels of dry matter losses noted at lower extrusion temperatures could be attributed to insufficient gelatinisation of carbohydrates in the feed.

The slopes of the regression equations express the rate of dry matter loss from the feed. The slopes of all the treatments differed significantly (P<0.05) from T_5, while the slopes of T_3 and T_4 also differed significantly (P<0.05) from that of T_1.

For the T_5 treatment, the average pre-extrusion temperature was 42.96°C with an average post-extrusion temperature of 68.88°C. A standing period (post-mixing with water) may be necessary to allow sufficient interaction between substrate and enzyme before the possible denaturing of the enzyme during processing as temperatures near 70°C are attained. Treatment T_5 lost dry matter at a rate significantly slower (P<0.05) than the other

treatments and thus had a significantly better ($P<0.05$) WS than the other treatments. This means that for the other feeds, there will be significantly less dry matter reaching the abalone, resulting in fewer nutrients being consumed. The excessive loss of dry matter and nutrients from feed can directly lead to economic losses for producers as well as the potential degradation of the aquatic environment on the farm. For the animals, this may lead to increased stress, increased risk of disease and decreased growth (Tacon, 1997).

In order to ensure good pellet binding and starch gelatinisation, using water hotter than 80°C in mixing would therefore prove highly beneficial in relation to water stability. However, in using water of such a high temperature, one would need to address the question of how enzymes should be added to the feed under these conditions. Enzymes could be mixed in a smaller quantity of warmer water (<60°) after the separate addition of "hot" water. The "cooler" enzyme water could then be slowly poured into the feed whilst mixing. Due to the fact that the pre-extrusion temperature, after five minutes mixing with 80°C water, had an average temperature of 42.96 ± 3.1°C (a temperature suitable for enzyme activity), improved enzyme activity could be expected when using this method (Walsh *et al*., 1993). The hot water would provide a good base for adequate binding of the feed post-extrusion, whilst the cooler water and warm feed would not denature the enzymes.

3.5 Conclusion

The results obtained from this study show that extrusion temperature has a significant effect ($P<0.05$) on feed water stability. Feed extruded at 68.88 ± 1.28°C (T_5) proved to be significantly ($P<0.05$) more water stable than the other four treatments (T_1, T_2, T_3 and T_4 extruded at 57.34 ± 1.31 °C, 61.88 ± 2.33°C, 65.38 ± 1.26°C and 68.20 ± 1.42°C respectively), also losing dry matter at a rate significantly slower ($P<0.05$) than these four treatments.

Water temperature above 80°C is recommended for use during pelleting to ensure the best possible water stability. Enzymes, though, should be mixed into the feed using cooler (<60°C) water after the addition of the hot water. The pre-extrusion temperature of such a mixture would be conducive for enzyme activity (<60°C). An enzyme conditioning phase is suggested to allow enzymes to actively interact with their substrate as, although enzyme activity is possible, it cannot be guaranteed throughout the extrusion process.

3.6 References

Barrows, F. T., Stone, D. A. J. & Hardy, R. W., 2006. The effects of extrusion conditions on the nutritional value of soybean meal for rainbow trout (*Oncorhynchus mykiss*). Aquaculture. (265): 244-252.

Bautista-Teruel, M. N., Fermin, A. C. & Koshio, S. S., 2003. Diet development and evaluation for juvenile abalone, *Haliotis asinina*: Animal and plant protein sources. Aquaculture. (219): 645-653.

Chamberline, G. W. 2004. Re-emergence of extruded shrimp feed. Aqua Feeds; Formulation and Beyond. 1(2): 18-20.

Inborr, J., & Bedford, M. R. 1994. Stability of feed enzymes to steam pelleting during feed processing. Animal Feed Science Technology 46(3-4): 179-196.

Jayaram, M. G., & Shetty, H. P. C. 1981. Formulation, processing and water stability of two new fish feeds. Aquaculture. (23): 355-359.

Kraugerud, O. F. & Svihus, B. 2010. Effects of online pretreatment of plant ingredients on processing responses and physical properties in extruded fish feed. Animal Feed Science Technology In Press, Corrected Proof.

Obaldo, L. G., Divakaran, S. & Tacon, A. G. 2002. Method for determining the physical stability of shrimp feeds in water. Aquaculture Research. (33): 369-377.

Ratnayake, W. S. & Jackson, D. S. 2006. Gelatinisation and solubility of corn starch during heating in excess water: New insights. Journal Agriculture Food Chemistry (54): 3712-3716.

Sinha, A. K., Kumar, V., Makkar, H. P. S., De Boeck, G. & Becker, K. 2011. Non-starch polysaccharides and their role in fish nutrition – A review. Food Chemistry. 127(4): 1409-1426.

Sørensen, M., Stjepanovic, N., Romarheima, O. H., Krekling, T. & Storebakken, T. 2008. Soybean meal improves the physical quality of extruded fish feed. Animal Feed Science and Technology. (149): 149-161.

Tacon, A. G. J. 1997. Review of the state of world aquaculture. 2011(12 July): 163.

Walsh, G. A., Power, R. F. and Headon, D. R. 1993. Enzymes in the animal-feed industry. TIBTECH. (11): 424-429.

Chapter 4

Evaluation of dry matter leaching in fish meal replaced and enzyme treated abalone feed

4.1 Abstract

This study was comprised of two phases: a fish meal replacement phase (Phase A) and an enzyme treatment phase (Phase B). Diets used in Phase A consisted of a control fish meal diet (Control=22%FM, 0%SBM), a fish meal-soybean meal diet (FMSBM=20%FM, 15%SBM), a soybean meal-low diet (SBMlow=0%FM, 15%SBM) and a soybean meal diet (SBM=0%FM, SBM30%). In Phase B, the FM diet and SBM diet were used as basal diets (FME0 and SBME0). These diets were then treated with three commercial enzyme products, namely, a β- glucanase (FME1 and SBME1), xylanase (FME2 and SBME2) and α-D-galactosidase (FME3 and SBME3). Subsequently, all three enzymes were combined to make two treatments (FME123 and SBME123). In each phase the 22% fish meal (FM) diet acted as the positive control. Results of Phase A showed no significant differences in the rate of dry matter loss between treatments over sixteen hours. The water stability of SBM and FMSBM differed significantly from the FM control over eight hours. In Phase B, the rate of dry matter loss of the control diet (FME0) was significantly slower in comparison to the FME1, SBME1, SBME3 and SBME123 diets. Differences were observed over four, eight and sixteen hour time periods as well. A significant loss of ash, neutral detergent fibre and nitrogen-free carbohydrates (NFC) in the fish meal diet, and ash and neutral detergent fibre in the soybean diet, were noted in pre- and post- sixteen hour water exposed feeds. Enzyme treated soybean meal results in a more rapid loss of dry matter than most fish meal diets, but soybean meal does not significantly reduce sixteen hour water stability. Therefore, soybean meal could be used as a fish meal replacement without significant effects on water stability. Further work is required to accurately quantify the extent of nutrient leaching.

Key words: water stability, nutrients, water exposure, pellet, non-starch polysaccharides, *Haliotis midae*,

4.2 Introduction

Abalone are slow moving nocturnal feeders (Britz, 1996), exhibiting slow feeding behaviour. This behaviour, coupled with the fast physical disintegration of conventional pelleted feeds, means that these pellets are unsuitable for use as abalone feed. Water stable feeds with the ability to remain stable for extended periods are thus important for optimal feed intake (Britz, 1996; Fleming *et al*., 1996).

Feed water stability refers to the percentage dry pellet recovered after immersion in water for a given amount of time (Fleming *et al*., 1996; Friedman, 1996; Denstadli *et al*., 2011). The stability required is between 16-36 hours for abalone, with feed losing over 30% of its dry-matter weight during this period (Fleming *et al*., 1996; Chamberline, 2004; Sales, 2004). Water soluble nutrients like, sugars, starches, salts, amino acids (AA's) and water-soluble vitamins are easily lost due to this degradation (Gatlin III, 2003; Lunger *et al*., 2005; Suresh, 2006; Li *et al*., 2009).

Due to sustainability awareness and its finite supply, fish meal (FM) as a primary protein source in abalone feeds is sought to be replaced by plant-protein alternatives, such as soybean meal (Welch *et al*., 2010). Complete replacement of fish meal in abalone diets with viscera-soybean meal (Guzm´an & Viana, 1998) and soybean meal (Sales, 2004), have shown significantly poorer water stability when compared to a control fish meal diet (Knauer *et al*., 1993; Guzm´an & Viana, 1998; Bautista-Teruel *et al*., 2003).

The impact of soybean meal replacement on water stability is still, however, not fully known and needs to be investigated to establish confidence in its use (Glencross, 2007; Refstie, 2007). The aim of this trial was to evaluate the water stability of both fish meal replaced and enzyme treated abalone diets as quantified by leaching and disintegration losses over a simulated time period. The trial was divided into two phases. Phase A tested water stability and leaching parameters in fish meal replaced diets, whilst Phase B tested these parameters in enzyme supplemented fish meal and soybean meal diets.

4.3 Materials and methods

4.3.1 Treatments and design

This experiment was performed at the Welgevallen experimental farm, Stellenbosch University. The trial consisted of two phases. For Phase A, four experimental diets were formulated and produced in an experimental feed mill. Diets used in Phase A consisted of a control fish meal diet (Control=22%FM, 0%SBM), a fish meal-soybean meal diet (FMSBM=20%FM, 15%SBM), a soybean meal-low diet (SBMlow=0%FM, 15%SBM) and a soybean meal diet (SBM=0%FM, SBM30%). In Phase B, the FM diet and SBM diet were used as basal diets (FME0 and SBME0). These diets were then treated with three commercial enzyme products, namely, a β- glucanase (FME1 and SBME1), xylanase (FME2 and SBME2) and α-D-galactosidase (FME3 and SBME3). Subsequently, all three enzymes were combined to make two treatments (FME123 and SBME123). The treatments used in Phase A and B are given in Tables 4.1 and 4.2 respectively.

Table 4.1 Treatment diets used in Phase A: fish meal replacement phase.

Treatment composition	Treatment code
SBM0 (FM22, SBM0)	FME0 (control)
S15 (FM20, SBM15)	FMSBM
SBM15 (FM0, SBM15)	SBMlow
SBM30 (FM0, SBM30)	SBME0

Fish meal (FM), soybean meal (SBM), Fish meal and soybean meal (FMSBM) and low soybean meal (SBMlow), number indicates percent inclusion of ingredient. FM,SBM: refers to % inclusion of fish meal and soybean meal

Table 4.2 Phase B: enzyme treatment phase.

Treatment composition	Treatment code			
	β-glucanase (E1) (200ppm)	Xylanase (E2) (200ppm)	α-galactosidase (E3) (100ppm)	Combination (E123)
SBM0 (FM22, SBM0)	FM E1	FM E2	FM E3	FM E123

SBM30 (FM0, SBM30)	SBM E1	SBM E2	SBM E3	SBM E123

Fish meal (FM), soybean meal (SBM), E (enzyme), number indicates percent inclusion of ingredient. FM,SBM: refers to % inclusion of fish meal and soybean meal

In Phase A, four treatments in the form of fishmeal reduced diets were used, whilst a total of ten enzyme treated diets were included as treatments in Phase B. The compositions of the test diets are given in

Table 4.3 Composition of experimental diets and proximate analysis of diets (%).

	Diet S0 (F22)[1]	S30 (F0)[2]	S15 (F20)[3]	S15 (F0)[4]
Formulations				
Fish meal 65	22	0	20.28	0
Soybean 46	0	30	15	15
Maize	40	26	34.56	30.75
Abalone binder	22	22	22	22
Poultry by-product meal 65	9.99	15.49	5.94	21.79
Hamlet protein 300	2.07	0	0	4.38
L-lysine HCL	2	0.84	0	0.78
Proximate analysis				
Dry matter	100	100	100	100
Ash	7.87	8.57	9.27	9.68
Crude fiber	3.25	3.70	2.61	5.19
Crude fat	3.88	3.25	3.96	3.07
Crude protein	35.76	35.69	38.72	36.80
Nitrogen free extract	46.98	46.68	42.49	44.01

*Vitamin and mineral premix ingredient composition is proprietary information
Diet superscripts 1 & 2 indicate enzyme treated feeds, whilst superscripts 1, 2, 3 & 4 indicate fish meal replaced feeds compositions

4.3. Listed in the table are the four treatments used in Phase A (fish meal replacement). Diet S0 (F22) and diet S30 (F0) were used in Phase B (enzyme treatment phase) and treated with the three individual enzymes (E1, E2 and E3) as well as the three enzymes in combination (E123). In both phases an abalone binder was included at 22%.

Table 4.3 Composition of experimental diets and proximate analysis of diets (%).

	Diet S0 (F22)[1]	S30 (F0)[2]	S15 (F20)[3]	S15 (F0)[4]
Formulations				
Fish meal 65	22	0	20.28	0
Soybean 46	0	30	15	15

Maize	40	26	34.56	30.75
Abalone binder	22	22	22	22
Poultry by-product meal 65	9.99	15.49	5.94	21.79
Hamlet protein 300	2.07	0	0	4.38
L-lysine HCL	2	0.84	0	0.78
Proximate analysis				
Dry matter	100	100	100	100
Ash	7.87	8.57	9.27	9.68
Crude fiber	3.25	3.70	2.61	5.19
Crude fat	3.88	3.25	3.96	3.07
Crude protein	35.76	35.69	38.72	36.80
Nitrogen free extract	46.98	46.68	42.49	44.01

*Vitamin and mineral premix ingredient composition is proprietary information
Diet superscripts 1 & 2 indicate enzyme treated feeds, whilst superscripts 1, 2, 3 & 4 indicate fish meal replaced feeds compositions

The standard fish meal diet with no enzyme addition acted as the positive control in both experiments. The feed was weighed using an OHAUS SD75L bench scale and UWE HGS-300 scale. A commercial mixer was also used and the feed was cold extruded through a 10x10mm die-head (<72°C ± 5.2, die head temperature). The feed was dried for twelve hours at 60°C in a convection oven. The three enzyme products used were : endo -1, 3(4) - β- glucanase (E1, Pentopan® Mono BG, 2500FXU-W/g), endo-1,4-xylanase (E2, Ultraflo® Max, 250 FXU-S/g) and. α-D-galactoside (E3, Alpha-Gal™ 600L, 600 GALU/g). These enzymes were sourced from Novozymes, South Africa, and were included at levels recommended by the supplier: 200g/ton for β-glucanase and xylanase and 100g/ton for α-galactosidase.

4.3.2 Physical analysis of feed

The water stability of the feed was determined over zero, four, eight and sixteen hours respectively. Five replicates (10 ± 0.05 grams) of each of the twelve treatments were placed into individual mesh canisters of known mass. Canisters (approximately 200g, 10cm diameter, 1mm mesh gauze) were marked and submersed in an aerated water bath, with nozzles beneath each canister of the water stability evaluation unit. The water temperature was recorded at 18 ± 0.2°C and the salinity (<0.05ppm) was that of freshwater (Obaldo *et al*., 2002).

After each of the submersion periods the samples were carefully removed from the tank and transferred to a convection oven for twelve hours at 70°C. After drying, they were then weighed to determine the percentage weight loss of feed to water. The water stability was

determined by calculating the percentage difference of weight lost from pre- to post- water exposure as shown in Equation 2.

Equation 4.2 Formula for the calculation of water stability

$$\boldsymbol{Water\ stability\ (WS)} = \left(\frac{\boldsymbol{Final\ feed\ weight\ (g) - Initial\ feed\ weight\ (g)}}{\boldsymbol{Initial\ feed\ weight\ (g)}}\right) \times \mathbf{100}$$

In this trial the Rapid Water Stability test of Viljoen (2007) was used, as it amplifies differences. Figure 4.1 and **Error! Reference source not found.**4.2 show the feed pre-submersion, the water bath and aerated water as well as the feed post sixteen hour exposure.

Figure 4.1 From left-to-right; the water bath used and close-up view of the aeration chambers.

Figure 4.2 From left-to-right; fish meal, fish meal-soybean meal, soybean meal low and soybean meal diets after sixteen hour water exposure.

4.3.3 Chemical analysis of feed

The chemical analysis was performed in the laboratory of the Department of Animal Science, Stellenbosch University. Proximate analyses of the pooled feed treatments for various nutrients were analysed as prescribed by the Association of Official Analytical Chemists (AOAC). Crude fibre, crude fat, ash, moisture, protein, acid detergent fibre (ADF) and neutral detergent fibre (NDF) were determined (Goering & van Soest, 1970; AOAC, 2002a; AOAC, 2002b; AOAC, 2002c; AOAC, 2002d). Feed unexposed to water was analysed for the above mentioned nutrients as well as feed after sixteen hour water exposure. For Phase A, the four feeds were analysed (n=2), whilst in Phase B, the FME0 and SBME0 treatments were analysed (n=5) as individual treatments. Nutrient leaching remains largely undetermined when referring to water stability, with only the dry matter loss being emphasized (Shipton, 1999; Sales & Britz, 2001; Tsanigab, 2009; Green *et al*., 2011).

Three enzyme assay procedures (test kits) were obtained from Megazyme™, Ireland. These assays were used to test the in-feed NSP levels in the fish meal and soybean meal diets respectively. Levels of D-xylose, β-glucan and α-D-galactose were tested by procedures developed by McCleary (2003), with duplicate values for each assay obtained.

Reagents were obtained from the Department of Polymer Science, Stellenbosch University. Levels of NSP's in the fish meal and soybean meal diets are given in **Error! Reference source not found.**3.

4.3.4 Data analysis

Regression analyses were carried out on the individual time blocks using PROC REG. Thereafter, a comparison of the slopes between treatments was executed using PROC GLM. The model included the intercept as a covariate so that the results were adjusted for any differences in the intercept of the individual canisters. A one way ANOVA was also done to determine differences at each time point. All analyses were done using SAS for Windows version 9.1.3.

4.4 Results and discussion

4.4.1 In-feed non-starch polysaccharide levels

In-feed non-starch polysaccharide levels are given in Figure 4.3. The levels of non-starch polysaccharides in soybean meal were significantly higher ($P<0.05$) than those found in fish meal for all three non-starch polysaccharides tested.

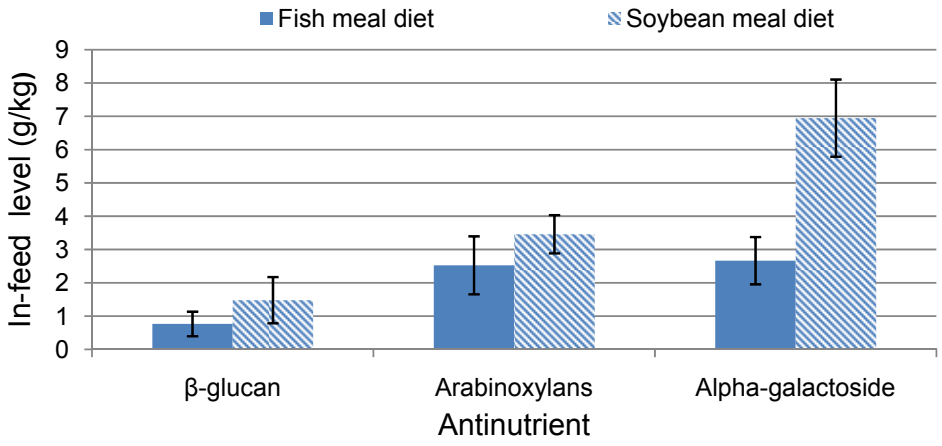


Figure 4.3 In-feed non-starch polysaccharide levels for fish meal and soybean meal based diets (± standard deviation indicated by bars; n=5).

4.4.2 Phase A: Fish meal replacement

4.4.2.1 Dry matter loss/ degradation

The results in **Error! Reference source not found.**4 and 4.5 and **Error! Reference source not found.**4.4 reveal that none of the Phase A diets, i.e. FM, FMSBM, SBMlow

and SBM, showed any significant difference (P>0.05) in relation to dry matter loss or water stability over the four, eight or sixteen hour intervals. Similarly, none of the regression slopes of the treatments differed significantly (P>0.05) from each other. It can therefore be concluded that the rate of dry matter loss of the fish meal replacement diets did not differ significantly (P<0.05) from that of the control fish meal diet.

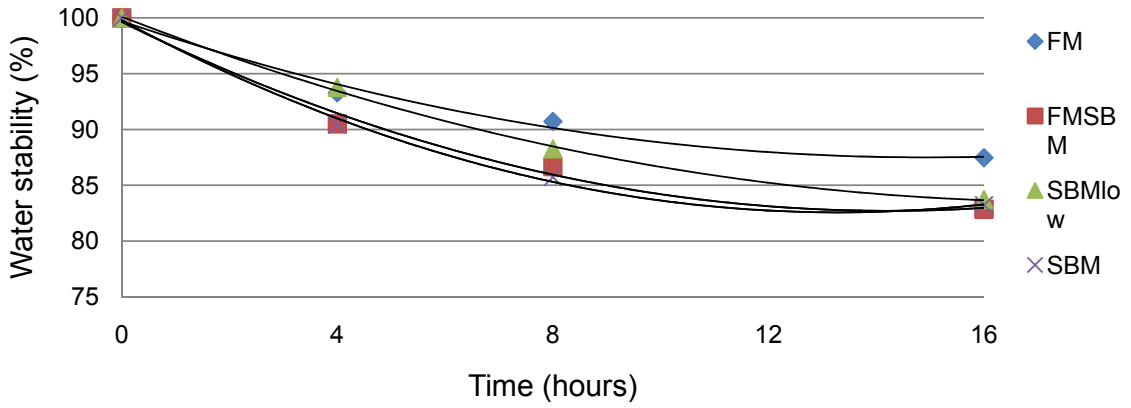


Figure 4.5 Dry matter loss of diets T1, T6, T7 and T8 over zero, four, eight and sixteen hours.

FM: fish meal, SBM: soybean meal, FMSBM: fish meal-soybean meal, SBMlow: low soybean meal.

Table 4.4 Regression analysis of fish meal-replaced diets over sixteen hours.

Treatment	Regression equation	R^2	Significance*
Fish meal (control)	$y=0.0546x^2-1.633x + 99.71$	0.99	A
Fish meal- Soybean meal	$y= 0,0837x^2-2.381x+99.64$	0.99	A
Soybean meal low	$y=0.0531x^2-1.878x + 100.12$	0.99	a
Soybean meal	$y= 0.0973x^2-2.587x+99.79$	0.99	a

*Slopes with different letters, differ significantly (P<0.05)

y= water stability %; x= time in hours

The FMSBM, SBMlow and SBM diets had 16.50%, 31.88% and 5.77% less animal-based protein than the FM diet. These diets also had 16%, 12.57% and 9.56% more plant-based protein sources than the FM diet. The results indicate that fish meal can be replaced by soybean meal over a wide range of inclusion levels without significantly affecting pellet water stability.

Table 4.5 Water stability (%) over time (zero, four, eight and sixteen hours) for fish meal replaced diets.

Treatment	N	Code	Time 0	4	8	16
Fish meal	5	FM	100 ± 0[a]	93.28 ± 1.78[a]	90.72 ± 1.30[a]	87.46 ± 2.64[a]
Fish meal- soy bean	5	FMSBM	100 ± 0[a]	90.50 ± 2.79[a]	86.67 ± 1.94[a]	82.86 ± 3.40[a]
Soybean low	5	SBMlow	100 ± 0[a]	93.77 ± 1.34[a]	88.26 ± 1.75[a]	83.72 ± 4.71[a]
Soybean meal	5	SBM	100 ± 0[a]	90.43 ± 1.23[a]	85.74 ± 1.73[a]	83.22 ± 4.15[a]
P-value			0.00	0.20	0.15	0.24

[a-b] Means within a column with a common superscript are not significantly different (P>0.05);n=5

The impact and importance of pellet quality (due to gelatinisation) has been described by Flemming *et al.*, (1996), Kraugerud & Svihus (2011), Denstadii *et al.*, (2011) and Sinha *et al.,* (2011), with specific emphasis placed on the significant differences (P<0.05) in pellet quality observed between cereal and legume based pellets (Denstadli *et al*., 2011; Sinha *et al*., 2011). It has been shown that legumes do not gelatinise as well as cereals and decreased gelatinisation results in poorer water stability (Kraugerud & Svihus, 2010; Krogdahl *et al*., 2010; Sinha *et al*., 2011). These findings were, however, not observed in this trial. This could be the case as a result of the effect of the starch binder at a 22% inclusion level which may have mitigated the effect of any improper gelatinisation.

The absence of significant differences (P>0.05) observed over four, eight and sixteen hours could also be as a result of the binder, as the effect of the binder has the largest effect on overall water stability as suggested by Flemming *et al*, (1996). During hot extrusion, 80-95% of starch can be gelatinised (Chamberline, 2004; Zhenhua, 2011), allowing good pellet binding, with the inclusion of binder into the formulation further improving pellet binding.

Binder compositions are mostly proprietary information and therefore comparisons between trials and various binders are difficult due to the secretive nature of the binder composition (Fleming *et al*., 1996).

4.4.2.2 Nutrient leaching

Error! Reference source not found.4.6 shows the proximate analysis of the diets pre- and post- sixteen hour water exposure. In all four diets, a decrease in ash was observed, whilst an increase in the concentration of neutral detergent fibre (NDF) and nitrogen-free carbohydrate (NFC) was observed. Ash contains the mineral component of the diet. These

mineral losses as well as the loss of water soluble vitamins are of concern in abalone diets as they are important in shell growth (Fleming *et al*., 1996). The increase in percentage of NDF could be due to the relative loss of soluble carbohydrates and other nutrient fractions which result in the insoluble portion (NDF) increasing in percentage feed fraction. No statistical analysis could be done because samples were pooled for analysis. This aspect warrants further investigation.

Table 4.6 Proximate analysis of fish meal replaced feeds pre- (zero hours) and post (sixteen hours) water exposure.

	FM 0	FM 16	FMSBM 0	FMSBM 16	SBMlow 0	SBMlow 16	SBM 0	SBM 16
Ash	7.87	7.34	9.27	8.19	9.68	6.47	10.03	8.36
Crude fibre	3.26	2.32	2.61	2.74	5.15	4.65	3.69	4.30
Crude fat	3.88	3.82	3.96	3.62	3.07	3.08	2.69	2.81
NDF	7.00	17.84	22.05	30.51	19.85	28.63	13.42	23.41
ADF	6.41	5.54	13.84	8.70	5.97	8.37	16.84	10.21
CP	35.76	34.42	38.72	42.34	36.80	39.89	35.69	37.49
NFC	49.23	52.11	45.44	43.11	45.30	45.90	47.90	47.04

FM=fish meal, SBM= soybean meal, FMSBM=fish meal-soybean meal combination and SBMlow= low soybean meal replacement; n=2.
NDF: neutral detergent fibre; ADF: acid detergent fibre; CP: crude protein; NFC: nitogen-free carbohydrate

4.4.3 Phase B: Enzyme treatment phase

4.4.3.1. Dry matter loss/ degradation

The results, expressed as regression equations, are presented in **Error! Reference source not found.**4.5 and **Error! Reference source not found.**showing the significant differences ($P<0.05$) between the diets in the rate of dry matter loss over time.

The rate of dry matter loss for FME1 (β-glucanase) treatment, SBME1 (β-glucanase), SBME3 (α-galactosidase) and SBME123 (combination), were significantly higher ($P<0.05$) than the control (FME0) and SBME0 treatments. The significant ($P<0.05$) water stability differences over time for the fish meal and soybean meal diets occurred after four, eight and sixteen hours exposure. The control, FME0, differed significantly ($P<0.05$) from SBME1, SBME3 and SBME123 over four hours and FME3 and FME123 differed from SBME123. FME0 differed significantly ($P<0.05$) from FME1, SBME1, SBME3 and SBME123, with FME3 differing from SBME2 and SBM123 over the eight hour exposure. Over the sixteen hour exposure, only FME0 and SBME123 differed significantly ($P<0.05$). The control treatment, FME0, showed the best water stability after sixteen hours, only losing 12.54% of its dry matter. Out of the ten feeds, SBME0 had the third best water stability after sixteen hours, only losing 16.78% of its dry matter and never differing

significantly from (P>0.05) FME0. This is surprising as legume based feeds were expected to have poorer water stability due to poor gelatinisation of their carbohydrates (Denstadli *et al*., 2011; Sinha *et al*., 2011). The worst performing feed over the sixteen hour period was SBME123, which lost 23.64% of its dry matter. The four, eight and sixteen hour differences correspond with the treatments which differed significantly (P<0.05) for the rate of dry matter loss.

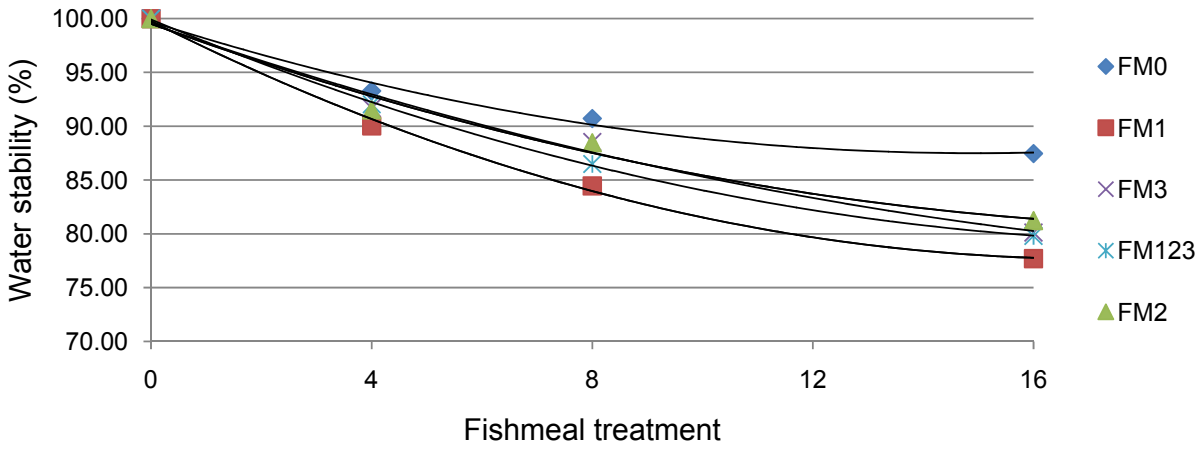


Figure 4.6 Water stability of enzyme treated fish meal diets over time.

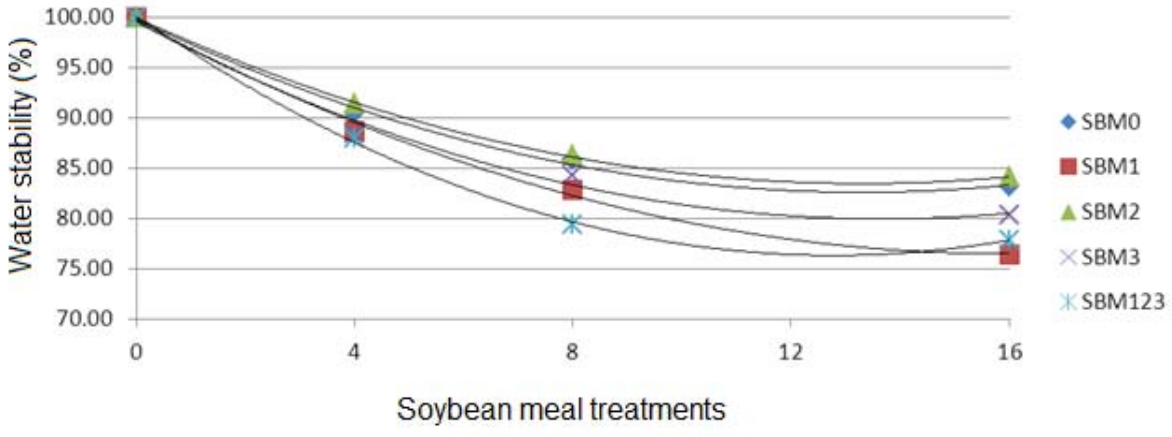


Figure 4.7 Water stability of enzyme treated soybean meal diets over time.

Table 4.7 Regression equations and R-squared values for the enzyme treated fish meal and soybean meal abalone feeds.

Treatment	Regression equation	R^2	Significant slope differences*
Fish meal no enzyme(control)	$y= 0.057x^2 - 1.70x + 100$	0.99	A
Fish meal enzyme 1 (β-glucanase)	$y= 0.077x^2 - 2.63x + 100$	0.99	B
Fish meal enzyme 2 (xylanase)	$y= 0.051x^2 - 1.98x + 100$	0.98	A
Fish meal enzyme 3 (α-galactosidase)	$y= 0.041x^2 - 1.89x + 100$	0.98	A
Fish meal enzyme 123 (combination)	$y= 0.056x^2 - 2.16x + 100$	0.99	A
Soybean meal no enzyme	$y= 0.099x^2 - 2.64x + 100$	0.99	A
Soybean meal enzyme 1 (β-glucanase)	$y= 0.094x^2 - 2.98x + 100$	0.99	B
Soybean meal enzyme 2 (xylanase)	$y= 0.094x^2 - 2.49x + 100$	0.99	A
Soybean meal enzyme 3 (α-galactosidase)	$y= 0.11x^2 - 2.98x + 100$	0.99	B

*Slopes with different letters, differ significantly ($P<0.05$)

y= water stability %; x= time in hours

The decreased rate of dry matter loss observed in FME1 and SBME1 may have occurred as a result of β-glucan hydrolyses in the feed. Gums abundant in β-glucans are often used as, or included in, binder formulations due to their viscous nature (O'Mahoney *et al*., 2011). The activity of the β-glucanases may have thus reduced the level of in-feed or binder β-glucans, subsequently decreasing viscosity. The SBME3 (α-galactosidase) may have affected the water stability of the soybean meal diets due to the very high level of soybean meal α-galactosides in the feed (**Error! Reference source not found.**). As a result, lots of substrate was available to be hydrolysed. This indicates that, although the legume NSP's are complex, singular α-galactosidase enzyme supplementation can affect the substrate, allowing for the breakdown of α-galactosides in the feed. These findings differ slightly from the findings of Denstadii *et al*., (2011) who saw no significant differences ($P>0.05$) in plant-based enzyme treated salmon feeds after two hour water stability. It must be noted however that salmon and abalone feeds differ in the way that they are processed due to their differing roles: salmon feeds are immediately consumed, whilst abalone feeds spend extended periods submersed in the water column. The validity of such a comparison is therefore questionable.

The SBME123 treatment's significantly (P<0.05) lower water stability in comparison to the control (FME0) and SBME0 feeds could be as a result of more soluble carbohydrates being cleaved and released from complex insoluble structures (cell walls) due to post-extrusion enzyme action. This indicates possible enzyme activity through processing, due to the fact that pre-extrusion enzyme effect would yield more complex carbohydrates as simple sugars (which would be gelatinised), consequently improving water stability. The continued enzyme activity post pelleting would lead to decreased water stability due to increased water soluble carbohydrates being cleaved by enzymatic hydrolysis. Since only SBME2 does not differ significantly (P>0.05) from the control (FME0) and SBME0, it can be concluded that enzyme inclusion does affect the rate of dry matter loss over time in abalone diets.

Table 4.8 Water stability of enzyme treated fish meal and soybean meal diets.

			Time			
Feed	Treatment	Code	0	4	8	16
FM	FM Control + (E0)	FM E0	100 ± 0	93.28±1.78^{a}	90.72±1.30^{a}	87.46±2.64^{a}
	B-glucan (E1)	FME1	100 ± 0	90.04 ± 0.85^{abc}	84.46 ± 3.73^{bcd}	77.70 ± 4.43^{ab}
	Xylanase (E2)	FME2	100 ± 0	91.52 ± 1.03^{abc}	88.47 ± 1.93^{ab}	81.24 ± 5.56^{ab}
	A-galac (E3)	FME3	100 ± 0	91.69 ± 2.32^{abc}	88.54 ± 2.25^{ab}	80.12 ± 3.53^{ab}
	Combo (E123)	FME123	100 ± 0	92.05 ± 2.18^{ab}	86.49 ± 1.02^{abc}	79.81 ± 4.62^{ab}
SBM	SBM Control (E0)	SBM E0	100 ± 0	90.43 ± 1.23^{abc}	85.74 ± 1.73^{abc}	83.22 ± 4.15^{ab}
	B-glucan (E1)	SBME1	100 ± 0	88.68 ± 1.68^{bc}	82.88 ± 3.43^{bcd}	77.87 ± 6.51^{ab}
	Xylanase (E2)	SBME2	100 ± 0	91.34 ± 0.96^{abc}	86.22 ± 3.22^{abc}	85.09 ± 2.06^{ab}
	A-galac (E3)	SBME3	100 ± 0	88.47 ± 0.64^{bc}	84.27 ± 1.87^{bcd}	80.34 ± 6.57^{ab}
	Combo (E123)	SBME123	100 ± 0	87.89 ± 2.48^{c}	79.40 ± 3.04^{d}	76.36^{b} ± 6.13^{b}
	P-value		0	0.0001	0.0001	0.0188

$^{a-b}$ Means within a column with a common superscript are not significantly different (P>0.05);n=5

FM= Fish meal; SBM= Soybean meal

It has been shown that single enzyme treatments may not be effective in degrading the complex legume NSP's sufficiently (Chesson., 1993; Walsh *et al*., 1993; Fourij, 2007; Sinha *et al*., 2011). Such findings were not observed in this trial since SBME1 and SBME3 diets resulted in significant differences (P<0.05) in dry matter being observedat four (SBME1) and eight (FME1 and SBME1) hours of treatment. The soybean meal diets

contained significantly (P<0.05) more β-glucans, arabinoxylans and α-galactosides than fish meal diets (Figure 4.3). The rate at which dry matter leaches into the water differs between plant-based and fish meal-based diets over four, eight and sixteen hours. After sixteen hours, the slope of the dry matter loss curve appears to plateau showing that pellet degradation may slow down, possibly due to the action of the binder and gelatinisation. It is possible that the effect of the binder largely mitigates the poor pelleting qualities of legumes that have been observed in literature (Kraugerud & Svihus, 2010).

There is currently no known literature on exogenous enzyme supplementation in abalone diets and their effect on water stability. The slow feeding behaviour of abalone requires the use of water stable pellets and high inclusion levels (22%) of a binder, which make pellet characteristics different to the enzyme effects observed by Denstadli *et al*. (2011) in salmon diets. Sales and Britz (2003) concluded that although soybean meal diets appeared to have good nutrient digestibility in abalone, poor water stability of such diets may negatively impact their potential for commercial use. A better understanding of specific non-starch polysaccharides which negatively influence digestion and water stability needs to be quantified (Sinha *et al*., 2011).

4.4.3.2 Nutrient leaching

The results of an analysis of the proximate nutrient compositions of the fish meal and soybean meal-based diets are presented in

The fish meal and soybean meal diets differed from each other in composition as reported in

Table 4.3 Composition of experimental diets and proximate analysis of diets (%).

	Diet S0 (F22)[1]	S30 (F0)[2]	S15 (F20)[3]	S15 (F0)[4]
Formulations				
Fish meal 65	22	0	20.28	0
Soybean 46	0	30	15	15
Maize	40	26	34.56	30.75
Abalone binder	22	22	22	22
Poultry by-product meal 65	9.99	15.49	5.94	21.79
Hamlet protein 300	2.07	0	0	4.38
L-lysine HCL	2	0.84	0	0.78
Proximate analysis				
Dry matter	100	100	100	100

Ash	7.87	8.57	9.27	9.68
Crude fiber	3.25	3.70	2.61	5.19
Crude fat	3.88	3.25	3.96	3.07
Crude protein	35.76	35.69	38.72	36.80
Nitrogen free extract	46.98	46.68	42.49	44.01

*Vitamin and mineral premix ingredient composition is proprietary information
Diet superscripts 1 & 2 indicate enzyme treated feeds, whilst superscripts 1, 2, 3 & 4 indicate fish meal replaced feeds compositions

. The crude protein (CP) values did not differ significantly ($P<0.05$) between the fish meal and soybean meal diets for zero and sixteen hour water exposure. After sixteen hours of water exposure, the fish meal diet differed significantly ($P<0.05$) from the non-exposed fish meal diet for decreased ash and increased NDF and NFC levels in the feed. Ash was expected to be lost to water as it contains water soluble minerals (Fleming *et al*., 1996). The significant increase in NDF is likely to be due to the insolubility of this feed fraction (cell wall, hemi-cellulose, cellulose and lignin). The significant increase ($P>0.05$) in NFC in the feed is possibly due to the gelatinisation of the starch (maize and binder) in the diet, making the component insoluble in water. This component therefore proportionally increases relative to the other nutrients lost.

4.9.

The fish meal and soybean meal diets differed from each other in composition as reported in

Table 4.3 Composition of experimental diets and proximate analysis of diets (%).

	Diet S0 (F22)[1]	S30 (F0)[2]	S15 (F20)[3]	S15 (F0)[4]
Formulations				
Fish meal 65	22	0	20.28	0
Soybean 46	0	30	15	15
Maize	40	26	34.56	30.75
Abalone binder	22	22	22	22
Poultry by-product meal 65	9.99	15.49	5.94	21.79
Hamlet protein 300	2.07	0	0	4.38

L-lysine HCL	2	0.84	0	0.78
Proximate analysis				
Dry matter	100	100	100	100
Ash	7.87	8.57	9.27	9.68
Crude fiber	3.25	3.70	2.61	5.19
Crude fat	3.88	3.25	3.96	3.07
Crude protein	35.76	35.69	38.72	36.80
Nitrogen free extract	46.98	46.68	42.49	44.01

*Vitamin and mineral premix ingredient composition is proprietary information
Diet superscripts 1 & 2 indicate enzyme treated feeds, whilst superscripts 1, 2, 3 & 4 indicate fish meal replaced feeds compositions

. The crude protein (CP) values did not differ significantly ($P<0.05$) between the fish meal and soybean meal diets for zero and sixteen hour water exposure. After sixteen hours of water exposure, the fish meal diet differed significantly ($P<0.05$) from the non-exposed fish meal diet for decreased ash and increased NDF and NFC levels in the feed. Ash was expected to be lost to water as it contains water soluble minerals (Fleming *et al*., 1996). The significant increase in NDF is likely to be due to the insolubility of this feed fraction (cell wall, hemi-cellulose, cellulose and lignin). The significant increase ($P>0.05$) in NFC in the feed is possibly due to the gelatinisation of the starch (maize and binder) in the diet, making the component insoluble in water. This component therefore proportionally increases relative to the other nutrients lost.

Table 4.9 Proximate analysis of fish meal and soybean meal based diets from zero to sixteen hours.

Diet	Ash	Crude fibre	Crude fat	*NDF	*ADF	Crude Protein	NFC*
F0	$8.52^{ab} \pm 0.70$	$3.12^{ab} \pm 0.90$	$3.63^{a} \pm 0.75$	$7.49^{d} \pm 1.73$	$6.97^{b} \pm 2.45$	$34.55^{a} \pm 2.11$	$50.18^{b} \pm 2.90$
F 16	$7.01^{c} \pm 0.50$	$2.62^{b} \pm 0.53$	$3.11^{a} \pm 3.18$	$20.43^{b} \pm 2.04$	$6.82^{b} \pm 1.31$	$36.16^{a} \pm 1.95$	$51.13^{a} \pm 1.97$
S0	$9.07^{a} \pm 0.62$	$3.88^{ab} \pm 0.51$	$2.90^{a} \pm 2.24$	$15.56^{c} \pm 2.70$	$11.96^{a} \pm 4.16$	$35.16^{a} \pm 1.17$	$52.87^{b} \pm 1.01$
S 16	$7.79^{bc} \pm 0.75$	$4.14^{a} \pm 0.34$	$2.78^{a} \pm 0.78$	$24.37^{a} \pm 0.90$	$9.51^{ab} \pm 1.21$	$36.32^{a} \pm 1.95$	$48.97^{b} \pm 1.93$
P	>0.0005	0.0127	0.1103	>0.0001	0.0180	0.0003	>0.0001

[a-d] Means within a column with a common superscript are not significantly different (P>0.05);n=5.

F0 :Fish meal not water exposed; F16: Fish meal water exposed for 16 hours; S0: Soybean meal not water exposed; S16: Soybean meal water exposed for sixteenhours. *NDF: neutral detergent fibre and ADF: acid detergent fibre; NFC: Nitrogen free carbohydrates

The sixteen hour exposed soybean meal diet differed significantly (P<0.05) from the unexposed soybean meal diet for ash and NDF only. It is surprising to note that there was no significant loss (P>0.05) in CP, as crystalline amino acids (AA's) were added to the diets. Possible interactions with the complex legume carbohydrates may have a role in this (Sinha *et al*., 2011). In crayfish diets, similar losses have been observed over eight hour exposure (D'Agaro & Mecatti, 2006). Shrimp diets have been found to have significantly lower concentrations of ash and CP over eight hour exposure as well (Tolomei *et al*., 2003).

Proximate analyses are not adequate in determining observed changes in nutrients, CP, CF, CF and NDF, as they do not allow specific nutrient changes to be observed, preventing identification of the reason for an observed change. As mentioned, AA analysis should be performed in follow up experiments since CP is not sensitive enough to report significant AA changes. It is clear though, that changes do occur in nutrient compositions of feeds due to water exposure, and that the losses differ between feed types. Further research in this area is required so that probable nutrient uptakes and losses can be quantified more accurately. Nutrient delivery (bio-availability), rather than nutrient inclusion, needs to be the formulation focus in aquaculture diets (Suresh, 2006).

4.5 Conclusion

The lack of significant differences (P>0.05) for the rate of dry matter loss and water stability observed between treatments in Phase A, could possibly be explained by the inclusion of the binder (22% inclusion in all diets), which could have mitigated the poor pelleting characteristics of the soybean meal-based diets. In phase B, it appears that the use of β-glucanase (E1), α-galactosidase (E3) or combination enzymes (E123) in legume containing diets, significantly affects binding, resulting in reduced water stability and an increased rate of dry matter loss. The effect these enzymes have on water stability could be due to their interaction with the binder component, possibly reducing its viscosity and thus binding effect.

Proximate analyses are not specific in showing the source or reason for the observed change of nutrients leached. It is thus important that more specific analytical procedures should be used to investigating the rate and level of specific nutrient loss.

The study indicates that fish meal replacement does not affect the water stability of abalone feeds, but supplementation of β-glucanases (E1, in fish meal and soybean meal diets), α-galactosidase (E3) or combination enzymes (E123) does reduce water stability. Nutrient leaching requires more specific analysis to be correctly quantified.

4.6 References

AOAC ed., 2002a. Association of Official Analytical Chemists Inc. Ash of Animal Feed. Official methods of analysis (17th edition) ed. AOAC Official Method 942.05. Arlington, Virginia, USA.

AOAC ed., 2002b. Association of Official Analytical Chemists Inc. Fat (Crude) Or Ether Extract in Animal Feed. Official methods of analysis (17th edition) ed. AOAC Official Method 920.39. Arlington, Virginia, USA.

AOAC ed., 2002c. Association of Official Analytical Chemists Inc. Fibre (Crude) in Animal Feed and Pet Food. Official methods of analysis (17th edition) ed. AOAC Official Method 962.09. Arlington, Virginia, USA.

AOAC ed., 2002d. Association of Official Analytical Chemists Inc. Protein (Crude) in Animal Feed and Pet Food. Official methods of analysis (17th edition) ed. AOAC Official Method. Arlington, Virginia, USA.

Bautista-Teruel, M. N., Fermin, A. C. & Koshio S. S., 2003. Diet development and evaluation for juvenile abalone, *Haliotis asinina*: Animal and plant protein sources. Aquaculture. (219): 645-653.

Britz, P. J., 1996. The nutritional requirements of *Haliotis midae* and development of a practical diet for abalone aquaculture. Doctoral dissertation, Rhodes University Editor.

Chamberline, G. W., 2004. Re-emergence of extruded shrimp feed. Aqua Feeds; Formulation and Beyond. 1(2): 18-20.

Chesson., A., 1993. Feed enzymes. Animal Feed Science Technology 45, (1993): 65-79.

D'Agaro, E., and Mecatti, M. 2006. Comparison among pre-gelatenized starches of different botanical origins used in noble crayfish *Astacus astacus* diets. Bullitin. Francais. Peche Pisioi. (391): 1261-1267.

Denstadli, V., Hillestad, M., Verlhac, V., Klausen, M. & Øverland, M., 2011. Enzyme pretreatment of fibrous ingredients for carnivorous fish: Effects on nutrient utilisation and technical feed quality in rainbow trout (*Oncorhynchus mykiss*). Aquaculture. In Press, Corrected Proof.

Fleming, A. E., Van Bameveld, R. J. & Hone, P. W., 1996. The development of artificial diets for abalone: A review and future directions. Aquaculture. (140): 5-53.

Fourij, J. L., 2007. The effect of multiple-enzyme combinations in maize-soya diets for broiler chickens. Doctoral dissertation, Stellenbosch University.

Friedman, M., 1996. Nutritional value of proteins from different food sources. A review. Journal Agriculture and Food Chemistry. (44): 6-29.

Gatlin III, D. M., 2003. Nutrition and Fish Health In: Fish Nutrition (Third Edition). Eds. John E. Halver, and Ronald W. Hardy Academic Press, San Diego. pp. 671-702.

Glencross, B., 2007. Strategies for assessing feed grains for fish. Harvesting the benefits of grain in aquaculture feeds, Freemantle, Australia.

Goering, H. K. & Van Soest, P.J., 1970. Forage Fibre Analyses (Apparatus, Reagents, Procedures and some Applications). Agricultural Handbook Number 379. USDA-ARS. Washington, USA.

Green, A. J., Cliford, L. W. & Britz, P. J., 2011. The protein and energy requirements of farmed South African abalone *Haliotis midae L*. cultured at optimal and elevated water temperatures. Aquaculture Research. (23): 1-11.

Guzm´an, J. M. & Viana, M. T., 1998. Growth of abalone H*aliotis fulgens* fed diets with and without fish meal, compared to a commercial diet. Aquaculture. (165): 321-331.

Knauer, J., Britz, P. J. & Hecht, T., 1993. The effect of seven binding agents on 24-hour water stability of an artificial weaning diet for the South African abalone, *Haliotis midae*. Aquaculture. 115(3-4): 327-334.

Kraugerud, O. F. & Svihus, B., 2010. Effects of online pretreatment of plant ingredients on processing responses and physical properties in extruded fish feed. Animal Feed Science Technology. In Press, Corrected Proof.

Krogdahl, A., Penn, M., Thorsen, J., Refstie, S. & Bakke, A., 2010. Important antinutrients in plant feedstuffs for aquaculture: An update on recent findings regarding responses in salmonids. Aquaculture Research. 41: 331-344.

Li, P., Mai, K., Trushenski, J. & Wu, G., 2009. New developments in fish amino acid nutrition: Towards functional and environmentally oriented aquaculture feeds. Amino Acids. (37): 43-53.

Lunger, A. N., Craig, S. R. & McLean, E., 2005. Replacement of fish meal in cobia (*Rachycentron canadum*) diets using an organically certified protein. Aquaculture. 257: 393-399.

McCleary, B. V. 2003. Dietary fibre analysis. Nutrition Society. 62: 3-9.

Obaldo, L. G., Divakaran, S. & Tacon, A. G., 2002. Method for determining the physical stability of shrimp feeds in water. Aquaculture Research. (33): 369-377.

O'Mahoney, G., Mouzakitis J., Doyle J., & Burnell G. 2011. A novel konjac glucomannan-xanthan gum binder for aquaculture feeds: The effect of binder configuration on

formulated feed stability, feed palatability and growth performance of japanese abalone, *Haliotis discus hanai*. Aquaculture Nutrition. (10): 1-13.

Philminaq., 2008. Improvement of aquaculture feeds in the Phillipines for better profitability and reduced environmental impact: 1-26.

Refstie, S., 2007. Soybean in aquaculture feeds. Harvesting the benefits of grain in aquaculture feeds, Freemantle, Western Australia.

Sales, J., 2004. Aqua feeds: Formulation & beyond. Aqua Feeds: Formulation and Beyond. 1(3): 23-26.

Sales, J. & Britz, P. J., 2001. Research on abalone *(Haliotis midae L*.) cultivation in South Africa. Aquaculture Research. 32: 863-874.

Shipton, T. A., 1999. The protein requirements of the South African abalone, *Haliotis midae*. Doctoral dissertation, Rhodes University Editor.

Sinha, A. K., Kumar, V., Makkar, H. P. S., De Boeck, G., & Becker, K., 2011. Non-starch polysaccharides and their role in fish nutrition– A review. Food Chemistry. 127(4): 1409-1426.

Suresh, A. V., 2006. Improving nutrient delivery in aqua feeds: Implications for nutritionists and formulators. Feed Technology Update. 1(4): 1-32.

Tolomei, A., Crear, B., & Johnston, D., 2003. Diet immersion time: Effects on growth, survival and feeding behavior of juvenile Southern rock lobster, *Jasus edwardsii.* Aquaculture. 219(1-4): 303-316.

Tsanigab, S. M., 2009. Evaluating Namibian macrophytic algae as a dietary source for South African abalone. Doctoral dissertation, Stellenbosch University, Department of Food Science.

Walsh, G. A., Power, R. F. & Headon, D. R., 1993. Enzymes in the animal-feed industry. TIBTECH. 11: 424-429.

Welch, A., Hoenig R., Stieglitz J., Benetti D., Tacon A., Sims N. & O'Hanlon C. 2010. From fishing to the sustainable farming of carnivorous marine finfish. Reviews in Fisheries Science. 18(3): 235-247.

Zhenhua, Z., 2011. Solution for extrusion aquafeed production. My View Integrated Solution Provider. 4(2): 1-11.

Chapter 5

Effect of dietary fish meal replacement with soybean meal on the intestinal morphology of South African abalone, *Haliotis midae*

5.1 Abstract

Treatments used in this study consisted of a control fish meal (FM) diet (FM=22%FM, 0% SBM), a soybean meal (SBM) diet (SBM=0%FM, SBM30%), a SBME (SBME) diet (SBME=0%FM, SBM30%, β- glucanase, xylanase and α-D-galactosidase) and a commercial animal protein-free diet (ECO). The intestine of the abalone consists of five regions. In this trial the intestinal regions were grouped into two intestinal regions (II-IV and V) which were scored using a semi-quantitative method. In region II-IV the intestinal morphology of abalone fed on the soybean meal diet differed significantly from the abalone fed on a commercial diet. In relation to region V the abalone fed on a soybean meal diet differed significantly from the abalone fed on a soybean meal enzyme treated diet. Artificial diets have an impact on abalone intestinal morphology, untreated soybean diets more so.

Key words: abalone, fishmeal-replacement, soybean meal, non-starch polysaccharides, Uran score, intestinal enteritis

5.2 Introduction

During the past 10 years the inclusion of plant feedstuffs in fish feeds, as fish meal (FM) replacements have increased markedly (Krogdahl *et al*., 2010). One such plant feed is soybean meal (SBM), which is often used as a fish meal replacement due to its suitable amino acid profile, its inclusion has been reported in various aqua feeds (Drew *et al*., 2007; Knudsen *et al*., 2007; Urán *et al*., 2009; Krogdahl *et al*., 2010; Welch *et al*., 2010). However, according to Lim *et al*. (2010) the use of soybean meal has often been restricted in aquaculture feeds due to the abundant antinutritive factors (ANF) it contains. These antinutritive factors can cause intestinal damage and reduce growth performance in fish by their direct interactions with epithelial cells of the intestine and modulation of digesta viscosity (Sinha *et al*., 2011).

The morphological response of the gut to antinutritive factors has been termed intestinal enteritis. Enteritis is a non-infections sub acute condition well documented in finfish species (mainly salmonoids) that are fed soybean meal and other legume based diets (Baeverfjord & Krogdahl, 1996; Kraugerud *et al*., 2007; Carmona, 2008; Knudsen *et al*.,

2008; Uran, 2008; Øverland *et al*., 2009; Krogdahl *et al*., 2010; Lim *et al*., 2010; Sinha *et al*., 2011; Sørensen *et al*., 2011). It is thought to be caused by the heat-stable allergenic compounds present in these ingredients, as well as possible oligosaccharide interactions with nutrient digestion, but the exact causes are still unknown (Krogdahl *et al*., 2010).

Enteritis is characterized by shortening of primary and secondary mucosal folds and widening of the *lamina propria*, which is also infiltrated by a mixed population of inflammatory cells (Baeverfjord & Krogdahl, 1996; Refstie, 2007; Krogdahl *et al*., 2010). Prolonged consumption of soluble non-starch polysaccharides has shown to result in significant digestive changes in poultry and pigs (Choct, 1997; Alexis & Nengas, 2001). No conclusive information is however available on the direct effects of non-starch polysaccharides on fish intestinal histology (Sinha *et al*., 2011).

Literature published on abalone gut morphology and histology is incomprehensive (Campbell, 1965; Bevelander, 1988; Harris *et al*., 1998a; Edwards *et al*., 2003). Currently there is no published work focusing on morphology and histology of the species *Haliotis midae* in particular. Existing anatomical literature mainly investigated the digestive gland, prevalent diseases and juvenile endogenous enzyme activities of Haliotids (Knauer *et al*., 1996; Erasmus *et al*., 1997; Serviere-Zaragoza *et al*., 1997; Long-Bo *et al*., 2001; Macey & Coyne, 2005; Garcia-Esquivel & Felbeck, 2006; Mouton & Gummow, 2011). Kemp (2001) emphasized the importance of research required on the influence of the diet on intestinal morphology in abalone, especially with regard to the effect of artificial feeds as already seen in certain fish and monogastric livestock species (Choct & Kocher, 2001; Francis *et al*., 2001; Kemp, 2001).

The aim of the study was therefore to investigate the long-term (more than 70 days, Flemming (1996)) response of abalone intestinal morphology to soybean meal as a replacement for fishmeal. This was done by scoring the gut morphology of abalone fed on different diets, using an intestinal enteritis scoring method (Uran et al., 2007).

5.3 Materials and Methods

5.3.1 Animals

Abalone were kept at the Department of Science and Technology Pilot Project at Hondeklipbaai (30°31'S; 17°27'E). A total of 500 animals per treatment (initial length: 59.46 ± 6.51 mm; initial mass: 35.24 ± 7.01 g) had been fed the various test diets for eight

months, as part of a growth study. This helped ensure the animals' guts were adapted to the specific diets.

Error! Reference source not found. and **Error! Reference source not found.** present the trial diets and their proximate composition. Diets consisted out of a control fish meal (FM) diet (22%FM, 0% SBM), a soybean meal (SBM) diet (0%FM, SBM30%), a SBM enzyme (SBME) diet (0%FM, SBM30%, β- glucanase, xylanase and α-D-galactosidase) and a commercial animal protein-free diet (ECO).

Table 5.1 Description of the experimental diets used in the trial.

Treatment	Treatment description
FM	Fish meal based control feed – 22% fish meal inclusion
SBM	Soybean meal - 30% soybean meal inclusion
SBME	Soybean meal with three enzymes (xylanase, β-glucanase and α-D-galactosidase) - 30% soybean meal inclusion
ECO	Commercial animal-protein free diet

FM: Fish meal; SBM: soybean meal; SBME soybean meal with enzyme; ECO: commercial diet

The three treatment feeds were individually formulated and prepared at the University of Stellenbosch's experimental feed mill located on Welgevallen experimental farm. Ingredients were sourced from NutroScience, Malmesbury and the three enzymes products were sourced from Novozymes (Johannesburg, South Africa). A fourth animal protein-free commercial enzyme pre-treated soybean diet was also obtained from NutroScience, Malmesbury.

Table 5.2 Compositions of experimental diets and their proximate compositions.

Ingredient and % inclusion in feed	SBM	SBME	FM	Eco
Maize	26	26	40	-
Fish meal 65	0	0	22	-
Soybean 46	30	30	0	-
Abalone binder**	22	22	22	-
Ash	8.57	8.57	7.87	5.90
Crude protein	35.69	35.69	35.76	34.63
NFC	46.68	46.68	46.98	50.4
Crude fat	3.25	3.25	3.88	6.00

* Eco diet is a commercial diet and formulation is proprietary information, it is devoid of any animal protein source however. SBM: Soybean meal; FM: Fish meal; Eco *: Commercial diet. Abalone binder*: Proprietary information

5.3.3 Sampling

Six animals were randomly selected on basis of shell length (length: 67.02 ± 0.94 mm; mass: 48.22 ± 4.96 g) from each of the four treatments. Animals were selected by randomly sampling one animal from each of the five treatment baskets, with one basket sampled twice (n=6). Food was withheld from animals prior to dissection, to ensure minimal food in the gut and also because empty gut animals stain more intensely in formalin fixed samples (Harris *et al*., 1998a).

5.3.4 Microprobe analysis

Crop and stomach pH readings of the animals were taken immediately after shucking. After that the excision tissue pieces were washed in 15% saline solution until mostly cleared of particulate matter and were then placed in a 10% formalin solution.

The pH meter was calibrated using on a two-point system, using a buffer solution with a pH of 7.00 and 4.00, at 20°C. Measurements were performed at 18°C. The same 24 abalone sacrificed for the histology scoring were used in the trial. Once shucked, the integument was removed exposing the gut which was punctured with a scalpel allowing insertion of the probe. All measurements were recorded after the pH probe was inserted into the gut lumen and the response had stabilized for more than five seconds. Feed pH was measured mixing five grams of dry feed with 15ml distilled water to form a wet paste, excess water was decanted out. The pH values of the six replicates per feed were recorded and the means and standard deviations are presented.

Due to the differences published in literature on dissection techniques and their complexity (Campbell, 1965; Bevelander, 1988; Harris *et al*., 1998a; Johnston *et al*., 2005; Joly, 2011), a comprehensive and simple technique was adapted from Edwards *et al*., (2003) and Joly (2011). It was chosen for its ease of use and specific targeting of regions II, III and V of the intestine (the proposed areas of absorption). It was hypothesized that feed effect would be visible in these absorptive regions. Animals were shucked, a sharp scalpel was used to remove the foot and then the required tissue was cut from the viscera (Figure 5.1). The intestinal region was removed by making cuts next to either side of the shell muscle, on the ventral side of the muscle the cut was made just above the stomach sac.

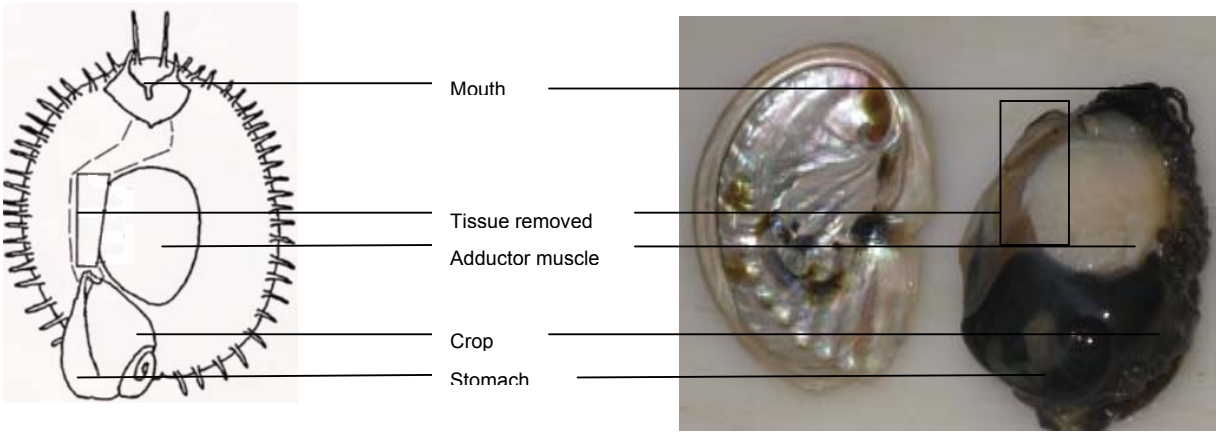


Figure 5.1 Schematic diagram of an abalone (Harris *et al*,. 1998), showing the location of incisions into the integument.

5.3.5 Histology and Scoring

Fixation and slide preparation was done at the Biomedical Sciences' Department of Anatomy and Physiology of Stellenbosch University's Tygerberg campus. Sections of 5µm were cut and stained with standard haematoxylin and eosin (H&E) staining. The transverse section was cut from the area of the viscera attached to the left part of the shell muscle across through the gills (the four region cut, shown in Figure 5.1). This was done to ensure the same section of the gut regions would be examined. Slides were examined under an Olympus CH 30 light microscope at 100x magnification. Cell identification was aided by Mandi Albas, Department of Biomedical Sciences, Division of Anatomy & Histology, Tygerberg and the works of Harris *et al*., (1998b); Johnston *et al*., (2005) and Uran (2008). Pathomorphological changes in abalone associated with soybean meal fed salmon were scored on principles laid out by the semi-quantitative Uran scoring system, shown in **Error! Reference source not found.**.

Table 5.3 Quantitative salmon gut scoring system adapted from Uran *et al.*, (2007) and Knudsen *et al*., (2007).

	Score	Description
Mucosal folds (MF)	1	Basal length/ long and thin
	2	Some shrinking and bloating/ medium length, complex folds thicker
	3	Diffused shrinkage and onset of tissue disruption/ short to medium
	4	Diffused tissue disruption/ Thick and short
	5	Total tissue disruption
Supranuclear vacuoles (SNV)	1	Basal SNV size/ Occupy almost all of apical part of enterocytes
	2	Some size reduction/ Medium sized vacuoles, occupy less than half enterocytes
	3	Diffused size reduction/ small vacuoles present in some enterocytes
	4	Onset of extinction/ scattered small vacuoles
	5	No SNV
Goblet cells (GC)	1	Scattered cells
	2	Increased number of sparsely distributed
	3	Diffused number widely spread
	4	Densely grouped
	5	High abundant and tightly packed
Sub mucosa (SM)	1	Thin layer between folds and stratum compactum / almost none
	2	Slightly increased amount below the MF/ slight increase below some folds
	3	Clear increase below the MF
	4	Thick layer of CT below many folds
	5	Extremely thick layer of CT below some folds
Lamina propria (LP)	1	Normal size LP / very thin delicate core of connective tissue
	2	Increased size of LP/ slightly more distinct in some folds
	3	Medium size LP/ markedly increased in most folds
	4	Larger LP/ thick in many folds
	5	Largest LP/ very thick in many folds

Images used as score examples and for assistance can be viewed at: http://library.wur.nl/WebQuery/catalog/lang/1872717.

This scoring system focuses on intestinal responses to dietary soybean meal that are evaluated separately:

(1) Presence and size of supranuclear vacuoles (SNV)
(2) Degree of widening of the *lamina propria* (LP) of simple folds
(3) Amount of sub mucosa (SM) between base folds and stratum compactum,
(4) Degree of mucosal fold (MF) thickening and
(5) Goblet cell (GC) proliferation.

Each of these effects are ranked according to a scale of 1 to 5, where '1-2' represents normal morphology, '3' distinct morphological signs of inflammation and '5' represents severe enteritis (Knudsen *et al*., 2007; Uran, 2008) an example is shown in **Error! Reference source not found.**. Histological evaluations were blind and repeated twice, selected sections were photographed with a Nikon digital sight camera and were analysed in Nikon NIS-Elements BR 3.1 Software. Tissue was examined and evaluated using both the Uràn score and the adaption of it as used by Knudsen *et al.,* (2007).

5.3.6 Statistical analysis

The data pH and gut data were analysed using a one-way analysis of variance (PROC ANOVA) and multiple comparison Bonferoni t-tests. All analyses were done using SAS for Windows version 9.1.3.

5.4 Results and discussion

5.4.1 Intestinal pH

The pH observations in this trial are in line with those of general molluscan pH values of 4.6-5.4 for the crop and 5.4-6 for the stomach (Yonge, 2005). **Error! Reference source not found.** and **Error! Reference source not found.** show the pH of the feed, crop and stomach of the abalone sampled in the trial.

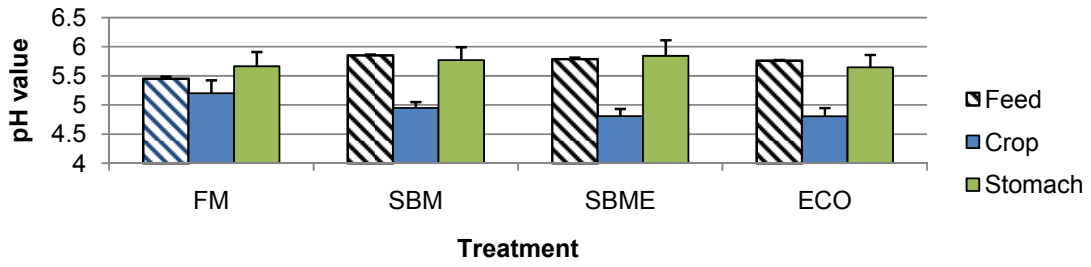


Figure 5.2 pH values of the feed, crop and stomach, standard deviation indicated with bars

FM: fish meal, SBM: soybean meal, SBME: SBMEs, ECO: commercial diet.

Table 5.4 The pH values of the crops and stomachs of abalone fed various test diets.

Treatment	Region pH		
	Feed	Crop	Stomach
Fish meal (control)	5.45 ± 0.04[d]	5.22 ± 0.30[a]	5.67 ± 0.24[a]
Soybean meal	5.86 ± 0.01[a]	4.95 ± 0.10[ab]	5.77 ± 0.22[a]
Soybean meal enzymes	5.79 ± 0.03[b]	4.81 ± 0.12[b]	5.84 ± 0.27[a]
Eco	5.76 ± 0.01[c]	4.81 ± 0.14[b]	5.64 ± 0.21[a]
P-value	0.0001	0.003	0.461

[a-b] Means within a column with a common superscript are not significantly different (P>0.05);n=6

Crop pH's of various molluscs range from 4.6-5.4, whilst stomachs have a pH range of 5.4-5.6. The crop and stomach are known to have the lowest pH of the gastropod digestive tract (Yonge, 2005). Harris *et al*. (1998) found the pH of the crop and stomach of seven green lip abalone to be 5.3 ± 0.1 and 5.5 ± 0.1 respectively, whilst Edwards (2003) stated that in abalone these regions had a pH of about 5. The observed results differed to those of Harris *et al*. (1998) and Edwards (2003), but were still within the molluscan ranges specified by Yonge (2005).

All of the feed pH values differed significantly (P<0.05). The crop of the FM fed abalone differed significantly (P<0.05) from the crop pH of the other treatments, whilst there are no significant differences (P>0.05) between the stomach pH values. The significant difference (P<0.05) in crop pH of the FM diet could be have been caused by pre-shucking stress, as they were exposed to air for a longer period of time (five minutes) than the other treatments due to the longer collection period for this specific treatment. It has been suggested that non-starch polysaccharides have the ability to lower gut pH as they are able to alter gut micro flora populations which are often largely responsible for gut pH (Sinha *et al*., 2011). This was not observed in this trial as the soybean meal diet did not significantly differ from any of the treatments. Although the crop pH of the FM diet significantly differed significantly (P<0.05) from the other treatment, the biological significance of this may be negligible as the pH is still in the range (4.6-5.4) of that for mollusk crops as suggested by Yonge (2005). Thus, it is possible to suggest that no

significant changes exist between the treatments for crop and stomach pH-values, on a biological level.

The acidic pH in these regions decreases the viscosity of the mucus allowing gut contents to mix more readily. The crop is the most acidic region in Haliotids, this region is thought to act as a food storage and digestion organ due to large food pieces and fine particulate matter found there. The stomach collects food and secretions from the salivary gland, caecum and digestive gland, with microbial and enzyme activities dependent on the low pH conditions. The low pH of these regions also helps select against pathogenic bacteria not suited to these extremes (Harris *et al*., 1998b). Very little is still known of the pH of the gut of Haliotids (Gomez-Pinchetti & Garcia-Reina, 1994; Harris *et al*., 1998b).

5.4.2 Gut scoring

Clarity on exact intestinal nomenclature and location needs to be established. Bevelander (1988) and Harris *et al*. (1998) have what appear to be the most comprehensive and descriptive works on abalone gut morphology. However, their descriptions still differ with regard to the exact location of intestinal sections and function as shown in **Error! Reference source not found.** (emphasis on region II-III and V in particular). Scoring was assumed on the intestinal regions identified by Harris *et al.,* (1998a) as their descriptions were similar to the observations of this study.

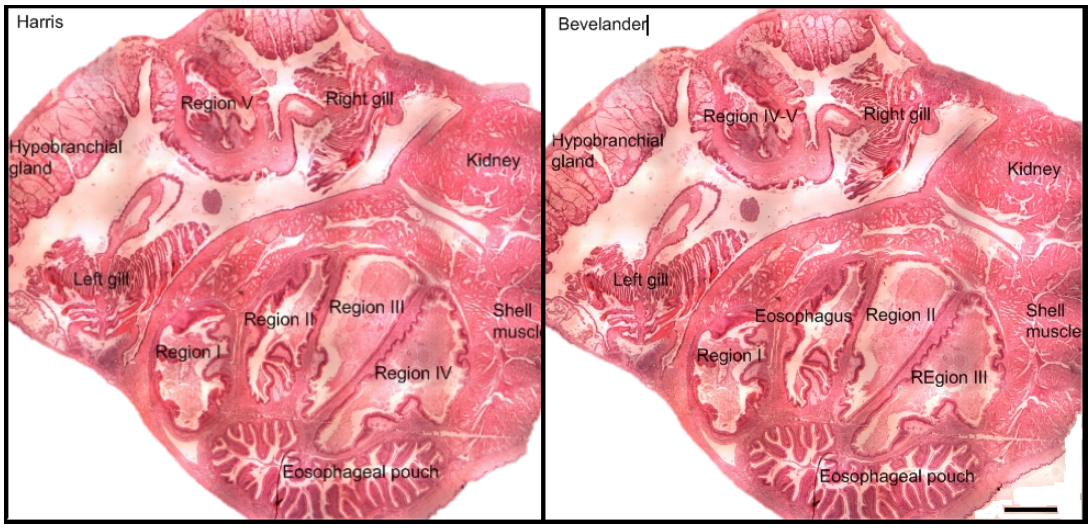


Figure 5.3 Transverse section through the intestinal region of *Haliotis midae,* from this study, illustrating the difference in naming of intestinal regions according to the authors Harris *et al.,* (1998) and Bevelander (1988). Bar is 1000 µm. Shadowlike appearance on images is as a result of image stitching.

Error! Reference source not found. shows the contrasting scores from region II, III & IV for FM and SBM diets as an example of how scores were allocated. The intestinal region was scored under live microscope viewing at 4x, 10x and 65x magnifications depending on the view required for the criteria being scored (SNV and GC require 10x and 65x). Except for the typhlosole region, the entire section was factored into the scoring.

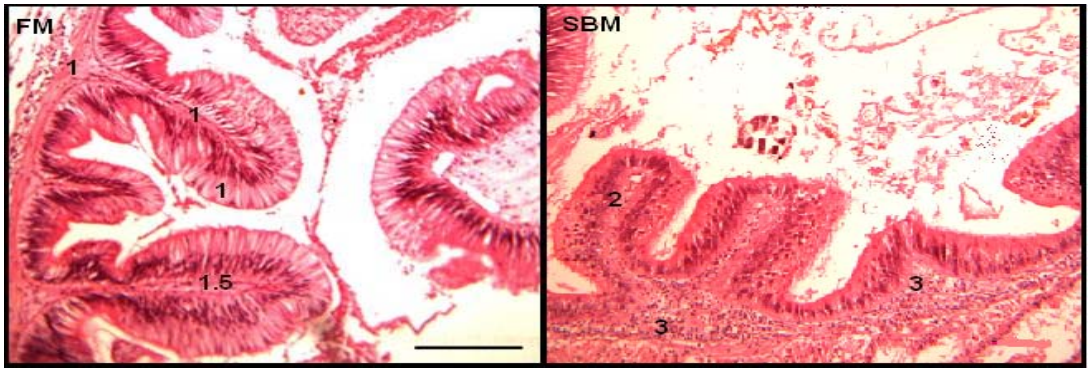


Figure 5.4 Examples of scores (1-5) allocated in region III of the abalone intestine; FM: Fish meal; SBM: Soybean meal. In the SBM photo the mucosal folds (MF) appear wider as well as the sub-mucosa (SM) and lamina propria (LP). The SM and LP are also infiltrated with inflammatory granulocytes. Bar is 50µm.

Initially region III was viewed in isolation but after closer inspection, evaluation regions II and IV were included to accommodate a more accurate score. The areas are largely morphologically similar, primarily differing in cell height and abundance.

The average gut scores for region II, III and IV and region V are given in

and Table 5.6. The commercial diet (ECO), FM (control) and SBME diets yielded better gut scores than the standard SBM diet. For region II, III and IV, ECO yielded the lowest gut score, with SBME having the lowest score in region V.

Table 5.5 Histological evaluation of the intestine region II, III & IV.

	N	Mucosal folds	Sub Mucosa	Lamina Propria	Goblet cells	Supra nuclear vacuoles	Average Scores
Eco	6	1.33 ± 0.61[a]	2.33 ± 0.61[a]	1.59 ± 0.38[a]	2.08 ± 0.38[a]	1.91 ± 0.66[a]	1.82 ± 0.28[a]
FM (+)	6	1.50 ± 0.32[ab]	3.08 ± 0.49[ab]	1.67 ± 0.26[ab]	2.58 ± 0.86[ab]	1.25 ± 0.41[a]	1.90 ± 0.23[a]
SBME	6	2.17 ± 0.41[ab]	2.75 ± 0.42[ab]	2.08 ± 0.38[ab]	2.42 ± 0.49[ab]	1.83 ± 0.41[a]	2.15 ± 0.19[ab]
SBM	6	2.33 ± 0.75[b]	3.42 ± 0.58[b]	2.50 ± 0.89[b]	3.17 ± 0.52[b]	2.08 ± 0.58[a]	2.57 ± 0.49[b]
P-value		0.011	0.0134	0.028	0.033	0.38	0.0025

Fish meal (control): FM (+); Soybean meal: SBM; Soybean meal enzyme: SBME; Commercial Eco diet: Eco.

A score of "1-2" represents normal morphology while a score of "5" represents severe enteritis, as described in **Error! Reference source not found.**.

[a-c] Means followed by a common superscript are not significantly different (P>0.05); n=6

In the low scoring slides, mucosal folds, where present, appeared longer and more finger like and the lamina propria were thin cell layers underlying the epithelium. Well distributed supra nuclear vacuoles were visible, goblet cells were evenly distributed (in II, III and IV), and the sub mucosa was found between the basal part of the *stratum compactum*.

In region II, III and IV the ECO diet differed significantly (P<0.05) from the SBM diet for MF, SM, LP and GC in region III, but it did not differ significantly from FM and SBME. The average scores of ECO and FM differed significantly (P<0.05) from SBM but not from SBME. These significant differences indicate a negative impact on morphology of the intestinal tract due to antinutritive factors and possibly non-starch polysaccharides found in the SBM diet. These negative changes are not associated with a fully developed enteritis condition as the average score is still below three, but they do show a tendency towards an inflammatory morphological change. None of the treatments' average scores were above three for intestinal region II, III and IV. This is important to note as gut scores are associated with enteritis if they are larger than three. The SBME did not differ significantly (P>0.05) from the SBM or ECO treatments and so it can thus be said that enzymes do not have an effect in reducing SBM induced morphological changes.

Table 5.6 Histological evaluation of intestinal region V.

	N	Mucosal folds	Sub Mucosa	Lamina Propria	Goblet cells	Supra nuclear vacuoles	Average Scores
Eco	6	2.30 ± 0.45^{a}	3.60 ± 0.41^{a}	2.00 ± 0.71^{a}	3.80 ± 0.27^{ab}	2.00 ± 0.35^{a}	2.58 ± 0.34^{ab}
FM (+)	3	1.50 ± 0.00^{a}	3.67 ± 0.29^{a}	2.67 ± 0.58^{ab}	3.83 ± 0.29^{b}	2.67 ± 1.15^{a}	2.69 ± 0.19^{ab}
SBME	5	2.00 ± 0.61^{a}	3.50 ± 0.35^{a}	2.30 ± 0.57^{a}	3.20 ± 0.45^{a}	1.90 ± 0.42^{a}	2.43 ± 0.19^{a}
SBM	5	2.30 ± 0.44^{a}	4.00 ± 0.35^{a}	3.50 ± 0.50^{b}	4.00 ± 0.35^{b}	1.80 ± 0.27^{a}	3.03 ± 0.02^{b}
P-value		0.122	0.202	0.008	0.017	0.201	0.012

Fish meal (control): FM (+); Soybean meal: SBM; Soybean meal enzyme: SBME; Commercial Eco diet: Eco.

A score of "1-2" represents normal morphology while a score of "5" represents severe enteritis, as described in **Error! Reference source not found.**.

$^{a-c}$ Means followed by a common superscript are not significantly different (P>0.05); n=3,5,6

In region V replicates for the FM, SBME and SBM treatments were lost due to the loose attachment of the rectal area to the rest of the intestine. Taking extra caution during dissection and removal of this section is therefore important. The FM treatment was excluded from statistical analysis for this region due to insufficient replicates (n=3). The scores of both the ECO and SBME differed significantly (P<0.05) from SBM for LP, whilst SBME differed significantly (P<0.05) from SBM for GC. The average score of differed significantly (P<0.05) from SBME, but ECO did not differ significantly (P>0.05). With regard to the other parameters, the treatments showed no significant difference (P>0.05).

The common factor in these observations is soybean meal, which differs significantly (P<0.05) from the other scores. The reason the average score of SBME is significantly different from SBM in this region is primarily due to the increased GC score. The soybean meal, LP, GC and average scores of region V were significantly (P<0.05) higher than those of region II, III and IV. It is interesting to note that SBME does not differ significantly (P>0.05) from ECO and FM for region II, III and IV and yet does differ significantly (P<0.05) from SBM in region V, thus showing that enzymes have an effect in reducing the morphological effect of SBM in the diet of abalone. It is still not clear what all of the

enteritis inducing factors may be (Uran, 2008; Sinha *et al*., 2011), but non-starch polysaccharides targeted by the enzymes (β-glucans, α-galactosidase and arabinoxylans) may contribute to the onset of enteritis. The enzyme treated SBM diet's score is lower than the gut score from that of the standard SBM diet.

Proliferation of GC in region V is inherent as shown by Bevelander (1988) and Harris e*t al*., (1998), and may not be due to inflammation. The abundance of GC thus skews the average score between region V and region I, II and IV. The average scores of region V differed significantly ($P<0.05$) from region II, III and IV's average score along with the MF, GC, MUC and LP scores. An explanation for these results could either be that an inflammatory response is greater in this region due to more epithelial interaction with nutrients, or that inherent morphological differences exist. It is of the opinion of the researcher that the latter is true. In future it is not recommended that the score of region V should be used as an indication of enteritis or gut condition unless future research indicates it is essential in nutrient absorption. Campbell (1965) suggests possible absorption in this region whilst Harris *et al*., (1998) suggest region II, III and IV as primary absorptive regions. The abundant goblet (mucous) cells of region V (**Error! Reference source not found.**) may allude to a more excretory function in this region (Harris *et al*., 1998a). Region III is also the longest area in the intestine (possibly to increase its absorptive surface) (Harris *et al*., 1998a). Photographs of the intestinal regions III and V from x40 and x400 magnification can be seen in **Error! Reference source not found.**, **Error! Reference source not found.**, **Error! Reference source not found.** and **Error! Reference source not found.** with general identification annotations made. In order to detect the prevalence and visibility of goblet (mucous) cells in region V (in comparison to region III), Figures 5.6 and 5.7 should be held in contrast to one another.

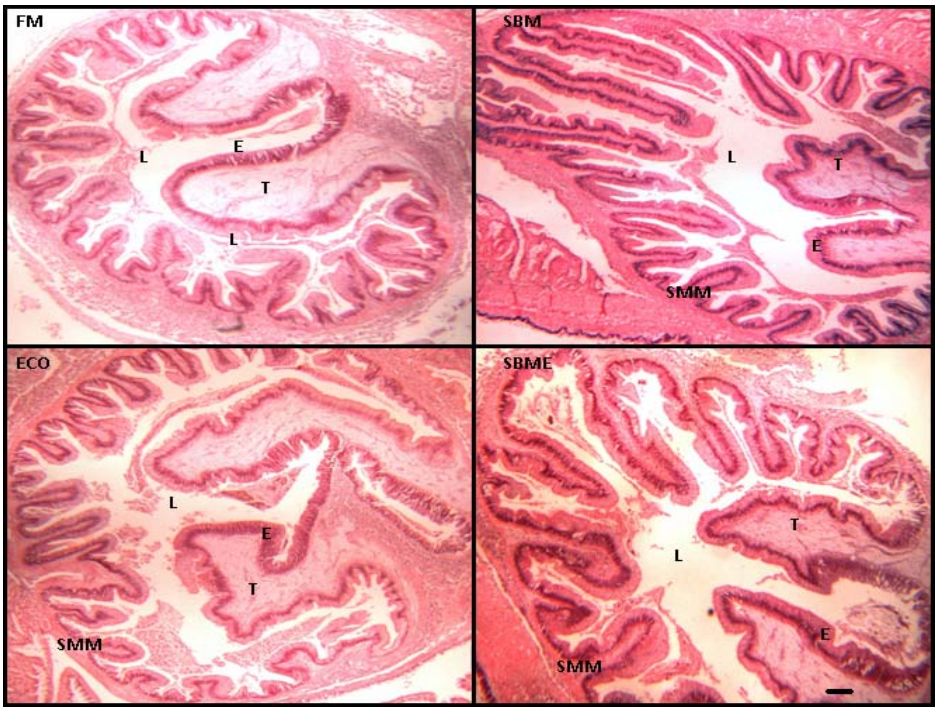


Figure 5.5 Region II, III and IV (magnification x 40) of the intestine of; FM: Fish meal; SBM: Soybean meal; ECO: Commercial and SBME: Soybean meal enzyme. L: lumen; E: epithelium cells; SM: sub mucosa; T: typhlosole. Bar is 100µm.

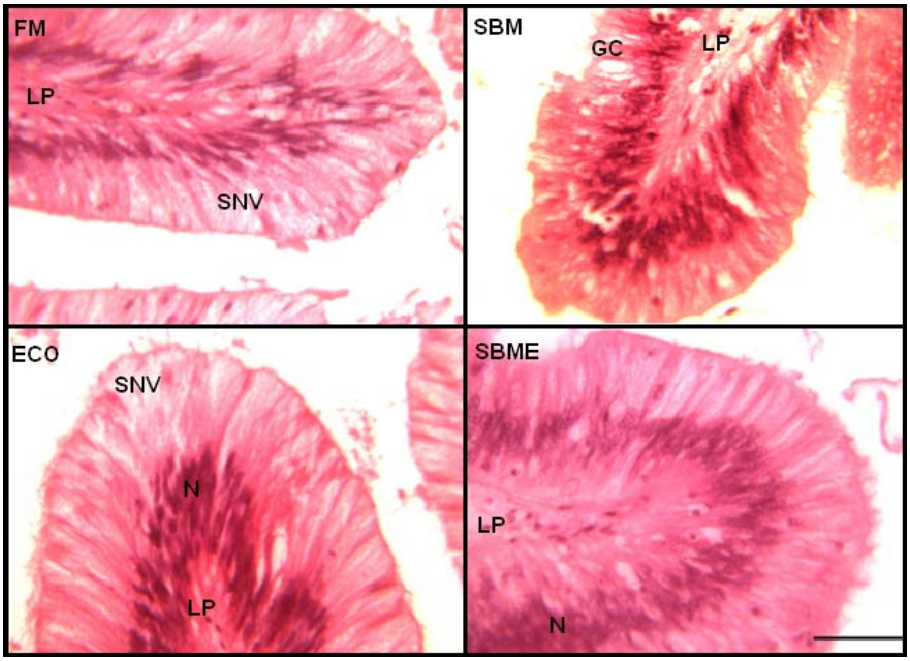


Figure 5.6 Region II, III and IV (magnification x 40) of the intestine of; FM: Fish meal; SBM: Soybean meal; ECO: Commercial and SBME: Soybean meal enzyme. LP: lamina propria; N: Nucleus; SNV: supra nuclear vacuoles; GC: goblet cells. Bar is 50 µm.

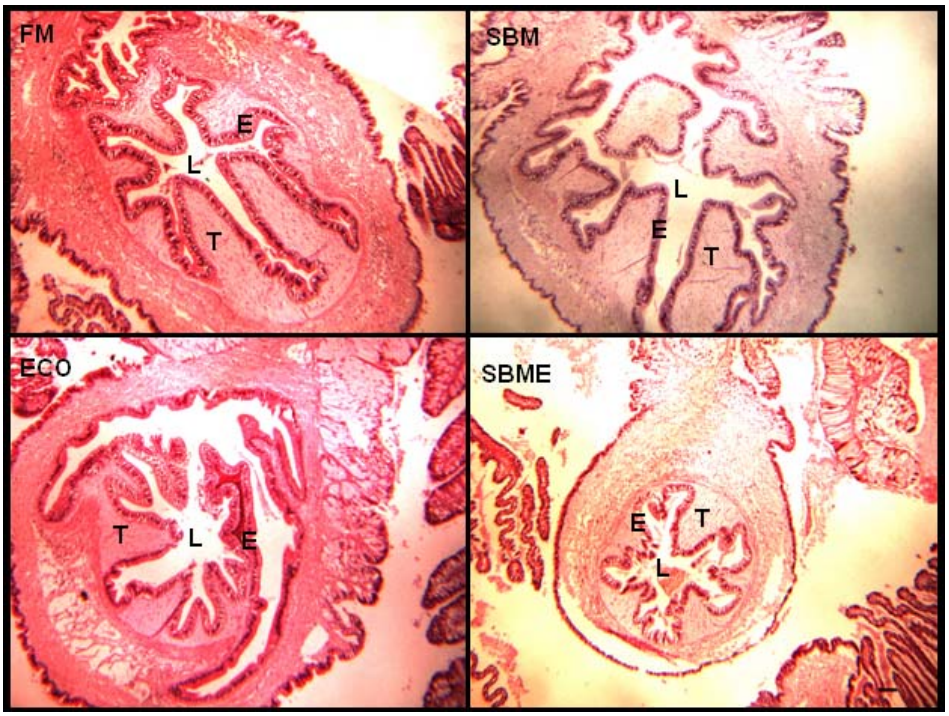


Figure 5.7 Region V of the intestine of the intestine; FM: Fish meal; SBM: Soybean meal; ECO: Commercial and SBME: Soybean meal enzyme. L: lumen; E: epithelium cells; SM: sub mucosa; T: typhlosole. Bar is 100µm.

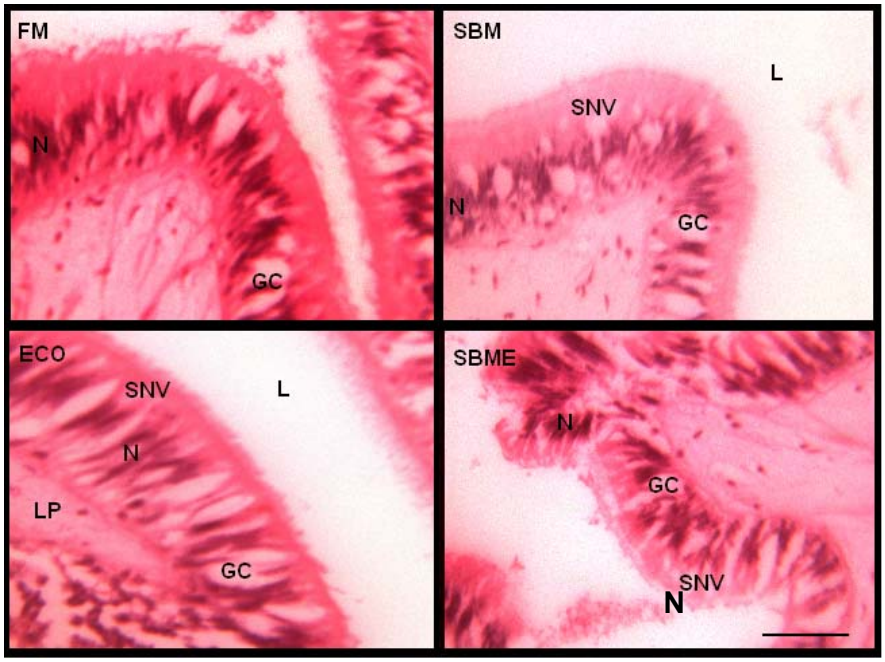


Figure 5.8 Region V of the intestine; FM: Fish meal; SBM: Soybean meal; ECO: Commercial; SBME: Soybean meal with enzymes. SNV: supra nuclear vacuole; GC: goblet cells; N: nucleus. Bar is 50µm.

Irrespective of the possible functions of these gut regions, it is interesting to note that none of the treatments for both regions II, III and IV and region V received an average score of below 1.5. It can thus be seen that artificial diets cause slight changes in morphology of the abalone digestive tract as suggested by Kemp (2001). A natural diet should have been added as a control in this trial to add further weight to this statement, as was done by Kemp (2001). The changes in morphology of the abalone digestive tract include an increase in mucus cover over the epithelium and more centrally located nuclei, as well as a decrease in supra nuclear vacuoles. These are all signs of a morphological response and can be attributed to the antinutritive factors in artificial feeds (Kemp, 2001).

These changes are not significant in production performance as abalone fed on artificial feed still outperform abalone fed a natural diet (Britz, 1996; Fleming *et al*., 1996). Reasons for this could be due to the endogenous enzyme activities of abalone which include cellulose, alginatelyase, lyase, laminarinase and carrageenase (Erasmus *et al*., 1997). These enzymes are either produced by secretory cells in the digestive gland or by gut bacterial populations (Garcia-Esquivel & Felbeck, 2006). Certain abalone species have been shown to adapt their activity according to the dietary composition of feed (Erasmus *et al*., 1997; Garcia-Esquivel & Felbeck, 2006). This adaption of polysaccharide digesting enzymes may increase in soybean meal fed animals breaking down harmful non-starch polysaccharides to a level that is not fully inflammatory.

Furthermore, the scoring system used should be evaluated for its suitability to abalone. This is necessary as physiological features like the presence of a typhlosole in the intestine (**Error! Reference source not found.**), which is unique in certain invertebrates and lower vertebrates, could be used as a scoring parameter. A specific intestinal region could be identified as a focal point of scoring, as cellular structures vary between regions (Harris *et al*., 1998a) and may respond differently to antinutritive factors.

Some bacterial cells were evident in region V of the intestine and are thought to be predominantly associated with the mucous of this region rather than the epithelium (Harris *et al*., 1998a). Protozoa were also observed attached to the intestinal epithelium as observed by Mouton (2008). Micro flora is inherent to the abalone digestive tract and is thought to be involved in cellulosic hydrolysis to some extent (Erasmus *et al*., 1997; Garcia-Esquivel & Felbeck, 2006). Oligosaccharide production has been found in Vibrio bacteria isolated from abalone (Zhang *et al*., 2004). The binding of protozoa to the gut wall may also facilitate an inflammatory response of some kind as seen on the far right in

Error! Reference source not found.. This, however, remains speculation and requires further investigation.

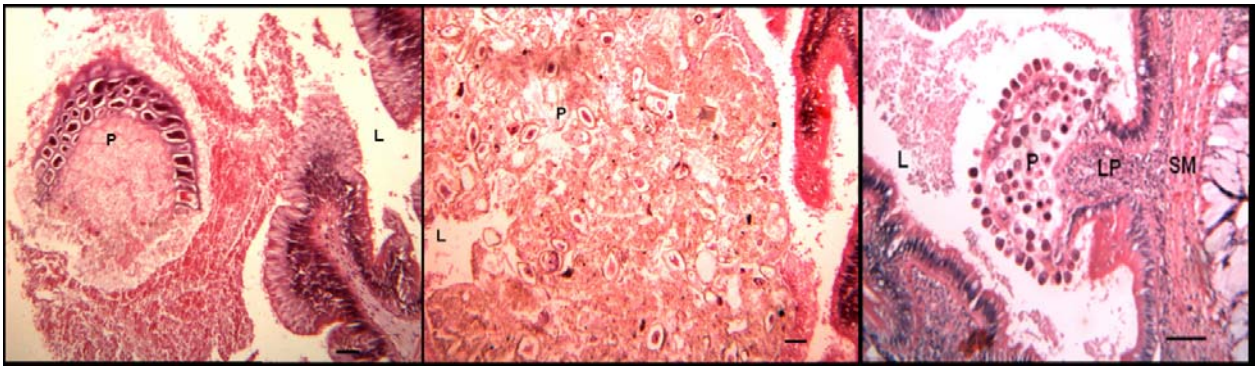


Figure 5.9 Protozoa in the lumen and bound to the gut wall inducing inflammation, L: Lumen; P: Protozoa; LP: Lamina propria; SM: Sub mucosa. Bar is 50µm.

5.5 Conclusion

The influence of soybean meal on abalone intestinal morphology was investigated. Similar observations as to that of Kemp (2001) were noted, in that artificial feeds have an effect on abalone intestinal morphology. The effect of the different diets tested appears to have a negligible effect on crop and stomach pH. Untreated soybean meal-based diets, have a significant ($P<0.05$) negative impact on gut morphology when compared to fish meal and treated plant diets, with morphological changes similar to an early enteritis-like condition observed. Treatment of soybean meal with a combination of β-glucanase, xylanase and α-galactosidase enzymes has proven to significantly ($P<0.05$) reduce the damaging impact of soybean meal on the gut region V and maintain a comparative condition to the other tested diets for region II, III and IV. Recommendation from this study for further research include the evaluation of different cellulosic enzymes and other soybean meal treatment methods that may also hold potential in improving gut morphology. Investigation of gut specific gut function is also required to be able to scrutinize the effect of the above mentioned diets on the specific are of absorption.

5.6 References

Alexis, M. N. & Nengas, I., 2001. Current state and knowledge of soy products in diets for feeding sea bass and sea bream and needs for the future. Doctoral dissertation, National Centre for Marine-Research, Athens, Greece.

Baeverfjord, G. & Krogdahl, A., 1996. Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar*, distal intestine: A comparison with the intestines of fasted fish. Journal of Fisheries. 19(5): 375-387.

Bevelander, G., 1988. Abalone, gross and fine structure. First ed. The Boxwood Press. 183 Ocean View Boulevard, Pacific Grove, California 93950.

Britz, P. J., 1996. The nutritional requirements of *Haliotis midae* and development of a practical diet for abalone aquaculture. Doctoral dissertation, Rhodes University Editor.

Campbell, J. L., 1965. The structure and function of the alimentary canal of the black abalone, *Haliotis cracherodii* (leach). Transactions of the American Microscopial Society. 84(3): 376-395.

Carmona, P. A. U., 2008. Etiology of soybean-induced enteritis in fish. Doctoral dissertation, University of Wagenen.

Choct, M. & Kocher, A., 2001. Non-starch carbohydrates: Digestion and its secondary effects in monogastrics. Doctoral dissertation, Department of Animal Science, University of New England, Armidale, NSW, 2351.

Choct, M., 1997. Feed non-starch polysaccharides: Chemical structures and nutritional significance. Feed Milling International. (June): 13-26.

Choct, M., Dersjant-Li, Y., McLeish, J. & Peisker, M., 2010. Soy oligosaccharides and soluble non-starch polysaccharides: A review of digestion, nutritive and anti-nutritive effects in pigs and poultry. Journal of Animal Science. 23(10): 1386-1398.

Drew, M. D., Borgeson, T. L. & Thiessen, D. L., 2007. A review of processing of feed ingredients to enhance diet digestibility in finfish. Animal Feed Science and Technology. 138: 118-136.

Edwards, S., Geraghty, D. & Ralph, J., 2003. Pharmacological and histological assessment of gut muscle movement in blacklip abalone, *Haliotis rubra* (leach). Aquaculture Research 34(5): 383-388.

Erasmus, J. H., Cook, P. A. & Coyne, V. E., 1997. The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. Aquaculture. 155(1-4): 377-386.

Fleming, A. E., Bameveld, R. J. V. & Hone, P. W., 1996. The development of artificial diets for abalone: A review and future directions. Aquaculture. 140: 5-53.

Francis, G., Makkar, H. P. S. & Becker, K., 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture. (199): 197-227.

Garcia-Esquivel, Z. & Felbeck, H., 2006. Activity of digestive enzymes along the gut of juvenile red abalone, *Haliotis rufescens*, fed natural and balanced diets. Aquaculture. 261(2): 615-625.

Gomez-Pinchetti, J. L. & Garcia-Reina, G., 1994. Acid deoxyribonuclease activity in crude extracts from marine phycophages used for seaweed protoplast isolation. Scientia Marina. 58(3): 133-236.

Harris, J. O., Burke, C. M. & Maguire, G. B., 1998a. Characterization of the digestive tract of greenlip abalone, *Haliotis laevigata*, morphology and histology. Journal of Shellfish Research. 17(4): 979-988.

Harris, J. O., Burke, C. M. & Maguire, G. B., 1998b. Characterization of the digestive tract of greenlip abalone, *Haliotis laevigata*, micro-environment and bacterial flora. Journal of Shellfish Research. 17(4): 989-994.

Johnston, D., Moltschaniwskyj , N. & Wells, J., 2005. Development of the radula and digestive system of juvenile blacklip abalone (*Haliotis rubra*): Potential factors responsible for variable weaning success on artificial diets. Aquaculture. 250(1-2): 341-355.

Joly, J. P., 2011. Molluscs Processing for Diagnosis by Histology In: Ifremer. 3rd ed. European Union Reference Laboratory for Mollusc Diseases, France. pp. 1-7

Kemp, R., 2001. The effect of diet on intestinal structure and function in the abalone *(Haliotis laevagata*). Doctoral dissertation, University of Adelaide.

Knauer, J., Britz, P. J. & Hecht, T., 1996. Comparative growth performance and digestive enzyme activity of juvenile South African abalone, *Halioits midae*, fed on diatoms and a practical diet. Aquaculture. (140 (1996)): 75-85.

Knudsen, D., Jutfelt , F., Sundh, H., Sundell, K., Wolfgang, K. & Frokiaer, H., 2008. Dietary soya-sapponins increase gut permeability and play a key role in the onset of soybean-induced enteritis in Atlantic salmon (S*almo salar*). British Journal of Nutrition. (100): 120-129.

Knudsen, D., Uran, P., Arnous, A., Koppe, W. & Frokler, H., 2007. Saponin-containing subfractions of soybean molasses induce enteritis in the distal intestine of Atlantic salmon. Journal of Agriculture and Foof Chemistry. (55): 2261-2267.

Kraugerud, O. F., Penn, M., Storebakken, T., Refstie, S., Krogdahl, A. & Svihus, B., 2007. Nutrient digestibilities and gut function in Atlantic salmon (*Salmo salar*) fed diets with cellulose or non-starch polysaccharides from soy. Aquaculture. 273: 96-107.

Krogdahl, A., Penn, M., Thorsen, J., Refstie, S. & Bakke, A., 2010. Important antinutrients in plant feedstuffs for aquaculture: An update on recent findings regarding responses in salmonids. Aquaculture Research. 41: 331-344.

Lim, S., Kim, S., Ko, G., Song, J., Oh, D., Kim, J. & Lee, K., 2010. Fish meal replacement by soybean meal in diets for tiger puffer, *Takifugu rubripes*. Aquaculture.

Long-Bo, C. U. I., Chuan-Lin, L. I. U., Xun, L. I. U. & Yao-Hua, L. U., 2001. Structure and function of mucous epithelium of the intestine in haliotis discus hannai. Acta Zoological Sinica. 47(3): 324-338.

Macey, B. M. & Coyne, V. E., 2005. Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment. Aquaculture. 245(1-4): 249-261.

Mouton, A., 2008. An epidemiological study of parasites infecting the South African abalone (*Haliotis midae*) in Western Cape aquaculture facilities. Doctoral dissertation, University of Pretoria.

Mouton, A. & Gummow, B., 2011. The occurrence of gut associated parasites in the South African abalone, *Haliotis midae*, in Western Cape aquaculture facilities. Aquaculture. 313(1-4): 1-6.

Øverland, M., Sørensen, M., Storebakken, T., Penn, M., Krogdahl, Å. & Skrede, A., 2009. Pea protein concentrates substituting fish meal or soybean meal in diets for Atlantic salmon (*Salmo salar*)—Effect on growth performance, nutrient digestibility, carcass composition, gut health, and physical feed quality. Aquaculture. 288(3-4): 305-311.

Refstie, S., 2007. Soybean in aquaculture feeds. Harvesting the benefits of grain in aquaculture feeds, Freemantle, Western Australia.

Serviere-Zaragoza, E., Toro, M. A. N. & Garcia-Carrefio, F. L., 1997. Protein-hydrolyzing enzymes in the digestive systems of the adult Mexican blue abalone, *Haliotis fulgens* (gastropoda). Aquaculture. (157): 325-336.

Sinha, A. K., Kumar, V., Makkar, H. P. S., De Boeck, G. & Becker, K. 2011., Non-starch polysaccharides and their role in fish nutrition – A review. Food Chemistry. 127(4): 1409-1426.

Sørensen, M., Penn, M., El-Mowafi, A., Storebakken, T., Chunfang, C., Øverland, M. & Krogdahl, Å., 2011. Effect of stachyose, raffinose and soya-saponins supplementation on nutrient digestibility, digestive enzymes, gut morphology and growth performance in Atlantic salmon (*Salmo salar*). Aquaculture. 314(1-4): 145-152.

Uran, P. A., 2008. Etiology of soybean-induced enteritis in fish. Doctoral dissertation, University of Wageningen.

Urán, P. A., Schrama, J. W., Rombout, J. H. W. M., Taverne-Thiele, J. J., Obach, A., Koppe, W. & Verreth, J. A. J., 2009. Time-related changes of the intestinal morphology of Atlantic salmon, *Salmo sala*r, at two different soybean meal inclusion levels. Journal of Fisheries. 32(9): 733-744.

Welch, A., Hoenig, R., Stieglitz, J., Benetti, D., Tacon, A., Sims, N. & O'Hanlon, C., 2010. From fishing to the sustainable farming of carnivorous marine finfish. Reviews in Fisheries Science. 18(3): 235-247.

Yonge, C. M., 2005. The hydrogen ion concentration in the gut of certain lamellibranchs and gastropods. 938-952.

Zhang, Z., Yu G., Guan H., Zhao X., Du Y., and Jiang X., 2004. Preparation and structure elucidation of alginate oligosaccharides degraded by alginate lyase from vibro species. 510. Carbohydrate Research. 339(8): 1475-1481.

Chapter 6

The effect of fishmeal replacement and dietary enzyme supplementation on the production performance of South African abalone, *Haliotis midae*

6.1 Abstract

A trial was conducted to investigate the effect of fish meal replacement and enzyme supplementation of soybean meal-based diets on the production performance of South African abalone *Haliotis midae.*

The study was comprised of two phases: a fish meal replacement phase (Phase A) and an enzyme treatment phase (Phase B). Diets used in Phase A consisted of a control fish meal diet (Control=22%FM, 0%SBM), a fish meal-soybean meal diet (FMSBM=20%FM, 15%SBM), a soybean meal-low diet (SBMlow=0%FM, 15%SBM) and a soybean meal diet (SBM=0%FM, SBM30%). In Phase B, the FM diet and SBM diet were used as basal diets (FME0 and SBME0). These diets were then treated with three commercial enzyme products, namely, a β- glucanase (FME1 and SBME1), xylanase (FME2 and SBME2) and α-D-galactosidase (FME3 and SBME3). Subsequently, all three enzymes were combined to make two treatments (FME123 and SBME123). With regard to the gut morphology and growth trials, a thirteenth energy enhanced commercial animal protein-free diet (ECO) was used The thirteen treatments were replicated five times, filling individual commercial grower baskets with one hundred abalone of 58.29 ± 3.76 mm shell length, and 33.69 ± 6.54 g body weight. Twenty animals from each basket were weighed and measured every sixty days.

In Phase A of the experiment, soybean meal replaced fish meal effectively at all levels without any negative effect on production performance parameters. In Phase B, enzyme addition did not result in increased production performance parameters. Soybean meal has the potential to partially and completely replace fish meal in abalone diets, but further work is required to evaluate the production and cost benefit of enzymes in abalone diets.

6.2 Introduction

Abalone are one of the most valuable aquaculture species globally, representing over 51% of the local aquaculture sector (Sales, 2004; Mowlana, 2007). On average, South African abalone, *H. midae,* take forty two to fifty four months to reach a marketable size, making

growth rate and efficient feed conversion important aspects of their production (Mouton & Gummow, 2011).

The use of protein sources that are not only sustainable, but also crucial in improving growth rate (and thus reducing production time), would be of significant economic benefit for abalone production. Soybean meal (SBM) already holds great potential as a fish meal (FM) replacement in fin-fish species (Francis *et al*., 2001; Drew *et al*., 2007; Glenncross *et al*., 2007; Krogdahl *et al*., 2010; Sinha *et al*., 2011) and it should therefore be thoroughly investigated as a possible complete fish meal replacement for abalone diets as well. Viscera-soybean (Guzm´an & Viana, 1998), defatted soybean (Bautista-Teruel *et al*., 2003), soybean-casein, soybean-fish meal (Fleming *et al*., 1996) and soybean meal (Britz, 1995) are all forms of partial fish meal replacement in abalone diets. These diets have been shown to only be able to partially replace fish meal. The afore mentioned studies all used juvenile animals (< 20mm),since sub- adult animals (30-50 mm) have been shown to have different growth rates and feed responses (Fleming *et al*., 1996; Tsanigab, 2009). It is of interest to investigate the effect of these diets in sub-adults.

Heat stabile and heat labile antinutritive factors are found in soybean meal. The latter are easily removed during extrusion and heat processing, whilst the former require further treatments and are of greater concern (O'Keef, 2003). Enzymes have been suggested as possible cheaper alternatives to aqueous and ethanolic extraction in removing heat-stabile antinutritive factors from soybean meal (Refstie, 2007). Enzymes have already been successfully used in poultry, pigs and fish, but they are yet to be tested in abalone diets (Fleming *et al*., 1996; Sinha *et al*., 2011) Addition of enzymes to soybean meal diets is a relatively new topic. Supplementation in legume diets appears to be more effective when enzyme cocktails (multiple enzyme treatments) are added instead of single enzyme treatments, due to the complex carbohydrate structure of legumes (Chesson., 1993; Walsh *et al*., 1993; Karr-Lilienthal *et al*., 2005; Fourij, 2007).

Abalone produce endogenous enzymes in their digestive glands and native microflora (Erasmus *et al*., 1997; Garcia-Esquivel & Felbeck, 2006; Kumagai & Ojima, 2009). Several oligosaccharides have been isolated and purified from vibrio bacteria found in abalone (Zhang *et al*., 2004). β-1-3-glucanase has been found in pacific abalone and has been shown to be effective in degrading certain celluloses, but is ineffective in degrading xylans (Kumagai & Ojima, 2009). The activities of abalone carbohydrases are lower than the enzyme activities found in terrestrial herbivorous species (Garcia-Esquivel & Felbeck,

2006), thus their ability to digest terrestrial carbohydrates is uncertain and enzyme supplementation may be necessary to aid in their digestion.

The aim of this study was to investigate the way in which abalone cultured on fish meal and soybean meal diets are affected. The experiment was divided into two phases (Phase A and Phase B). In Phase A, a control fish meal diet was compared to three unique soybean meal diets as well as to a commercial fish meal-free diet. In Phase B, a control fish meal diet was compared to enzyme treated fish meal, soybean meal diets, as well as a fish meal-free commercial diet. For both of these trials, the effects of the treatments on abalone weight and length growth rate, feed conversion ratio (FCR) and condition (Britz factor, Britz (1996)) were evaluated.

6.3 Materials and methods

A growth trial was conducted at The Department of Science and Technology pilot project in Hondeklipbaai (30°31'S; 17°27'E) over a 240 day period, from April to November 2011. Thirteen treatment diets were replicated five times each and water temperature was measured daily. Treatments were placed in baskets from 1-13. Twelve baskets occupied a block, meaning that an odd number of treatments were sequentially placed in an even number of baskets per block, ensuring randomized design within the system.. Measurements were taken every sixty days, but animals were not graded during the trial period as the sub-adults had been graded according to their rate of growth prior to the onset of the trial. At the end of the trial, 7800 length and 7800 weight measurements had been made, all of which have been used for the construction of length-weight regressions to establish growth isometrics for the validation condition factor.

6.3.1 Animals

A total of 6500 sub-adult animals were used in this study and were divided into thirteen treatment groups (n=500) with five replicates per group (n=100). Animals were graded by length and distributed to limit variation between treatments (58.29 ± 3.76 mm shell length; 33.69 ± 6.54 g live weight; mean ± SD). Animals had been fed a standard abalone grower diet (AquaNutro Classic Abalone grower, NutroScience, Malmesbury) prior to the commencement of the trial over an acclimatization phase (Goosen, 2007) of eight weeks. The mean biomass of each basket was 3.38 ± 0.39 kg at the start of the trial.

6.3.2 Rearing facilities

The system was comprised of a continuous flow through system of parallel tanks (See **Error! Reference source not found.**). Water was pumped directly from the sea and the water temperature in the system was 13.84 ± 1.12°C over the duration of the trial. Each tank contained twelve baskets with an area of 3.5m^2, with seven vertical plastic plates and a horizontal 'feeding plate' positioned approximately 8cm below the water surface. An inlet was located at each side of the tank with a drain in the centre of the tank. For baskets near the centre of the tank, water was of a poorer quality. However, the thirteen treatment block design ensured treatment replicates were not grouped to a specific location in a tank but spread throughout.

Figure 6.1 Tank with six baskets in each row and inlets at each side (twelve baskets total)

6.3.3 Feeding trial

Each basket of abalone was fed to apparent satiety daily, with an experienced feeder feeding animals according to consumption (approximately 1-3% of body weight) to avoid wastage. Feed was adapted and consistently fed to all tanks, placed out at 4pm daily. Uneaten feed was unable to be recovered and thus feed fed was considered consumed. Food consumption was recorded for each basket (n=65) and uneaten feed was flushed from the system. The tanks were also cleaned weekly to maintain water quality. Every two months the animals were weighed and measured, except for the final two measurements (t_{210} and t_{240}) which were taken after one month. Animals were anaesthetized with $MgSO_4^-$

(5-10%) according to farm procedure prior to handling in order to prevent injury during weighing and measuring (Frik Venter, personal communication). On day 180, six animals were removed from each treatment, one per basket, for dissection.

6.3.4 Experimental diets

Error! Reference source not found., **Error! Reference source not found.** and Table 6.3 show the experimental design and crude nutritional composition of the feeds used. The trial consisted of two phases with two experimental feeds respectively: fish meal reduced diets (five treatments) and enzyme treated diets (eleven treatments).

Table 6.1 Composition of experimental diets and proximate analysis of diets used in the growth trial

	Diet (% inclusion)				
	S0 (F22)[1]	S30 (F0)[2]	S15 (F20)[3]	S15 (F0)[4]	*ECO
Formulations					
Fish meal 65	22	0	20.28	0	-
Soybean 46	0	30	15	15	-
Maize	40	26	34.56	30.75	-
*Binder	22	22	22	22	-
Poultry by-product meal 65	9.99	15.49	5.94	21.79	-
Hamlet protein 300	2.07	0	0	4.38	-
L-lysine HCL	2	0.84	0	0.78	-
Mono-calcium phosphate	1.14	3.5	1.32	3.35	-
Vitamin and mineral premix	0.8	0.8	0.8	0.8	-
Oil-sunflower	-	1.28	-	10.59	-
DL- methionine	-	0.09	0.11	0.08	-
Proximate analysis					
Dry matter	100	100	100	100	100
Ash	7.87	8.57	9.27	9.68	5.90
Crude fiber	3.25	3.70	2.61	5.19	3.40
Crude fat	3.88	3.25	3.96	3.07	6.00
Crude protein	35.76	35.69	38.72	36.80	34.63
Nitrogen free extract	46.98	46.68	42.49	44.01	50.40

*Proprietary information
Diet superscripts 1-2 indicate enzyme treated feeds, whilst superscripts 1-4 indicate FM replaced feeds compositions

A random block design was used for these two phases. Diets used in Phase A consisted of a control fish meal diet (Control=22%FM, 0%SBM), a fish meal-soybean meal diet (FMSBM=20%FM, 15%SBM), a soybean meal-low diet (SBMlow=0%FM, 15%SBM) and a

soybean meal diet (SBM=0%FM, SBM30%). In Phase B, the FM diet and SBM diet were used as basal diets (FME0 and SBME0). These diets were then treated with three commercial enzyme products, namely, a β- glucanase (FME1 and SBME1), xylanase (FME2 and SBME2) and α-D-galactosidase (FME3 and SBME3). Subsequently, all three enzymes were combined to make two treatments (FME123 and SBME123). The thirteen treatment diets, shown in **Error! Reference source not found.**, were individually formulated and prepared at the University of Stellenbosch's experimental feed mill located on Welgevallen experimental farm. Ingredients were sourced from NutroScience, Malmesbury and the three enzyme products used were: BG (endo -1, 3(4) - β- glucanase (E1, Pentopan® Mono, 2500FXU-W/g), endo-1,4-xylanase (E2, Ultraflo® Max, 250 FXU-S/g) and α-D-galactosidase (E3, Alpha-Gal™ 600L, 600 GALU/g). These enzymes were sourced from Novozymes, South Africa, and were included at levels recommended by the supplier: 200 g/ton for β-glucanase and xylanase and 100 g/ton for α-galactosidase.

Table 6.2 Treatment diets used in Phase A: fish meal replacement phase.

Treatment composition	Treatment code
SBM0 (FM22, SBM0)	FME0 (control)
S15 (FM20, SBM15)	FMSBM
SBM15 (FM0, SBM15)	SBMlow
SBM30 (FM0, SBM30)	SBME0

Fish meal (FM), soybean meal (SBM), Fish meal and soybean meal (FMSBM) and low soybean meal (SBMlow), number indicates percent inclusion of ingredient. FM,SBM: refers to % inclusion of fish meal and soybean meal

Table 6.3 Phase B: enzyme treatment phase.

Treatment composition	Treatment code			
	β-glucanase (E1) (200ppm)	Xylanase (E2) (200ppm)	α-galactosidase (E3) (100ppm)	Combination (E123)
SBM0 (FM22, SBM0)	FM E1	FM E2	FM E3	FM E123
SBM30 (FM0, SBM30)	SBM E1	SBM E2	SBM E3	SBM E123

Fish meal (FM), soybean meal (SBM), E (enzyme), number indicates percent inclusion of ingredient. FM,SBM: refers to % inclusion of fish meal and soybean meal

Proximate analysis of the feed was performed in the Department of Animal Science, Stellenbosch University. The methods prescribed by the Association of Official Analytical Chemists (AOAC) were used. Crude fibre, crude fat, ash, moisture, protein, acid detergent fibre (ADF) and neutral detergent fibre (NDF) were determined (Goering & van Soest, 1970; AOAC, 2002a; AOAC, 2002b; AOAC, 2002c; AOAC, 2002d).

Three enzyme assay procedures (test kits) were obtained from Megazyme™, Ireland. These assays were used to test the in-feed non-starch polysaccharide levels in the fish meal and soybean meal diets respectively. Levels of D-xylose, β-glucan and α-D-galactose were tested according to procedures developed by McCleary (2003), with duplicate values for each assay obtained. Reagents were obtained from the Department of Polymer Science, Stellenbosch University.

6.3.5 Abalone growth performance

Food was withdrawn from animals twelve hours before weighing to ensure empty-gut weighing. Shell length was measured using an electronic calliper to the nearest 0.01 mm and weighed to the nearest 0.01 g using a pan-top electronic balance (UWE HGS-300). Measurements were made on days 1, 60, 120, 180, 210 and 240 respectively. The condition factor was only calculated for days 0 and 240 as prescribed by Britz (1996). Sampled animals were marked with bee tags to avoid repeated measures.

Britz Condition factor

Describes the relationship between the weight of the abalone per unit shell length (Britz, 1996).

Equation 6.3 Formula for the calculation of the condition factors (Britz, 1996)

$$\boldsymbol{Condition\ factor\ (Britz) = \left(\frac{Body\ weight\ (g)}{Shell\ length\ (mm)^{2}\text{^}99}\right) x\ 5575}$$

Feed conversion ratio

Feed conversion ratio (FCR) over the 240 (t_0-t_{240}) day trial is given in Equation 6.2, calculated as follows:

Equation 6.4 Formula for the calculation of feed conversion ratio

$$\boldsymbol{Feed\ conversion\ ratio\ (FCR) = \left(\frac{Feed\ intake\ (g)}{Final\ wet\ weight\ (g) - Initial\ wet\ weight\ (g)}\right)}$$

Specific growth rate (SGR)

It has been shown that the growth of Haliotids can be non-linear and SGR models should be used if observed. Britz (1997) stated that animals < 70mm generally follow a linear growth curve, whereas larger animals may grow non-linearly (Britz & Hecht, 1997). Trials that are of a long duration relative to the animals life span generally fit non-linear growth curves as opposed to short duration trials which are modelled linearly (Goosen, 2007). The growth curve of the trial is presented in **Error! Reference source not found.**. Specific growth rate is the growth over a period starting t_0 and ending t_1 and is shown in Equation 6.3.

Equation 6.5 Formula for the calculation of specific growth rate

$$\boldsymbol{Specific\ growth\ rate = \left(\frac{\ln\left(\text{weight at } \frac{x}{\text{weight}} \text{ initially}\right)}{\text{time at } x - \text{time initially}}\right) x\ 100}$$

Where W is animal weight and t is time in days

Average daily growth rate and shell length

The average daily growth rates (ADG), shown in Equation 6.4 and Equation 6.5, in terms of daily body weight (ADGW) and shell length (ADGSL) were calculated as follows:

Equation 6.6 Formula for the calculation of average daily gain in weight

$$\boldsymbol{Average\ daily\ gain\ in\ body\ weight = \left(\frac{Final\ wet\ weight\ (g) - \ initial\ wet\ weight\ (g)}{Time\ (days)}\right)}$$

Equation 6.7 Formula for the calculation of average daily gain in shell length

$$\boldsymbol{Average\ daily\ gain\ in\ shell\ length} = \left(\frac{\boldsymbol{Final\ shell\ length\ (mm)} - \boldsymbol{\ initial\ shell\ length\ (mm)}}{\boldsymbol{Time\ (days)}}\right)$$

Average growth rates are linear descriptions of growth and are equal to the slope of the regression line. To decide on the most suitable function to explain growth rates in this trial, a graph was plotted to the weight measurements recorded throughout the duration of the trial.

6.3.6 Data analysis

Data were analysed using an ANOVA, and a regression was fitted for each basket for each parameter over the trial duration and interpreted by means of a general linear model. SAS Enterprise guide (2004) statistical software was used.

6.4 Discussion and Results

6.4.1 Weight-length relationship

Growth in this trial was not isometric (b≠3), as has been observed in *H. midae* by Goosen (2007). As a result, the Fulton condition factor was not used, but rather the Britz factor, which is better suited to describe the animals' condition under these circumstances (b<3). The regression equation for all the animals is shown in **Error! Reference source not found.**.3 and **Error! Reference source not found.**, given by log (mass) = -2.99 + 2.56 log (length), R^2: 78.6%

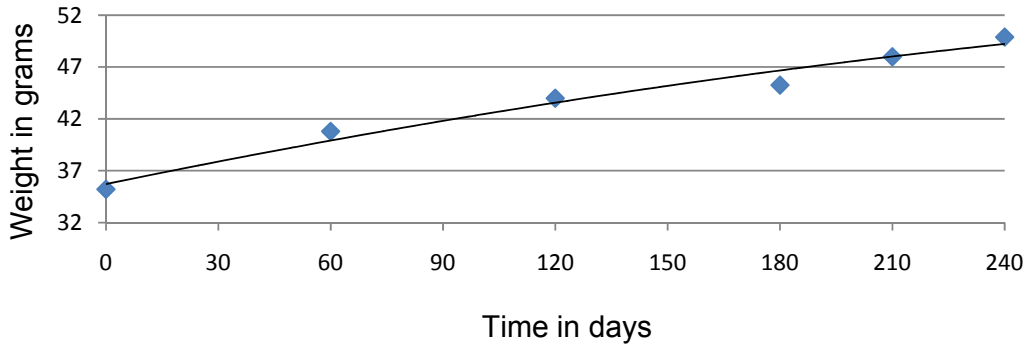


Figure 6.2 The non-linear fit of the line to the average weight gain of animals in over 240 days

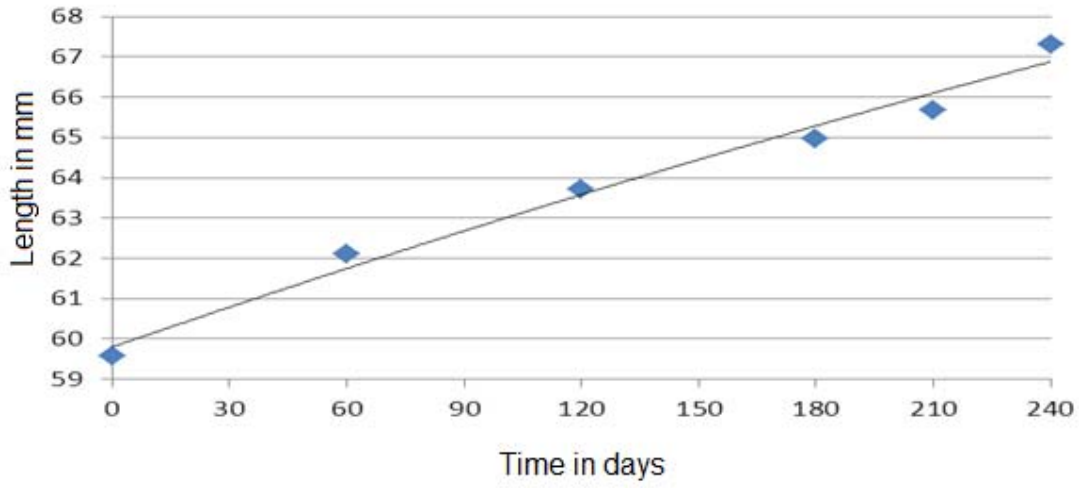


Figure 6.3 The non-linear fit of the line to the average length increase of animals over 240 days

Equation 8.6 Formula for weight gain and length increase curves are given by

$$\boldsymbol{Weight\ gain = -0.8E-05x2 + 0.075x + 35.72}$$

$$\boldsymbol{Length\ increase = 2E-05x2 + 0.034x + 59.80}$$

Where X= time in days

Animals were fairly large near the end of the trial (average length: 65.68 ± 4.27 mm), showing that the growth may have started to fit a more non-linear curve as average weight approached approximately 70 mm, as predicted by Britz (1996).

6.4.2 Water temperature

Error! Reference source not found. shows the average temperature throughout the duration of the trial, recorded as prescribed by Flemming (1996). The water temperature on average was 12.01 ± 1.16°C at 08:30 a.m. and 14.16 ± 1.25°C at 16:00 p.m. Optimal temperature for *H.midae* growth is between 12-20°C (Britz, 1996).

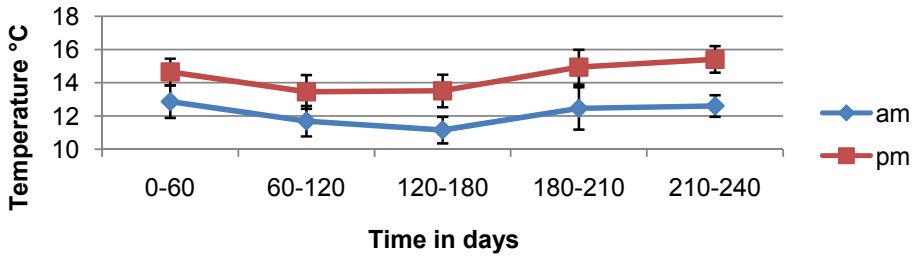


Figure 6.4 Average monthly water temperatures from April to November 2011, as recorded at 08:00 a.m. and 16:00 p.m.

During a period of sixty days (from day 120 to day 180), electricity lines in the district were re-laid, resulting in severe power outages. This meant that for about six weeks, the Hondeklipbaai area was without a constant electricity supply. In addition to these complications, the night time water temperatures were below optimal temperatures for abalone growth, as seen in the 08:30 a.m. readings. As a result, animals were very stressed during this period. Due to the power outages, water and aeration supplies were also often cut, compounding the stress further. Water quality was poor as tanks could not be cleaned as the stress of handling was deemed detrimental to animal health. Feeding was thus also greatly restricted and animals did not feed for several days at a time (Frik Venter, Farm manager, Personal communication). Consequently, animal growth was grossly impaired during this period and is apparent in the results over T_{120}-T_{180}.

6.4.3 In-feed antinutirent levels

The in-feed non-starch polysaccharides levels are shown in **Error! Reference source not found.**. The soybean meal diets had significantly higher ($P<0.05$) non-starch polysaccharide levels than the fish meal diets.

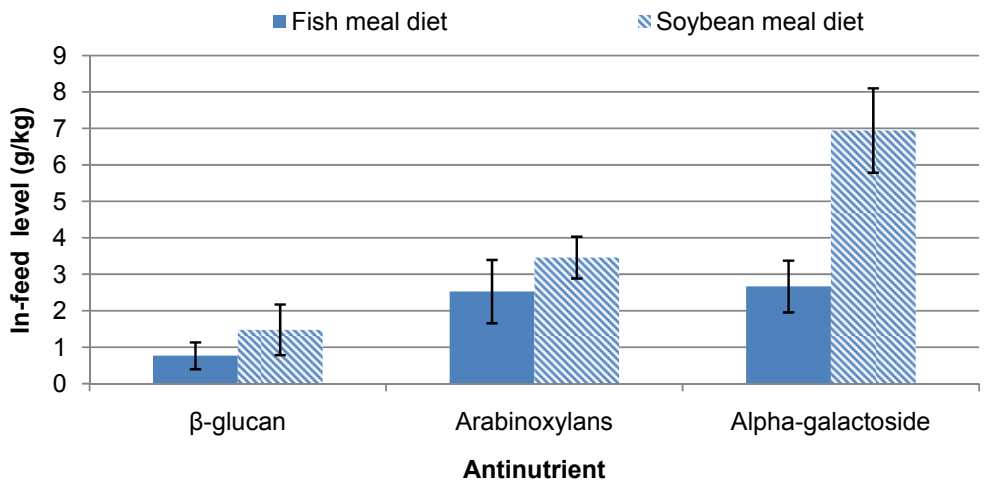


Figure 6.5 In-feed non-starch polysaccharide levels for fish meal and soybean meal based diets (± SD indicated by bars; n=5)

6.4.4 Phase A: Fish meal replacement phase

The treatment means for mass increase, length increase, average daily weight gain and length (ADGW and ADGL), specific growth rate (SGR), feed conversion ratio (FCR), Britz condition and percentage length and weight increase for the diets are reported in Figures 6.6-6.7 and Tables 6.4-6.7.

Table 6.4 Initial and final weight (g), shell length (mm) and standard growth rate (SGRW), feed conversion ratio (FCR) plus minus standard deviation of abalone over 240 days

Code	W_0 (g)	W_{240} (g)	L_o (mm)	L_{240} (mm)	B_{240}	SGRW (mg/day)	FCR
FM (C+)	34.93 ± 6.83[a]	50.10 ± 8.20[b]	59.26 ± 4.27[a]	65.66 ± 4.63[ab]	0.98 ± 0.13[ab]	0.18 ± 0.03[a]	1.21 ± 0.11[a]
FMSBM	35.72 ± 6.44[a]	50.29 ± 9.85[b]	59.28 ± 6.68[a]	64.71 ± 4.51[b]	0.98 ± 0.11[ab]	0.16 ± 0.05[a]	1.62 ± 1.16[a]
SBMlow	35.99 ± 7.23[a]	48.74 ± 8.56[b]	59.76 ± 6.51[a]	66.28 ± 4.21[ab]	0.95 ± 0.08[ab]	0.14 ± 0.05[a]	1.79 ± 0.68[a]
SBM	35.80 ± 6.95[a]	48.41 ± 8.25[b]	59.70 ± 3.87[a]	65.60 ± 4.17[b]	0.94 ± 0.09[b]	0.14 ± 0.03[a]	1.33 ± 0.40[a]
Eco	34.86 ± 5.34[a]	56.64 ± 7.56[a]	59.46 ± 6.33[a]	67.69 ± 3.74[a]	1.03 ± 0.14[a]	0.23 ± 0.02[a]	0.85 ± 0.11[a]
P-value	0.823	0.0001	0.797	0.0034	0.0001	0.078	0.321

[a-b] Means within a column with a common superscript are not significantly different (P>0.05);n=100Fish meal: FM, Fish meal soybean meal: FMSBM, low soybean meal: SBMlow, Soybean meal: SBM, Commercial Eco diet: Eco; E1: β-glucanase; E2: xylanase; E3: α-galactosidase

L: length, W: weight, B: Britz condition, SGRW: specific growth rate in weight, FCR: feed conversion rate, W_0: initial weigh, W_{240}: end weight, L_0: length initial, L_{240}: length final, B_{240}: Britz final

Table 6.5 Phase one weight measurements in grams and average daily gain in weight (ADGW) plus minus standard deviation over the 240 day trial period, ± standard deviation

Code	W_0 (g)	W_{60} (g)	W_{120} (g)	W_{180} (g)	W_{210} (g)	W_{240} (g)	ADGW (mg/day)
FM (C+)	34.93 ± 6.33^{a}	39.61 ± 6.33^{bdc}	44.14 ± 6.33^{bdc}	46.55 ± 9.00^{abc}	48.46 ± 8.49^{b}	50.10 ± 8.20^{b}	62.07 ± 8.84^{a}
FMSBM	35.71 ± 6.66^{a}	42.49 ± 6.07^{abc}	45.74 ± 6.07^{abc}	45.36 ± 8.47^{abc}	46.60 ± 9.36^{b}	50.29 ± 9.85^{b}	48.01 ± 9.00^{a}
SBMlow	35.99 ± 7.76^{a}	42.65 ± 7.08^{ab}	43.66 ± 7.08^{bdc}	47.14 ± 7.42^{ab}	48.63 ± 7.40^{b}	48.74 ± 8.56^{b}	56.00 ± 3.20^{a}
SBM	35.79 ± 6.71^{a}	38.43 ± 6.91^{d}	40.26 ± 6.91^{d}	42.98 ± 8.45^{c}	45.99 ± 8.35^{b}	48.41 ± 8.25^{b}	44.07 ± 8.84^{a}
Eco	34.85 ± 5.85^{a}	42.39 ± 5.09^{abc}	48.52 ± 5.09^{a}	49.28 ± 7.15^{a}	54.90 ± 8.22^{a}	56.64 ± 7.56^{a}	65.90 ± 8.81^{a}
P-value	0.823	0.0001	0.0001	0.0001	0.0001	0.0001	0.127

$^{a-b}$ Means within a column with a common superscript are not significantly different (P>0.05);n=100

Fish meal: FM, Fish meal soybean meal: FMSBM, low soybean meal: SBMlow, Soybean meal: SBM, Commercial Eco diet: Eco; E1: β-glucanase; E2: xylanase; E3: α-galactosidase
W_0: initial weigh, W_{240}: end weight, L_0: length initial, L_{240}: length final, B_{240}: Britz final, , ADGW: average daily gain in weight

Table 6.6 Phase one shell length measurements and average daily gain in length (ADGL) plus minus standard deviation in mm over the 240 day trial period, ± standard deviation

Code	SL_0 (mm)	SL_{60} (mm)	SL_{120} (mm)	SL_{180} (mm)	SL_{210} (mm)	SL_{240} (mm)	ADGL (µm/day)
FM (C+)	59.40 ± 4.39^{b}	61.38 ± 3.96^{c}	63.09 ± 3.80^{b}	64.58 ± 4.57^{ab}	65.66 ± 4.62^{ab}	66.73 ± 4.65^{b}	28.23 ± 4.38^{a}
FMSBM	59.95 ± 3.79^{ab}	62.68 ± 3.88^{abc}	64.10 ± 4.14^{b}	64.46 ± 3.94^{b}	64.72 ± 4.51^{b}	66.60 ± 4.58^{ab}	22.64 ± 4.39^{a}
SBMlow	59.96 ± 3.78^{ab}	63.19 ± 3.94^{bc}	63.65 ± 4.00^{a}	65.98 ± 3.62^{a}	66.28 ± 4.21^{ab}	66.68 ± 3.93^{ab}	30.59 ± 4.39^{a}
SBM	59.70 ± 3.87^{b}	60.79 ± 3.64^{c}	62.35 ± 3.84^{b}	64.47 ± 4.4^{b}	65.6 ± 4.17^{b}	66.76 ± 4.22^{b}	27.28 ± 4.39^{a}
Eco	59.86 ± 2.86^{ab}	62.89 ± 3.64^{a}	65.72 ± 3.55^{a}	67.01 ± 3.96^{a}	67.69 ± 3.74^{a}	68.53 ± 3.32^{a}	37.72 ± 4.37^{a}
P-value	0.823	0.0001	0.0001	0.0001	0.0001	0.0001	0.393

$^{a-b}$ Means within a column with a common superscript are not significantly different (P>0.05);n=100

Fish meal: FM, Fish meal soybean meal: FMSBM, low soybean meal: SBMlow, Soybean meal: SBM, Commercial Eco diet: Eco; E1: β-glucanase; E2: xylanase; E3: α-galactosidase
SL: shell length; ADGL: average daily gain in length

At the beginning of the trial, T_0, animal weight and length did not differ significantly ($P>0.05$) between the treatments. The ECO diet fed animals had gained the most weight by the end of the 240 day trial period, differing significantly ($P<0.05$) from the FM (control) and all the other treatments over days 120, 180, 210 and 240. The SBMFM and SBMlow diets differed significantly ($P<0.05$) from the SBM diet at day 60 for weight. The SBMlow diet showed a significant difference ($P<0.05$) from the SBM diet again on day 180 for weight. The FM (control) diet did not differ significantly ($P<0.05$) from the SBM diet for weight over the duration of the trial. By the end of the trial (day 240) animals fed the ECO diet had gained 18.73% (55.54 ± 9.00g) more body weight than the animals fed the SBM diet (48.41 ± 8.25g) since the onset of the trial. The rate of weight gain and the length of the abalone over the trial period are shown in **Error! Reference source not found.** and **Error! Reference source not found.**. The regression equations are given in **Error! Reference source not found.**7.

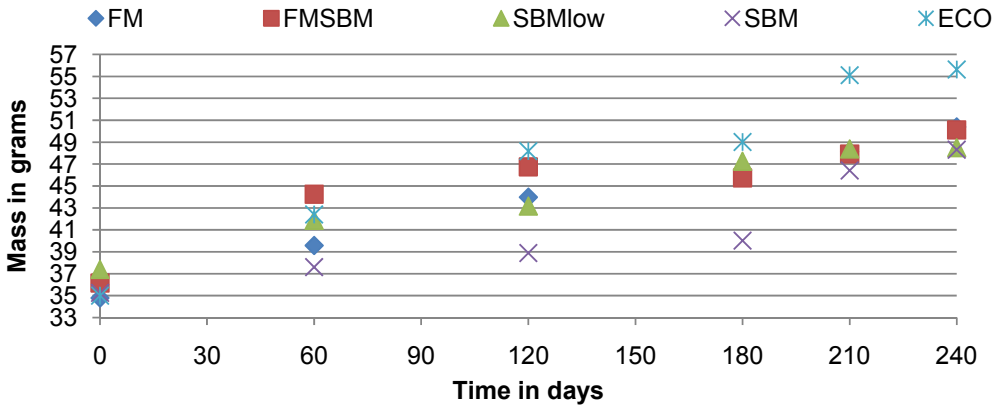


Figure 6.6 Weight gain (g) of fish meal reduced diets over 240 days; FM: fish meal, SBM: soybean meal, FMSBM: fish meal- soybean meal, SBMlow, soybean low and ECO: commercial diet

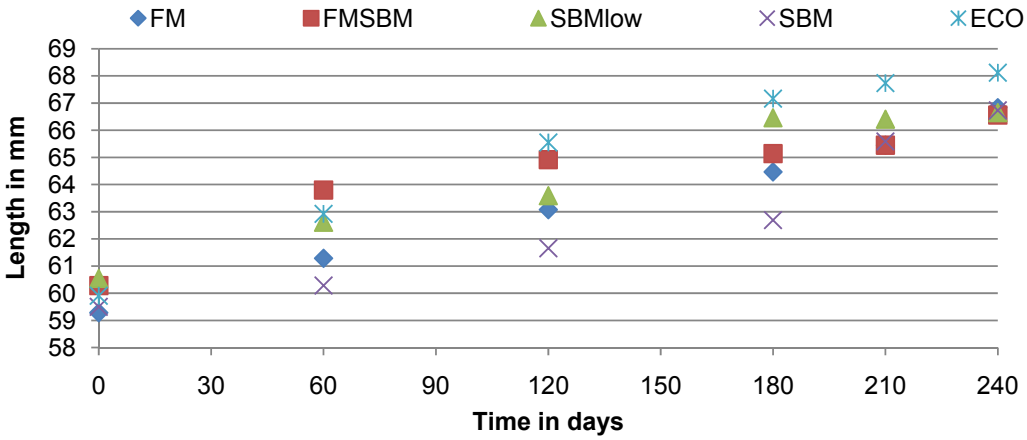


Figure 6.7 Length increase (mm) of enzyme treated diets over 240 days; FM: fish meal, SBM: soybean meal, FMSBM: fish meal- soybean meal, SBMlow, soybean low and ECO: commercial diet

Table 6.7 Regression equations for the fish meal replaced diets weight gain and shell length increase over 240 days.

	Mass		Length		
Treatment	Regression equation	R^2	Regression equation	R^2	Differences*
FM (C+)	$y = 7E\text{-}05x^2 + 0.0803x + 34.929$	0.99	$y = 5E\text{-}06x^2 + 0.0286x + 59.393$	1.00	a
FMSBM	$y = -0.0002x^2 + 1.003x + 37.195$	0.88	$y = -0.0001x^2 + 0.0468x + 60.66$	0.98	a
SBMlow	$y = -7E\text{-}05x^2 + 0.0642x + 37.557$	0.97	$y = -4E\text{-}05x^2 + 0.0357x + 60.50$	0.95	a
SBM (C-)	$y = 0.0002x^2 + 0.0067x + 35.905$	0.93	$y = 0.0001x^2 + 0.0006x + 59.645$	0.99	a
ECO	$y = -0.0001x^2 + 0.1127x + 35.448$	0.99	$y = -0.0001x^2 + 0.0588x + 59.87$	0.99	a

*Slopes with different letters, differ significantly (P<0.05)

y= Mass and length; x= growth rate; FM: fish meal, SBM: soybean meal, FMSBM: fish meal- soybean meal, SBMlow, soybean low and ECO: commercial diet

The length of animals fed the ECO diet differed significantly (P<0.05) from the FM (control) and SBM fed abalone after 60 days. The ECO fed animals also differed significantly (P<0.05) from all other treatment animals after 120 and 180 days. After 210 days however, the ECO diet only differed significantly (P<0.05) from the SBM and FMSBM diets. At the end of the trial, no significant differences (P>0.05) between shell lengths existed for any of the treatments. Shell length has often been considered to be less important than weight gain and was rather used as a diagnostic tool for farmers to determine the time of harvest (Fleming *et al*., 1996). However, it is now possible to model mass from length as described by Goosen (2007).

The ADGW and ADGL did not differ significantly (P>0.05) between any treatments. Studies on juvenile abalone have shown weight growth rates to be comparable between fish meal and soybean meal diets (Fleming et al., 1996; Guzm´an & Viana, 1998; Bautista-Teruel et al., 2003; Sales., 2004). Long term differences in growth rates have been observed between these diets in fin-fish, and thus soybean meal inclusion remains a concern due to long term morphological impacts of soybean meal on the intestine (Francis *et al*., 2001). No long term growth (<160 days) differences were observed in this study and it can therefore be said that these morphological impacts, if present, did not affect growth

rate over time. The stress conditions between days 120-180 may also have had an effect here.

The SGR of weight gain did not differ significantly either ($P>0.05$) in terms of the weight and length growth rates of the trial. The SBM diet fed abalone had the poorest condition as determined by the Britz condition factor, whilst the ECO diet had the best condition. The ECO diet differed significantly ($P<0.05$) from the SBMlow and SBM diets for the Britz condition. It is has been suggested that soybean meal based diets lack essential nutrients which must be supplemented. This raises concerns over nutrient leaching and delivery to the animal, if true soybean meal fed animals should have been in poorer condition than the other treatment fed animals (Cruz-Suárez *et al*., 2009). However, no other treatments differed significantly ($P>0.05$) from the SBM diet, suggesting that the commercial ECO diet may improve condition as opposed to SBM, which causes condition loss. The energy of the ECO diet was not iso-energetic when compared to the other diets; this may have resulted in the difference in performance.

The FCR's obtained in this trial varied, but were within the ranges (0.44-3.00) stipulated by the relevant literature (Britz, 1996; Knauer *et al*., 1996; Bautista-Teruel & Millamena, 1999; Goosen, 2007; Green *et al*., 2011). Although the FCR's ranged from 0.85-1.21 in this trial, statistically significant ($P>0.05$) differences were not observed on the 5% level. Again, the large variation within the treatment groups masked statistical differences.

The absence of a significant difference ($P>0.05$) between the SBM and FM (control) diet is of importance. The negative impacts of the SBM non-starch polysaccharides do not seem to significantly impair the length and weight gain of abalone, as they have been credited in doing in many fin-fish species (Dersjant-Li, 2002; Krogdahl *et al*., 2010; Sinha *et al*., 2011). Bautista-Tereuel (2003) and Cho (2011) observed that abalone fed a combination of animal and plant proteins, may, in some cases, perform better than animals fed a single plant or animal protein source. These findings were not observed in this trial, as no difference between any of the FM-replaced treatments was consistently observed. Comparisons between different trials are difficult as stocking density, animal age and water temperature are all important factors in growth trials (Sales & Britz, 2001).

The ECO and control FM diet were the only two diets to differ significantly ($P<0.05$) from the SBM diet for periodical weight measurements (60, 120, 180 and 210). The ECO diet differed significantly ($P<0.05$) from the FM diet. Thus, animals fed on partial or complete FM replaced diets displayed lower weight gain over the 240 day trial, although the rate of

gain did not differ significantly($P>0.05$). The treated commercial diet was thus the best performing diet, also showing significant differences ($P<0.05$) in weight and shell length in comparison to the other treatments at different times. FM, SBM, FMSBM and SBMlow diets did not differ significantly for SGR, weight gain or shell length increase over the 240 day trial. Soybean meal replaced diets can therefore potentially be used to replace fish meal diets. In addition, future research into this topic should be aimed at reducing initial sample variation by grading animals according to weight and the trial should also run for at least 240 days.

6.4.5 Phase B: Enzyme treatment phase

The treatment means for mass and length increase, ADGW, ADGL and SGR, FCR and Britz, for the diets are reported in Figures 6.8-6.9 and Tables 6.8-6.11. At the beginning of the trial, weight, length and Britz did not differ significantly ($P>0.05$).

The ECO diet fed animals differed significantly ($P<0.05$) from both the animals fed the positive control (FME0) and all the other diets over 120, 180, 210 and 240 days for weight, but did not differ significantly ($P>0.05$) from the FME0 control fed animals over 60 days. Over 60 days, ECO, FME123 and SME123 fed animals differed significantly from the SBME0 diet fed animals, with only ECO and SBME123 also differing from the positive control (FME0) for weight. Over 120 days, ECO, FME1, FME3, FME123 and SBME123 fed animals differed significantly ($P<0.05$) from the SBME0 fed animals diet for weight. After 180 days, ECO fed animals only differed from the SBME0 diet, whilst the FM control differed significantly ($P>0.05$) from the FME1, FME2, FME123, SBME1, SBME2 and SBME3 fed animals for weight.

Shell length was longest again in the ECO fed animals, differing significantly ($P<0.05$) from animals fed FME1, FME2, FME123, SBME0 and SBME1 diets for every measurement period after the onset of the trial (60, 120 and 180). After 210 days, the ECO diet fed animals differed significantly ($P<0.05$) from SBME0 animals, and after 240 days, the ECO diet fed animals differed significantly ($P<0.05$) from FME1, SBME1 and SBME3 fed animals for length.

Table 6.8 Initial and final weight, shell length, Britz (B_{240}), standard growth rate (SGR) and feed conversion ratio (FCR) plus minus standard deviation of abalone over 210 days

Feed type	Code	W_0 (g)	W_{240} (g)	SL_0 (mm)	SL_{240} (mm)	B_{240}	SGR (d^{-1})	FCR
Fish meal	FME0 (C+)	34.93 ± 6.83^{a}	50.10 ± 8.20^{b}	59.26 ± 4.27^{a}	66.73 ± 4.65^{ab}	0.98 ± 0.13^{abc}	0.18 ± 0.03^{a}	1.21 ± 0.11^{a}
	FME1	35.72 ± 5.91^{a}	48.93 ± 8.92^{b}	59.19 ± 3.64^{a}	65.95 ± 3.82^{b}	0.99 ± 0.11^{ab}	0.15 ± 0.06^{a}	1.65 ± 0.76^{a}
	FME2	35.57 ± 7.51^{a}	51.45 ± 10.52^{b}	59.29 ± 3.83^{a}	66.21 ± 3.90^{ab}	0.97 ± 0.14^{abc}	0.18 ± 0.06^{a}	1.43 ± 0.43^{a}
	FME3	35.95 ± 7.29^{a}	50.41 ± 9.60^{b}	59.99 ± 4.28^{a}	67.53 ± 4.59^{ab}	0.94 ± 0.07^{c}	0.16 ± 0.02^{a}	1.24 ± 0.26^{a}
	FME 123	34.39 ± 6.74^{a}	48.82 ± 10.15^{b}	59.32 ± 4.30^{a}	66.48 ± 4.72^{ab}	0.96 ± 0.08^{bc}	0.17 ± 0.03^{a}	1.23 ± 0.35^{a}
Soybean meal	SBME0	35.8 ± 6.95^{a}	48.41 ± 8.25^{b}	59.70 ± 3.87^{a}	66.76 ± 4.22^{ab}	0.94 ± 0.09^{c}	0.14 ± 0.03^{a}	1.33 ± 0.40^{a}
	SBME1	36.02 ± 7.15^{a}	47.74 ± 9.14^{b}	59.79 ± 4.03^{a}	66.05 ± 4.79^{b}	0.96 ± 0.10^{bc}	0.13 ± 0.07^{a}	1.36 ± 0.32^{a}
	SBME2	35.10 ± 6.16^{a}	48.04 ± 8.48^{b}	59.19 ± 3.47^{a}	66.57 ± 4.51^{ab}	0.94 ± 0.10^{c}	0.15 ± 0.02^{a}	1.31 ± 0.30^{a}
	SBME3	35.52 ± 7.20^{a}	48.95 ± 7.85^{b}	59.54 ± 3.96^{a}	66.57 ± 4.12^{b}	0.98 ± 0.11^{abc}	0.15 ± 0.02^{a}	1.26 ± 0.23^{a}
	SBME 123	34.86 ± 5.76^{a}	49.69 ± 8.19^{b}	59.48 ± 6.33^{a}	67.11 ± 3.85^{ab}	0.95 ± 0.10^{bc}	0.17 ± 0.03^{a}	1.13 ± 0.26^{a}
Commercial	Eco	34.86 ± 5.34^{a}	56.64 ± 7.56^{a}	59.46 ± 6.33^{a}	68.13 ± 4.78^{a}	1.03 ± 0.14^{a}	0.23 ± 0.02^{a}	0.85 ± 0.21^{a}
	P-value	0.823	0.0001	0.879	0.034	0.0001	0.078	0.323

$^{a-b}$ Means within a column with a common superscript are not significantly different (P>0.05);n=5

Fish meal: FME0, Fish meal with enzyme: FME1-123, Soybean meal: SBME0; Soybean meal with enzyme: SBME1-123, Commercial Eco diet: Eco; E1: β-glucanase; E2: xylanase; E3: α-galactosidase. W_{0-240}: initial and final weight; SL_{0-240}: initial and final shell length; ratio

Table 6.9 Phase two weight measurements and the average daily gain in weight and average daily gain (ADGW) plus minus standard deviation in grams over the 240 day trial period, ± standard deviation

Code	W_0 (g)	W_{60} (g)	W_{120} (g)	W_{180} (g)	W_{210} (g)	W_{240} (g)	ADGW (mg/day)
FME0 (C+)	34.61 ± 6.83^{a}	39.61 ± 6.92^{cd}	44.15 ± 6.90^{cd}	46.55 ± 9.00^{abc}	48.46 ± 8.49^{bc}	50.10 ± 8.20^{b}	62.07 ± 8.84^{a}
FME1	35.72 ± 5.91^{a}	41.03 ± 6.53^{abc}	43.49 ± 7.74^{c}	44.79 ± 8.84^{bc}	47.85 ± 7.99^{bc}	49.12 ± 8.92^{b}	52.05 ± 8.81^{a}
FME2	35.57 ± 7.51^{a}	41.59 ± 8.92^{abc}	42.29 ± 8.69^{cd}	44.92 ± 9.02^{bc}	47.98 ± 7.38^{bc}	51.45 ± 10.52^{b}	51.95 ± 8.80^{a}
FME3	35.95 ± 7.29^{a}	41.53 ± 8.43^{abc}	44.77 ± 8.23^{ab}	45.42 ± 9.72^{abc}	48.34 ± 9.63^{bc}	50.41 ± 9.60^{b}	53.77 ± 8.82^{a}
FME 123	34.39 ± 6.74^{a}	42.61 ± 9.34^{a}	45.28 ± 7.65^{ab}	44.73 ± 9.15^{bc}	46.95 ± 8.12^{bc}	48.82 ± 10.15^{b}	51.61 ± 8.81^{a}
SBME0	35.72 ± 6.95^{a}	38.44 ± 7.58^{d}	40.26 ± 8.15^{d}	42.98 ± 8.45^{c}	45.99 ± 8.35^{bc}	48.41 ± 8.25^{b}	44.07 ± 8.84^{a}
SBME1	35.99 ± 7.15^{a}	39.63 ± 6.95^{cd}	43.02 ± 9.01^{cd}	44.60 ± 8.45^{bc}	45.40 ± 8.25^{c}	47.74 ± 9.14^{b}	44.75 ± 8.80^{a}
SBME2	35.10 ± 6.16^{a}	39.56 ± 9.15^{cd}	43.49 ± 8.61^{cd}	45.04 ± 6.30^{bc}	47.02 ± 8.45^{bc}	48.04 ± 8.48^{b}	53.61 ± 8.82^{a}
SBME3	35.52 ± 7.20^{a}	38.81 ± 5.77^{cd}	42.29 ± 9.37^{cd}	45.58 ± 8.38^{bc}	48.27 ± 9.15^{bc}	48.95 ± 7.85^{b}	57.00 ± 8.85^{a}
SBME 123	34.86 ± 5.76^{a}	43.65 ± 8.09^{a}	46.31 ± 8.47^{a}	46.40 ± 8.51^{abc}	49.85 ± 7.68^{b}	49.69 ± 8.19^{b}	60.89 ± 8.82^{a}
Eco	34.86 ± 5.34^{a}	42.40 ± 7.42^{ac}	48.53 ± 7.78^{a}	49.28 ± 7.15^{a}	54.9 ± 8.22^{a}	56.64 ± 7.56^{a}	65.90 ± 8.81^{a}
P-value	0.823	0.0001	0.0001	0.0001	0.0001	0.0001	0.127

$^{a-b}$ Means within a column with a common superscript are not significantly different (P>0.05);n=5
Fish meal: FME0, Fish meal with enzyme : FME1-123, Soybean meal: SBME0; Soybean meal with enzyme: SBME1-123, Commercial Eco diet: Eco; E1: β-glucanase; E2: xylanase; E3: α-galactosidase
W_{0-240} Initial weight to final weight

Table 6.10 Phase two shell length measurements and average daily gain in length and average daily gain in shell length (ADGSL) plus minus standard deviation in mm over the 240 day trial period

Code	SL_0 (mm)	SL_{60} (mm)	SL_{120} (mm)	SL_{180} (mm)	SL_{210} (mm)	SL_{240} (mm)	ADGL (µm/day)
FME0 (C+)	59.40 ± 4.39^{a}	61.38 ± 3.96^{bcd}	63.09 ± 3.80^{c}	64.58 ± 4.57^{bc}	65.66 ± 4.62^{ab}	66.73 ± 4.65^{ab}	61.38 ± 3.96^{a}
FME1	59.19 ± 3.64^{a}	61.69 ± 3.90^{bcd}	63.55 ± 4.05^{bc}	64.64 ± 3.78^{bc}	65.42 ± 3.73^{b}	66.01 ± 4.85^{b}	61.69 ± 3.90^{a}
FME2	59.29 ± 3.83^{a}	62.08 ± 4.32^{abcd}	62.76 ± 4.16^{c}	63.91 ± 3.97^{c}	65.49 ± 3.91^{b}	67.22 ± 4.59^{ab}	62.08 ± 4.32^{a}
FME3	59.99 ± 4.28^{a}	61.99 ± 4.37^{abcd}	63.89 ± 4.27^{abc}	64.77 ± 4.52^{bc}	66.05 ± 4.60^{ab}	67.53 ± 4.6^{ab}	61.99 ± 4.37^{a}
FME 123	59.32 ± 4.30^{a}	62.95 ± 4.63^{abc}	64.07 ± 4.68^{abc}	64.82 ± 4.58^{bc}	65.21 ± 4.73^{b}	66.6 ± 4.56^{ab}	62.95 ± 4.63^{a}
SBME0	59.7 ± 3.87^{a}	60.79 ± 3.64^{cd}	62.35 ± 3.84^{c}	64.47 ± 4.40^{bc}	65.6 ± 4.17^{b}	66.76 ± 4.22^{ab}	60.79 ± 3.64^{a}
SBME1	59.79 ± 4.03^{a}	61.15 ± 3.9^{cd}	64.2 ± 3.42^{abc}	64.19 ± 4.64^{bc}	64.68 ± 4.51^{b}	66.05 ± 4.79^{b}	61.15 ± 3.90^{a}
SBME2	59.11 ± 3.47^{a}	61.41 ± 4.91^{cd}	63.49 ± 3.98^{bc}	65.12 ± 3.34^{abc}	65.73 ± 3.94^{ab}	66.57 ± 4.12^{ab}	61.41 ± 4.91^{a}
SBME3	59.54 ± 3.96^{a}	61.4 ± 3.41^{bcd}	62.67 ± 3.93^{c}	64.62 ± 3.83^{bc}	65.46 ± 4.36^{b}	66.15 ± 3.92^{b}	61.40 ± 3.41^{a}
SBME 123	59.48 ± 3.43^{a}	63.94 ± 4.08^{a}	65.18 ± 3.84^{ab}	65.65 ± 4.22^{abc}	65.83 ± 3.80^{ab}	67.11 ± 3.85^{ab}	63.94 ± 4.08^{a}
Eco	59.86 ± 2.86^{a}	62.89 ± 3.64^{abc}	65.72 ± 3.55^{a}	67.01 ± 3.96^{a}	67.69 ± 3.74^{b}	68.53 ± 3.32^{a}	62.89 ± 3.64^{a}
P-value	0.796	0.0001	0.0001	0.0001	0.0002	0.0034	0.393

$^{a-b}$ Means within a column with a common superscript are not significantly different (P>0.05);n=5

Fish meal: FME0, Fish meal with enzyme : FME1-123, Soybean meal: SBME0; Soybean meal with enzyme: SBME1-123, Commercial Eco diet: Eco; E1: β-glucanase; E2: xylanase; E3: α-galactosidase; $SL_{0\text{-}240}$: Initial to final shell length

The ADGW and ADGL, determined from the slopes of the regression lines, did not differ significantly ($P<0.05$) at any point between any of the treatments. Graphically however, tendencies of increased growth were observed (by ECO and SBME123 in particular). The specific growth rates for mass and length of the treatments did not differ significantly ($P>0.05$) over the duration of the trial. SGR may be more suited to description of growth, especially in trials of longer duration (Fleming *et al*., 1996; Goosen, 2007).

For the 240 day trial, the Britz value of the SBME0, SBME2 and FME123 fed animals, was significantly lower ($P<0.05$) than the ECO and FME2 treatment animals. No other differences were observed however. This shows that enzyme supplementation may affect the ability of animals to digest nutrients conducive to growth. The low Britz values indicate leaner animals as opposed to 'meatier' animals, observed in the ECO fed animals. Again, the higher energy value of the ECO diet may have been responsible for the improved performance observed in this trial. The weight gain and length of the abalone over the trial period are given in **Error! Reference source not found.** and **Error! Reference source not found.**. The regression equations are given in **Error! Reference source not found.**.

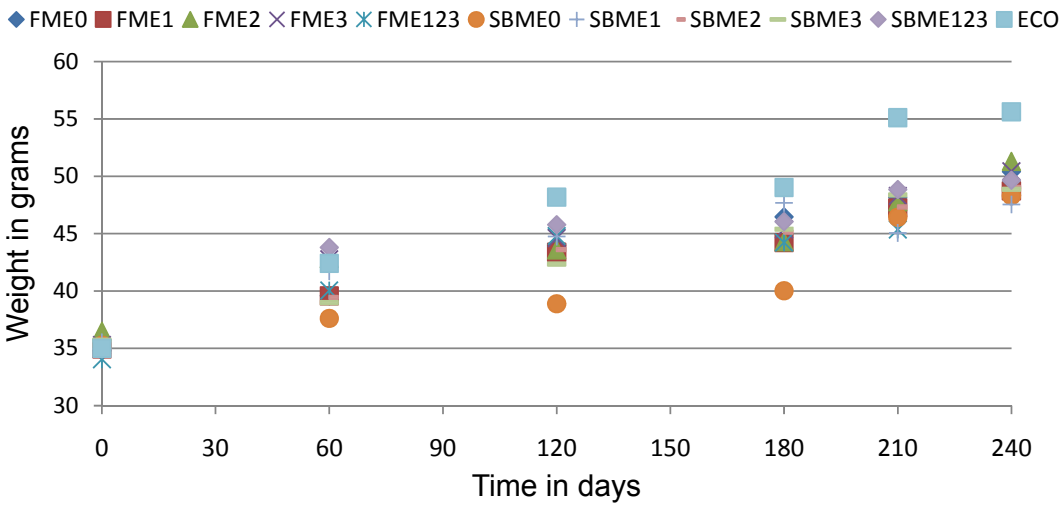


Figure 6.8 Weight gain of fish meal and soybean meal fed abalone over 240 days; Fish meal: FME0, Fish meal with enzyme: FME1-123, Soybean meal: SBME0; Soybean meal with enzyme: SBME1-123, Commercial Eco diet: Eco; E1: β-glucanase; E2: xylanase; E3: α-galactosidase.

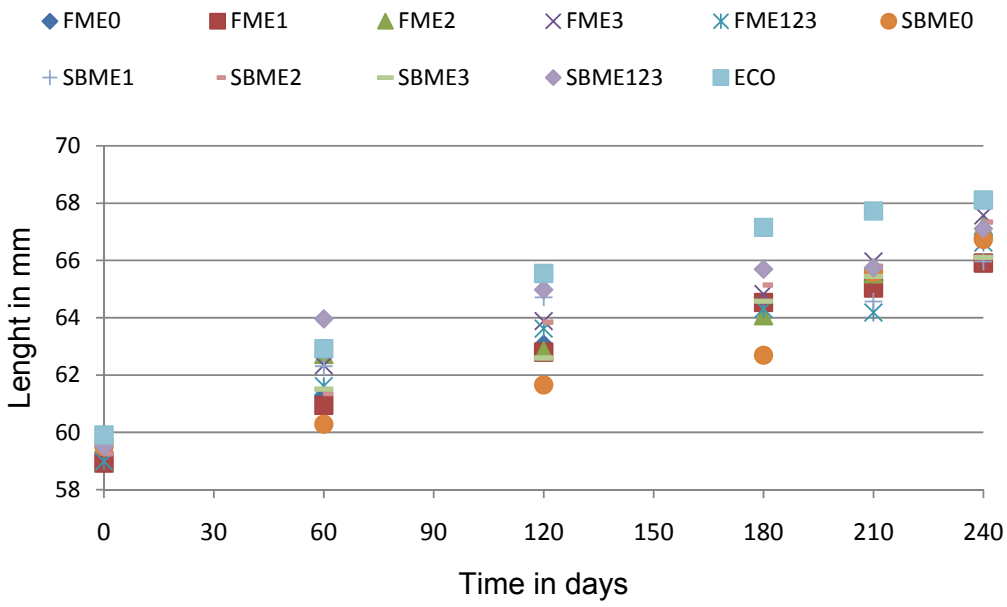


Figure 6.9 Length increase of enzyme treated diets over 240 days; Fish meal: FME0, Fish meal with enzyme: FME1-123, Soybean meal: SBME0; Soybean meal with enzyme: SBME1-123, Commercial Eco diet: Eco; E1: β-glucanase; E2: xylanase; E3: α-galactosidase.

Table 6.11 Regression equations for the enzyme treated diets weight gain and shell length increase over 240 days.

	Mass		Length		
Treatment	Regression equation	R^2	Regression equation	R^2	Differences*
FME0 (C+)	$y = -7E-05x^2 + 0.0803x + 34.929$	0.99	$y = 5E-06x^2 + 0.0286x + 59.393$	1	a
FME1	$y = -0.0001x^2 + 0.0799x + 36.066$	0.97	$y= -0.336x^2 + 55.91$	0.99	a
FME2	$y = -7E-05x^2 + 0.0694x + 36.152$	0.96	$y= -0.982x^2 +2.069x + 57.64$	0.96	a
FME3	$y = -0.0001x^2 + 0.0849x + 36.3$	0.97	$y= -0.195x^2 + 2.65x +57.54$	0.99	a
FME123	$y= -1.098x^2 + 9.43x + 26.75$	0.96	$y= -0.484x^2 + 4.279x +55.73$	0.98	a
SBME0	$y = 0.0002x^2 + 0.0067x + 35.905$	0.99	$y = 0.0001x^2 + 0.0006x + 59.645$	0.99	a
SBME1	$y= -0.358x^2 + 4.69x + 31.73$	0.98	$y=-0.305x^2 + 3.24x + 56.603$	0.96	a
SBME2	$y= -0.452x^2 + 5.703x + 29.86$	0.99	$y= -0.299x^2 + 3.518x + 55.79$	0.99	a
SBME3	$y= -0.0877x^2 + 3.657x + 31.917$	0.99	$y= -0.0578x^2 + 1.869x + 57.75$	0.99	a
SBME123	$y = -0.0003x^2 + 0.1225x + 35.601$	0.96	$y= -0.005x^2 + 0.0237x +59.635$	0.99	a
ECO	$y = -0.0001x^2 + 0.1127x + 35.448$	0.96	$y = -0.0001x^2 + 0.0588x + 59.87$	0.99	a

*Slopes with different letters, differ significantly (P<0.05); y= mass or length; x= growth rate; Fish meal: FME0, Fish meal with enzyme: FME1-123, Soybean meal: SBME0; Soybean meal with enzyme: SBME1-123, Commercial Eco diet: Eco; E1: β-glucanase; E2: xylanase; E3: α-galactosidase.

The animals fed fish meal diets supplemented with enzymes (FME1-123) did not differ significantly (P>0.05) from the FM (control) diet fed animals after 180, 210 and 240 days for weight. Only animals fed FME123 differed significantly (P<0.05) from FME0 and SBME0 after 60 and 120 days, and only from SBME0 animals after 210 days. None of the other SBME1-3 fed animals showed a significant difference (P>0.05) in weight from the control fed animals over the entire trial duration. The effect of enzymes in fish meal diets is of less consequence, as seen in soybean

meal-based diets, where SBM123 fed animals consistently differed from SBME0 animals for weight. An oversupply of β-glucanase (and possibly other fibrolytic enzymes) has been shown to create excess monosaccharide production in poultry, especially in diets with a low level of oligosaccharides. This creates an osmotic effect by fluid retention which in turn affects the feed passage rate, as well as having a negative effect on nutrient absorption (Schutte, 1990; Irish & Balnave, 1993). Thus, the need or use of enzymes in fish meal diets should be further evaluated for animals with low variation, in order to investigate this if enzymes could be oversupplied. It is doubtful that this happened however, due to the fact that FME3 and FME123 fed animals showed improved weight gain after 60 and 120 days.

Carbohydrates in soybean meal are largely available as oligosaccharides in the form of sucrose, raffinose and stachyose. Sucrose is largely available to aquatic species, but raffinose and stachyose are not. These carbohydrates require an α-galactosidase activity to break them down and this enzyme is largely absent in aquatic species (Gatlin, 2007). The SBME123 diet resulted in the heaviest animals of the soybean meal fed animals, with a consistent trend of significantly increased weight over W_{60}-W_{210}, but not W_{240}.

Reasons for the few observed differences in this trial include the poor growth experienced during the 120-180 day period due to the power outages. Over this time, performance was grossly stunted, along with the fact that the large variation within the treatments made statistical differences difficult to observe. Future trials must place emphasis on reducing variation within treatments as expressed by Goosen (2007) and trials should run for a minimum of 180 days. Animals should also be split into groups according to their sizes (as appears in research done by Shipton (1999) and Tsanigab (2007)) to determine possible size or age effects. Further investigation into enzyme treated soybean meal diets is also required as soybean meal diets treated by enzymes may lead to cost effective saving and increased production performance. The final weights of the soybean meal diets, though not significant, were lower than those of the fish meal diets. The slightly slower growth rate may be as a result of the poorer digestibility of the soybean meal proteins as well as the inherent protein deficiencies in the soybean meal diet (O'Keef, 2003; Gatlin, 2007).

6.6 Conclusion

The objective of this trial was to investigate the effect of fish meal replacement and enzyme treatment on the production performance of abalone.

No differences were found between the treatment growth rates and FCR measurements in Phase A of the trial (replacement diets). However, the commercial ECO diet fed animals did differ significantly ($P<0.05$) for shell length and final weight from all the other animals in Phase A. In Phase B (enzyme treatment phase), the ECO fed animals differed significantly ($P<0.05$) from all the other treatment fed animals for final weight, also differing from FME1, SBME1 and SBME3 for final length. The significantly improved performance of the ECO diet can be attributed to its higher energy content, and the diet's significantly better production performance is therefore to be expected. Focus should be placed on the soybean meal diets that did not perform worse than the fish meal diets, showing potential as replacements. Despite the fact that weight gain tendencies were shown, enzyme treatment did not significantly improve growth in abalone. With specific reference to Phase B, the large variation within treatments observed in this trial may have masked some significant parameter differences.

The lack of differences between the fish meal and soybean meal fed abalone indicate that soybean meal-based diets could potentially be used as a replacement for fish meal based feeds without compromising production performance. The use of enzymes did not result in significant ($P<0.05$) production performance increases. It is highly recommended that further research into this topic should be conducted.

6.6 References

AOAC ed., 2002a. Association of Official Analytical Chemists Inc. Ash of Animal Feed. Official methods of analysis (17th edition) ed. AOAC Official Method 942.05. Arlington, Virginia, USA.

AOAC ed., 2002b. Association of Official Analytical Chemists Inc. Fat (Crude) Or Ether Extract in Animal Feed. Official methods of analysis (17th edition) ed. AOAC Official Method 920.39. Arlington, Virginia, USA.

AOAC ed., 2002c. Association of Official Analytical Chemists Inc. Fibre (Crude) in Animal Feed and Pet Food. Official methods of analysis (17th edition) ed. AOAC Official Method 962.09. Arlington, Virginia, USA.

AOAC ed., 2002d. Association of Official Analytical Chemists Inc. Protein (Crude) in Animal Feed and Pet Food. Official methods of analysis (17th edition) ed. AOAC Official Method. Arlington, Virginia, USA.

Bautista-Teruel, M. N., Fermin A. C. & Koshio S. S. 2003. Diet development and evaluation for juvenile abalone, *Haliotis asinina*: Animal and plant protein sources. Aquaculture. (219): 645-653.

Bautista-Teruel, M. N. & Millamena O. M. 1999. Diet development and evaluation for juvenile abalone, *Haliotis asinina*: Protein energy levels. Aquaculture. (178): 117-126.

Britz, P. J. 1996. The nutritional requirements of *Haliotis midae* and development of a practical diet for abalone aquaculture. Doctoral dissertation, Rhodes University.

Britz, P. J. & Hecht T. 1997. Effect of dietary protein and energy level on growth and body composition of South African abalone, *Haliotis midae*. Aquaculture. 156(3-4): 195-210.

Cho., S.H. 2010. Effect of fishmeal substitution with various animal and/or plant protein sources in the diet of *Haliotis discus hannai* . Aquaculture. (41): 587-593.

Chesson., A. 1993. Feed enzymes. Animal Feed Science Technology. 45, (1993): 65-79.

Cruz-Suárez, L. E., Tapia-Salazar M., Villarreal-Cavazos D., Beltran-Rocha J., Nieto-López M. G., Lemme A. & Ricque-Marie D. 2009. Apparent dry matter, energy, protein and amino acid digestibility of four soybean ingredients in white shrimp *Litopenaeus vannamei* juveniles. Aquaculture. 292(1-2): 87-94.

Dersjant-Li, Y. 2002. The use of soy protein in aquafeeds. Advances in aquaculture nutrition, Cancun, Mexico.

Drew, M. D., Borgeson T. L. & Thiessen D. L. 2007. A review of processing of feed ingredients to enhance diet digestibility in finfish. Animal Feed Science Technology. 138: 118-136.

Erasmus, J. H., Cook P. A. & Coyne V. E. 1997. The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. Aquaculture. 155(1-4): 377-386.

Fleming, A. E., Bameveld R. J. V. & Hone P. W. 1996. The development of artificial diets for abalone: A review and future directions. Aquaculture. 140: 5-53.

Fourij, J. L. 2007. The effect of multiple-enzyme combinations in maize-soya diets for broiler chickens. Doctoral dissertation, Stellenbosch University.

Francis, G., Makkar H. P. S. & Becker K. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture. 199: 199-227.

Garcia-Esquivel, Z. & Felbeck H. 2006. Activity of digestive enzymes along the gut of juvenile red abalone, *Haliotis rufescens*, fed natural and balanced diets. Aquaculture. 261(2): 615-625.

Gatlin, D. M., Barrows F. T., Brown P., Dabrowski K., Gaylord T. G., Hardy R. W., Herman E., Hu G., Krogdahl Å., Nelson R., Overturf K., Rust M., Sealey W., Skonberg D., J Souza E., Stone D., Wilson R. & Wurtele E. 2007. Expanding the utilization of sustainable plant products in aquafeeds: A review. Aquaculture Research 38(6): 551-579.

Glenncross, B., C. Carter. & S. Refstie. 2007. Harvesting the benefits of grain aquafeeds. Soybean in aquafeeds, Freemantle, Western Australia.

Goosen, N. J. 2007. Organic acids as potential growth promoters in abalone diets. Doctoral dissertation, Stellenbosch University.

Green, A. J., Cliford L. W. & Britz P. J. 2011. The protein and energy requirements of farmed South African abalone *Haliotis midae*, cultured at optimal and elevated water temperatures. Aquaculture Research. 1: 1-11.

Guzm´an, J. M. & Viana M. T. 1998. Growth of abalone *Haliotis fulgens* fed diets with and without fish meal, compared to a commercial diet. Aquaculture. (165): 321-331.

Irish, G. G. & Balnave D. 1993. Non-starch polysaccharides and broiler performance on diets containing soybean meal as the sole protein concentrate. Australian Journal of Agriculture Research (44): 1483-1483.

Karr-Lilienthal, L. K., Kadzere C. T., Grieshop C. M. & Fahey J., G.C. 2005. Chemical and nutritional properties of soybean carbohydrates as related to nonruminants: A review. Livestock Production Science. 97(1): 1-12.

Knauer, J., Britz P. J. & Hecht T. 1996. Comparative growth performance and digestive enzyme activity of juvenile South African abalone, *Halioits midae*, fed on diatoms and a practical diet. Aquaculture. (140 (1996)): 75-85.

Krogdahl, A., Penn M., Thorsen J., Refstie S. & Bakke A. 2010. Important antinutrients in plant feedstuffs for aquaculture: An update on recent findings regarding responses in salmonids. Aquaculture Research. 41: 331-344.

Kumagai, Y. & Ojima T. 2009. Enzymatic properties and the primary structure of a β-1,3-glucanase from the digestive fluid of the pacific abalone *Haliotis discus hannai*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 154(1): 113-120.

McCleary, B. V. 2003. Dietary fibre analysis. Nutrition Society. 62: 3-9.

Mouton, A. & Gummow B. 2011. The occurrence of gut associated parasites in the South African abalone, *Haliotis midae*, in Western Cape aquaculture facilities. Aquaculture. 313(1-4): 1-6.

Mowlana, R. 2007. Marine aquaculture in South Africa. Department Environmental Affairs and Tourism. (1): 1-16.

O'Keef, T. 2003. Plant protein ingredients for aquaculture feeds: Use considerations & quality standards. Doctoral dissertation, American Soybean Association.

Refstie, S. 2007. Soybean in aquafeeds. Harvesting the benefits of grain in aquaculture feeds, Freemantle, Western Australia.

Sales, J. 2004. Aqua feeds: Aqua Feeds: Formulation and Beyond. 1(3): 23-26.

Sales, J. & Britz P. J. 2001. Research on abalone (*Haliotis midae*) cultivation in South Africa. Aquaculture Research. 32: 863-874.

Schutte, J. B. 1990. Nutritional implications and metabolisable energy value of d-xylose and L-arabinose in chicks. . Poultry Science. (69): 1724—1730.

Sinha, A. K., Kumar V., Makkar H. P. S., De Boeck G. & Becker K. 2011. Non-starch polysaccharides and their role in fish nutrition – A review. Food Chemistry. 127(4): 1409-1426.

Tsanigab, S. M. 2009. Evaluating Namibian macrophytic algae as a dietary source for South African abalone. Doctoral dissertation, Stellenbosch University, Department of Food Science.

Walsh, G. A., Power R. F. & Headon D. R. 1993. Enzymes in the animal-feed industry. TIBTECH. 11: 424-429.

Welch, A., Hoenig R., Stieglitz J., Benetti D., Tacon A., Sims N. & O'Hanlon C. 2010. From fishing to the sustainable farming of carnivorous marine finfish. Reviews in Fisheries Science. 18(3): 235-247.

Zhang, Z., Yu G., Guan H., Zhao X., Du Y. & Jiang X. 2004. Preparation and structure elucidation of alginate oligosaccharides degraded by alginate lyase from vibro species. Carbohydrate Research. 339(8): 1475-1481.

Chapter 7

General conclusion

The objective of this trial was to evaluate the suitability of a fish meal (FM) replaced diet in comparison to a soybean meal (SBM) diet for abalone culture in South Africa. Feed pellet water stability, feed effect on abalone intestinal morphology and growth performance parameters were used to evaluate the suitability of these diets.

The effect of extrusion temperatures on water stability showed that water stability is significantly ($P<0.05$) affected by extrusion temperature. Feed extruded at 68.88°C (T5) was 54.44% more water stable than feed extruded at 57.34°C (T_1) after sixteen hour water exposure.

In Phase A (FM replacement) of the water stability trial, no significant differences ($P>0.05$) occurred between the diets with regard to the rate of dry matter (DM) loss or water stability over time. In Phase B (enzyme treated), the rate of DM loss for the FME1, SBME1, SBME3 and SBME123 significantly differed ($P<0.05$) from the positive control (FM). Three of the four enzyme treated soybean meal diets (SBME1, SBME3 and SBME123) resulted in a significantly ($P<0.05$) lower water stability than the SBME0 diet by 6.43, 3.46 and 8.24% respectively. Enzyme supplementation in soybean meal diets therefore had a negative effect on water stability. The FME1, SBME1 and SBME 123 diets all contained β-glucanase, which shows that certain enzymes (such as β-glucanase) may affect the viscosity of the binder, resulting in impaired binding.

Artificial feeds were found to have a negative impact on the intestine of abalone. Signs of morphological effects were observed in the intestinal regions, yet the effects were never severe enough to describe an enteritis or acute inflammatory condition (scores ranged between: 1.82 – 3.03). In region II, III and IV, the FM and ECO diet resulted in significantly better ($P<0.05$) gut condition scores than the SBM diet (1.82 ± 0.28 and 1.90 ± 0.23 vs. 2.57 ± 0.49). The SBME diet did not differ significantly ($P>0.05$) from any of the treatments in this region (2.15 ± 0.19). In region V, the SBME diet had a significantly improved ($P<0.05$) score from the SBM diet (2.43 ± 0.19 vs. 3.03 ± 0.02). The FM and ECO treatments did not differ significantly ($P>0.05$) from any treatments in region V.

Literature varies on the exact functioning of specific intestinal regions and even certain intestinal nomenclature varies between authors. A unique method to score gut parameters must be adapted for abalone due to the differing nature of the molluscan intestinal tract (typhlosole, abundant mucous cells and varying epithelial length). There is large scope for research in this area and great potential to improve production performance by reducing the negative dietary effect on gut health. Further investigation and research is thus required in this field.

In Phase A of the growth trial, it was found that the ECO diet fed animals yielded significantly ($P<0.05$) heavier (mass in grams) animals compared to the FM (control) and soybean meal diet fed animals at the end of the 240 day growth trial period (9.80% and 12.84%). No differences ($P>0.05$) were noted for final length (mm), feed conversion ratios (FCR) and specific growth rates (SGR) for mass and length between animals for all of the treatments. The ECO diet fed animals showed significantly ($P<0.05$) improved condition (Britz) in comparison to the soybean meal diet fed animals (7.77%). The ECO diet was thus the best performing diet. No significant differences ($P>0.05$) were observed between the FM and three fish meal-replaced diet fed animals.

In Phase B of the growth trial, after 240 days, the ECO diet fed animals exhibited, once again, a significantly better ($P>0.05$) final weight than the FME0 and SBME0 diet fed animals (9.80% and 12.84%, as well as the animals fed on the other diets). Again, no significant differences ($P>0.05$) occurred in FCR and SGR for mass and length between treatments. In terms of final length on day 240, the ECO diet fed animals were significantly longer than the FME1, SBME1 and SBME3 fed animals (3.20%, 3.06% and 2.23%). By the end of the trial, the ECO diet fed animals were also in significantly better condition than those fed the soybean meal diet, as well as FME3 and FME123 fed animals. The large variation within all the treatments may, however, have masked some of the differences. The ECO diet was not iso-energetic to the fish meal or soybean meal diets and thus the favourable results obtained in this study are not surprising. The comparative performance of the soybean meal and fish meal diets is thus of primary focus.

Enzyme treated soybean meal (SBME123) yielded poorer water stability than the soybean meal or fish meal feeds. It did however yield comparable (region II, III and

IV) and even improved (region V) intestinal morphology. Although significant growth differences ($P>0.05$) were not observed on the 5% level, SBME123 fed animals had the second longest shell length overall, the best soybean meal diet growth and the fourth best SGR overall. Enzyme treated soybean meal is a relatively cheap feed alternative when compared to FM or the ECO diet, and thus holds potential for commercial capacity.

Untreated soybean meal had comparative water stability and animal growth to the fish meal-based diet, and is thus a potential fish meal-replacement, although it did have a significant effect ($P<0.05$) on the intestine of abalone. The significant ($P<0.05$) intestinal effects, noted in the soybean meal treatments, never translated into significant ($P>0.05$) decreased production performance effects. The soybean meal diets used in this trial did not perform significantly ($P>0.05$) worse than the fish meal diets. Consequently, there is great potential for the replacement of fish meal with soybean meal in abalone diets.

Further work is required to validate the potential of exogenous enzymes in abalone diets and focus on animal weight variation is required when conducting a growth trial with mollusks as variation masks results. The slow growth of these animals also results in long and often impractical trial durations, allowing time for seasonal effects and other unforeseeable factors to influence trial production performances.