THE EFFECT OF ALGINATE SUPPLEMENTATION ON THE GROWTH, FEED UTILIZATION, DIGESTIVE ENZYME ACTIVITY LEVELS, AND INTESTINAL MORPHOLOGY OF JUVENILE SOUTH AFRICAN ABALONE (*HALIOTIS MIDAE*) FED FORMULATED FEEDS

A thesis submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

of

RHODES UNIVERSITY

by

THATO TLAKEDI

March 2020

ABSTRACT

Alginate occurs abundantly in kelp and while a few studies have quantified the effect of kelp inclusion in formulated *Haliotis midae* feeds, none have researched the effect of alginate inclusion on the growth of abalone fed this feed. Feeding kelp to cultured abalone affects gut structure and modulates gut bacteria, aiding digestion by upregulating enzyme activity. This farm-based experiment compared the growth, feed utilization, digestive enzyme activity levels and intestinal villi structure between abalone (1-year-old, 10 - 20 mm shell length) fed kelp-supplemented (BK) or alginate-supplemented (BA) diets and abalone fed basal (B) and fresh kelp (K) diets for a period of eight months from June 2017 to February 2018. Growth and enzyme activities were also compared between abalone that experienced dietary changes and abalone that remained on their initial diets. The tested diets were: base diet (B, 34% protein), the same base diet supplemented with fresh minced kelp (0.90% kelp [dry mass, equivalent to 0.20% alginate]; BK), base diet supplemented with sodium alginate (0.20% alginate; BA) and a fresh kelp diet (K).

Alginate or kelp inclusion in a formulated feed had no significant effect on the specific growth rates (SGR), linear shell growth rates (LGR), daily percentage feeding rates (PFR), feed conversion ratio (FCR), percentage mass gain (MG) and condition factor (CF) over the eight month trial period, compared to abalone fed the base diet. In October 2017, four months since the start of the trial in June, all the groups fed formulated feeds showed significantly higher average weight gain (8.42 ± 0.72 g to 8.86 ± 0.63 g) compared to the kelp-fed group (6.68 ± 0.43 g). However, no significant differences in the average weight gain were observed between abalone fed kelp and formulated feeds at the end of this study. Furthermore, there were no significant differences in the FCR and PFR between abalone fed the base or alginate diets and abalone fed the fresh kelp diet. However, the SW diet produced significantly lower FCR (1.00 ± 0.13) and PFR (0.78 ± 0.10%) compared to the kelp diet (10.6 ± 1.40% and 8.12 ± 1.01%, respectively). All formulated diets produced non-significant LGR, MG and CF compared to the kelp diet at the end of this study.

Enzyme activity levels were compared between abalone fed the test diets in July and August 2017 and February 2018. There were no significant differences in the average alginate lyase specific activity levels between abalone fed formulated diets in July and August 2017. At the end of the trial, however, the alginate diet induced significantly higher alginate lyase specific activity levels (4.89 ± 1.64 $mg.mg^{-1}$ protein) compared to the base diet (1.57 ± 0.98 $mg.mg^{-1}$ protein), but showed no significant differences compared to the SW (3.78 ± 0.41 $mg.mg^{-1}$

protein) and fresh kelp-only diets (4.00 ± 0.62 $mg.mg^{-1}$ protein). The SW diet showed no significant differences in the alginate lyase specific activity levels compared to the base diet. There were no significant differences in the chymotrypsin activity levels between the test diets throughout this study.

Alginate or kelp inclusion also had no significant effect on the intestinal structure compared to the base or fresh kelp diets. This was despite a higher degree of variability in microvilli height and width being observed in abalone fed the kelp-only diet, with microvilli visibly wider and longer than in abalone fed formulated feeds. Switching abalone between some diets significantly affected the average weight gain, MG, FCR and PFR, alginate lyase, chymotrypsin and cellulase activity levels compared to abalone kept on their initial diets.

The inclusion levels of kelp and alginate used in this study were likely too low to affect abalone growth as higher inclusion levels, particularly of alginate, have been found to improve fish growth. This study concluded that minced kelp and alginate inclusion in a formulated *H. midae* diet did not affect the SGR, FCR and intestinal morphology, but significantly affected alginate lyase and chymotrypsin activity levels. Furthermore, changes in growth and digestive enzyme activity levels coincided with dietary changes. Consequently, higher inclusion levels, particularly of sodium alginate, and the effect of dietary changes on abalone growth and digestive enzymes must be investigated in future studies.

DECLARATION

I declare that the all the work contained in this thesis is my own original work, that I am the sole author thereof and that I have acknowledged original authors where necessary. This thesis has also not been submitted for a degree at any other university.

ACKNOWLEDGEMENTS

Funding for this project was provided by the National Research Foundation's Research and Technology Fund, HIK Abalone Farm (Pty) Ltd, Marifeed (Pty) Ltd and Aqunion (Pty) Ltd, to whom I am grateful for their generosity.

I would like to thank my two supervisors, Dr. Aldi Nel and Prof. Cliff Jones for their guidance and support during my postgraduate studies. I would like to thank Cliff for always finding ways to secure finance for me to undertake this project and for being calm with me when I messed up. I would like to particularly thank Aldi for the long hours she dedicated to travelling to the farm and spending long hours standing in the lab to help me with enzyme and stats analyses and gut morphology. This project would not have been possible without your assistance and you taught me skills that I feel I could confidently apply anywhere in this field.

I would also like to thank Jason and Sanele for helping me with data collection. Those long, cold and rainy winter days you dedicated to helping me weigh and measure these "giants" will never be forgotten. To Dr. Wilbert Kadye, thank you for your advice on stats analyses. To Matthew Naylor, thank you for the mentorship you provided me with during my stay at the HIK.

I would also like to extend my gratitude to the Department of Microbiology, particularly Prof. Pletschke, for allowing me to use his lab and chemicals for my enzyme assays. Taryn from the South African Institute for Aquatic Biodiversity (SAIAB) is also thanked for providing facilities and equipment for this project.

To my parents, Telang and Matlotliso, I could never thank you enough for all these years of support in all ways possible. It has indeed been a long way and I have not found words good enough to express my gratitude to you. This thesis is dedicated to you both for always believing in me and dedicating your entire lives to making sure that my siblings I become the best we could ever be.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANFs	anti-nutritional factors
CF	condition factor
CP	crude protein
DO	dissolved oxygen
FCR	feed conversion ratio
GIT	gastrointestinal tract
LGR	linear shell growth rate
LMWSA	low molecular weight sodium alginate
MG	percentage mass gain
PFR	daily percentage feeding rate
PER	protein efficiency ratio
PUFAs	Polyunsaturated fatty acids
SD	standard deviation
SGR	specific growth rate
VSCFA	volatile short-chain fatty acids

CHAPTER 1

GENERAL INTRODUCTION

The use of formulated feeds in abalone aquaculture

Abalone aquaculture is a highly intensive form of farming that started in the 1950s and 1960s in Japan with the purpose of restocking overexploited wild abalone stocks (Britz *et al*. 1994). China is now the largest abalone producer, with over 300 farms in 2010 producing over an estimated 115 397 mt/y of abalone in 2015 (Cook 2016). In South Africa, the abalone industry began in the 1990s following the initial work of Genade *et al*. (1988), who reported that *Haliotis midae* could be spawned and cultured successfully under controlled and semi-controlled conditions. As of 2015, the South African abalone industry had grown steadily, producing 1660 mt of abalone that year alone worth $US73 434 900 (Krohn *et al*. 2016), up from a 500 - 800 mt production in 2001 (Sales and Britz 2001).

Studies on the development of formulated diets for the abalone *H. midae* were initially based on research on protein and amino acid profiles of feeds for Japanese abalone (Ogino and Ohta 1963; Ogino and Kato 1964; Uki *et al*. 1985a; Uki *et al*. 1985b; Uki *et al*. 1986a; Uki *et al*. 1986b; Uki and Watanabe 1992). Consequently, proximate analyses of abalone tissues for *H. midae* was conducted to ensure that formulated diets developed for the weaning and grow-out phases of this species contain optimal protein and amino acid levels (Britz *et al*. 1994; Knauer 1994; Britz 1995; Knauer *et al*. 1995). Juvenile *Haliotis midae* exhibited optimal growth at 34% fishmeal protein level and sub-adults grew optimally at 44% fishmeal protein level (Britz and Hecht 1997).

The successful development of practical diets for *H. midae* in the 1990s culminated in their suitability for optimal growth of this species being tested. Britz *et al*. (1994) developed a formulated practical diet for *H. midae* and reported faster feed intake in abalone offered this diet over those fed a natural diet. Prior to that, Dixon (1992) and Wee *et al*. (1992) had reported on the high digestibility of formulated abalone diets under culture conditions. Due to the high protein concentration of formulated diets, high growth rates of cultured abalone fed these diets, compared to those fed natural diets, have been reported for different abalone species (Uki *et al*. 1985a; Britz 1996a). Britz (1996a) evaluated the suitability of different protein sources for inclusion in a formulated diet for *H. midae*, against the natural diet kelp. The sources tested were; casein, low-temperature processed fish meal, soya oil cake and torula yeast. Fish meal was found to be more suitable for inclusion than casein in a formulated *H. midae* diet.

Moreover, abalone fed this diet displayed better growth performance with higher weight gain, protein efficiency ratio (PER), condition factor (CF) and lower feed conversion ratio (FCR) than abalone fed fresh kelp (Britz 1996a).

Macroalgae often contain relatively low levels of macronutrients such as protein. However, certain benefits have been identified when fresh macroalgae are fed to cultured abalone, including improved marketability, health and feed intake (Bansemer *et al*. 2014). Bansemer *et al*. (2016) investigated the effect of supplementing a formulated diet with dietary *Ulva sp*. and *Gracilaria sp*. (5, 10 and 20%, dry weight) on the growth performance and enzyme activities of greenlip abalone, *Haliotis laevigata*. The authors reported significantly higher feed consumption, growth rate, final weight and biomass gain and trypsin activity levels when inclusion levels of macroalgae of *Ulva sp*. and *Gracilaria sp*. were increased to 10 and 20% levels, respectively.

Viera *et al.* (2015) evaluated the nutritional value of a mixed dried macroalgae meal as the main ingredient for the abalone *H. tuberculata coccinea*. Abalone fed a formulated feed with a supplement of mixed *Ulva lactuca* (15%), *Gracilaria cornea* (16%) and *P. palmata* (12%) meal displayed higher shell growth rates, specific growth rates, and weight gain, condition index and dietary protein utilization compared to those fed a mixed fresh algae diet and other mixed algae meal diets. In general, all diets supplemented with dried macroalgae meal had superior PER and FCR than those fed a mixed fresh macroalgae diet. Several other studies have reported improved growth rates in abalone fed formulated feeds supplemented with different species of macroalgae compared to abalone fed formulated feeds only (O'Mahoney *et al*. 2014; Bansemer *et al*. 2016; Nel *et al*. 2017a; Kemp 2017).

The digestive mechanism of abalone has attracted much interest as attempts to improve feeding efficiency in cultured abalone continue. Feed costs contribute significantly to operating costs in abalone aquaculture (Britz 1996). Thus, an understanding of digestive enzymes in abalone could help improve feed utilization by cultured abalone. Enzymes help improve protein digestion and absorption (Bedford 2002; Karr-Lilienthal 2005; De Villiers 2012). In *H. midae* fed a formulated feed, total protease and amylase activities increased significantly after feeding, peaking at 12 and 18 hours (Britz *et al*. 1996). Using different enzymatic techniques to describe proteolytic activity in *H. fulgens*, Picos-Garcia *et al*. (2001) reported highest proteolytic activity in the hepatopancreas. Chymotrypsin, trypsin and acid phosphatase activities were also detected in the abalone gut (Picos-Garcia *et al*. 2001). Conducting a similar

study, Serviere-Zaragoza *et al*. (1997) detected protease activity in the intestine, rectum, hepatopancreas and crop-stomach regions. Chymotrypsin was highest in the stomach and rectum compared to other regions. This may well suggest that most of the protein absorption in the abalone gut occurs in these regions.

Studies on the use of alginate as a dietary ingredient for cultured abalone suggest that alginate is fermented in the gut to provide energy for growth in the form of volatile short chain fatty acids (VSCFA) (Sawabe *et al*. 2003; Iehata *et al*. 2009). Sawabe *et al*. (1995) found that alginolytic bacteria isolated from the guts of abalone and sea urchins, *H. discus hannai* and *Stongylocentrotus intermedius* and *Stongylocentrotus nudus*, respectively, were dominated by fermentative, non-motile bacteria that could degrade both the PolyM and PolyG blocks of alginate. The potential use of sodium alginate as a novel prebiotic to promote growth and improve health in cultured herbivorous fish was reported by Van Doan *et al*. (2016a). Van Doan *et al*. (2016a) reported that a basal diet supplemented with a combined administration of low molecular weight sodium alginate (LMWSA) and *Lactobacillus plantarum* significantly enhanced immune response, disease resistance and growth of the Nile tilapia *Oreochromis niloticus* exposed to a pathogenic *Streptococcus agalactiae*. This may be a significant finding for abalone, herbivorous marine gastropods that feed on kelp, a main source of alginate. However, no studies on the growth and/or disease resistance of *H. midae* fed an alginate-supplemented diet have been published.

The structure of the gastrointestinal tract of different haliotid species has been described previously (Crofts 1929 for *Haliotis tuberculate coccinea*; Campbell 1965 for *H. cracherodii*; Johnston *et al*. 2005 for *Haliotis rubra*; Nel 2016 and Kemp 2017 for *H. midae*). In abalone, the crop acts as fermentation vessel; here, small fragments of feed are stored and digested over several hours following ingestion (Foale and Day 1992; Day and Cook 1995) and hydrolyzed by bacteria (Erasmus *et al*. 1997). The crop has a volume half that of the stomach (Kemp 2001) and exhibits an anaerobic environment where VSCFAs are produced (Day and Cook 1995; Harris *et al.* 1998). Production of VSCFA in the crop is aided by bacteria (Sawabe *et al*. 2003) whereas enzymatic digestion of ingested lipids, carbohydrates and proteins occurs in the hepatopancreas (Bevelander 1988).

The effects of diets on the gastrointestinal tract (GIT) of abalone are largely unknown but have been investigated in a few studies. Schaefer *et al*. (2013) compared histological differences in the GIT of *H. laevigata* fed different dietary crude protein (CP) levels of soya bean meal. High

CP level feeds had significant deleterious effects on the epithelial thickness of the crop for sub-adults compared to low CP level feeds. Kemp (2001) reported varied effects of legume diets on the structure and function of the intestine in cultured versus wild *H. laevigata.* These ranged from changes in the pattern of intestinal development and function to changes in secretory cells and digestive enzyme activity levels between reared and wild abalone.

Evidence from previous studies suggests that abalone can adapt their enzyme profiles (Fleming *et al*. 1996; Knauer *et al*. 1996; Serviere-Zaragoza *et al*. 1997; Picos-Garcia *et al*. 2001) and/or intestinal/GIT structure to adjust to dietary changes (Johnston *et al*. 2005; Schaefer *et al*. 2013, Kemp 2001). Thus, there is evidence to suggest that switching or changing diets may result in similar changes in the gut/intestinal morphology of cultured abalone. These changes may range from intestinal development and function, villi height and width, mucus and secretory cells structure, digestive enzyme activity levels, nutrient assimilation and ultimately, abalone growth. Nel (2016) found no significant effect of kelp-supplementation in a 34%-protein diet on the crop villi structure of *H. midae*. However, no studies have investigated the direct effects of switching diets on the GIT of cultured abalone. This necessitates the need for research on the GIT of *Haliotis midae* as just two studies (Nel 2016, thesis; Kemp 2017, thesis) have to date been conducted on the effect of diet on the GIT of this species.

OVERALL AIM

The aim of this study was to investigate the effect of alginate and minced kelp (*Ecklonia maxima*) supplementation on the growth and feed utilization, digestive enzyme activity levels and intestinal morphology of juvenile *Haliotis midae* fed formulated diets. Minced kelp was chosen because it has previously been found to improve the growth of juvenile *H. midae* (Nel *et al*. 2017a) while alginate was chosen to determine due its high concentration in kelp. Additionally, the effect of switching diets on the growth and digestive physiology of the abalone was investigated to further assess the impact of diet on digestive activities and gut function. The following questions were addressed;

1. Does alginate supplementation of a formulated abalone feed influence the growth, feed consumption, digestive enzyme activity levels and intestinal morphology of the abalone *Haliotis midae*?
2. Does kelp *Ecklonia maxima* influence the growth, feed consumption, digestive enzyme activity levels and intestinal morphology of the abalone *Haliotis midae* when minced kelp is included in a formulated abalone feed?

3. Does altering/switching the dietary alginate or kelp supplementation in a formulated feed influence the growth, feed consumption and digestive enzyme activity levels in the abalone *Haliotis midae*?

To address these questions, the following objectives were evaluated;

1. The growth, feed utilisation, digestive enzyme activity and the intestinal villi surface areas were compared when *Haliotis midae* were fed either: a (a) formulated base diet (B), (b) the same formulated diet supplemented with minced kelp (BK); (c) the same formulated diet supplemented with alginate at the same inclusion level as the alginate in the minced kelp above (BA, 0.20%); and (d) a fresh kelp only diet (K). The BK diet was a pelleted short base diet (10 mm x 10 mm x 1.2 mm) supplemented with fresh minced kelp added to the base diet as a component. Kelp was minced finely using industrial machinery and mixed with the other ingredients while wet prior to extrusion.
2. The growth, feed utilization and digestive enzyme activity levels were compared when abalone were fed either the same diet (i.e. B, BK, BA or K) to those subjected to sudden change in diet (B to BK; BK to B or BA to K; K to BA).

CHAPTER 2

The effect of alginate supplementation on the growth and feed utilization, digestive enzyme activity levels and intestinal morphology when using formulated feeds

2.1 INTRODUCTION

Haliotis midae is a slow-growing marine gastropod belonging to the family Haliotidae and is the only species of abalone cultured in South Africa (Barkai and Griffiths 1986; Olin 1994; Dlaza 2006). The culture of *H. midae* has expanded greatly since the 1990s due to successful propagation of spat and replication of land-based aquaculture systems. In the wild, *H. midae* are generalist herbivores that feed on a variety of feeds found in their environment. They feed primarily on benthic microalgae as early juveniles, and on *Ecklonia maxima* on the West and southwest coasts and on *Rhodophytes* on the south coast (Barkai and Griffiths 1988; Britz 1995; Wood and Buxton 1996).

Most abalone farmers employ nutrient-enriched formulated feeds for faster growth of abalone (Wee *et al*. 1992; Britz *et al*. 1994; Kemp *et al*. 2015; O'Mahoney *et al*. 2014; Nel *et al*. 2017; Kemp 2017). However, the potential benefits of supplementing formulated diets with either fresh or dried macroalgae meal as a component of formulated feeds to further enhance abalone growth, enzyme activities and disease resistance have been investigated in macroalgae-utilizing countries such as Australia, Chile, Japan and South Africa (Cheng and Yu 2013; Bansemer *et al*. 2014, 2016; Kemp *et al*. 2015; Kemp 2017). Macroalgae have up to 50% reserve and structural carbohydrates but this differs between species and is influenced by environmental conditions such as season and locality (Bansemer *et al*. 2014). Nonetheless, they remain an invaluable source of natural feed and potential artificial feed supplements for abalone. Kemp *et al*. (2015) reported that fresh macroalgae-supplementation of formulated diets of *H. rufescens* significantly improved meat and viscera yields, growth rates and PER at an inclusion level 7.5% bw.day^{-1} (wet weight). Although a mixed diet of *Ulva, Gracillaria* and kelp performed better, a combination diet of kelp and base produced better growth in *H. midae* compared to a base-only diet (Naidoo *et al*. 2006).

The high costs of formulated diets have prompted abalone farmers to invest in research on alternative, cheaper vegetable-based ingredients. Although it may not fully replace protein-rich ingredients such as fish meal in formulated abalone feeds, dried macroalgae meal has shown potential benefits as a feed ingredient/supplement in formulated abalone feeds. Abalone fed formulated diets with dietary inclusions of dried macroalgae meal previously displayed

improved growth rates compared to abalone fed basal feeds (O'Mahoney *et al*. 2014). According to Bansemer *et al*. (2016), *H. laevigata* fed formulated feeds supplemented with 10 and 20% *Ulva* and *Gracilaria cliftonii* showed higher feed consumption, growth rate, final weight, biomass gain and enzyme activities compared to basal feeds. O'Mahoney *et al*. (2014) and Viera *et al.* (2015) have also found improved growth in *Hliotis discus hannai* and *Haliotis tuberculata coccinea*, respectively, fed macroalgae-supplemented feeds.

Combination diets of natural and artificial feeds have also been offered to the *H. midae* in South Africa with promising results. Dlaza *et al*. (2008) and Naidoo *et al*. (2006) found that post-weaning and juvenile abalone *H.midae*, respectively, fed formulated diets supplemented with fresh wild seaweeds displayed significantly higher growth rates compared to abalone fed non-supplemented feeds. Kemp (2017) found improved growth rates in *H. midae* fed a combination diet of a formulated feed and fresh macroalgae, in addition to improved protein efficiency ratio (PER) when the proportion of fresh macroalgae in the diet was increased to 7.5% bw.day^{-1}.

Macroalgae lack the nutritional pedigree offered by formulated feeds but contain a wealth of marine minerals, bioactive phytochemicals and structural and complex storage polysaccharides that formulated feeds lack such as alginates, fucans and cellulose and laminarins (Lahaye 1991; Mabeau and Fleurence 1993; Britz *et al*. 1994; O'Sullivan *et al*. 2010; Kemp 2017). Brown macroalgae offer low protein (5 - 15 %) due to high water content (68 - 83%) (Hahn 1989b; Erasmus *et al*. 1997; Fleurence 1999; Troell *et al*. 2006). The digestive tract of abalone is conducive to bacterial development that aids a host of activities, but mostly the digestion of complex structural polysaccharides found in kelp that are indigestible to abalone (Sawabe *et al*. 1995; Erasmus *et al*. 1997; Harris *et al*. 1998; Monje and Viana 1998; Sawabe 2006). These symbiotic bacteria are hypothesized to produce alginases, polyguluronate-specific lyases, laminarinases and carboxypeptidases that hydrolize fibers in macroalgae, improving feed digestion and absorption by abalone (Harris 1993; El-Shanshoury *et al*. 1994; Erasmus 1996; Erasmus *et al*. 1997; Hernandez-Santoyo *et al.* 1998; Monje and Viana 1998; Macey and Coyne 2005; Sawabe 2006). Thus, bacterial activity in the host gut appears to be particularly important for animals that feed on nutritionally poor diets (Vitalis *et al*.1988). Furthermore, the results of these studies suggest that the inclusion of macroalgae in formulated abalone feeds may increase feed utilization through bacteria-induced modifications such as altered enzyme production.

Brown macroalgae contain lower protein concentrations compared to formulated feeds. However, they are rich stores of carbohydrates. The use of carbohydrates in abalone drives

energy metabolism (Picos-Garcia *et al*. 2000). Digestive enzymes such as carbohydrates and proteases play a key role in feed digestion. In the abalone *Haliotis fulgens*, chymotrypsin activity has been found to be significantly higher in the intestine than in other parts of the digestive system, suggesting that protein and carbohydrate digestion occurs mainly in the intestine (Serviere-Zaragoza *et al.* 1997). The digestive gland of abalone also plays a significant role in feed utilization and *H. fulgens* fed formulated feeds displayed significant enzyme activities in this region (Garcia-Esquivel and Felbeck 2006). The composition of diets affects not only affects an organism's digestive potential, but also on an organism's innate ability to use exogenous or endogenous enzymes to digest such diets (Garcia-Esquivel and Felbeck 2006). Serviere-Zaragoza *et al*. (1997) found significantly higher chymotrypsin activity in abalone fed macroalgae. Kemp (2017) also found improved protein utilization in *H. midae* fed formulated feeds supplemented with kelp. Thus, upregulation of chymotrypsin was expected in the current study.

The possible effects of alginate supplementation on the growth and digestive enzyme activities in abalone

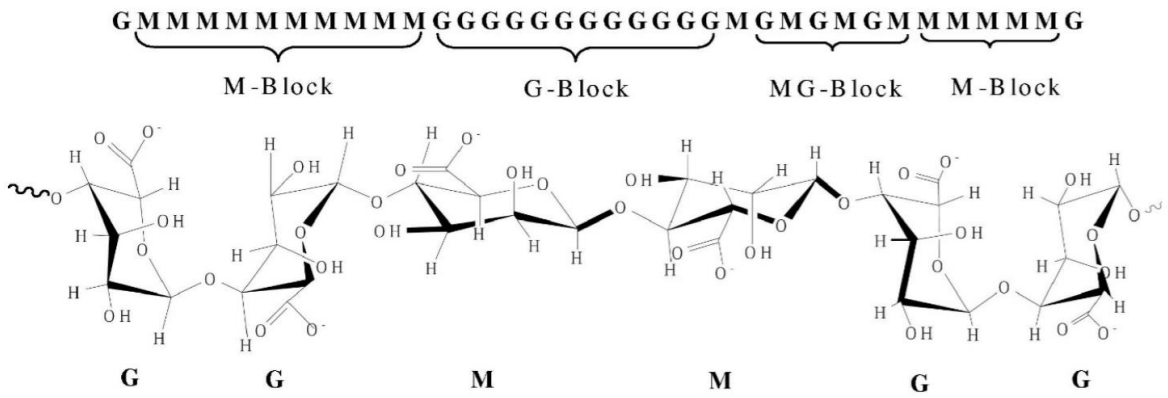

Figure 2.1: The chemical structure of alginate showing the chain sequence and molecular conformation of the PolyM and PolyG blocks (Labowska *et al*. 2019).

Kelp contains high amounts of important structural polysaccharides such as alginate, which accounts for over 30% of the content on a dry weight basis in *Ecklonia maxima* (Von Holdt *et al*. 1955). Alginate is a long-chain alginic acid and is present in kelp as calcium salt (Labowaska *et al*. 2019). It occurs in all species of kelp and consists of two basic building blocks arranged in either homopolymeric or heteroplymeric blocks, namely; poly-α-L-guluronate (polyG) or poly-ß-D-mannuronate (polyM) blocks (Figure 1; Gacesa 1988; *O'*Sullivan *et al.* 2010; Łabowska *et al*. 2019). Alginate is characterized by β-1, 4-linkages and

upon hydrolysis, yields 2,3-dimethyl D-mannuronide as a by-product (Monje and Viana 1998). Alginolytic bacteria hydrolyze alginate in the abalone gut by breaking down PolyM and PolyG blocks, providing oxidizable energy for the abalone host (Sawabe *et al*. 2003; Iehata *et al*. 2009). By hydrolyzing sodium alginate to volatile short-chain fatty acids (VSCFA) in the abalone gut, Sawabe *et al*. (2003) suggested that *Vibrio haliocticoli* contributes significantly toward abalone nutrition by providing oxidizable metabolic energy for growth.

Alginases associated with marine bacteria have long been detected in the GIT of the abalone *H. discus hannai, H. rufescens* and *H. corrugata*, respectively (Tsujino and Saito 1962; Nakada and Sweeny 1967) and their properties described (Doubet and Quatrano 1984). Nakada and Sweeny (1967) purified and separated two alginic acid eliminases from the hepatopancreas of two Pacific abalones, *H. rufescens and H. corrugata*. Both of these enzymes were able to digest alginate. Sawabe *et al*. (1995) isolated alginolytic bacteria from the guts of abalone and sea urchins and these hydrolized both the PolyM and PolyG blocks of alginate.

The potential use of sodium alginate as a novel prebiotic to improve growth and health in cultured fish and abalone has also been investigated. In the herbivorous grouper *Epinephelus bruneus*, significant improvements in the immune system's activation of the alternate complement pathway, natural haemagglutination and phagocytic activities have been achieved against a pathogenic *Streptococcus iniae* compared to a basal diet (Harikrishnan *et al*. 2011). Van Doan *et al*. (2016a) reported that combined administration of low molecular weight sodium alginate (LMWSA) and *Lactobacillus plantarum* significantly enhanced immune response (increased serum lysosome activity), disease resistance (protection against *Streptococcus agalactiae*) and growth compared to a basal diet alone, of the omnivorous Nile tilapia *Oreochromis niloticus*. Similar findings have been made in several other studies (Fujiki and Yano 1997; Yeh *et al*. 2008; Van Doan *et al*. 2016b). These results may have some implications for abalone health. Whereas the Nile tilapia are omnivorous (Jihulya 2014), *E. bruneus* are herbivorous, hence they are also known as "kelp grouper." Kelp contains alginate and since *H. midae*, herbivorous marine gastropods, naturally consume kelp as their main source of feed in some parts of South Africa, it can be hypothesized that kelp consumption by this species may improve disease resistance through alginate utilization. However, research is required to understand the effect of alginate on the health and growth of this species.

The prebiotic effect of sodium alginate as a feed ingredient in abalone culture is yet to receive the same attention it has received in fish culture. Consequently, investigating the prebiotic effects of sodium alginate on cultured abalone seem to have been limited to just one study in

which Cheng and Yu (2013) reported that Taiwanese abalone *H. diversicolor supertexta* exposed to a pathogenic *Vibrio parahaemolyticus* showed significantly high survival rates, phenoloxidase activity and respiratory burst when abalone were fed diets supplemented with 1.0, 2.0 and 3.0 $g.kg^{-1}$ of sodium alginate. Furthermore, increasing the sodium alginate inclusion level to 2.0 and 3.0 $g.kg^{-1}$ diet significantly increased phagocytic activity and clearance efficiency of *V. parahaemolyticus* by abalone. However, no studies have evaluated the effect of alginate inclusion on the growth of cultured abalone.

The effect of diet on the gut morphology of cultured abalone

The most complete anatomical account of haliotids was that by Crofts (1929). In this book, Crofts provided detailed drawings of the structure of *Haliotis tuberculata*, including a general dissection of the digestive system showing different regions of the GIT. In the book "Abalone: gross and fine structure", Bevelander (1988) describes in detail the structure of the alimentary canal of the abalone *Haliotis tuberculata* while Campbell (1965) described the structure and function of the alimentary canal of the black abalone, *Haliotis cracherodii*. Basic methods of dissection and histology have been employed to elucidate the structure of the alimentary canal and its associated structures in other abalone species (Harris *et al*. 1998 and Schaefer *et al*. 2013 on *Haliotis laevigate*; Johnston *et al*. 2005 on *Haliotis rubra*). Using an acrylic resin cast model, Kemp (2017) confirmed that the gut structure of the South African abalone, *Haliotis midae,* conforms to descriptions of the GIT of other abalone species, although he found minor and insignificant differences, the nature of which he did not describe.

Croft's (1929) drawings show in detail, parts of the abalone digestive system, including the buccal cavity, esophagus, crop, stomach, cecum, digestive gland, style sac, and intestine. Within these structures are secretory cells involved in enzymatic digestion (Bevelander 1988; Harris *et al*. 1998). A wide variety of these enzymes have been studied in detail; Knauer *et al*. (1996) and Nel *et al*. (2017) on *H. midae*; Harris *et al*. (1998) on *H. laevigata*; Picos-Garcıa *et al*. (2000), Garcıa-Carreno *et al*. (2003) and Garcia-Esquivel and Felbeck (2006) on *H. fulgens*.

Feed-induced histological changes and enzyme activities in abalone have revealed mixed results. Schaefer *et al*. (2013) compared histological differences in the GIT of juvenile and sub-adult abalone *H. laevigata* fed different dietary protein levels of solvent-extracted soya bean meal. The epithelial thickness of the crop was reduced by increased dietary protein level in sub-adult abalone, but no significant effect of this change was observed on abalone health and growth.

Kemp (2001) evaluated the effects of feeding legume diets to commercially reared *H. laevigata* on the intestinal structure and function of this species. Anti-nutritional factors (ANFs) found in legumes had significant deleterious effects on the intestinal structure and interfered with digestive physiology. Furthermore, commercially reared abalone fed this diet, in comparison to wild abalone, displayed signs described as "inflammatory responses." Similarly, abalone fed the commercial, soya flour and legume diets showed signs of enzyme inhibition due to the inhibitory components, such as phytate in legumes, of these feeds which are not found in natural feeds. Morphological differences were varied but generally indicated significant deleterious changes in commercially reared versus wild abalone. These ranged from changes in villus cell structure and height, lamina propria, thickening of the crypt region, intestinal columnar cells, goblet cells and mucus layers. Commercially reared abalone also showed signs of decreased feed intake, digestion and higher infection rates compared to wild abalone, which typically feed on macroalgae.

Currently, there is a dearth of information on the effects of diets on the gut morphology of the South Africa abalone *Haliotis midae*. As observed in abalone (Schaefer *et al*. 2013; Kemp 2001) and fish studies (Krogdahl *et al*. 2003), some diets can have detrimental effects on the gut morphology of cultured animals, resulting in reduced digestive enzyme activity levels, reduced capacity for nutrient absorption across the gut wall due to thinning of the GIT and consequently, depressed growth rates (Harris *et al*. 1998). This knowledge, obtained through histological and scanning electron microscopy techniques, may strengthen our knowledge of the normal growth patterns of the cultured abalone as they transition from one feed source to another during their growth, and enable us to develop better growth-promoting feeds for the culture of this species.

The literature review in this chapter has found that *Haliotis midae* fed formulated feeds that are supplemented with *Ecklonia maxima* show improved growths compared to abalone fed basal feeds. However, none have indicated which structural component of kelp may be responsible for this improved growth of abalone. Further literature review on kelp indicated that alginate constitutes over 30% of kelp on a dry weight basis and when included in fish feed as a prebiotic, alginate resulted in improved health and growth. However, no studies have investigated the effect of alginate-supplemented feeds on the growth of abalone. Thus, it remains unknown which component of kelp likely improves growth in *H. midae* fed kelp-supplemented feeds. This chapter investigates the question; does alginate inclusion in a

formulated feed influence the growth, feed utilization and digestive enzyme activity levels in *Haliotis midae*?

2.2 AIMS AND OBJECTIVES

Experiment 1: The aim of this experiment was to investigate the effect of alginate and kelp supplementation, respectively, on the growth, feed utilization, digestive enzyme activity levels and intestinal morphology of the South African abalone (*Haliotis midae*) fed formulated feeds. The growth and feed utilization, alginate lyase specific activity and chymotrypsin activity levels were compared between abalone fed a base diet and those fed the same base diet supplemented with either alginate or minced kelp. The alginate-supplemented diet (BA) was chosen for comparison with the kelp-supplemented diet (BK). Fresh kelp was finely minced and added to the base diet as a feed component to produce the kelp-supplemented diet (BK).

Experiment 2: The aim of this experiment was to investigate the effect of fresh kelp on the growth, feed utilization and digestive enzyme activity levels of *Haliotis midae*. The growth and feed utilization, alginate lyase specific activity and chymotrypsin activity levels were compared between abalone fed: (a) base diet; (b) the same base diet supplemented with alginate; (c) the same base diet supplemented with minced kelp or (d) a fresh kelp-only diet.

In addition, this study employed histological and microscopic techniques to evaluate the effect of diets on the intestinal structure of abalone fed formulated diets versus a kelp diet. The intestinal villi height, width and surface area were compared between abalone fed a: (a) base diet; (b) the same base diet supplemented with alginate; (c) the same base diet supplemented with minced kelp or (d) a fresh kelp diet only.

2.3 METHODS AND MATERIALS

Experiments 1 and 2 were conducted simultaneously in the same culture system and were subjected to the same culture conditions.

2.3.1 Experimental site and culture system

This study was conducted at the HIK Abalone Farm Pty (Ltd) in Hermanus, Western Cape (34°26′04.35″S; 19°13′12.51″E) over a period of eight months from June 2017 to February 2018, under commercial husbandry conditions (Naylor *et al*. 2013). One-year-old experimental abalone between 10 and 20 mm in shell length were used for this trial. Seawater was gravity fed to the flow-through abalone production tanks from a header tank that receives seawater

from a pump-ashore system on the farm. As per farming practice, the abalone tanks had two airlines mixing and aerating the water. The randomized block design experimental system consisted of four 2000 L tanks, each with five 0.13 m^2 oyster mesh baskets per treatment, placed in series within each production tank and stocked at 10% of the available surface area. Within each basket were vertical plastic racks providing surface area for attachment of abalone and a feeder plate placed horizontally above the racks (Nel *et al*. 2017). As per normal farm practice, the experimental tanks were cleaned weekly and the baskets cleaned once per month.

Seawater quality parameters (temperature, dissolved oxygen and pH) were recorded two days a week between 08:30 and 09:30 AM. Temperature and dissolved oxygen (DO ; $mg.L^{-1}$ and % saturation) were measured using a hand-held DO meter, whereas the pH was measured using a pH meter (OxyGuard International A/S, Birkerod, Denmark) during the experiment. During this study, the temperature averaged 14.70 ^{o}C, DO averaged 9.47 $mg.L^{-1}$ (115.58% air saturation) and the pH averaged 7.97. For the dietary switch trial (Chapter 3; February - June 2018), the temperature averaged 14.41 ^{o}C, DO averaged 8.01 $mg.L^{-1}$ (94.30%) and the pH averaged 7.56.

2.3.2 Experimental abalone and stocking

Hatchery-reared juvenile abalone from the same spawning (12-months-old, 10 - 20 mm shell length), were chosen as test animals. The abalone were spawned in the farm hatchery at 18 $^{\circ}C$ and reared in aerated flow-through tanks (2-h exchange rate) at ambient temperatures. Twenty oyster mesh baskets were each stocked at 10% of the available surface area at the start of the trial.

2.3.3 Experimental diets and feeding

Experiment 1: Experimental abalone were subjected to three different diets, comprising of the non-supplemented base (control), the same base diet supplemented with 0.20% alginate and the same base diet supplemented with 0.90% (dry mass) minced *Ecklonia maxima* (Table 1). The kelp-supplemented (BK) and alginate-supplemented (BA) diets were the same as the base diet (10 mm x 10 mm x 1.2 mm). For the BK diet, fresh wet kelp was finely minced into fragments approximately 1 x 4 mm and mixed with dry ingredients prior to extrusion. The 0.90% kelp component of the BK diet was based on a previous study by Nel *et al*. (2017) which found significantly high abalone growth with a kelp-supplementation level of 0.88% versus a base diet. The alginate component of the

BK diet. Sodium alginate was mixed with dry ingredients of the base diet prior to extrusion to produce the BA diet.

Experiment 2: Experimental abalone were subjected to four different diets, comprising of the fresh wild kelp diet (control), a non-supplemented base diet, the same base diet supplemented with 0.20% alginate and the same base diet supplemented with 0.90% minced *Ecklonia maxima* (Table 2.1).

For both experiments, each treatment had five replicates (five baskets), each of which was randomly assigned among five experimental culture tanks. The treatments were supplied once every two days, between 16:00 and 17:00 hours, except for the wild fresh kelp diet that was supplied as 1 kg per replicate per week. Kelp was purchased from a local supplier who harvested it from the wild.

Table 2.1: The composition and formulation of the pelleted diets of abalone included a non-supplemented base diet as the control for experiment 1, the same base diet supplemented with alginate (BA), the same base diet supplemented with minced kelp (BK) and a fresh kelp diet as a control (K) for Experiment 2.

		Base (B)	Base + Alginate (BA)	Base + Kelp (BK)
Dietary ingredients (%)	Fishmeal and soya*	50.4	50.4	50.2
	Kelp**	0.0	0.0	0.9
	Starch*	48.7	48.6	48.5
	Vitamin/mineral mix*	0.1	0.1	0.1
	Vegetable oil***	0.8	0.7	0.3
	Alginate****	0.0	0.2	0.0
Total		100.0	100.0	100.0
Formulation	Total alginate	0.0	0.2	0.2
	Protein	34.0	34.0	34.0
	Lipid	5.0	5.0	5.0

* Supplied by Marifeed Pty Ltd
** Ocean harvested *Ecklonia maxima* supplied by Taurus Cape Kelp (Pty) Ltd
*** Sunflower oil supplied by Pick ‘n Pay (Pty) Ltd
**** Sodium alginate supplied by Sigma-Aldrich (Pty) Ltd

2.3.4 Growth measurements and feed utilization

To reduce injury to the foot muscle during growth measurements, abalone were anaesthetized in a magnesium sulfate salt solution (0.012 $kg.L^{-1}$). Experimental abalone were reared for a total of eight months and weighed three times; at the start trial in June 2017, in October 2017 and at the end of the trial in February 2018. The abalone were individually measured to the nearest 1.0 mm using vernier calipers and weighed to the nearest 0.01 g using an electronic scale after blotting out excess water. The condition factors (CF), linear shell growth rate (LGR), specific growth rate (SGR) and percentage mass gain (MG) were determined at the end of the trial for the individually measured and weighed abalone using the equations;

(1) $CF = \frac{weight\ (g)}{length\ (mm)\text{^}2.99} X\ 5575$ (Britz 1996)

(2) $LGR\ (mm/day) = \frac{final\ shell\ length\ (mm) \div initial\ shell\ length\ (mm)}{time\ (days)}$

(3) SGR (% body weight day per day) $= \frac{[Ln\ final\ weight - Ln\ initial\ weight]}{time\ (days)} * 100.$

(4) MG (%) $= \frac{average\ final\ mass - average\ initial\ mass}{average\ initial\ mass} * 100$

The abalone were fed every two days between 16:00 and 17:00 hours. The percentage daily feeding rate (PFR) was determined using Ebert and Houk's (1984) formula:

(5) PFR (% body weight per day) $= \frac{C}{T*W} * 100$, where C represents the amount of feed offered, T represents the number of days and W is the final + initial weight of abalone/2. In addition, the feed conversion ratio (FCR) was calculated using the formula;

(6) $FCR = \frac{dry\ weight\ of\ feed\ applied}{weight\ gain\ per\ basket}$. The FCR was not corrected for leaching and uneaten feed as the mesh baskets did not permit collection of uneaten feed.

2.3.5 Digestive enzyme activity levels: sample collection and preparation

A subsample of 30 abalone per treatment (6 per replicate) was randomly selected from the baskets between 7:00 and 8:00 hours at the end of July and August 2017 and February 2018. This twelve hour period from the onset of the last feeding has been reported as the peak period of digestive enzyme activity in *Haliotis midae* (Britz *et al*. 2996). The sampled abalone were snap-frozen on dry ice on-farm and weighed and measured individually using an electronic balance (TR-104, Denver Instrument Company, New York, USA) and a vernier caliper.

Alginate lyase and chymotrypsin activities were measured from the entire digestive system, including the mouth, stomach, digestive gland and rectum, in this study. In the laboratory, five abalone per treatment were cleaned with 75% ethanol, their pooled digestive systems homogenized with a mortar and pestle after being cut into small pieces on ice using a sterilized scalpel. Each sample was then homogenized in 5.0 ml of chilled 0.1 M citric acid buffer with pH 5.2. Each sample was subsequently centrifuged for 45 min at 13000 x *g* at 4 ˚C and then 1.5 ml aliquots of the supernatant were stored at -30 ˚C until needed. Whole digestive systems were used for enzyme analyses.

The enzyme essays were run at room temperature and triplicates of enzyme activities from each replicate were obtained for each assay. All enzyme assays were conducted using colorimetric end-point techniques for which absorbance was determined using a PowerWave X-1 Microplate Spectrophotometer reader with KC Junior™ software (BioTek® Instruments; Winooski, Vermont, USA).

2.3.5.1 Alginate lyase specific activity levels

The alginate lyase specific activity was assayed using a modified dinitrosalicylic acid (DNS) method (Nel *et al*. 2017). A standard curve was generated using β-D-mannuronic acid (R^2 = 0.998) in the concentration range of 0.1 - 1.2 $mg.ml^{-1}$. Basically, a total reaction mixture volume of 400 µl consisted of 275 µl of phosphate buffer (pH 6.9), 25 µl enzyme extract, and 100 µl of buffered substrate (W201502, Sigma Aldrich). The mixture was incubated at room temperature for 30 min prior to centrifugation at 13 000 × *g* for 5 min to separate undigested substrate from the supernatant. The supernatant was then mixed with a DNS colour reagent, boiled for 5 min and then cooled for 5 min on ice and the absorbance was recorded at 540 nm. Cellulase activity was measured using this method, with the exception that cellulose was used as a substrate.

For the alginate lyase and cellulase assays, the concentration of the product was calculated from its absorbance at 540 nm using a linear standard curve equation (y = mx + c) plotted for concentration against absorbance. The average absorbance of triplicate readings was used to establish the hydrolysis end-product concentration, and the enzyme-specific activity expressed as the amount of end product per milligram (mg) of protein in the essay for a given time period (mg product/mg^{-1} protein).

2.3.5.2 Chymotrypsin activity levels

Chymotrypsin activity was assayed using a modified method of Picos-Garcia *et al*. (2000), using succinyl- Ala -Pro-Phe-p-nitroanilide (SAPNA; S7388, Sigma Aldrich) as a substrate. The assay was run at room temperature. A Tris-HCl buffer was used for this assay. Twenty-five microliters (25 µl) of enzyme preparation were mixed with 120 µl of 0.001 mM SAPNA solution and 100 µl of buffer (pH 7.5) and then the absorbance was recorded after 5 min at 410 nm. Chymotrypsin activity was expressed in SAPNA units/mg as [(A_{410nm} /min X 1000 X volume of the reaction mixture)/ 8800 X mg of protein in the assay)], where 8800 is the extinction coefficient of p-nitroaniline.

2.3.6 Intestinal morphology: sample collection and preparation

At the end of the study, three abalone per replicate were sampled for histological investigation. The abalone were anesthetized in magnesium sulfate to reduce injury to the foot muscle before being weighed and measured individually as described previously. They were then preserved whole (or the shell was removed if too big) in Davidson's fixative (Amanzi Biosecurity, Hermanus) and stored at room temperature in the laboratory until required. In the laboratory, the abalone were cleaned with 75% ethanol and dissected cold to obtain their whole guts. The abalone shells were excised using surgical blades after which small incisions were made on the intestine to expose the lining of the intestine.

Transverse section of the intestine were cut dorsoventrally below the base of the gills. The intestines, with their linings exposed, were sent to the University of Pretoria (Department of Paraclinical Sciences, Section Pathology) where the gut slides were prepared. Fixed tissue samples were dehydrated by a graded series of ethanol and chloroform concentrations, embedded with paraffin wax and then sectioned at 4 - 5 µm with a rotary microtome. The prepared intestine sections were mounted onto microscope slides, dried and stained with a Haematoxylin-Eosin solution. At Rhodes University (Department of Physics and Electronics), the slides were viewed under a compound light microscope (Olympus BX50) at 10X magnification and photographs of the villi taken using an Olympus Soft Imaging Solutions software (Tokyo, Japan). Measurements of the intestinal villi height and length were taken from the basal lamina to the gut lumen per slide within a rectangle of a standardized surface area (126 260 μm^2) using the ImageJ Plus version 1.46r software (National Institutes of Health, USA) and the surface area calculated using the equation; S.A. = villi height x villi width

(Schaefer *et al*. 2013). Nine measurements were taken per treatment. The measurements for determining the surface area were taken as shown in Figure 2.

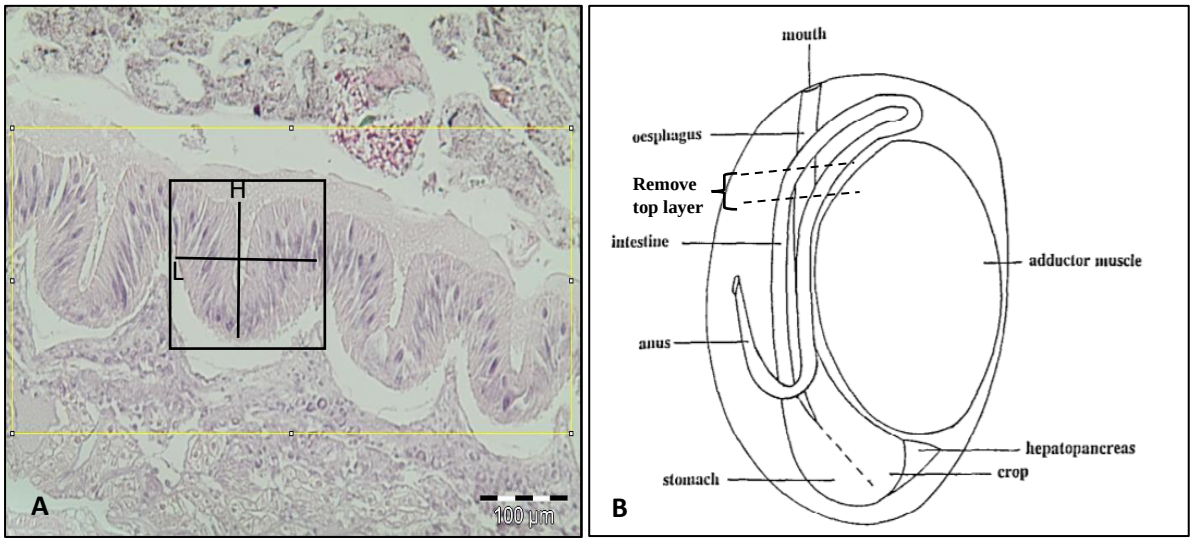

Figure 2.2: A) The yellow rectangular area indicates the surface area over which measurements were taken per slide, while the smaller black rectangular area indicates the side from which the measurements were taken and how the measurements (height, H and length, L) were taken. B) The transverse section of the intestine was obtained by carefully removing the outermost layer and exposing the internal lining of the intestine along the two dashed lines. B is extracted from Erasmus *et al*. (1997).

2.3.7 Statistical analyses

Statistica for Windows (version 13.3.704.1; TIBCO Statistica, Tulsa, USA) was used for all statistical analyses. The assumptions of homogeneity of variances and normal distribution were tested using the Levene's and Shapiro-Wilk tests, respectively. The One-Way ANOVA (Tukey post-hoc) was used to compare the growth, feed utilization, digestive enzyme activity levels and intestinal villi height, width and surface area between abalone fed the four different diets. Where the assumptions of these parametric tests were violated, and transformation of the data failed, the nonparametric Kruskal-Wallis test was performed as an alternative test to the One-Way ANOVA tests. A significant level of $p < 0.05$ was used for all statistical tests. The figures and tables were generated using Excel and the results presented as mean ± standard deviation.

2.4 RESULTS

2.4.1 The growth, feed conversion ratio, feed utilization and enzyme activity levels of abalone

Experiment 1

Growth results indicating comparison of the average weight and different growth indices between abalone fed the base (B), kelp-supplemented base (BK) and alginate-supplemented base diets (BA) are shown in Figure 2.3 and Table 2.2 below.

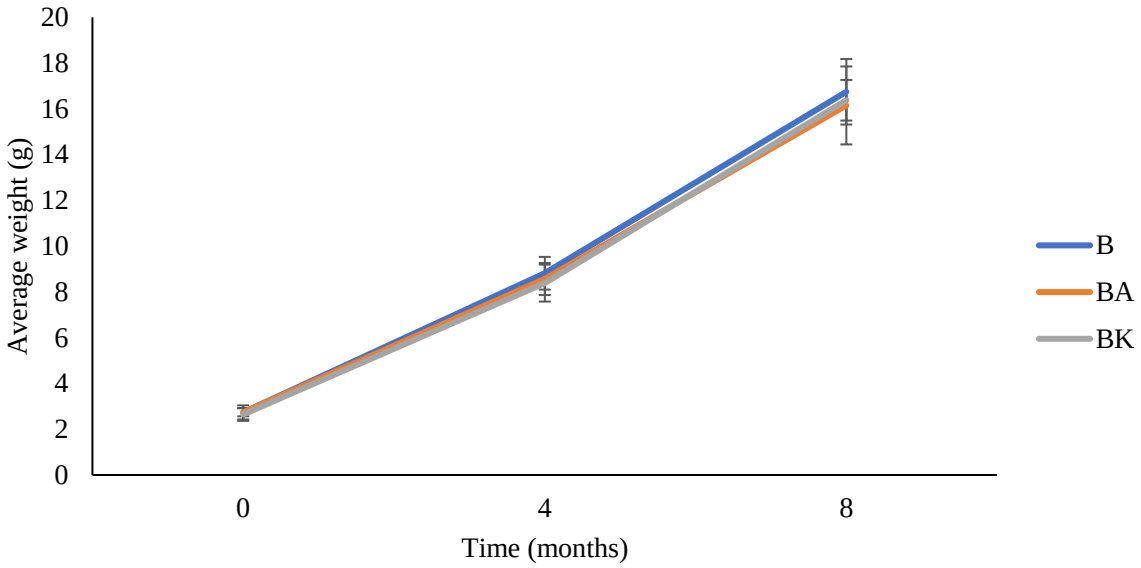

Figure 2.3: Comparison of the average (± s.d.) weight of abalone fed an alginate-supplemented (BA) feed versus abalone fed the kelp-supplemented (BK) and non-supplemented base (B) feeds between June (0) and October (4) 2017 and February 2018 (8).

At the beginning of this study (i.e. June 2017), there were no significant differences in the average weight between abalone fed the B, BA and BK diets ($F = 0.21$, $p = 0.82$; Figure 2.3). There were also no significant differences in the average weight of abalone fed the base diet compared to those fed the BA and BK diets in October 2017 ($F = 0.49$, $p = 0.62$). The results indicate that overall (i.e. in February 2018), alginate or kelp supplementation of a formulated feed had no significant effect on the average weight of abalone fed these diets compared to those fed the control diet ($F = 0.45$, $p = 0.65$).

Table 2.2: Comparison of the average (± s.d.) growth indicators between abalone fed an alginate-supplemented feed (BA), a kelp-supplemented diet (BK) and abalone fed the non-supplemented base diet (B) between June 2017 and February 2018.

	B	BA	BK	p
Growth indicators				
FCR	1.09 ± 0.17	1.11 ± 0.28	1.00 ± 0.13	0.72
PFR	0.84 ± 0.13	0.86 ± 0.20	0.78 ± 0.10	0.71
SGR	1.13 ± 0.04	1.10 ± 0.05	1.11 ± 0.02	0.40
MG	521 ± 75.9	484 ± 34.7	526 ± 96.4	0.63
LGR	0.06 ± 0.03	0.07 ± 0.00	0.07 ± 0.00	0.06
CF	1.45 ± 0.07	1.52 ± 0.10	1.47 ± 0.07	0.38

FCR = feed conversion ratio; PFR = percentage daily feeding rate (% body weight/day); SGR = specific growth rate (% body weight/day); MG (%) = percentage mass gain; LGR = linear shell growth rate (mm/day); CF = condition factor.

At the end of this experiment (February 2018), there were no significant differences in the FCR ($F = 0.36$, $p = 0.72$), PFR ($F = 0.35$, $p = 0.71$), SGR ($H = 0.82$, $p = 0.40$) and MG ($F = 0.47$, $p = 0.63$) between abalone fed either the BK, B or BA diets compared to each other (Table 2.2). Furthermore, there were no significant differences in the LGR ($H = 5.66$, $p = 0.06$) and CF ($F = 1.05$, $p = 0.38$), respectively, between the different treatments. Overall, these results indicate that alginate or kelp supplementation of a formulated feed has no significant effect on the growth of abalone fed either of these feeds compared to those fed the base feed (Table 2.2).

Experiment 2

Growth results indicating comparison of the average weight and different growth indices between abalone fed the base, kelp-supplemented base (BK), alginate-supplemented base diets (BA) and a fresh kelp diet are shown in Figure 2.4 and Table 2.3 below.

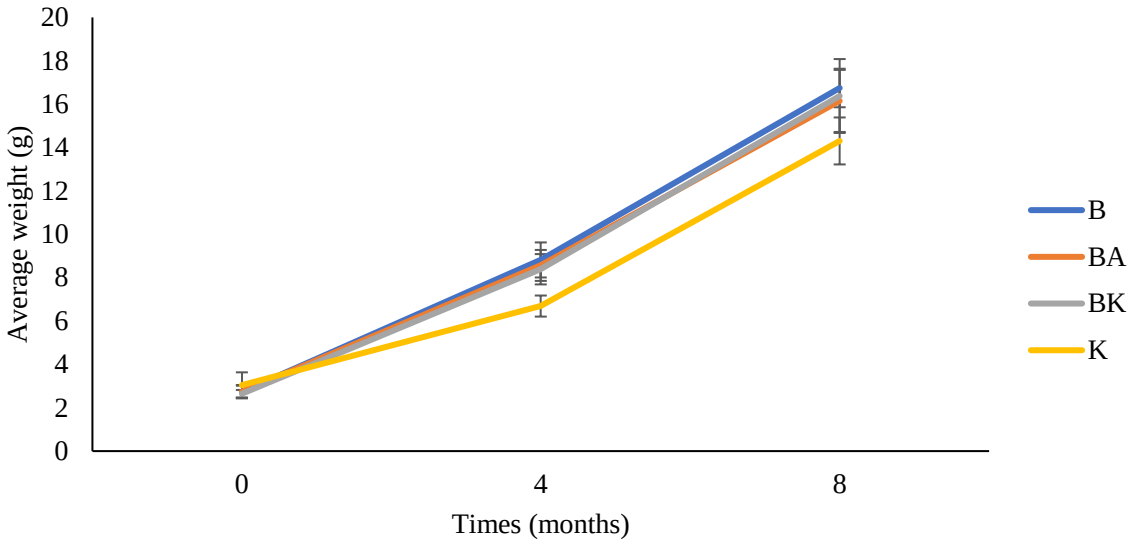

Figure 2.4: Comparison of the average (± s.d.) weight in abalone fed a minced kelp supplemented (BK) feed, a non-supplemented base feed (B), an alginate supplemented feed (BA) and a fresh kelp feed (K) between June (0) and October 2017 (4) and February 2018 (8).

In June 2017, there were no significant differences in the average weight of abalone fed either the B, BA or BK diets compared to those fed the K feed ($F = 0.94$, $p = 0.44$). In October 2017, all the groups fed formulated feeds showed significantly higher average weight compared to the K-fed group ($F = 10.07$, $p < 0.001$; Figure 2.4). At the end of this trial, the Tukey HSD post-hoc test showed that the K-fed abalone were not significantly different in their average weight compared to the abalone fed either the BA ($F = 3.61$, $p = 0.93$) or BK ($F = 3.61$, $p = 0.10$) diets but displayed significantly lower average mass than the B-fed abalone ($F = 3.61$, $p = 0.03$).

Table 2.3: Comparison of the average (± s.d.) growth indices between abalone fed an alginate-supplemented base feed (BA), a kelp-supplemented base diet (BK), a non-supplemented base diet (B) and a fresh kelp (K) diet between June 2017 and February 2018. Means that carry an asterisk superscript within each row are significantly different from each other (One-Way ANOVA/Kruskal-Wallis, $p < 0.05$).

	B	BA	BK	K
Growth indicators				
FCR	1.09 ± 0.17	1.11 ± 0.28	1.00 ± 0.13*	10.6 ± 1.40*
PFR	0.84 ± 0.13	0.86 ± 0.20	0.78 ± 0.10*	8.12 ± 1.01*
SGR	1.13 ± 0.04*	1.10 ± 0.05	1.11 ± 0.02	1.05 ± 0.04*
MG	521 ± 75.9	484 ± 34.7	526 ± 96.4	386 ± 118
LGR	0.06 ± 0.03	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.01
CF	1.45 ± 0.07	1.52 ± 0.10	1.47 ± 0.07	1.39 ± 0.14

FCR = feed conversion ratio; PFR = percentage daily feeding rate (% body weight/day); SGR = specific growth rate (% body weight/day); MG (%) = percentage mass gain; LGR = linear shell growth rate (mm/day); CF = condition factor.

At the end of the trial, there were no significant differences in the FCR between the K-fed abalone and abalone fed either the B (Kruskal-Wallis, $z = 2.46$, $p = 0.08$) or BA diets ($z = 2.57$, $p = 0.06$; Table 2.3). Moreover, there were no significant differences in the PFR between abalone fed the K diet compared to those fed the BA diet ($z = 2.57$, $p = 0.06$) or the B diet ($z = 2.46$, $p = 0.08$). In comparison, however, the BK-fed abalone showed significantly lower FCR ($z = 2.99$, $p = 0.02$) and PFR ($z = 2.99$, $p = 0.02$) compared to the K-fed abalone. At the end of the trial, only the B-fed abalone showed significantly higher SGR than the K-fed abalone ($F = 3.99$, $p = 0.03$) while the K-fed abalone showed no significant differences in their SGR compared to abalone fed the BA diet ($F = 3.99$, $p = 0.15$) or the BK diet ($F = 3.99$, $p = 0.07$).

The kelp-fed abalone showed no significant differences in their MG at the end of the trial compared to abalone fed the formulated feeds ($F = 2.78$, $p = 0.08$). There were also no significant differences in the LGR of abalone fed the kelp diet compared to those fed formulated diets in both October 2017 ($H = 6.63$, $p = 0.09$) and February 2018 ($H = 4.69$, $p = 0.20$). Furthermore, no significant differences in the CF were found between abalone fed the kelp diet compared to those fed formulated feeds at the end of the trial ($F = 1.69$, $p = 0.21$).

Experiment 1

Digestive enzyme activity levels for abalone fed the alginate-supplemented and kelp-supplemented feeds (K) versus those fed the base (B) feed were compared for the three different time points between July and August 2017 and February 2018. The results for these comparisons are presented in the figures below.

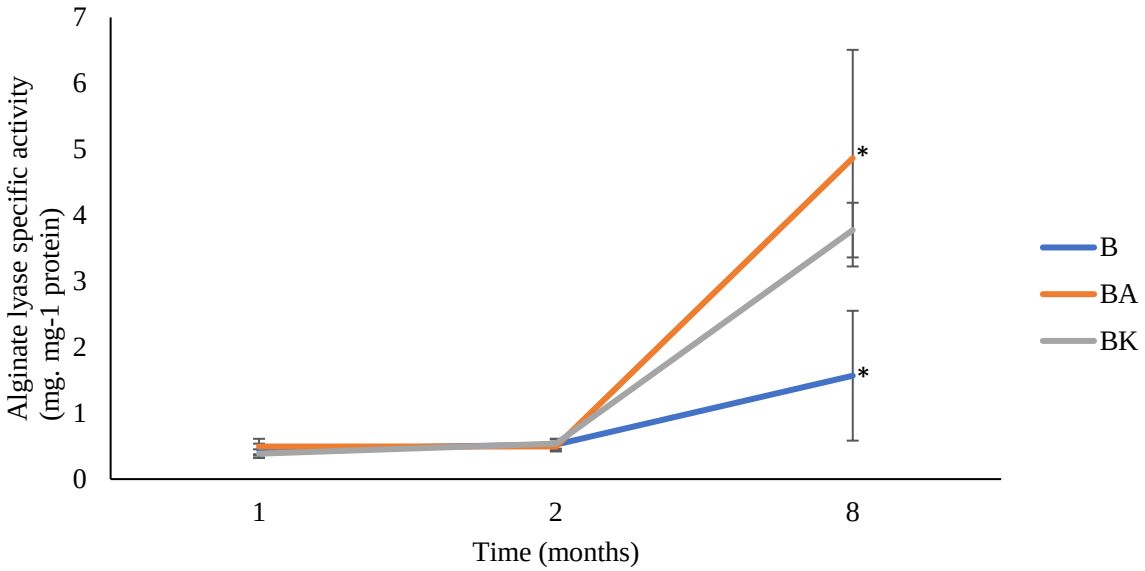

Figure 2.5: Comparison of the average (± s.d.) alginate lyase specific activity levels in abalone fed either an alginate-supplemented diet (BA) or a kelp-supplemented diet (BK) and a non-supplemented base diet (B) in July (1) and August 2017 (2) and February 2018 (8). Significant differences between treatments are shown by an asterisk (*).

There were no significant differences in the average alginate lyase specific activity levels in abalone fed the BA diet compared to those fed the B and BK diets in both July ($F = 1.68$, $p = 0.23$) and August 2017 ($F = 0.30$, $p = 0.75$). In February 2018, however, abalone fed the BA diet displayed significantly higher alginate lyase specific activity levels compared to abalone fed the B diet ($z = 2.99$, $p = 0.02$), but showed no significant differences compared to the BK-fed abalone ($z = 0.53$, $p = 1.00$). The BK-fed abalone further displayed no significant differences in alginate lyase specific activity levels compared to the B-fed abalone ($z = 2.46$, $p = 0.08$; Figure 2.5).

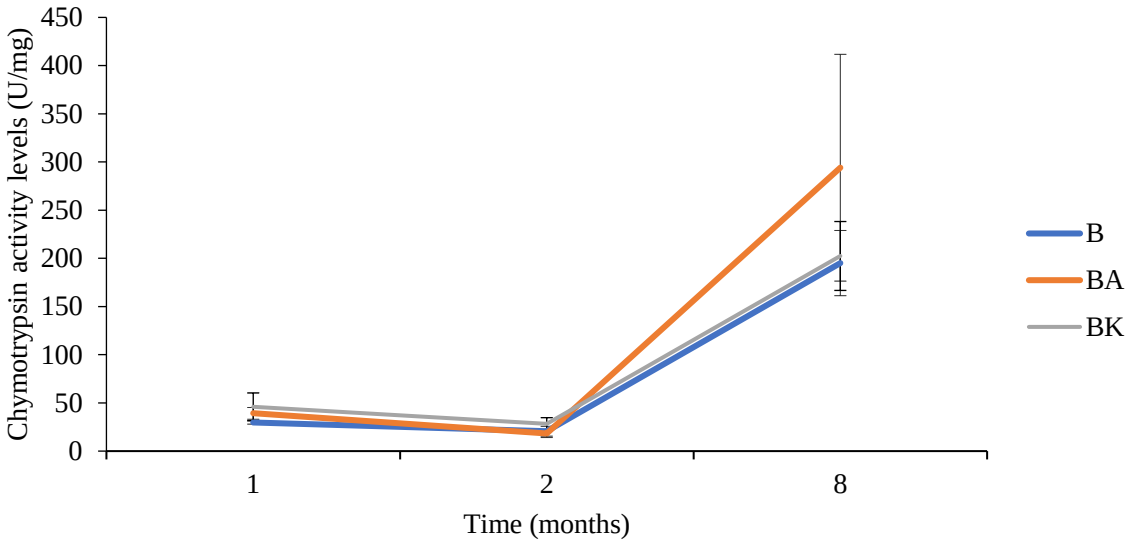

Figure 2.6: Comparison of the average (± s.d.) chymotrypsin activity levels in abalone fed with either an alginate-supplemented diet (BA) or a kelp-supplemented diet (BK) and a non-supplemented base diet (B) in July (1) and August 2017 (2) and February 2018 (8).

In July 2017, the abalone fed with the BK diet displayed significantly higher chymotrypsin activity levels than the abalone fed with the B diet ($z = 2.40$, $p = 0.04$; Figure 2.6). Chymotrypsin activity levels were not significantly different between abalone fed with the BA diet and those fed with either the B ($z = 2.26$ $p = 0.07$) or BK diets ($z = 0.14$, $p = 1.00$). However, no significant differences in chymotrypsin activity levels were found between the B-fed and BK-fed ($F = 4.84$, $p = 0.09$) abalone and between the base-fed and alginate-fed abalone in August 2017 ($F = 4.84$, $p = 0.77$). Contrary to results for July 2017, the BK-fed abalone displayed significantly higher chymotrypsin activity levels compared to the BA-fed abalone in August 2017 ($F = 4.84$, $p = 0.03$). However, no significant differences in chymotrypsin activity levels were found between the treatments in February 2018 ($F = 1.43$, $p = 0.27$).

Experiment 2

Digestive enzyme activity levels for abalone fed the alginate-supplemented (BA) and kelp-supplemented (BK) feeds versus those fed the base (B) and fresh kelp (K) feeds were compared for the time points in July and August 2017 and February 2018. The results for these comparisons are presented in the figures below.

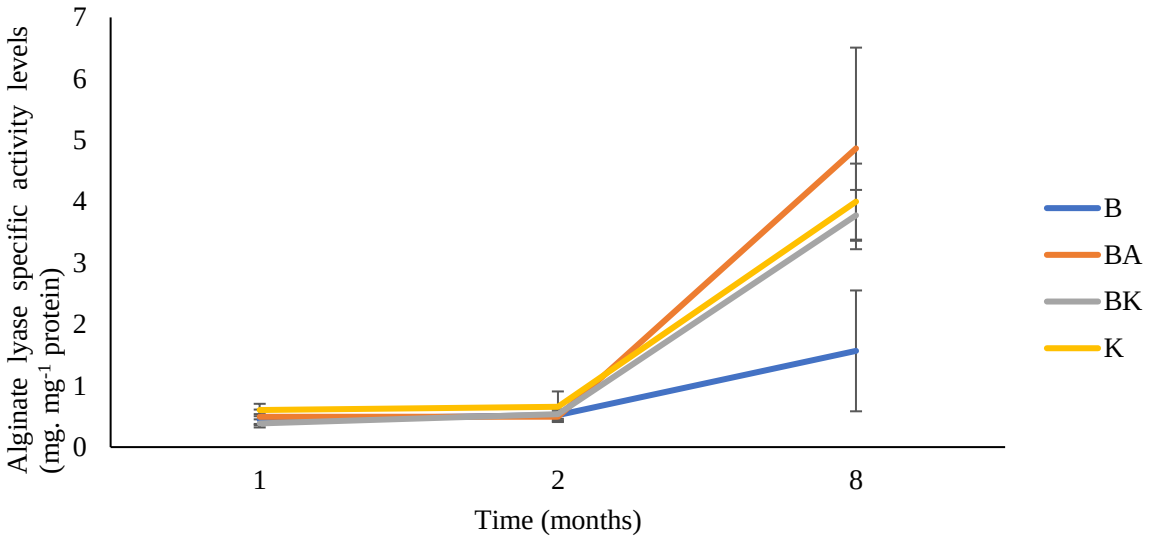

Figure 2.7: Comparison of the average (± s.d.) alginate lyase specific activity levels in abalone fed with either an alginate-supplemented (BA) diet, a kelp-supplemented diet (BK) or a non-supplemented base diet (B) and abalone fed a fresh kelp diet (K) in July (1) and August 2017 (2) and February 2018 (8).

In July 2017, the kelp-fed abalone showed significantly higher alginate lyase specific activity levels than the BK-fed abalone ($F = 4.67$, $p = 0.01$) but were not significantly different from either the base-fed ($F = 4.67$, $p = 0.08$) or the BA-fed abalone ($F = 4.67$, $p = 0.30$; Figure 2.7). There were no significant differences in the average alginate lyase specific activity levels between the treatments in August 2017 ($F = 2.02$, $p = 0.15$). At the end of this trial, there were no significant differences in the average alginate lyase specific activity levels between abalone fed the kelp diet and those fed the base diet ($z = 2.57$, $p = 0.06$), BA diet ($z = 0.43$, $p = 1.00$) or BK diet ($z = 0.10$, $p = 1.00$).

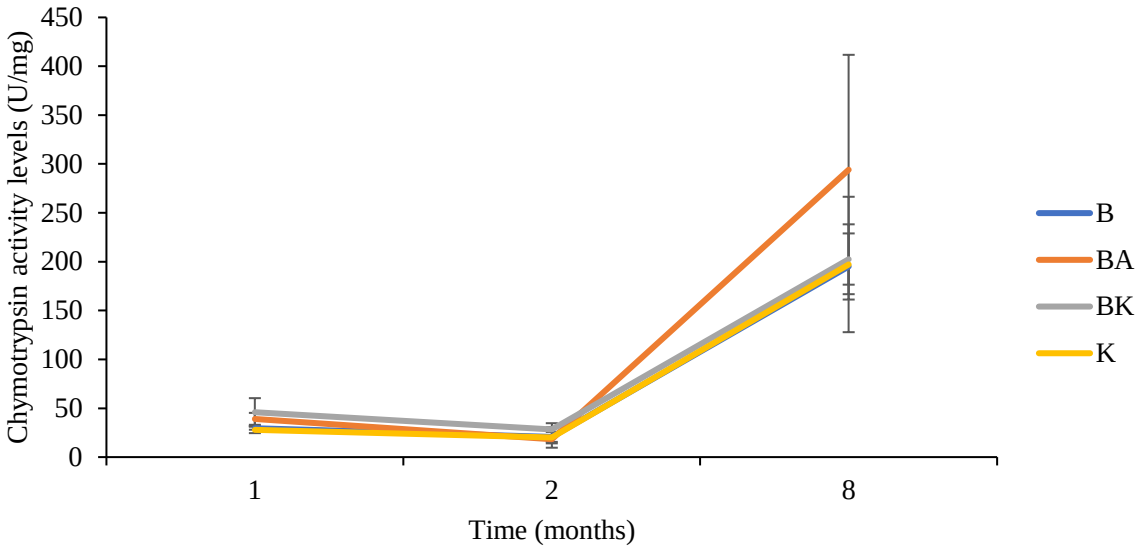

Figure 2.8: Comparison of the average (± s.d.) chymotrypsin activity levels in abalone fed with either an alginate-supplemented base diet (BA), a kelp-supplemented base diet (BK) or a non-supplemented base diet (B) and abalone fed with a fresh kelp-only diet (K) in July (1) and August 2017 (2) and February 2018 (8).

In July, there were no significant differences in the chymotrypsin activity levels between the abalone fed with the kelp diet compared to those fed with either the base ($z = 0.728$, $p = 1.00$), BA ($z = 2.62$, $p = 0.05$) or BK diets ($z = 2.62$, $p = 0.05$; Figure 2.8). There were also no significant differences in the chymotrypsin activity levels between abalone fed the kelp and formulated diets in August 2017 ($F = 2.37$, $p = 0.11$) and at the end of the trial ($F = 1.43$, $p = 0.27$).

Table 2.4: Comparison of the average (± s.d.) cellulase activity levels for abalone fed a minced kelp-supplemented feed (BK) and abalone fed the non-supplemented base feed (B), an alginate-supplemented feed (BA) and a fresh kelp-only feed (K) in February 2018.

	B	BA	BK	K
Cellulase activity levels ($mg.mg^{-1}$ protein)	0.82 ± 0.20	0.60 ± 0.34	1.11 ± 0.42	1.02 ± 0.49

There were no significant differences in the cellulase activity levels between abalone fed the different treatments at the end of this study ($F = 1.78$, $p = 0.19$; Table 2.4).

2.4.2 The intestinal morphology of abalone fed different experimental feeds

Photographs of the intestinal villi were analyzed: the villi height and width were measured using computer software and the intestinal villi surface area was calculated using a formula (Schaefer et al. 2013).

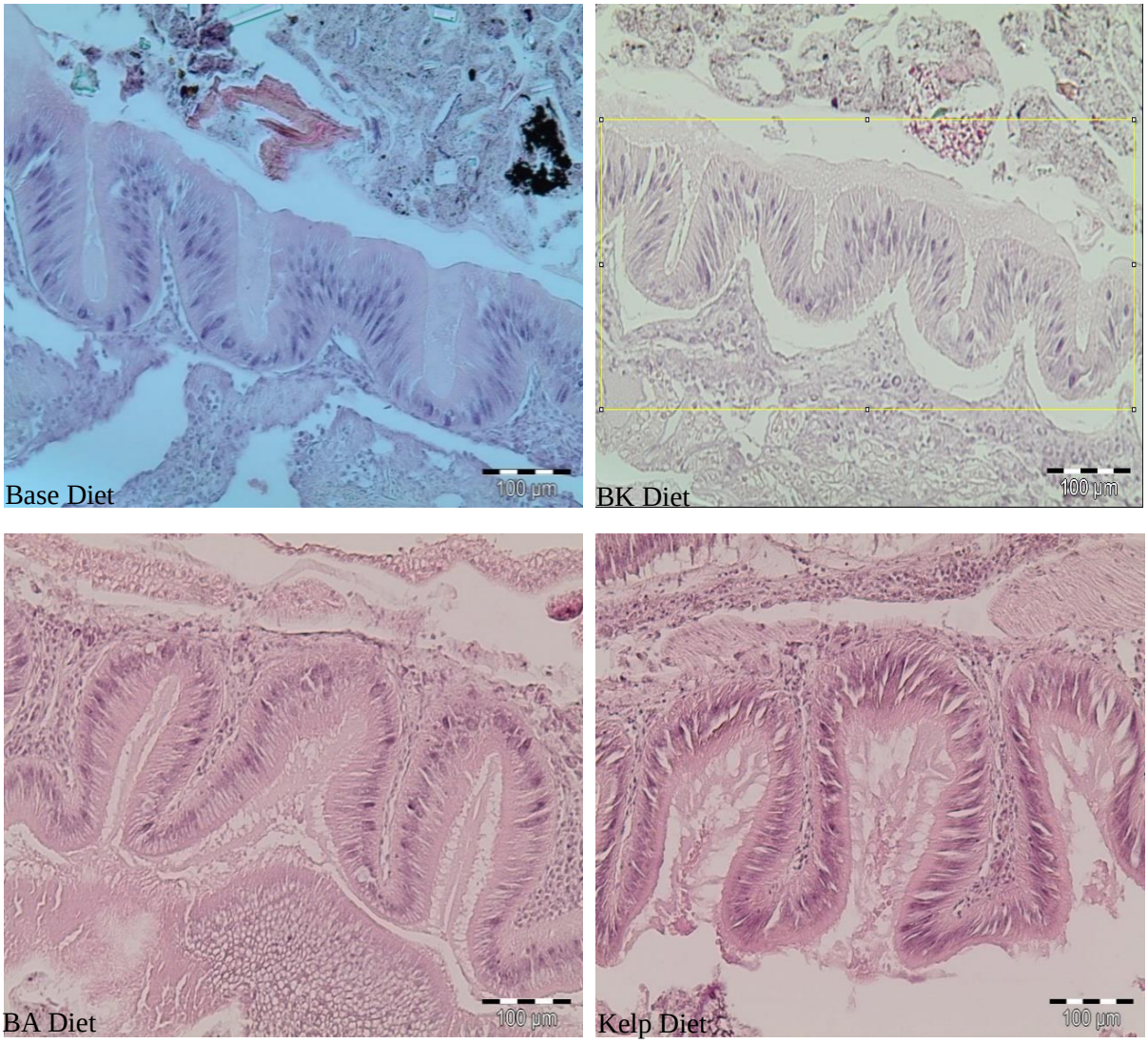

Figure 2.9: Photographs of the intestinal villi obtained from abalone fed different diets. Measurements of the villi height (H) and length (L) were recorded from the longest point on the villi within a rectangle of a standardized length and height as shown in slides 1 and 2, respectively.

Table 2.5: Comparison of the average (± s.d.) intestinal villi surface area, height and width for abalone fed a minced kelp-supplemented feed (BK) versus abalone fed the non-supplemented base feed (B), an alginate-supplemented feed (BA) and a fresh kelp-only feed (K).

	B	BK	BA	K	Anova
Height	151 ± 24.6	154 ± 45.6	184 ± 45.1	197 ± 78.9	0.43
Width	28.5 ± 33.4	134 ± 27.6	149 ± 63.5	160 ± 49.3	0.55
Surface area (μm^2)	25,030.3 ± 7,262.2	7,328.5 ± 10,593.2	28,025.0 ± 17,535.1	33,126.1 ± 17,210.2	0.68

There were no significant differences in the average intestinal villi surface area between abalone fed with the different diets ($F = 0.51$, $p = 0.68$; Figure 2.9, Table 2.5). Furthermore, no significant differences in the intestinal villi height ($F = 0.96$, $p = 0.43$) and width ($F = 0.72$, $p = 0.55$), respectively, were observed. When kelp was added to the base diet, there was greater variation in the surface area compared to the base diet. The standard deviation of the surface area of the villi in abalone fed the base diet was 28% of the total surface area, whereas this increased to 51% for fresh kelp, 63% for alginate and 145% for the BK diet. Thus, there was a clear contribution of kelp and/or its constituent, alginate, to the surface area of the villi (Table 2.5).

2.5 DISCUSSION

This chapter investigated the effect of minced kelp and alginate inclusion in a formulated feed for juvenile *Haliotis midae* on the growth, feed utilization, digestive enzyme activity levels and intestinal villi structure compared to a basal and fresh kelp diets. Kelp and alginate inclusion had no significant effect on the abalone percentage mass gain, FCR, PFR, LGR, SGR, CF and intestinal structure compared to the base and kelp diets, but kelp inclusion resulted in significantly lower FCR and PFR compared to the kelp diet. Alginate inclusion resulted in significantly higher alginate lyase activity compared to the base diet but none compared to the BK and kelp diets, whereas both kelp and alginate inclusion had no significant effect on chymotrypsin activity compared to the base and kelp diets at the end of this study.

Four months into the trial, all abalone fed formulated feeds showed significantly higher average weight gain compared to animals fed fresh kelp. However, the kelp group improved significantly over the last four months, showing no significant differences compared to abalone fed formulated feeds by the end of this study. These findings contradict published literature on the effect of kelp-supplementation of formulated feeds with fresh macroalgae or dried

macroalgae meal mixed with dry ingredients prior to extrusion in *H. midae*, *H. rufescens* or *H. discus hannai* (Dlaza *et al*. 2008; Naidoo *et al*. 2006; O'Mahoney *et al*. 2014; Kemp *et al*. 2015; Kemp 2017; Nel *et al*. 2017). Naidoo et al. (2006) found improved growth rates in *H. midae* fed Abfeed® (34.6% protein) was supplemented with fresh kelp. Nel *et al*. (2017) found that sub-adult *H. midae* fed a 26% protein Abfeed® diet supplemented with up to 3.54% minced kelp displayed significantly higher basket mass gain compared to abalone fed a non-supplemented Abfeed® diet. Similarly, Kemp (2017) reported improved growth in *H. midae* fed a 34%-protein diet supplemented with 7.5% fresh kelp. This effect has also been reported for post-weaning *H. midae* fed Abfeed® supplemented with fresh wild *Ecklonia maxima* and *Ulva lactuca* (Dlaza *et al*. 2008). Furthermore, Nel *et al*. (2017) found improved growth rates in sub-adult *H. midae* even at low inclusion levels of kelp (0.44%) than the 0.90% level used in this study. Improved body weight and shell length have also been achieved in *H. laevigata* fed a pelleted diet supplemented with 10% *Arthrospira maxima* or *Dunaliella salina* (Dang *et al*. 2011). From the cited literature, it is evident that the effect of macroalgae-supplementation not only depends on species, but also on the inclusion level chosen.

Nel *et al*. (2017) had supplemented a 26%-protein feed with minced kelp and found improved FCR in sub-adult abalone fed this diet compared to those fed a formulated diet only, a 34%-protein diet was used in this study. Rowan Yearsley (Marifeed (Pty) Ltd, personal communication) notes that *H. midae* fed this 34%-protein feed and supplemented with kelp had produced no significant increase in growth compared to abalone fed the same non-supplemented diet in a farm trial. However, Kemp (2017) used this diet in combination with fresh kelp and found significantly improved growth and feed utilization in juvenile *H. midae*. Thus, it evident that protein concentration in formulated feeds for *H. midae* influences growth even when these feeds are supplemented with kelp.

Low molecular weight sodium alginate inclusion in formulated fish feeds has been found to promote better growth rates (Fujiki and Yano 1997; Yeh *et al*. 2008; Van Doan *et al*. 2016b Van Doan *et al*. 2016b). However, the 0.20% level used in this study did not have this effect on abalone growth and feed utilization compared to the base, fresh kelp or the BK diets. The following two reasons are proposed for the non-significant effects of alginate- and/or kelp-supplementation on abalone growth in the current study:

Using stable isotope biomarkers and Bayesian mixing models, Kemp (2017) found that abalone fed a formulated diet-only displayed poor feed utilization compared to abalone fed a

combination diet of kelp and a formulated feed due to poor mixing of fishmeal as a protein source in the former feed. The degree to which alginate mixes with other components when included in formulated feeds is influenced by its structure, concentrations of PolyM and PolyG. The size of fragments of minced kelp may also affect the mixing of kelp with other components. As such, the properties of alginate, including its gelling properties, must be considered when it is included in formulated feeds.

Bansemer *et al*. (2014) wrote that feeding abalone with macroalgae may produce improved feed intake, health and marketability. Macroalgae, including kelp, are rich stores of bioactive compounds suck as eckol and phloroglucinol, sulfated polysaccharides, fucoidans, xylomannans, calcium spirulan and many more (Dang *et al*. 2011; Aremu *et al*. 2015) and reports suggest that these compounds possess strong antirival, anti-infection, antimicrobial, anticancer, antioxindant and prebiotic properties (O'Sullivan *et al*. 2010; Chojnaka *et al*. 2012). Dang *et al*. (2011) found that abalone *H. laevigata* fed a formulated feed supplemented with *Ulva lactuca* and *Spyridia filamentosa* showed improved growth and antibacterial activity against *Vibrio anguillarum* compared to abalone fed formulated feed-only diets.

The current study proposes that alginate- or kelp-supplemented diets offer no direct benefits for abalone growth or feed utilization and that supplementation of formulated feeds with macroalgae results in improved abalone health (e.g. antimicrobial activity, Dang *et al*. 2011). Although no significant differences existed, this study found that alginate- and kelp-supplemented base diets showed increased condition factors compared to the base diet. Nel *et al*. (2017a) found no significant differences in the CF between abalone fed a base diet and those fed the same diet supplemented with 0.44 - 3.54% minced kelp. There is also evidence from other studies that alginate- or macroalgae-supplemented base diets improve the health of cultured fish or abalone (Harikrishnan *et al*. 2011; Viera *et al.* 2015; Van Doan *et al*. 2016a). Viera *et al*. (2015) found that in *H. tuberculate* fed a 35%-protein diet supplemented with *Palmaria palmate*, abalone displayed improved growth, condition index and protein utilization. On the contrary, supplementation with *Laminaria digitata* significantly reduced the efficiency of dietary protein, resulting in abalone displaying lower nutritional index, protein deposition in the foot muscle as well as meet-to-shell ratio. This literature suggests that a certain inclusion level of macroalgae in a formulated feed that is necessary to achieve a certain aspect of growth, may be inadequate to achieve an optimal level of another aspect. As such, the growth of BA and BK-fed abalone may have been insignificant compared to the B-fed abalone in the current study due to the inclusion levels of alginate or kelp used.

In many papers on the effect of kelp-supplementation of artificial feeds on abalone growth, compounds that are responsible for improved abalone growth are unknown and as such, remain largely undescribed. In addition to laminarin, fucoidan, alginate and cellulose, kelps also contain small amounts of lipids, marine minerals (Ca, Mg, Zn, Na etc), proteins, vitamins and bioactive compounds such as phlorotannins. For example, the nutraceutical industry uses polyunsaturated fatty acids (PUFAs) from kelp for their antifungal, antimicrobial and antibacterial properties (Michalak and Chojnacka 2015). In pigs, it has been found that piglet feed supplemented with fucoidan and laminarin from *Laminaria spp*. increased feed digestibility and reduced *Escherichia coli* counts in feces (O'Doherty *et al*. 2010). Although the digestive system of pigs varies significantly from that of abalone and since brown seaweeds contain a wide range of bioactive compounds and other components, these cited studies suggest that in order to fully understand the effect of kelp-supplementation on abalone, it may be necessary to isolate, characterize and fully investigate the effect of some of the individual components such as laminarin or fucoidan on the growth of cultured abalone.

Perhaps the gelling properties of alginate should also be considered when it is included in formulated feeds for cultured animals. Storebakken (1985) and Storebakken and Austreng (1987) reported that when used in formulated feeds as binders and depending on their viscosity, alginates reduced the feed intake, digestibility of protein, analytical residue (dry matter - sum of crude protein), nitrogen, ash, fat and calcium while increasing the moisture content of the feed of the rainbow trout *Oncorhynchus mykiss*. It appears that this viscosity depends on the amount of guluronic and mannuronic acids found in the alginate (Storebakken and Austreng 1987; Suzuki *et al*. 1993). The two acids form bonds of different strengths Storebakken and Austreng (1987) and consequently, the amount of each in a feed may exert a limiting factor on the digestibility of the feed in which alginate is included.

Alginates have been used successfully in different industries such as pharmaceuticals, agriculture, textiles, biomedical and food industries (Michalak and Chojnacka 2015; Labowska *et al*. 2019). Properties of alginate such as those of antimicrobial, antioxidant, anticancer, immunostimulatory and free radical-scavenging nature, are due to the structure, alkaloid, amino acid, phenol, tannin and flavonoid contents of alginate (Mazumder *et al*. 2016; Janarthanan and Kumar 2018). However, the purpose for which alginate is applied must take into account several factors; morphology, the conformation of functional groups on the chemical structure, purity of the final product as well as chemical content of the product (Labowska *et al*. 2019). Furthermore, the PolyM and PolyG contents of the final product must

be understood as products with high G (guluronic acids) contents have higher gelling properties while those with higher M (manuronic acid) contents lead to higher viscocity (Fertah *et al*. 2017). Several studies have used alginate as a binder for abalone fed (Britz *et al*. 1994; Knauer *et al*. 1993; Viera *et al*. 2015). The efficiency of an abalone diet containing alginate is affect by a wide range or properties and these must be addressed if the purpose for which alginate is used is to be achieved. Making up to 40% of dry matter in brown macroalgae, a natural diet of *Haliotis midae* in South Africa, it is unlikely that alginate would present significant problems, if any at all, for abalone when used as a feed supplement. However, this literature review suggests that a combination of alginate, when its properties are not adequately considered for this application, with other components of formulated feeds, maybe problematic.

For instance, during an *in vitro* digestion study, Ramirez *et al*. (2015) noted that the addition of 1.0 and 2.0 g of sodium alginate per 100 g of potato starch resulted in significantly lower starch hydrolysis, highlighting the importance of molecular interaction between alginate and different components of feed formulations. The addition of alginate affected the viscosity of the alginate-starch mixture, resulting in alginate playing a protector role on the potato starch granules and causing low starch hydrolysis (Ramirez *et al*. 2015). Perhaps this protector role of alginate could explain the observations of Storebakken and Austreng (1987) in the rainbow trout *Salmo gairdneri*. In rats, Suzuki *et al*. (1993) found low feed intake and poor growth in rats fed a diet supplemented with alginate compared to a basal diet, particularly an alginate diet rich in guluronic acid. However, it is not known whether and to what extent alginate would have the same effect in abalone as abalone and rats are morphologically and physiologically different species and the current study found that the alginate diet produced higher feed utilization compared to the base and BK diets.

Digestive enzymes aid in efficient feed utilization by hydrolyzing ingested feed prior to absorption across the intestinal wall (Bedford 2002; Karr-Lilienthal 2005; De Villiers 2012) and have been studied extensively (Britz *et al*. 1996; Picos-Garcia *et al*. 2001; Serviere-Zaragoza *et al*. 1997). At the end of this trial, there were no significant differences in the average alginate lyase specific activity levels between abalone fed the kelp diet and those fed the base, BA or BK diets whereas abalone fed the BA diet displayed significantly higher alginate lyase specific activity levels compared to abalone fed the base diet, but showed none compared to the BK-fed abalone. The BK-fed abalone further displayed no significant differences in alginate lyase specific activity levels compared to the base-fed abalone.

The digestion of kelp and its constituents in the abalone gut is aided by exogenous enzymes of bacterial origin (Erasmus *et al*. 1997; Vitalis *et al.*1988; Sawabe *et al*. 2003; Macey and Coyne 2005; Sawabe 2006; Iehata *et al*. 2009; Nel *et al*. 2017a) and when alginate is included in the feed, this may have resulted in increased utilization by bacteria. This is the first study to investigate alginate-supplementation in abalone and comparisons with other literature could not be conducted. Nel *et al*. (2017a) also found no significant effect of kelp-supplementation in sub-adult abalone compared to a base diet. However, ten Doeschate and Coyne (2008) found significantly higher alginate lyase activity in *H. midae* fed kelp supplemented with probiotics compared to non-supplemented kelp. Thus, it is possible that alginate in the current study provided conducive conditions for rapid bacterial colonization of the abalone gut, resulting in higher alginate lyase activity compared to the base diet whereas kelp in its fresh form and in the BK diet would require longer periods of bacterial colonization prior to hydrolysis.

Chymotrypsin is one of the well-studied enzymes in cultured abalone due to its important role in the pool of digestive enzymes found in the abalone gut. Chymotrypsin activity levels were not significantly different between abalone fed formulated diets and fresh kelp at the end of this study. Nel *et al*. (2017a) also found no significant effect of kelp-supplementation (0.90%) on the chymotrypsin activity of sub-adult *H. midae* (30.21 g/abalone), compared to a basal diet (27% protein). This low kelp inclusion level could result in similar macronutrient composition between formulated feeds and consequently, similar protease activity levels between diets. Furthermore, Garcia-Esquivel and Felbeck (2006) also found no significant differences in chymotrypsin activity between abalone fed fresh kelp and formulated diets. However, Macey and Coyne (2005) reported significantly higher protease activity in abalone fed a probiotic diet compared to a basal diet. Kelp and its components are digested with the aid of exogenous enzymes of bacterial origin in the abalone gut. However, the degree to which this applies is not fully understood as alginate and kelp had no significant effect on chymotrypsin activity in this study.

Nel *et al*. (2017a and 2017b) showed that kelp inclusion in formulated *H. midae* feeds influenced the diversity of gut microbiota, and that feed utilization and digestive enzyme activity levels were influenced by the presence of kelp. However, evidence of this was not found in this study as the BK-fed abalone showed similar alginate lyase, chymotrypsin and cellulase activity levels compared to the base-fed abalone. It was strange that the base diet produced similar alginate lyase specific activity levels to the BK and fresh kelp diets. However,

Garland *et al.* (1985) noted that the post-larvae of *H. rubra* ingest bacteria growing on the surface of the culture system. According to Erasmus *et al*. (1997) and Wong *et al*. (2000), alginate lyases can also be produced by kelp-utilizing bacteria. Since the culture system used in this study utilizes a pump-ashore water system, the tanks were in sunlight, and there was evidence of algal films growing on the surfaces to which the abalone were attached, it is possible that this water and algal productivity in the tank could have been the source of kelp-utilizing bacteria. These bacteria could have propagated in the culture tanks and may have been consumed by abalone, resulting in the detection of alginate lyase in the base-fed abalone. This hypothesis will need to be tested in future work. It is also possible that the culture system may have harbored a source of alginate unknown to the author.

At the end of the trial, cellulase activity levels were also compared between abalone fed the different diets to determine if this enzyme plays any key role in the pool of digestive enzymes found in the abalone gut. However, no significant differences were found in cellulose activity between abalone fed the four diets. Monje and Viana (1998) and Garcia-Esquivel (2006) suggested that the presence of cellulose in kelp promotes the development of bacteria that produce cellulase in the abalone gut. However, there is evidence that these bacteria played an important role in the current study, perhaps depending on the amount of cellulose in the feed. In fact, Garcia-Esquivel and Felbeck (2006) found significantly low cellulase activity in juvenile *H. rufescens* fed a basal diet compared to those fed kelp. However, when abalone *H. fulgens* were fed a diet containing 1% alginate and 19% cellulose versus 20% sodium alginate, Monje and Viana (1998) found that cellulase activity was highest for the cellulose diet compared to the alginate diet. The current study expected kelp to produce higher cellulase activity than the other diets. Since abalone require exogenous enzymes to digest cellulose (Monje and Viana 1998), the lack of higher cellulase activity for the kelp-fed group in the present study may suggest that a longer period of physiological adaptation is required for juvenile *H. midae* as animals were fed a formulated diet prior to the start of the trial. The required timespan for adaptation is unknown.

Abalone are active nutrient absorbers, utilizing energy to drive nutrient absorption across the intestinal wall (Crofts 1929). Abalone fed the fresh kelp diet had longer and wider intestinal villi height and surface area compared to abalone fed formulated feeds, with no significant statistical differences. The large intestinal villi surface area found in abalone fed fresh kelp, compared with that found in abalone fed formulated feeds, combined with long retention time of kelp in the abalone gut (Foale and Day 1992; Day and Cook 1995), may necessitate high

energy expenditure for extended periods of nutrient absorption. However, Kemp (2017, personal communication) found wider tubules in the digestive diverticulum of *H. midae* in response to formulated feeds compared to fresh kelp and combination diets. Whether these observations were due to feed type is uncertain as this could have been a part of the structural tissue development. However, Nel (2016) found that kelp-supplementation, as compared to a basal diet, had no observable effect on the crop epithelial cell structure due to similar macronutrient composition of the diets. The abalone digestive system is diverse and different parts of the digestive tract may react differently to different diets. The current study found no significant differences in the intestinal villi height and width, respectively, between abalone fed different diets. Nonetheless, these studies provide scope for further investigations on the effect of diet on the gut morphology of *H. midae*.

In rats, Suzuki *et al*. (1993) reported that a basal diet supplemented with either mannuronic or guluronic acids (which form either polyM or polyG building blocks of alginate; O'Sullivan *et al*. 2010) resulted in significantly lighter liver and spleen while the caecum, small and large intestines became significantly heavier compared to rats fed the basal diet. Ikegami *et al*. (1990) reported that supplementing a rat feed with 5% sodium alginate reduced feed digestion and absorption and caused an enlargement of digestive organs. The alginate content applied by Ikegami *et al*. (1990) is significantly higher than the 0.20% level used in the current study. It is worth noting that these components are not natural diets of rats as opposed to abalone which get them by ingesting their natural kelp diet. Again, however, no negative effects of feeding BA, BK or fresh kelp diets (all of which contained alginate) compared to the base diet on the abalone intestine were observed in the present study. Although the physiology and growth conditions of rats and abalone differ considerably, it might demonstrate the effect of alginate inclusion on the GIT. Considering that kelp differs significantly in macronutrient composition from formulated feeds used in this study, it is possible that the effects of diets are region-specific and/or depend on the inclusion level. Thus, it is hypothesized that since the abalone intestine is mainly concerned with absorption and some digestion, feed likely does not have a significant effect on this region of the gut. However, this must be verified with further research by comparing the effect of diet on different regions of the abalone gut.

Perhaps also worthy of consideration when studying gut morphology in abalone is the type of ingredient added to a formulated feed. Kemp (2001) reported deleterious effects of legumes on the intestinal structure and function in Australian abalone. Due to anti-nutritional factors such as antigenic proteins found in legumes, abalone GIT displayed an increase in mucus over the

epithelium, an abundance of secretory cells, inhibition of digestive enzymes and poor growth rates. Feeds such as kelp or formulated diets used in this study have no recorded history of major negative effects on the GIT of abalone. Although the intestinal villi in abalone fed kelp were relatively wider and longer, microscopically, than in other feeds, there does not seem to have been any negative reaction to this feed as growth was similar between all the diets. Alternatively, it is suggested that further studies be conducted to identify the cause of this difference.

2.7 CONCLUSION

Kelp or alginate supplementation of the formulated diet of the cultured South African abalone had no growth or feed utilization benefits for abalone at 0.90% or 0.20% dietary inclusion levels, respectively. Furthermore, whereas alginate-supplementation resulted in increased alginate lyase activity levels, kelp-supplementation had no significant effect on the alginate lyase specific activity levels compared to the base diet. Both alginate and kelp-supplementation had no significant effect on chymotrypsin and cellulase activity levels and intestinal villi height, width and surface area in abalone fed these diets compared to abalone fed the base or fresh kelp diets. This study concluded that the structural carbohydrate of kelp, i.e. alginate, did not affect the growth, feed utilization, intestinal morphology and chymotrypsin activity levels, but affected alginate lyase activity levels in the South African abalone *Haliotis midae* at the supplementation levels used. Consequently, it is unknown which component of kelp is responsible for improved growth of abalone when kelp was included in formulated feeds in other studies. It is likely that other less studied components of kelp such as fucoidan or carbohydrates might promote growth in abalone when kelp is included in formulated abalone feeds, or that the level tested in this study was insufficient to induce any observable changes in growth. The fact that sodium alginate increased alginate lyase activity provides the scope for further investigation of this diet.

CHAPTER 3

The effect of switching diets on the growth, feed utilization and digestive enzyme activity levels in juvenile abalone fed formulated feeds

3.1 INTRODUCTION

The reproductive and feeding biology of abalone have been described for different species (Shepherd and Laws 1974; Shepherd and Hears 1983, for *Haliotis cyclobates*, *Haliotis laevigata*, *Haliotis roei*, *Haliotis ruber* and *Haliotis scalaris*; Keesing and Wells 1989, for *Haliotis roei*; Wood and Buxton 1996a, 1996b, for *Haliotis midae*). Briefly, following external fertilization, abalone eggs develop into trochophore larvae, after which they develop into veliger larvae (Genade *et al*. 1984). During this period, juvenile Australian abalone (*H. laevigata, H. roei, H. ruber, H. cyclobates and H. scalaris*) and the Japanese abalone *H. discus hannai* feed on mucus and biofilm (Shepherd 1973; Kawamura *et al*. 2001) while *H. midae* retain the egg yolk on which they depend for metabolic energy and growth (Genade *et al*. 1984). Post-larvae of *H. discus hannai* and *H. midae*, respectively, develop a radula at about 0.8 mm and 5 - 8 mm shell length and start feeding on diatoms, before transitioning to macroalgae at about 5 - 10 mm for *H. discus hannai*, while in *H. midae*, feeding on the seaweeds *Plocamium sp*. and *Ulva fasciata sp.* occurred in abalone longer than 10 mm (Genade *et al*. 1984; Kawamura *et al*. 2001).

The feeding transitions identified in abalone (Genade *et al*. 1984 in *H. midae*; Picos-Garcia *et al*. 2000 in *H. fulgens* and Kawamura *et al*. 2001 in *H. discus hannai*) are associated with radula development (Johnston *et al*. 2005). Abalone feed on a wide variety of diets, including diatoms as early juveniles and macroalgae as they grow (Genade *et al*. 1984; Britz 1995; Knauer *et al*. 1996). Naturally, *H. midae* feed on different species of macroalgae, but feed particularly on the *E. maxima* in the wild (Barkai and Griffiths 1988) and rhodophytes (Wood and Buxton 1996), depending on locality. Consumption of *Ulva* is also prominent in this species, depending on location (Naidoo *et al*. 2007; Dlaza *et al*. 2008; Kemp 2017). In *H. discus hannai*, gut microflora changed coincident with dietary change (Tanaka *et al*. 2003). Thus, there is evidence of changes in abalone digestive physiology in response to different diets (Britz *et al*. 1996; Knauer *et al*. 1996; Monje and Viana 1998; Takami *et al*. 1998). Therefore, the transition from one diet to another as abalone grow suggests that these animals may possess physiological mechanisms that need to be better understood.

The digestive system of abalone is biochemically suited to utilize a wide range of feeds (Garcıa-Carreno *et al*. 2003; Nel 2016) and exhibits a high degree of phenotypic and physiological flexibility under different nutritional settings, allowing these animals to successfully exploit a wide range of diets (Johnston *et al*. 2005; Garcia-Esquivel and Felbeck 2006; Schaefer *et al*. 2013). The digestive enzymes of these animals change to conform to alterations in nutritional settings (Britz *et al*. 1996; Knauer *et al*. 1996; Monje and Viana 1998; Takami *et al*. 1998). Observations on feeding transitions in abalone provide an opportunity to study the effects of altering diets on gut morphology and digestive enzymes. As such, the effects of changing diets on digestive enzyme activities and gut morphology of cultured abalone have been investigated for some abalone species.

Kawamura *et al*. (2001) and Kawamura *et al*. (1999) employed scanning electron microscopy methods to investigate diet-associated developmental changes in the radula of *H. discus hannai* and *Haliotis iris*, respectively, from the larval stage to the adult stage. An increase in the number of rows baring lateral teeth as abalone transition between different diets was observed in both studies. Johnston *et al*. (2005) found that as juvenile *H. rubra* grew, the digestive systems became more complex, with increased number and density of tubules. According to the authors, this was consistent with the need for higher enzyme production and more efficient feed utilization (Johnston *et al*. 2005).

Tanaka *et al*. (2003) showed that changes in the gut microbiome of *H. discus hannai* can be induced through dietary alterations. Hatchery-reared abalone fed diatoms for three months were transitioned to an artificial diet and a macroalgal diet, and changes in their gut bacterial composition analyzed. Coincident with the dietary change from diatoms to macroalgae, the gut microbiome changed significantly with populations being dominated by facultative anaerobes of polysaccharide-degrading bacteria (Tanaka *et al.* 2003). For the diatom-to-artificial feed switch, there was an increase in bacterial count. Since bacteria are associated with exogenous enzyme production in the abalone gut (Erasmus *et al*. 1997; Sawabe *et al*. 1997; Macey and Coyne 2005, 2006; Iehata *et al*. 2009; Jiang *et al*. 2013; Nel *et al*. 2017b; Nel *et al*. 2018), it is plausible that digestive enzyme activity levels may have also changed due to the dietary switches. Indeed, Onitsuka *et al*. (2015) found that in the sea urchin *Strongylocentrotus intermedius*, animals experienced significantly different growth rates and digestive enzyme activity levels due to dietary switches. Such diet-induced changes in enzyme

activity levels have also been reported in fish and rats (Infante and Cahu 1994; Frances and Yudkin 1963).

The structure and function of the gastrointestinal tract (GIT) of abalone has been described previously (Campbell 1965; Harris *et al*. 1998; Johnston *et al*. 2005; Schaefer *et al*. 2013) and the literature review in Chapter 2 suggests that changes in gut structure may influence changes in production and secretion of digestive enzymes (Kemp 2001). As Kemp (2001) found in *H. laevigata*, some diets can have detrimental effects on the gut and secretory cell structure. The results of these studies suggest that altering diets in abalone culture may lead to changes in the GIT structure. Subsequently, diet-induced changes in the structure of secretory cells can lead to changes in the concentrations of enzymes. This, in turn, may lead to changes in the digestion and absorption efficacy in the intestine and consequently, affect the resulting growth of cultured abalone. Currently, no quantitative studies have been conducted to investigate the effect of dietary alterations on the growth, feed utilization and digestive enzyme activity levels in the abalone *Haliotis midae*. Thus, it is essential that the mechanisms underlying dietary switch-induced changes in growth and digestive enzyme activity levels in *H. midae* be investigated as this is a common practice in the culture of this species.

3.2 AIMS AND OBJECTIVES

The aim of this chapter was to determine the effect of altering/switching the dietary alginate or kelp supplementation in a formulated feed on the growth, feed utilization and digestive enzyme activity levels in the abalone *Haliotis midae*. The growth and feed utilization, alginate lyase specific activity and chymotrypsin activity levels were compared between abalone fed their respective diets for eight months, after which they were subjected to dietary switches for four months and were compared to abalone that were maintained on the original diets for another four months (total of twelve months on the initial diet). Dietary switches were conducted between base (B) and kelp-supplemented (BK) diets and between alginate- supplemented base (BA) and natural (fresh kelp *Ecklonia maxima*, K) diets.

3.3 METHODS AND MATERIALS

3.3.1 Site and culture system

The site and culture system used for this experiment are described in Chapter 2.

3.3.2 Diet switching protocol

The abalone were fed on their respective diets for eight months as described in Chapter 2 (June 2017 to 12 February 2018), after which the diets were altered and supplied for 4 months (12 February to 19 June 2018). Each treatment had a total of five randomly distributed replicates prior to the dietary switches. At the end of eight months, two replicates (baskets) of each treatment that remained on the initial diets were fed their respective diets for a further four months and those replicates were fed the initial diets for a total of 12 months (ending June 2018). At the end of the first eight months, three replicates per treatment were suddenly switched with three replicates of another treatment as follows; to determine if switching diets between base and minced kelp-supplemented (BK) diets would affect growth and digestive enzymes, three replicates of the base diet were randomly switched with three replicates of the BK diet and three replicates of the BK diet randomly switched with three replicates on the base diet (Table 3.1).

To determine if switching diets between formulated and natural diets affected growth and digestive enzymes, three replicates (baskets) of the alginate-supplemented diet (BA) were randomly switched with three replicates of the fresh kelp diet (K) and three replicates of the K diet randomly switched with three replicates of the BA diet (Table 3.1). The same procedure was followed for the base (B) and BK (kelp-supplemented base) diets. For each dietary switch (Treatments 5, 6, 7 and 8 in Table 3.1), abalone growth, feed utilization and digestive enzyme activity levels were compared with abalone that did not experience any dietary switch (Treatments 1, 2, 3 and 4 in Table 3.1).

Table 3.1: Growth, feed utilization and digestive enzyme activity levels were compared between abalone that experienced dietary switches and abalone that remained on initial treatments.

Initial Treatments (12 months)	Dietary Switch Treatments (4 months)
1. Base (B)	5. B to BK
2. BK (base + kelp)	6. BK to B
3. BA (base + alginate)	7. BA to K
4. Fresh Kelp (K)	8. K to BA

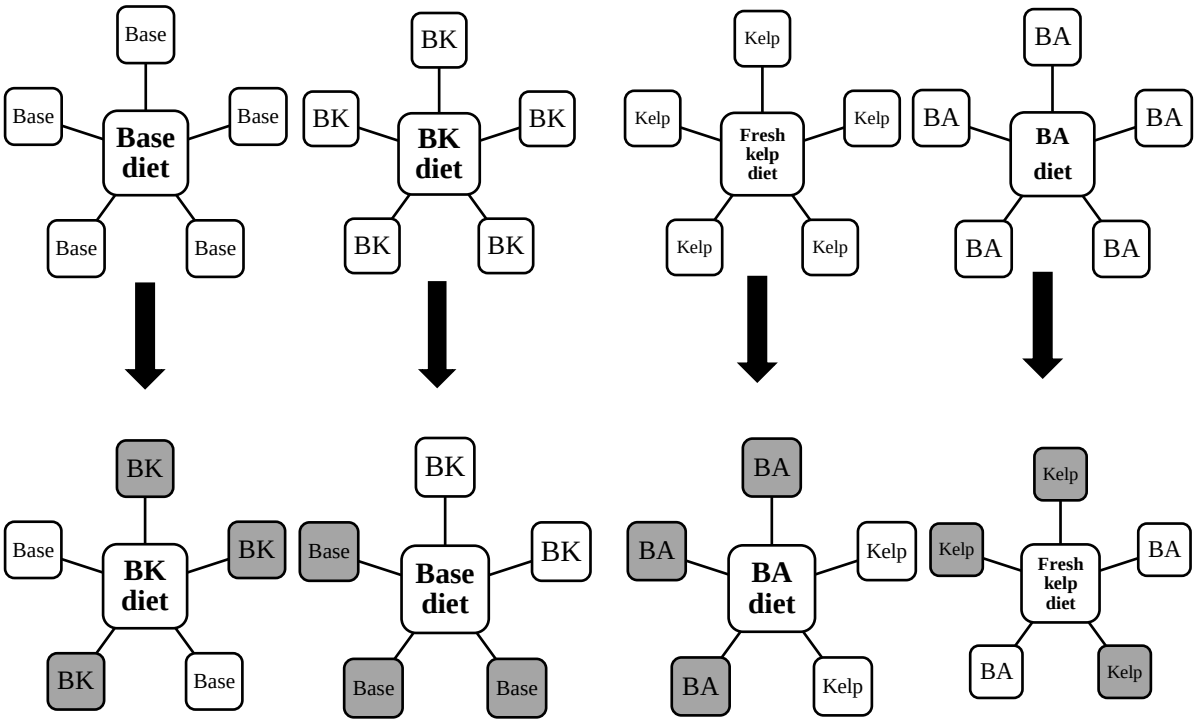

Figure 3.1: The dietary switching process used for in the study. After feeding for eight months, three replicates (baskets; gray boxes) of each treatment were swopped with three replicates of another diet, while the other two replicates remained unchanged. At the end of the trial in June 2018, unchanged baskets fed their respective diets for twelve months were compared with the three replicates changed to a different.

3.3.3 Growth, digestive enzyme activity levels

Growth measurements and calculations and sample collection for digestive enzyme activity levels were conducted as explained in Chapter 2.

3.3.4 Statistical analyses

Statistica for Windows (version 13.3.704.1; TIBCO Statistica, Tulsa, USA) was used for all statistical analyses. The assumptions of homogeneity of variances and normal distribution were tested using the Levene's and Shapiro-Wilk tests, respectively. The independent samples t-test was used to compare the growth, feed utilization and digestive enzyme activity levels between abalone switched from one diet to another and those kept on the initial diets. Where the assumptions of this parametric test were violated, and transformation of the data failed, the

nonparametric Mann-Whitney U test was performed as an alternative test. A significant level of $p < 0.05$ was used for all statistical tests. The figures and tables were generated using Excel and the results presented as mean ± standard deviation.

3.4 RESULTS

3.4.1 The growth of abalone switched between different diets

Growth was compared between abalone that experienced dietary switches for four months and abalone that remained on the same diet for twelve months and significant differences are represented with an asterisk (*). Data were collected at the end of twelve months for animals that remained on unchanged diets (example; BK unchanged) and after four months for animals that experienced dietary changes (example; BK to B). Bars that share the same color represent animals that were compared when kept on their initial diets and when they were switched to a different diet.

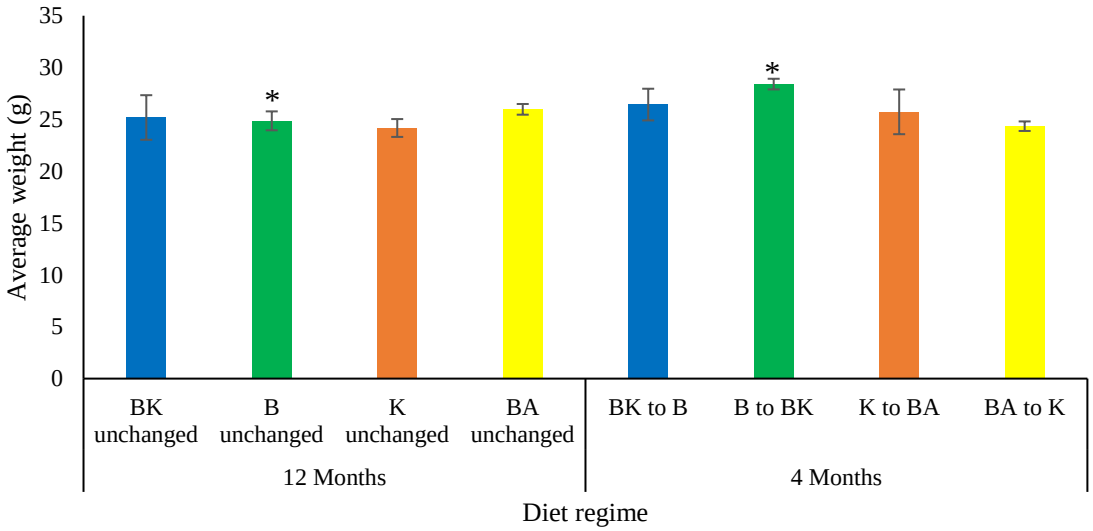

Figure 3.2 Comparison of the average weight (± s.d.) at the end of the trial between abalone that experienced a dietary switch between a kelp-supplemented diet (BK) and a base diet (B) and between an alginate-supplemented diet (BA) and fresh kelp diet (K) in the last four months, and abalone kept on the initial (unchanged) diets for the full 12 months. These animals were significantly different from each other if they share an asterisk (*; t-test/Mann-Whitney U-test, $p < 0.05$).

Switching abalone from the BK diet to the base diet had no significant effect on the average weight of abalone ($t = 0.60$, $p = 0.59$; Figure 3.2). However, abalone switched from the base diet to the BK diet displayed significantly higher average weight compared to abalone kept on the base diet ($t = 4.06$, $p = 0.03$). There were no significant effects of switching abalone from

the kelp diet BA diet on the average weight (t = 1.08, p = 0.36). Similarly, changing from the BA diet to the kelp diet had no significant effect on the average weight of abalone (Mann-Whitney U = 2.00, p = 0.77; Figure 3.2).

Table 3.2: Comparison of the average (± s.d.) condition factor (CF), linear shell growth rates (LGR), specific growth rates (SGR), daily feeding rate (PFR), feed conversion ratio (FCR) and percentage mass gain (MG) between abalone that experienced dietary switches and those kept on their initial diets. Comparisons are made between abalone switched between the base (B) and kelp-supplemented (BK) feeds and between an alginate-supplemented (BA) and fresh kelp (K) diets, respectively. Comparisons are made from top to bottom within each column and significant differences between treatments are shown with an asterisk.

Initial Treatments		KB	B	K	BA
	CF	1.38 ± 0.01	1.40 ± 0.03	1.25 ± 0.04	1.46 ± 0.06
	LGR	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
	SGR	2.39 ± 0.03	1.72 ± 0.67	2.36 ± 0.02	2.40 ± 0.03
	PFR	0.38 ± 0.01*	0.40 ± 0.04*	3.34 ± 0.31*	0.40 ± 0.02*
	FCR	1.59 ± 0.04*	1.18 ± 0.27	12.4 ± 2.79	1.28 ± 0.25
	MG	50.2 ± 2.13*	61.6 ± 2.79	63.4 ± 5.9	65.2 ± 13.4
Dietary Switch Treatments		BK to B	B to BK	K to BA	BA to K
	CF	1.34 ± 0.07	1.46 ± 0.03	1.40 ± 0.05	1.33 ± 0.03
	LGR	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
	SGR	2.43 ± 0.07	2.49 ± 0.03	2.41 ± 0.05	2.36 ± 0.08
	PFR	0.81 ± 0.06*	0.59 ± 0.04*	0.67 ± 0.22*	3.89 ± 0.76*
	FCR	0.71 ± 0.29*	0.94 ± 0.76	1.57 ± 0.75	31.8 ± 10.7
	MG	64.8 ± 4.21*	60.8 ± 1.89	89.6 ± 16.5	50.7 ± 2.4

FCR = feed conversion ratio; PFR = percentage daily feeding rate (% body weight/day); SGR = specific growth rate (% body weight/day); MG (%) = percentage mass gain; LGR = linear shell growth rate (mm/day); CF = condition factor.

Switching abalone from the BK diet to the base diet had no significant effect on the CF (t = 0.52, p = 0.64), LGR (t = 1.86, p = 0.16) and SGR (t = 0.60, p = 0.59) but resulted in significantly lower FCR (t = -3.29, p = 0.05) and significantly higher PFR (t = 7.55, p = 0.01) and MG (t = 3.49, p = 0.04) compared to abalone kept on the BK diet (Table 3.2). Switching abalone from the base diet to the BK diet had no significant effect on the CF (t = 1.57, p = 0.22), FCR (t = -0.33, p = 0.76), LGR (t = -1.24, p = 0.30), MG (t = - 0.32, p = 0.77) and SGR (U = 0.00, p = 0.15) but resulted in significantly higher PFR (t = 4.64, p = 0.02).

Abalone switched from the kelp diet to the BA diet showed no significant differences in their FCR ($U = 0.00$, $p = 0.15$), CF ($t = 2.88$, $p = 0.06$), LGR ($t = 0.55$, $p = 0.62$), MG ($t = 1.67$, $p = 0.19$) and SGR ($t = 1.16$, $p = 0.33$) but resulted in significantly lower PFR ($t = -8.77$, $p < 0.01$) compared to abalone kept on the kelp diet (Table 3.2). In comparison, abalone switched from the BA diet to the kelp diet also showed no significant difference in the FCR ($t = 3.13$, $p = 0.05$), CF ($U = 0.00$, $p = 0.15$), LGR ($t = -0.28$, $p = 0.80$), MG ($U = 2.00$, $p = 0.77$) and SGR ($t = 0.60$, $p = 0.59$) but showed significantly higher PFR ($t = 5.01$, $p = 0.02$) compared to abalone kept on the BA diet.

3.4.2 Digestive enzymes activity levels of abalone switched between different diets

Results for digestive enzyme activity levels were compared between abalone that were fed the initial (unchanged) diets for twelve months and abalone that experienced dietary switches and raised for four months. Significant differences between treatments are shown with an asterisk (*) in Figure 3.3.

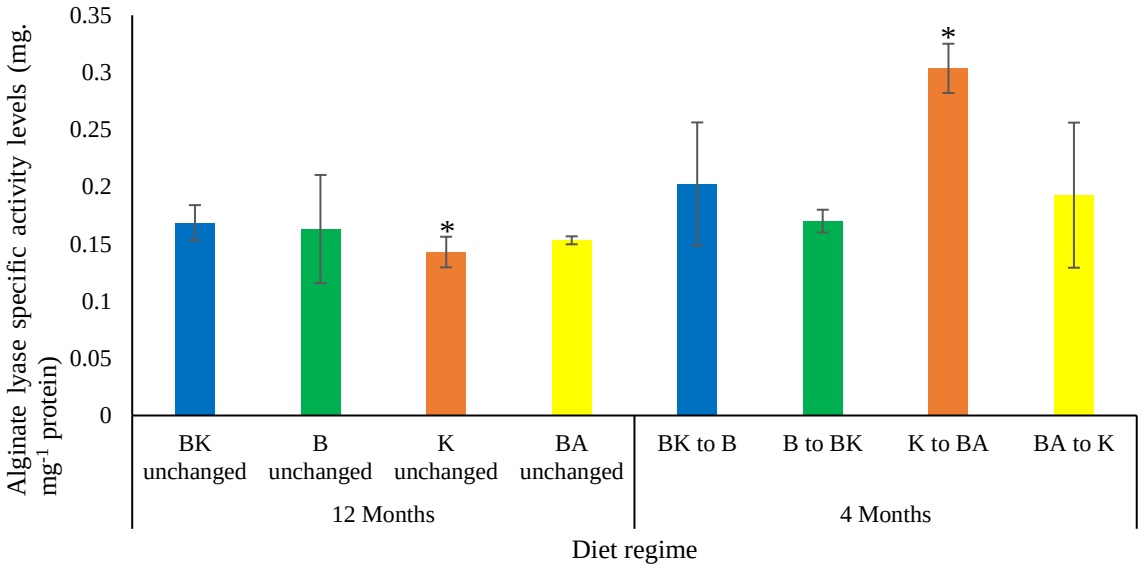

Figure 3.3: Comparison of the average (± s.d.) alginate lyase specific activity levels between abalone that experienced dietary switches between kelp-supplemented (BK) and base (B) diets and between alginate-supplemented (BA) and fresh kelp (K) diets, respectively, and abalone kept on the initial (unchanged) diets. These animals were significantly different from each other if they share the same colored bars and an asterisk (*; t-test/Mann-Whitney U-test, $p < 0.05$).

Switching abalone from the BK diet to the base diet or from the base diet to the BK diet had no significant effect on the alginate lyase specific activity levels compared to abalone kept on the BK diet ($U = 2.00$, $p = 0.77$) or those kept on the base diet ($t = 0.56$, $p = 0.62$; Figure 3.3).

Switching abalone from the BA diet to the kelp diet had no significant effect on the alginate lyase activity levels ($t = 0.93$, $p = 0.42$). However, switching from the kelp diet to the BA diet resulted in significantly higher alginate lyase specific activity levels ($t = 3.23$, $p = 0.04$).

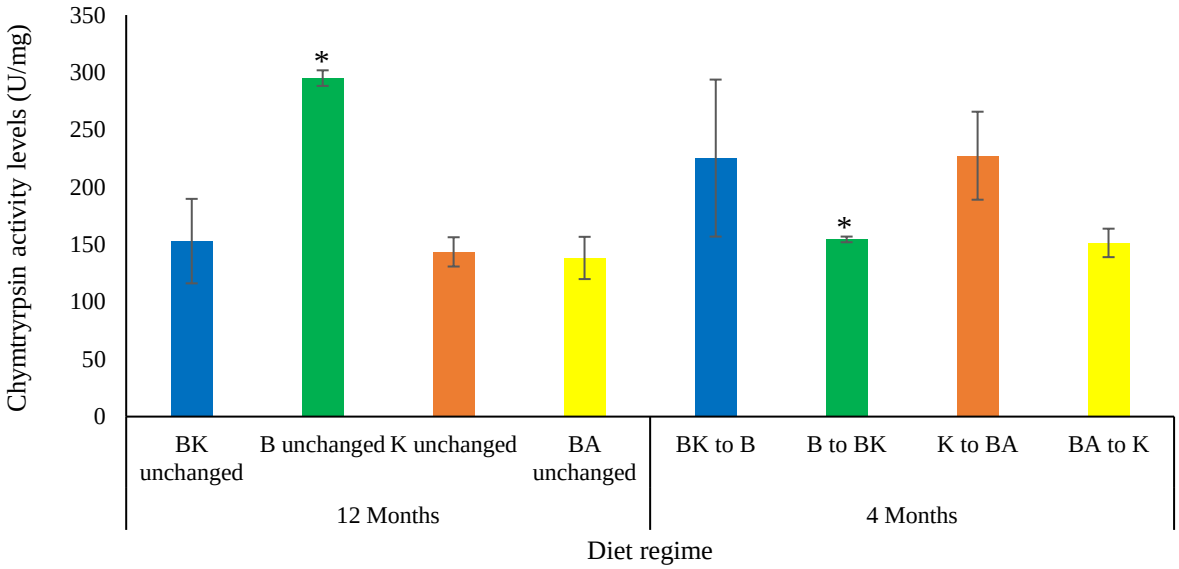

Figure 3.4: Comparison of the average (± s.d.) chymotrypsin activity levels between abalone that experienced dietary switches between kelp-supplemented and base diets and between alginate-supplemented and fresh kelp diets, respectively, and abalone kept on the initial (unchanged) diets. These animals were significantly different from each other if they share the same colored bars and an asterisk (*; t-test/Mann-Whitney U-test, $p < 0.05$).

Abalone switched from the BK diet to the base diet, from the kelp diet to the BA diet or from the BA diet to the kelp diet for four months, respectively, did not show any significant differences in their chymotrypsin activity levels compared to abalone kept on the BK ($t = 2.13$, $p = 0.12$), kelp ($t = 1.34$, $p = 0.27$) or BA diets for twelve months ($t = 0.36$, $p = 0.74$; Figure 3.4). However, switching abalone from the base diet to the BK diet resulted in significantly lower chymotrypsin activity levels compared to abalone kept on the base diet ($t = -7.81$, $p = 0.004$).

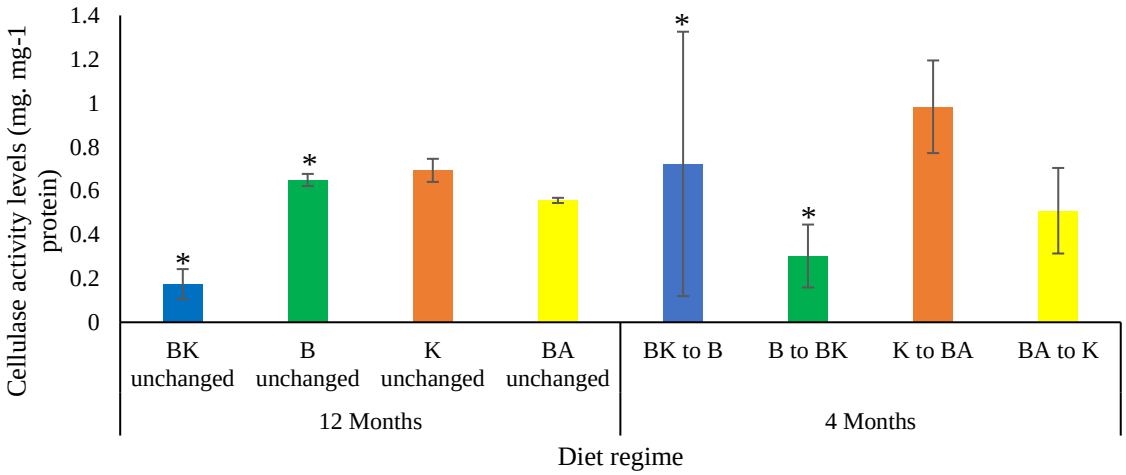

Figure 3.5: Comparison of the average (± s.d.) cellulase activity levels between abalone that experienced dietary switches between kelp-supplemented (BK) and base (B) diets and between alginate-supplemented (BA) and fresh kelp-only (K) diets, respectively, and abalone kept on the initial (unchanged) diets. Cellulase activity levels of these animals were significantly different from each other if they share the same colored bars and an asterisk (*; t-test/Mann-Whitney U-test, $p < 0.05$).

Abalone switched from the BK diet to the base diet displayed significantly higher cellulase activity levels compared to abalone kept on the BK diet ($t = 8.34$, $p < 0.01$; Figure 3. 5) while abalone switched from the base diet to the BK diet showed significantly lower cellulase activity levels compared to abalone kept on the base diet ($t = - 7.09$, $p = 0. 01$). Furthermore, switching abalone from the kelp diet to the BA or from the BA diet to the kelp diet had no significant effect on the cellulase activity levels compared to abalone kept on the kelp diet ($t = 0.39$, $p = 0.72$) or those kept on the BA diet ($t = -2.00$, $p = 0.86$).

3.5 DISCUSSION

This chapter investigated the effect of dietary switches on the growth, feed utilization and digestive enzyme activity levels between abalone fed basal and kelp-supplemented (BK) diets and between abalone fed formulated (alginate) and natural diets (fresh kelp *Ecklonia maxima*). Switching abalone from the BK to the base diet had no significant effect on the average weight gain, condition factor (CF), linear shell growth rates (LGR) and specific growth rates (SGR) of abalone but significantly decreased feed conversion ratio (FCR) and increased percentage mass gain (MG) and percentage daily feeding rates (PFR). Switching abalone from the base to the SW diet significantly increased the average weight gain but had no significant effect on the CF, LGR, SGR and contrary to the previous switch, significantly increased PFR while having no effect on the FCR and MG. Formulated feeds supplemented with macroalgae improve

health and feed utilization in Australian abalone (Bansemer *et al*. 2014). Although abalone switched from the base diet to the BK diet showed improved PFR as expected, no significant improvements in the CF, FCR and MG were observed. These observations contradict results of previous studies on kelp-supplementation in *H. midae*. Nel *et al*. (2017a) found significantly faster feeding response and improved FCR in abalone fed kelp-supplemented diets over basal diets. Chapter 2 of this thesis found no significant differences in the growth of abalone fed the base versus BK diets. Since Nel *et al*. (2017a) used sub-adult abalone, however, and juveniles were used in the current study, it is clear that the developmental stage of animals must be accounted for in addition to other factors such as environmental variability and protein concentration. Whereas a 26% protein diet supplemented with kelp has been found to improve abalone growth (Nel *et al*. 2017a), a 34% protein diet used in this study failed to produce similar results in an on-farm trial (Rowan Yearsley, Marifeed, personal communication).

Digestive enzymes play a crucial role in the efficiency of feed utilization by abalone (Serviere-Zaragoza *et al*. 1997; Picos-Garcia *et al*. 2000) and a dietary change will affect the activity of these enzymes (Howard and Yudkin 1963; Onitsuka *et al*. 2015). Switching abalone from the base to the BK diet or from the BK to the base diet had no significant effect on the alginate lyase specific activity levels, whereas the former switch resulted in significantly lower chymotrypsin activity levels. Seaweeds hydrolyzed in the abalone gut is aided by exogenous bacterial enzymes and kelp have been reported to modulate gut microbiome in the abalone gut (Erasmus *et al*. 1997; Tanaka *et al*. 2003; Daume 2006; Michel *et al*. 2006; Nel *et al*. 2017b). In *H. discus hannai*, the gut microflora of juveniles fed diatoms was similar to microflora collected from seawater (Tanaka *et al*. 2003). However, the gut microbiome changed coincident with a dietary change from diatoms to algal pellets, with the dominant microflora being alginolytic in nature.

Although Tanaka *et al*. (2003) did not study digestive enzymes, the presence of alginolytic bacteria would be expected to affect alginate lyase activity. However, a dietary switch from a base to a kelp-supplemented diet did not improve alginate lyase activity in the current study. In fact, the presence of kelp reduced chymotrypsin activity levels. The presence of kelp likely increases bacterial enzymes such as laminarinases and alginases, interfering with the activity of other enzymes such as chymotrypsin. Nel *et al*. (2017b) found no significant effects of kelp-supplementation on protease activity in sub-adult *H. midae*. In the black tiger prawn, *Penaeus monodon*, digestive enzymes changed with age, with trypsin and chymotrypsin activity levels

increasing with increase in age. Abalone switched from the base diet to the BK diet showed significantly lower chymotrypsin activity levels compared to abalone kept on the base diet. This study hypothesizes that although both diets had the same amount of protein, this switch may have resulted in kelp-utilizing bacteria replacing protein-digesting bacteria in the abalone gut. This has been suggested as a possibility in studies of abalone probiotics (Chi *et al.* 2014; Faturrahman *et al*. 2015; Ghosh *et al*. 2003; Jiang *et al*. 2013; Maliza 2014). Although the digestive physiology of *P. monodon* and *H. midae* vary considerably, this study suggests that digestive enzymes be investigated in older abalone fed kelp-supplemented diets.

The effect of changing diets is probably more significant when it involves protein-rich and protein-poor diets. Of all the growth indicators studied in the current study, only the PFR was significantly affected by a dietary switch between alginate and fresh kelp diets. The reason for the markedly high or low daily feeding rates in abalone switched from the alginate to the kelp diet or from the kelp to the alginate diet can be linked to the difference in protein concentration between the two diets. The alginate diet contains a higher protein concentration than the kelp diet. Thus, the latter dietary switch provides sufficient protein for growth, whereas the former reduces protein availability, requiring increased feed consumption to maintain metabolism. Previously, abalone or fish fed alginate-supplemented formulated diets displayed improved health or growth (Yeh *et al*. 2008; Harikrishnan *et al*. 2011; Cheng and Yu 2013). However, the condition factors of abalone switched from the alginate to the kelp diet or from the kelp to the alginate diet were not significant in this study. This was despite the alginate diet having a lower concentration of alginate compared to the fresh kelp diet.

Significant changes in digestive enzyme activity levels were also observed as abalone switched from kelp to alginate displayed significantly higher alginate lyase specific activity levels. Abalone switched from the kelp diet to the alginate diet produced significantly higher alginate lyase specific activity levels, whereas abalone switched from the alginate diet to the kelp diet showed no significant differences in alginate lyase compared to abalone kept on the initial diets. This result is thought to be an artefact of the administration of alginate in the two diets. The direct administration of alginate in the alginate-supplemented diet likely boosted the alginate lyase activity levels when abalone were switched to this diet.

In chapter 2, however, it was reported that no significant differences in the alginate lyase activity levels between abalone fed the alginate diet compared to those fed the kelp diet. Thus, it would appear that abalone gut microbiome require more time to adapt to kelp than they need

to adjust to alginate following this dietary switch. Erasmus *et al*. (1997) found that in *H. midae*, alginate lyase activity changed in response to a dietary change, being higher in abalone fed kelp compared to abalone fed *Gracilaria verrucosa*. Since the cell wall of *E. maxima* is mainly composed of alginate, abalone fed kelp mainly utilize this substrate for growth. Thus, the direct application of alginate in the formulated diet provided a compensatory mechanism between the ingested alginate and alginate lyase, resulting in matching enzyme profiles between alginate and fresh kelp in the present study. Indeed, abalone fed fresh kelp would compensate against the alginate diet by increasing digestive enzyme activity levels, resulting in higher efficiency with which kelp is utilized and matching growth rates, as observed in this chapter. This has been observed for *Calanus helgolandicus* (Harris *et al*. 1986). Corring (1980) found that when the nutritional requirements, relating to protein are met, adaptation is not useful to an animal. However, when nutritional requirements are not met, the adaptation of enzymes may be advantageous to an animal. Indeed, this would be the reason fresh kelp produced similar growth and enzyme activity levels to the formulated feeds in this study.

The effect of changing diets in this study can be regarded as having little consequences because significant effects on feeding rates and digestive enzyme activity levels did not significantly affect abalone growth. However, these results indicate that changes in dietary composition may significantly affect the type and activity of digestive enzymes, probably due to changes in the composition of gut microbiota (Erasmus *et al.* 1997; Serviere-Zaragoza *et al*. 1997; Tanaka *et al*. 2003; Bansemer *et al*. 2016). The degree of this effect likely depends on the nutritional profiles of the feeds involved and the developmental stage of the test animals.

The lack of observable changes in abalone growth following dietary switches may be also indicative of the ability of abalone to adapt to a wide range of changes in diet (Schaefer *et al*. 2003), particularly in the absence of anti-nutritional factors that are known to trigger negative effects on growths in cultured animals (Kemp 2001; Krogdahl *et al*. 2003). As Corring (1980) notes, adaptation may not be as useful to animals whose nutritional requirements (mainly protein) are met as it is to animals experiencing a nutritional deficiency. The author further notes that substrate decrease may equate to a dietary deficiency, resulting in enzyme adaptation being more advantageous than a nutritional adaptation. Thus, although the alginate diet was expected to produce better growth in abalone previously fed the fresh kelp diet, this was not the case as kelp-fed abalone likely developed a mechanism to "counter" this effect.

As kelp generally differs in nutritional profile compared to formulated feeds, increased enzyme production as an adaptive mechanism is likely advantageous to abalone fed this diet, allowing them to compete favorably with abalone fed formulated feeds. Switching diets from the alginate diet to the kelp diet had no significant effect on abalone growth, alginate lyase, and chymotrypsin and cellulase activity levels but resulted in significantly higher feeding utilization in the current study. Based on Corring's (1980) note on adaptation, this study hypothesizes that increased enzyme production by abalone fed kelp to match enzyme production by abalone and/or their microbiome fed formulated diets is an adaptive strategy which allows them to sufficiently utilize this poor nutritional feed. Thus, increased enzyme production enables better feed utilization and abalone fed kelp are able to attain similar growth to abalone fed formulated feeds.

3.6 CONCLUSION

Since there were no significant differences in the specific growth rates, percentage mass gain and feed conversion ratios between supplemented and non-supplemented feeds and between formulated and natural feeds following dietary switches, this study suggests that *Haliotis midae* is able to adapt to different feed sources without negative effects on growth. One way that these abalone are able to do this may be through adjustments in feeding activity as dietary switches appeared to significantly affect daily feeding rates.

As switching diets between formulated feeds had no significant effect on the alginate lyase specific activity levels, this study suggests that abalone were able to use their own endogenous enzymes to utilize the alginate added as a prebiotic in one of the feeds and that the alginate level used in the present study was insufficient to have any effect on alginate lyase through exogenous enzymes. However, the dietary switch from the base diet to the BK diet revealed that chymotrypsin activity levels dropped significantly, indicating the potential shift in the role of gut bacteria associated with kelp hydrolysis through exogenous enzymes. This needs to be confirmed by studies of more digestive enzymes associated with kelp digestion.

Significant effects of these dietary switches on cellulase activity levels also revealed the importance of this enzyme in the hydrolysis of these feeds. As switching abalone from the BK diet to the base diet resulted in significantly higher cellulase activity while the opposite switch resulted in significantly lower cellulase activity, this study hypothesizes that cellulase activity in kelp-supplemented feeds likely plays a minor role as digestion becomes dominated by exogenous enzymes associated with kelp-utilizing gut microbiome.

The kelp-to-alginate dietary switch resulted in significantly higher alginate lyase specific activity levels while the alginate-to-kelp dietary switch had no significant effect on the alginate lyase specific activity levels. However, none of these two dietary switches had a significant effect on chymotrypsin and cellulase activity levels. Thus, the effect on alginate lyase associated with the kelp-to-alginate dietary switch appears to be an artefact of the mode of application of the prebiotic alginate and not necessarily the dietary shift itself.

In conclusion, switching diets had no significant effect on abalone growths and this study suggests that *Haliotis midae* are able to maintain growth following dietary switches by using digestive enzymes associated with the hydrolysis of the new feed as a buffer against negative changes in growth. As such, there were no significant effects of switching between base and kelp-supplemented diets and between alginate-supplemented and fresh kelp diets.

CHAPTER 4

CONCLUDING DISCUSSION

This research has demonstrated that the inclusion of alginate and minced kelp in a formulated feed had no significant effect on the growth of juvenile *Haliotis midae*. Alginate-supplementation resulted in significantly higher alginate lyase specific activity levels compared to the non-supplemented base diet, but had no significant effect compared to the kelp-supplemented and fresh kelp diets. Furthermore, no significant differences in chymotrypsin and cellulase activity levels were observed between these diets. Thus, it appears that the high alginate lyase specific activity level associated with alginate-supplementation is a result of the "direct" mode of application of this component in a formulated feed. Alginate applied in this manner is hypothesized to present a higher substrate surface area for enzyme hydrolysis, whereas the cell wall of kelp, composed of cellulose, must be broken down prior to the utilization of the alginate.

The literature review in Chapter 2 summarized the potential benefits of including alginate and/or kelp (*Ecklonia maxima*) as components of formulated abalone feeds. This review suggested that both alginate and kelp may improve the growth rates and enzyme activity levels in cultured *Haliotis midae* compared to non-supplemented feeds (O'Mahoney *et al*. 2014; Kemp *et al*. 2015; Kemp 2017; Viera *et al.* 2015; Nel *et al*. 2017a). It also suggested that formulated feeds may result in changes on the intestinal structure of cultured abalone (Kemp 2001; Johnston *et al*. 2005; Schaefer *et al*. 2013) and that switching diets would result in changes on the growth, feed utilization and digestive enzyme activity levels (Kemp 2001; Tanaka *et al*. 2003; Onitsuka *et al*. 2015). However, the effects of diets on the intestinal structure or dietary switches on the growth and digestive enzymes in *H. midae* had never been investigated prior to this study.

Therefore, this study investigated a series of nutritional aspects in cultured *Haliotis midae*, aimed at finding evidence that: 1) supplementing a formulated diet with either alginate or minced kelp (BK diet) would improve the growth and feed utilization against a basal (control) and fresh kelp diets; 2) supplementing a formulated diet with either alginate or minced kelp would improve digestive enzyme activity levels compared to a basal and fresh kelp diets; 3) different diets have different effects on the intestinal villi structure in abalone; and 4) switching diets would result in changes in the growth, feed utilization and digestive enzyme activity levels.

The on-farm trial established that both alginate and kelp inclusion in a formulated base diet had no significant effect on the specific growth rates (SGR), feed conversion ratios (FCR), percentage mass gain (MG), linear shell growth rates (LGR), daily feeding rates (PFR) and condition factor (CF) compared to the base and fresh kelp diets. This result differed from those reported for sub-adult *H. midae* fed kelp-supplemented or formulated plus fresh kelp combination diets (Kemp 2017; Nel *et al*. 2017a) and other abalone species (Kemp *et al*. 2015; Bansemer *et al*. 2016). These studies had reported improved abalone growth when formulated feeds were supplemented with macroalgae. However, the BK diet produced significantly lower FCR and PFR compared to the base diet in the current study. Previous literature on fish and abalone nutrition suggests that kelp and alginate may serve as prebiotics when fed to cultured abalone, improving abalone health and growth (O'Sullivan *et al*. 2010; Cheng and Yu 2013; Van Doan *et al*. 2016a). However, this study did not find evidence of this at the inclusion levels used as both the BK and alginate diets produced similar CFs to the base and fresh kelp diets. It is likely that these levels (0.20% of dry mass) were insufficient to induce such effects as the results of the previous studies were based on higher inclusion levels (Kemp 2017; Cheng and Yu 2013). It is worth noting that a kelp-supplemented 26% protein diet produced significantly higher growth rates when Nel (2016) used 0.44% inclusion level in sub-adult *H. midae*. Thus, it is also possible that other factors, such as time of sampling (seasonality), protein concentration and even age, affect the growth of abalone.

The literature review also suggested that due to bacterial colonization, alginate and/or kelp inclusion would up-regulate digestive enzyme activity levels and promote higher alginate lyase, cellulase and chymotrypsin activities compared to the base and fresh kelp diets (Harris 1993; El-Shanshoury *et al*. 1994; Erasmus 1996; Erasmus *et al*. 1997; Hernandez-Santoyo *et al.* 1998; Monje and Viana 1998; Macey and Coyne 2005; Sawabe 2006). However, the results for digestive enzyme activity levels are not consistent with this as the alginate and BK diets produced similar chymotrypsin and cellulase activity levels compared to the base and kelp diets. The alginate diet, however, produced significantly higher alginate lyase specific activity levels compared to the base diet. Although the sampling period followed sufficient time for abalone to enzymatically adjust to their respective diets following the start of the trials, it is possible that the similar macronutrient compositions of these feeds played a role in keeping enzyme activity levels similar between diets. Adaptation is more important in animals fed nutrient-deficient feeds (Corring 1980). Thus, kelp-fed abalone likely adapt to this diet by elevating enzyme production, resulting in digestive enzyme activity levels in abalone fed this

diet being similar to those of abalone fed nutrient-rich formulated feeds (Corring 1980). However, conditions that favor this are unknown as fresh kelp produced similar cellulase and chymotrypsin activity levels to the formulated feeds.

The microscopic study of the intestinal villi structure indicated that although formulated feeds showed no significant effect on the intestinal villi height, width and surface area, there was greater variability in the intestinal villi surface area when abalone were fed fresh kelp. Previously, *H. midae* fed combination diets of formulated and fresh kelp diets displayed increased tubules of the digestive diverticulum compared to fresh kelp diets (Kemp 2017, personal communication). Nel *et al*. (2016) also found no significant effect of kelp-supplementation on the crop villi structure in *H. midae* (Nel 2016). Due to the dearth of literature on the effects of diets on the gastrointestinal structure of this species, the cause of the observations on the intestinal villi structure when abalone were fed fresh kelp in the current study are unknown. However, this study hypothesizes that due to abalones' phenotypic plasticity in gut morphology (Schaefer *et al*. 2013), the high variability observed in the intestinal structure of kelp-fed abalone might be a response to protein deficiency of kelp. The intestinal villi in kelp-fed abalone is likely to be a mechanism that enables these animals to absorb as much nutrients as possible from kelp. Similar to digestive enzymes, this adaptation would be particularly relevant to kelp-fed abalone since this diet contains a considerably lower protein concentration compared to formulated feeds (Corring 1980).

The effects of diets on digestive enzyme activities were more variable than they were on growth following dietary switches. Switching abalone between the BK and base diets did not affect the SGR, LGR and CF but resulted in significantly lower FCR and significantly higher PFR and MG. On the contrary, abalone switched from the base to the BK diet ad no significant effect on all the growth indices with the exception of the significantly high PFR. Clearly kelp-supplementation had no benefit for abalone growth following this dietary change. Although it is well known that the presence of kelp in the abalone gut modulates gut bacteria (Erasmus *et al*. 1997; Sawabe *et al*. 1997; Tanaka *et al*. 2003; Iehata *et al*. 2009; Jiang *et al*. 2013; Nel *et al*. 2018), the period required for bacterial colonization of the gut following a dietary switch is unknown. The gut microbiome of post-settlement abalone fed fresh kelp displayed a relatively low diversity compared to abalone fed 34% protein basal or kelp-supplemented diets (Nel *et al*. 2018). Consequently, it could be deduced that kelp-utilizing bacteria in the abalone gut appear to be specialists rather than generalists and such bacteria would require extended

periods of adaptation following a dietary change. Thus, studies of dietary changes must be coupled with studies of changes in gut microbiome populations.

Switching abalone between the alginate and fresh kelp diets produced similar results, with no significant effects on the SGR, FCR, LGR, CF and MG. However, the kelp-to-alginate dietary switch resulted in low feed utilization and the alginate-to-kelp dietary switch resulted in significantly high feed utilization. This is probably caused by the difference in protein concentration between these feeds. The former switch resulted in significantly high alginate lyase activity levels but had no effect on the chymotrypsin and cellulase activity levels. On the contrary, the latter switch did not affect the alginate lyase, chymotrypsin or cellulase activity levels. Thus, it is likely that the high alginate lyase activity levels observed in the former alginate-to-kelp switch are merely an artefact of the mode of application of alginate. Direct application of alginate likely increases the surface area for increased digestion as opposed to kelp that must be broken down prior to the utilization of the alginate.

This research has shown that kelp and alginate cannot be used efficiently for growth as dietary supplements at the levels used in the current study. However, the high alginate lyase activity levels observed in abalone fed the alginate-supplemented diet compared to the base diet provides a scope for further investigations with higher inclusion levels of either alginate or kelp. It is hypothesized, based on the results of previous studies, that the "correct" inclusion levels would be beneficial for abalone gut health. *Ecklonia maxima* contains a host of marine biochemical such as phlorotannins (eckol, phloroglucinol, dieckol and eckmaxol) phenolic compounds, fucoidan and laminarin whose health benefits include antibacterial, antitumor, anticancer and anti-inflammatory properties (Lahaye 1991; Mabeau and Fleurence 1993; O'Sullivan *et al*. 2010; Bansemer *et al*. 2014). Thus, higher inclusion levels, added particularly to lower protein feeds than the one used in this study, could be beneficial to abalone. This research has further demonstrated that although a dietary change may not necessarily affect abalone growth, it may affect feed consumption, the efficiency with which feed is converted into body tissue and digestive enzyme activity levels, all of which are critical to abalone growth. Since this is a common practice in abalone aquaculture, growth trials must be coupled with studies of digestive enzymes and gut microbiome to understand the full scope of the dietary change.

REFERENCES

Aremu O, Masondo NA, Rengasamy KRR, Amoo SO, Gruz J, Bíba O, Šubrtová M, Pěnčík A, Novák O, Doležal K and Van Staden J. 2015. Physiological role of phenolic biostimulants isolated from brown seaweed *Ecklonia maxima* on plant growth and development. *Planta*, 241: 1313 - 1324.

Bansemer MS, Qin JG, Harris JO, Howarth GS and Stone DAJ. 2014. Nutritional requirements and use of macroalgae as ingredients in abalone feed. *Reviews in Aquaculture*, 5: 1 - 15.

Bansemer MS, Qin JG, Harris JO, Duong DN, Hoang TH, Howarth GS and Stone DAJ. 2016. Growth and feed utilisation of greenlip abalone (*Haliotis laevigata*) fed nutrient-enriched macroalgae. *Aquaculture*, 452: 62 - 68.

Bedford MR. 2002. The role of carbohydrases in feedstuff digestion. In: Poultry feedstuffs supply, composition and nutritive value. *CABI Publishing*, 319 - 331.

Bevelander G. 1988. Abalone: gross and Fine Structure. *Boxwood Press*, 24 - 39.

Barkai R and Griffiths CL. 1986. Diet of the South African abalone *Haliotis midae*. *South African Journal of Marine Science*, 4: 37 - 44.

Britz PJ, Hecht T, Knauer J and Dixon MG. 1994. The development of an artificial feed for abalone farming. *South African Journal of Science*, 90: 7 - 8.

Britz PJ. 1995. The nutritional requirements of *Haliotis midae* and development of a practical diet for abalone aquaculture. PhD Thesis, Rhodes University, South Africa.

Britz PJ. 1996a. The suitability of selected protein sources for inclusion in formulated diets for the South African abalone, *Haliotis midae*. *Aquaculture*, 140: 63 - 73.

Britz PJ. 1996b. Effect of dietary protein level on growth performance of South African abalone, *Haliotis midae*, fed fishmeal-based semi-purified diets. *Aquaculture*, 140: 55 - 61.

Britz PJ and Hecht T. 1997. Effect of dietary protein and energy level on growth and body composition of South African abalone, *Haliotis midae*. *Aquaculture*, 156: 195 - 210.

Britz PJ, Hecht T and Mangold S. 1997. Effect of temperature on growth, feed consumption and nutritional indices of *Haliotis midae* fed a formulated diet. *Aquaculture*, 152: 191 - 203.

Campbell J. 1965. The structure and function of the alimentary canal of the blacklip abalone, *Haliotis cracherodii* Leach. *Transactions of the American Microscopic Society*, 84:376 - 395.

Cheng W and Yu J. 2013. Effects of the dietary administration of sodium alginate on the immune responses and disease resistance of Taiwan abalone, *Haliotis diversicolor supertexta*. *Fish and Shellfish Immunology*, 34: 902 - 908.

Chi C, Liu JY, Fei SZ, Zhang C, Chang YQ and Liu XL. 2014. Effect of intestinal autochthonous probiotics isolated from the gut of sea cucumber (*Apostichopus japonicus*) on immune response and growth of *A. japonicus*. *Fish and Shellfish Immunology*, 38: 367 - 373.

Cho DM, Pyeun JH, Byun DS. 1983. Proteolytic activity of the crude enzyme extracted from the digestive tract of marine gastropods. *Korean Journal of Fisheries and Aquatic Society*, 16: 216 - 224.

Chojnaka KW, Wiykowska Z, Saeid A and Tuhy L. 2012. Biologically active compounds in seaweed extracts - the prospects for the application. *The Open Conference Proceedings Journal*, 3: 20 - 28.

Cook PA. 2016. Trends in worldwide abalone production. *Journal of Shellfish Research*, 35: 581 - 583.

Corring T. 1980. The adaptation of digestive enzymes to the diet: its physiological significance. *Reproductive Nutritional Development*, 20: 1217 - 1235.

Crofts DR. 1929. *Haliotis*. *Liverpool Marine Biology Community Memoirs*, 29: 1 - 174.

Dang VT, Li Y, Speck P and Benkendorff K. 2011. Effects of micro and macroalgal diet supplementations on growth and immunity of greenlip abalone, *Haliotis laevigata*. *Aquaculture*, 320: 91 - 98.

Day RW and Cook P. 1995. Bias towards brown algae in determining diet and food preferences: The South African abalone *Haliotis midae*. *Marine and Freshwater Res*earch, 46: 623 - 627.

De Villiers CM. 2012. Application of exogenous enzymes in *Haliotis midae* diets with soybean meal as fish meal replacement. Masters Thesis, Stellenbosch University, South Africa.

Dixon MG. 1992. The effects of temperature and photoperiod on the digestive physiology of the South African abalone *Haliotis midae*. Masters Thesis, Rhodes University, South Africa.

Dlaza TS, Maneveldt GW and Viljoen C. 2008. Growth of post-weaning abalone *Haliotis midae* fed commercially available formulated feeds supplemented with fresh wild seaweed. *African Journal of Marine Science*, 30: 199 - 203.

Doubet RS and Quatrano RS. 1984. Properties of alginate lyases from marine bacteria. *Applied and Environmental Microbiology*, 47: 699 - 703.

Daume S. 2006. The role of bacteria and micro and macro algae in abalone aquaculture: a review. *Journal of Shellfish Research*, 25: 151 - 157.

Ebert E and Houk JJ. 1984. Elements and innovations in the cultivation of red abalone *Haliotis rufescens*. *Aquaculture*, 39: 375 - 392.

El-Shanshoury AR, Mona MH, Shoukr FA, and E1-Bossery AM. 1994. The enumeration and characterization of bacteria and fungi associated with marine wood-boring isopods, and the ability of these microorganisms to digest cellulose and wood. *Marine Biology*, 119: 321 - 326.

Erasmus JH. 1996. The role of enteric bacteria in the abalone *Haliotis midae*. Masters Thesis, University of Cape Town, South Africa.

Erasmus JH, Cook PA and Coyne VE. 1997. The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae. Aquaculture*, 155: 377 - 386.

Fertah M, Belfkira A, Montassir DE, Taourirte M and Brouillette F. 2017. Extraction and characterization of sodium alginate from Moroccan *Laminaria digitata* brown seaweed. *Arab Journal of Chemistry*, 10: 3707 - 3714.

Foale S and Day R. 1992. Recognizability of algae ingested by abalone. *Marine and Freshwater Research*, 43: 1331 - 1338.

Fleming AE, Van Bameveld RJ, Hone PW. 1996. The development of artificial diets for abalone: A review and future directions. *Aquaculture*, 140: 5 - 53.

Fleurence J. 1999. Seaweed proteins: biochemical, nutritional aspects and potential uses. *Trends in Food Science and Technology*, 10: 25 - 28.

Fleurence J, Morancais M, Dumay J, Decottignies P, Turpin V, Munier M, Garcia-Bueno N and Jaouen P. 2012. What are the prospects for using seaweed in human nutrition and for marine animals raised through aquaculture? *Trends in Food Science and Technology*, 27: 57 - 61.

Fujiki K and Yano T. 1997. Effects of sodium alginate on the non-specific defense system of the common carp (*Cyprinus carpio* L.). *Fish and Shellfish Immunology*, 7: 417 - 427.

Faturrahman, Rohyati IS and dan Sukiman. 2015. Improved growth rate of abalone *Haliotis asinine* fed pudding probiotic-enriched protein. *Procedia Environmental Science*, 23: 315 - 322.

Frances H and Yudkin J. 1963. Effect of dietary change upon amylase and trypsin activities of the rat pancreas. *British Journal of Nutrition*, 17: 281.

Gacesa P. 1988. Alginates. *Carbohydrate polymers*, 8: 161 - 182.

Garcıa-Carreno FL, del Toro M de los A and Serviere-Zaragoza E. 2003. Digestive enzymes in juvenile green abalone, *Haliotis fulgens*, fed natural food. *Comparative Biochemistry and Physiology*, 134:143 - 150.

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

Garland CD, Cook SL, Grant JF and McMeeken TA. 1985. Ingestion of the bacteria on the cuticle of crustose (non-articulated) coralline algae by postlarval and juvenile abalone (*Haliotis ruber* Leach) from Tasmania waters. *Journal of Experimental Marine Biology and Ecology*, 91: 137 - 149.

Genade AB, Hirst AL and Smit CJ. 1988. Observations on the spawning, development and rearing of the South African abalone *Haliotis midae* Linn. *South African Journal of Marine Science*, 6: 3 - 12.

Ghosh K, Kumar S and Ray AK. 2003. Supplementation of an isolated fish gut bacterium *Bacillus circulans*, in formulated diets for rohu, *Labelo rohita*, fingerlins. *The Israeli Journal of Aquaculture*, 55: 13 - 21.

Howard F and Yudkin J. 1963. Effect of dietary change upon the amylase and trypsin activities of the rat pancreas. *British Journal of Nutrition*, 17: 281.

Hahn KO. 1989b. Nutrition and growth of abalone. In: Hahn, K.O. (Ed.), Handbook of Culture of Abalone and Other Marine Gastropods. *CRC Press*, 135 - 156.

Harikrishnan H, Kim M, Kim J, Han J, Jang I, Balasundaram C and Heo M. 2011. Immunomodulatory effect of sodium alginate enriched diet in kelp grouper *Epinephelus brneus* against *Streptococcus iniae*. *Fish and Shellfish Immunology*, 30: 543 - 549.

Harris JM. 1993. The presence, nature, and role of gut microflora in aquatic invertebrates: a synthesis. *Microbial Ecology*, 25: 195 - 231.

Harris JO, Burke CM and Maguire GB. 1998. Characterization of the digestive tract of greenlip abalone, *Haliotis laevigata* Donovan: I. Morphology and histology. *Journal of Shellfish Res*earch, 17:979 - 988.

Harris RP, Samain JF, Moal J, Martin-Jézéquel V and Poulet SA. 1986. The effect of algal diet on digestive enzyme activity in *Calanus helgolandicus*. *Marine Biology*, 353 - 361.

Hernandez-Santoyo H, Hernandez-Arana A, Arreguın-Espinosa R and Rodrıguez-Romero A. 1998. Purification and characterization of several digestive proteases from the blue abalone, *Haliotis fulgens*. *Aquaculture*, 159: 203 - 216.

Iehata S, Inagaki T, Okunishi S, Nakano M, Tanaka R, Maeda H. 2010. Improved gut environment of abalone *Haliotis gigantea* through *Pediococcus sp*. Ab1 treatment. *Aquaculture*, 305: 59 - 65.

Ikegami S, Tsuchihashi F, Harada H, Tsuchihashi N, Nishide E and Innami S. 1990. Effect of viscous indigestible polysaccharides on pancreatic-biliary secretion and digestive organs in rats. *The Journal of Nutrition*, 120: 353 - 360.

Infante JLZ and Cahu C. 1994. Development and response to a diet change of some digestive enzymes in sea bass (*Dicentrarchus labrax*) larvae. *Fish Physiology and Biochemistry*, 12: 399 - 408.

Janarthanan M and Kumar MS. 2018. Extraction of alginate from brown seaweeds and evolution of bioactive alginate film coated textile fabrics for wound healing application. *Journal of Indian Textile History*, 0: 1 -24.

Jiang HF, Liu XL, Chang YQ, Liu MT and Wang GX. 2013. Effects of dietary supplementation of probiotic *Shewanella colwelliana* WA64, *Shewanella olleyana* WA65 on the innate immunity and disease resistance of abalone, *Haliotis discuss hannai Ino*. *Fish and Shellfish Immunology*, 36: 86 - 91.

Johnston D, Moltschaniwskyj N and 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: 341 - 355.

Jihalya NJ. 2014. Diet and feeding ecology of Nile tilapia, *Oreochromis niloticus* and Nile perch, *Lates niloticus* in protected and unprotected areas of Lake Victoria, Tanzania. *International Journal of Scientific and Technology Research*, 3: 280 - 286.

Karr-Lilienthal LK, Kadzere CT, Grieshop CM and Fahey JGC. 2005. Chemical and nutritional properties of soybean carbohydrates as related to non-ruminants: A review. *Livestock Production Science*, 97: 1 - 12.

Kawamura T, Roberts RD and Nicholson CM. 1999. Growth and survival of postlarval abalone (*Haliotis iris*) in relation to development and diatom diet. *Journal of Shellfish Research*, 18: 243 - 250.

Kawamura T, Takami H, Roberts RD and Yamashita Y. 2001. Radula development in abalone *Haliotis discus hannai* from larval to adult in relation to feeding transitions. *Fisheries Science*, 67: 596 - 605.

Kemp R. 2001. The effect of diet on intestinal structure and function in the abalone (*Haliotis laevegata*). PhD Thesis, Adelaide University, Australia.

Kemp JOG, Britz, PJ and Toledo Aguero PH. 2015. The effect of macroalgal, formulated and combination diets on growth, survival and feed utilization in the red abalone *Haliotis refescens*. *Aquaculture*, 448: 306 - 314.

Kemp JOG. 2017. Aspects of the nutritional physiology of the abalone species *Haliotis midae* (L.) and *H. rufescens* (Swainson). PhD Thesis, South Africa.

Keesing JF and Wells FE. 1989. Growth of the abalone *Haliotis roei* Gray. *Australian Journal of Marine and Freshwater Research*, 40: 199 - 204.

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

Krogdahl A, Bakke-McKellep AM and Baeverfjord G. 2003. Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). *Aquaculture Nutrition*, 9: 361 - 371.

Krohn R, Britz P, Brenner L and Chigumira G. 2016. Maximising niche markets: South African abalone. *TIPS*.

Łabowska MB, Michalak I and Detyna J. 2019. Methods of extraction, physicochemical properties of alginates and their applications in biomedical field - a review. *Open Chemistry*, 17: 738 - 762.

Lahaye M. 1991. Marine algae as sources of fibres: Determination of soluble and insoluble dietary fibre contents in some sea vegetables. *Journal of the Science of Food and Agriculture*, 54: 587 - 594.

Mabeau S and Fleurence J. 1993. Seaweed in food products: biochemical and nutritional aspects. *Trends in Food Science and Technology*, 4: 103 - 107.

Macey BM and Coyne VE. 2005. Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment. *Aquaculture*, 245: 249 - 261.

Maliza S. 2014. The use of probiotics in the diet of farmed South African abalone *Haliotis midae L*. Masters Thesis, Rhodes University, South Africa.

Mazumder A, Holdt SL, De Francisci D, Alvarado-Morales M, Mishra HN and Angelidaki I. 2016. Extraction of alginate from *Sargassum muticum*: process optimization and study of its functional activities. *Journal of Applied Phycology*, 28: 3625 - 3634.

Michalak I and Chojnacka K. 2015. Algae as production systems of bioactive compounds. *Engineering in Life Sciences*, 15: 160 - 176.

Michel G, Nyval-Collen P, Barbeyron T, Czjzek M and Helbert W. 2006. Bioconversion of red seaweed galactans: a focus bacterial agarases and carrageenases. *Applied Microbiology and Biotechnology*, 71: 23 - 33.

Monje H and Viana MT. 1998. The effect of cellulose on the growth and cellulolytic activity of abalone *Haliotis fulgens* when used as an ingredient in formulated artificial diets. *Journal of Shellfish Research*, 17: 667 - 671.

Naidoo K, Maneveldt G, Ruck K and Bolton JJ. 2006. A comparison of various seaweed-based diets and formulated feed on growth rate of abalone in a land-based aquaculture system. *Journal of Applied Phycology*, 18: 437 - 443.

Nakada HI and Sweeny PC. 1967. Alginic Acid Degradation by Eliminases from Abalone Hepatopancreas. *The Journal of Biological Chemistry*, 242: 845 - 851.

Nel A. 2016. The effect of kelp supplementation in formulated feed on the production performance and gut microbiota of South African abalone (*Haliotis midae*). PhD Thesis, Rhodes University, South Africa.

Nel A, Pletschke BI and Britz PJ. 2017a. The effect of low-level kelp supplementation on digestive enzyme activity levels in cultured abalone *Haliotis midae* fed formulated feeds. *African Journal of Marine Science*, 39: 175 - 182.

Nel A, Pletschke BI, Jones CLW, Kemp J, Robinson G and Britz PJ. 2017b. Effects of kelp *Ecklonia maxima* inclusion in formulated feed on the growth, feed utilisation and gut microbiota of South African abalone *Haliotis midae*. *African Journal of Marine Science*, 39: 183 - 192.

O'Doherty JV, Dillon S, Figat S and Callan JJ. 2010. The effects of lactose inclusion and seaweed extract derived from *Laminaria spp*. on performance, digestibility of diet components and microbial populations in newly weaned pigs. *Animal Feed Science Technology*, 157: 173 - 180.

Ogino C and Ohta E. 1963. Studies on the nutrition of abalone. I. Feeding trials of abalone, Haliotis discus with artificial diets. *Bulletin of the Japanese Society of Scientific Fisheries*, 29: 691 - 694.

Ogino C and Kato N. 1964. Studies on the nutrition of abalone. Il. Protein requirements for growth of abalone, *Haliotis discus*. *Bulletin of the Japanese Society of Scientific Fisheries*, 30: 523 - 526.

Olin P. 1994. Abalone Culture in Hawaii: *Haliotis fulgens* and *Haliotis diversicolor supertexta*. *Center for Tropical and Subtropical Aquaculture*.

Onitsuka T, Niwa K, Unuma T and Umezu Y. 2015. Dietary shifts in the juvenile sea urchin *Strongylocentrotus intermedius* associated with the development of digestive enzymes. *Marine Biology*, 162: 869 - 880.

O'Sullivan L, Murphy B, McLoughlin P, Duggan P, Lawlor PG, Hughes H, and Gardiner GE. 2010. Prebiotics from marine macroalgae for human and animal health applications. *Marine Drugs*, 8: 2038 - 2064.

Picos-Garcıa C, Garcıa-Carreno FL and Serviere-Zaragoza E. 2000. Digestive proteases in juvenile Mexican green abalone, *Haliotis fulgens*. *Aquaculture*, 181: 157 - 170.

Ramirez C, Millon C, Nunez H, Pinto M, Valencia P, Acevedo C and Simpson R. 2015. *Food and Hydrocolloids*, 44: 328 - 332.

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

Sang VT, Hung ND and Se-kwon K. 2019. Pharmaceutical properties of marine polyphenols: an overview. *Acta Pharmaceutica Sciencia*, 57.

Sawabe T, Oda Y, Shiomi Y and Ezura Y. 1995. Alginate degradation by bacteria isolated from the gut of sea urchins and abalones. *Microbial Ecology*, 30: 193 - 202.

Sawabe T, Onitsuka M and Ezura Y. 1997. Novel alginate lyases from marine bacterium *Alteromonas sp*. strain H-4. *Carbohydrate Research*, 304: 69 - 76.

Sawabe T, Setoguchi N, Inoue S, Tanaka R, Ootsubo M, Yoshimizu M and Ezura Y. 2003. Acetic acid production of *Vibrio halioticoli* from alginate: a possible role for establishment of abalone-*V. halioticoli* association. *Aquaculture*, 219: 671 - 679.

Sawabe T. 2006. The mutual partnership between *Vibrio halioticoli* and abalones. *The Biology of Vibrios*.

Schaefer EN, Harris JO, Howarth GS, Bansemer MS and Stone DAJ. 2013. Comparative histological changes in the greenlip abalone *Haliotis laevigata* gastrointestinal tract in response to water temperature, different dietary protein levels, and animal age. *Journal of Shellfish Research*, 32: 131 - 141.

Serviere-Zaragoza E, Navarrete del Toro MA and García-Carreño FL. 1997. Protein-hydrolyzing enzymes in the digestive systems of the adult Mexican blue abalone (Gastropoda). *Aquaculture*, 157: 325 - 336.

Shepherd SA. 1973. Studies on southern Australian abalone (Genus *Haliotis).* I. Ecology of five sympatric species. *Australian Journal of Marine and Freshwater Research*, 24: 217 - 257.

Shepherd SA and Laws HM. 1974. Studies on Southern Australian Abalone (Genus Haliotis) II. Reproduction of Five Species. *Australian Journal of Marine and Freshwater Research*, 25: 49 - 62.

Shepherd SA and Hears WS. 1983. Studies on Southern Australian Abalone (Genus Haliotis). IV Growth of *H. laevigata* and *H. ruber*. *Australian Journal of Marine and Freshwater Research*, 34: 461 - 75.

Storebakken T and Austreng E. 1987. Binders in fish feeds II. Effect of different alginates on the digestibility of macronutrients in rainbow trout. *Aquaculture*, 60: 121 - 131.

Stott AE, Takeuchi T, Koike Y and Imada O. 2003. The effect of three different application methods for an artificial micro particle diet on the survival and growth rate of post-larval abalone *Haliotis discus* (Reeve). *Seafood*, 51: 197 - 203.

Suzuki T, Nakai K, Yoshie Y, Shirai T and Hirano T. 1993. Effect of sodium alginates rich in guluronic and manuronic acids on cholesterol levels and digestive organs of high-cholesterol-fed rats. *Nippon Suisan Gakkaishi*, 59: 545 - 551.

Takami H, Kawamura T and Yamashita Y. 1998. Development of polysaccharide degradation activity in post-larval abalone *Haliotis discus hannai*. *Journal of Shellfish Research*, 17:723 - 727.

Tanaka R, Sugimura I, Sawabe T, Yoshimizu M, Ezura Y. 2003. Gut microflora of abalone *Haliotis discus hannai* in culture changes coincident with a change in diet. *Fisheries Science*, 69: 951 - 958.

ten Doeschate KI and Coyne VE. 2008. Improved growth rate in farmed *Haliotis midae* through probiotic treatment. *Aquaculture*, 284: 174 - 179.

Troell M, Robertson-Anderson D, Anderson RJ, Bolton JJ, Maneveldt G, Halling C and Probyn T. 2006. 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*, 257: 266 - 281.

Tsujino I and Saito T. 1962. Studies on Alginase Part II, a new unsaturated uronide isolated from alginase hydrolysate. *Agricultural and Biological Chemistry*, 26: 115 - 118.

Uki N, Kemuyama A and Watanabe T. 1985a. Nutritional evaluation of several protein sources in diets for abalone *Haliotis discus hannai*. *Bulletin of the Japanese Society of Scientific Fisheries*, 51: 1835 - 1839.

Uki N, Kemuyama A and Watanabe T. 1985b. Development of semi-purified test diets for abalone. *Bulletin of the Japanese Society of Scientific Fisheries*, 51: 1825 - 1833.

Uki N, Kemuyama A and Watanabe T. 1986a. Optimum protein levels in diets for abalone. *Bulletin of the Japanese Society of Scientific Fisheries*, 52: 1005 - 1012.

Uki N, Sugiura M and Watanabe T. 1986b. Requirements of essential fatty acids in the abalone *Haliotis discus hannai*. *Bulletin of the Japanese Society of Scientific Fisheries*, 52: 1013 - 1023.

Uki N and Watanabe T. 1992. Review of the nutritional requirements of abalone (*Haliotis spp*.) and development of more efficient artificial diets. In: Shepherd SA, Tegner MJ and Guzmart del Proo SA (Editors). Abalone of the World. Biology, Fisheries and Culture. *Fishing News Books*, 504 - 517.

Van Doan H, Tapingkae W, Moonmanee T and Seepai A. 2016a. Effects of low molecular weight sodium alginate on growth performance, immunity and disease resistance of tilapia, *Oreochromis niloticus*. *Fish and Shellfish Immunology*, 186 - 194.

Van Doan H, Hoseinifar SH, Tapingkae W, Tongsiri S and Khamtavee P. 2016b. Combined administration of low molecular weight sodium alginate boosted immunomodulatory, disease resistance and growth enhancing effects of *Lactobacillus plantarum* in Nile tilapia (*Oreochromis niloticus*). *Fish and Shellfish Immunology*, 58: 678 - 685.

Viana MT, Lopez LM and Salas A. 1993. Diet development for juvenile abalone *Haliotis fulgens*: evaluation of two artificial diets and macroalgae. *Aquaculture*, 117: 149 - 156.

Viera MP, Courtois de Viçose G, Robaina L, Izquierdo MS. 2015. First development of various vegetable-based diets and their suitability for abalone *Haliotis tuberculata coccinea* Reeve. *Aquaculture*, 448: 350 - 358.

Vitalis TZ, Spence MJ and Carefoot TH. 1988. The possible role of gut bacteria in nutrition and growth of the sea hare *Aplysia juliana*. *The Veliger*, 30: 333 - 341.

Von Holdt MM, Ligthelm SP and Nunn JR. 1955. South African seaweeds: seasonal variations in the chemical composition of some phaeophyceae. *Journal of the Science of Food and Agriculture*, 6: 193 - 197.

Wee KL, Maguire GB and Hindrum SM. 1992. Methodology for digestibility studies with abalone. Comparisons of markers in an artificial diet for blacklip abalone *(Haliotis rubra)* and greenlip abalone *(H. laevigata). Third Asian Fisheries Forum*, 152 - 155.

Wong TY, Preston LA and Schiller NL. 2000. Alginate lyase: review of major sources and enzyme characteristics, structure-function analysis, biological roles and applications. *Annual Review of Microbiology*, 54: 289 - 340.

Wood AD and Buxton CD. 1996a. Aspects of the biology of the abalone *Haliotis midae* (Linne, 1758) on the east coast of South Africa. 1. Feeding biology. *South African Journal of Marine Science*, 17: 61 - 68.

Wood AD and Buxton CD. 1996b. Aspects of the biology of the abalone *Haliotis midae* (Linne, 1758) on the east coast of South Africa. 2. Reproduction. *South African Journal of Marine Science*, 17: 61 - 68.

Yeh SP, Chang CA Chang CY, Liu CH and Cheng W. 2008. Dietary sodium alginate administration affects fingerling growth and resistance to *Streptococcus* sp. and *iridovirus*, and juvenile non-specific immune responses of the orange-spotted grouper, *Epinephelus coioides*. *Fish and Shellfish Immunology*, 25: 19 - 27.