Ulva as a functional feed: A practical investigation into the effects of *Ulva lacinulata* on the growth, consumption, health and gut microbiota of the farmed abalone *Haliotis midae*

Thesis presented for the degree of Doctor of Philosophy in the Department of Biological Sciences

By Morgan John Brand

University of Cape Town

30 January 2023

I hereby:

(a) grant the University free license to reproduce the above thesis in whole or in part, for the purpose of research;

(b) declare that:

(i) the above thesis is my own unaided work, both in conception and execution, and that apart from the normal guidance, I have received no assistance apart from that stated below;

(ii) except as stated below, neither the substance or any part of the thesis has been submitted in the past, or is being, or is to be submitted for a degree at this University or any other University.

(iii) I am now presenting the thesis for examination for the Degree of PhD.

Signed: Signed by candidate

Dedication

Dedicated to John Williams for his unwavering passion and commitment to friendship, this thesis is a testament to the influence of being my father's son. Eureka!

And to, the animals and microbes in my life who have allowed me to perceive the world in a more connected way.

Acknowledgments

Man who is his own master is a fool!

Gratitude runs deep as I reflect on the journey that led me here. Katie, Sam, Mom, Dad, and my sisters, your unwavering support propelled me to the finish line of this project. Thank you Simon for being the silent orchestrator who helped me flip the switch to finished..

Life's collaborative tapestry is woven with countless threads, and I'm indebted to those who made this work possible. In particular, my time at Rhodes University introduced me to Martin Davis, Brett Pletshke, Peter Britz, and Cliff Jones, deserving of my heartfelt thanks.

Upon my move to Cape Town, a new chapter unfolded in my academic journey. John Bolton and Brett Macey were the pillars of wisdom and patience that guided my steps. Pavs Pillay, Sharon Bosman, Emlyn Balarin, Anusuya Chinsamy-Turan, Gilly Smith and the entire crew from the Department of Biological Sciences, your support sustained me.

Special mention to Abagold (Pty) Ltd. and Specialized Aquatic Feeds—your facilities and Chris Parker's expertise in extruding quality pellets were indispensable.

A debt of gratitude extends to Michael Joubert for sharing my dream in this endeavour. To friends, both online and in person—Alick Hendricks, Mark Cyrus, Jonathan Dean, Ross English, William Krummack, Gareth Nicholson, and Nick Riddin—your friendship made the solitary moments brighter.

AJ Smit, Henning Winker, and Greg Duckworth, your statistical expertise added a layer of depth and colour to my journey.

Milo, your silent companionship witnessed the highs and lows, and I am forever grateful for your existence.

A somber note accompanies my gratitude—an apology to the abalone whose lives were sacrificed for this research. The weight of that decision lingers, and I carry it with me to this day.

Abstract

The intensification of aquaculture production methods has created a dependency on commercially formulated feeds that contain high-quality protein and readily digestible carbohydrates. A high-quality protein, like that from fishmeal, has been considered an essential component in formulated feeds, where it has been used to promote growth while carbohydrates are considered the most economical energy source in animal diets. High levels of digestible carbohydrates in the diet of humans, fish, crustaceans, and gastropods have however been associated with decreased metabolic efficiencies while the continued use of fishmeal is limited due to the high environmental burden which that has resulted in a global search for alternative protein sources.

The majority of formulated feeds utilized by South African abalone farms contain a high protein content derived from fishmeal along with high levels of readily digestible maize starch as the primary carbohydrate. In the wild, abalone are herbivorous and consume a mixed diet of seaweeds, including *Ulva*, that contain a range of complex carbohydrates and a diverse community of microbiota. Since around 2000, there has been increased adoption of *Ulva* cultivation by the South African abalone aquaculture industry using IMTA systems. The *Ulva* that is grown in abalone effluent serves as a biofilter, which allows partial recirculation, and the co-produced *Ulva* is often used as a supplementary feed. Farms that use fresh *Ulva* as a supplement to formulated feed have reported benefits, such as a reduction in the volume of formulated feed required to achieve optimal growth of abalone and enhanced profitability of the system. The supplementation of *Ulva* into the feeding regime of other organisms has also demonstrated the capacity to improve glycaemic regulation and enhance the immune response of the cultivated organisms, while positively contributing to the overall function of the IMTA systems. Improving the aquaculture system efficiency and overall sustainability of aquaculture practices through the inclusion of feeds capable of providing benefits beyond major nutritional components can be a useful tool in the optimisation of feeding regimes used on abalone farms. However, there are no standard methods available for integration into production systems partially due to a lack of empirical data describing how *Ulva* enhances the growth and possibly the health of cultivated abalone when fed as a dietary supplement.

To gain a better understanding of the role of IMTA-grown *Ulva* as a feed ingredient for the abalone *Haliotis midae*, this study investigated how varying feeding regimes that included IMTA-grown *Ulva*, or specific components thereof, that could be used to reduce the farms reliance on formulated feeds, enhance feed consumption, improve growth rates and product quality, and positively influence the immune and metabolic state of the abalone, while also impacting the gut microbial ecology. The objectives of this study were addressed by: (1) offering IMTA-grown *Ulva* alongside dry formulated feeds to quantify the consumption, growth, condition and determine the extent to which *Ulva* can be used to replace formulated feeds for abalone under farm conditions, (2) determining whether dietary *Ulva* will have an effects on the abalone glycaemic regulation and immune response under simulated challenges, and (3) feeding abalone IMTA-grown *Ulva*, or specific components thereof (dried *Ulva*, ulvan or glucuronic acid) incorporated into a dry feed, offered under laboratory conditions and measuring the effects on growth, physiology and gastrointestinal microbial patterns.

This study demonstrated that the inclusion of fresh IMTA-grown *Ulva* into the feeding regime of abalone, when offered alongside Abfeed™ S34®, increased the total daily feed consumption by 90 % when compared to abalone fed single diets of both *Ulva* or Abfeed™ S34®. In a farm scale trial the partial replacement (up to 60 %) of the high fishmeal based formulated feed Abfeed™ S34® with fresh effluent-grown *Ulva* or wild collected kelp in the feeding program of *H. midae* did not compromise the growth (SGR and MISL) or condition of abalone. The results of this study also show that when abalone were fed a combination of Abfeed™ S34® and *Ulva* the muscle glycogen levels were not statistically different, which is considered a beneficial trait for high quality canned or live transported abalone products. This study is the first to show that *Ulva* can successfully reduce reliance on dry formulated feed, without negatively affecting the condition or growth of abalone under aquaculture conditions.

The assay used in this study for monitoring haemolymph glucose concentrations during an induced glycaemic response demonstrated a significantly higher basal haemolymph glucose concentration and a prolonged period of hyperglycaemia in abalone fed a formulated feed when compared to abalone fed on fresh *Ulva*. The immune response of abalone fed fresh *Ulva* was not significantly different to that of abalone fed the formulated feed but there was more pronounced activity from stimulated haemocytes

following challenge with an injected dose of *Vibrio anguillarum*. These data were correlated with higher levels of bacterial clearance from the haemolymph indicating a more efficient immune response by the haemocytes of abalone fed fresh *Ulva*.

In a controlled laboratory experiment, abalone were maintained on a diet of fresh IMTA-grown *Ulva* (FU) and grew at a rate not significantly different from abalone maintained on a commercially formulated feed (AB). *Ulva* fed abalone were found to have significantly elevated tissue moisture and reduced muscle glycogen when compared to abalone fed the formulated feed (AB). The muscle glycogen content of abalone fed fresh *Ulva* (40 %), as a partial replacement for a formulated feed (60 %), was similar to that of abalone fed on the formulated feed AB. Abalone fed the mixed diet (ABFU) had significantly improved SGR and MISL when compared to abalone fed either diet individually. The denaturing gradient gel electrophoresis (DGGE) data clearly separated the gut microbial fingerprints into two groups, with one cluster consisting mainly of abalone maintained on formulated feeds and the other cluster comprised of abalone fed fresh *Ulva* diets. The non-metric multidimensional scaling and analysis of specific bands within the DGGE data indicate that abalone fed fresh *Ulva* diets, and its components, produced significant associations in their intestinal bacterial patterns. Abalone that were fed a formulated feed that had been fortified with glucuronic acid had specific associations of the microbial pattern shared with abalone fed *Ulva* as well as significantly higher SGR and improved feed conversion ratios compared to abalone fed a formulated feed.

This study shows that the feeding of fresh *Ulva* can be used to partially replace formulated feeds, enhance the growth and consumption rate of abalone while also significantly effecting the abalone's health, physiology and gut microbial ecology. Dietary *Ulva* is likely contributing to the enhanced growth of abalone through enhanced consumption and modifications to the gut microbiome which can improve metabolic and immune homeostasis. The changes to abalone consumption, physiology and microbiome, when fed on a diet of fresh *Ulva* and its components, can be used to better design finishing feeds to enhance the quality of canned or live abalone products which may have an impact on the associated human health benefits. The general methods for abalone cultivation have been well understood but there remains a lack of knowledge on the abalone metabolome which may account for the high variability in growth under

commercial conditions. Collectively, these findings provide a basis for further investigation into specific roles of extracts obtained from fresh *Ulva* on the growth, health, physiology and microbiome of aquaculture species.

Table of Contents

List of Tables

List of Figures

Chapter 1 - General Introduction

1.1 Context and topic

The world's population has reached 8 billion people and is growing, which has created profound changes in the global food system. Some of the anthropogenic pressures driving the shift in production are; changes in consumption patterns linked to urbanisation, improved/diminished financial capacity, nutritional values and environmental concerns (Rosenzweig et al., 2020). The current complex global human food supply chain has created suboptimal food availability where a large proportion of the land resources are used in the production of livestock and aquaculture feeds (Sandström et al., 2022). Currently, about half of the available land on earth is used for intensive agriculture (Ritchie & Roser, 2017) with 40 % of farmed land being used for the production of crops for their inclusion into formulated feeds. Approximately 30 % of all cereal crops are produced for their inclusion into formulated feeds with an increasing amount of other food system by-products being upcycled as alternative sources of nutritional components (Sandström et al., 2022). The food system is a major driver of anthropogenic changes to the landscape (Rosenzweig et al., 2020) and is under pressure to optimize the production of food products used as feeds for livestock and aquaculture feeds (Molden et al., 2010; Falkenmark, 2013; Cameira & Pereira, 2019; Sandström et al., 2022).

In 2016 aquaculture was reported to be the fastest growing food production sector globally (Ratcliff et al., 2016) and in 2018 recorded a total fish production volume of 82 million tons. In 2018 the global capture fisheries accounted for 54 % of the global fisheries produced and was calculated to be 97 million tons (FAO, 2020). Of the overall total fish produced, 156 million tons were used for human consumption with the remaining 22 million tons destined for non-food products, including fishmeal for the production of formulated feeds used to feed livestock and aquaculture species (FAO, 2020). As a consequence of the increased production from aquaculture the proportion of fishmeal destined for non-food products for use within aquaculture increased substantially; where for example in 1980 only 10 % of fishmeal production from wild fisheries was used for aquaculture (Tacon et al., 2008), inclusion of fishmeal has subsequently increased to 69 % in 2016 (Naylor et al., 2021). The successful

formulation of feeds for aquacultured species along with the intensification of aquaculture production methods has created a dependency on commercial formulated feeds (Kwasek, Thorne-Lyman & Phillips, 2020) that are designed to promote growth and may improve the profitability of a farming operation.

Since continued use of fishmeal may be limited in the future, its inclusion in aquaculture feeds and its utilization on farms must be optimized and ultimately reduced to help improve the environmental sustainability of the industry (Klinger & Naylor, 2012; Rosenzweig et al., 2020). A reduction on the reliance of fishmeal based formulated feeds, or an improvement in its utilization, to maintain aquaculture growth is a global concern, where individuals and organizations are looking for novel solutions for the production of feeds used in growing aquacultured species (Rana, Siriwardena & Hasan, 2009; Olsen, 2011; Klinger & Naylor, 2012; Naylor et al., 2021). The formulations of feed used in aquaculture have seen an increased use of soybean meal as a partial protein replacement of fishmeal and now represents approximately 66 % of the global contribution to the protein portion of formulated feeds (Heuzé, Tran & Kaushik, 2020). The aquaculture sector has achieved significant progress in the past 20 years with increased feed efficiencies alongside an increased use of plant-based ingredients that are produced on land or as by-products of land-based agricultural systems (Naylor et al., 2009; Silva et al., 2014; Naylor et al., 2021; Campanati et al., 2021).

Aquaculture effluents present as valuable by-products of aquaculture systems that can be optimized for the cascading of materials through integrated trophic levels that are able to produce co-products alongside the primary human-consumable products. For example, ocean-based systems exploit the traits of various marine species using the environmental conditions to allow for vertical integration of effluent streams in the production of multiple products destined for human consumption. High nutrient loads found in land-based effluent flows can be recaptured using integrated multi-trophic aquaculture (IMTA) systems where algal integration with the primary culture organism, can enhance the overall performance of an aquaculture operation and reduce the environmental burden of production (Chopin et al., 2001; Nobre et al., 2010; Bolton et al., 2016; Cameira & Pereira, 2019). The bioremediation of waste waters using IMTA systems for the production of valuable food system by-products is a critical component for the transition towards circular food systems (Sandström et al., 2022). There are a

number of algae that show promise for their potential inclusion for production in IMTA and for use as a supplementary feed or alternative feed ingredient however, this study sought to focus on land-based marine aquaculture systems that are feeding formulated feeds in conjunction with seaweeds to the cultured South African abalone *Haliotis midae*. The alga investigated here is effluent-grown *Ulva lacinulata* (Bachoo, 2021), which has been cultivated in IMTA systems in South Africa for more than two decades.

1.2 Scope and focus

The abalone aquaculture industry is similar to other forms of intensive agriculture where optimal health and maximum yield of the cultured organisms is a priority to maintain profitability (Sales, 2001; Mulvaney, 2016). The global production of farmed abalone for the year 2016 was calculated to be 160662 tons with 87 % of the total production coming from China (132697 tons) (Cook, 2019). In 2016, a total of 1703 tons of abalone were produced by 19 farms, which contributed 41 % of the total production weight for the marine aquaculture sector in South Africa (excluding seaweeds) (DFFE, 2021). The commercial South African abalone industry has grown rapidly since the 1990's and has increased by 218 % from 2003 – 2015, becoming the most lucrative aquaculture sector in the country contributing 75% of the total value of aquaculture in South Africa (DFFE, 2019). The South African abalone *H. midae* is a highly prized human food item with high market acceptance due to its flavour, texture and size (Cochet et al., 2013; Chigumira, 2016). Intensive abalone aquaculture operations almost always employ technologies which promote the rearing of animals at stocking densities that far exceed those occurring naturally (Kennedy et al., 2016), where research and development into feed formulations that promote the growth of abalone has been ongoing for the past 30 years (Hecht & Britz, 1992; Britz, 1996; Britz & Hecht, 1997; Britz, Hecht & Mangold, 1997; Shipton, 1999; Sales & Britz, 2003; Sales, Truter & Britz, 2003; Green, Jones & Britz, 2011b; Wu, Kaiser & Jones, 2019; Meusel et al., 2021).

The proximate analysis and nutritional profile of whole abalone has been used in formulating feeds that are balanced in the macronutrients considered essential for growth of farmed abalone (Knauer, Hecht & Duncan, 1994; Sales & Britz, 2003). The crude protein levels in abalone feeds that provide high growth potential (by weight) for abalone under culture conditions are reported to be between 28 – 35.8 % dry weight

(Sales, Truter & Britz, 2003). The commercial abalone feed Abfeed™ S34®, produced by Marifeed (Pty) Ltd, (http://www.marifeed.com/abfeed/) has been formulated to contain 34 % crude protein and is considered a benchmark feed for post-weaning of quality abalone by South African producers (pers. comm. Devin Ayres 2018). The total protein content of Abfeed™ S34® has been optimized to contain a balance of fish and plant-derived protein sources (Britz, 1996; Sales & Britz, 2003), where 73.1 % of the total protein comes from fishmeal/ oil and 26.9 % from defatted soybean meal (pers. comm. Abagold (Pty) Ltd, 2011). The successful formulation of feeds that promote growth have led to improved profits of *H. midae* under culture conditions and further commercialized the abalone aquaculture industry in South Africa (Britz et al., 1994, Sales, Truter & Britz, 2003).

A large proportion of South African abalone producers utilize formulated feeds, while there are still a number of producers, particularly along the southwest and west coasts, who continue to make use of wild harvested kelp (*Ecklonia maxima*) as a feed source for abalone. Farms that fall within the biogeographic range of the kelp *E. maxima* go to considerable efforts to source and transport the macroalga, where it is added directly to the culture environment of abalone in commercial systems (Sales, 2001; Troell et al., 2006). Abalone farms that use fresh kelp, and when fed in conjunction with dry formulated feed(s), have found it can reduce pest loads and enhance growth rates of abalone (Simon et al., 2005; Naidoo et al., 2006; Troell et al., 2006; Dlaza, Maneveldt & Viljoen, 2008). Wild harvested kelp is however near the limits of sustainable harvesting in certain kelp Concession Areas (Troell et al., 2006), which will restrict further growth and development of abalone farms that are reliant on the resource. Furthermore, kelp is not readily available to all producers as it does not occur throughout the South African coastline. Collectively, these constraints have stimulated research and investment in alternative feed crops which can be locally grown by integrating farming practices into a more circular movement of energy forms, such as the integrated production of alternative feed sources (co-products).

In the year 2000, the primary production of *Ulva* began in South Africa for its use as a supplementary source of feed for abalone by a farm which fell outside of the biogeographic range for *E. maxima* (Bolton et al., 2006; Robertson-Andersson et al., 2008; Bolton et al., 2009). There are now several abalone producers in South Africa

which grow the macroalga *Ulva* using IMTA systems (Neveux et al., 2018), which has led to *Ulva* ranking as South Africa's largest aquacultured product by weight, with 2155 tons (wet weight) produced per annum (Cai et al., 2021). Part of *Ulva*'s attractiveness as an alternative feed crop on South African abalone farms comes from; (1) its ability to thrive unattached in aquaculture conditions, (2) its affinities for growth in environments with high nitrogen concentrations, and (3) the relative size and shape make it fairly easy to harvest fresh (Bolton et al., 2016). The South African abalone farms that have adopted the integration of *Ulva* production may benefit from; the bio-remediation capacity of *Ulva* on the nutrient loads in discharged water and increased profits under partial recirculation (Nobre et al., 2010), 50 - 100 % recirculation which reduces pumping cost (Nick Loubser, pers. comm. 2022), while also providing a feed supplement that can be locally produced (Bolton et al., 2009; farms later described in Chapter 2). However, the large-scale production of *Ulva* in South Africa awaits broader acceptance in the industry partially as a result of biosecurity concerns related to its use with the existing integrated systems (Bolton et al., 2009, de Jager, 2019) and the lack of empirical data on specific benefits of its use as a supplementary feed in the production of abalone.

The management practices of the farms utilizing *Ulva* for biofiltration or as a supplementary feed will play an important role in maintaining biosecurity of the system where it could become compromised if an unfavourable stressor/pathogen were allowed to maintain a steady population (Pulkkinen et al., 2010; Mulvaney, Winberg & Adams, 2013). There can be variable operating parameters for individual farms where the management of feeding can lead to increased biotic and abiotic stressors. When commercially grown abalone are confronted with significant bacterial pathogens or extreme abiotic stressors it can lead to general die-offs of animals, particularly when the animals are stressed (Loker, 2010). Feeds and feeding regimes can be developed which contribute to the overall health of the cultured abalone and may reduce the susceptibility of abalone to stressors (Macey & Coyne. 2005; Braid et al., 2005; Zhao et al., 2018; Cicala et al., 2022) where benefits beyond nutrition are considered. To the author's knowledge there are no examples of *Ulva* being a vector in the transmission of any pathogens, where it has been included into feeding regimes for abalone, in the South African context for more than two decades.

Species of the green seaweed *Ulva* are currently grown for human food in some parts of the world (Andrade et al., 2009) and were identified as a potential feed supplement for abalone farms more than 40 years ago (Tenore, 1976). The combination of high stocking densities and the concurrent use of formulated feeds used in flow-through abalone aquaculture systems creates waste-water effluent flows that are higher in nutrients than inflowing seawater (Probyn et al., 2017) making them well suited to the cultivation of *Ulva*. The nutritional profile of *Ulva* grown in high nitrogen culture environments like that of abalone effluent water, is enriched with phenolics, increased tissue nitrogen and starch content, high antioxidant activity, optimal fatty acid profiles (McCauley et al., 2018; Prabhu et al, 2019; Shpigel et al., 2019) and is seasonally consistent when compared to non-effluent grown *Ulva* (Shuuluka, Bolton & Anderson, 2013; Laramore, Wills & Hanisak, 2022). Effluent-grown *Ulva* can maintain high levels of protein (Queirós, 2021) that range from 7.8 – 25.8 % (Robertson-Andersson, Maneveldt & Naidoo, 2011; Shuuluka, Bolton & Anderson, 2013; Queirós et al., 2021) where the protein has a comparable amino acid profile to that of fishmeal (Figure 1-1), a long praised high-quality protein source considered essential for growth of most aquaculture species (Fleming, Barneveld & Hone, 1996). The favourable amino acid profile and enhanced nutritional profile of effluent-grown *Ulva* make it a valuable co-product from IMTA systems. Furthermore, the *Ulva* grown in abalone effluent waters has been reported to have the same growth rate as microalgae per unit area for outdoor systems making it an ideal co-product for local feed production in South Africa (Bolton et al., 2016).

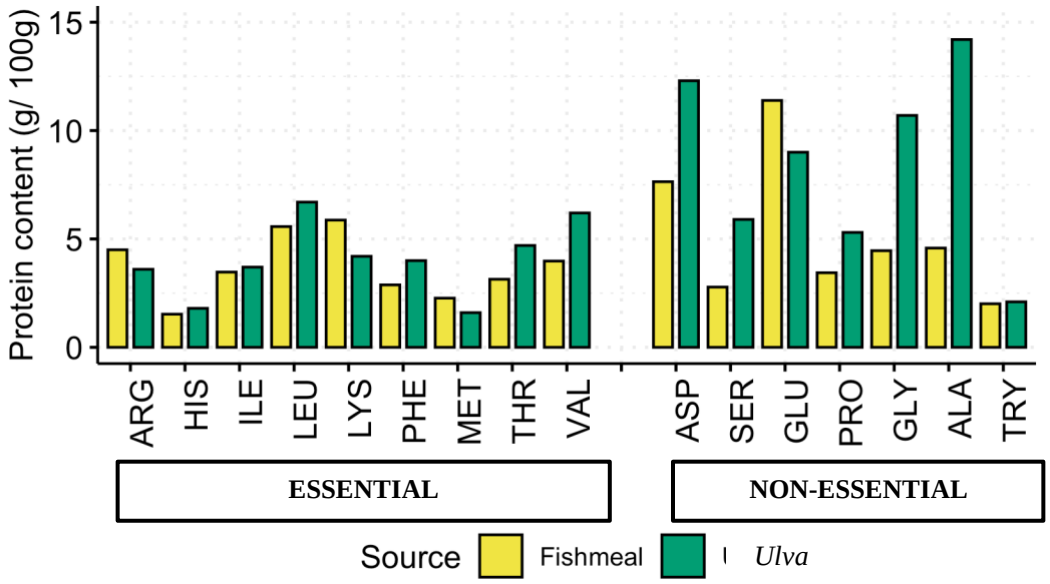

Figure 1-1 Amino Acid profiles for fishmeal (yellow) used in the development of abalone aquafeeds compared to that of *Ulva* (green) grown in farm effluent water. Individual amino acids are abbreviated using the standard 3-letter code and are represented as grams per 100 g of protein. Amino acids are grouped into essential and non-essential and presented side-by-side for comparison. The data used for the amino acid profile of fishmeal was obtained from Sales & Britz (2003) and for *Ulva* the data are from Shuuluka, Bolton & Anderson (2013).

Fresh *Ulva* has already been successfully used as a fresh feed supplement in abalone aquaculture systems to improve growth and survival for *H. laevigata* (Bansemer et al., 2016), *H. rufescens* (Kemp, Britz & Agüero, 2015), *H. rubra* × *H. laevigata* (Mulvaney, Winberg & Adams, 2013; Mulvaney et al., 2015) and *H. midae* (Naidoo et al., 2006). Dried *Ulva* meal has also been successfully included into a formulated feed at up to 30 % and used to grow *H. laevigata* and *H. asinine*, where the *Ulva* meal was used as a partial replacement for a combination of solvent extracted soybean meal, wheat flour and dehulled lupin (Bansemer et al., 2016; Duong et al., 2021). The inclusion of *Ulva* into the formulated feed for *H. laevigata* as a partial replacement of solvent extracted soybean meal, wheat flour and dehulled lupin significantly improved the absorbed energy rates from the feed when compared to a control feed (Duong et al., 2021). Enriched *Ulva pertusa* has also been included into a formulated feed as a partial protein replacement for up to 30 % of the fishmeal protein component without any significant differences in the growth metrics measured when fed to *H. asinina* (Santizo-Taan,

Bautista-Teruel & Maquirang, 2020). The replacement of ingredients that are used in the making of formulated feeds must however be balanced to provide optimal digestibility to achieve high feed conversion ratios, while it is also important to consider the costs for growing, drying and processing of the *Ulva*. Collectively, these studies have tested several iterations for the use and inclusion of *Ulva* into feeds and feeding regimes for abalone however, to date there has not been clarification on the direct mode of action or specific components within *Ulva* that are responsible for the benefits which have been reported for the use of *Ulva* as a supplementary feed or feed additive.

The co-production of *Ulva* in abalone effluent water has led to the availability of a high-quality feed source that is locally available and can be included into feeding regimes of abalone, where it may enhance the overall performance of the system (Bolton et al., 2016). Farms that use fresh *Ulva* as a supplement for formulated feed may benefit from a reduction in the volume of formulated feed required to achieve optimal growth of abalone however, there are no standard methods available for integration into production systems, nor is there information to which formulated feeds can be replaced with fresh *Ulva* as a supplementary feed. Waste from feeding is a major source of pollution caused by aquaculture and through better management methods the productivity of aquaculture systems can be improved (Boyd, McNevin & Davis, 2022). The benefit of using fresh *Ulva* in feeding regimes can be realised through reduced logistics in terms of harvest/ processing that is required for formulated feeds. The formulation of feeds that contain *Ulva* may be done as a partial replacement for the protein portion of formulated feeds or it can be included where specific benefits beyond nutrition may be achieved. This thesis will present *Ulva* as an important functional component in the feeding of *H. midae* and experimentally determine its' capacity as a partial replacement for formulated feeds used in commercial abalone aquaculture systems.

1.3 Importance and relevance

The feeds and feeding regimes utilized by abalone producers can have implication for the health and quality of marketable abalone (Øiseth et al., 2013; Zhao et al., 2018). In the wild, *H. midae* typically are slow growing gastropods that consume a diet high in complex polysaccharides from several macroalgae and are entirely herbivorous, feeding preferentially in South Africa on the kelp *E. maxima*, where it is available

(Barkai & Griffiths, 1986). The gut of *H. midae* contains enzymes that are capable of digesting complex polysaccharides where approximately 70 - 90 % of the enzymatic potential in the gut is a result of endogenous bacterial secretions from the abalone gut microbiota (Erasmus, Cook & Coyne, 1997). The primary source of the carbohydrates in the commercially available formulated feed used by the South African abalone industry is maize starch that comprises ~40 % of the total feed composition and is rapidly digested by *H. midae* (Sales & Britz, 2002). Maize starch is processed from land-based cereal crops and the primary carbohydrates differ in their structural complexity from marine equivalents (Helbert, 2017). In humans (McKeown et al., 2004), crayfish (Radford et al., 2005), fishes (Moon, 2001), and gastropods (Rossi et al., 1993), the consumption of refined starch, like that in the formulated feeds used for abalone, causes a sharp increase in the blood glucose concentration (hyperglycaemia) and alters the hosts metabolic efficiencies. In humans these metabolic alterations can be related to lifestyle dietary choices and are commonly referred to as the metabolic syndrome (McKeown et al., 2004; Ruiz-Núñez et al., 2013). The metabolic syndrome, which remains largely a concept in human biology, affects similar evolutionary conserved energetic mechanisms and due to the ancestral nature of these metabolic pathways they share a common thermodynamic inheritance across all organisms (Schlegel & Stainier, 2007; Imanikia & Stürzenbaum, 2013; Dierking & Pita, 2020). The metabolic conditions that are characteristic of the metabolic syndrome share similar defining features to those described in humans when investigated in other mammals and invertebrates (Schlegel & Stainier, 2007; Imanikia & Stürzenbaum, 2013). Collectively these disorders negatively impact the overall efficiency of the host metabolism and are linked to chronic low-grade cellular inflammation (Ruiz-Núñez et al., 2013).

Marine derived polysaccharides can mediate beneficial alterations to the intestinal structure and microbial composition that can have a direct impact on host metabolism (Mohan et al., 2018; Safavi et al., 2019; Zarei et al., 2022). For example, marine polysaccharides have been used successfully in the form of prebiotics for the management of hyperglycaemia and other metabolic disorders where it is suspected to beneficially regulate glucose metabolism, and influence the gut microbiota in other animal models (Geurts et al., 2014; Seong et al., 2019; Li et al., 2021). *Ulva* has been shown to contain long chain sulphated polysaccharides known as ‘ulvan’ (Wang et al.,

2014, Yu-Qing et al., 2016, Well et al., 2018, Li et al., 2018, Mo'o et al., 2020, Gotteland et al., 2020, Ponce et al., 2020) that comprise 8 – 34 % of the dried seaweed (Silva et al., 2019). Ulvan has been identified as a prebiotic capable of promoting immune function in fishes (Ponce et al., 2020; Safavi et al., 2019) and improve the intestinal integrity/structure of broiler chickens (Cañedo-Castro, 2019). Prebiotics, and their role in the modulation of the microbiome, are a powerful emerging tool that could be used in the formulation of functional foods and the modulation of the gut microbiome (Guerreiro, Oliva-Teles & Enes, 2018; Mohan et al., 2018; Guerreiro et al., 2019; Lopez-Santamarina et al., 2020; Perry et al., 2020; Yuan et al., 2020; Zheng, Chen & Cheong, 2020; Rajeev et al., 2021).

Foods that demonstrate the ability to beneficially affect functions within the body beyond the nutritional effects are regarded as functional foods (Roberfroid, 2002, Holdt & Kraan, 2011, Wells et al., 2016, Ringo et al., 2016, Marta, Marta & Marco, 2017, Bullon, Seyfoddin & Alfaro, 2022). The digestive tract of abalone species (*H. laevigata, H. laevigata* × *rubra, and H. tuberculata*) has been identified as a niche environment with unique selective pressures that allows for the establishment of a 'core' microbiota (Gobet et al., 2018; Wang, Pyecroft & Barton, 2020; Wang et al., 2020; Danckert et al., 2021). The surface of *Ulva* has been found to provide a microhabitat which is inhabited by various microbial species which are different from those in the surrounding environment (Tujula et al., 2010; de Jager, 2021). The combination of fresh *Ulva* and the associated bacterial hosts found on its surface, when used as dietary supplements for abalone have significant potential to contribute towards microbial recruitment involved in the digestion and assimilation of dietary components (Erasmus, Cook & Coyne, 1997; Gobet et al., 2018; Wang, Pyecroft & Barton, 2020; Danckert et al., 2021). The microbiota in the gut of abalone play an important role in the digestion and assimilation of nutrients (Danckert et al., 2021) where abiotic factors such as culture conditions and/ or diet can change the structure/ function of the gut microbiota that will impact the host metabolic efficiencies for growth and health of the organism (Wang et al., 2020).

Collectively, these studies suggest that *Ulva* has considerable capacity to act as a functional food when included into the feeding regimes of cultured abalone that could be used in the dietary intervention of *H. midae* to better meet the market requirements

and improve the economic profitability of abalone farms. In the case of *H. midae*, the likely source of prebiotics would come from the ingestion of ulvan found in *Ulva*, which forms part of their natural diet (Barkai & Griffiths, 1986, Wijesekara, Pangestuti & Kim, 2011) where the *Ulva* is host to specialised communities of bacteria (Califano et al., 2020). The inclusion of *Ulva*, or specific components of *Ulva*, may be a viable tool that could be used to improve feeding efficiency, the metabolic capacity and ultimately the health of cultured abalone.

1.4 Aims and Objectives

The aim of this study was to take a practical approach to understanding the role of effluent-grown *Ulva* as a feed ingredient for the abalone, *H. midae*, which will have relevance to industry application. A greater understanding of the range of benefits that *Ulva* can have on the growth, health and physiology of abalone will have commercial relevance for further feed development as well as improve the scientific understanding of the underlying mechanisms.

It was hypothesised that *Ulva* could be used as a feed supplement to partially replace formulated feeds that would improve the overall growth, consumption and health of abalone. Some of the improvements to abalone culture and function cited by previous authors (Naidoo et al., 2006; Mulvaney, Winberg & Adams, 2013; Kemp, Britz & Agüero, 2015; Bansemer et al., 2016, Santizo-Taan, Bautista-Teruel & Maquirang, 2020) may be linked to the polysaccharide fraction of *Ulva* serving as a prebiotic for the cultured abalone. In an attempt to further understand the perceived benefits of *Ulva* as a functional ingredient, *Ulva*, and its carbohydrate component, were included into feeds and feeding regimes to investigate effects on the growth, consumption, health, physiology and gut microbial patterns of the cultured abalone *H. midae*.

The specific objectives of this study were formulated to test these hypotheses under a combination of farm scale and controlled laboratory conditions, which were:

(1) Investigate the effects of *Ulva*, when offered alongside dry formulated feeds, on the consumption, growth, condition and determine the extent to which *Ulva* can be used to replace formulated feeds for abalone under farm conditions;

(2) Determine whether dietary *Ulva* will have an effect on the abalone glycaemic regulation and immune response under simulated challenges;

(3) Incorporate *Ulva*, or specific components thereof, into a dry feed offered under laboratory conditions to measure the effects of specific components of *Ulva* (dried *Ulva*, ulvan, glucuronic acid) on growth, physiology and gastrointestinal microbial patterns.

Chapter 2 - Growth, condition and consumption rates of the cultured abalone *H. midae* fed using a formulated feed and/ or seaweed (*Ulva lacinulata* or *Ecklonia maxima*)

2.1 Introduction

2.1.1 Context and topic

The South African abalone *Haliotis midae* was considered a species on the threshold of commercial production in 1992 (Hecht & Britz, 1992) and in 2019 contributed 75 % to the total value for aquaculture in South Africa (DFFE, 2021). The majority of South African abalone producers nowadays rely on dry formulated feeds which have been optimized for improved growth of the abalone *H. midae* under culture conditions (Britz, 1996; Britz & Hecht, 1997; Britz, Hecht & Mangold, 1997; Shipton, 1999; Sales & Britz, 2003; Sales, Truter & Britz, 2003; Green, Jones & Britz, 2011a; Wu, Kaiser & Jones, 2019; Meusel et al., 2021). The collaborative research between industry and Rhodes University was instrumental to this success (Britz et al., 1994). The feed conversion ratios and apparent consumption of formulated feeds are published by feed developers (Britz, Hecht & Mangold, 1996) leaving it up to the farms to develop feeding regimes. The methods of feeding, although informed by scientific process, can be considered a skill, with farmers determining feed intake based on observation, and is often dependant on the site's current environmental conditions (Cho & Bureau, 2001). Along with the complicated management of feeding regimes, producers must also balance feeding with apparent satiation observed on the farms when macroalgae (*Ecklonia maxima*) is available from wild harvests as a feed supplement (Francis, Maneveldt, & Venter, 2008; Michael Joubert, pers. comm. 2011).

The supplementation of macroalgae into the feeding regime of cultured abalone that are fed on dry formulated feeds has been shown to enhance growth rates when compared to either feed source fed individually (Dlaza, Maneveldt & Viljoen, 2008; Mulvaney, Winberg & Adams, 2013; Kemp, Britz, & Agüer, 2015; Mulvaney et al., 2015). The kelp *Ecklonia maxima* has been relied upon extensively as a feed supplement by

abalone producers, particularly at the farm where this study took place (Abagold (Pty) Ltd) where the kelp is harvested from the wild in areas where the farms fall within the biogeographic range of the kelp. The use of *Ulva* has also demonstrated capacity to improve the growth of abalone (Naidoo et al., 2006; Mulvaney, Winberg & Adams, 2013; Kemp, Britz & Agüero, 2015; Bansemer et al., 2016, Santizo-Taan, Bautista-Teruel & Maquirang, 2020) which has led to *Ulva* ranking as South Africa's largest aquacultured product by weight, with 2155 tons (wet weight) produced per annum (Cai et al., 2021). The majority of South African abalone producers that currently cultivate *Ulva* incorporate the *Ulva* into mixed feeding regimes where it has been observed to enhance the performance of abalone production systems (Devin Ayres pers. comm. 2022; John J. Bolton, pers. comm. 2022; Nick Loubser, pers. comm. 2022). Each farm has unique motivation for the integration of macroalgae and to date there remains a lack of published data on the extent to which the supplementation of abalone diets with effluent-grown *Ulva* or wild harvested kelp can be maintained without compromising abalone growth. The supplementation of macroalgae into the diets of farmed abalone is done *ad lib* under standard production methods while this study used standardised feeding regimes to experimentally quantify consumption, growth and condition of abalone being fed different ratios of either effluent-grown *Ulva* or the wild harvest kelp. This study sought to focus on identifying feeding regimes that included *Ulva* to improve feeding efficiency along with reductions in the quantity of formulated feeds required to grow abalone at a rate not different to the standard commercial production system at Abagold (Pty) Ltd.

2.1.2 Scope and focus

There are currently five farms in South Africa that are testing or have adopted the integration of *Ulva* into their farming practices. The motivation for the adoption of this integrated multi-trophic aquaculture (IMTA) approach/technology differs for each farm and ranges from (1) production of an alternative feed crop; (2) improving the quality of farm effluent water; (3) reducing pumping costs and consequent electrical consumption; and (4) to allowing for the partial or complete re-circulation of water, the latter specifically during harmful algal blooms. Wild Coast Abalone (Pty) Ltd. (32°45'04.3" S 28°16'27.9" E), situated on the south-east coast of South Africa, was one of the first farms to adopt this technology and are able to produce in excess of 2

tons of *Ulva* per day in large D-ended raceway systems, which circulate the water using a large paddle wheel (Bolton, 2006). *Ulva* is harvested from the D-ended raceways that is used as a feed supplement, where it is added directly into the abalone culture systems. Irvine and Johnson (I&J) Cape Abalone located at Danger Point on the southwest coast (34°37'35.1" S 19°17'47.6" E) utilize similar technology to that of Wild Coast Abalone but have also taken advantage of *Ulva*'s biofiltration efficiency to enable partial recirculation and have as a result improved overall farm profits (Nobre et al., 2010). More recently, Buffeljags Abalone Farm on the southwest coast (34°45'14.7" S 19°36'51.9" E) and Diamond Coast Aquaculture at Kleinsee on the northwest coast (29°39'59.8" S 17°03'03.2" E) have expanded this system to full farm design, taking advantage of *Ulva*'s efficient biofiltration ability and partially recirculating abalone effluent water (50 % recirculation), which reduces the costs of pumping (~ 40 % cost saving) and increases water temperatures (by ~ 1°C), with excess *Ulva* fed directly back to the abalone as a supplementary feed (Nick Loubser, pers. comm. 2022). The latter systems have been built as modular units, called platforms, consisting of 'micro-farms', each connected to an effluent pond in which all water recirculation and feeding are contained and isolated from one platform to another so as to address any potential biosecurity concerns linked with feeding effluent-grown *Ulva* back to abalone. Similarly, Abagold (Pty) Ltd. (the site of this experiment) on the southwest coast (34°26'02.8" S 19°13'17.7" E) have been investigating the potential to convert current infrastructure (flowthrough raceway systems) into integrated production raceways (abalone/*Ulva*) to help reduce pumping costs on the farm and provide an alternative/supplementary feed for the cultured abalone (Chris Parker, pers. comm., 2015). This particular farm has not implemented the latter technology, partly due to the perceived biosecurity threats associated with feeding effluent-grown *Ulva* back to abalone, where *Ulva* may serve as a potential vector for spread of disease within the farm (Bolton et al., 2009); but also because there is a lack of empirical data on the extent to which formulated feeds can be substituted with the seaweed without compromising growth and condition of cultured abalone. IMTA uses the cascading of materials through integrated trophic levels that are able to produce co-products alongside the primary production system and in all the farms mentioned above, formulated feeds have

a crucial role in the feeding of abalone as well as the maintenance of integrated *Ulva* production systems.

2.1.3 Importance and relevance

The high nitrogen (predominantly ammonia) content of abalone effluent water (Probyn et al., 2017) enables the consistent production of a valuable feed supplement which has an enhanced nutritional profile and higher protein levels compared to the wild harvested kelp *E. maxima* and/ or *Ulva* (Robertson-Andersson, Maneveldt & Naidoo, 2011; Shuuluka, Bolton & Anderson, 2013; McCauley et al., 2018; Prabhu et al, 2019; Shpigel et al., 2019; Queirós et al., 2021). The crude protein levels of effluent-grown *Ulva* range from 7.8 – 25.8 % (Robertson-Andersson, Maneveldt & Naidoo, 2011; Shuuluka, Bolton & Anderson, 2013; Queirós et al., 2021) and is consistently higher when compared to that of wild harvested kelp (9.73 %), which is utilised as a supplementary feed on many South African farms (Robertson-Andersson, Maneveldt & Naidoo, 2011). Enriched *Ulva* (27.7 % crude protein) has been used as a dietary ingredient for the abalone *H. laevigata*, where it enhanced energy metabolism and somatic growth compared to a formulated feed that contained 35 % crude protein (Duong et al., 2020; Duong et al., 2021). It has been calculated that only 5 % of the ingested energy for *H. midae* is available for growth and reproduction (Barkai, & Griffiths, 1988) making feed efficiency an essential consideration. The feeding regimes of abalone can be optimised to contain other sources of protein and/ or through improved feeding efficiency of the feed that is already available.

Marine gastropods typically rely on chemoreception in feeding where they are attracted by the release of secondary metabolites (Kohn, 1961; Croll, 1983). A feed that can enhance the feed palatability may be a key component in the development of efficient feeds and feeding regimes. The abalone *H. midae* have a distinctive diurnal feeding rhythm from late afternoon to the early hours of the morning, where the abalone have demonstrated consumption preference for both *Ecklonia maxima* and *Ulva* (Barkai, & Griffiths, 1987). The inclusion of other seaweeds (*Ecklonia radiata* and *Sargassum linearifolium*) as a supplementary diet for sea urchin has been shown to enhance overall consumption and or stimulate feeding as volatile organic compounds are released into the seawater that surrounds the seaweeds (Dworjanyn, Pirozzi & Liu, 2007). Akakabe

& Kajiwara (2008) showed that the snail (*Lunella coronatea*) recognized *Ulva pertusa* via chemoreception and fed preferentially on it when compared to other marine algae. In contrast, when *Ulva* is grazed on, DMS is released into the seawater and DMS is reported to be a herbivore deterrent to some marine herbivores (Alstyne & Houser, 2003; Peckol, 2017). The inclusion of specific dietary ingredients in formulated feeds may impact feed palatability through the release of metabolites into the surrounding water when feeds are grazed on thereby impacting the efficiency of certain feeding regimes.

Food availability may be the most important factor for the growth of *H. midae*, which exhibits high individual variability in growth rates (Tarr, 1995). Feeding is associated with an increase in oxygen consumption (Bayne & Newell, 1983) and at higher temperatures the consumption rate and relative energy consumed by individual abalone increases (Barkai, & Griffiths, 1988; Britz, Hecht & Mangold, 1997). When water temperatures exceed 22 °C the feeding of protein rich formulated feeds can be detrimental to the health of the cultured abalone; leading to a condition known as "bloat" caused by an increase in microbial activity in the digestive tract of abalone (Macey & Coyne, 2005). Green, Jones & Britz (2011a) demonstrated it is possible to reduce mortalities of abalone during elevated temperatures by reducing the protein content to 22 % in the formulated feeds. Furthermore, a local abalone farm occurring where water temperatures periodically exceed 22 °C has demonstrated a reduction in 'bloat' when abalone are fed *Ulva* instead of formulated feeds (John J. Bolton pers. comm. 2021) which is supported in the findings from Lange et al. (2014) for *H. laevigata* fed *Ulva*.

The large muscular foot of the abalone is the main marketable product and the potential to adjust specific characteristics of the intended product through nutritional interventions can become a useful tool. Glycogen is the primary energy molecule in the abalone foot tissue and is heavily relied on for energy reserves (Chiou, Lai & Shiau, 2001; Laas & Vosloo, 2010). Abalone have a carbohydrate-based metabolism and store large amounts of glycogen in the foot muscle tissue, which they utilize preferentially under times of locomotion and starvation (Carefoot, 1991; Venter et al., 2018). The foot of an abalone makes up approximately 66 % of the wet body mass (not including the shell) (Vosloo & Vosloo, 2006) and higher glycogen reserves are beneficial for survival

of live transported abalone where it is used as a source of energy during aerobic metabolism (Laas & Vosloo, 2010). The tissue glycogen content of the abalone *H. diversicolor* fed a formulated feed was reported to be significantly higher than that reported in abalone fed macroalgae (Chiou, Lai & Shiau, 2001). Similarly, high starch diets have been found associated with higher tissue glycogen in the marine snail *Babylonia areolata* (Zhang, Zhou & Cheng, 2009). The molecular structure of glycogen allows for the retention of water at a rate of 2 - 4 g of water per gram of glycogen (Fernández-Elías et al., 2015; Kemp, Britz & Agüero, 2015) making glycogen content an important consideration for the development of feeding regimes for the production of canned abalone (Kemp, 2018) which are an important export product from commercial abalone farms in South Africa. The feeds and feeding regimes used for the optimal growth of abalone use high carbohydrate diets that can have a positive impact on the yield of canned abalone however, different end products may require different feeding regimes.

2.1.4 Objectives and questions

Ulva has demonstrated capacity to positively influence the cultivation of abalone under intensive farming conditions, but specific feeding regimes for promoting growth, while minimizing costs through improved feed efficiencies or increased dietary supplementation of formulated feeds with seaweeds, have yet to be developed. The aim of this study was to investigate the effects of dietary supplementation with effluent-grown *Ulva* on the feed consumption, growth and physiology of abalone *H. midae*. The experiment was conducted on a commercial abalone farm, under the standard commercial operating conditions at Abagold (Pty) Ltd., where abalone were fed a combination of macroalgae (*Ulva* or *Ecklonia maxima*) and/ or varying levels of a locally formulated abalone feed Abfeed™ S34® (34.6 % protein; 43.3 % carbohydrate; 5.3 % fat; 1.2 % crude fibre; 5.7 % ash and ~10 % moisture; Marifeed (Pty) Ltd (http://www.marifeed.com/abfeed/). The specific objectives of this study were addressed in two separate experiments which were;

> Experiment 1 - determine the consumption rate and feed preference when fed dietary *Ulva* as a partial or complete replacement of formulated feed Abfeed™ S34®.

Experiment 2 - determine the effects of the dietary supplementation with *Ulva* or kelp (*Ecklonia maxima*) on the growth and condition of abalone fed graded levels of the formulated feed Abfeed™ S34®.

2.2 Material and methods

2.2.1 Farm operational parameters

Details of the water supply, systems and standard farming/management practices at this facility are provided in greater detail below.

2.2.1.1 Farm Location and water supply

The research was conducted at the commercial abalone facility Abagold (Pty) Ltd, Hermanus, South Africa (34°26'02.8"S 19°13'17.7" E). The 20-year average (1989 – 2011) water temperature for the Atlantic Ocean coastal environment in Hermanus, where the farm extracts its seawater, is 15.6 °C. The temperature ranged from an absolute minimum of 9 °C to an absolute maximum of 23.5 °C over the 20 year period (Figure 2.1). Monthly means vary relatively little during the year over this period (14.7 °C – 16.6 °C).

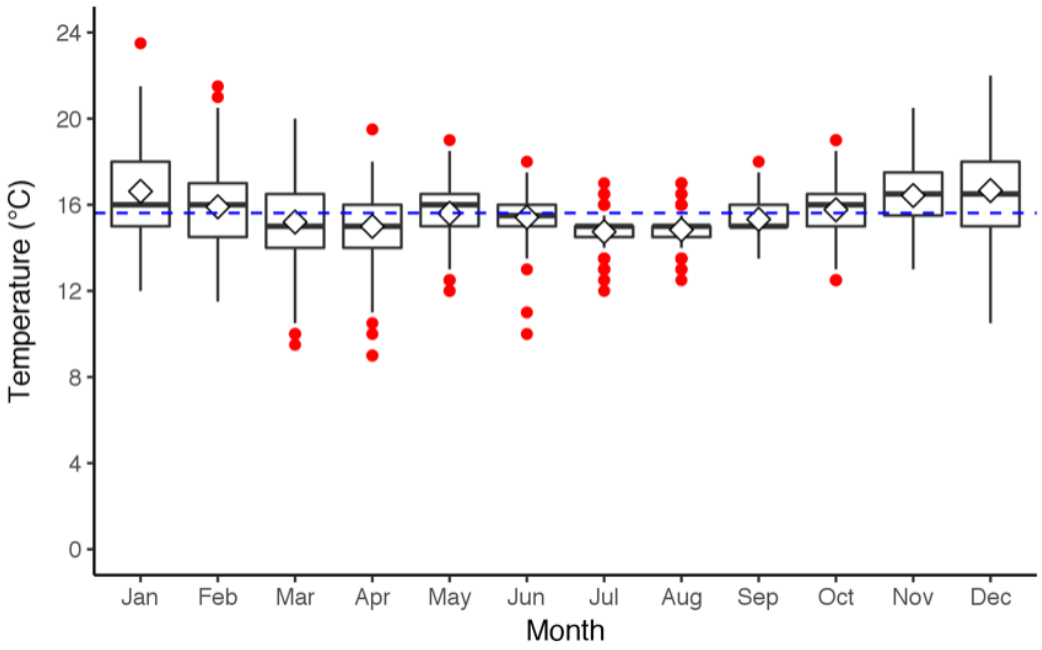

Figure 2-1 Box and whisker plot for monthly sea temperatures that were captured using hand held thermometers in Hermanus and the data sourced from the South Africa Weather Service (SAWS, 2011). The annual mean temperature is represented by the dashed blue line. The monthly variation is visible by the relative position of each box and the mean denoted by the white diamond in each box. Outliers are marked in red.

All sea water for the production of abalone at Abagold (Pty) Ltd. is pumped directly from the Atlantic Ocean from behind a barrier in Walker Bay, which was erected to help protect the pump house from rough seas. As a result of being exposed to variable ocean conditions, the pump rate will vary depending on daily environmental changes with a maximum pumping rate of 12 million litres per hour (Chris Parker, pers. comm. 2011). Sea water is passed through drum filters (500 μm) prior to entering a settlement tank, from where it is further distributed throughout the farm and to the culture tanks.

2.2.1.2 System and system management

The standard farm production tank at Abagold (Ltd) Pty. is termed a 'raceway' and consists of a 3490 L concrete structure with internal dimensions of L x W x H: 4.83 x 0.84 x 0.86 m. Each raceway is supplied with filtered water at a rate of 25 - 40 $L.min^{-1}$ (one tank exchange at least every 2.3 h). A single PVC pipe, with holes drilled every 30 cm, is fitted along the bottom of each raceway, along its entire length, to provide moderate aeration. Animals in each raceway are housed in baskets that are suspended in the tanks, with each basket containing 9 submerged vertical plates, a feeder plate approximately 10 cm sub-surface, and a cover that is floated within the basket. The baskets are constructed from oyster mesh (L x W x H: 800 x 500 x 550 mm) and house the abalone for the entire grow-out stage of production. Each raceway is cleaned at least once every two weeks by washing and scrubbing the raceway with fresh water. The baskets are temporarily removed from the raceway while it is cleaned and then returned to the cleaned raceway.

2.2.1.3 Abalone and sorting

Abalone grow-out at Abagold (Pty) Ltd. relies on the production of spat from wild collected broodstock animals which are batch spawned on site within their hatchery. Abalone remain within the nursery/hatchery for approximately eight months before entering the grow-out stage of production. Once abalone enter the production stage of grow-out, they are graded and sorted approximately every four months into size classes (small, medium, large, or rejects) and stocked at 10 kilograms of abalone per basket. Damaged and/or diseased abalone are grouped as rejects, which are then removed from the general production for alternative uses. The density per basket is readjusted following every sorting event to account for abalone growth, which is projected to be

approximately 1 kg.basket^{-1}.month^{-1}. These adjustments are made in order to optimize space allocated to growing abalone and the management of feeding regimes to ensure maximum yields as measured by weight of the abalone produced.

2.2.1.4 Diets and feeding regime

Abagold (Pty) Ltd uses Abfeed™ S34® fed three times a week (Mondays, Wednesdays and Fridays), at the discretion of a trained farm worker, and abalone diets are further supplemented with wild harvested kelp (*Ecklonia maxima*), fed once a week when available. The feeding involves inspecting the feeder plate for any remaining Abfeed™ S34® and offering more feed accordingly. The daily feeding rate of Abfeed™ S34® and kelp were calculated from a three-month record of weekly feeding amounts for 1584 farm baskets. The average feeding rate was 0.22 percent body weight (including shell) per day (% BW.day^{-1}) of Abfeed™ S34®, with an additional 0.21 % BW.day^{-1} of kelp (dry weight approximation) totalling 0.43 % BW.day^{-1}. The addition of kelp was only done when available where the formulated feeds are fed constantly to abalone when water temperatures are favourable.

2.2.1.5 *Ulva* production and harvesting

At the time of this study, the production of effluent-grown fresh *Ulva lacinulata* (hereafter referred to as '*Ulva'*) at Abagold (Pty) Ltd. was limited to an experimental section within the production infrastructure. The *Ulva* in this section of the farm was grown in integrated production raceways (abalone/*Ulva*), in which effluent water from a raceway stocked with abalone is serially passed through an identical raceway containing *Ulva*, but no abalone, before it exits the farm. The *Ulva* grown in effluent in these systems was then used as feed for the experiments described below. *Ulva* stock cultures were maintained in separate isolated culture tanks and were fertilized once a week using a mixture of ammonium sulphate and mono-ammonium phosphate at a ratio of 2:1. The raceways containing the effluent-grown *Ulva* were cleaned and re-stocked with 22 kg of *Ulva*, from the *Ulva* stock cultures, every 6 weeks. Nutrient dynamics and the proximate analysis formed part of a separate study and are not available to the author.

2.2.2 Experiment 1 - Abalone consumption rate and feed preference when fed dietary *Ulva* as a partial or complete replacement of formulated feed.

To empirically determine consumption rates for abalone fed a locally formulated feed (Abfeed™ S34®), *Ulva*, or a mixed diet consisting of *Ulva* and Abfeed™ S34®, a consumption trial was conducted in an experimental farm system at Abagold (Pty) Ltd.

2.2.2.1 System details

Three standard farm production raceways were used to house the experimental animals. The baskets used in this experiment were custom made with a mesh at the bottom and the sides of 3 mm and 6 mm, respectively. This was done to prevent sheets of *Ulva* or formulated feed (size ~ 10 x 40 mm) from falling through the baskets. Six baskets per raceway were used, which had dimensions of L x W x H: 400 x 400 x 400 mm and a total available surface area of 0.8 m^2 (Figure 2.2). The flow rate in the raceways was set to a maximum of 20 $L.min^{-1}$.

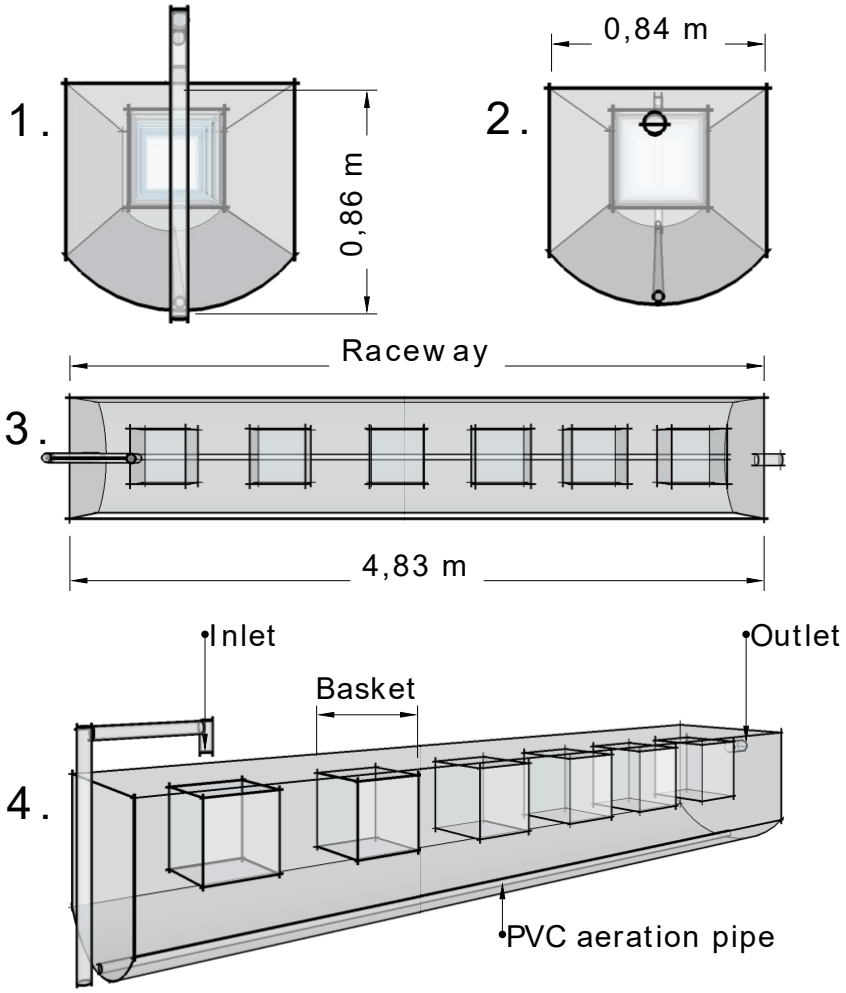

Figure 2-2 Schematic of abalone production raceway used for the quantification of consumption showing (1) front view, (2) back view, (3) top view, and (4) isometric projection. Each component within the raceway is labelled in (4) and includes; water inlet, water outlet, abalone housing basket, and the PVC aeration pipe.

2.2.2.2 Abalone

The abalone were obtained from a single cohort (batch spawning) and were graded by weighing individuals to the nearest 0.01 g using an electronic balance (MODEL: Scout Pro SPU402) and measuring to the nearest 0.01 mm with electronic digital Vernier callipers (MODEL: Grip). Animals of similar weight, no more than 10 g difference, were chosen and had a mean (±SD) weight and length of 67.30 ± 5.49 g and 70.84 ±

2.96 mm, respectively. Each experimental basket was stocked with ten abalone, which were acclimated to their respective diets for 2 weeks prior to the start of the experiment.

2.2.2.3 Diets and feeding regimes

Each time fresh *Ulva* was offered as feed, it was spun free of excess water, since it was found that the sheet-like structure of *Ulva* trapped water and prevented accurate and consistent measurements of the mass of fresh *Ulva* for feeding. A standard protocol was developed, taking advantage of the centrifugal properties of a household salad spinner (Checkers, South Africa), by spinning the *Ulva* free of excess water. The weight loss of the harvested *Ulva* was determined by weighing the container after every fifth revolution (five complete turns of the handle) and the percent weight loss was plotted as a function of number of rotations (Figure 2-3). Based on the results of this experiment, fresh *Ulva* was spun a total of 30 rotations with a salad spinner prior to recording wet weight for feeding purposes. The dry weight of *Ulva* was determined by first recording the wet weight to the nearest 0.01 g, as described above, before oven drying each sample at 60 °C to a constant weight (24 - 48 hours) and reweighing the dried samples to the nearest 0.01 g. The difference between the wet and dry weight was used to calculate a moisture content of *Ulva*, which had a mean (±SD) of 81.6 ± 1.3 % over the experimental period.

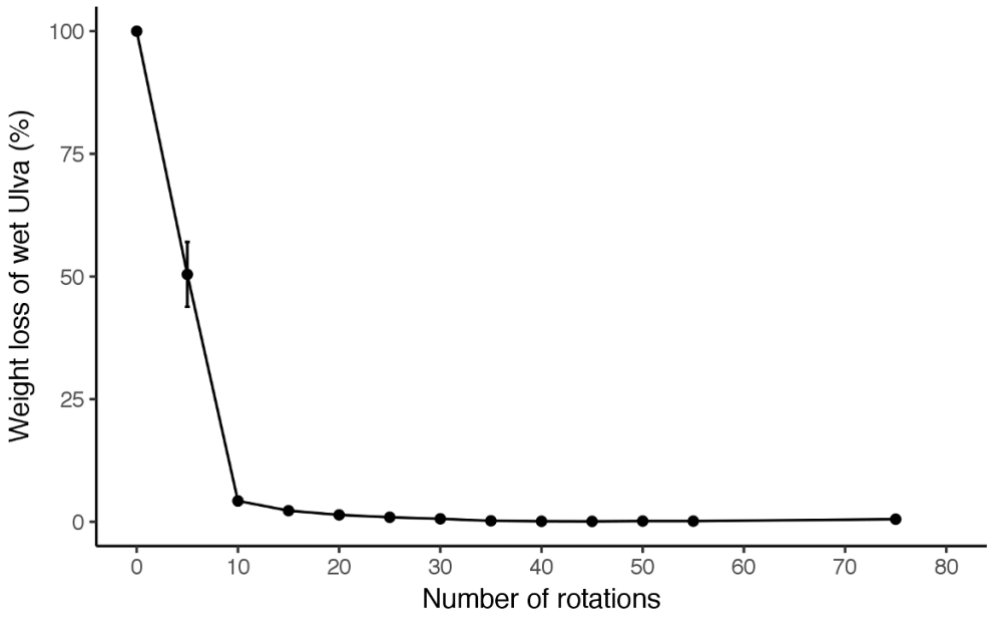

Figure 2-3 Mean (±SE) percent weight loss for fresh *Ulva* as a function of the number of rotations when being spun dry using a household salad spinner. Weight loss is calculated as the percentage decrease from the initial weight multiplied by 100.

The effects of five diets/ diet mixtures (hereafter referred to as feeding regimes) on total consumption by abalone were tested and consisted of graded levels of Abfeed™ S34® and *Ulva* (Figure 2-4). Feeding rations were calculated to feed equal amounts, calculated on a dry weight basis. Total feed was offered in excess, at a mean daily rate of 1.27 percent body weight (% BW.day^{-1}) to ensure that feed availability did not limit consumption. Thus, abalone were fed with either Abfeed™ S34® at a rate of 1.27 % BW.day^{-1} (100 % Abfeed™ S34® control) or with graded levels of Abfeed™ S34® (75, 50, 25 and 0 % of the control group feeding rate), with the balance of the feed constituting *Ulva*. The feeding regimes were as follows;

(1) 100A = 100 % Abfeed™ S34® (Control);
(2) 75A25U = 75 % Abfeed™ S34® and 25 % *Ulva*;
(3) 50A50U = 50 % Abfeed™ S34® and 50 % *Ulva*;
(4) 25A75U = 25 % Abfeed™ S34® and 75 % *Ulva*; and
(5) 100U = 100 % *Ulva*.

A sixth basket (blank) with no abalone was also included in each raceway to allow for quantification of *Ulva* growth and dry feed leaching; which may have an impact on the calculation of consumption data. The baskets in each raceway were positioned so that treatments with the lowest *Ulva* content (e.g. 100A, followed by 75A25U) were placed closest to the water inlet and treatments with a higher *Ulva* content (e.g. 25A75U, followed by 100U) were placed incrementally closer to the outlet (Inlet: 100A, 75A25U, 50A50U, 25A75U, 100U, blank). This was deliberately done to minimize any potential feeding stimulus possibly caused by fresh *Ulva* or compounds leaching from fresh *Ulva*.

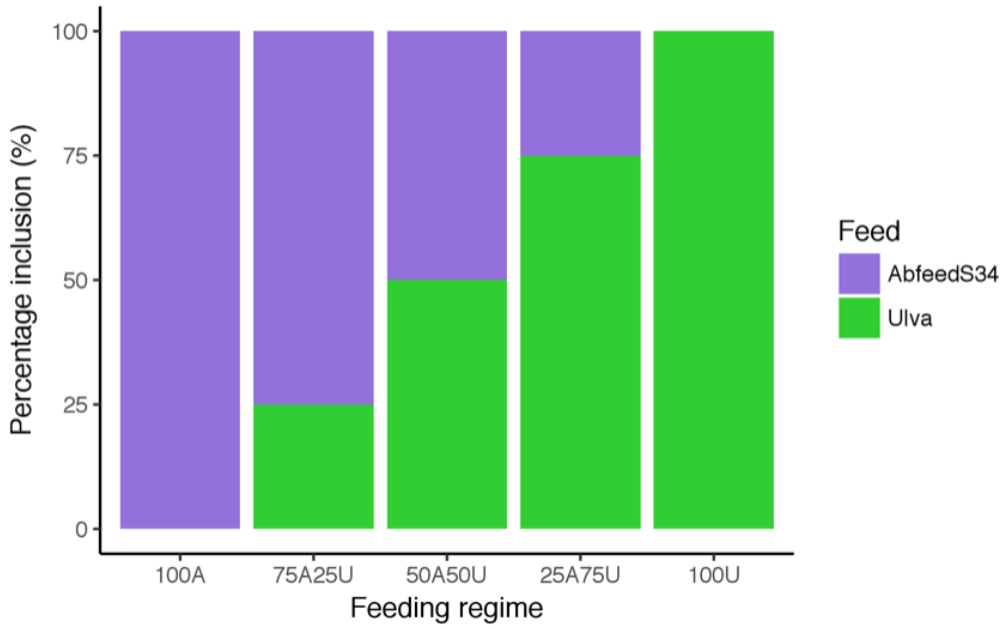

Figure 2-4 Feeding regimes expressed as percentage inclusion of Abfeed™ S34® and fresh *Ulva*. The total feeding ration per basket was offered at 1.27 % BW.day^{-1}, calculated on a dry weight basis for both Abfeed™ S34® and *Ulva*. The feeding regimes were: 100 % Abfeed™ S34® control (100A); 75 % Abfeed™ S34® and 25 % *Ulva* (75A25U); 50 % Abfeed™ S34® and 50 % *Ulva* (50A50U); 25 % Abfeed™ S34® and 75 % *Ulva* (25A75U); and 100 % *Ulva* (100U). All treatments were offered in triplicate.

The feed portion for each feeding regime was pre-weighed prior to feeding and offered consistently in the afternoons after collection of uneaten feed. Abalone were maintained on the feeding regimes from 14 June 2011 - 11 July 2011 (28 days). Each basket was

colour coded using plastic indicators attached to the outer rim of the basket to allow for identification of the specific feeding regime. All feeds were offered to in triplicate. For diet mixtures that had both Abfeed™ S34® and *Ulva*, a mean moisture content of *Ulva* was used to approximate the dry weight equivalent of *Ulva* so as to maintain equal feeding rates across treatments. The *Ulva* was fed after being spun free of excess water and weighed as described in Figure 2-3. On feeding days, all uneaten feed was removed prior to feeding.

2.2.2.4 Data collection

Uneaten feed from each basket was collected by hand at 14h00 after removing the basket from the raceway, without disturbing the abalone that remained attached to the basket walls/ bottom. The uneaten Abfeed™ S34® or fresh *Ulva* from each basket was transferred to separate, labelled, and colour coded containers for each of the three replicate baskets. Uneaten Abfeed™ S34® was dried at 60 °C to a constant weight (24 h) and weighed to the nearest 0.01 g. Uneaten *Ulva* was spun free of excess water and weighed to the nearest 0.01 g. The wet weight of *Ulva* was converted to a dry weight equivalent based on the specific moisture content of *Ulva* calculated at each feeding as previously described in section 2.2.1.4.

Growth of *Ulva* was calculated by placing a known mass of *Ulva*, which had been spun free of excess water, in a basket (blank) at each feeding (basket held no animals and served as a control) and this *Ulva* was then collected, processed and weighed as described above along with uneaten food. An additional control was included to account for the loss of solid matter through leaching for Abfeed™ S34®. This was estimated by placing a known mass of pellets into a basket that held no abalone (the same basket used for assessing *Ulva* growth). Pellets were placed in this basket at each feeding and subsequently removed, dried and weighed along with uneaten food as described above. At the same time, a known weight of Abfeed™ S34®, not offered as feed, was dried and weighed to account for any ambient moisture in the formulated feed.

Consumption of Abfeed™ S34® for each of the three replicate treatments was expressed as a percentage of body weight per day (% $BW.day^{-1}$), corrected for leaching and moisture, according to equation 2.1 of Britz and Hecht (1997):

$$C = \frac{(FL - R)}{TN} \quad \textbf{2.1}$$

where C is consumption (g); F is dry weight of feed offered (g); R is dry weight of remaining feed (g); L is leaching factor; N is number of abalone per replicate; and T is experimental period (days).

Consumption of *Ulva* for each replicate was also expressed as a percentage of body weight per day (% BW.day^{-1}), corrected for growth and moisture, and calculated using equation 2.2:

$$C = \frac{\left(\left(\left(\frac{U_i}{U_m}\right) * \left(\frac{C_f}{C_i}\right)\right) - U_f\right)}{NT} \quad \textbf{2.2}$$

where C is consumption (g); U_i and U_f were the initial and final mass of the *Ulva* in a basket, respectively; U_m was the moisture content of the *Ulva*; C_i and C_f were the initial and final mass, respectively, of the control *Ulva* in the basket with no abalone; N is number of abalone per replicate basket; and T is experimental period (days).

The total consumption for mixed diets was calculated as a combination of both Abfeed™ S34® and *Ulva* and expressed as total feed consumed as a percent body weight per day (% BW.day^{-1}).

Preferential consumption of different feed types, namely Abfeed™ S34® and *Ulva*, for mixed treatments was determined using equation 2.3 that approximates an electivity index (Q) and is described fully in Jacobs (1974) and adapted from Jenkins (1979):

$$Q = \frac{r_i(1 - p_i)}{p_i(1 - r_i)} \quad \textbf{2.3}$$

where r_i is the proportion of feed consumed for feed type *i* and p_i is the proportion of feed available which belonged to feed type *i*. An electivity index, Q, greater than 0 to infinity implies preference for a feed type, a value less than 0 to negative infinity implies selection against the feed type (Jenkins, 1979)

2.2.2.5 Statistical analysis

All statistical analyses were done using R (R Core Team, 2016). Consumption data were normalized to a daily rate, measured as a percent body weight consumed (% $BW.d^{-1}$), for each basket and each feeding. For mixed diets, the combined consumption of Abfeed™ S34® and *Ulva* were used. These data were then summarized with the replicate as the factor level and analysed using a one-way analysis of variance (ANOVA) with significance determined at $p \leq 0.05$. Significant differences were further tested using a post hoc Tukey test. All data were tested for normality and equal variance using Shapiro-Wilk test for normality of residuals and the Bartlett Test of homogeneity of variances ($p = 0.05$).

Jacobs electivity index (Q) was calculated using equation 2.3 for each feeding and the results aggregated using replicate as the factor level. For differences in electivity of each feed type (*i*), the mean Q were tested using the Kruskal-Wallis non-parametric test, as assumptions for normality of residuals and homogeneity of variances were not met. Significant difference in electivity is found with $p \leq 0.05$.

2.2.3 Experiment 2 – Effects of the dietary supplementation with *Ulva* or kelp (*Ecklonia maxima*) on the growth and condition of abalone fed graded levels of formulated feed

To assess the extent to which Abfeed™ S34® could be replaced by supplementation with fresh effluent-grown *Ulva* or wild harvested kelp, without compromising the growth, and condition factor of cultured abalone, a one-year growth trial was conducted at Abagold (Pty) Ltd. To address this objective, abalone were fed with either Abfeed™ S34® at a rate of 0.27 % $BW.day^{-1}$ (Abfeed™ S34® control) or with graded levels of Abfeed™ S34® (100, 80, 60, 40 % of the control group feeding rate) supplemented with fresh effluent grown *Ulva* or wild harvested kelp. All treatment groups except the control, were supplemented with macroalgae at a rate of 0.21 % $BW.day^{-1}$ (DW) where the *Ulva* was grown in abalone effluent water. Abalone were graded to a similar size range before the start of the growth trial to reduce the inter-animal size variability which were managed in line with standard farming practices. No further sorting of abalone was used for the study animals and spanned three sorting periods (not including the initial sorting at the start of the study) from August 2011 - August 2012 (Period 1: 17

Aug – 7 Dec 2011 (112 days); Period 2: 7 Dec 2011 – 27 Mar 2012 (111 days); Period 3: 27 Mar – 17 Aug 2012 (143 days)). The use of fresh kelp forms part of standard farm practices and was included to compare trends between abalone fed on kelp vs *Ulva*. Each experimental period was determined by farm operations and, as a result, Period 3 was one month longer than Periods 1 and 2, which was normalized for calculation of growth parameters.

2.2.3.1 System details

Standard farm production raceways (Figure 2-5) that were stocked with six out of eight oyster mesh production baskets which housed abalone were used for this experiment. Each raceway was cleaned at least once every two weeks and contained six oyster mesh baskets which housed the abalone.

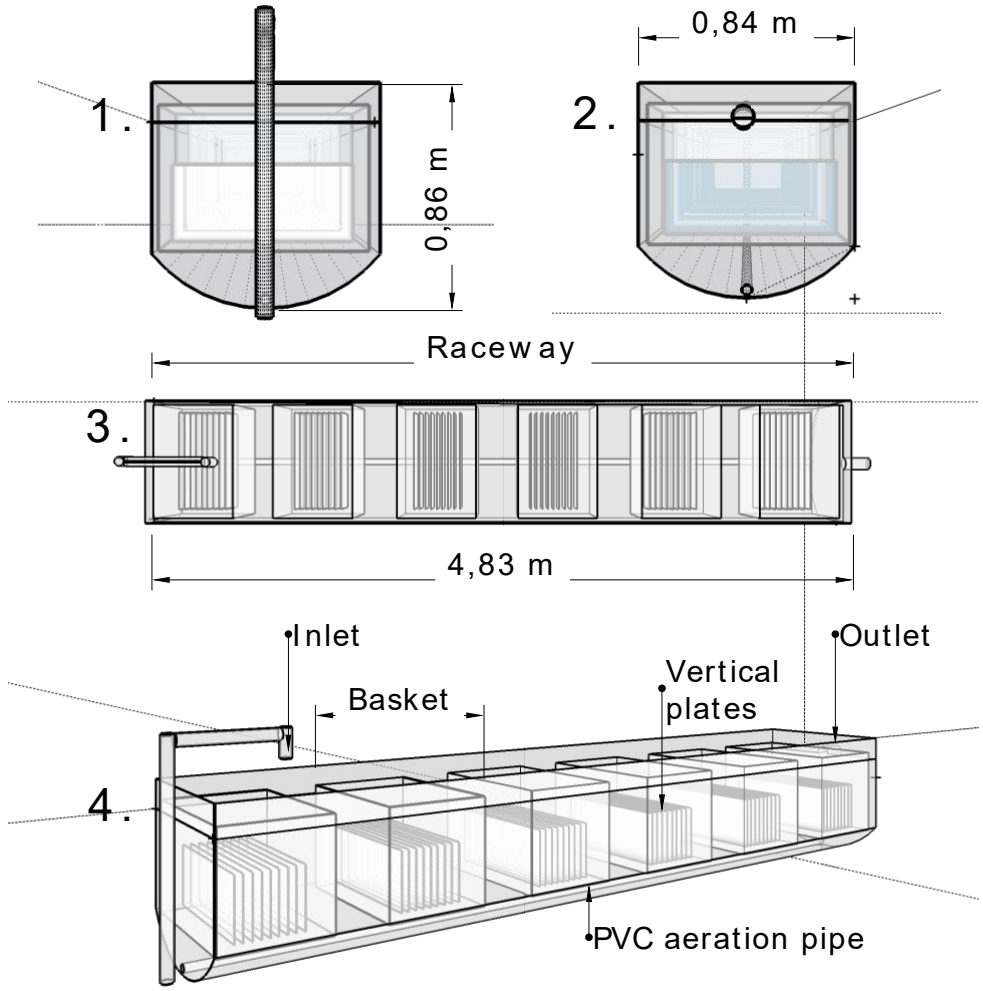

Figure 2-5 Schematic of an abalone production raceway used in the growth trial with; (1) front view, (2) back view, (3) top view, and (4) isometric projection. Each component within the raceway is labelled in (4).

2.2.3.2 Abalone

Abalone that were two years old with a weight of 50 ± 5 g were obtained from two separate farm cohorts (batch spawnings that use different broodstock animals) spawned 2 weeks apart. Due to the large variation in abalone size within cohorts more than 10000 abalone were weighed from each cohort to get two uniform batches of 3600 abalone each. Abalone from the separate cohorts were not mixed and were assigned to either the *Ulva* or kelp experimental group (U and K, respectively) that were treated as

independent experiments and identically. At the start of the growth trial, and after each period, 50 abalone from each basket were weighed and measured and their mean (±SD) weight and length for abalone at the start of the growth trials was (U) 51.10 ± 3.09 g and 64.24 ± 2.07 mm and (K) 51.23 ± 3.43 g and 64.82 ± 2.41 mm. In each experimental group, the abalone were housed in 18 replicate baskets containing 200 individual abalone in each basket. The stocking density in each experimental basket was readjusted to 10 kg by removing animals until the basket weighed approximately 10 kg without sorting (i.e. separating and grouping animals according to size) to maintain random selection. This was done to account for growth from the previous period for optimal system function of the flow-through raceways used for the cultivation of abalone.

2.2.3.3 Diets and feeding regimes

Six dietary feeding regimes were tested and consisted of an Abfeed™ S34® (A) control diet fed at a rate of 0.27% BW.day^{-1}, or graded levels of Abfeed™ S34® (100, 80, 70, 60 and 40 % of the control feeding rate) supplemented with either *Ulva* (U) or kelp (K). The *Ulva* and kelp were fed to animals in the latter 5 treatment groups at a rate of 0.21 % BW.day^{-1} (dry weight approximation). Each experimental group (U and K) consisted of three separate raceways, as described in Figure 2-5, and were set up in a complete randomized block design, with each raceway considered a single replicate and each dietary regime represented only once per raceway. All basket positions were randomised at the start of the growth trial and the relative position within a tank remained constant throughout the growth trial.

Abalone were fed Abfeed™ S34® on Mondays, Wednesdays, and Fridays using feeding cups designed to deliver the desired amount of feed (specified above) for each treatment. The Abfeed™ S34® was offered as a double ration on Mondays and Wednesdays to compensate for the two day feeding intervals (i.e. 0.54 % of the basket weight for the Abfeed™ S34® control and 100, 80, 70, 60 and 40 % of the control feeding rate for the remaining treatments) and a triple ration was offered on Fridays (i.e. 0.81 % of the basket weight for the Abfeed™ S34® control and 100, 80, 70, 60 and 40 % of the control feeding rate for the remaining treatments) for the weekend. This was done in order to conform with the standard farming practices of Abagold (Pty) Ltd.,

as previously outlined in section 2.2.1.4. above. Kelp was offered once a week, as per standard farm practice, whilst the Ulva was offered on Monday, Wednesday and Friday together with the Abfeed™ S34®. The feeding cups used to deliver Abfeed™ S34® were adjusted every two months for both groups (U and K) to account for an increase in basket weight (baseline farm projections which anticipate a growth rate of 1 kg.basket^{-1}.month^{-1}). These feeding rates were readjusted approximately every four months when the stocking densities were readjusted.

2.2.3.4 Data collection

2.2.3.4.1 Temperature

Throughout the growth trial, daily water temperatures for incoming seawater were measured in the settlement tank, prior to being pumped to the farm raceways.

2.2.3.4.2 Growth indices

Over the duration of the growth trial, starting in August 2011 and finishing in August 2012 (366 days), a total of 7200 abalone were weighed and measured after the initial sorting. Abalone were sorted approximately every four months, during which time the length and weight of abalone was determined by randomly selecting 50 abalone from each replicate treatment/basket, weighing the abalone to the nearest 0.01 g and measuring them to the nearest 0.01 mm. The weight and length data collected at each sampling point was used to calculate the following growth parameters:

1) Specific growth rate;
2) Monthly increment in shell length; and
3) Condition factor.

Specific growth rate (SGR as % growth.day^{-1}) was calculated from mean weight of abalone in each treatment (n = 3 replicates) using equation 2.4:

$$SGR = \left(\frac{ln(W_f) - ln(W_i)}{t}\right) * 100 \qquad \textbf{2.4}$$

where $\ln(W_f)$ is the natural log of the mean final weight of abalone, $\ln(W_i)$ is the natural log of the mean initial weight of abalone, and *t* is time in days.

Monthly increment in shell growth (MISL, in mm) was calculated from mean length of abalone in each treatment (n = 3 replicates) using equation 2.5:

$$MISL = \left(\frac{SL_f - SL_i}{t}\right) * 30 \qquad \textbf{2.5}$$

where SL_f is the mean final length, SL_i is the mean initial length, *t* is the time in days, and 30 days was considered one month.

The condition factor (CF) was calculated according to the equation derived by Britz (1996) which quantifies the average size of an abalone shell in relation to the body of an abalone. A CF of '1' is considered average and above '1' is considered above average. The CF was calculated from mean length and weight of abalone in each treatment (n = 3 replicates) using equation 2.6:

$$CF = \left(\frac{Weight\ (g)}{Length\ (mm)^{2.99}}\right) * 5575 \qquad \textbf{2.6}$$

2.2.3.4.3 Tissue glycogen

Tissue glycogen samples were taken at the end of the growth trial from abalone. For each replicate unit, three abalone were processed in an identical way from each replicate treatment. The selection of abalone was done randomly and the abalone were collected from their respective treatments and were placed shell side down on a bed of ice. The foot muscle was dissected away from the shell and a portion of the abalone foot marked as 'A' in Figure 2-6 was stored in sterile 1.5 mL micro centrifuge tubes that were flash frozen in liquid nitrogen. These samples were then stored at -80°C until they were used for the quantification of muscle glycogen content using the phenol-sulphuric method for quantification of glucose (S1).

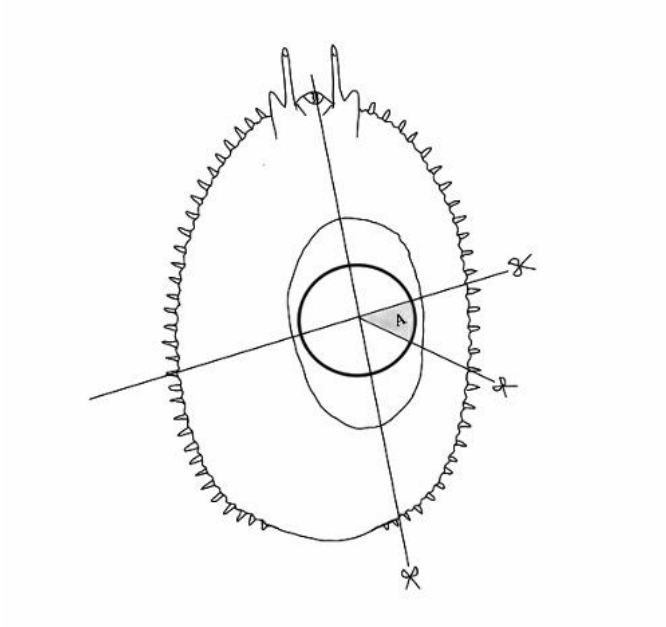

Figure 2-6 Schematic representation of the abalone dissection for muscle tissue glycogen collection. The sample was taken from the area labelled 'A', highlighted in grey.

2.2.3.5 Statistical analysis

All statistical analyses were carried out using R (R Core Team, 2016). The effect of treatment on specific growth (SGR) rate and monthly increment in shell length (MISL) for the entire period were compared using a one-way ANOVA with Treatment as a factor. The effect of time on SGR/MISL within each treatment was compared using a one-way ANOVA with Time as a factor. For comparison of condition factor (CF), a one-way ANOVA was used to determine significance for the initial and final CF separately, as growth is assumed to change as animals age and the relative ratio will not be comparable. A paired students t-test was used to compare differences between periods within each treatment group. Differences in tissue glycogen content for abalone maintained on different dietary treatments were compared using a one-way ANOVA. Assumptions of normality of distribution of the residuals and homogeneity of variance were met and significance was determined with $p \leq 0.05$. A power analysis was not performed prior to data collection. A retrospective power analysis to indicate power for outcomes already observed is conceptually flawed and analytically misleading (Zhang et al., 2019) therefore the post hoc test was not used for this data.

Further comparison of abalone weight were made using linear mixed-effects analysis using the nlme package (Pinheiro, Bates & R-core, 2016) for the relationship between weight and treatment over time. In contrast to a more traditional approach, with data aggregation and repeated-measures ANOVA analysis, this analysis allowed for control of the variance associated with random factors without data aggregation. For fixed effects, time in days and treatment (without interaction term) were used in the final model that included data for all four sampling points across one year. For random effects the individual basket nested within replicate was considered the most appropriate slope structure. Furthermore, an effect of basket variance for the effect of time was used to account for heteroscedasticity. Visual inspection of residual plots revealed obvious deviations from homoscedasticity (Figure 2-7 A) and a log transformation of weights was used in an attempt to improve the residual spread. However, this alone did not improve spread (Figure 2-7 B), and the variance structure was further controlled by allowing a unique variance at each time point, which achieved homoscedastic spread of residuals (Figure 2-7 C).

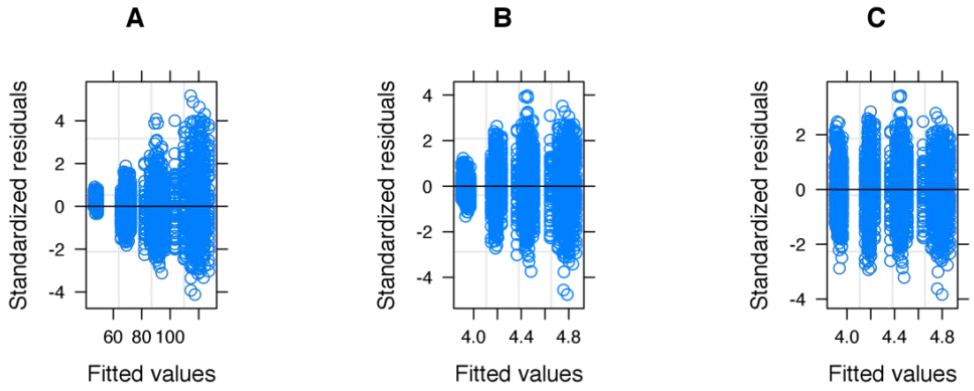

Figure 2-7 Plots of residual for the models under development (log_weight ~ days * treatment) in which heteroscedacity was evident for plot A and B. The data were; (A) untransformed, (B) log transformation of raw data, and (C) weighted variance structure for time. The final model included both log transformation and a weighed variance structure to account for heteroscedastic data plotted in (C).

The initial model included beyond-optimal conditions that included all explanatory variables and interactions and used a stepwise procedure to find the optimal random

slope structure for each random intercept under restricted maximum likelihood (Barr et al., 2013). The maximum-likelihood estimation was used with a backwards stepwise procedure to test the fixed effects and this process was repeated until no additional explanatory variables could be removed. To assess the optimal random effects structure, all nested effects were included and a top-down strategy for model selection was performed as described in the steps below:

- Step 1 - For the model selection, the fixed effects (time and treatment) were included to contain beyond-optimal interactions of Time:Treatment.
- Step 2 - The beyond-optimal model for fixed effects was then tested to determine the optimal structure of the random component. The model was fitted to contain no random effects using generalized least squares, random effects with random slope, and random effects with random intercept and slope using linear mixed-effects models.
- Step 3 - Several models were tested and compared using Akaike Information Criteria (AIC) to identify the ideal variance structure using Restricted Maximum Likelihood Estimation (REML).
- Step 4: The best fit model based on the lowest AIC was then fitted and assumptions were tested. The residuals were normally distributed but, there was evidence of heteroscedasticity as the residual spread is larger for larger fitted values.
- Step 5 - The response variable (weight) was log transformed to address the increase in spread of residuals and the model variance structures were weighted by time to account for different variance at each time point.
- Step 6 - Comparison of the new model, with a modified variance structure was compared to the old model by ANOVA.
- Step 7 - The optimal fixed structure for the model was tested using Maximum Likelihood (ML) estimation and comparison of the interaction by ANOVA.
- Step 8 - The optimal model was refitted with REML and the outputs validated.

2.3 Results

2.3.1 Experiment 1: Abalone consumption rate and feed preference when fed dietary *Ulva* as a partial or complete replacement of formulated feed

The incorporation of small amounts (25 %) of *Ulva* in the dietary regime 75A25U significantly improved total feed consumption by approximately 90% (0.38 ± 0.10 % BW.day^{-1}), when compared with the 100 % Abfeed™ S34® (100A; 0.20 ± 0.08 % BW.day^{-1}) and 100 % *Ulva* (100U; 0.2 ± 0.02 BW.day^{-1}) dietary regimes ($F = 4.034$, $p = 0.0335$; Figure 2-8). However, additional replacement of Abfeed™ S34® with higher amounts of *Ulva* (50 % and 75 %) did not lead to an additional increase in total consumption, but instead there was an apparent decrease in the mean total consumption from the 25 % *Ulva* inclusion. The total consumption of the latter two mixed diets was not significantly different from the 100 % Abfeed™ S34® or 100 % *Ulva* controls. In the treatment 50A50U abalone were found to consume *Ulva* at a rate of 0.19 % BW.day^{-1} and they were able to consume a comparable amount of Abfeed™ S34® (0.21 % BW.day^{-1}) as the abalone that were fed only Abfeed™ S34® 0.20 % BW.day^{-1}). Furthermore, there was no significant difference in total feed consumption between the 100 % Abfeed™ S34® or 100 % *Ulva* controls.

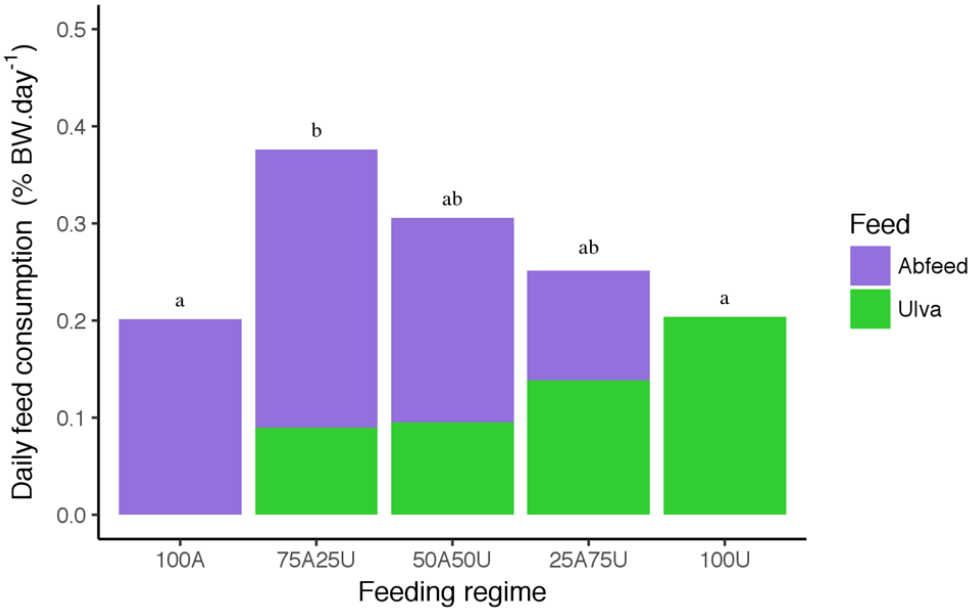

Figure 2-8 Mean daily consumption by abalone (mean (±SD) weight of 67.30 ± 5.49) given the five feeding regimes. Total feed (Abfeed™ S34®, *Ulva*, or a combination of each) was offered at a mean daily rate of 1.27 % BW.day^{-1}. The feeding regimes were; 100 % Abfeed™ S34® control (100A), 75 % Abfeed™ S34® and 25 % *Ulva* (75A25U), 50 % Abfeed™ S34® and 50 % *Ulva* (50A50U), 25 % Abfeed™ S34® and 75 % *Ulva* (25A75U), and 100 % *Ulva* only (100U). The contribution of Abfeed™ S34® to the total consumption is represented by the top stack of each bar for mixed feed (greyscale correction). The post-hoc Tukey HSD test was used to determine significance levels which are denoted by the letters above the bars. A different superscript denotes a significant difference at p = 0.05.

2.3.1.1 Jacobs Index

Both feed types, Abfeed™ S34® and *Ulva*, elicited a significant positive selection (Kruskal-Wallis, p = 0.027) when assessed using the Jacobs electivity index (equation 2.3) described in section 2.2.2.4. As the incorporation of Abfeed™ S34® was decreased from 75 % to 25 %, through graded replacement with *Ulva*, there was a significant increase in the abalone's electivity for Abfeed™ S34®. In contrast, as the incorporation of *Ulva* was increased from the 25 % treatment to the 75 % treatment, there was a significant decrease in the abalone's electivity for *Ulva* albeit still positive selection. Although a significant effect of treatment was observed, all treatments displayed positive selection for both feed types and in treatment 75A25U, abalone displayed a

greater elective feeding preference for *Ulva*, with Q = 1.09 compared with Q = 0.36 for the Abfeed™ S34® (Figure 2-9). When abalone were offered both feeds in equal quantities (dry weight) the abalone showed a high selection for Abfeed™ S34® of Q = 1.46 compared to Q = 0.17 for *Ulva*.

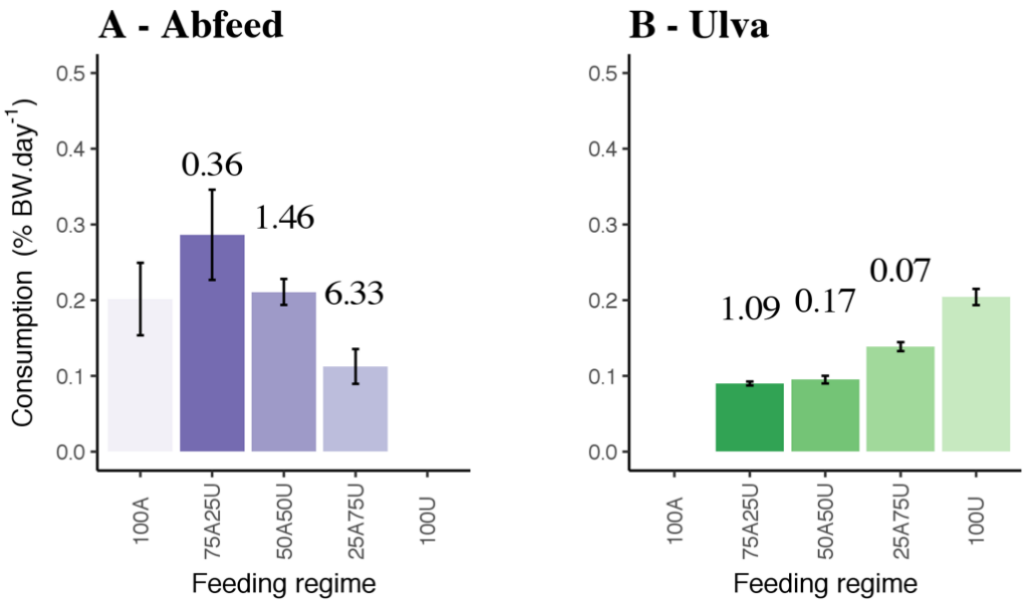

Figure 2-9 Mean (±SE) daily consumption of (A) Abfeed™ S34® and (B) *Ulva* by abalone in the five feeding regimes. Feeding regimes were Abfeed™ S34® only (100A), a mixture of Abfeed™ S34® and *Ulva* in which Abfeed™ S34® was replaced with *Ulva* at graded levels (75A25U, 50A50U, 25A75U), or *Ulva* only (100U). All dietary treatments were offered in triplicate. Standard error is displayed on the bars. Jacobs electivity index is represented above the bars and correspond to either Abfeed™ S34® (A) or *Ulva* (B) in the different feeding regimes.

2.3.2 Experiment 2: Effects of the dietary supplementation with *Ulva* or kelp (*Ecklonia maxima*) on the growth and condition of abalone fed graded levels of formulated feed

2.3.2.1 Farm water temperatures

The farm growth trial was conducted over one year, from August 2011 to August 2012, with three growth periods as highlighted in the temperature profile of Figure 2-10. The daily temperature marked by the solid grey line showed at least one upwelling event for each growth period in the months of November 2011 (Period 1: 17 Aug – 7 Dec 2011),

January 2012 and February 2012 (Period 2: 7 Dec 2011 – 27 Mar 2012), and in April 2012 (Period 3: 27 Mar – 17 Aug), which consisted of a periodic decrease in temperatures. No mortalities were reported during any of these upwelling events. Maximum and minimum recorded temperatures for the year, indicated by the red and blue dashed lines were 21.2 ° C and 9.8 ° C respectively, with an average temperature of 15.72 ° C denoted by the dashed black line over the duration of the study period. The annual pattern was visualized using a locally weighted regression loess smooth as described by Cleveland (1979) and marked by the solid black line. The loess smooth was fitted to the raw data as a visual aid and the standard error is displayed as the grey ribbon on the line.

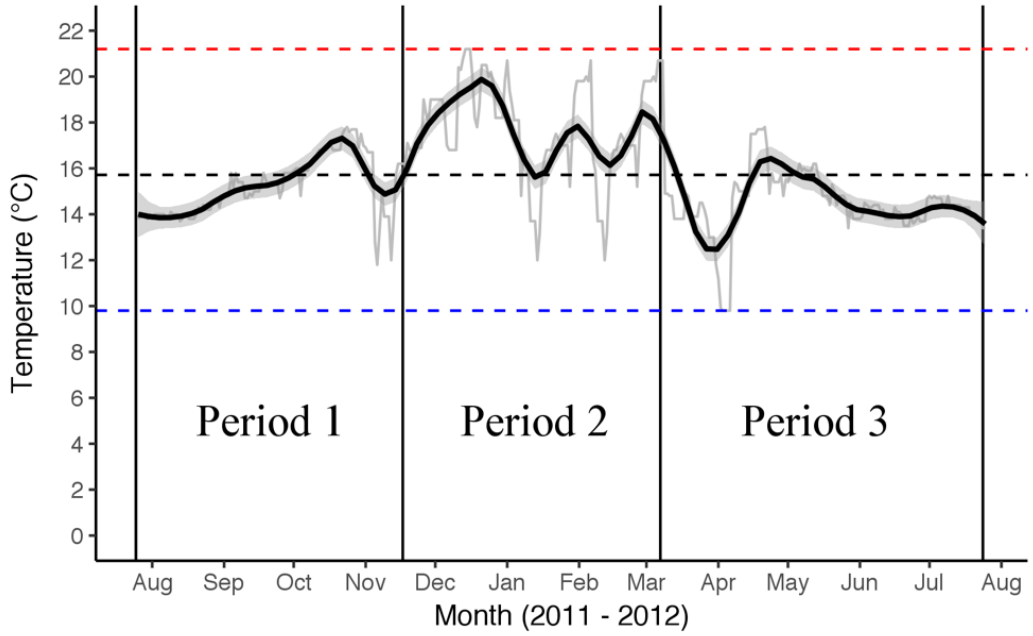

Figure 2-10 Daily farm temperature recordings from Abagold (Pty) Ltd for the year 20 August 2011 - 20 August 2012. Maximum and minimum temperatures are marked by the red and blue dashed lines respectively, with the solid black line illustrating the annual pattern by plotting a locally weighted regression loess smooth fitted to the raw data. Vertical lines indicate the periods between sampling and correspond to the farm's sorting cycle. The cumulative days from start are; Period 1 = 111 days, Period 2 = 226 days and Period 3 = 366 days. Period 3 was 20 days longer than Period 1 due to unavoidable farm sorting logistics.

2.3.2.2 Effects of macroalgae supplementation on abalone growth parameters

2.3.2.2.1 Specific growth rate (SGR)

The specific growth rate (SGR), measured as percentage weight gain per day, was not statistically significantly different between any of the dietary treatments supplemented with either *Ulva* (Figure 2-11 A; $F = 0.51$, $p = 0.764$) or kelp (Figure 2-11 B; $F = 0.729$, $p = 0.615$); when compared using a one-way ANOVA for the entire growth trial. Conversely, abalone in all treatments supplemented with macroalgae outperformed their respective Abfeed™ S34® controls, with the best performing dietary treatment recorded being 70AU (for the *Ulva* trial) and 80AK (for the kelp trial); which attained a mean (±SE) SGR of 0.24 ± 0.01 % BW.day^{-1} and 0.26 ± 0.01 % BW.day^{-1} respectively. The SGR for abalone maintained on 100AU for Period 2 (112 – 226 days) was 0.25 ± 0.01 % BW.day^{-1} and was significantly higher than the recorded SGR during Period 1 (0 – 111 days) of 21.2 ± 0.01 % BW.day^{-1} (Figure 2-11 C). No significant differences in SGR within dietary treatments for abalone supplemented with kelp were recorded between sampling periods (Figure 2-11 D).

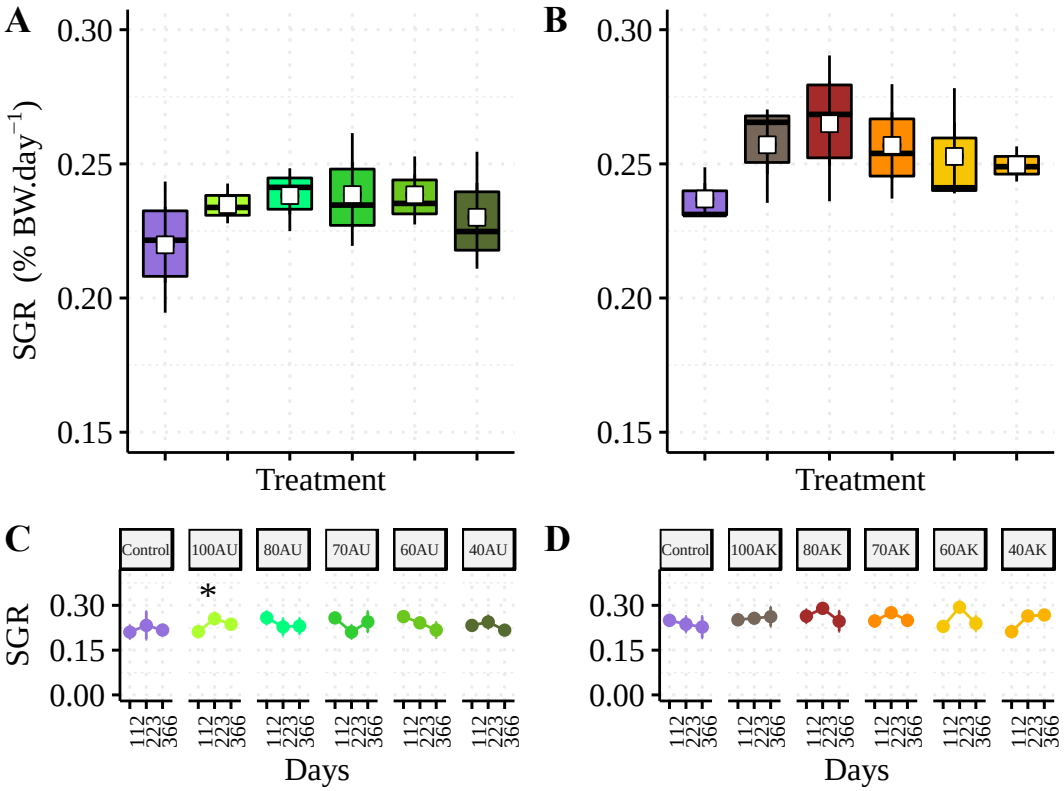

Figure 2-11 (A & B) Mean (white square), median indicated by the thick black line with the box extending to the 1st (lower) and 3rd (upper) quartiles, whiskers represent 2.5 time the interquartile range with outliers indicated by black dots of abalone weights measured between August 2011 and August 2012. (C & D) Mean (±SE) specific growth rate (SGR) for abalone over the growth period from August 2011 - August 2012. Six feeding regimes were compared; an Abfeed™ S34® only Control, followed by varying the amount of Abfeed™ S34® whilst supplementing with either *Ulva* (A) or kelp (B). The amount of Abfeed™ S34® offered to the treatment diets was 100, 80, 70, 60, 40 percent of the Abfeed™ S34® control with the addition of macroalgae. All treatments were offered in triplicate with significant differences indicated by the *.

2.3.2.2.2 Monthly increment in shell length (MISL)

Abalone shell growth, calculated as a monthly increment in length (MISL), showed a similar trend to the percent weight gain and was not statistically significantly different between any of the treatments supplemented with either *Ulva* (Figure 2-12 A; one-way ANOVA, F = 0.455, p = 0.802) or kelp (Figure 2-12 B; F = 0.614, p = 0.691). The best performing dietary treatments in each experiment were the 70AU and 80AK treatments, which attained a mean (±SD) MISL of 1.97 ± 0.12 mm.month^{-1} and 2.08 ± 0.16

mm.month^{-1}, respectively. In both experiments (U and K treatments) all treatments supplemented with macroalgae outperformed their respective Abfeed ®S34 controls, but these differences were not statistically significant.

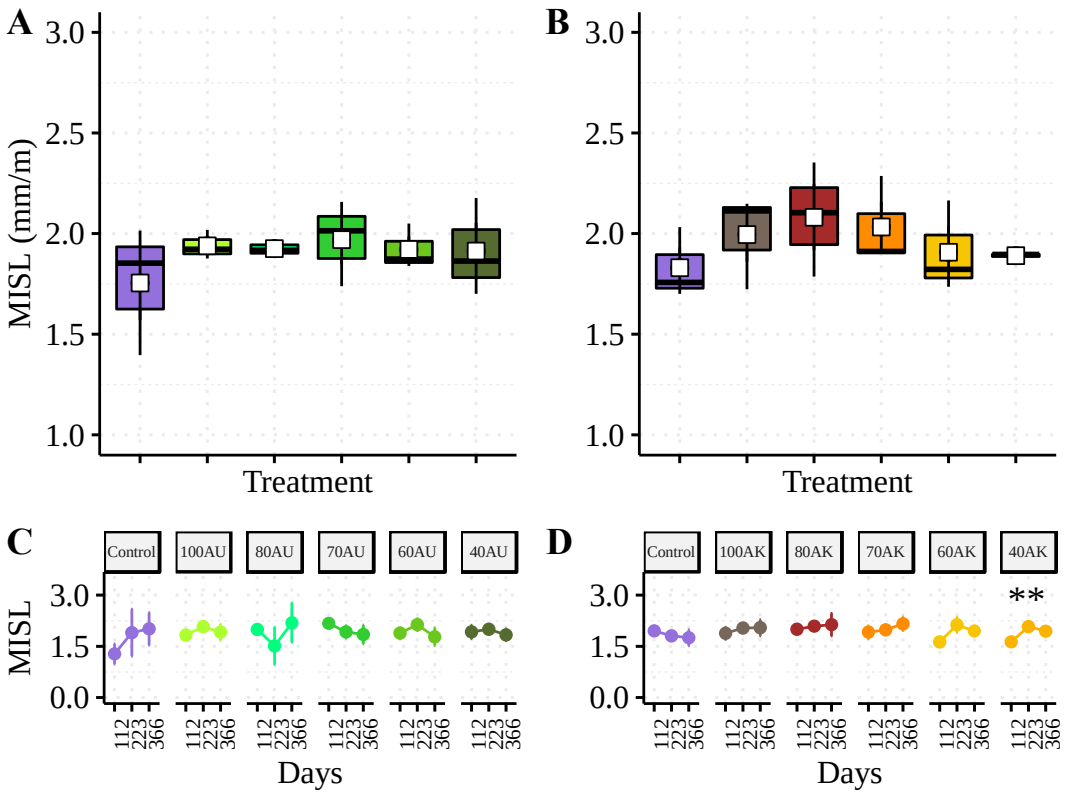

Figure 2-12 Mean (±SE) monthly increment in shell length (MISL) for abalone over the growth period from August 2011 - August 2012. Six dietary treatments were compared; an Abfeed™ S34® only Control, followed by varying the amount of Abfeed™ S34® whilst supplementing with either *Ulva* (A) or kelp (B). The amount of Abfeed™ S34® offered to the treatment diets was 100, 80, 70, 60, 40 percent of the control with the addition of macroalgae. All treatments were offered in triplicate and no significant differences were recorded.

2.3.2.2.3 Condition factor (CF)

The CF for abalone was compared between feeding regimes within each treatment group (U or K) at the start of the experiment and at after each period (Figure 2-13). No statistically significant differences were recorded between feeding regimes at either the initial or final measurements for abalone supplemented with either *Ulva* (Figure 2-13 A) or kelp (Figure 2-13 B), when compared using a one-way ANOVA. In contrast, the

CF of abalone in all treatment groups supplemented with *Ulva* were significantly lower ($p \leq 0.001$) at the end of the experiment when compared to the initial assessment. The abalone in treatment 70AK and 40AK had significant effect of time on the CF however, there were no statistically significant difference between initial and final CF for all treatments (Figure 2-13 B). The mean CF of all abalone in this study always remained above average (CF > 1).

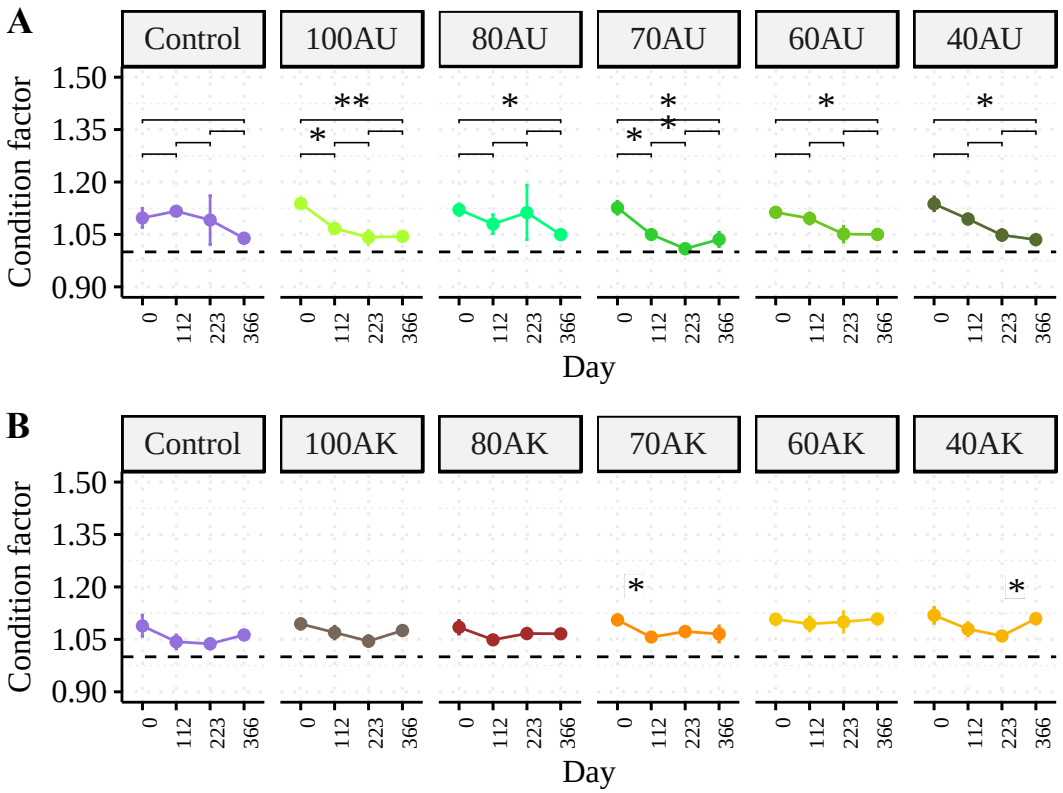

Figure 2-13 Mean (±SE) condition factor for abalone at the start (August 2011) and end (August 2012) of the feeding trial. Six feeding regimes were compared; an Abfeed™ S34® only Control, followed by varying the amount of Abfeed™ S34®, whilst supplementing with either *Ulva* (A) or kelp (B). The amount of Abfeed™ S34® offered to the treatment diets was 100, 80, 70, 60, 40 percent of the Abfeed™ S34® control with the addition of macroalgae. All treatments were offered in triplicate. The significance is denoted by the stars with; $p \leq 0.001$ marked as ***, $p \leq 0.01$ marked as **, $p \leq 0.05$ marked as *.

2.3.2.3 Effect of time and treatment on the weight of abalone

The weights of abalone recorded at each sampling point during the growth trial were analysed using linear mixed-effects regression for both the U and K experimental groups. For abalone in the experimental group U, there was a significant effect of time on weight increase with $F(1, 3581) = 3480.5$, $p < 0.001$ (Table 1). However, the effect of treatment was not significant with $F(5, 12) = 0.2$, $p = 0.966$.

Table 1 Summary table for the final mixed effects model used to determine the effect of time and treatment on the growth of abalone fed diets supplemented with *Ulva* as a measure of weight. Six feeding regimes were compared; an Abfeed™ S34® only Control, followed by varying the amount of Abfeed™ S34® whilst supplementing with *Ulva*. The amount of Abfeed™ S34® offered was 100, 80, 70, 60 and 40 percent of the control portion with the addition of Ulva. The significance is denoted by the stars in the right-hand column with; p ≤ 0.001 marked as *.**

Fixed effect	Value	Std.Error	DF	t.value	p.value	
(Intercept - Control)	3.928	0.012	3581	339.382	<0.001	***
Days	0.002	0.000	3581	58.996	<0.001	***
Treatment: 100AU	0.002	0.016	12	0.095	0.926	
Treatment: 80AU	0.001	0.016	12	0.052	0.959	
Treatment: 70AU	0.006	0.016	12	0.358	0.727	
Treatment: 60AU	0.013	0.016	12	0.797	0.441	
Treatment: 40AU	0.003	0.016	12	0.159	0.877	

Similarly, for abalone in the experimental group K, there was a significant effect of time on weight increase, with $F(1, 3573) = 3937.7$, $p < 0.001$ (Table 2.2). In contrast to experimental group U, a significant effect of treatment was found, with $F(5, 12) = 3.5$, $p = 0.0347$, and abalone within treatment 80AK were found to be significantly larger in weight when compared to the Abfeed™ S34® control (Table 2).

Table 2 Summary table for the final mixed effects model used to determine the effect of time and treatment on the growth of abalone fed diets supplemented with kelp as a measure of weight. Six feeding regimes were compared; an Abfeed™ S34® only Control, followed by varying the amount of Abfeed™ S34® whilst supplementing with kelp. The amount of Abfeed™ S34® offered to the treatment diets was 100, 80, 70, 60, 40 percent of the control portion with the addition of kelp. The significance is denoted by the stars in the right-hand column with; p ≤ 0.001 marked as ***, p ≤ 0.01 marked as **, and p ≤ 0.05 marked as *.

Fixed effect	Value	Std.Error	DF	t.value	p.value	
(Intercept - Control)	3.920	0.008	3573	511.523	<0.001	***
Days	0.003	0.000	3573	62.751	<0.001	***
Treatment: 100AK	0.011	0.011	12	1.039	0.319	
Treatment: 80AK	0.029	0.011	12	2.751	0.018	*
Treatment: 70AK	0.019	0.011	12	1.799	0.097	
Treatment: 60AK	0.006	0.011	12	0.607	0.555	
Treatment: 40AK	-0.011	0.011	12	-1.006	0.334	

There was a higher mean final weight for all feeding regimes supplemented with *Ulva* (Figure 2-14 A) when compared to the Abfeed™ S34® control diet, albeit not statistically significant. The best performing feeding regime, in terms of mean final weight, was 60AU with a mean (±SD) final weight of 122.52 ± 8.11 g. There was a marginally greater recorded mean final weight for abalone fed diets supplemented with *Ulva* in the order of 60AU > 70AU > 80AU > 100AU when compared to the Abfeed™ S34® control. Although the latter trend was not evident at all sampling points, treatment 60AU consistently attained the greatest mean weight for each sampling point, with the Abfeed™ S34® control outperforming 100AU in growth Period 1 (see Figure 2-10 for periods).

Similarly, abalone growth (mean weight) for all feeding regimes supplemented with kelp were greater than the Abfeed™ S34® control, with the feeding regime 80AK being statistically significant from all other treatment groups (Figure 2-14 B; p = 0.018). Similar to the abalone in the *Ulva* experiment, greater mean weights were recorded for

dietary regimes supplemented with kelp; with the exception of dietary regime 40AK, which only outperformed the Abfeed™ S34® control by the final sampling.

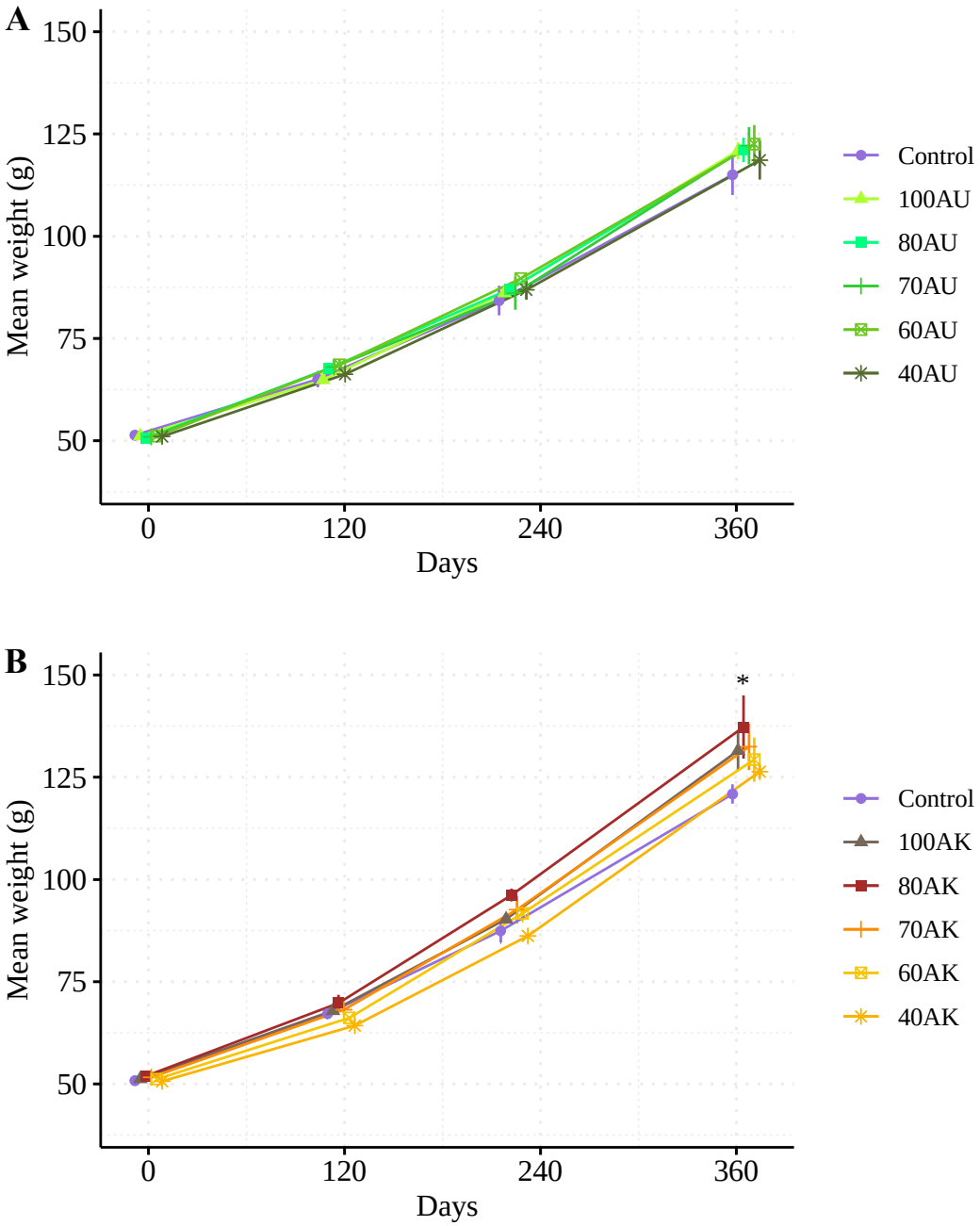

Figure 2-14 Mean (±SE) weight of abalone measured every four months from August 2011 - August 2012. Six dietary treatments were compared; an Abfeed®S34 only Control, followed by varying the amount of Abfeed®S34 whilst supplementing with either *Ulva* (A) or kelp (B). The amount of Abfeed®S34 offered to the treatment diets was 100, 80, 70, 60, 40 percent of the Abfeed®S34 control with the addition of macroalgae. All treatments were offered in triplicate. Points are separated for clarity; the time of sampling was the same for treatments in each group at each sampling time/point.

2.3.2.1 Effect of treatment on muscle glycogen content

The mean glycogen content in the muscle tissue of abalone was compared between feeding regimes within the treatment group supplemented with fresh effluent grown *Ulva* at the end of the experimental period. No statistically significant differences for mean glycogen content were recorded between feeding regimes for abalone supplemented with *Ulva* when compared using a one-way ANOVA (Figure 2-15). The mean tissue glycogen content across all treatment groups ranged from 65.3 – 92.5 mg of glycogen per gram of muscle tissue with the lowest/highest recorded values in 70AU (18.6 mg/g) and the highest in 60AU (137.4 mg/g).

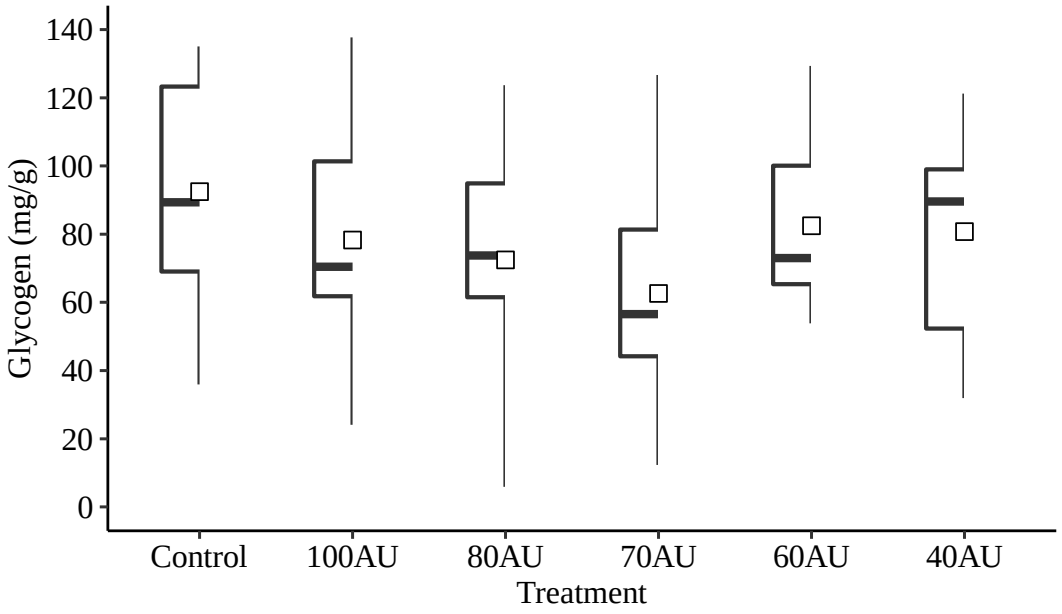

Figure 2-15 Mean (white square), median indicated by the thick black line with the box extending to the 1st (lower) and 3rd (upper) quartiles, whiskers represent 2.5 time the interquartile range, and the scatter points plotted on each treatment (a jitter was applied to the points as to ensure no overlapping occurs in the plotting) represents individual abalones tissue glycogen content measured in August 2012. Six dietary treatments were compared; an Abfeed™ S34® only Control, followed by varying the amount of Abfeed™ S34® whilst supplementing with *Ulva* (A). The amount of Abfeed™ S34® offered to the treatment diets was 100, 80, 70, 60, 40 percent of the control with the addition of macroalgae. All treatments were offered in triplicate and no significant differences were recorded.

2.4 Discussion

2.4.1 Summary

This study demonstrated that dietary supplementation with *Ulva* or kelp can be used to reduce the reliance on the use of the formulated diet Abfeed™ S34® as a feed for abalone *Haliotis midae*, without compromising growth or condition of abalone under culture conditions. As much as 60 % of the fixed feeding ration for Abfeed™ S34® (0.27 % BW.day^{-1}) could be supplemented with either *Ulva* or kelp over a one year feeding experiment without negatively altering specific growth rate (SGR), monthly increment in shell length (MISL), or condition factor (CF) of the abalone. The inclusion of fresh *Ulva* into the feeding regime of abalone, when offered alongside Abfeed™ S34®, increased the daily feed consumption by 90 % when compared to abalone fed single diets of either *Ulva* or Abfeed™ S34®. The results of this study also show that when abalone were fed a combination of Abfeed™ S34® and *Ulva* (25A75U) they showed increased preference for the Abfeed™ S34®. Previous literature has reported improved growth of *H. midae* when fed effluent-grown *Ulva* as part of a mixed diet (Naidoo et al., 2006; Dlaza, Maneveldt & Viljoen, 2008; Robertson-Andersson, Maneveldt & Naidoo, 2011) however, this study is the first to show that *Ulva* can successfully reduce reliance on dry formulated feed, without negatively affecting the condition or growth of abalone under culture conditions.

2.4.2 Interpretations

The evaluation of a feed for an aquaculture species must consider its palatability and consumption by the desired species (Glencross, Booth & Allan, 2007). A study by Bansemer et al. (2016), in which *Ulva* protein content was enriched (process not clear), found that *H. laevigata* fed with enriched *Ulva* had higher consumption rates than those fed with wild *Ulva*. A study by Angell et al. (2012) tested the effects of *Ulva*, and a range of macroalgae with varying nutritional profiles, on the feeding preference of *H. asinina* and found it is unlikely that the nutritional value of *Ulva* is acting as a feeding cue. Work done by Cyrus et al. (2015) demonstrated that the sea urchin *Tripneustes gratilla* exposed to a feeding cue released by *Ulva lacinulata* (previously known as *Ulva rigida*) exhibited a positive chemosensory response to the feed. The above studies present *Ulva* as positive feeding cue and in this study abalone demonstrated positive

selectivity for both *Ulva* as well as Abfeed™ S34® in mixed diets with a preference for a high Abfeed™ S34® to *Ulva* ratio (75:25). In contrast *Ulva fenestrata* has been shown to produce large amounts of acrylic acid in response to feeding, which has been shown to deter feeding in the sea urchin *Strongylocentrotus droebachiensis* (Alstyne et al., 2003). Given that Wild Coast Abalone have been growing large amounts of *Ulva* for feed since 2000 (Bolton et al., 2009), coupled with the significant increase in overall consumption when supplemented with fresh *Ulva* that has been documented in this study, it appears that *H. midae* is not deterred by chemical defences of *U. lacinulata*. The increase in mean total consumption rate of abalone when being offered a relatively small portion of *Ulva* (75A25U) compared with abalone fed single diets of both *Ulva* or Abfeed™ S34®, suggests that dietary supplementation with fresh *Ulva* improves overall consumption and may enhance the feeding efficiency of abalone.

Formulated feeds are prone to a high rate of nutrient leaching during the prolonged stay of feed in water (Sales & Janssens, 2004) where changes in feeding behaviour of abalone will have implications for the management of feeding regimes. The high leaching rates reported for formulated feeds means that the feeding efficiency of abalone can have an impact on the system productivity. Currie et al. (2016) demonstrated that abalone fed fresh *Ulva* initiate feeding more rapidly, spend more time feeding and move less frequently than abalone feeding on formulated feeds alone, where abalone moved frequently and grazed on the softened portion of formulated feed only partially consuming pellets before moving on. The ingestion rates of formulated feeds by the abalone *H. laevigata* × *H. rubra* were found to be very low when compared to macroalgal diets and that may impact the cumulative amount of feed consumed in a single feeding event (Mulvaney, Winberg & Adams, 2013). The findings from the above studies in conjunction with findings from this study indicate abalone may exhibit different feeding behaviours for different feed types, yet they are able to consume comparable amounts of feed based on a dry weight comparison. For the feeding regime 75A25U, abalone had a greater selectivity for *Ulva* (Q = 1.09) compared to Abfeed™ S34® (Q = 0.36) and this trend was reversed when the amount of Abfeed™ S34® was decreased to 25 % (25A75U) of the total feed mixture with abalone showing a high consumption and selection for the Abfeed™ S34® (Q = 6.33; 0.11 % BW.day^{-1}) compared to the *Ulva* (Q = 0.07; 0.14 % BW.day^{-1}). Additional dietary replacement of

total feed with effluent-grown *Ulva* (50A50U, 25A75U, 100U feeding regimes) did not cause any further increase in consumption by abalone in this study, but instead mean daily consumption tended to decrease with increasing *Ulva* supplementation beyond 75A25U (Figure 2-8). Based on the findings of Currie et al. (2018) it is hypothesised that abalone consuming more *Ulva* would spend extended periods of time feeding on the macroalgae in order to consume comparative amounts of the formulated feeds. As the amount of *Ulva* is increased, the effect on abalone feeding behaviour may reduce the benefits of improved consumption that was reported in the feeding regime 75A25U. This is further supported by the reduction in formulated feed consumed when abalone were offered increasing amounts of *Ulva* relative to the Abfeed™ S34® portion. These results indicate that the inclusion of *Ulva* into the feeding regime of abalone improves the preference of abalone for Abfeed™ S34® and enhances their feeding efficiency when the abalone are fed on the mixed diets with a preference for a high Abfeed™ S34®:*Ulva* ratio.

The contribution of dietary ingredients to the growth of an organism may vary between species. For example, a study by Peckol & Putnam (2017) demonstrated that *Ulva* was preferentially grazed upon by the snail *Littorina littorea*, but the snail exhibited relatively slow growth rates when compared to the control. The authors of the above study suggest that there may be antinutritional impacts of *Ulva* which reduce the digestibility and assimilation of nutrients and therefore negatively impacted growth rates. Abalone in the treatments supplemented with macroalgae in this study achieved the highest SGR, albeit not statistically different to the others. Similarly Dlaza et al. (2008) recorded the highest SGR of 1.05 % BW.day^{-1} for small *H. midae* (0.2 - 9 g) when fed a combination of Abfeed™, *Ulva*, and kelp compared to a abalone fed on Abfeed™ alone. The results of this study, along with those of Dlaza et al. (2008), show that the combination of Abfeed™ and macroalgae in feeding regimes for abalone can improve growth rates. The improved growth rates reported by Naidoo et al. (2006), Dlaza et al. (2008) and Robertson-Andersson, Maneveldt & Naidoo (2011) for small *H. midae* (0.2 – 27.8 g) fed with *Ulva* and/ or kelp, alongside the results for SGR in this study, indicate that any antinutritional components in *Ulva* are likely not negatively affecting growth rates of abalone across a wide weight range.

During Period 2 of this study the abalone in the 100AU treatment had a significant increase in SGR from 0.21 % BW.day^{-1} to 0.25 % BW.day^{-1} with no other significant increases in SGR reported for the same time period. In a study by Nel et al. (2017) the inclusion of dried kelp into formulated feeds was able to improve the feed conversion ratios and protein efficiency in *H. midae* when compared to an Abfeed™ control and resulted in the development of a more stable gut microbial community (Nel et al., 2018). Formulated feeds offered to *Haliotis rufescens* led to increased carbohydrase activity in the gut when compared to kelp, which was reported to be due to the high surface area of the formulated feeds promoting high numbers of bacteria that facilitate its digestion (Garcia-Esquivel & Felbeck, 2006). The water temperature in this study varied from 9.8 °C - 21.2 °C and during the second sampling period the average water temperature remained consistently above average (15.72 °C; Period 2; Figure 2-10) which is within the ideal temperature for the intensive culture of abalone (12 – 20 °C) (Britz, Hecht & Mangold, 1997). It is possible that the warmer temperatures reported during Period 2 led to an increase in gut microbial abundance (Macey & Coyne, 2005) and increased digestive efficiency for the abalone in the treatment 100AU, which had a higher relative abundance of feed available. An enhanced feed efficiency may be able to improve the amount of harvestable energy derived from feeds that could be allocated to growth.

Although the above studies reported significantly different specific growth rates, the shell growth reported (MISL) for of abalone of various size classes is comparable. The shell of a gastropod is an extracellular structure, where the shell growth may be a more appropriate measure of metabolic efficiency and can be uncoupled from weight gain (Lewis & Cerrato, 1997). The energy allocated to shell growth in the abalone *H. laevigata* was reported to be higher for abalone fed macroalgal diets when compared to a formulated feed (Duong et al., 2020). The MISL reported in Robertson-Andersson, Maneveldt & Naidoo (2011) for abalone fed *Ulva* and kelp was 1.79 mm.month^{-1} and is comparable to abalone fed Abfeed™ S34® in the current study (U-Control = 1.76 mm.month^{-1}; K-Control = 1.83 mm.month^{-1}). Abalone in the 70AU and 80AK treatments attained a MISL of 1.97 mm.month^{-1} and 2.08 mm.month^{-1} respectively, which is comparable to figures reported for smaller abalone fed a mixed diet of *Ulva*, kelp and *Gracilaria gracilis* (1.98 mm.month^{-1}; Naidoo et al., 2006) or Abfeed™, *Ulva* and kelp (1.89 mm.month^{-1}; Dlaza et al., 2008). The high MISL for abalone fed

treatment 80AK in this study, along with significantly higher mean final weight may indicate that this feeding regime may enhance the harvestable energy from dietary ingredients. The significantly elevated MISL for abalone in the 40AK treatments of this study for periods two and three may be due to a greater contribution of energy to shell growth or enhanced metabolic efficiency where relatively more dietary kelp was available compared to Abfeed™ S34®. There were no significant differences for CF of the abalone fed kelp between initial and final reported values while the CF for abalone in all treatments supplemented with *Ulva* significantly decreased over the study but always remained above 1 suggesting they were in good condition (Britz, 1994). In the current study the differences between the two groups (U and K), in terms of observable trends for SGR, MISL, CF and the higher final weights for abalone supplemented with kelp, may be a result of a reduced digestibility of *Ulva* (Duong et al., 2021) compared to kelp and/ or an artifact of the genetic profile for broodstock abalone that are better suited for production parameters with the inclusion of kelp.

A protein content of 35 % in formulated feeds has been used to grow other abalone species where the *Ulva* has been used to improve digestibility of the formulated feed (Bansemer et al., 2016; Bates et al., 2017; Duong et al., 2021). The farm where this study was conducted was feeding on average 0.22 % BW.day^{-1} of Abfeed™ S34 with the addition of fresh kelp at approximately 0.21 % BW.day^{-1} when available. The nature of on-farm feeding is that there are fluctuations in feeding volumes that are related to environmental conditions and overall performance of the system which can be highly variable. The feeding ration of Abfeed™ S34® used in this study was higher than the farm average for Abfeed ™ S34® however, the combined feeding ration of the farm would be 0.43 % BW.day^{-1} when kelp was available having a higher relative feeding ration than was used in this study. A study by Britz, Hecht & Mangold (1997) reported *H. midae* of a similar size and within the temperatures of this study had a consumption rate for formulated feed of 0.35 – 0.4 % BW.day^{-1} which is also higher than this farms average feeding rate. Francis, Maneveldt & Venter (2008) found that abalone fed on kelp consumed approximately 0.48 % BW.day^{-1} which is comparable to the mixed diets used in this study with no replacement of formulated feeds (100AU/K) of 0.48 % BW.day^{-1}. The lower average feeding rates used in this study may be a condition of the specific culture systems and it is unlikely that abalone were being underfed. This is

further supported by the lack significant differences in growth rates of abalone fed treatment 100AU/K compared to treatments in which the formulated feed contribution was reduced by 60 % (40AU/K). The inclusion of *Ulva* into the feeding regimes of abalone may reach a limit where growth benefits are no longer realised and feed efficiency is reduced.

The replacement of natural foods for cultivated organisms with high starch dry formulated feeds has been found to elevate haemolymph glucose concentrations in the crab (*Chasmagnathus granulate;* Kucharski & Silva, 1991), snail (*Babylonia areolate;* Zhang, Zhou & Cheng, 2009) and in abalone (Chiou, Lai & Shiau, 2001; Kemp, 2018; Guo et al., 2022) while maintaining high tissue glycogen stores. In this study, there were no significant differences in the muscle tissue glycogen content across treatments, which is an indicator that the dry formulated feeds are able to maintain comparable levels of tissue glycogen even under reduced feeding amounts of formulated feed. Kemp (2018) demonstrated that when *H. rufescens* were fed increasing proportions of fresh macroalgae alongside formulated feeds there was a significant positive relationship with the protein efficiency while an increasing starch portion of the feed also led to elevated deposition of muscle glycogen for the abalone. Guo et al. (2022) investigated the effects of different dietary carbohydrate levels on the growth and physiology of *H. discus hannai* where high carbohydrate diets (45.55%) negatively influenced the glucose and lipid metabolism. Diets that are high in digestible starch, like the maize starch in Abfeed ™ S34® (43.3 % carbohydrate), may be contributing to an enhanced growth rate of abalone due to its high starch digestibility while also negatively impacting the metabolic performance of the abalone through changes to the lipid and protein digestion and assimilation (Kemp, 2018; Guo et al., 2022). Furthermore, by changing the biochemical profile of the marketable products, such as tissue glycogen content in abalone, the farm could specifically enhance the production quality for canned or live transported products that may directly impact farm profitability.

2.4.3 Implications

Several studies have alluded to improved growth of abalone when fed mixed diets. Abalone growth rates (SGR and MISL) are important metrics for the design of feeding

regimes considering that the highest volumes of feed are required for the feeding of abalone during the post-weaning where abalone are marketable from approximately 30 g and upwards. Commercial aquaculture operations rely on predictable feed quality and feeding management to ensure maximal outputs are achieved (Glencross, Booth & Allan, 2007). As an example, we can consider the financial requirements for a commercial abalone facility, in terms of feed costs, for the cost of feeding different treatments for one year. For these calculations, a hypothetical 100 ton abalone farm which uses a fixed feeding regime of Abfeed™ S34® at 0.27 % BW.day^{-1} is compared to a 60 % reduction of formulated feed when supplemented with either *Ulva* or kelp at 0.21 % BW.day^{-1}. The treatments are; 40AU/K (Abfeed™ S34® = 0.11 % BW.day^{-1}; *Ulva*/ kelp = 0.21 % BW.day^{-1}) and 100A (Abfeed™ S34® = 0.27 % BW.day^{-1}) that will be considered for comparisons. Using the following prices a simple cost analysis can be performed; Abfeed™ S34® cost ZAR 32.51.kg^{-1} (October 2022), kelp (79 % moisture) could be bought at ZAR 2.95 .kg^{-1} (July 2022) and it is approximated that *Ulva* (81.6 % moisture) can be produced at ZAR 3.kg^{-1} fresh weight in paddle-raceways (Michael Joubert, pers. comm. 2016). The total volume of Abfeed™ S34® required for the 100A treatment is 98.6 tons and would cost ZAR 3.21 million. Using the fixed feeding rates for the treatment 40AU/K it is calculated that 40.15 tons of Abfeed™ S34® would be required costing a total of ZAR1.31 million. To account for the cost of macroalgae, one must take into account the cost of *Ulva* production for a total of 375 tons (wet weight) per year to supplement the feeding rate of *Ulva* determined in the 40AU treatment which would cost ZAR 1.13 million per year for *Ulva* production. Approximately 247 (wet weight) tons of wild harvested kelp are fed per year, which amount to a total cost of ZAR 0.73 million. For 100 tons of abalone fed Abfeed™ S34® and *Ulva* (40AU), the total cost of feed per annum would amount to ZAR 2.44 million, which is 19 % more than if they were fed Abfeed®S34 and kelp (40AK) at the same feeding rate (ZAR 2.04 million) and 24 % less than feeding only Abfeed™ S34® (100A).

Considering the economics of the above-mentioned example, it would cost a farmer 24 % less in feed costs to supplement with effluent-grown *Ulva* in 40AU and 36 % less to supplement with kelp (40AK) while achieving comparable growth performance for abalone greater than 50 g. Furthermore, the difference in feed costs may also be viewed

as a 60 % reduction in the total amount of a formulated feed (Abfeed®S34) required to commercially produce abalone. There are concerns about the consistency and reliability of kelp supplies whereas effluent-grown *Ulva* could be produced year-round in integrated systems described in Bolton et al. (2009). This calculation does not consider the benefits form integrated production identified by Nobre et al. (2010) in which a further 1.5 – 5 % increase in farm profits were predicted if the IMTA configuration was adopted. In order to successfully manage a farming environment a sound understanding of the implications of a feed type on the animals' wellbeing is needed.

Effluent-grown *Ulva* is host to a unique microbiome (Tujula et al., 2010, de Jager, 2021) where the surface of *Ulva* is in constant production of a range of secondary metabolites that may be important feeding cues for abalone and could influence the feeding regimes that are developed. The specific mode of action which is responsible for the enhanced consumption remains unclear and should be explored further where there is scope for product development with commercial application. The sulphated polysaccharide derived from *Ulva* has been found to provide beneficial immune modulation in some aquacultured species (Safavi et al., 2019; Fumanal et al., 2020; Ponce et al., 2020) and modulate the development of gut microbial communities in other abalone (Gobet et al., 2018; Wang et al., 2020). The use of fresh *Ulva* as a feed supplement for abalone may improve feed efficiency by improving the abalones overall health under intensive culture conditions, by changing the gut microbial communities and/ or as a result of enhanced feed consumption of mixed diets. From the literature and based on results presented within this study, it is hypothesized that the improved consumption is related to chemical cues produced by *Ulva*, which illicit a feed response, and if the feed presented is palatable it will improve consumption. It is recommended that abalone farmers, wishing to improve feeding rates and efficiency of abalone, should include fresh effluent-grown *Ulva* into the dietary regime of their animals, even if it is only available and provided to the animals in small quantities.

The findings from this study show the potential for *Ulva* to be incorporated as a dietary ingredient that can positively impact the marketability of live and canned abalone products while reducing the overall economic burden of the current production system. It is clear that *Ulva* has a positive impact on consumption and growth of abalone and it

would thus be beneficial to determine which chemical component(s) of *Ulva* are responsible for the observed effects. The source of dietary ingredients used can influence the biochemical make-up of the cultured organisms that may have unfavourable repercussions on the consumer health or farm profitability (Nelson, 2002; Mulvaney et al., 2016; Fry, 2016). This study did not measure the fatty acid profiles or DMSP content of tissue in the tissue of abalone that were supplemented with *Ulva* which are important parameters for product development. The inclusion of macroalgae has demonstrated potential to alter the fatty acid profile in abalone meat (Nelson, 2002; Mulvaney et al., 2016) and can have an impact on the overall health benefits derived by the consumer by altering the nutritional profile of the farmed product (Fry et al., 2016). The feeding regimes for farms should be dynamically adjustable through the application of scientific understanding, which will enable a more informed approach for the inclusion of *Ulva* grown on farms into the daily management of an existing industry. Collectively, these findings suggest that besides the benefits on feed consumption, dietary *Ulva* supplementation may also have other health/ physiological implication for the culture of *H. midae*.

Chapter 3 - Antihyperglycaemic and immunostimulatory effects of *Ulva lacinulata* as a feed for the abalone *Haliotis midae*

3.1 Introduction

3.1.1 Context and topic

The nutritional value of feed is measured by comparing a complex suite of physiological interactions that collectively form an organism's nutritional physiology (Kemp, 2018). Adequate nutrition is the main factor ensuring fast growth where dietary ingredients and genetic capacity can have impacts beyond basic nutrition in abalone (Morash & Alter, 2015; Venter et al., 2018). Carbohydrates are the most economical energy source in animal diets, where an increase in the proportion of carbohydrates can decrease feed costs and improve the digestibility of a feed (Sales & Britz, 2002; Guo et al., 2022). Maize starch, which is included in Abfeed™ S34®, has been shown to be highly digestible to *H. midae*, with high feed stability at up to 60 % inclusion in extruded feeds (Sales & Britz, 2002). Decreased growth and feed efficiency have been reported in abalone species when the level of starch inclusion is above ~50 % however, abalone demonstrate the capacity to deal with high carbohydrate loads (Guo et al., 2022). Research on humans (McKeown et al., 2004), crayfish (Radford et al., 2005), and many fishes (Moon, 2001), indicate that the consumption of highly digestible carbohydrates are associated with prolonged hyperglycaemia (Furukawa et al., 2004; Termizy & Mafauzy, 2009; Tas et al., 2011), which may adversely affect the growth and health of the organism, extending beyond basic nutrition.

3.1.2 Scope and focus

Gastropods convert excess glucose to tissue glycogen for storage as an important molecule under times of limiting food, stress and/ or spawning (Watanabe, Yamanaka & Yamakawa, 1992; Carefoot, Taylor & Land, 2000; Chiou, Lai & Shiau, 2001; Braid et al., 2005; Polakof et al., 2012), where it can be rapidly utilized via enzymatic hydrolysis as an intracellular energy reserve (Plisetskaya & Joosse, 1985; Laas & Vosloo, 2010). Short (a few hours) to medium-term (a few days) periods of starvation

are a normal occurrence for abalone in their natural environment, which makes it important for them to have sufficient energy reserves and the appropriate physiological mechanisms to mobilize these reserves. The most quickly affected stores are tissue glycogen (in *H. kamtschatkana*; Carefoot, 1991), then non-essential amino acids (in *H. fulgens*; Viana, D'Abramo & Gonzalez, 2007), and only after approximately 70 days of induced starvation does lipid metabolism occur (in the sea hare *Aplysia dactylomela*; Carefoot, 1991). High carbohydrate diets have been shown to correlate with high tissue glycogen content and elevated haemolymph glucose concentrations in other cultured animals (Veldhuijzen ; 1974; Díaz & Nakagawa, 1990; Kucharski & DaSilva, 1991; Rossi & DaSilva, 1993; Zhang, Zhou & Cheng, 2009; Kemp, 2018), where the metabolic pathways for energy metabolism are impacted (Guo et al., 2022). Changes to the basal energy metabolism of abalone may likely also result in alterations in their mechanisms of feeding (Harvey Anderson & Woodend, 2003).

In 1953 the glucostatic theory was proposed, which hypothesised that low blood glucose concentrations trigger the onset of feeding and that high blood glucose levels signal satiety and the termination of feeding (Mayer, 1953). Carbohydrates can be classified according to a glycaemic index, a method developed by Jenkins et al. (1981) for quantifying carbohydrates according to the intensity and duration of the glucose response it elicits. Maize starch is a plant storage carbohydrate that has a high amylopectin to amylose ratio and increases the digestibility along with the related glycaemic index (Singh, Kaur & Shevkani, 2014). The glycaemic index is not a linear relationship between food and response as it can vary according to the extent of processing, quality of the starch, and the ingredients ingested with it (Jenkins et al., 1981; Foster-Powell, Holt & Brand-Miller, 2002; Singh, Dartois & Kaur, 2010). The formulated feed used for *H. midae*, in this study, contains 47.17 % pre-gelatinized maize starch, which has been hailed a beneficial ingredient by feed formulators for providing energy and promoting growth (Sales & Britz, 2002; Lee, 2004). Pre-gelatinized maize starch has a higher glycaemic index after being extruded in the formulated feeds where heat/ force induced changes to the starch are able to increase the accessibility of enzymes to the starch (Bornet, 1989). The continued ingestion of high glycaemic (rapid digestion) carbohydrates, that are able to induce prolonged periods of high blood glucose have been associated with negative health effects that are

reported in the onset of the metabolic syndrome and obesity in humans (Liu, 2002; Caceres & Legendre, 2021).

The carbohydrate-insulin model has recently been presented as a working hypothesis for the mechanism which partially drives the onset of obesity where rapidly digestible carbohydrates cause increased fat deposition and drive a positive energy balance (Ludwig et al., 2021). A study by Guo et al. (2022) on the glucose metabolism of the abalone *H. discus hannai* showed that high carbohydrate diets led to increased insulin production which culminated in insulin resistance above 45.55 % carbohydrate inclusion. Insulin resistance has also been associated with the onset of the metabolic syndrome in humans (Termizy & Mafauzy, 2009) with one of the earliest factors in the etiology of type-2 diabetes being decreased insulin sensitivity in insulin responsive tissues (Styskal et al., 2012). Venter et al. (2018) investigated the metabolome of fast vs. slow growing abalone under commercial conditions and observed significant differences in their energy pathways via insulin production where fast-growing abalone predominantly use their central carbon metabolism for energy production. Li et al. (2021) showed that the inclusion of vitamin D_3 into a formulated feed which contained high digestible carbohydrates for the abalone *H. discus hannai* significantly reduced circulating haemolymph glucose concentrations by promoting glucose transport and insulin pathway activation. Abalone have unique evolutionary adaptations, with regards to their biochemical and metabolic abilities, that make them suited to their ecology (Venter et al., 2018) and may also enhance their metabolic capacity for high carbohydrate diets.

The identification of dysglycaemia (prediabetic elevated blood glucose) in humans can be reliably measured using a standardise glucose tolerance test where the subject is administered a dose of glucose and the ability to clear glucose is measured (Jagannathan, 2020). Oxidative stress can occur following extensive glucose metabolism, and unregulated production of reactive oxygen species is thought to be one of the major drivers for the onset of metabolic disorders in humans (Tas et al., 2011). Prolonged incidences of diet-induced oxidative stress plays a critical role in the pathogenesis of conditions associated with the metabolic syndrome, which can impair the functioning of glucose metabolism (Brownlee, 2001; Cornish & Garbary, 2010). A prolonged glycaemic response in the Atlantic cod (*Gadus morhua*) fed high

carbohydrate diets was associated with a stress related change to the carbohydrate metabolism (Hemre, Lambertsen & Lie, 1991). Alterations to energy pathways, including diet-induced hyperglycaemia (Liu, 2002), can create an increase in energy substrates, which are utilized by the mitochondria that then produce reactive oxygen species increasing the overall oxidative stress (Furukawa et al., 2004). Glucose has been used as a reliable 'end-point' indicator of biological stress in crustaceans (Schock et al., 2010) and changes to the glucose homeostasis of organisms can have far reaching consequences for the host energy metabolism (Venter et al., 2018).

The increase in high energy substrates that from part of digestion and assimilation of haemolymph glucose may have implications for the metabolic function of immunity which is dominated by respiratory burst of oxygen radicals (Coyne, 2011). Molluscan haemocytes kill engulfed pathogens via oxidative burst reactions, in which toxic reactive oxygen species are generated (Sokolova, 2009). In *H. midae*, phagocytosis of foreign particles requires increased oxidative activity within the haemocyte which is likely to originate in the mitochondria (Coyne, 2011) and results in an increase in cellular biochemical activity (Donaghy et al., 2010). The immune response in *H. midae* activates several energetically expensive processes, where specialized cells known as haemocytes are involved in the clearance of circulating pathogens and can be significantly retarded in compromised abalone (Hooper et al, 2007, Sokolova, 2009; Coyne, 2011). Challenge assays have been used to assess the effects of environmental stressors and diet on the innate immune response of a variety of marine invertebrates, including shrimp (Scholz et al., 1999; Burgents et al., 2005), crabs (Macey, Rathburn, et al., 2008; Ikerd, Burnett & Burnett, 2015), lobster (Knapp et al., 2019) and oysters (Macey, Achilihu, et al., 2008). A study by Kurtz (2002) demonstrated that the phagocytic capacity of *Panorpa vulgaris* can be accurately predicted by, and correlates to, the number of haemocytes in circulation. The activation and reallocation of energy reserves to the immune response can have significant costs on total energy stores (Bashir-Tanoli & Tinsley, 2014) and any enhancements to this mechanism, through diet, will be a useful tool for feed developers to address.

3.1.3 Importance and relevance

Studies investigating the effects of dietary seaweed inclusion on the metabolic syndrome of mammals have shown that several *Ulva* species are able to induce

beneficial alterations to energy metabolism (Termizy & Mafauzy, 2009; Tas et al., 2011; Ramirez-Higuera et al., 2014). Due to the lack of available literature citing alterations to the fundamental energy pathways in gastropods, evidence from other invertebrates and mammals will be used to speculate potential connections, which will require further investigation. *Ulva* has previously been shown to have strong inhibitory effects on the enzymes involved in starch digestion (BelHadj et al., 2013; Trentin et al., 2020) and it has also been associated with other anti-hyperglycaemic effects (Celikler et al., 2009; Tas et al., 2011; Sharifuddin et al., 2015; Gómez-Zorita et al., 2020). The reported anti-hyperglycaemic properties of *Ulva* are largely attributed to either (1) the phenolic fraction, which is extracted in the polar solvent ethanol (Celikler et al., 2009) or (2) the highly sulphated water-soluble sulphated polysaccharide ulvan (Sathivel et al., 2008). Water soluble sulphated polysaccharides that can be extracted from *Ulva,* known as Ulvans, have demonstrated the ability to enhance the immune response of the Senegalese sole (Fumanal et al., 2020; Ponce et al., 2020), rainbow trout (Safavi et al., 2019), and shrimp (Akbary & Aminikhoei, 2018). *Ulva* and its components have demonstrated capacity for significantly improving metabolic function in several organisms (Tas et al., 2011; Sharifuddin et al., 2015; Majee et al., 2018; Wang et al., 2018; Guerreiro et al., 2019; Fumanal et al., 2020; Gómez-Zorita et al., 2020; Martin-Gallausiaux et al., 2020; Ponce et al., 2020; Duong et al., 2021), which are likely derived from multiple components of *Ulva*.

Communication between organs is crucial for controlling energy homeostasis and when it is disturbed it can illicit changes in food intake, energy utilization and the development of insulin resistance in humans (Geurts et al., 2014). Improving the efficiencies in production by eliminating the variability in growth rates through improved metabolic function will ultimately lead to reduced production costs for commercial abalone producers (Venter et al., 2016). Inflammation, even at a cellular level is merely the unnecessary stimulation of metabolic pathways that results in the depletion of energy reserves that may otherwise be utilised in the sustained growth of a cultured species and these interactions can reduce the overall farm profitability (Butler, Btaiche & Alaniz, 2005).

3.1.4 Objectives and questions

Diet induced changes to the metabolic function may act as a biotic stressor that could negatively impact the production of abalone through increased susceptibility to pathogens. In light of the high content of digestible carbohydrate that is used in formulated feeds for abalone along with its impact on the glucose metabolism in other animals the aim of this study was to investigate the glucose metabolism, and determine the differences in immune and metabolic responses to a bacterial challenge, of abalone fed formulated feed versus fresh *Ulva*. The end-point analysis of glucose was hypothesised to be a useful indicator of the underlying metabolic condition of abalone.

The specific objectives of this study were to:

1) to determine the effect of diet on glucose metabolism of abalone by:
   a. assessing the abalone's ability to maintain glucose homoeostasis under induced starvation,
   b. induce hyperglycaemia via injection into the cephalic arterial sinus, and
   c. quantify the abalone's basal circulating haemolymph glucose, including the ability to return to basal glycaemia, after an induced glycaemic response.
2) to determine the immune and metabolic response of abalone following a bacterial challenge by:
   a. quantifying the bacterial clearance efficiency (following injection of a known dose of live *Vibrio anguillarum* into the cephalic arterial sinus),
   b. quantifying circulating haemocyte counts pre- and post-bacterial injection, and
   c. measuring the glycaemic expense of an immune response for abalone maintained on either Abfeed™ S34® or *Ulva* for six months.

The findings from this study will provide information on the effects of diet on glucose metabolism and the innate immune response of *H. midae*. A thorough exploration for the potential benefits of *Ulva* have been investigated however, identification of the risks remains essential to the maintenance of efficient biosecurity protocols under real farm conditions (Stentiford et al., 2017). Natural immunostimulants that are locally produced can be a sustainable option of controlling pathogens in aquaculture systems (Safavi et al., 2019) if strong biosecurity protocols are in place.

3.2 Materials and methods

3.2.1 Experimental site and water supply

The research was carried out at the Department of Forestry, Fisheries and the Environment (DFFE) Marine Research Aquarium (MRA), Cape Town, South Africa (33° 55' 6.492" S, 18° 22' 52.572" E). All sea water utilized by the MRA is pumped directly from the Atlantic Ocean via pipes running into the subtidal zone of a semi-sheltered bay directly in front of the aquarium. Sea water entering the aquarium is pumped through a drum filter and sand filters prior to entering a sump tank at the highest point in the MRA, where after it is gravity fed to the various systems within the aquarium. The 40-year average water temperature for the coastal environment in Sea Point, where the Research Aquarium extracts its seawater from, is 13.08 °C. The temperature ranges from an absolute minimum of 8.7 °C to an absolute maximum of 23 °C.

3.2.2 Source of macroalgae

All *Ulva* used in this study was sourced from Irvin and Johnson, a commercial abalone farm in Gansbaai in the Western Cape Province of South Africa (34° 37' 35.238" S, 19° 17' 47.2164" E). *Ulva* on this farm was grown in abalone effluent waters and was delivered when required to the MRA. At the MRA the *Ulva* was stocked into a 1000 L polyethylene tank provided with moderate aeration and water flow (2 $L.min^{-1}$). The *Ulva* was maintained under artificial light (24 h light regime) and harvested by hand for feeding. Fresh kelp was offered as a maintenance feed for abalone that were housed in non-experimental systems at the MRA and was collected form the intertidal zone around the MRA.

3.2.3 Determination of haemolymph glucose concentrations

Haemolymph glucose concentrations were determined following the phosphorylation of glucose by adenosine triphosphate (ATP), generating glucose-6-phosphate (G6P), and the subsequent oxidation of G6P to 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) (Glucose HK Assay kit, Sigma-Aldrich GAHK20). Specific reactions are listed in Equations 3.1 and 3.2:

$$Glucose + ATP \xrightarrow{Hexokinase} Glucose6Phosphate + A \quad 3.1$$

$$Glucose6Phosphate + NAD \xrightarrow{G6PDH} 6Phosphogluconate + NADH \quad 3.2$$

During this oxidation, an equimolar amount of NAD is reduced to NADH and the increase in absorbance at 340nm is directly proportional to the glucose concentration in the sample.

3.2.4 Quantifying the glucose metabolism of abalone

3.2.4.1 Glucose homeostasis of abalone under induced starvation

Preliminary assessments revealed that haemolymph glucose concentrations of abalone varied greatly. It was hypothesised that a period of induced starvation would reduce this variability, allowing for a more accurate measure of basal glucose levels in the haemolymph of abalone. The ability of abalone to maintain glucose homoeostasis under a period of starvation was therefore assessed to determine an appropriate sampling point for subsequent experiments. The experimental system and detailed methods are provided below.

3.2.4.1.1 System and system management

The experimental culture system consisted of three identical replicate plastic tanks (L x W x H: 1 x 1 x 0.7 m) supplied with filtered seawater at a rate of 6 L. min^{-1} and constant aeration. Each tank housed a single submerged oyster mesh basket (L x W x H: 0.4 x 0.4 x 0.4 m) that contained vertical plates to increase the surface area for attachment of abalone. The tanks were cleaned once a week of any particulates, which accumulated on the tank bottom, via siphoning to waste.

3.2.4.1.2 Experimental animals, feeding, and acclimation

Abalone with a mean (±SD) weight of 84.41 ± 16.32 g and length of 75.45 ± 6.11 mm, were used in this experiment. These animals were sourced from the Sea Point Marine Research Aquarium and were maintained on a diet of fresh kelp (*Ecklonia maxima*) for approximately two years prior to their use in this study and had not been used for other experimental purposes in that time. A total of 20 abalone were stocked in each of the three replicate baskets and were acclimated to the experimental system for a period of 2 weeks. Fresh kelp was offered once a week, after removing old kelp from the baskets

and siphoning the tank bottoms. At the start of the experimental period (Day 0) all kelp was removed, and the basket remained free of any feed for a period of 24 days.

3.2.4.1.3 Experimental protocol

The effect of starvation on glycaemic homoeostasis was determined by monitoring circulating blood glucose concentration of three replicate groups of abalone over a period of 24 days on days 0, 2, 6, 9, 13, 17 and 24. At each sampling, six randomly selected animals (n = 2 abalone from each replicate tank, which were averaged as technical replicates for each treatment) were blotted dry, weighed to the nearest 0.01 g using an electronic balance (MODEL: Scout Pro SPU402) and measured to the nearest 0.01 mm with electronic digital Vernier callipers (MODEL: Grip). Haemolymph (± 200 μL) was collected from the cephalic arterial sinus of each abalone using a 1 mL syringe fitted with a 26-gauge × ½ inch needle. Separate syringes and needles were used for each animal. Sampled haemolymph was immediately transferred to a sterile 1.5 mL microcentrifuge tube and all samples were placed on ice while they were processed for quantification of blood glucose. Sampling was consistently performed between 10h00 and 12h00 and each sampled abalone was removed from the system and transferred to a separate holding tank to ensure that no animal was re-sampled. Haemolymph glucose concentrations were quantified using the Glucose Hexokinase kit (Sigma-Aldrich; described in Section 3.2.3).

3.2.4.1.4 Statistical analysis

All statistical analysis was done using R (Team, 2016). The haemolymph glucose concentration for abalone (the average of technical replicates) was considered a replicate. For differences in the mean haemolymph glucose concentrations at each time point a Kruskal-Wallis non-parametric test was used, as assumptions for normality of residuals and homogeneity of variances were not met. Significant difference in haemolymph glucose concentration at each time was determined at $p \leq 0.05$.

3.2.4.2 Effect of induced hyperglycaemia on the abalone's ability to return to basal haemolymph glucose concentrations

Induced hyperglycaemia has been used as a reliable indicator of disglycaemia in humans and a similar protocol, which requires abalone to process a glycaemic dose and return to basal circulating haemolymph glucose concentrations, was developed. The

dose of glucose required to induce a glycaemic response was approximated from (Vosloo & Vosloo, 2006), where the authors calculated that 43 % of an abalones body mass, including the shell, is haemolymph. Using the value for the approximate haemolymph volume of an abalone from Vosloo & Vosloo (2006), the approximate amount of glucose circulating in the haemolymph of a 50 g abalone with ±21.5 mL haemolymph would range from 280 to 1120 µg of glucose for an un-starved animal. Based on preliminary findings along with results from Section 3.2.4.1, it was decided to starve abalone for a period of six days prior to injection with a dose of 1000 µg (concentration = 20 mg. mL^{-1}) of glucose into the cephalic arterial sinus to induce a glycaemic response and in order to decrease inter-animal variability.

3.2.4.2.1 System and system management

An identical system to that described in section 3.2.4.1.1 was used with one exception; only one tank was used that housed an independent group of abalone.

3.2.4.2.2 Experimental animals, feeding, and acclimation

Abalone with a mean (±SD) weight of 57.78 ± 4.15 g and length of 69.41 ± 1.96 mm, were used in this experiment. These animals were sourced from the Sea Point Marine Research Aquarium and were maintained on a diet of fresh kelp (*E. maxima*) for approximately two years prior to their use in this study and had not been used for other experimental purposes in that time. A total of 12 abalone were stocked in an oyster mesh basket and were acclimated to the experimental system for a period of 2 weeks. Fresh kelp was offered once a week, after removing old kelp from the baskets and siphoning the tank bottoms. At the start of the experimental period (Day 0) all kelp was removed, and the basket remained free of any feed for a period of six days before the abalone were administered a dose a glucose to induce a glycaemic response.

3.2.4.2.3 Injecting a glycaemic dose into the haemolymph of abalone

The dose of glucose was injected using a 50 µL glass Hamilton syringe fitted with a 26-gauge by 1½-inch needle, directly into the cephalic arterial sinus (Jorgensen, Ware & Redmond, 1984) and animals were then monitored over a period of 180 minutes for circulating haemolymph glucose levels. The site of injection was thoroughly cleaned with 100% ethanol immediately before and after injection and the dose was injected slowly to ensure that the entire bolus entered haemolymph circulation and there was no

leakage from the site of injection. Control animals were injected with an equal volume of sterile distilled water.

3.2.4.2.4 Experimental protocol

The abalone were processed in batches of six. Each abalone was placed in separate individually marked baskets within the same tank. All abalone were treated identically and independently. An initial sample of ± 80 µL haemolymph was withdrawn from the cephalic arterial sinus using a 1 mL syringe fitted with a 26-gauge × 1½-inch needle and immediately transferred to a sterile 1.5 mL micro-centrifuge tube and kept on ice. These data were used for basal haemolymph glucose concentration, prior to injection. After the initial haemolymph sample was taken, each abalone was injected with 50 µL of a glucose solution (20 $mg.mL^{-1}$) into the cephalic arterial sinus.

The process of injecting each abalone was staggered by 1.5 minutes to allow sufficient time for sampling. Haemolymph samples (± 80 µL) were taken from each abalone at 0, 15, 30, 45, 60, 120 and 180 minutes and immediately transferred to individually labelled 1.5 mL micro-centrifuge tubes after the sample was taken. All the samples on hand were taken to the lab where they were centrifuged at 6000 × g for 2 minutes at 4 °C. For each sample, a 50 µL aliquot of the supernatant was pipetted into a 1.5 mL micro centrifuge tube, labelled and stored at -80 °C for processing at a later stage using the glucose hexokinase kit.

3.2.4.3 Effect of diet on basal haemolymph glucose concentrations and the induced glycaemic response of abalone

In order to test for dysglycaemia in abalone maintained on different diets the glycaemic response developed in Section 3.2.4.2 was used.

3.2.4.3.1 System and system management

An identical system to that described in section 3.2.4.1.1 was used with one exception; only two tanks were used, each housing an independent group of abalone that were maintained on a different diet.

3.2.4.3.2 Experimental animals and acclimation

All abalone for this experiment were sourced from Abagold (Pty) Ltd. and were obtained from a single cohort (single batch spawning event), with a size range of 40 – 50 g (± 2.5 years old). Abalone had been maintained on a diet of Abfeed™ S34® supplemented with kelp under standard grow-out conditions as those described in Chapter 2. After acclimation, abalone were maintained on their respective diets of Abfeed™ S34® or fresh *Ulva* (fed *ad libitum*) for a period of 6 months, prior to experimentation, and were stocked at approximately 50 abalone per basket. Abalone had a mean (±SD) weight and length of 62.60 ± 6.62 g and 69.31 ± 2.91 mm, respectively, at the time of sampling. Abalone (n = 12 from each treatment) were moved to independent purge tanks for each treatment group (and not fed) for a period of six days prior to testing basal blood glucose and inducing a glycaemic spike via cephalic arterial sinus injection of a known dose (1000 µg) of glucose as described in Section 3.2.4.2.4. At the end of the experiment, the injected abalone were returned to separate holding tanks to ensure that the same animal was not sampled more than once.

3.2.4.3.3 Statistical analysis

All statistical analysis was done using R (Team, 2016). The haemolymph glucose concentration for each individual abalone was considered a replicate, which was determined by the average of three assay technical replicates per sample. For differences in the mean basal haemolymph glucose concentrations the data were then tested using a Welch Two Sample t-test for between group differences and significance determined at $p = 0.05$. For differences in mean haemolymph glucose concentrations at each time point during an induced glycaemic spike, within treatment data were compared using one-way analysis of variance with significance set at $p \leq 0.05$. Significant differences were further tested using a post hoc Tukey test. All data met the assumptions and were confirmed using the Shapiro-Wilk test for normality of residuals and the Bartlett Test of homogeneity of variances ($p \leq 0.05$).

3.2.5 Quantifying the innate immune response of abalone

In order to test the effect of a stressor and/or diet on the innate immune response in abalone a bacterial challenge protocol, which provides a holistic "*in situ*" overview of an animal's ability to deal with an 'infection' for abalone, was used. Briefly, the abalone, *H. midae* were injected with a known dose of a strain of GFP labelled *Vibrio anguillarum* 5677 that had been transfected with a stable *Vibrio* derived plasmid coding

for Chloramphenicol (Cm) and kanamycin (Kan) antibiotic resistance to allow for selective re-isolation following bacterial challenge (Knapp et al., 2019). Bacteria were injected directly into the cephalic arterial sinus of each abalone. The site of injection was chosen as this is a large sinus that is easily located posterior of the mouth and the anterior aorta terminates into this sinus, which then pumps haemolymph into several smaller arteries and two large arteries supplying the foot muscle. The ability of the animal to inactivate (render non-culturable) the injected dose of bacteria was assessed.

3.2.5.1 Optimization of the time-point for discerning the impact of a stressor on the immune response of abalone

The ability of abalone to clear an injected dose of bacteria with time was assessed in order to determine the optimal time point for sampling haemolymph following exposure to a stress or specific dietary treatment; where the number of haemocytes and culturable bacteria that remain in circulation can be accurately quantified following an exposure to a known dose of injected of live bacteria.

3.2.5.1.1 Animals

For this experiment, abalone (n=8) with a mean weigh of approximately 16.15 ± 1.43 g (in-shell weight) were used. These animals were sourced from the Marine Research Aquarium and were maintained on a diet of fresh kelp (*E. maxima*) for approximately two years prior to their use in this experiment. Approximately 24 hours before being injected, animals were transferred to pre-acclimation glass tanks, in a temperature-controlled room maintained at 16 °C, that contained filter (0.22 µm) sterilised seawater.

3.2.5.1.2 Bacterial preparation

A strain of *Vibrio anguillarum* 5677, previously isolated from diseased *Haliotis midae* by Dr Anna Mouton (Amanzi Biosecurity, Hermanus, South Africa) (published in Macey, 2015), was used for injecting abalone in the challenge trials as the primary stressor. The bacterium was transfected with a stable *Vibrio*-derived plasmid (plasmid pEVS146; sourced from Prof. Eric Stabb, University of Georgia) coding for chloramphenicol (Cm) and kanamycin (Kan) antibiotic resistance, and the expression of green fluorescent protein (GFP). For each injection, *V. anguillarum* from a single, frozen, working stock was streaked onto tryptic soy agar (TSA) plates supplemented with 2.0 % NaCl (w/v), 100 µg.mL^{-1} Kan A and 5 µg.mL^{-1} Cm and grown overnight at

30 °C. Following incubation, a small amount of bacteria were re-suspended in 5 mL of sterile 10 mmol.L^{-1} HEPES buffered saline supplemented with 2.5 % NaCl (w/v) and vortexed thoroughly to resuspend the bacteria. The optical density (OD) was adjusted to 0.1 ± 0.005 at a wavelength of 540 nm, which equates to a bacterial concentration of 4 x 10^7 colony-forming units (CFU) per millilitre (Knapp et al. 2019). The bacterial preparation used for injection was replicated for each experimental batch of animals. No mortalities were observed in any of the injected animals throughout the study period.

3.2.5.1.3 Optimization protocol and data collection

Following the acclimation period described in 3.2.5.1.1, animals were injected with 20 µL of a 4 × 10^7 CFU.mL^{-1} dose of *V. anguillarum* (at 0 min) and haemolymph was subsequently sampled from the cephalic arterial sinus of each animal at 10, 20, 40 and 120 min, and at 24 hrs. An additional sample of haemolymph was also taken 10 min prior to the injection of bacteria to provide information on the basal number of haemocytes prior to injection. These time intervals were initially chosen since they have been shown to be to be optimal for measuring clearance of bacteria from the haemolymph of other molluscs (Macey, Achilihu, et al., 2008) and crustaceans (Burgents et al., 2004; Holman, Burnett & Burnett, 2004).

For each extraction, a sterile 1 mL syringe fitted with a 26-gauge x 1½ inch needle was used to withdraw approximately 100 µL of haemolymph from the cephalic arterial sinus. A 25 µL subsample was immediately mixed with 100 µL Alsevers buffer ((w/v) 2.08% $C_6H_{12}O_6$, 0.8% $C_6H_5Na_3O_7.2H_2O$, 0.336% EDTA, 2.24% NaCl, 12% HCHO), for the determination of total circulating haemocyte count (THC).mL^{-1}. Haemocytes were counted using a haemocytometer and a light microscope (100× magnification). Three separate aliquots (10 µl each) of the diluted haemocyte suspension were counted and averaged for each animal and the values expressed as the mean (±SE) THC.mL^{-1} haemolymph for abalone at each time point. A second subsample of haemolymph (50 µL) was diluted in 100 µL 10 mM HEPES-buffered saline supplemented with 2.5% NaCl and used to determine the culturable bacteria remaining in haemolymph circulation. A 100 µL aliquot of the diluted haemolymph was spread plated onto TSA plates supplemented with 2.0% NaCl (w/v), Cm (5 µg ml) and Kan (100 µg ml). All plates were incubated for 48 h at 30 °C, at which point the number of bacterial colonies were counted and recorded. Counts were averaged from the duplicate plates and data

expressed as the mean (±SE) culturable *V. anguillarum* mL^{-1} haemolymph for abalone at each time point. Haemolymph obtained from saline injected abalone (conducted randomly throughout the experimental period) were included as negative controls to ensure that there was no growth of bacteria on the selective media other than the injected *V. anguillarum*.

3.2.5.2 Effect of diet on the abalones immune and metabolic responses to a bacterial challenge

The impact(s) of diet, before and after bacterial challenge, on the metabolic status of abalone was also assessed by quantifying an immune response. The assay investigated overall removal of bacteria from circulation and generated information on the magnitude (glycaemic cost) of the immune response, bacterial clearance rates, and the response of the main immune effector cells, the haemocytes.

3.2.5.2.1 Experimental animals

A total of 12 abalone per dietary treatment with a mean (±SD) weight of 52.17 ± 3.39 g and length of 68.42 ± 1.86 mm which had been maintained on their respective diets of Abfeed™ S34® (A) or fresh *Ulva* (U) for a period of 6 months described in Section 3.2.4.2.2 were randomly selected from each basket and moved to the experimental system described below for the bacterial challenge trials. The abalone used for the trials were selected from the same baskets that are described in Section 3.2.4.3.2 with no resampling between experiments.

3.2.5.2.2 Experimental system and acclimation

Individual abalone from each dietary treatment were moved to individually marked 20 L glass aquaria in a temperature controlled room (16 °C) where they were kept for 24 h prior to the bacterial challenge. Each tank was provided with fresh filtered (2µm) seawater and constant aeration. Each tank was covered with a plastic lining to prevent the animals from escaping and to help prevent cross contamination. No food was provided during the 24 h acclimation period. The bacterial challenge was done in four batches that were processed on the same day, with six abalone per batch (n = 3 from each treatment group; total of 12 abalone per treatment) and with a 3 min waiting period between each abalone to ensure sufficient time for sampling and preparation.

3.2.5.2.3 Experimental protocol

Immediately before injecting an abalone with *V. anguillarum,* a 150 µL sample of haemolymph was withdrawn from the cephalic arterial sinus for the quantification of glucose, total haemocyte counts, and presence of culturable *V. anguillarum*. All samples were stored on ice. Animals from each treatment received an injection dose of 4×10^4 *V. anguillarum* (100 µL of a 4×10^7 CFU.mL^{-1} stock solution) using a 100 µL glass Hamilton syringe equipped with a 26-gauge × 1½ inch needle and were injected into the cephalic arterial sinus of each abalone over a period of approximately 10s. This equates to a circulating dose of approximately 1.7×10^5 bacteria mL^{-1} haemolymph for a 52 g abalone. The cardiac output for a 52 g abalone (22.62 mL haemolymph) is 14.43 mL.min^{-1} (Jorgensen, Ware & Redmond, 1984), which is equivalent to injecting 100 µL of the bacterial dose into 2.4 mL haemolymph moving through the cephalic arterial sinus in 10s. This was done deliberately to ensure sufficient dilution of the dose and to prevent localized clotting, due to possible clumping of the bacteria around the site of injection, as described by (Macey, Rathburn, et al., 2008).

Animals were then returned to the glass tanks for a period of 30 min before being sampled again to determine the total number of circulating haemocytes and the number of culturable bacteria remaining in circulation. The 30 min time-point was chosen as preliminary experiments (described in Section 3.2.5.1) indicated that it would be optimal for measuring the impact of a stressor or change in diet on the chosen immune parameters. The chosen time-point is similar to studies on other marine invertebrates (Scholz et al., 1999; Burgents et al., 2005; Macey, Achilihu, et al., 2008; Macey, Rathburn, et al., 2008; Ikerd, Burnett & Burnett, 2015; Knapp et al., 2019).

At 30 min post-injection, a 150 µL sample of haemolymph was withdrawn from the cephalic arterial sinus and placed on ice. A 25 µL subsample was immediately mixed with 100 µL Alsevers buffer for the determination of total circulating haemocyte count (THC).mL^{-1} (described in Section 3.2.5.1.3). For determining blood glucose, a 75 µL sample of the raw haemolymph was centrifuged at 6,000 × g for 2 minutes at 4 °C. Following centrifugation, a 50 µL aliquot of the supernatant was transferred to a new sterile 1.5 mL microcentrifuge tube and stored at -80 °C until the blood glucose concentration was determined according to Section 3.2.3. Finally, the number of culturable *V. anguillarum* remaining in the haemolymph of each abalone 30 min

following injection was determined by selective plating as described in Section 3.2.5.1.3.

3.2.5.2.4 Statistical analysis

All statistical analyses were done using R (R Core Team, 2016). Prior to statistical analysis the data were visually analysed for outliers using the ggplot2 package by Wickham and Chang (2017). The aim of this experiment was to identify an effect on immune response by dietary treatment and although outliers may pose interesting questions it was decided that a value which is 2.5 times greater than the inter quartile range (IQR) should be discarded. The total haemocyte counts (THC) for abalone contained data which were within 2.5 times the IQR and no outliers were identified. For haemolymph glucose concentrations, there was a single outlier for each group which was greater than 2.5 times the IQR and these data were discarded before statistical analyses were conducted. The data collected for bacterial counts of culture forming units (CFU) showed two outliers for abalone maintained on Abfeed™ S34® and one outlier for abalone maintained on *Ulva* and were removed before any further analysis.

After the removal of outliers from the data each abalone was considered an independent replicate and all technical replicates were averaged. The differences between initial (T_i) and final (T_f) mean values for abalone, both between and within groups, were compared using the following methods with significance determined at $p \leq 0.05$:

1) The difference in free circulating haemolymph glucose was tested for significance using a Two Sample t-test. Tests were done between treatment groups (A and U) at T_i (0 min) and T_f (30 min) and within groups for difference between T_i and T_f;
2) The difference in total haemocyte count (THC) was tested for significance using a Two Tailed t-test. Tests were done between treatment groups (A and U) at T_i (0 min) and T_f (30 min) and within groups for difference between T_i and T_f;
3) The difference in bacterial clearance was tested for significance in total culture forming units counted using a Welch Two Sample t-test between treatment groups (A and U) at T_f (30 min). No *V. anguillarum* was detected in haemolymph samples prior to injection.

3.3 Results

3.3.1 Glucose homeostasis of abalone under induced starvation

A period of induced starvation had no statistically significant effect on the circulating haemolymph glucose concentration of abalone over a period of 24 days ($p = 0.1319$; Figure 3-1) and ranged from 0.48 - 89.64 $\mu g.mL^{-1}$. The most extreme outlier was recorded on day 0 (89.64 µg glucose per mL^{-1} haemolymph; Figure 3-1). The lowest recorded values were on day 0 (4.63 $\mu g.mL^{-1}$) and day 2 (0.48 $\mu g.mL^{-1}$). The mean (±SD) haemolymph glucose concentration ranged was 19.72 ± 27.29 $\mu g.mL^{-1}$ over the first two days of starvation and from day 6 - 24 it was 22.67 ± 7.81 $\mu g.mL^{-1}$. The inter-animal variation in haemolymph glucose concentration for animals was reduced after a brief period of starvation and after six days of starvation, the variation observed in haemolymph glucose concentrations ranged from 15.47 - 22.49 $\mu g.mL^{-1}$.

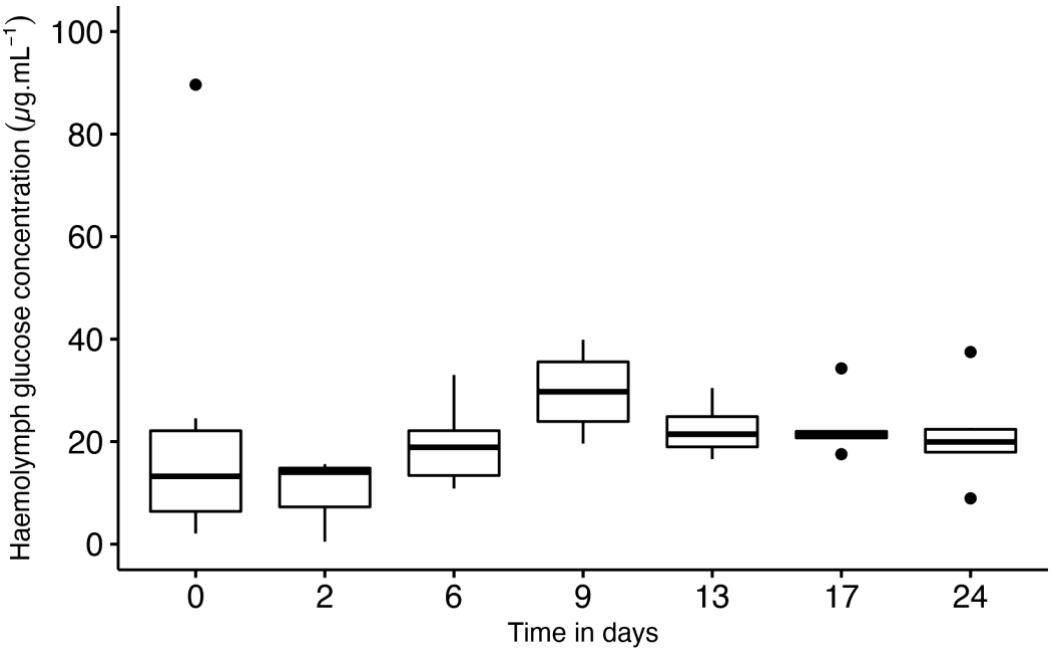

Figure 3-1 Box plots of haemolymph glucose concentrations over a 24 day period for abalone under a period of induced starvation. The box represents the lower and upper quartile (25 - 75 percent, respectively) of the data (interquartile range), the vertical lines reach out to 2.5 times the interquartile range, and outliers displayed as black dots (n=6 for each box).

3.3.2 Effect of induced hyperglycaemia in abalone

After a period of starvation the inter-animal variability of haemolymph glucose concentrations was reduced (results above) and allowed for repeatable inductions of a hyperglycaemic state for abalone. The injection of saline water into the cephalic arterial sinus did not induce a significant response in abalone, in terms of circulating haemolymph glucose concentrations, over 180 minutes (Figure 3-2-Control; $F = 0.314$; $p = 0.924$). In contrast, a dose of 1000 µg glucose injected into the cephalic arterial sinus induced a significantly elevated haemolymph circulating glucose concentration in abalone for 30 minutes post-injection (Figure 3-2-Dose; $F = 2.612$; $p = 0.037$).

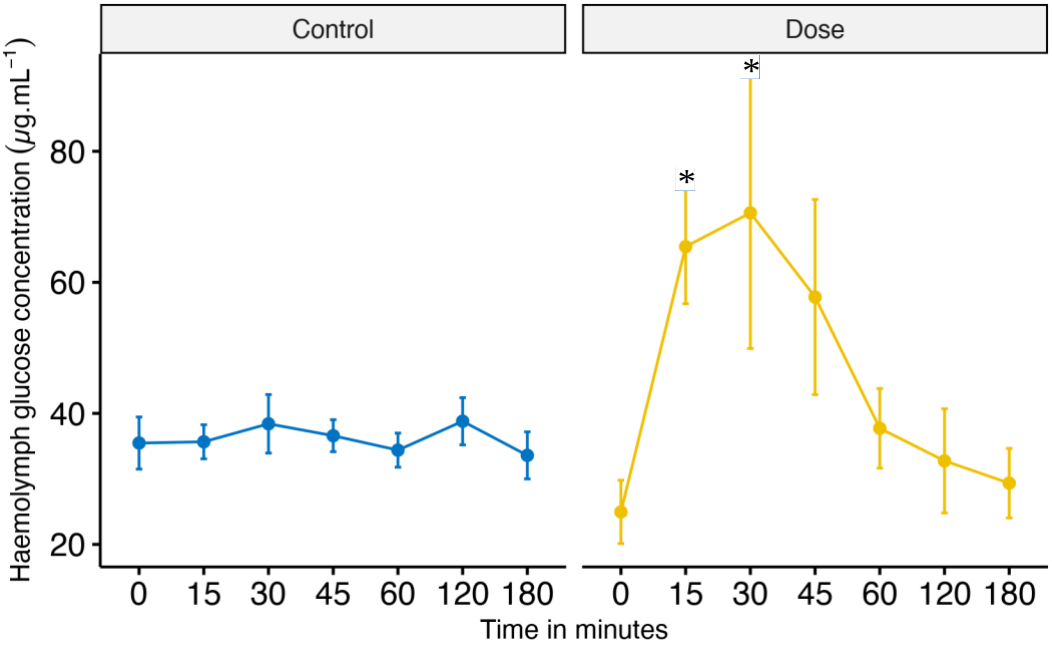

Figure 3-2 Mean (±SE) haemolymph glucose concentrations of abalone after receiving an injection of either distilled water (control) or a dose of 1000 µg glucose, injected into the cephalic arterial sinus. Abalone were starved for a period of six days prior to injection and were monitored at specific intervals (15, 30, 45, 60, 120, 180 minutes) over a 180-minute period. For the control, a total of five abalone were injected with distilled water and for the experimental group a total of 6 abalone were injected with the glucose dose to assess the effect of induced glycaemia on abalone haemolymph glucose concentrations/ clearance ability. The significance is denoted by the stars with $p \leq 0.05$ marked as *.

3.3.3 Effect of diet on basal haemolymph glucose concentrations and the induced glycaemic response of abalone

Abalone that were maintained on a diet of Abfeed™ S34® for a period of six months had significantly elevated circulating haemolymph glucose concentrations when compared to abalone maintained exclusively on a diet of fresh *Ulva* for the same period of time ($p = 0.001$; Figure 3-3). Abalone fed Abfeed™ S34® had a mean (±SD) haemolymph glucose concentration of 47.59 ± 5.01 $\mu g.mL^{-1}$, whereas abalone maintained on *Ulva* had a mean (±SD) haemolymph glucose concentration of 31.39 ± 11.6 $\mu g.mL^{-1}$ (Figure 3-3).

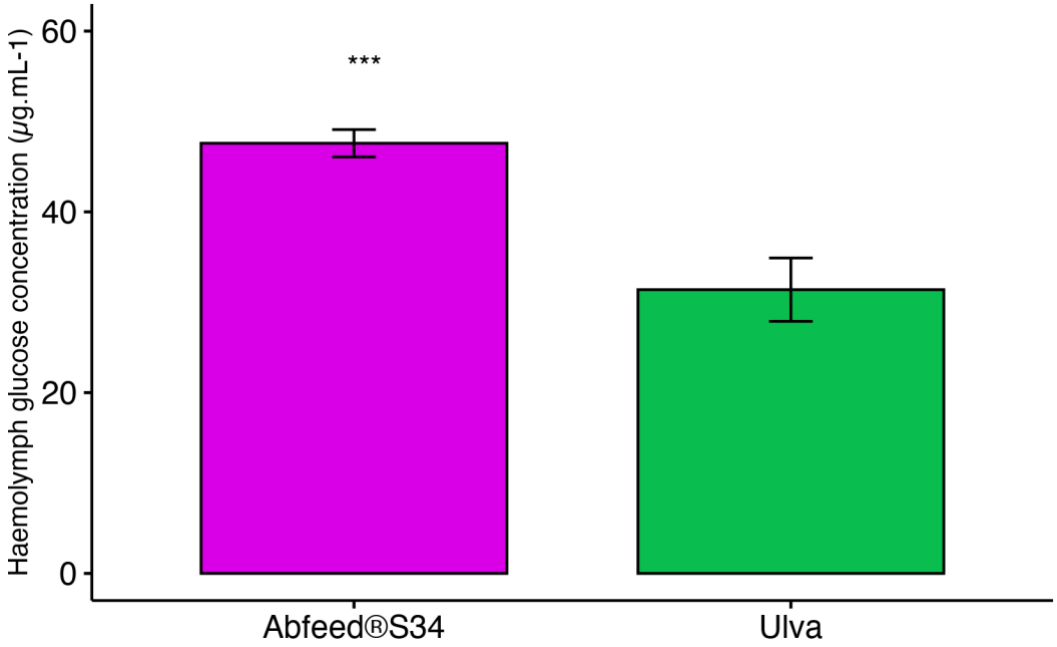

Figure 3-3 Mean basal (±SE) glucose concentrations in the haemolymph of starved abalone (6 days without food), maintained on either Abfeed™ S34® or *Ulva* for a period of six months (n=12 abalone per treatment) prior to the testing. Significance is indicated by * ($p<0.05$) for a comparison between means.**

Abalone maintained on either Abfeed™ S34® (Figure 3-4 A) or *Ulva* (Figure 3-4 B) were both able process the injected dose of glucose (1000 µg) within 180 minutes; with haemolymph glucose values returning to pre-injection level within this period. As noted previously (3.3.2 above), basal haemolymph glucose concentrations were higher in abalone maintained on the diet of Abfeed™ S34® compared with abalone maintained

on the diet of fresh *Ulva*. The duration of the glycaemic response for abalone fed *Ulva* appeared to be shorter, when compared with abalone fed the diet of Abfeed™ S34®. Abalone maintained on *Ulva* had significantly elevated mean (±SE) haemolymph glucose levels at 15 (52.83 ± 5.59 µg.mL^{-1}; p = 0.029) and 30 (51.80 ± 5.17 µg.mL^{-1}; p = 0.035) minutes post-injection of glucose, but by 45 (49.80 ± 5.09 µg.mL^{-1}) minutes glucose levels had returned to a level that were no longer statistically significant from their initial value (Figure 3-4B; F = 17.882, p = 0.078). Conversely, abalone maintained on Abfeed™ S34® had significantly elevated haemolymph glucose levels at 15 (p < 0.001), 30 (p = 0.001), 45 (p = 0.006) and 60 (p = 0.001) minutes post-injection of glucose and only returned to their basal level after 120 minutes (F = 18.057, p = 0.091; Figure 3-4A).

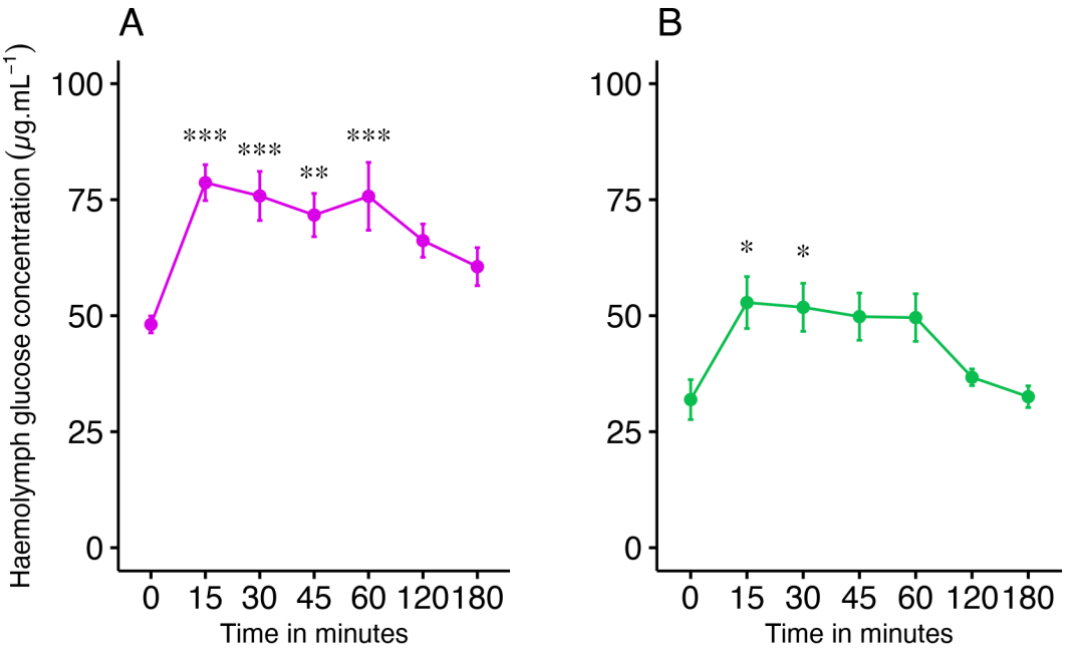

Figure 3-4 Mean (SE) circulating glucose levels recorded in the glycaemic response over 180 minutes in abalone maintained on a diet of either Abfeed™ S34® (A) or *Ulva* (B) for a period of six months before intravenous injection into the cephalic arterial sinus with 1000 µg of glucose. Twelve individual abalone were injected from each dietary treatment and the circulating glucose levels expressed as the mean (±SE) at each sampling time. A significant difference in glucose concentration at each time point from the initial sample (time 0) within each treatment is denoted by an asterisk on the top of the plot area (* p < 0.05 ; ** p < 0.01; *** p<0.005) .

3.3.4 Effect of time on the abalone's total haemocyte count and bacterial clearance efficiency used for the optimization of the bacterial challenge

3.3.4.1 Bacterial clearance

Injection of a known dose of bacteria (100 µl of a 4 x 10^7 CFU.mL^{-1} stock solution) into the haemolymph of *Haliotis midae* demonstrated the animal's ability to respond rapidly to an injected dose of bacteria, with more than 80% of the theoretical dose of *V. anguillarum* (~178,890 bacteria.mL^{-1} haemolymph for a 52 g abalone) in haemolymph circulation rendered non-culturable within first 20 min (Figure 3-5). By 24 hours, only 0.9 % of the original injected theoretical dose of *V. anguillarum* remained in haemolymph circulation.

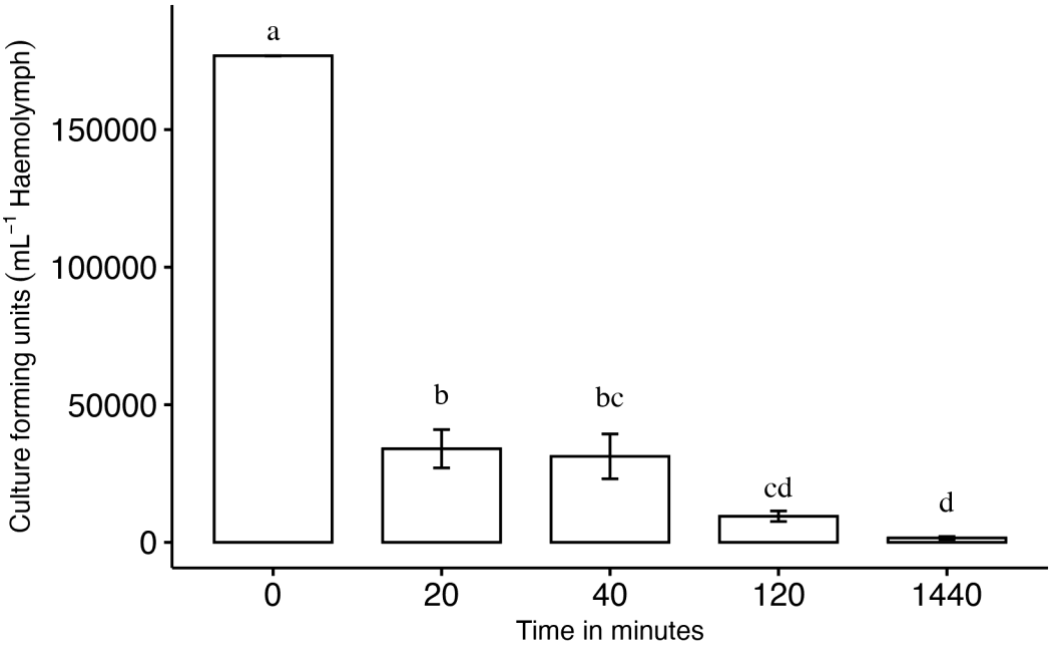

Figure 3-5 Mean (±SE) number of culturable bacteria recorded in abalone haemolymph (n = 8) at specific time points (20, 40, 120, and 1440 minutes) after injection of a known dose (at 0 minutes) of live bacteria (*V. anguillarum*). Each abalone tested negative for *V. anguillarum* pre-injection and clearance counts confirmed by spread plate technique confirmed the presence of *V. anguillarum* post-injection (n = 8). The theoretical injected dose of *V. anguillarum* (178890 CFU.mL^{-1} for a 52 g abalone) is indicated by the bar at 0 minutes. Significant differences between time points are denoted by different letters above the bars.

3.3.4.2 Total haemocyte count

Haemocyte data shows that the bacterial challenge elicits a clear immune response in apparently healthy abalone, but the animals recover quickly, with circulating haemocyte numbers returning to pre-injection levels within 24hrs following injection of the *V. anguillarum*. The mean number of circulating haemocytes in the haemolymph of abalone prior to the injection with *V. anguillarum* was 5096875 haemocytes.mL^{-1} haemolymph (Figure 3-6). By 20 minutes following injection of bacteria, the number of circulating haemocytes had declined significantly to 2242857 haemocytes.mL^{-1} haemolymph. However, by 40 minutes post-injection with bacteria, the numbers of circulating haemocytes were no longer significantly different from pre-injection levels and by 24 hours post-injection the number of circulation haemocytes had returned to 5220833 haemocytes.mL^{-1}, almost identical to pre-injection levels (Figure 3-6).

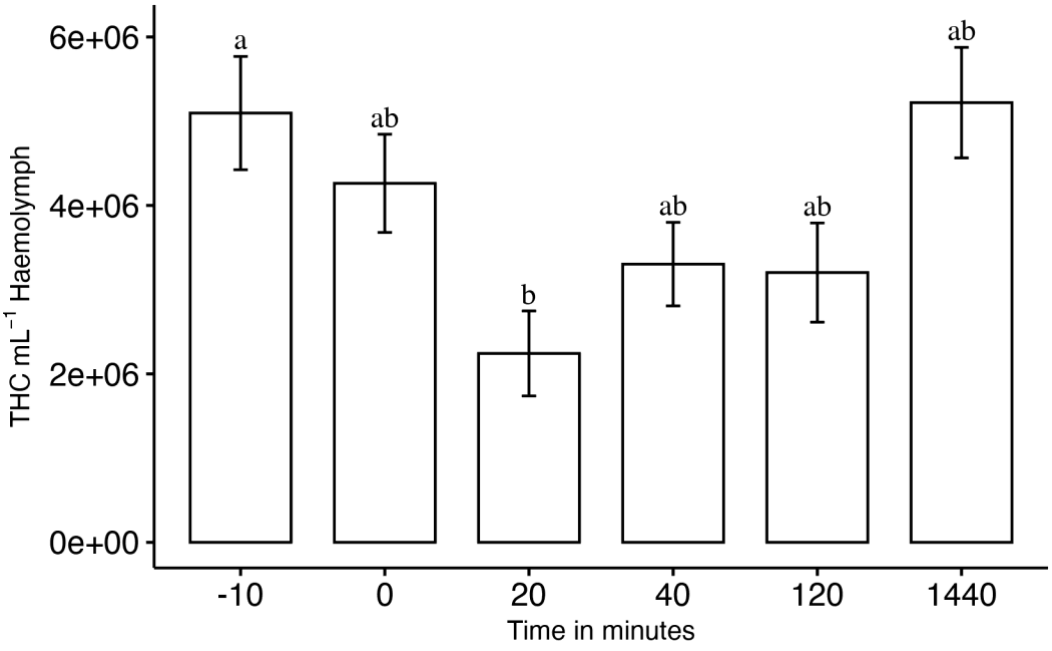

Figure 3-6 Mean (±SE) total haemocyte counts recorded from the haemolymph of abalone pre- (-10 minutes) and post- (10, 20, 40, 120, and 1440 minutes) injection with a known dose of live *Vibrio anguillarum* (n=8 abalone). Significance is denoted by different letters above the bars.

3.3.5 Effect of diet on the abalones immune and metabolic responses to a bacterial challenge

3.3.5.1 Haemolymph glucose concentration

The haemolymph glucose concentrations of abalone maintained on Abfeed™ S34® were higher, but not significantly different from abalone maintained on *Ulva* ($p = 0.094$; Figure 3-7) prior to the injection of *Vibrio anguillarum*. Conversely, at 30 minutes post-injection, the mean glucose concentration of abalone maintained on *Ulva* (24.75 µg.mL^{-1}) were significantly lower than the mean haemolymph glucose concentrations of abalone maintained on Abfeed™ S34® (47.20 µg.mL^{-1}); when tested using the Welch Two Sample t-test (Figure 3-7; $p = 0.041$). For both dietary treatments, the bacterial challenge does not have a significant impact on haemolymph glucose concentrations, with no significant differences recorded within treatments.

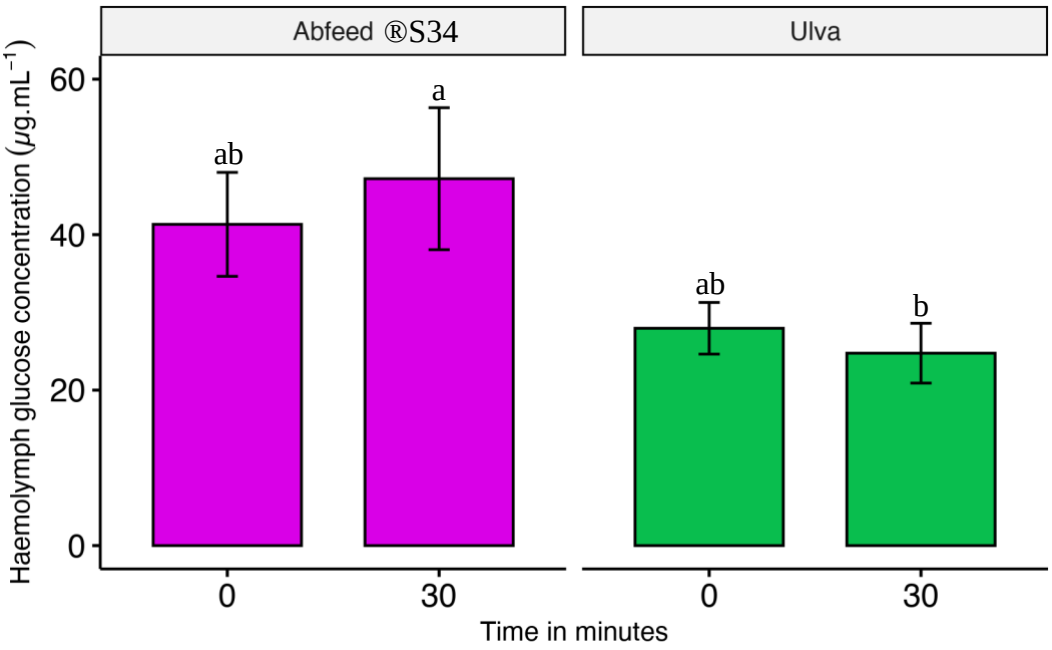

Figure 3-7 Mean (±SE) free circulating haemolymph glucose concentrations recorded from abalone fed Abfeed®S34 or *Ulva* (for a period of 6 months) pre- and post- (30 minutes) injection with a known dose of live *Vibrio anguillarum* (n=12 abalone per treatment group). Significance is denoted by different letters for within treatment tests.

3.3.5.2 Bacterial clearance

In both treatment groups, there was a rapid reduction in the number of culturable bacteria from the haemolymph within 30 min following injection of the challenge dose of *V. anguillarum* (Fig. 3.8). For abalone maintained on a diet of Abfeed™ S34® for a period 6 months, only 3.4 % (6027 ± 1860 bacterial CFU.mL^{-1} haemolymph) of the theoretical injected dose of *V. anguillarum* (176835 CFU.mL^{-1}) remained in the haemolymph at 30 minutes post-injection, whereas only 1.7 % (3060 ± 882 CFU.mL^{-1} haemolymph) remained in the haemolymph of abalone maintained on fresh *Ulva* (Figure 3-8). There was however no significant difference in the number of culturable bacteria remaining in the haemolymph of animals in the two treatment groups 30 min post-injection ($F = 1.441$, $p = 0.174$). Each abalone tested negative pre-injection for *V. anguillarum* and clearance counts confirmed by spread plate technique confirmed the presence of *V. anguillarum* post-injection.

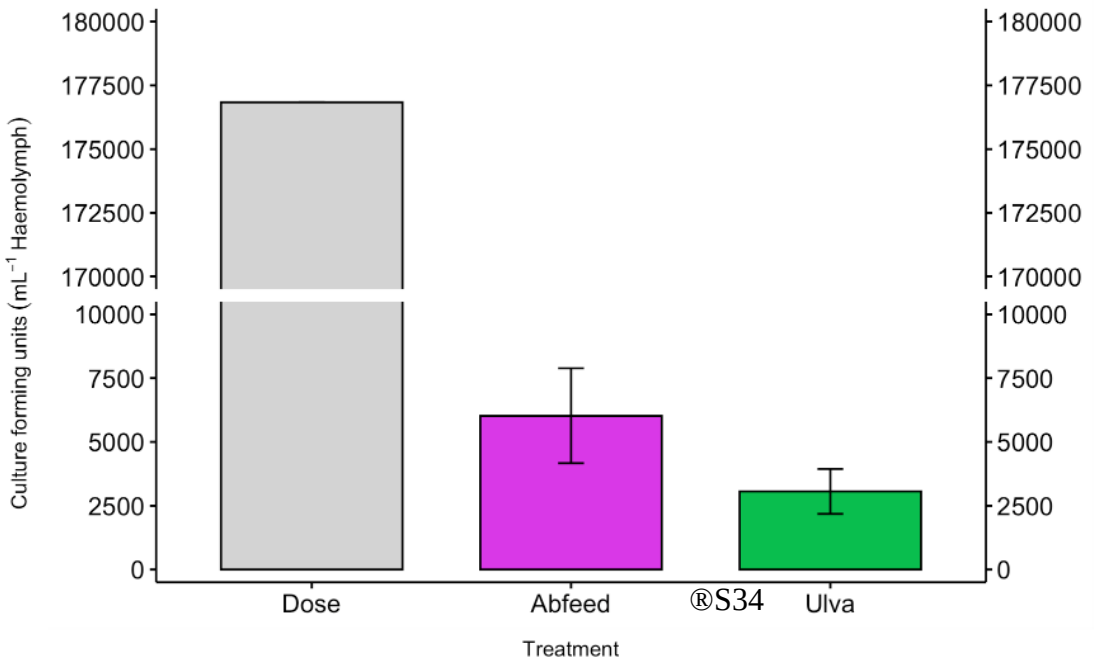

Figure 3-8 Mean (±SE) number of *Vibrio anguillarum* culture-forming units remaining in the haemolymph of abalone fed Abfeed®S34 or Ulva 30-minutes post-injection of a known dose of live bacteria (n=12 abalone per dietary treatment group). Data from n = 10 abalone maintained on Abfeed™ S34® and n = 9 maintained on *Ulva* were used after exclusion of outliers. The theoretical injected load of 176835 CFU.mL^{-1} is marked by the grey bar (Dose). The y-axis is broken into two sections (0 – 10000 and 170000 – 180000) as indicated by the break in the Dose bar.

3.3.5.3 Total haemocyte count

Pre-challenge circulating mean (SD) haemocyte numbers for abalone maintained on Abfeed®S34 and fresh *Ulva* for a period of six months were 6840152 ± 2664905 and 7447917 ± 1727898 THC.mL^{-1} haemolymph, respectively (Fig. 3-9). There was no statistically significant difference in the number of circulating haemocytes between treatment groups prior to injection with 100 µL of a 4 × 10^{7} CFU.mL^{-1} dose of *V. anguillarum* (t = -0.654; p = 0.519). Following injection, abalone that had been maintained on Abfeed™ S34® for six months had a lower mean (±SD) THC.mL^{-1} haemolymph at 30 minutes post-injection (6840152 ± 2664905 haemocytes.mL^{-1}) compared to pre-injection levels (5394886 ± 3132065 haemocytes.mL^{-1}), but this difference was not statistically significant (Figure 3-9 ; p = 0.258). Conversely, abalone

maintained on a diet of fresh *Ulva* had significantly lower numbers of circulating haemocytes at 30 minutes post-injection (5352462 ± 1726384 THC.mL^{-1} haemolymph) compared to the pre-injection levels denoted by the star in Figure 3-9 (7447917 ± 1727898 THC.mL^{-1} haemolymph; t = 2.907; p = 0.008).

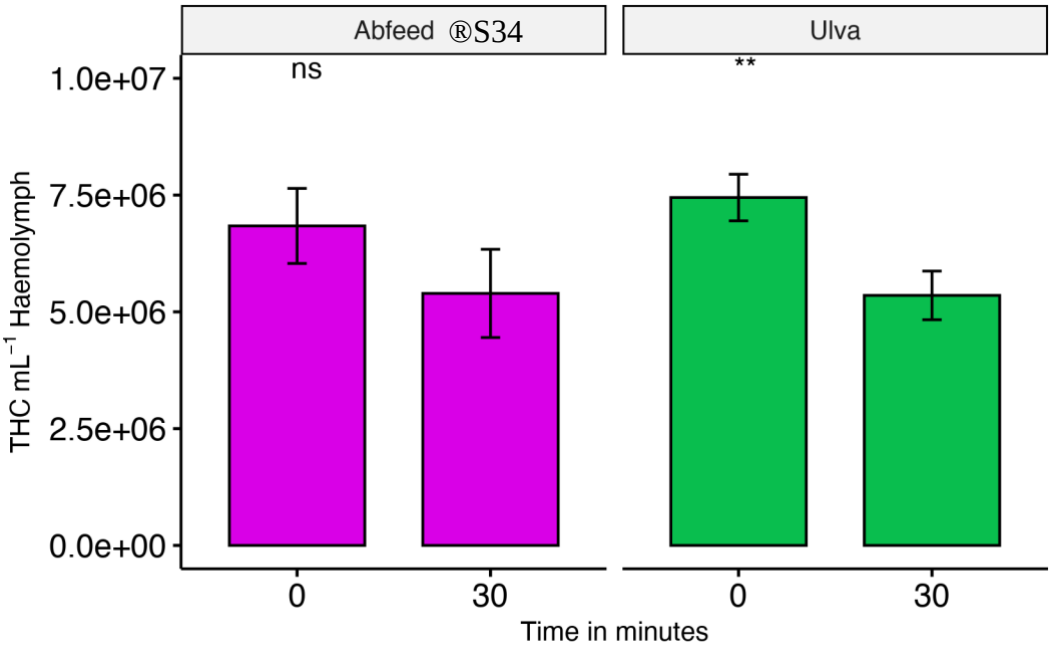

Figure 3-9 Mean (±SE) total haemocyte counts for abalone maintained on Abfeed®S34 or fresh Ulva for a period of 6 months pre- and post- (30 minutes) injection with a known dose of live *Vibrio anguillarum* (n=12 abalone per treatment group). Significance for within group differences is denoted an asterisks above the respective bar (p < 0.01). ns denotes no significant difference.**

3.4 Discussion

3.4.1 Summary

This study demonstrated that diet has a significant effect on the circulating haemolymph glucose concentrations of the abalone *Haliotis midae*. Specifically, abalone maintained on a diet of fresh *Ulva* for a period of 6 months had significantly lower haemolymph glucose concentrations than abalone maintained on a dry formulated feed, Abfeed™ S34®. To my knowledge, this is the first study to demonstrate an effect of diet on *H. midae* haemolymph glucose concentrations and is also the first study to illustrate how efficient farmed *H. midae*, fed both fresh *Ulva* and a dry formulated feed for an extended period of six months, are at rendering an injected dose of bacteria non-culturable, with more than 98% of the injected bacteria rendered non-culturable within a 30-minute period. The bacterial clearance efficiency of abalone fed Abfeed™ S34®, which contains a high content of maize starch as the primary carbohydrate (Sales & Britz, 2002) does not appear to compromise the bacterial clearance efficiency, whereas fresh *Ulva* appears to have an impact on the number of circulating haemocytes following the bacterial injection. Circulating haemocyte numbers of abalone fed the diet of fresh *Ulva* where significantly reduced 30 minutes post injection with live *Vibrio anguillarum*, compared with pre-injection levels, suggesting that the primary immune effector cells of abalone maintained on a diet of *Ulva* respond more efficiently to a challenge with non-self particles.

The results from the current study on haemolymph glucose concentrations under starvation show that the glucose titre for *H. midae* is around 20 $\mu g.mL^{-1}$ of haemolymph, which is possibly the physiological minimum level required for basal metabolic processes to occur (Brand, Bolton & Macey, 2013). The starvation curve for haemolymph glucose matches what is expected under endocrine control via insulin-like substances (Plisetskaya & Joosse, 1985). More recently, the metabolome of *H. midae* has been investigated which confirms that abalone actively participate in glycaemic regulation using evidence from the metabolites present in *H. midae* (Venter et al., 2016). Still, a more complete mechanism involving its specific metabolites is unknown and requires further research.

3.4.2 Interpretations

In this study, the basal haemolymph glucose concentrations recorded for *H. midae* ranged from 21 - 52 μg.mL^{-1} haemolymph (2.1 - 5.2 % w/v) with the diet high in digestible carbohydrates, Abfeed™ S34®, significantly increasing the basal haemolymph glucose concentration in abalone to 47.59 ± 5.01 μg.mL^{-1} haemolymph, when compared to abalone maintained on a diet of fresh *Ulva* (31.39 ± 11.62 μg.mL^{-1}). Elevated levels of glucose in the haemolymph of animals fed a diet high in digestible carbohydrates has been found to be indicative of decreasing efficiencies of insulin in the insulin-sensitive tissues (Guo et al., 2022). Insulin, the hormone responsible for stimulating the uptake of glucose into the cells, has a role in activating protein synthesis and the inhibition of protein catabolism, which favours growth and is an important characteristic for production. Decreased efficiencies of insulin in relation to diet may therefore adversely impact the growth/ production. Venter et al. (2018) speculated that fast growing *H. midae* are able to release insulin at a faster rate or that the released insulin results in a greater metabolic response. The upregulation of genes related to insulin production have been previously identified in faster growing abalone (Van Der Merwe, Franchini & Roodt-Wilding, 2011) and these observed differences may impact metabolic assimilation of nutrients from the diet (Venter et al., 2018).

A study conducted by Rossi & Silva (1993) found that a high carbohydrate diet fed to the land gastropod *Megalobulimus oblongus* resulted in significantly elevated haemolymph glucose concentrations compared to animals fed a control diet of lettuce. They also reported significantly greater glycogen concentrations in the mantle, digestive gland and foot muscle, that exhibited different rates of glycogen deposition when compared to *M. oblongus* maintained on a diet of lettuce (Rossi & Silva, 1993). Zhang, Zhou & Cheng (2009) offered the marine gastropod *Babylonia areolata* diets of increasing starch levels and found an increase in their soft body glycogen content, which correlated with a decrease in tissue lipid content. The marine crab *Chasmagnathus granulata* fed a high carbohydrate diet was found to have increased muscle glycogen and lipid levels, which was accompanied by significantly increased haemolymph glucose concentrations compared to crabs maintained on a high protein / low carbohydrate diet (Kucharski & Silva, 1991). These results are in line with the

findings of this study in which a high carbohydrate diet can significantly increase the haemolymph glucose concentrations in an animal, specifically *H. midae*.

The high glucose concentration in the haemolymph of abalone maintained on a diet of Abfeed™ S34® in this study may be an indication of alterations to energetic pathways which is onset by dietary provisioning of different diets. Data from metabolomic differences in fast and slow growing wild *H. iris* suggests that low haemolymph glucose concentrations are an indicator of a greater reliance on anaerobic glycolysis for locomotion (Venter et al., 2022). Venter et al. (2018) identified a reduction in mitochondrial function in slow growing individuals linked to their energy production pathways, while fast growing individuals showed greater abilities in the generation of an insulin response and improved mitochondrial activity. Oxidative stress is also associated with hyperglycaemia, whereby the increase in available glucose will generate more substrates entering into mitochondrial respiration, potentially generating higher levels of superoxide radicals (Furukawa et al., 2004). The end-point analysis of glucose levels in haemolymph circulation can be an important indicator of the complex regulation of consumption, where elevated post-prandial glucose levels are associated negatively with appetite (Harvey-Anderson & Woodend, 2003; Schock et al., 2010; Johansson et al., 2013).

The onset of feeding is thought to be linked to decreasing intracellular glucose concentrations (Mayer, 1953) which may occur less frequently for abalone in this study which were maintained on Abfeed™ S34®. Abalone maintained on Abfeed™ S34® for a period of six months and then starved for 6 days exhibited an elevated haemolymph glucose concentration to a maximum of 56.7 % increase for over 60 minutes after receiving an intravenous injection of glucose, returning to basal (pre-injection) levels after 120 minutes. Conversely, abalone maintained for the same period of time on a diet of fresh *Ulva* had a shorter but more intense glycaemic response (~ 30 minutes) which increased the total circulating glucose concentrations by 67.6 % following administration of the same glycaemic load, returning to basal (pre-injection) levels within 45 minutes. While there remains insufficient knowledge on the basic biochemical constituents of abalone under culture conditions (Sales & Janssens, 2004; Venter et al., 2016), ongoing research into the role that highly digestible carbohydrates play in abalone feed formulations should be carefully considered.

The effect of carbohydrates in the development of the metabolic syndrome are characterised when two or more of the following conditions are met; hypertriglyceridemia, reduced high-density lipoprotein, hypercholesterolemia, high blood pressure, hyperglycaemia and elevated fasting plasma glucose (Termizy & Mafauzy, 2009). Wang et al. (2009) evaluated semi-purified diets that were formulated to contain dextrin, heat-treated wheat starch, wheat starch, corn starch, tapioca starch and potato starch at the same percentage (33.5%) and their impact on serum triglyceride (TG) levels in *H. discus hannai* demonstrating a correlation between high starch digestibility and increased levels of TG. Similarly, when the abalone *H. discus hannai* was maintained on a diet with increasing carbohydrate content there was increased biosynthesis of fatty acids in the abalone tissue and beyond 45.55 % carbohydrate content the insulin signalling pathway was inhibited (Guo et al., 2022). The abalone that had been maintained on a diet of Abfeed™ S34® in this study displayed both hyperglycaemia and elevated fasting haemolymph circulating glucose levels, which are likely a result of the high carbohydrate digestibility and specific carbohydrate sources in the dry formulated feed. The results presented in the former studies (Wang et al., 2009; Guo et al., 2022), along with data presented in this thesis, would suggest that two of the conditions for the development of the 'metabolic syndrome' are met (hypertriglyceridemia and hyperglycaemia with elevated fasting plasma glucose) when highly digestible carbohydrates are used as a dietary ingredient.

It is apparent that diet can significantly influence the basic energy metabolism of abalone and may be considered a part of the reason that highly digestible carbohydrates are hailed as providing overall benefits to their commercial cultivation. Venter et al. (2018) reported that the metabolome of faster growing abalone has metabolites that indicate both increased glycolytic activity and decreased utilization of the β-oxidation pathway with high levels of mitochondrial activity. Specific improvements to the metabolic efficiencies of an organism through dietary modification can be a useful tool in the optimisation of feed efficiencies. Tas et al. (2011) demonstrated that *Ulva rigida* was able to improve the carbohydrate metabolism in streptozotocin-induced rats by lowering the circulating blood glucose, increasing insulin levels, and decreasing serum triglycerides when compared to controls. Sulphated polysaccharides extracted from *Ulva intestinalis* have been used to enhance growth and improve the immune function

of freshwater rainbow trout whereby lower activities of SOD were recorded in the group being administer *Ulva* extracted polysaccharides (Safavi et al., 2019). Alterations to the primary energy metabolism in organisms can impact the mobility of energy reserves available to an organism (Brown, 2000; Butler, Btaiche & Alaniz, 2005) and these changes can alter the efficacy of an immune response.

There is limited knowledge on the metabolome of the immune system response (the detectable metabolites that are related to immune activation) of abalone making overreaching conclusions difficult to define (Hooper et al., 2007; Beltran & Coyne, 2020). Similar bacterial challenge models to the one in this study have been used to assess the effects of a variety of environmental stressors on the overall immune response of the Atlantic blue crab *Callinectes sapidus* (Holman, Burnett & Burnett, 2004; Macey, Rathburn, et al., 2008; Ikerd, Burnett & Burnett, 2015), spiny lobster *Jasus lalandii* (Knapp et al., 2019), and the Eastern oyster, *Crassostrea virginica* (Macey, Achilihu, et al., 2008; Williams et al., 2009). The bacterial clearance rates of abalone in this study were rapid, with more than 80% (<40,000 CFU.mL^{-1} haemolymph remaining) of the theoretical injected load of bacteria (178,890 CFU.mL^{-1} haemolymph) rendered non-culturable within a 20 minute period. In contrast, the oxidative response in *Haliotis discus discus* is seen to have an apparent lag-phase after stimulation for 60 minutes followed by a regular increase in oxidative activity (Donaghy et al., 2010). For the abalone maintained on either *Ulva* or Abfeed™ S34® there were no significant differences in the bacterial counts post injection while both groups displayed profound clearance ability in just 30 minutes. Abalone which had been maintained on *Ulva* had an average of 0.6 % of the approximate injected bacterial load in circulation whereas those maintained on Abfeed™ S34® had 1.2 %, although this difference was not significant. These results demonstrate that a diet of Abfeed™ S34®, which contains a high content of pre-gelatinized maize starch as the primary carbohydrate, does not adversely compromise the bacterial clearance efficiency of farmed abalone when compared with animals fed fresh *Ulva*.

Data on the immune response in *H. midae* has shown that the mitochondrial respiratory chain is highly activated in the haemocytes, inducing an increase in biochemical activity

and a greater demand for high energy molecules adenosine triphosphate (ATP) (Rensburg & Coyne, 2009; Coyne, 2011). If a hypothetical abalone in the current study were required to double the concentration of glucose in the haemolymph, those maintained on Abfeed™ S34® would need to release 51.6% more glucose than abalone that were maintained on *Ulva* via glycogenolysis or glucogenesis. In this study, there was no significant difference in haemolymph glucose concentration between the two groups of abalone following injection of viable *V. anduillarum* directly into haemolymph circulation prior to the bacterial challenge. The lack of a significant decrease in circulating haemolymph glucose for the abalone suggest that they were metabolically responsive and/or that glycogen stores were able to provide adequate energy required for the response. This is an important finding for local abalone (*H. midae*) producers as it has been postulated, and demonstrated in some mammalian models (Roberts, 2000; Harvey-Anderson & Woodend, 2003; Kumar et al., 2015), that the inclusion of high amounts of highly refined starches in animal feeds can compromise the health and physiology of animals.

Stimulation of the immune system of *H. midae* via dietary manipulation has been demonstrated by Macey and Coyne (2005). The latter authors included three probiotic microorganisms, one bacterium (*Vibrio midae* SY9) and two yeasts (*Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1), into the formulated feed Abfeed™ and demonstrated a significant improvement in both the growth and innate immune (phagocytic) response of the abalone, as well as an improvement in survival following challenge with *V. anguillarum*. These authors postulated that the probiotic cells stimulated the immune system of *H. midae*, enabling the abalone to respond more rapidly to an infection when compared with non-immunostimulated animals. Following the bacterial challenge in this study, an initial decline in circulating total haemocyte numbers was observed, which were shown to be significantly lower than pre-injection levels at 20 minutes following the injection (Figure 3-6), before counts returned to the pre-injection levels within a 24-hour period. These findings are again in line with observations made for several other marine invertebrates that were challenged with a known dose of bacteria (Holman, Burnett & Burnett, 2004; Powell et al., 2006; Macey, Rathburn, et al., 2008; Knapp et al., 2019).

The initial and often rapid decline in circulating haemocyte numbers has been attributed to the movement of bacteria to the site of injection/ wounding (Powell et al., 2006) and following recognition of the non-self particles the immune cells are capable of employing a wide variety of functions, including phagocytosis, cytotoxic and inflammatory responses (Macey, 2005). This rapid innate response also results in the formation of loose aggregates of bacteria and haemocytes, and these aggregates then become trapped in the fine vasculature of numerous tissues/organs, especially the gill tissues which can reduce oxygen uptake (Schock et al., 2010), as has been clearly demonstrated in the Atlantic blue crab *C. sapidus* (Ikerd et al., 2015). Rensburg & Coyne (2009) demonstrated that dysfunctional mitochondria resulted in significantly less ATP production in *H. midae* haemocytes and that their phagocytic capacity was reduced. For abalone maintained on the diet of Abfeed®S34, the decline in total circulating haemocyte numbers was not significantly different between pre- and post-injection levels following challenge with the *V. anguillarum*. Conversely, abalone maintained on a diet of fresh *Ulva* exhibited a significant decrease in total haemocyte numbers 30 minutes after injection with *V. anguillarum*. The significant reduction in THC for *Ulva*-fed abalone suggests a more efficient response by haemocytes from this group of abalone. Conversely, abalone maintained on Abfeed™ S34® may have had impaired mitochondrial functioning due to the interactions of the high carbohydrate content with energy homeostasis (Venter et al., 2018). Research by Leiro et al. (2007) and Akbary & Aminkhoei (2017) demonstrated that the water-soluble polysaccharide extracted from *U. rigida* enhanced mouse macrophage activity and improved the growth and immune response of flathead grey mullet, *Mugil cephalus*. Castro et al. (2004) investigated the effects of a cold water extract made from *U. rigida* and found that it was capable of significantly increasing the respiratory burst activity of the fish *Psetta maxima* phagocyte cells, which was likely associated with the polysaccharides. The above studies highlight the immunostimulatory role of *Ulva* or specific extracts that can be made from *Ulva*, which are capable of enhancing the overall health of the cultured organism.

3.4.3 Implications

Haemocytes play a central role in the clearance of foreign particles and this study suggests haemocytes from *Ulva*-fed abalone were more primed for the defence against

injected *V. anguillarum* compared with abalone maintained on the diet of Abfeed™ S34®. The modulation of the gut microbiota through nutrient inclusion, as would be the case for the inclusion of prebiotics, has also been used as a treatment to reverse host metabolic interactions linked to gut dysbiosis in the human gut microbiome (Geurts et al., 2014). It is possible that *Ulva* or components associated with *Ulva*, elicited a similar immune-stimulatory response in abalone maintained on this diet in the present study, and further investigation of the effect of *Ulva* on the gut microbiome is thus required. It is apparent that seaweeds are highly dynamic ingredients and although the abalone producers continue to prefer the consistency of supply of a formulated feed, specifically a high carbohydrate feed, this study provides the first evidence that diet significantly alters the basal haemolymph glucose concentration in *H. midae*. Shifts in these evolutionarily conserved metabolic pathways should be further investigated when looking to enhance the functional ingredients of formulated feeds. The prolonged hyperglycaemia exhibited by abalone maintained on Abfeed™ S34® in conjunction with the glucostatic theory highlight a potential negative impact on feeding activity that could be counteracted with the inclusion of dietary *Ulva*. Furthermore, *Ulva* has also been reported to have antimicrobial properties and the bioactive sulphated polysaccharide, ulvan, has been shown to inhibit both gram-negative and gram-positive bacteria (Berri et al., 2016). To further optimize the nutritional impacts on the overall health of abalone, it is recommended that *Ulva* be investigated further for its potential as a functional food for the abalone *H. midae* which could further enhance the sustainability of an existing industry.

Chapter 4 - Comparison of dietary effects of whole *Ulva lacinulata*, and the prebiotic potential for specific components thereof, on the growth, physiology, and gut microbial patterns of the cultured abalone *Haliotis midae*

4.1 Introduction

4.1.1 Context and topic

The seaweed and its associated microbiota are defined as the meta-organism, which comprise the holobiont (Egan et al., 2013; Lachnit, Thomas & Steinberg, 2015; Gobet et al., 2018) where bacteria can be fundamental in the functioning of the seaweed (Lachnit, Thomas & Steinberg, 2015). *Ulva*, a green alga and a primary focus of this work, is host to a complex epiphytic microbiome with bacterial cells on the thallus of *Ulva australis* ranging in density from 10^6 - 10^7 cells.cm^{-2} (Tujula et al., 2010). The community composition of the epiphytic microbiome on *U. australis* is driven by environmental pressures and exhibits a functional selection for the specific organisms, rather than species level specificity forming distinct communities that are identifiable from the surrounding water (Burke et al., 2011; Egan et al., 2013). *Ulva rigida*, when grown in integrated multitrophic aquaculture systems (IMTA), is dominated by an epiphytic microbial community that is different from the wild type and significantly shaped by the composition of effluent-water microbial communities of seabass and seabream (Califano et al., 2020). Bacteria associated with seaweeds, when consumed, often play an important role in the establishment of a normal non-opportunistic microbial community in the digestive tract where they have the potential to contribute significantly towards digestion and the assimilation of dietary components (Gobet et al., 2018; Wang, Pyecroft & Barton, 2020; Danckert et al., 2021).

The occurrence of what appears to be a ‘core microbiome’ for *Haliotis tuberculata* (Gobet et al., 2018), *Haliotis laevigata* and *Haliotis laevigata* × *Haliotis rubra* (Danckert et al., 2021) has been identified using a combination of next generation

sequencing and functional bioinformatics. The microbiome sequenced from the digestive gland of *Haliotis tuberculata*, indicate a relationship between the algal polysaccharide fermenters and the algal diet of the abalone, which were dominated by the bacteria from the genera *Psychrilyobacter*, *Mycoplasma*, and *Vibrio* (Gobet et al., 2018; Danckert et al., 2021). *Vibrio halioticoli* strains, which have been documented as a major component of the abalone holobiont, including *H. midae*, and *Vibrio* spp. have the capacity for fermentation of algal polysaccharides into short chain fatty acids (SCFA), which would increase the amount of energy available for absorption in the intestinal tract (Sawabe, 2006; Gobet et al., 2018; Danckert et al., 2021). It is hypothesized that the *Ulva lacinulata* holobiont, and its carbohydrate components, can be used through dietary supplementation to modulate the microbiome and consequently, improve the overall growth and production of the cultured abalone *Haliotis midae*.

4.1.2 Scope and focus

There are numerous interactions along the journey food takes, however these can be simplified to absorption, distribution, metabolism, and excretion (Mo'o et al., 2020). Abalone feed on a diet of fresh seaweeds by grinding the algae thallus along the radula (foregut), where large portions of cellular materials containing ulvan (prebiotic), are made available for bacterial digestion within the mid- and hindgut. After foods have been sufficiently digested into their simplest building blocks, they must interact with the microbial inhabitants that forms a dense network of cells in the intestinal mucosal lining where they have the capacity to interact with food particles (Roberfroid et al., 2010; Geurts et al., 2014; Wells et al., 2016; Martin-Gallausiaux et al., 2020). During passage through the gastrointestinal tract (GIT), the prebiotics are presented to the microbiome in the mid- and hindgut where they are able to metabolize the food into SCFA prior to absorption (Wang et al., 2018), which are then transported into the abalone tissues (Majee et al., 2018). Many bacterial inhabitants of the gut are ubiquitous in the environment and have evolved remarkable metabolic and functional diversity that has enabled them to colonize a wide variety of surfaces/ substrates (Tarnecki et al., 2017), where they are ultimately excreted back into the surrounding environment of aquaculture systems.

Prebiotics have been used at an inclusion level of around 1 %, where they have had a significant impact on host health and improving metabolic efficiencies related to metabolic disorder (Zheng, Chen & Cheong, 2020). Fructooligosaccharides (FOS) have been used as a prebiotic to select for the growth of *Bifidobacteria* and *Lactobacillus* in rats, which are both associated with the production of SCFA that have been shown to improve gut and host health (Wilson & Whelan, 2017). Alternatively, the inclusion of specific bacterial strains into formulated feeds as dietary probiotics for *H. midae* has successfully demonstrated its ability to significantly improve the growth and health of cultured abalone (Macey & Coyne, 2005). The supplementation with beneficial bacterial partners along with diet-mediated microbiome modulation is presented as a strategy to improve aquaculture productivity through enhanced feed efficiencies (Rajeev et al., 2021).

Significant advancements have been made to improve our understanding of the architecture and function of intestinal microbiota (Xu et al., 2013; Fernández et al., 2016; Hindu et al., 2019; Lopez-Santamarina et al., 2020; Rajeev et al., 2021) and one of the current interests of research for seaweeds are their bioactive polysaccharides which exhibit chemical, physicochemical and fermentation characteristics that are not found among higher plant carbohydrates (O'Sullivan et al., 2010; Wang et al., 2014; Aguilar-Briseño et al., 2015; Wells et al., 2016; Safavi et al., 2019). *Ulva* has been reported to contain approximately 38 - 54 % (w/w) dietary fiber (Ray & Lahaye, 1995; Gao et al., 2018; Neto et al., 2018; Mo'o et al., 2020) of which a large portion are highly sulphated polysaccharides called ulvan. Ulvans are a group of water-soluble anionic polysaccharides which comprise 9 – 36 % of the polysaccharides from dried *Ulva* and those extracted from *Ulva lactuca* have been reported to yield the following monosaccharide composition; rhamnose (8.2 – 60.8 %), glucuronic acid (2.6 – 52 %), xylose (0 – 35.4 %), iduronic acid (5 – 6 %), glucose (2 – 87.2 %), galactose (0 – 1.9 %) and uronic acid (16.8 – 47.1 %) (Kidgell et al., 2020). There are distinct features across sources of *Ulva* where the general trends in biological activities relative to its structural features remains ambiguous (Kidgell et al., 2020) however, their prebiotic action has been well documented (Wells et al., 2016; Chi et al., 2018; Cañedo-Castro et al., 2019; Lopez-Santamarina et al., 2020).

Ulvans are poorly fermented by human faecal bacteria (Bobin-Dubigeon, Lahaye & Barry, 1997; Ai et al., 2017) however, a bacterium (GH105) isolated from the faeces of the sea slug (*Aplysia punctata*) was shown to be capable of metabolising ulvan (Hehemann, Boraston & Czjzek, 2014) indicating that this capacity may be more widespread in the marine environment. The abalone gut is characterized as a microaerophilic niche environment (Harris, Burke & Maguire, 1998) with unique selective pressures that clearly form associations with genera of bacteria that have the capacity to ferment the algal polysaccharides/prebiotics into SCFAs that the abalone can utilize for metabolic homeostasis (Nel et al., 2017; Gobet et al., 2018; Danckert et al., 2021). Individuals of the same size and living within the same environment can attain differing growth rates if their metabolic potential for assimilation of nutrients and maintenance rates differ (Gurney & Nisbet, 2004), where the microbiome may play a significant role in feed efficiency (Gobet et al., 2018; Danckert et al., 2021).

There are a number of molecular independent techniques which have been used with varying levels of success in trying to understand the connections between bacterial diversity and organismal function (Hamady & Knight, 2009). Two of these approaches are; (1) next generation sequencing platforms that investigate genome variation in the DNA (Ansorge, 2009), or (2) fingerprinting of communities represented by the bacterial DNA markers, such as the 16S rDNA (Ogier et al. 2004, Marzorati et al. 2008). Both of these approaches are subject to sampling bias at a number of levels (Roden et al. 2000, Klindworth et al. 2013, Hahn et al. 2016) which are not standardized. The fingerprinting of communities involves amplification of DNA by genus-specific primers targeting the 16S rDNA sequences, followed by separation/differentiation of this DNA in a polylacrylamide gel containing a linear denaturing gradient (DGGE). The genetic fingerprints obtained through DGGE provide complex band profiles that are a representative of the genetic structure or diversity of the complex microbial community (Muyzer & Smalla, 1998; Ranjard, Poly & Nazaret, 2000). The advantage of this method is that it does not require any previous knowledge of the microbial populations and that it can provide a visual profile of changes occurring in microbial communities subjected to different treatments. DGGE has been successfully used in several studies of the soil microbiome in an attempt to identify community level changes under variable land management practices and the differentiation of intestinal

microbiomes in aquacultured organisms maintained on varying dietary treatments (Bossio et al., 2005; Fry et al., 2006; Shimano, Sambe & Kasahara, 2012; Trabal et al., 2012; Nel et al., 2017; Osimani et al., 2019). DGGE is a useful platform for high throughput sample analysis and comparison of changes in microbial community profiles provided that the shortfalls, including the potential for sample bias (Janse, Bok & Zwart, 2004; Sigler, Miniaci & Zeyer, 2004; Neilson, Jordan & Maier, 2013), are considered during the generation and interpretation of the data. These studies support its use as a tool for identifying differences in microbial communities of abalone fed diets containing specific algal prebiotics.

4.1.3 Importance and relevance

The relatively high concentration of glucuronic acid found within ulvan is interesting, where glucuronic acid participates in the elimination of toxic metabolites (Martínez-Leal, Ponce-García & Escalante-Aburto, 2020) and has a role in biomineralization of calcium carbonate ($CaCO_3$) (Arias & Fernández, 2008; Rao et al., 2014; Pellock & Redinbo, 2017). Ulvan has been used as a feed supplement for the poultry industry, with improvements reported for egg-shell thickness/ strength and yolk colour, when used at a 1 % inclusion level for laying hens (Li et al., 2018). Glucuronic acid has also been found in small amount in the shells of the articulated brachiopods Rhynchonelliformea (Gaspard et al., 2007), where it likely has a role in the biomineralization of $CaCO_3$ (Arias & Fernández, 2008; Rao et al., 2014). The shell matrix is a complex environment that utilizes organic molecules, including mucopolysaccharides, secreted from the mantle, which has been shown to control the deposition of calcium carbonate ($CaCO_3$) in the abalone *Haliotis tuberculata* (O'Neill et al., 2013).

The mucus on the foot and in the gastrointestinal tract of the abalone is a complex mix of mucopolysaccharides containing hyaluronic acid (a linear polymer of repeating units of N-acetylglucosamine and glucuronic acid) (Lopes-Lima et al., 2005; Schiraldi, La & De, 2010; Chen et al., 2012; Wang et al., 2017). The abalone is covered by a layer of mucus which is involved in several important functions including lubrication, locomotion, protection, and adhesion to the substrate (Portela et al., 2012) where glucuronic acid is an important component in the formation of mucopolysaccharides

(Peck et al., 1987; Harris, Bruke & Maguire, 1998). The mucosal barrier forms a critical component of the immune repertoire of the animal (Dawood et al., 2017; Perry et al., 2020) and mucosal production contributes significantly to the energy budget of *Haliotis tuberculata* accounting for 29.1 % of total energy expenditure in 50 g abalone (Peck et al., 1987). Calculations for the rate of mucus production for a stationary *H. midae* exposed to simulated air transport are reported to produce 8.67 % of their body mass in mucus in 36 hours requiring significant energy allocations (Vosloo & Vosloo, 2006). Considering the fact that mucus can have a water content of ~90% (Davies & Williams, 1995) the capacity for mucosal production and specific water content of abalone tissue may be an important route of water loss for the abalone and can impact the abalone holobiont.

The water content of tissues can be influenced by activity and evidence in humans suggests that increased aerobic exercise increases muscular moisture content by an increasing size and spacing between muscle fibres (Mora-Rodríguez et al., 2016). Tissue glycogen, which is stored as an energy reserve in abalone and used during exercise, has been inversely correlated to available tissue moisture in the disk abalone *H. discus* (Watanabe, Yamanaka & Yamakawa, 1992) and the abalone *H. diversicolor* (Chiou, Lai & Shiau, 2001). High tissue moisture can have an impact on weight loss during transportation of live abalone, during canning and in the drying process (Vosloo & Vosloo, 2006; Kemp, 2018). Muscle glycogen content in the abalone *H. diversicolor* showed significant differences between abalone fed dry formulated feeds when compared to those fed exclusively on *Gracilaria* sp. along with high seasonal variations for the group fed formulated feeds (Chiou, Lai & Shiau, 2001). Glycogen is an important storage molecule used in reproduction where peak spawning activity in abalone is associated with depleted glycogen reserves and generally occurs from September – March for locally produced *H. midae* (Ayres, 2013). The glycogen content of meat is commonly considered tasteless, but it is able to significantly impact a range of marketable traits for the abalone such as appearance, canning and transportability (Vosloo & Vosloo, 2006; Brown et al., 2008, Kemp, 2018).

During the canning process abalone meat is thermally processed and sterilized in a brine solution, which results in a decrease in the overall weight of the product (Young &

Olley, 1974). In the meat of tuna, the canning process can cause interactions between tissue glycogen and available lysine that ultimately changes the nutrient composition of the final product (Castrillon, Navarro & Garcia-Arias, 1996) where fast growing abalone have significantly more tissue lysine than slow growing (Venter et al., 2018). The refined starch ingredients used in this study have previously been shown to be readily available for digestion (Sales & Britz, 2002) and are associated with high tissue glycogen content in other organisms (Veldhuijzen ; 1974; Díaz & Nakagawa, 1990; Kucharski & DaSilva, 1991; Rossi & DaSilva, 1993; Zhang, Zhou & Cheng, 2009; Kemp, 2018). Feeds that are able to impact the biochemical components within the marketable product can be designed to enhance the market acceptance of various products destined for consumption.

4.1.4 Objectives and questions

It is hypothesised that the bacterial fermentation of specific dietary ingredients in the intestine of abalone improves overall abundance of specific microbiota and enhances the metabolic capacity of abalone. Members of the genus Mycoplasma are members of the core microbiome identified in abalone that are facultative anaerobes and have the capacity to ferment algal polysaccharides into SCFAs (Nel et al., 2017; Wang, Pyecroft & Barton, 2020; Choi et al., 2021; Danckert et al., 2021) where they activate the energy metabolism in abalone (Gobet et al., 2018).

The broad aim of this research was to investigate the effects of dietary inclusion of *Ulva*, and its carbohydrate components separately, on the growth, physiology and gut microbial patterns of cultured *Haliotis midae*. In a study by Duong et al. (2021) where the abalone *H. laevigata* was fed a diet which was formulated to contain 30 % dried *Ulva* a negative impact on digestibility suggesting a maximum level for inclusion into formulated feeds exists. In this study, feeds were formulated to contain increasingly refined fractions of *Ulva* that were included as substrates for bacterial fermentation and assimilation. The process of drying *Ulva* at 40 °C diminishes its antioxidant activity and has a negative impact on the recovery of phenolics while it enhances the recovery of extracted ulvan making dried *Ulva* an ideal ingredient for inclusion into a formulated feed for its carbohydrate fraction (Silva et al., 2019). Diets were formulated to contain 10 % dried *Ulva*, 1 % ulvan extracted from the same source of dried *Ulva* and 0.1 %

pure glucuronic acid. The ulvan that was extracted from *Ulva* yielded 11.8 % which approximates to a 1 % inclusion at an equivalent level of a 10 % dried inclusion. The glucuronic acid was included at 0.1 % to represent an equivalent amount to what may be available in the 10 % died *Ulva* and the 1 % ulvan feeds. These feeds contained specific components of *Ulva* that were used to explore how these elements are able to influence the overall growth, physiology and intestinal microbial patterns of the cultured abalone.

The specific objectives of this study were to:

1) modify a commercially available abalone feed by incorporating dried *Ulva* or components thereof (ulvan at 1 % and glucuronic acid at 0.1%) into the feed at their relative proportions found comparable to the 10 % dried *Ulva* inclusion;
2) investigate the effect of diet on the growth, physiology, and feed conversion ratio for abalone under experimental conditions during a simulated growth trial over 7.2 months; and
3) compare the effect of diet on the lower intestinal bacterial 16S rDNA patterns for each dietary regime using Denaturing Gradient Gel Electrophoresis.

This research will provide a glimpse into the interaction of diet and microbiome for the cultured abalone, *H. midae*, as well as various functional effects that can be elicited. Farmed abalone are primarily sold as live, canned and dried products from the South African farms and diet can elicit beneficial characteristics for each product. The feeds with specific functional components in abalone aquaculture could further increase the sustainability of the industry by providing specific beneficial traits which may not only be growth. *Ulva* has proven itself to be an ideal candidate for the integration into IMTA systems and understanding the scope of its uses beyond bioremediation should be of vital significance (Bolton et al., 2016).

4.2 Material and methods

4.2.1 Experimental site and water source

The research was carried out at the Department of Forestry, Fisheries and the Environment (DFFE), Marine Research Aquarium (MRA), Sea Point (Cape Town), South Africa (33°91'84.7"S 18°38'12.7"E). All sea water utilized within the Research Aquarium is pumped directly from the Atlantic Ocean via pipes running from the subtidal zone of a semi-sheltered bay. Sea water entering the aquarium is pumped through a drum filter and sand filters prior to entering a sump tank at the highest point in the MRA, whereafter it is gravity fed to the various systems within the aquarium. The 40-year average water temperature for the coastal environment in Sea Point, where the Research Aquarium extracts its seawater from, is 13.08 °C. The temperature ranges from an absolute minimum of 8.7 °C to an absolute maximum of 23 °C.

4.2.2 Source of *Ulva*

All *Ulva* used in this study was sourced from Irvin and Johnson (I&J) Cape Abalone, a commercial abalone farm in Gansbaai in the Western Cape Province of South Africa (34° 37' 35.238" S, 19° 17' 47.2164" E). *Ulva* on this farm was grown in abalone effluent waters that are described in Nobre et al. (2010). The *Ulva* was delivered fresh when required and stocked into a 1000 L polyethylene tank provided with moderate aeration and water flow (2 $L.min^{-1}$). The *Ulva* was maintained under artificial light (24 h light regime) and harvested by hand for feeding.

4.2.2.1 Extraction of ulvan

A crude extract of the water-soluble carbohydrate ulvan was made from a 1.4 kg portion of dried *Ulva* following the basic protocol for "Sample 1" described in (Costa et al., 2012). Briefly, 500 g of dried *Ulva* was added to 4.5 L distilled water and autoclaved to extract the water-soluble fraction. The remaining particulates were removed via filtration through cheese cloth and discarded. The supernatant was concentrated by submerging 5 L beakers containing the supernatant in a heat bath set at 90 °C, with a desk fan blowing air over the surface of the beakers to facilitate evaporation. Once the supernatant was concentrated to approximately a quarter of its original volume, 96 % ethanol was used to precipitate the carbohydrate fraction at a ratio of 3 parts ethanol to

1 part concentrate. The precipitate was collected via centrifugation at 1,000 ×g and the supernatant was discarded. The precipitate was then dried under vacuum and a hammer mill was used to grind the final dried extract to a fine powder, which attained a final yield of 11.8 %.

4.2.3 System design and water supply

The experimental system allowed for control of variables such as feeding amounts, light, water flow, aeration, and stocking density. Sea water, supplied by the main sump (Section 4.2.1), was first passed through a solids settlement tank after which it was actively pumped to the system using a 0.35 kW impeller pump. To maintain a consistent water temperature of 16 °C, a heat exchanger was coupled to the system post solids settlement.

The system contained 36 independent and identical plastic tanks (L x W x H: 390×300×140 mm) which were all housed in two raised fibreglass raceways with dimensions of L x W x H: 3210 × 1000 × 200 mm (Figure 4-1). Each tank contained two pieces of PVC half rounds with a radius of 90 mm and a length of 150 mm to provide shelter and increase the available surface area for attachment of abalone. The fibreglass raceways had an incline on the bottom, which allowed for drainage of effluent water from each plastic tank to a single common point in the large fiberglass raceway.

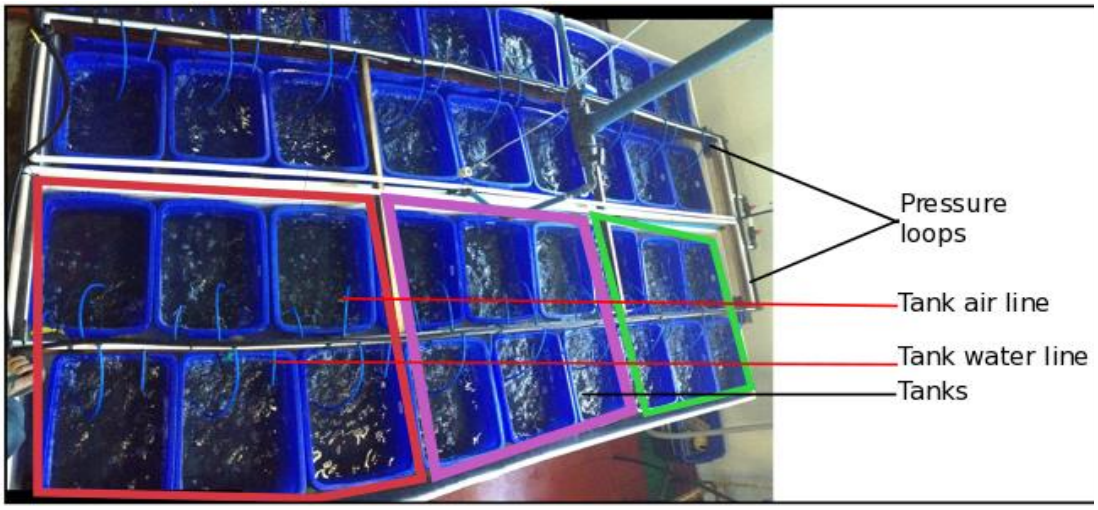

Figure 4-1 Top view of the experimental system used for the growth trial. Each plastic tank received sea water, maintained at 16 °C, provided by two separate pressure loops and aeration from a single air loop. Groups of six tanks were considered as single replicate, units as marked by the coloured squares.

The water flow to each tank was controlled by two identical pressure loops which were each controlled by a single tap; to enable better control of flow-rates into each tank. Each experimental tank (~16 L) was supplied with fresh sea water, delivered to the left-hand side of each tank approximately halfway along its length, with the flow rates maintained at 300 $mL.min^{-1}$; equating to a water exchange rate of one tank volume per 54 minutes (Figure 4-1).

The experimental system was divided into six replicate areas with six tanks per area. For the purposes of this experiment, only the combination of six tanks in a replicate is defined as a single replicate unit. Each replicate unit was covered by a roof, which was constructed to hinge open and was made using 20 mm PVC pipe covered with correx plastic supplied by Maizeys, Paarden Island, Cape Town (Figure 4-2). Each tank was cleaned twice a week by carefully removing the PVC half rounds, gently brushing the edges of the tank and inverting the contents to ensure complete removal of particulates. Each tank was scraped clean every three months using a paint scraper to remove any calcitic growth on the sides of the tank during the sampling protocol, which is detailed in Section 4.2.6.

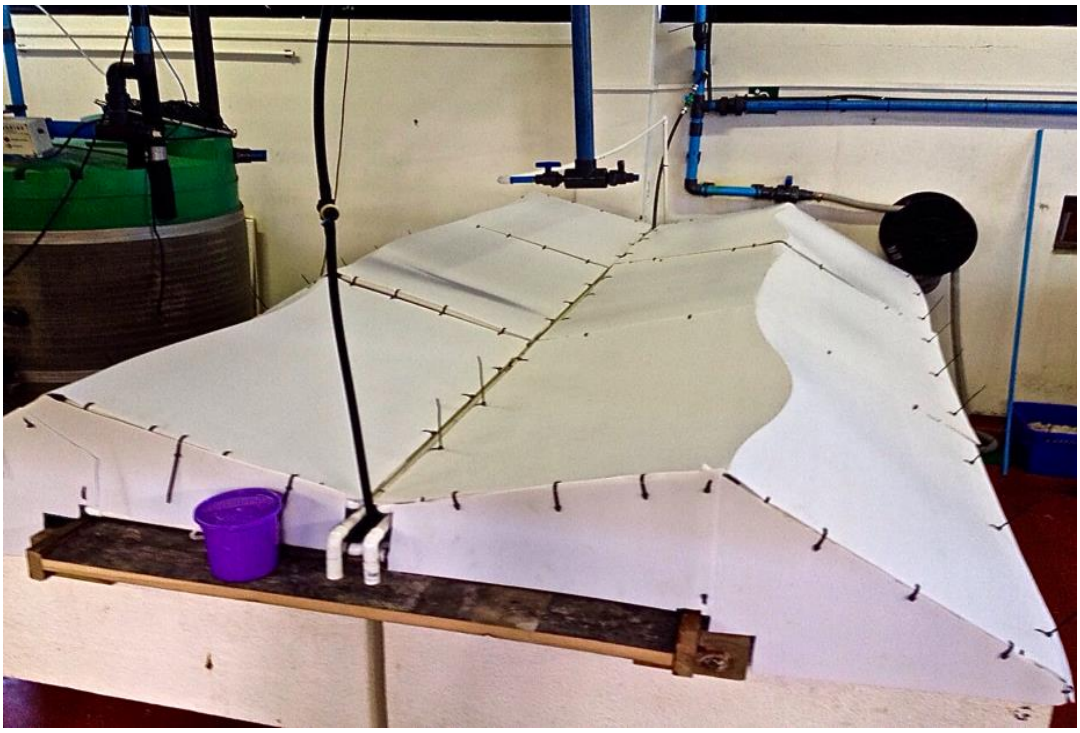

Figure 4-2 Experimental system used for the growth trial, with the covers down to protect the abalone from ambient light and disturbance. Each cover was designed to hinge open over a single replicate unit and minimize light inception, reduce visible moment, and limit external disturbance. The lights in the experimental area were kept off at night to maintain day night cycles.

4.2.4 Animals, acclimation and tagging

All abalone for this experiment were sourced from Abagold (Pty) Ltd, Hermanus, South Africa (34°26'02.8"S 19°13'17.7" E) and were obtained from a single cohort that was batched-spawned on the 6 September 2012. Abalone in the size range of 20 – 30 g were selected for inclusion by individually weighing animals to the nearest 1 g using an electronic scale on the farm. The abalone were transported in cotton netting bags, which were packed into sealed plastic bags inflated with pure oxygen and placed into polystyrene cooler boxes with ice packs for transport from Hermanus to the Sea Point MRA in Cape Town. Upon arrival at the MRA, abalone were immediately removed from the cotton net bags and placed into the experimental tanks where they were allowed to acclimate for one month on the same formulated feed that was provided/fed to abalone at Abagold (SAF4000, https://www.specialisedaquaticfeeds.co.za/aquatic-feeds). During the acclimation period, abalone were randomly shuffled between the

replicate tanks to allow for mixing of the various groups, which may have been an artefact of the farm sorting process.

After the one-month acclimation period, each abalone was tagged using a variety of bead types (shapes and colours; n = 30), which were purchased from Beads for Africa in Cape Town. A number between 1 and 30 was allocated to each unique bead type (Figure 4-3). In order to adhere the beads to the abalone shells the replicate tanks were submerged in a larger container of fresh sea water that received a steady supply of carbon dioxide, bubbled through a large air stone, in order to anaesthetise the animals which is in line with standard farm procedure at sorting. Following anaesthesia, each abalone was carefully removed from the tank and the shell blotted dry with paper towel before gluing a bead onto the shell using a combination of sodium bicarbonate ($NaHCO_3$) and super glue (cyanoacrylate). Once the abalone were tagged, they were stocked at 30 animals per replicate tank, which was approximately 2.4 % of the available surface area.

Figure 4-3 Tagging system developed to provide more statistically powerful growth data by marking each abalone with unique beads glued onto their shells. For each replicate, a single bead from all 30 bead types was pooled in test tubes with the respective replicate codes (left side) and were used in the tagging of abalone. A number chart was developed which referenced each bead by a unique number prefixed by the replicate (right side).

4.2.5 Experimental feed formulations, diets and feeding

The experimental feeds were formulated by Specialized Aquatic Feeds (SAF) in Hermanus, South Africa using a proprietary commercial feed recipe (https://www.specialisedaquaticfeeds.co.za/aquatic-feeds). Following production, each feed formulation was packaged and frozen at -20 °C to maintain the integrity of the ingredients. For the purposes of this experiment, the base feed will be referred to as AB, as it differed slightly from the SAF4000 commercial recipe after being balanced according to the amino acid profile of *Ulva*.

4.2.5.1 Preparation of the formulated feeds

For the inclusion of *Ulva* and its carbohydrate components an attempt was made to maintain relative proportions of fresh *Ulva* to dried *Ulva*, ulvan and also glucuronic acid. Dried *Ulva* used for inclusion into the formulated feed was dried and supplied by Specialized Aquatic Feeds. The crude extract of ulvan, which was prepared according to Section 4.2.2.1 from the dried *Ulva*, yielded 11.8 %. This is lower than median values reported for *U. lactuca* but it is within the range that have been reported (Kidgell et al., 2018).

4.2.5.1.1 Formulated feed supplemented with dried Ulva

An inclusion level for dried *Ulva* of 10 %, where 1 kg of dried *Ulva* was added to 9 kg of premix AB prior to extrusion. The feed was labelled AB10U to indicate the 10 % level of inclusion for dried *Ulva*. The raw ingredients used were calculated after balancing the amino acid profile between AB and AB10U.

4.2.5.1.2 Formulated feed supplemented with ulvan

A 100 g portion of the dried ground ulvan was added to 9.9 kg of premix AB prior to extrusion, which represented an approximately equivalent amount of ulvan present in the AB10U feed formulation. The ulvan supplemented feed was labelled AB1U to indicate the 1 % level of inclusion for ulvan.

4.2.5.1.3 Formulated feed supplemented with glucuronic acid

One of the major polysaccharides forming the backbone of ulvan is glucuronic acid, which represents 2.6 – 52 % of the total water-soluble polysaccharide. A 100 g portion

of D-glucuronic acid was sourced from Sigma-Aldrich (Cat# G5269) and added to 9.99 kg of premix AB prior to extrusion. This was intended to represent an approximately equivalent amount of glucuronic acid that would be present in the AB10U and AB1U feed formulations.

4.2.5.2 Experimental diets

The diets formulated and tested in this study were: (1) AB offered as the only feed; (2) a 60:40 dry weight combination of AB and fresh *Ulva* (ABFU); (3) AB with a 10 % level of inclusion for dry *Ulva* (AB10U); (4) AB with a 1 % level of inclusion for ulvan (AB1U); (5) AB with a 0.1 % level of inclusion for glucuronic acid (AB0.1U); and (6) fresh *Ulva* only (FU). Each formulated diet underwent the standard feed quality control checks and near-infrared spectroscopy was used to determine the relative protein, fat, moisture and ash content of each diet (Table 3).

Table 3 The relative protein, fat, moisture and ash content of the diets formulated by Specialized Aquatic Feeds for this study, as determined by near-infrared spectroscopy. Each feed was made in 10 kg batches and kept in a walk-in freezer until needed, thereby minimizing loss of nutritional value. During the feed trials, colour coded feed buckets for each formulation were filled every three weeks and kept at the experimental system.

Formulation	Protein	Moisture	Fat	Ash
AB	35.67	11.38	4.71	6.58
AB10U	36.07	11.49	4.28	7.36
AB1U	35.58	11.12	4.51	6.58
AB0.1U	34.24	11.31	3.81	6.96

4.2.5.3 Feeds and feeding

A standard feeding cup was used for administering dry feeds to each tank, which when full contained 6 - 7 g of feed and was offered to the abalone three times a week on Mondays, Wednesdays and Fridays from February 2015 to September 2015. The size of the feeding cup was adjusted after 4 months to contain between 7 - 8 g of dry feed to account for growth of abalone. The feeding ration was calculated to be approximately 0.34 - 0.4 percent of the combined abalone body weight in a tank per day (% $BW.d^{-1}$).

Each diet was colour coded by using coloured feeding buckets, which were refilled every three weeks. The feeding containers were weighed prior to being filled and these data were used to calculate total feed offered over the experimental period.

Each tank in a replicate unit was randomly assigned a colour by placing plastic tags on the tanks that corresponded to a specific diet formulation. Each replicate unit contained one replicate tank for each diet. Abalone fed a combination of AB and *Ulva* (ABFU) were offered the mixed diet at a ratio of 60:40 AB to fresh *Ulva* (dry weight equivalents). Abalone fed the *Ulva* only diet were fed at a rate approximately equivalent to all other diets on a dry weight basis (average moisture content of *Ulva* calculated to be 84 %) on the same days. To maintain the appropriate feeding portions for fresh *Ulva*, the rations for each replicate were weighed to equal portions, after being spun free of excess water, and placed into feeding buckets that were offered to each replicate in the treatment.

Feed stability was determined by placing a known amount of each dry feed diet into tanks without abalone for a period of 48 hours to account for the loss of solid matter through leaching. After 48hrs, the feeds were collected and dried to a constant weight (~24h) at 100 °C. At the same time, a known weight of each feed was dried and weighed to account for any ambient moisture in the formulated feeds. The difference, after accounting for ambient moisture, in dry weight of each feed before and after the leaching period was used to calculate a leaching factor, which was specific to each dry feed and later used in the calculation of feed conversion ratios.

4.2.6 Abalone sampling

Abalone were sampled and data were collected around day 0, 105 and 215. Sampling was split over a period of two weeks at each time due to the need for both the collection of morphological and physiological data and gut intestinal sampling. In the first week n = 2 abalone were randomly sampled from each replicate, which is further described in section 4.2.6.1 (below). In the second week, all remaining abalone were weighed and measured (described in detail in section 0).

4.2.6.1 Abalone dissections and processing of physiological measurements

Abalone were purged for 24 hours prior to sample collection, after the tanks were emptied of any remaining food, to standardise the fed state of individual abalone. For each replicate unit, the abalone were processed in an identical way by collecting one abalone from each of the dietary treatments. The selection of abalone was done by randomly selecting six coloured beads from a pool of 30 beads, which represented a specific number. Once the abalone were collected from their respective treatments, they were placed shell side down on a bed of ice within a coloured ring that corresponded to their treatment and immediately transported to the dissections table. All the required tools and sample containers were prepared in advance and pre-labelled.

Each abalone was weighed to the nearest 0.01 g and measured to the nearest 0.01 mm and photographed. After the growth data was captured and haemolymph drawn (as described in section 3.2.4.1.3), a sharp sterile scalpel was used to sever the pallial sinus, which was considered the most humane form of euthanasia for the sampling procedure.

4.2.6.1.1 Quantification of the relative shell weight contribution

Once an abalone had been euthanized, the shell was carefully removed by sliding the flat edge of a weighing spoon between the site of attachment for the adductor muscle to the shell and gently detaching the shell. The shell was weighed, placed on a pre-labelled aluminium weigh boat and dried at 100 °C until constant weight for ~48 h for quantification of the relative contribution of the shell to the total abalone weight.

4.2.6.1.2 Abalone intestinal microbiota

After the shell was removed the abalone was pinned onto a sterile dissection tray that had been rinsed with 96 % ethanol. The gastrointestinal tract was carefully dissected away from the foot muscle and gently separated into intestine (blue) or stomach (purple) as indicated in Figure 4-4. A clamp was placed between the stomach/crop and intestine to keep the contents separated which is indicated by the straight lines separating the intestine stomach/crop (see Figure 4-4). The opposite end of the lower intestinal tract, the anus, remained intact which prevented the loss of intestinal contents. The fluids lost from the stomach/crop were collected for enzymatic analysis and stored in 2 mL micro-centrifuge tubes at -10 °C (samples used in other work). The intestinal samples were

stored in sterile pre-labelled 2 mL centrifuge tubes, whereas the stomach/crop samples were stored in 5 mL sterile pre-labelled centrifuge tubes and both samples were immediately flash frozen in liquid nitrogen. The different sections of the digestive tract of abalone have been found to contain significantly different microbial inhabitants with the lower intestinal tract being more selective for fermentation of food products (Guo, 2017) which was the reason for only processing the intestinal samples for this work. The lower intestine is represented by the lower end of the digestive process where the largest degree of bacterial fermentation has been reported to occur. The crop of the greenlip abalone has very few ciliated cells within the crop indicating a low surface area of attachment for bacterial cells (Harris, Bruke & Maguire, 1998). Samples of foot muscle, mantle tissue and left/right gills were also taken for future research into the metabolome and all samples were stored at -80 °C before being processed as described below.

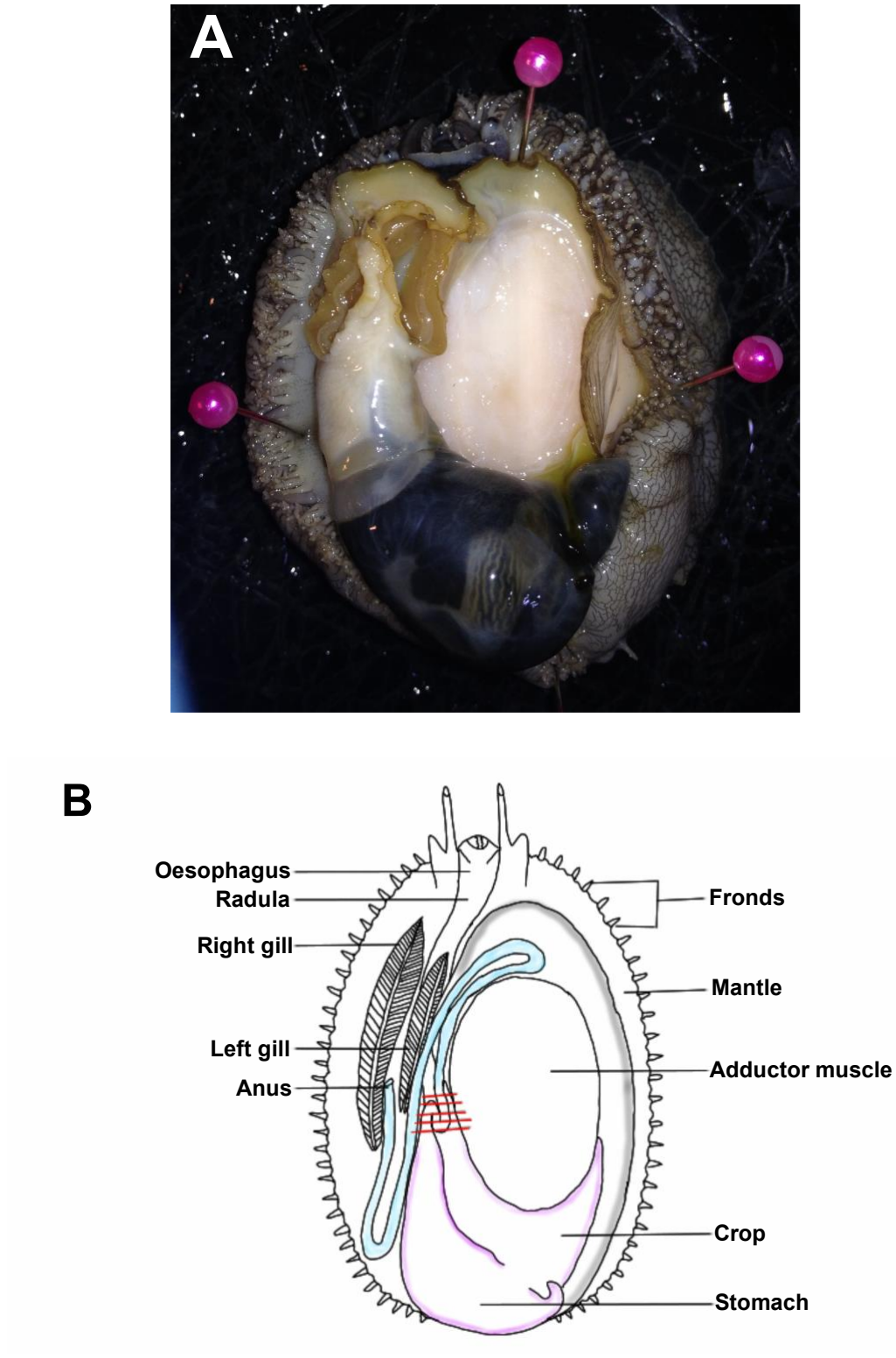

Figure 4-4 Abalone dissection (A) and anatomy (B). Labels indicate the various organs which can be identified on the abalone. Samples of left gill, right gill, mantle, adductor muscle, crop/stomach and the radula were processed for further research.

4.2.6.1.3 Abalone foot muscle moisture and glycogen content

The remaining foot muscle was further dissected for quantification of tissue moisture and muscle glycogen content. Abalone in this study were approximately 30 g and the foot muscle of each abalone was cut into quarters as indicated in Figure 4-5. The entire quarter labelled Section '1' Figure 4-5 was weighed to the nearest 0.01 g before being placed in an aluminium weighing boat and dried at 100 °C until constant weight for quantification of muscle moisture content. Sections '2' and '3', which were smaller sections marked by the inner circle in Figure 4-5 were placed in sterile pre-labelled micro-centrifuge tubes, flash frozen in liquid nitrogen and stored at -80 °C until they could be used for the quantification of muscle glycogen content using the phenol-sulphuric method for the quantification of free glucose (S1) adopted from Bennett et al. (2007) or for future metabolomic research, respectively.

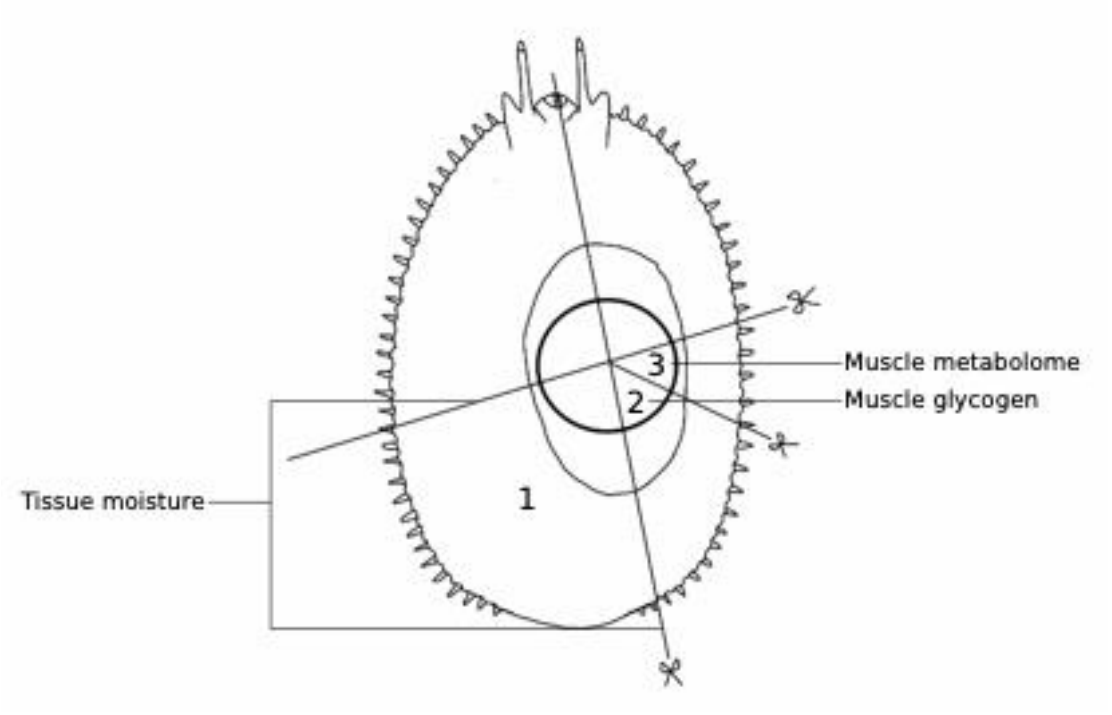

Figure 4-5 Schematic representation of the abalone dissection for muscle tissue collection.

4.2.7 Abalone growth indices and feed conversion

To ascertain whether the diets formulated and tested in this study affected the growth of abalone, all abalone from each replicate in each dietary treatment were sampled at time zero, 105 days and 215 days. Abalone were anaesthetised prior to sampling by submerging each replicate tank in a larger tank containing sea water lightly bubbled with carbon dioxide (CO_2). Following anaesthetisation, individual abalone were photographed, and their unique identifier noted by matching the bead to the chart (Figure 4-3). Each abalone was weighed to the nearest 0.01 g using an electronic balance and length measured to the nearest 0.01 mm using digital Vernier callipers. If a bead was not present the abalone length and weight were recorded and these data were not included in the statistical tests for growth, but were used to calculate feed conversion ratios. The weight and length data collected at each sampling point were used to calculate the following growth parameters:

- Specific growth rate;
- Monthly increment in shell length; and
- Condition factor.

4.2.7.1 Specific growth rate

Specific growth rate (SGR as % growth.day^{-1}) was calculated for each individually marked abalone in each treatment at each sampling period and for the total period using equation 4.1:

$$SGR = \left(\frac{ln(W_f) - ln(W_i)}{t}\right) * 100 \qquad 4.1$$

where $ln(W_f)$ is the natural log of the final weight of abalone, $ln(W_i)$ is the natural log of the initial weight of abalone, and *t* is time in days.

4.2.7.2 Monthly increment in shell length

Monthly increment in shell length (MISL, in mm) was calculated for each individually marked abalone in each treatment at each sampling period and for the total period using equation 4.2:

$$MISL = \left(\frac{SL_f - SL_i}{t}\right) * 30 \qquad 4.2$$

where SL_f is the abalone final length, SL_i is the abalone initial length, *t* is the time in days, and 30 days was considered one month.

4.2.7.3 Condition factor

The condition factor (CF) was calculated according to Britz (1996) for each individually marked abalone. The CF was calculated using equation 4.3 for abalone at the beginning and for each sampling period using equation 4.3:

$$CF = \left(\frac{Weight\ (g)}{Length\ (mm)^{2.99}}\right) * 5575 \qquad \textbf{4.3}$$

4.2.7.4 Feed conversion ratio

In order to calculate feed conversion ratios (FCR), the total amount of feed offered over the experimental period was carefully recorded for each dietary replicate as described in section 4.2.5.3. The total feed offered was corrected for leaching before being used to calculate the FCR for each diet. Fresh *Ulva* was converted to a dry weight equivalent and in the case of diet ABFU the two dietary components were added. FCR was calculated using equation 4.4 and included weights of all abalone from each replicate:

$$FCR = \left(\frac{Wet\ feed\ offered\ (g) * Leaching\ factor}{total\ replicate\ weight\ gain\ (g)}\right) \qquad \textbf{4.4}$$

4.2.8 Denaturing gradient gel electrophoresis SSU rDNA ladder development for normalization of gels

To allow for comparisons between samples run on multiple gels, DNA reference ladders were formulated and loaded on each gel for gel normalization and gel-to-gel comparisons (Ogier et al., 2004). Previous work by Sawabe et al. (2003) identified high concentrations of *Vibrio halioticoli* (40 – 65 %) in the gut of *Haliotis midae*, *Haliotis discus discus*, *Haliotis diversicolor aquatilis*, and *Haliotis diversicolor diversicolor* indicating it may be a member of the 'core' microbiome present in *Haliotis* spp. For this reason, n=10 isolates of *V. halioticoli* were used for the creation of a small subunit DNA reference ladder. A second SSU rDNA reference ladder, which contained SSU

DNA products amplified from bacteria association with/ isolated from *Ulva lacinulata*, was also created for this study.

4.2.8.1.1 Isolates of Vibrio halioticoli

Isolates of *Vibrio halioticoli* (n = 10) were kindly donated by Dr Tomoo Sawabe (Faculty of Fisheries Sciences Marine Life Science, Marine Biotechnology and Microbiology, Hokkaido University, Japan); described in Sawabe (2003). Upon arrival at the DFFE molecular lab in Cape Town, each isolate was maintained on Tryptic Soy Agar supplemented with polysorbate 80 (5 g/L), lecithin (0.7 g/L) (Sigma-Aldrich; # 51414) and 2.5 % sodium chloride and grown at 24 °C for 36 h. After 2 weeks of incubation, a single colony of each isolate was individually picked using a sterile inoculating loop and used to inoculate a fresh plate for the preparation of glycerol stocks. The newly inoculated plates were incubated as before. Following the second incubation period, glycerol stocks were prepared by using a flamed loop to scrape colonies of each plate and transfer them into separate sterile solutions of Tryptic Soy Broth (Sigma-Aldrich; #22092) supplemented with 25 % NaCl (g/v) and 15 % glycerol (v/v) for long term storage. A total of five glycerol stocks were produced for each isolate and stored at -80 °C until required.

Table 4 *Vibrio halioticoli* isolates with strain identifiers used for the creation of a small subunit rDNA ladder for DGGE normalization (Sawabe et al., 2003). The strain identified with accosiation in *H. Midae* in indicated in bold (Sawabe, 2006).

Strain Code
HT 2-1 gal
RW 14
G3-2 F sup
GHG 2-1
HM 3-4
HDD 3-1
ZAM 14967 hali
RW 22
HDS 1-1
HM 1-4

4.2.8.1.2 Isolates of Ulva*-associated bacteria*

Bacterial species found growing on *Ulva* and capable of utilizing ulvan or glucuronic acid (GlucA) as a substrate were isolated by culturing bacteria from *Ulva* on selective media with the latter two substances as the main carbon source. For each substrate (Ulvan/GlucA) duplicate broths were prepared, which were made with 0.1 % yeast extract (Sigma-Aldrich), 0.05 % of either substrate (Ulvan/GlucA), and sterile artificial sea water (ASW: 30 g NaCl, 1.079 g $MgCl_2$, 0.3 g KCl, distilled water to 1000 mL). Bacteriological agar (1%, w/v) (Sigma-Aldrich) was included in the broth for the preparation of agar plates and stored at 16 °C until required.

A 5 g sample of effluent-grown *Ulva* thallus was lightly rinsed in distilled water to remove any transient epiphytic bacteria before being vortexed for 10 min in 50 mL ASW. A 1 mL aliquot of the above rinse solution was used as an inoculum for each 250 mL substrate specific broth, which was incubated on a shaking platform (set to 80 rpm) in an incubator set at 23 °C for a 48 hour period. A second round of selection was

performed by inoculating fresh sterile broths (250 mL) with 1 mL of each respective culture from the previous 48 incubation period and incubating the cultures for a further 48 h.

A 100 µL sample of each broth was serially diluted to $1x10^{-6}$ in phosphate buffered saline (PBS) before being spread plating a 100 µL aliquot of each dilution on the selective agar media containing either Ulvan or GlucA mentioned above. The plates were incubated at 24 °C for 48 h to allow for sufficient growth prior to picking of individual colonies based on visual differences (e.g. colony shape, colour, size, texture and margin). Picked colonies were transferred to PBS and a 100 µL aliquot was used as an inoculant for spread plating onto fresh selective media after dilution (as described above). This step was repeated until there appeared to be one colony type which grew in each selective medium based on the colony morphology viewed at 40 × magnification under a light microscope. For isolates which grew on both substrates, only colonies that grew on plates supplemented with GlucA were further maintained, as the latter media was considered a more selective media than the crude extract of ulvan. Each of the final 12 isolates were cryopreserved in n = 5 replicates of Tryptic Soy Broth (Sigma-Aldrich) supplemented with 2.5 % NaCl (g/v) and 15 % glycerol (v/v) and stored at -80 °C until required.

4.2.9 Bacterial genomic DNA extraction

For the extraction of genomic material from the bacterial isolates described above (see Section 4.2.8), each isolate was grown from cryopreserved working stocks on Tryptic soy agar, supplemented with polysorbate 80 (5 g/L), lecithin (0.7 g/L) (Sigma-Aldrich; # 51414) and 2.5 % sodium chloride, at 24 °C for 36 h. Cells from a single colony of each isolate were carefully transferred using a sterile pipette tip to 25 µL TE buffer in separate sterile 50 µL PCR reaction tubes. The tubes were thoroughly vortexed to re-suspend the bacterial cells before transfer to a heat block set to 95 °C for 5 minutes to lyse the bacterial cells and release the genomic DNA. Thereafter, tubes were centrifuged at 18,000 × g at 4 °C for 2 minutes to remove cellular debris. An aliquot of 15 µL of supernatant, containing genomic DNA, was moved into a new 50 µL PCR reaction tube, which was then used directly as template for the polymerase chain reaction (PCR) described in section 4.2.11 below.

4.2.10 Total genomic DNA extractions from abalone gut tissue samples

Samples were processed in batches, with all samples being treated identically and all equipment sterilized between preparations to prevent any cross contamination of samples. Total genomic DNA was extracted using the method provided by Qiagen for the QIAamp Fast DNA Stool Mini Kit (#51604 - Quick start protocol). Briefly, approximately 150 - 200 mg of intestinal sample was ground in liquid nitrogen and transferred into a pre-cooled sterile 1.5 mL micro-centrifuge tube. A pre-heated (50 °C) aliquot (1 mL) of InhibitEX Buffer was immediately added to the sample, which was thoroughly vortexed and allowed to incubate for one hour at 25 °C. Finally, a 5-minute heat lysis was performed at 95 °C in a heating block to ensure complete lysis of bacterial cells. Following lysis, samples were processed further according to the genomic DNA extraction protocol described in the QIAamp Fast DNA Stool Mini Kit Quick Start Protocol. Each sample's genomic DNA was eluted in 200 µL Buffer ATE, provided in the kit, and stored at -20 °C until needed. The quality and concentration of genomic DNA in each sample was determined using a nano-drop spectrophotometer (Jenway Genova Nano, Bibby Scientific) and the integrity was checked using 0.8 % agarose gel electrophoresis

4.2.11 Amplification of the 16S rRNA gene using polymerase chain reaction

The concentration of DNA in each sample was standardised to range from 75 - 125 ng.µL^{-1}. KapaTaq ReadyMix (Kapa Biosystems; Cat#KK1006) was used for all PCR reactions. The PCR reaction mixtures (25 µL) consisted of 1 uL of genomic DNA (75 - 125 ng), 12.5 µL KapaTaq ReadyMixo, 0.5 µL (200nM) of the forward and reverse primers, respectively, and 10.5 µL PCR grade distilled water. All PCR reactions were performed in a hot start state, by preheating the thermocycler to 95 °C prior to loading the samples and commencing with the thermal cycling programme, using a Labnet Multigene™ Thermal Cycler (Labnet International Inc.).

4.2.11.1 Primer selection and PCR thermal programs

For the amplification of bacterial genomic DNA from the intestinal samples, a nested touchdown polymerase chain reaction (PCR) program was used, which is in line with previous attempts to understand microbial diversity using PCR-DGGE as a

fingerprinting tool (Park & Crowley, 2010; Shimano, Sambe & Kasahara, 2012; Zhao et al., 2012; Alberoni et al., 2018; Dignam et al., 2018). In the first round of PCR, the almost entire (1500-bp) 16S rRNA gene, containing the variable region V3, was amplified using universal PCR primers 16SF1 (5'–AGA GTT TGA TCC TGG CT CAG–3') and 16SR5 (5 –ACG GCT ACC TTG TTA CGA CTT–3'). The cycling conditions used for the first round of the nested PCR approach consisted of an initial denaturation of 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 1 minute, 55 °C for 30 seconds, 72 °C for 1 minute and a final extension of 72 °C for 30 minutes. An aliquot (0.5 uL) from the first round of PCR was used as template in the second touchdown PCR using primers designed by Ariefdjohan, Savaiano & Nakatsu (2010), which included a GC-clamp added to the 5' end of the forward primer to prevent DNA from being completely denatured into single strands in the denaturing gradient polyacrylamide gel. The primers used in the touchdown PCR were forward primer BA338f-GC (5' **CGC CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG** GAC TCC TAC GGG AGG CAG CAG 3'; GC-clamp is in boldface) and reverse primer UN518r (5' ATT ACC GCG GCT GCT GG 3'). The touchdown PCR cycling conditions consisted of an initial denaturation of 94 °C for 5 min, followed by 10 touchdown cycles of 94 °C for 30 seconds, 66 °C (1 °C decreased per cycle) for 1 minute, 72 °C for 3 minutes, followed by 20 cycles of 94°C for 30 seconds, 56 °C for 1 minute, and 72°C for 3 minutes, with a final extension at 72°C for 30 minutes.

4.2.12 Denaturing gradient gel electrophoresis

4.2.12.1 Gel pouring

Gel electrophoresis was performed using a DCode system (Bio-Rad) with 8% polyacrylamide gels poured to contain a linear denaturing gradient from 30 % to 55 %. Each polyacrylamide gel was prepared with 16 lanes for samples. The outer 2 lanes were not utilized, due to the temperature induced 'smiling' effect often associated with DGGE gels that will impact gel-to-gel comparisons and analysis/interpretation of results. Both front and back gels of the DCode system were run in a single operation.

4.2.12.2 Gel loading and runtime

The DCode system was filled with 7 L of 1×TAE buffer, which was pre-heated to 60 °C. Gels were submerged in the pre-heated buffer and allowed 30 minutes to come to

temperature prior to loading of samples and reference ladders. For each gel, 16S rDNA PCR products amplified from 6 individual abalone intestinal tract samples (as described in 4.2.11.1 above) were gently mixed and carefully loaded into lanes 5, 6, 7, 10, 11, and 12 of the polyacrylamide gel. Lanes 3, 4, 8, 9, 13, and 14 were used for the loading of the SSU rDNA reference ladders described in section 4.2.12.3 below. Each of the aforementioned lanes on a gel were loaded with 20 µL of sample, containing 10 µL of PCR product from the touchdown PCR reaction mixed with 10 µL of DCode dye solution (0.05 % Bromophenol Blue, 0.05 % Xylene cyanol, 70 % glycerol and 29.9 % distilled water). Each polyacrylamide gel contained samples that were representative of a single abalone from each dietary treatment and the front and back gel combination during each run contained the SSU rRNA gene products from 12 individual abalone sampled from each replicate unit (n = 2 abalone per replicate). The polyacrylamide gels were electrophoresed for 5 h at 60 °C and 200 V.

4.2.12.3 Ladder reference lanes

Bacteria associated with either the abalone gut (Sawabe, 2006) or as epiphytes found on *Ulva* were selected for use as reference genetic material for the denaturing gradient gel electrophoresis (DGGE). On each gel, lanes 3, 9, and 14 were loaded with the SSU rDNA reference ladder consisting of 16S rDNA products amplified from bacteria (n = 12) isolated from *Ulva* (see section 4.2.8.1.2), whereas lanes 4, 8, and 13 were loaded with the SSU rDNA reference ladder developed from the ten *V. halioticoli* strains (Figure 4-6). The PCR 16S rDNA products from of each of the 12 *Ulva* bacterial isolates or the 10 *V. halioticoli* strains were mixed into separate 50 µL PCR reaction tubes by adding equal volumes (1 µL) of each isolates genomic material and then diluting to a final volume of 11.5 µL with distilled water. The two separate (one containing isolates of *V. halioticoli* and the other containing isolates of *Ulva* epiphytes SSU rDNA ladders) would be used for gel normalization in the DGGE (detailed procedures described below).

The 16S SSU rDNA products of the 10 *V. halioticoli* strains, which formed one of the two SSU rDNA reference ladders, generated 12 well-defined bands following polyacrylamide gel electrophoresis that were selected for normalization (indicated in red on Figure 4-6). The 16S SSU rDNA products amplified from bacteria associated

with *Ulva* generated 6 well-defined bands that were distributed mainly in the lower half of the gel, following electrophoresis. The 18 bands generated from both 16S SSU rDNA reference ladders were selected for normalization to improve accuracy when comparing banding profiles within and between DGGE gels (marked in green on Figure 4-6). The banding profile for the SSU rDNA ladders did not span the entire length of the gel, as is evident in Figure 4-6. As a consequence, only those bands which fall within the range of the bands selected in the reference lanes were considered for the community analysis to improve reliability and accuracy of community profiling within and between gels.

4.2.12.4 Staining of gels

DGGE gels were stained for 30 min by submerging them in a plastic dish containing 300 mL TAE buffer supplemented with 30 µL (10 $mg.mL^{-1}$) of ethidium bromide. Following staining, gels were de-stained for 30 min in deionized water prior to imaging. Gel images were photographed on a UV transilluminator using a DNR Bio-Imaging System (MiniBis Pro) and recorded digitally as TIFF files. Each lane in a DGGE gel represents the 16S rDNA signature or 'fingerprint' of one intestine sample and these were used for comparison of microbial communities between different dietary treatments.

4.2.12.5 Image processing

DGGE fingerprints on each polyacrylamide gel were analysed for similarities via digital image analysis using the BioNumerics software package GelCompare2 (BioNumerics, Applied Maths, Inc., Austin, TX). Each image was processed using the same 4-step approach. Briefly (1) Definition of the area of the gel to be analysed was selected, (2) correction of background noise, (3) gel normalization after defining the reference lanes, and (4) band identification in the fingerprints using automated identification and refined by reader selection (Figure 4-6). After processing and photographing the gel, the lanes were added to the database and the individual key (Time, Diet, Replicate, Individual ID) was assigned as descriptive information before further analysis.

It was possible to further create averaged fingerprint profiles using GelCompare2 6.0 Scripts, which can be found on the Applied Maths webpage (http://www.applied-maths.com/gelcompar-ii). An averaged fingerprint was made for each replicate

treatment by combining the banding profile from the16S rDNA products of both the front and back gels for abalone from a single dietary treatment. These average fingerprints are described further in section 4.2.13.2.

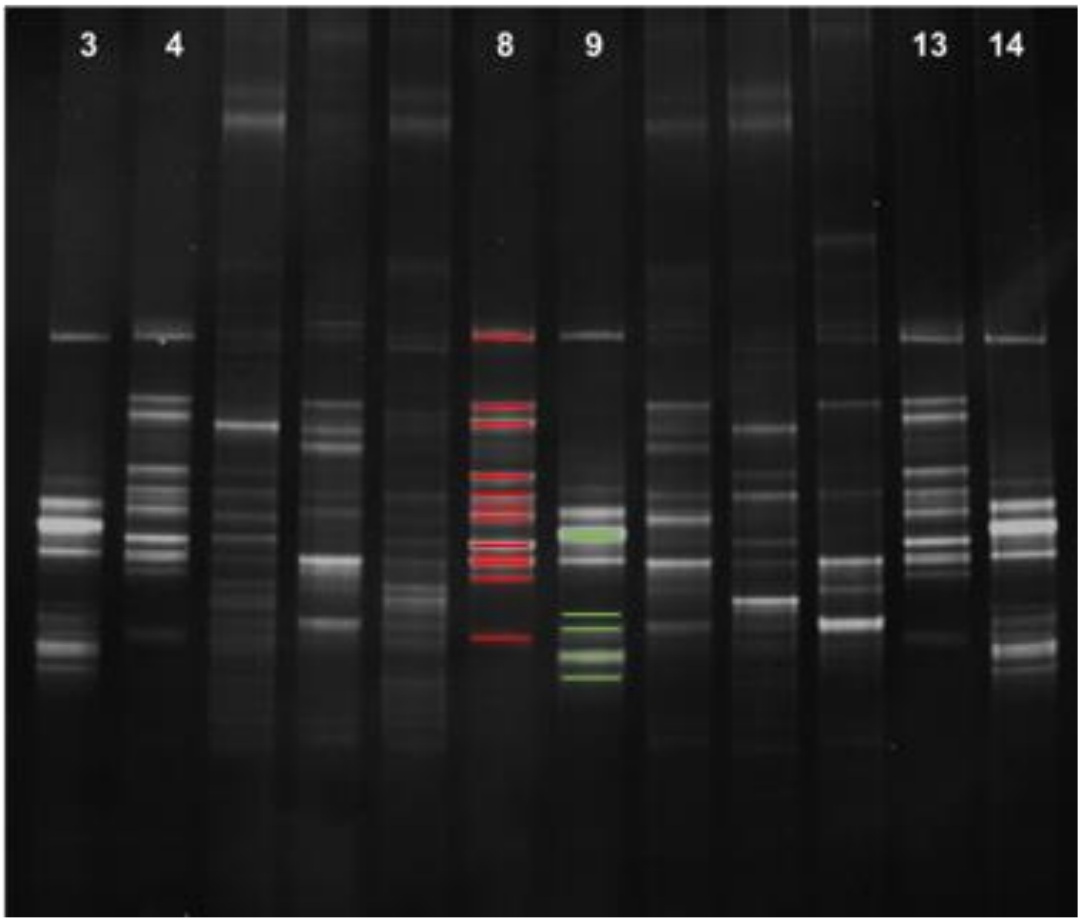

Figure 4-6 Denaturing gradient gel electrophoresis (DGGE) fingerprints before image processing and normalization, as viewed through the UV transilluminator. Lanes 3, 9, and 14 were loaded with 16S rDNA nested PCR products amplified from bacteria isolated from *Ulva* and lanes 4, 8, and 13 were loaded with 16S rDNA PCR products amplified from bacteria isolated from *Vibrio halioticoli*. Only bands which were within the range of the selected bands of the two reference ladders were included in the analysis. Bands marked in red and green highlight the bands from each reference sample selected for gel normalization.

4.2.13 Statistics

All statistical analyses were carried out using R (R Core Team, 2016) except for the dendrogram from DGGE profiles in which digital images of the gels were compared

using BioNumerics software package GelCompare2 (BioNumerics, Applied Maths, Inc., Austin, TX) with the band matching tables being further analysed in R. Significance is determined when $p \leq 0.05$.

4.2.13.1 Growth and physiological comparisons

For each replicate, the specific growth rate (SGR), monthly increment in shell length (MISL), and condition factor (CF) were calculated using individual abalone data made possible by the bead tagging system. Abalone which were not identified by a bead were not included in the analysis for SGR, MISL and CF. The mean SGR, MISL and CF were calculated from data captured for each individually marked abalone in each replicate treatment for each period (105 and 215 days) and the SGR and MISL for the entire period was also calculated for each individually marked abalone. The mean SGR and MISL from individually marked abalone for the entire growth period were used for statistical analysis and comparisons between treatment. For the physiological data (tissue moisture, relative shell weight contribution, and tissue glycogen), a mean for each replicate was calculated from abalone dissected over that period (i.e., at day 105 and 215) which were used for statistical analysis and comparisons.

The effect of diet on MISL, mean initial CF, and CF at 105 days, mean relative shell weight contribution (%) at 215 days, mean abalone tissue moisture (%), and mean abalone tissue glycogen ($g.kg^{-1}$) at 105 days were compared using a one-way ANOVA with diet as the grouping factor using Tukeys HSD post hoc comparisons for individual differences. The effect of diet on SGR, mean feed conversion ratios and the final CF, mean final tissue glycogen content, and the final mean shell weight contribution were compared using a non-parametric Kruskal-Wallis test with post-hoc analysis using Dunn's pairwise comparisons and p-values adjusted for multiple comparisons using Benjamini-Hochberg methods.

4.2.13.2 PCR-DGGE fingerprint analysis using UPGMA

The calculation of similarities between fingerprint profiles is based on the Pearson (product-moment) correlation coefficient and produced a distance matrix. Cluster analyses were performed using Bionumerics2.0. The clustering algorithm of Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to calculate

dendrograms for fingerprint samples from multiple gels. The structural diversity of the microbial community was examined by the Shannon index of general diversity (H') using a script provided by GelCompare2 6.0 Scripts. The number of bands and H' were each compared using a one-way ANOVA.

4.2.13.3 PCR-DGGE band quantification of relative abundance and analysis of unique identifiers

The PCR-DGGE band pattern generated by BioNumerics2.0 contained information on the position of each band in the fingerprint and a relative abundance for the band and were treated similarly to the data analysis in Pimentel et al. (2017). These data were uploaded to R Core Team (2016), converted into relative abundance and data were log(x+1) transformed using the Hellinger methods (Legendre & Gallagher, 2001). Using the metaMDS function in the "vegan" package in R (Oksanen et al., 2020), with Bray-Curtis similarity coefficient, a non-metric multidimensional scaling (NMDS) was performed in order to visualize and interpret the similarities between bacterial communities from abalone maintained on different diets. The NMDS procedure was iterative with the lowest number of axis (n = 3) selected based on the criteria for a stress level < 0.25. To test if there were statistical differences in the relative abundance data of microbial patterns between the dietary treatments, a dissimilarity matrix was made using the vegdist{vegan} package and tested using the ANOSIM{vegan} in R. The betadisper{vegan} function in R, is a multivariate analogue of Levene's test for homogeneity of variances and was used to test the assumption of homogeneity of the dissimilarity matrix.

The 'multipatt' function from the "indicspecies" package in R (Caceres & Legendre, 2021) was used to test for indicator species in the relative abundance of bands in the PCR-DGGE microbial fingerprints using point biserial correlation coefficient. This made it possible to highlight bands which had significant associations with the bacterial fingerprints. The individual bands identified as being 'indicator species' were extracted from the abundance data frame and plotted as boxplots of relative abundance in each dietary treatment to further visualise and interpret similarities between gut microbial patterns of abalone maintained on different diets.

4.3 Results

4.3.1 Effect of diet on morphological and physiological data collected during dissections

4.3.1.1 Relative shell weight contribution

The mean relative shell weight contribution, measured as a percentage of the total abalone weight, showed significant differences between diets when compared using a one-way ANOVA at 105 days ($H^2 = 17.34$, $p = 0.004$) denoted by the red letters above the error bars and at 215 days ($F = 2.965$, $p = 0.017$) denoted by the black letters below the error bars (Figure 4-7). There were no significant differences within treatments for data collected at day 105 and 215. Abalone which had been maintained on AB0.1U had the highest mean (±SE) relative shell weight contribution for both sampling points (105 and 215 days). After 215 days the relative shell weight contribution of abalone maintained on AB0.1U was 31.26 ± 0.69 %, significantly greater than those maintained on FU (27.78 ± 0.71 %, $p = 0.037$) when compared using Tukeys HSD post hoc analysis. Dietary supplementation with fresh *Ulva* (ABFU) and inclusion of dried *Ulva* (AB10U), ulvan (AB0.1U) and fresh *Ulva* (FU) did not result in significant differences in the relative shell weight contribution for abalone after 215 days, when compared to the control diet (AB) or between treatments (Figure 4-7). The black dashed line represents the mean relative shell weight contribution for n = 10 abalone that were tested after the acclimation period and before sorting.

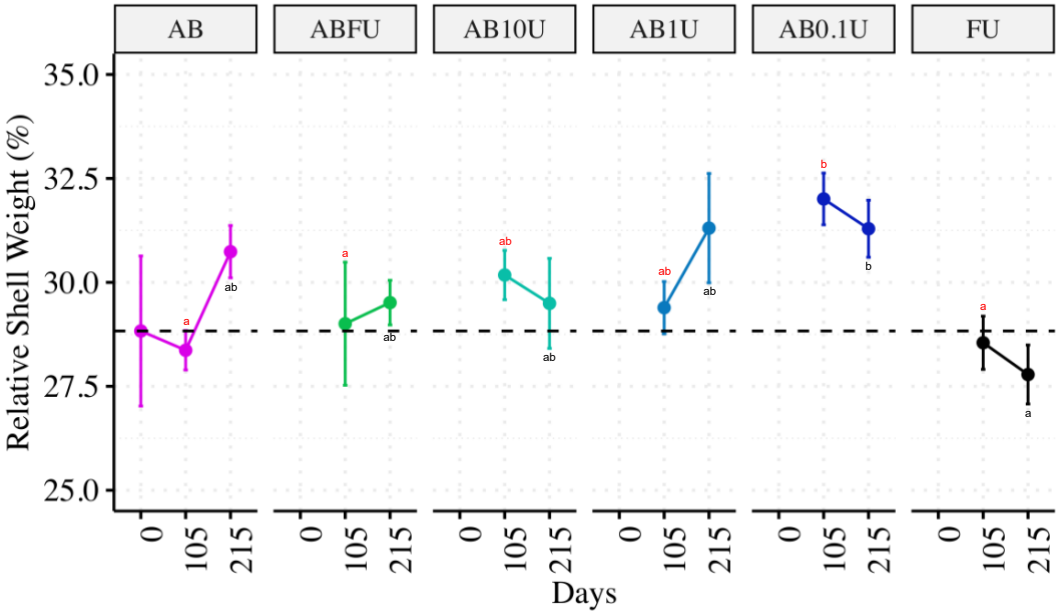

Figure 4-7 Mean (±SE) relative shell weight contribution to whole abalone measured as a percentage of body weight for each period (105 days and 215 days). The dashed black line represents the relative shell weight contribution of abalone at the start of the experiment. The effect of six diets were compared which were; a formulated feed (AB), a 60:40 combination of AB and fresh *Ulva* (ABFU), AB with a 10 % dry *Ulva* inclusion (AB10U), AB with a 1 % ulvan inclusion (AB1U), AB with a 0.1 % glucuronic acid inclusion (AB0.1U), and a fresh *Ulva* diet (FU). The dashed line represents the mean relative shell weight contribution to the whole abalone at the start of the growth trial, Day 0. All treatments have n=5 independent replicates. Significant differences between treatments at day 105 are indicated by red letters above the error bars and for differences between treatments at time 215 significance is indicated by black letters below the error bars, with different letters indicating significance ($p \leq 0.05$).

4.3.1.2 Abalone foot muscle moisture and glycogen content

Two separate sections of the abalone foot muscle were used to measure mean muscle moisture as a percentage (Figure 4-8 A) and mean muscle glycogen content in grams per kilogram (Figure 4-8 B). The data showed significant differences between diets at each sampling period (denoted by letters in Figure 4-8) and within treatments over the sampling periods 105 and 215 days (indicated by the stars in Figure 4-8). The black dashed line represents the mean for n = 10 abalone that were tested after the acclimation period and before sorting.

The abalone maintained on diet AB, AB10U, and AB1U showed significantly reduced tissue moisture for within treatment comparisons between days 105 and 215, indicated by the stars in Figure 4-8 A, when compared using an unpaired t-test. By day 215, abalone maintained on diet AB1U had the lowest recorded mean (±SE) muscle moisture of 70.65 ± 0.84 %, significantly less than that of abalone maintained on diets AB, ABFU, and FU (Figure 4-8 A). Abalone which had been maintained on diet FU had the highest recorded mean (±SE) muscle moisture of 77.9 ± 0.77 %, significantly greater than all treatments other than ABFU (75.08 ± 0.93 %; Figure 4-8 A; $F = 8.87$, $p < 0.001$).

Abalone which had been maintained on diet FU had the lowest recorded mean (±SE) muscle glycogen content by day 215 of 17.67 ± 2.39 $g.kg^{-1}$, significantly less than all treatments other than AB0.1U (38.01 ± 4.04 $g.kg^{-1}$; Figure 4-8 B; $H^2 = 28.68$, $p < 0.001$). Abalone maintained on diet AB0.1U had a mean (±SE) muscle glycogen content that was significantly lower than the glycogen content of abalone maintained on diets AB and AB1U. The abalone maintained on diets AB, ABFU, AB1U, and AB0.1U all showed significantly increased muscle glycogen content for within treatment comparisons between the sampling periods at days 105 and 215, indicated by the asterisk in Figure 4-8 B, when compared using an unpaired t-test. Abalone maintained on the positive control diet AB had the highest recorded mean (±SE) muscle glycogen content of 72.06 ± 9.4 $g.kg^{-1}$, not significantly different from diets ABFU, AB10U, and AB1U (Figure 4-8 B).

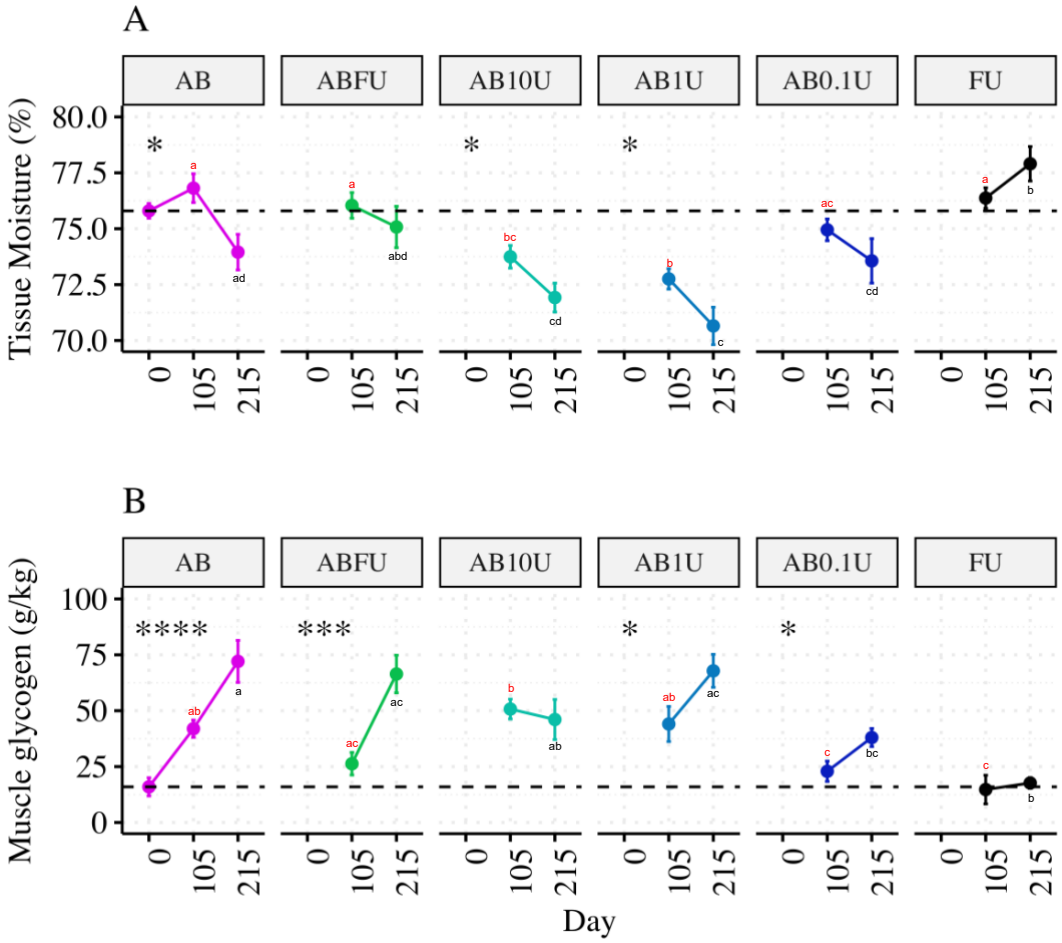

Figure 4-8 Mean (±SE) tissue moisture (A) and muscle glycogen (B) of abalone foot muscle tissues that were dissected and dried for tissue moisture or processed for the calculation of muscle glycogen. The dashed black line represents the relative shell weight contribution of abalone at the start of the experiment. The effect of six diets were compared which were; a formulated feed (AB), a 60:40 combination of AB and fresh *Ulva* (ABFU), AB with a 10 % dry *Ulva* inclusion (AB10U), AB with a 1 % ulvan inclusion (AB1U), AB with a 0.1 % glucuronic acid inclusion (AB0.1U), and a fresh *Ulva* diet (FU). All treatments were offered in quintuplicate. The mean tissue moisture and muscle glycogen for day 0 are indicated by the dashed line. Significant differences between treatments at day 105 are indicated by red letters above the error bars and for differences between treatments at time 215 significance is indicated by black letters below the error bars, with different letters indicating significance ($p \leq 0.05$). Significant differences within treatments are indicated by the asterisk in the panels (*≤0.05, **≤0.01, *≤0.001, ****≤0.0001).**

4.3.2 Effect of diet on abalone growth parameters

4.3.2.1 Specific growth rate

The specific growth rate (SGR) of abalone maintained on different diets, measured as percentage weight gain per day, was found to be statistically different between treatments over the entire period when compared using a non-parametric Kruskal-Wallis test (Figure 4-9; $H^2 = 123$, $p < 0.001$). Dunn's pairwise test showed that abalone maintained on ABFU had the highest recorded mean (±SE) SGR of 0.21 ± 0.005 % BW.day^{-1}, significantly higher than diets AB ($p < 0.001$), AB10U ($p < 0.001$), and FU ($p < 0.001$; Figure 4-9). The second highest mean (±SE) SGR was recorded for abalone maintained on diet AB0.1U was 0.20 ± 0.004% BW.day^{-1}, significantly higher than the SGR of abalone maintained on diets AB (0.16 ± 0.005% BW.day^{-1}) and AB10U (0.16 ± 0.006% BW.day^{-1}; Figure 4-9). The individual SGR for each abalone, represented by the scatter for individual abalone, illustrate the data spread within treatments.

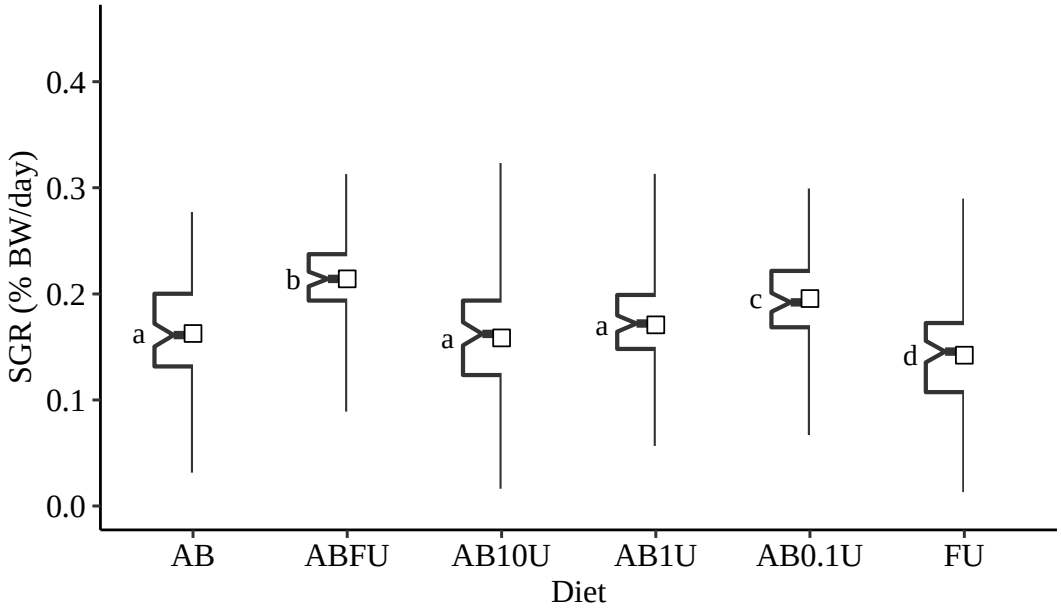

Figure 4-9 Mean (white square), median indicated by the notch in the box which extend to the 1st (lower) and 3rd (upper) quartiles, whiskers represent 2.5 time the interquartile range, and the scatter points plotted on each treatment (a jitter was applied to the points as to ensure no overlapping occurs in the plotting) represents individual abalones specific growth rate (SGR) over the growth period from February 2015 - September 2015. The effect of six diets were compared which were; a formulated feed (AB), a 60:40 combination of AB and fresh *Ulva* (ABFU), AB with a 10 % dry *Ulva* inclusion (AB10U), AB with a 1 % ulvan inclusion (AB1U), AB with a 0.1 % glucuronic acid inclusion (AB0.1U), and a fresh *Ulva* diet (FU). All treatments were offered in quintuplicate and significant differences are indicated by letters on the left of the mean with different letters indicating significance ($p \leq 0.05$).

4.3.2.2 Monthly increment in shell length

The monthly increment in abalone shell length (MISL), measured as growth (mm) per month (30 days), was found to be statistically different between treatments over the entire period (Figure 4-10; $F = 7.55$, $p < 0.001$). Abalone maintained on ABFU had the highest recorded mean (±SE) MISL of 1.388 ± 0.033 mm.month^{-1}, significantly higher than the MISL of abalone maintained on diets AB ($p = 0.002$), AB10U ($p = 0.005$), AB1U ($p = 0.003$), and FU ($p < 0.001$; Figure 4-10). The second highest mean (±SE) MISL was recorded for abalone maintained on AB0.1U of 1.24 ± 0.026 mm.month^{-1}, significantly higher than abalone maintained on diets AB (1.089 ± 0.034 mm.month^{-1})

and AB10U (1.091 ± 0.04 mm.month^{-1}; Figure 4-10). The individual MISL for each abalone, represented as the jitter plot, illustrate the data spread within treatments.

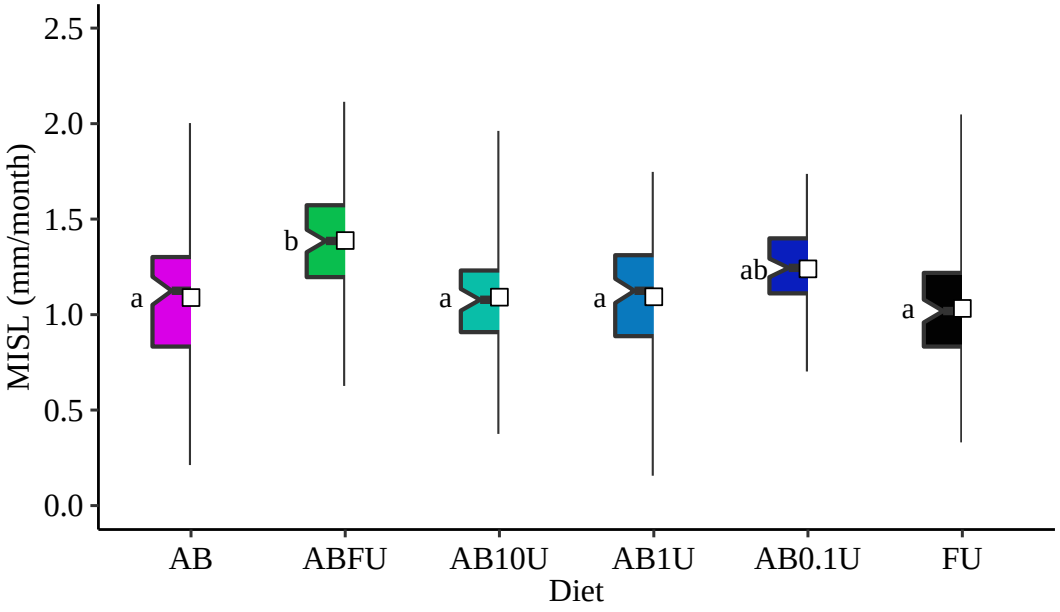

Figure 4-10 Mean (white square), median indicated by the notch in the box which extend to the 1st (lower) and 3rd (upper) quartiles, whiskers represent 2.5 time the interquartile range, and the scatter points plotted on each treatment (a jitter was applied to the points as to ensure no overlapping occurs in the plotting) represents individual animal's monthly increment in shell length (MISL) over the growth period from February 2015 - September 2015. The effect of six diets were compared which were; a formulated feed (AB), a 60:40 combination of AB and fresh *Ulva* (ABFU), AB with a 10 % dry *Ulva* inclusion (AB10U), AB with a 1 % ulvan inclusion (AB1U), AB with a 0.1 % glucuronic acid inclusion (AB0.1U), and a fresh *Ulva* diet (FU). All treatments were offered in quintuplicate and significant differences are indicated by letters on the left of the mean with different letters indicating significance ($p \leq 0.05$).

4.3.2.3 Condition factor

The condition factor (CF) for abalone was compared between diets at the start of the experiment in February 2015, at day 105 and 215. No statistically significant differences were recorded between abalone maintained on different diets at day 0 when compared using a one-way ANOVA ($F = 1.48$, $p = 0.232$) or day 215 when compared using non-parametric Kruskal-Wallis test ($H^2 = 10.83$, $p = 0.055$). The CF of abalone in all diets other than those maintained on FU decreased significantly over the

experimental period (Figure 4-11). In the final measurement (day 215), abalone maintained on fresh *Ulva* had the greatest variation in CF, with a mean (±SE) of 1.102 ± 0.26. Although there was a significant decrease in CF with time, the mean CF of all abalone in this study always remained above 1 (Britz, 1996; Figure 4-11).

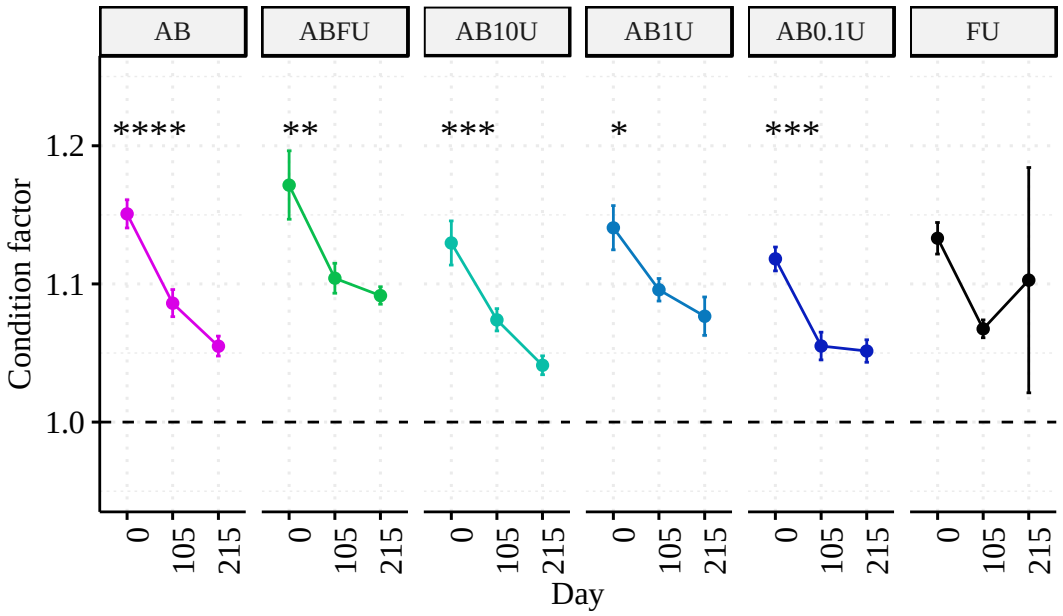

Figure 4-11 Mean (±SE) condition factor for abalone over the growth period from February 2015 - September 2015. The effect of six diets were compared; a formulated feed (AB), a 60:40 combination of AB and fresh *Ulva* (ABFU), AB with a 10 % dry *Ulva* inclusion (AB10U), AB with a 1 % ulvan inclusion (AB1U), AB with a 0.1 % glucuronic acid inclusion (AB0.1U), and a fresh *Ulva* diet (FU). All treatments were offered in quintuplicate and the average 'fatness' (CF=1) is indicated by the dashed line. Significant differences within treatments are indicated by the asterisk in the panels (*≤0.05, **≤0.01, *≤0.001, ****≤0.0001).**

4.3.3 Effect of diet on feed conversion ratio

The feed conversion ratio (FCR), calculated as the amount of feed required to grow 1 kg of abalone, was found to be statistically different between treatments when compared using a non-parametric Kruskal-Wallis test (Figure 4-12 ;H^2 = 14.105, p = 0.015). Dunn's pairwise test showed that abalone maintained on AB0.1U had a significantly lower mean (± SE) FCR of 1.34 ± 0.05 than the FCR of abalone maintained on either AB (2.51 ± 0.31, p = 0.043) or FU (2.77 ± 0.45, p = 0.038). Abalone

maintained on diet FU had the highest recorded mean FCR and also the largest recorded standard error (Figure 4-12).

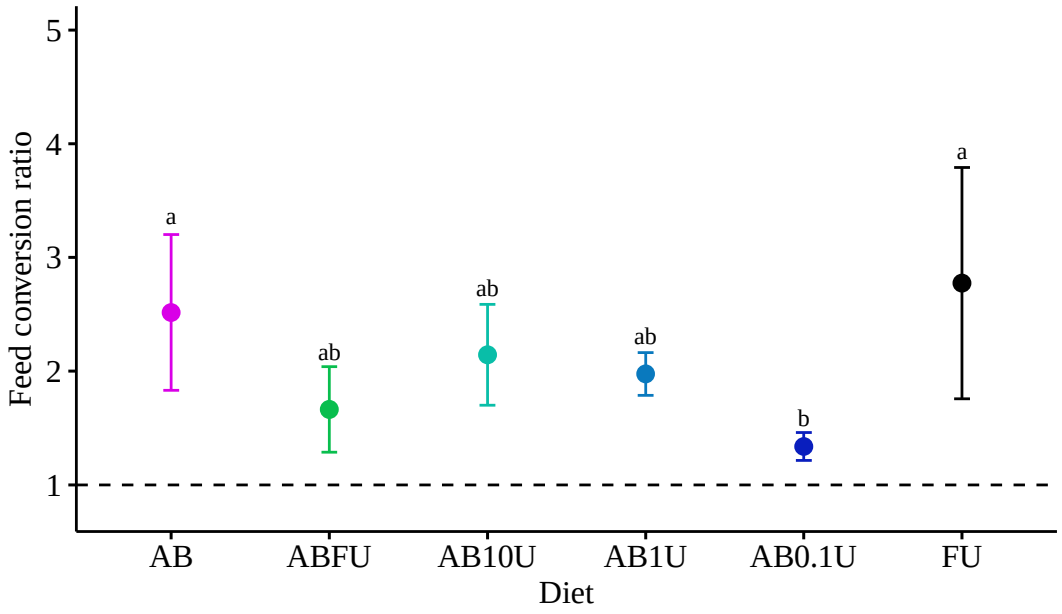

Figure 4-12 Mean (±SD) feed conversion ratios (FCR) for abalone over the growth period from February 2015 - September 2015. The effect of six diets were compared which were; a formulated feed (AB), a 60:40 combination of AB and fresh *Ulva* (ABFU), AB with a 10 % dry *Ulva* inclusion (AB10U), AB with a 1 % ulvan inclusion (AB1U), AB with a 0.1 % glucuronic acid inclusion (AB0.1U), and a fresh *Ulva* diet (FU). All treatments were offered in quintuplicate and significant differences are indicated by letters above the error bars, with different letters indicating significance ($p \leq 0.05$).

4.3.4 Effect of diet on the abalone intestine 16S rDNA gut microbial fingerprints

4.3.4.1 PCR-DGGE fingerprint analysis using UPGMA

The PCR-DGGE patterns of gut microbial patterns revealed different band profiles among abalone fed a diet of either *Ulva lacinulata* and components thereof or those maintained on the formulated feed, diet AB (Figure 4-13). The averaged PCR-DGGE profiles of the microbial consortia associated with the intestine of abalone maintained on different diets for a period of 215 days revealed four clear clusters indicated by the pink, green, grey, and yellow highlights (71 % global cophenetic correlation). Two of

the main clusters contain 86.7 % of the fingerprints. The two main clusters show a separation between diets which contained fresh *Ulva* and those which were a dry formulated feed (cophenetic statistic = 68 %) indicated by the pink (formulated feeds only) and green (all diets containing *Ulva* of components thereof) highlights in Figure 4-13. All except one averaged PCR-DGGE fingerprint from the abalone maintained on AB clustered together with only other formulated feeds. The only fingerprint for abalone maintained on AB which is found in the cluster which also contained the fingerprints from abalone maintained on fresh *Ulva* was grouped with diets AB10U and AB0.1U (cophenetic statistic of 51 %). The comparisons of number of bands and the Shannon-Wiener index showed no statistical differences between diets when compared using a one-way ANOVA.

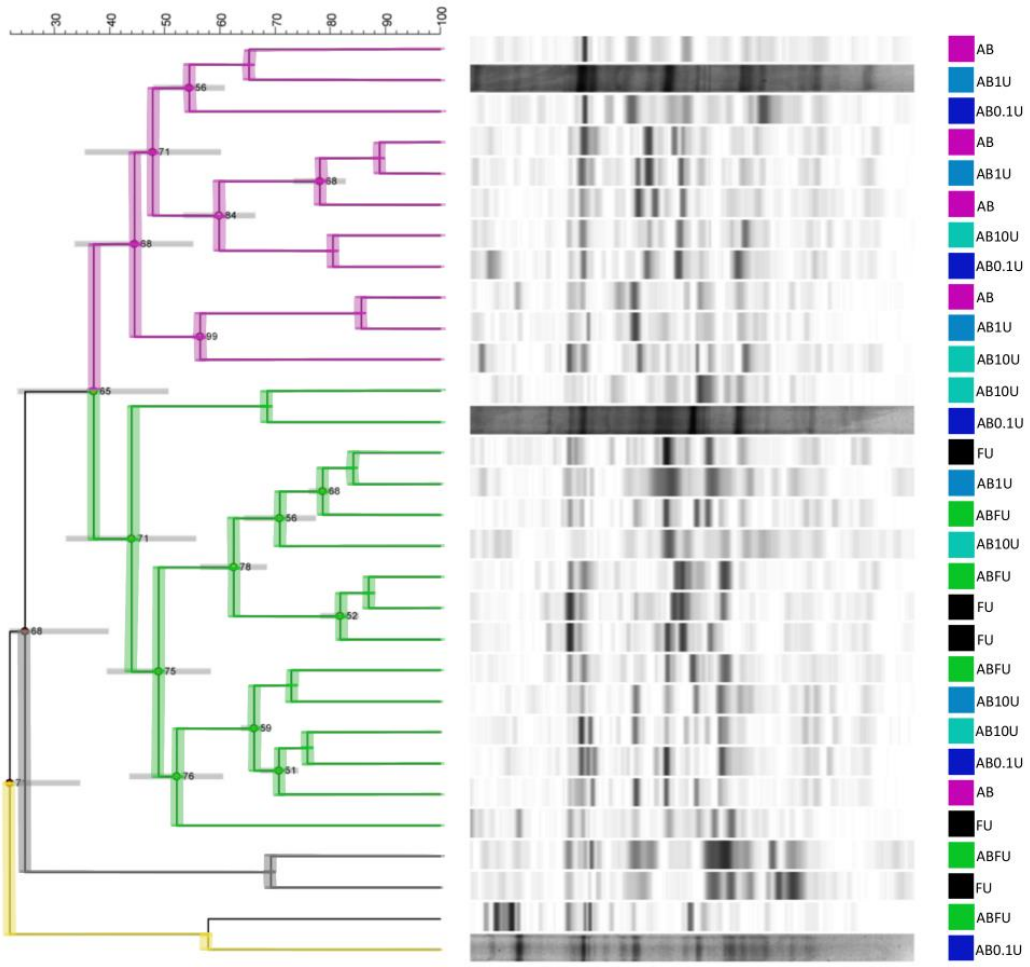

Figure 4-13 Dendrogram obtained through cluster analysis of Pearson correlation similarity matrix using the unweighted pair group method with algorithmic means algorithm (UPGMA) of averaged PCR-DGGE fingerprint patterns obtained from 16S rDNA products generated from abalone intestine sections. Abalone were maintained on different diets for 215 days prior to sampling and diets are indicated in the legend above. Diets were; (AB) formulated feed, (ABFU) a 60:40 combination of AB and fresh *Ulva* , (AB10U) AB with a 10 % dry *Ulva* inclusion, (AB1U) AB with a 1 % ulvan inclusion, (AB0.1U) AB with a 0.1 % glucuronic acid inclusion, (FU) and a fresh *Ulva* diet. Similarity is expressed as a cophenetic correlations on the branches with values near 100 indicating maximum similarity

4.3.4.2 Non-metric multidimensional scaling of PCR-DGGE fingerprints

The non-metric multidimensional scaling (NMDS) revealed that the gut microbial patterns associated with the intestinal tract of abalone maintained on a diet of fresh *Ulva*

(FU) were poorly described by NDMS1. The NDMS2 plotted against NDMS3 clearly separated the abalone maintained on FU into the positive values of NDMS3 with associations between FU and ABFU with a stress value of 0.17 (Figure 4-14 B). The three-dimensional space for Figure 4-14 A demonstrates grouping for abalone maintained on diets ABFU, FU, AB0.1U and AB1U with associations between FU and AB0.1U.

The multilevel pattern analysis for indicator species using the log transformed relative abundance of bands produced a total of 43 bands/bacterium with seven bands highlighted as significant indicators in the bacterial PCR-DGGE fingerprints ($p = 0.001$, $R^2 = 0.124$; Figure 4-14 C). Indicator bands at a relative front (Rf) Rf54.4 and Rf34.7 showed significant associations with PCR-DGGE fingerprints from abalone maintained on diets FU ($p = 0.001$) and ABFU ($p = 0.026$), as indicated by the matching star above the boxplots for each treatment in Figure 4-14 C. The band Rf34.7 was detected in all intestine samples contributing up to 33.6 % for abalone fed FU. The band Rf54.4 was found to contribute up to 46.6 % of the relative abundance of bacterial pattern for abalone fed ABFU and it was not detectable in the intestine of abalone fed diet AB. It was detected in the diets containing the components of *Ulva*, in order of decreasing relative abundance, FU, AB1U, AB10U, and AB0.1U. Abalone maintained on a diet of FU had a further two indicator bands at Rf32.4 ($p = 0.006$) and Rf23.3 ($p = 0.013$), which showed a significant association in the PCR-DGGE bacterial fingerprints. Abalone maintained on diet AB0.1U have two indicator bands, one unique to the Rf38.6 ($p = 0.037$) and another at Rf37.5. The indicator band at Rf37.5 also showed significant associations in the intestine samples from abalone maintained on diet AB ($p = 0.045$), and was present in all other formulated feeds but it is absent in the intestine samples of abalone fed fresh *Ulva* (FU). The band at Rf44.0 was significantly associated with abalone maintained on diet AB ($p = 0.009$) and contributed up to 54 % of the relative abundance of the bacterial pattern identified (Figure 4-14 C). The band Rf38.6 was found at 14.3 % and 15.3 % relative abundance in samples from abalone fed AB1U and AB0.1U respectively, but was not detected in other treatments. These results support the NDMS representation, which exhibits abalone maintained on fresh *Ulva* alone or formulated diets supplemented with FU as being similar in terms of bacterial patterns from the PCR-DGGE fingerprints.

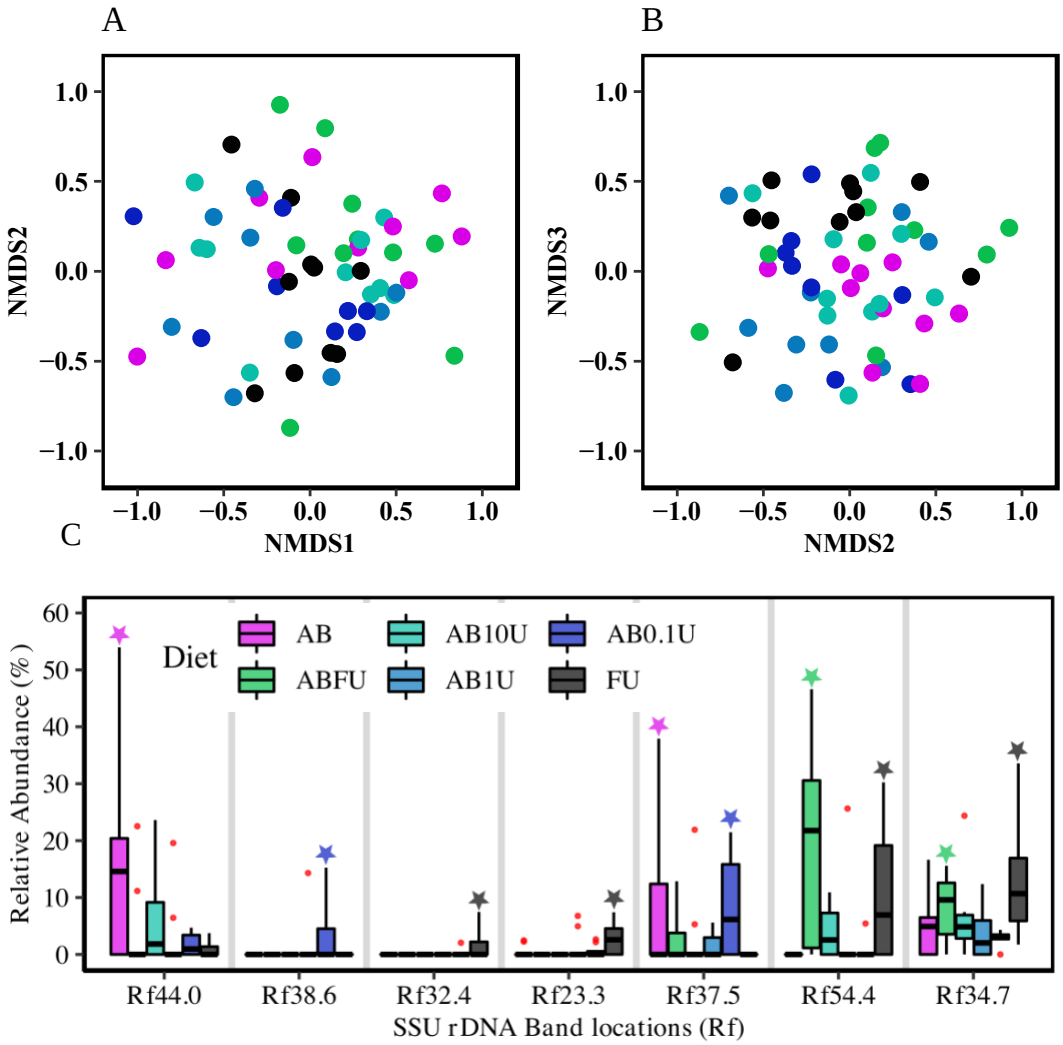

Figure 4-14 (A and B) Non-metric multidimensional scaling (NMDS) ordination for bacterial PCR-DGGE fingerprints. Each fingerprint pattern was obtained from the 16S rDNA extracted from individual abalone intestinal section and normalized according to the relative abundance of each band. NMDS quantified the data on 3-axis converging after 20 iterations with a stress value of 0.18. The MDS for axis 1 vs 2 (A) and 1 vs 3 (B) were plotted. (C) Boxplots of relative abundance for band locations represented as median, indicated by the line in the box which extend to the 1st (lower) and 3rd (upper) quartiles and whiskers representing 2.5 time the interquartile range. Outliers are plotted in red. Each fingerprint pattern was analysed for indicators 'species' which found seven bands with significant associations to diet. The star above the whisker indicates the diet in which significant associations were identified with the band for the individual PCR-DGGE bacterial fingerprint. Diets were; (AB) formulated feed, (ABFU) a 60:40 combination of AB and fresh *Ulva* , (AB10U) AB with a 10 % dry *Ulva* inclusion, (AB1U) AB with a 1 % ulvan inclusion, (AB0.1U) AB with a 0.1 % glucuronic acid inclusion, (FU) and a fresh *Ulva* diet.

4.4 Discussion

4.4.1 Summary

The findings from this study show that the inclusion of *Ulva* and/ or specific components of *Ulva* as dry feed ingredients for abalone can significantly affect the qualities associated with abalone products, overall growth of abalone. Also, the bacteria inhabiting the intestine form associations with diet. Significant improvements in growth rate (both SGR and MISL) were observed for abalone maintained on a dry formulated feed supplemented with fresh *Ulva* (ABFU treatment) compared to abalone fed the non-supplemented formulated feed (AB). In the latter treatment, abalone were fed 40 % less of the formulated feed (AB), with the remainder of the daily feeding ration (total feed provided at 0.34-0.4 % body weight per day) comprised of fresh *Ulva* (administered as a dry weight equivalent to all other diets). Furthermore, abalone maintained on a diet of fresh *Ulva* grown in an IMTA abalone/*Ulva* system, grew at a rate not statistically different to those maintained on a fishmeal based formulated feed, suggesting that farmed abalone can be maintained on a diet consisting only of fresh *Ulva* during the on-growing phase of production. When fresh *Ulva* was fed as the only source of food (FU) abalone were found to have significantly elevated tissue moisture along with reduced tissue glycogen. In contrast, abalone fed a diet with ulvan at a 1 % inclusion (AB1U) were found to have the significantly lower tissue moisture than abalone fed on diet AB with comparable tissue glycogen content. The fact that abalone fed the formulated feed supplemented with 0.1% glucuronic acid had a comparable MISL to abalone fed ABFU, and had SGR's that were significantly greater than abalone fed AB alone, suggests that glucuronic acid may be one of the components within *Ulva* contributing towards growth of abalone.

The denaturing gradient gel electrophoresis (DGGE) data clearly separated the gut microbial fingerprints into two groups, with one cluster consisting mainly of abalone maintained on formulated feeds and the other cluster comprised of abalone fed fresh *Ulva* diets. The non-metric multidimensional scaling (NMDS) and analysis of "indicator species" based on the PCR-DGGE fingerprints indicates that abalone fed fresh *Ulva* diets, and its components, produced significant associations in their intestinal bacterial patterns; suggesting that specific bacterial species are selected for

and are associated with the digestive tract of abalone fed components of these feeds. The analysis of an indicator species identified seven bands that had significant associations with the various dietary treatments, where bands Rf34.7 and Rf44.0 were shown to be present in all samples which responded by increasing abundance in response to *Ulva* or formulated feeds respectively, suggesting they form part of the core microbiome of the abalone *Haliotis midae* and are influenced by diet. The positive selection for band Rf54.4 in the intestine of abalone fed *Ulva* and its components indicates the capacity for modulation of the microbiome in abalone and this modulation may be responsible for a dynamic shift in the metabolic efficiencies in the gut (Roberfroid et al., 2010; Hu et al., 2017; Martin-Gallausiaux et al., 2020).

4.4.2 Interpretations

Feeding the abalone *Haliotis rufescens* a formulated feed resulted in increased activity of carbohydrase in the gut when compared to kelp, which was linked to the high surface area of the formulated feeds that promoted high numbers of bacteria that facilitated its digestion (Garcia-Esquivel & Felbeck, 2006). An increased feed efficiency in the abalone *Haliotis discus hannai* was associated with significant differences in intestinal microbiota with enhanced enzyme activity for carbohydrates in the intestinal tract (Yu et al., 2022). Band Rf44.0 contributed up to 3.6 % of the total abundance for bacteria in the intestine of abalone fed fresh *Ulva*, whereas it contributed up to 54 % of the total bacterial abundance identified in the intestinal samples of abalone fed solely with the formulated feed (AB). Furthermore, band Rf44.0 contributed up to 23.5 % of the bacterial abundance identified in the intestinal samples of abalone fed a combination of fresh *Ulva* and formulated feed (ABFU). These findings suggest that the formulated feed may be contributing to the proliferation of a bacterium identified at Rf44.0 where the increased carbohydrase activity of abalone fed formulated feeds reported by Garcia-Esquivel & Felbeck (2006) may further add to the enhanced growth reported in abalone fed diet ABFU. The growth indices for abalone fed monospecific feeds in this study were significantly lower compared to abalone fed the combination diet ABFU, which is likely a result of the holistic impact that dietary *Ulva* is having on the metabolic abilities, energy metabolism and gut health in the abalone.

In a study where *Haliotis midae* were fed a diet supplemented with 0.9 % dried kelp they demonstrated improved FCR and protein efficiency ratios which was associated with higher levels of bacteria belonging to the genus *Mycoplasma* in the gastrointestinal tracts of abalone (Nel et al., 2017). The intestine of adult *Haliotis discus hannai* was found to contain a high ratio of Firmicutes to Bacteroidetes when fed an 'algal extruded pellet' (Choi et al., 2021) and the high number of Firmicutes has been associated with unregulated overproduction of high energy short chain fatty acids (SCFA) (Abenavoli et al., 2019). Abalone in this study that were fed a formulated feed supplemented with fresh *Ulva* (ABFU) shared two bands/bacteria with abalone fed fresh *Ulva*. One of the bands, Rf54.4, was only found in the intestines of abalone fed *Ulva* and its components while the band Rf34.7 was found in all treatments. The relative abundance of band Rf54.4 was up to 46.6 % and 30.2 % in the intestinal samples obtained from abalone fed diets ABFU and FU. Interestingly, band Rf54.4 was not detected in the intestine of abalone fed diet AB and it was only detected in one replicate treatment of the group of abalone fed diet AB0.1U, where it contributed to 5.4 % of the relative abundance of bacteria in the sample (see outlier in red illustrated in Figure 4-14). These data suggest that the bacterium, represented by band Rf54.4, may be a result of the inclusion of fresh *Ulva* that is also found to associate with refined fractions of *Ulva*.

The dominant fermenters in an abalone gut may have acquired algal polysaccharide degrading genes by lateral gene transfer from aerobic epiphytic algal microbiota themselves (Gobet et al., 2018). Facultative anaerobic bacteria belonging to the genera *Psychrilyobacter* formed a dominant group within the core microbial community associated with the digestive gland of the abalone *Haliotis tuberculata*, which demonstrated strong associations with algal diets for the capacity to ferment polysaccharides into SCFAs (Gobet et al., 2018). In this study the abalone fed on the diet ABFU were offered 40 % less formulated feed with the supplementation of fresh *Ulva* where their growth measurements significantly outperformed either monospecific dietary treatment (AB and FU). The enhanced growth reported for abalone in diet ABFU may be due to the increased production of SCFA by bacterial fermentation that may increase the amount of energy available for absorption in the intestinal tract (Sawabe, 2006; Gobet et al., 2018; Danckert et al., 2021) where a positive energy balance drives weight gains (Ludwig et al., 2021).

Diet induced changes to the appearance, composition and metabolic capacity of an abalone can influence the farm profitability as well as the market acceptance of the products (Smit et al., 2007; Qi et al., 2010; Hoang et al., 2016; Valente et al., 2016). In this study, abalone fed the formulated feed supplemented with 0.1 % glucuronic acid (AB0.1U) had a noticeable increase in their relative shell weight contribution, with shell weight being significantly elevated at the first sampling point (105 days) when compared to the control (AB) and fresh *Ulva* (FU) diets. It has been shown that abalone secrete shell building organic molecules containing glucuronic acid along the mantle (O'Neill et al., 2013), where they initiate the process of biomineralization (Arias & Fernández, 2008; Rao et al., 2014). Although the MISL data were not significant, abalone fed diets supplemented with 0.1 % glucuronic acid displayed the least inter-animal-to-animal variation for MISL, which is displayed in Figure 4-10. The observed improvement in SGR, FCR and relative shell weight in abalone maintained on a diet supplemented with glucuronic acid is possibly through the contribution of glucuronic acid to the total energy budget where the shell building process is enhanced. While a diet containing glucuronic acid can increase the relative shell weight contribution to an abalone total weight this is not considered favourable for the production of abalone where total yields are measured by weight.

This study identified a band in the PCR-DGGE fingerprints that was significantly associated with abalone maintained on diet AB0.1U, contributing up to 15.3 % of the total abundance, suggesting there is also a bacterium associated with the readily available dietary glucuronic acid that may be contributing towards the enhanced effects noted above. Abalone fed diets fed on diet AB0.1U had significant associations with the bacteria/bands Rf 38.6 and Rf 37.5 from intestine samples along with reduced FCR and a relatively high MISL, which was similar to the abalone fed diet ABFU. Hyaluronan, containing glucuronic acid, is an important component of the molluscan mucus that accounts for a significant proportion of the total energy budget for the abalone *Haliotis tuberculata,* where the total cost of pedal mucus production was 23.3 - 29.1 % for abalone fed *Ulva lactuca* (Peck et al., 1987). The production of complex glycosaminoglycans (mucus) which contain glucuronic acid, is crucial in providing beneficial shell building microenvironments or improved gut lining and gut health (Lopes-Lima et al., 2005; Portela et al., 2012; Caon et al., 2020). These data combined

indicate that the dietary glucuronic acid may directly be available to the host for assimilation where it may contribute to improved biomineralization and enhanced feed efficiency that may be associated with the bacteria/ band Rf 38.6. Glucuronic acid may be providing benefit to the abalone through one or many mechanisms such as, modulation of the gut microbiota, enhanced mucosal production, and improved capacity for shell building. However, there is little evidence on the simple inclusion of glucuronic acid into feeds, making it difficult to understand the range of interactions a dietary supplement may endure prior to absorption (Martínez-Leal, Ponce-García & Escalante-Aburto, 2020). The effects of glucuronic acid on the energy budget would be useful to determine how improvements in FCR are distributed in the energy balance model for abalone.

Ulvan has demonstrated the ability to increase the rate of hyaluronan synthesis in cultured dermal fibroblasts without increasing cell proliferation (Adrien et al., 2017). Abalone fed a diet with a 1 % inclusion of ulvan in this study had significantly lower muscle moisture content, and correspondingly high tissue glycogen concentrations, compared to abalone maintained on diets AB and ABFU. Similarly, high glycogen levels in the abalone *H. discus* and *H. diversicolor* were found to be inversely correlated to the moisture content in the tissues (Watanabe, Yamanaka & Yamakawa, 1992; Chiou, Lai & Shiau, 2001). Red sea bream that were fed on a diet high in available carbohydrates supplemented with the prebiotic β-glucan resulted in significant reduction in the tissue moisture, serum glucose and triglycerides (Dawood et al., 2017). Dawood et al. (2017) further demonstrated that fish that had been fed a diet supplemented with β-glucan had high bactericidal activity of the mucus and more mucosal secretion with increased body lipid content. In this study, dietary ulvan demonstrated capacity to significantly decrease tissue moisture content and maintain muscle glycogen level in abalone when compared to a formulated feed, which is a beneficial characteristic for canning. Furthermore, dietary *Ulva* has been shown to enhance the tissue lipid content in the abalone *Haliotis laevigata* × *Haliotis rubra* (Mulvaney et al., 2015). Feeding abalone the latter diet could be used to as part of a feeding regime where reduced moisture is favourable, such as in the process of drying abalone and the enhanced lipid content may further enhance the perceived human health benefits.

The high moisture content in the tissues of abalone fed FU may be directly related to the high moisture content of dietary *Ulva*, or an increased requirement for cellular water through higher levels of cellular DMSP that acts as an osmolyte (Motard-Côté & Kiene, 2015). The removal of DMSP from ulvan through processing and the increased exercise associated with abalone fed diet FU may be partially responsible for the observed contrast in tissue moisture content for abalone fed diets FU and AB1U in the present study. The high tissue moisture content of abalone fed diet FU would likely result in high water loss during canning, resulting in lower meat yields in the canning process. Conversely, abalone fed diets supplemented with ulvan (AB1U) had high muscle glycogen concentrations and low tissue moisture content that may be beneficial when canning abalone, since less water, and weight, is lost during canning, resulting in increased profits. A high glycogen content in the abalone prior to canning is thought to reduce water loss from the muscular foot where water-loss can significantly impact the meat yield and influence the profitability of the process (pers. comm. Devin Ayres 2018). The time taken to grow an abalone to marketable size can be between 3 – 7 years, which means any improvements in the market acceptance or production efficiencies of the product would have a benefit to the industry.

High starch diets have been correlated to higher tissue glycogen content in the gastropod *Babylonia areolata* (Zhang, Zhou & Cheng, 2009), and in this study, significantly elevated levels of muscle glycogen were recorded in the foot muscle of abalone maintained on the formulated diet AB, ABFU and AB1U when compared to abalone maintained on fresh *Ulva* alone. Muscle glycogen levels for abalone maintained on diet AB, ABFU and AB1U were all increasing over the experimental period (February – September 2015). These increases coincide with the pre-spawning period where spawning generally occurs between September – March for *Haliotis midae* (Ayres, 2013), where sexually mature animals would generally be storing energy reserves and building gonad for the upcoming spawning season. It should however be noted that the abalone used for trials in this study were relatively young abalone (starting weight of ~30g) and were not all fully sexually mature based on personal observations during sampling. The low relative levels of glycogen in the foot muscle of abalone fed fresh *Ulva* is likely a result of: low availability of starch, due to the more complex nature of carbohydrates in *Ulva*; the age of the animal, and the activity

required for feeding on *Ulva* (Currie et al., 2016). It is unlikely that the abalone maintained on fresh *Ulva* in this study were starving and in a state of depleted glycogen reserves, due to the comparative growth rates recorded for abalone fed FU compared with abalone maintained on diet AB.

The abalone fed a formulated feed with 10 % inclusion of dried *Ulva* (AB10U) grew equally well and with a marginally lower food conversion ratio than abalone fed diet AB (not significant). A 10 % inclusion of *Ulva* into the formulated feeds of the Australian abalone *Haliotis laevigata* has demonstrated enhanced growth compared to the control group of abalone fed the non-supplemented formulated feed which was included as a specific replacement by reducing solvent extracted soybean meal, wheat flour and de-hulled lupin levels (Bansemer et al., 2016). Abalone in this study fed AB10U were not found to grow significantly better than those fed AB, as was demonstrated for *H. laevigata* by Bansemer et al. (2016). This result may be related to the quality of diet where abalone in the latter study were fed Abgrow premium (Eyre Peninsula Aquafeeds (EPA), Lonsdale, Australia) and/ or the enriched *Ulva* which was included as a dried ingredient. *Ulva* has a high content of sulphated polysaccharides and through the act of drying *Ulva* at temperature above 40 ºC, the extractive potential of ulvan is increased (Silva et al., 2019). In this study, effluent-grown *Ulva* was dried at 60 ºC and used in the formulation of feeds which were balanced by amino acid content in proprietary formulations that were produced by Specialized Aquatic Feeds, Hermanus, South Africa. When *Ulva* is dried at temperature above 40 ºC it will likely lose the reported benefits that have been attributed to the phenolic component of *Ulva*, such as its anti-hyperglycaemic capacity (Celikler et al., 2009; Tas et al., 2011) while the prebiotic capacity may be enhanced.

4.4.3 Implications

For the cultured abalone *Haliotis tuberculata*, the digestive microbiota has been shown to form a stable dominant community during early development (in the first year of life) and feed type only accounts for a small proportion of the dominant microbiome variability (Gobet et al., 2018). The abalone used in this study were around 2 years old when they arrived at the Marine Research Aquarium and had previously been fed a formulated feed similar in composition to diet AB. There were no significant

differences in PCR-DGGE microbial fingerprints community structure for the abalone fed different dietary treatments but significant association between specific bands were identified. Similarly, different monospecific algal diets did not appear to cause a shift in the dominant microbiota of juvenile *H. tuberculata*, which was comprised of *Psychrilyobacter*, *Mycoplasma*, and *Vibrio*. Instead, a less abundant and diet-specific core microbiota developed featuring genera representing aerobic bacteria specific to the algal polysaccharides in the diet (Gobet et al., 2018).

Ulva rigida grown in an IMTA system develops unique epiphytic microbial communities that are characteristic of the primary culture environment (Califano et al., 2020). Some of these epiphytic microbes that are found on seaweed have unique capacity for the fermentation of polysaccharides which may contribute to the overall performance of integrated systems. Diet should therefore be considered as an important variable throughout the entire abalone aquaculture value chain, that includes processing and marketing, and not just for production. Collectively, the results of this study suggest that dietary supplementation with fresh *Ulva* can have a significant positive impact on overall growth of *H. midae* while also enhancing market related product traits. This data shows that dietary supplementation with effluent-grown *Ulva* can modulate the bacterial community of the gastrointestinal tract of abalone and alter specific physiological (glycogen, moisture content, and FCR) processes, clearly demonstrating that *Ulva* can provide benefit beyond just growth for the cultivation of abalone *Haliotis midae*.

Furthermore, specific components of *Ulva*, for example the inclusion of 0.1 % glucuronic acid, into a formulated feed of abalone was shown to significantly improved the SGR, relative shell weight contribution, and FCRs; providing valuable insight into the role of specific components of *Ulva* as a dietary supplement. While the mechanism of absorption and digestion of glucuronic acid remains unclear, it would be interesting to further investigate the influence of its incorporation on the microbiome of abalone through next generation sequencing and investigate the metabolome of selected tissues, specifically the mantle.

Chapter 5 - General Discussion

5.1 Context

This study sought to take a practical approach in understanding the role of effluent-grown *Ulva* as a feed ingredient for the abalone, *Haliotis midae*, which will have relevance to the aquaculture industry. It was hypothesised that *Ulva* could be used to reduce the reliance on fishmeal based formulated feeds or improve efficiency of its utilization, improve the overall growth and health of abalone while serving as a functional ingredient where some of the improvements to abalone culture and function cited by previous authors (Naidoo et al., 2006; Mulvaney, Winberg & Adams, 2013; Kemp, Britz & Agüero, 2015; Bansemer et al., 2016, Santizo-Taan, Bautista-Teruel & Maquirang, 2020) may be linked to the polysaccharide fraction of *Ulva* serving as a prebiotic for the cultured abalone. In an attempt to further understand the perceived benefits of *Ulva* as a functional ingredient, *Ulva*, and its carbohydrate component, were included into feeds and feeding regimes to investigate effects on the growth, consumption, health, physiology and gut microbial patterns of the cultured abalone *H. midae*. In order to provide feed formulators with information on the potential impacts of *Ulva* as an ingredient in aquaculture feeds, it is necessary to develop a qualitative and quantitative understanding of the effects of *Ulva* or its components when used as a dietary ingredient.

5.2 Findings

This study demonstrated that dietary supplementation with *Ulva lacinulata* produced in integrated multitrophic aquaculture (IMTA) systems can reduce an abalone producers reliance on fishmeal-based dry formulated feeds. The partial replacement (60 %) of the high fishmeal based formulated feed Abfeed™ S34® with fresh effluent-grown *Ulva* or wild collected kelp in the feeding program of *H. midae* did not compromise the growth (SGR and MISL) or condition of abalone in a farm scale trial. Conversely, when *Ulva* was used as a 40 % replacement of the formulated feed AB, abalone were found to have significantly enhanced growth under experimental conditions. Moreover, inclusion of fresh IMTA-grown *Ulva* into the feeding regime of abalone, when offered alongside Abfeed™ S34®, significantly increased the total daily feed consumption when compared to abalone fed single diets of both *Ulva* or Abfeed™ S34®. The assay

used in this study for monitoring haemolymph glucose concentrations during an induced glycaemic response demonstrated a significantly prolonged period of hyperglycaemia in abalone fed a formulated feed along with significantly elevated basal haemolymph glucose concentrations when compared to abalone fed *Ulva*. The immune response of the abalone fed fresh *Ulva* was not significantly different to that of abalone fed the formulated feed, indicating that replacement of Abfeed™ S34® with *Ulva* does not compromise immunity and disease response of abalone. In fact, there was a more pronounced activity from stimulated haemocytes in *Ulva*-fed animals following challenge with an injected dose of *Vibrio anguillarum* which also correlated with higher levels of bacterial clearance from the haemolymph. The culture independent molecular technique, which utilized denaturing gel electrophoresis (DGGE) to separate unique 16S rRNA gene fragments amplified from abalone gut samples, found that diet showed no significant effect on the overall structure of the gut microbial patterns. However, DGGE patterns that were generated from the 16S rRNA gene fragments from abalone gut contained specific bands/bacteria that significantly associated with dietary treatments. This is not the first investigation into the use of *Ulva* as an aquafeed and several studies have noted favourable outcomes following its inclusion as an alternative or supplementary feed for abalone (Makhande, 2008; Bolton et al., 2009; Angell et al., 2012; Mulvaney, Winberg & Adams, 2013; Bansemer et al., 2016; Currie et al., 2016; Hoang et al., 2016; Gobet et al., 2018; Santizo-Taan, Bautista-Teruel & Maquirang, 2020; Duong et al., 2021). However, this is the first study that demonstrated the potential for *Ulva* to be used as a feed supplement that can reduce the reliance on formulated feeds and can significantly enhance growth of abalone. Also, abalone that had increasingly refined fractions of effluent-grown *Ulva* incorporated into formulated feeds are found to form significant bacterial association in the microbial composition of the intestine between abalone fed these diets.

5.3 Interpretations

The use of dry formulated feeds, especially when fed to animals maintained under high stocking densities, can adversely affect water quality if their administration on farms is poorly managed and consequently may be detrimental to the health of animals on the farm (Irungu et al., 2018). In this study, fresh *Ulva* was used as a partial replacement (60 %) for formulated feed where abalone grew at a rate not different to the control. The reduction in formulated feeds required to grow abalone will be quantifiable in the

nutrient loads of effluent water where high protein feeds are linked to high nutrient loads of effluent flows (Probyn et al., 2017). While there may be benefits to the inclusion of *Ulva* into the feeding program of cultured abalone for enhanced growth there are secondary interactions that could impact the overall growth and health of abalone as well as the marketability of the final product.

The consumption of fresh *Ulva* by the abalone has been shown to increase concentrations of dimethylsulphoniopropionate (DMSP) in the tissue of abalone, which corresponds to negative taste profiles in the canned abalone products (Smit, Robertson-Andersson & Bolton, 2010). High levels of DMSP in the tissue are reversible through an exclusion diet (Smit et al., 2007) while the relative concentration of DMSP in *Ulva* may be reduced through drying, as has been shown for other algae (McLenon & DiTullio, 2012). In contrast, dried *Ulva* has been used as a flavour enhancer in the feeds for the fish barramundi (*Lates calcarifer*) with the flavour being attributed to the DMSP content in the flesh of the fish (Jones, Smullen & Carton, 2016). The abalone *H. midae* which contained a concentration of DMSP that was comparable to the levels found in wild caught abalone were considered to have an enhanced sensory quality (Smit, Robertson-Andersson & Bolton, 2010). Advancements in animal husbandry by improving the animals condition through specific aspects of nutrition can enhance the organisms metabolic capacity and positively impact the overall economic profitability of aquaculture systems (Roberts, Kawamura & Nicholson, 1999; Cha et al., 2008; Hooper et al., 2011).

The inclusion of 20 % dried *Ulva* into a formulated feed for the abalone *Haliotis leavigata* significantly increased the rate of nutrient absorption for this species (Duong et al., 2021) while the inclusion of dried *Ulva* in the feeds for *Haliotis asinine* increased the crude protein and reduced lipid contents of the abalone foot (Santizo-Taan, Bautista-Teruel & Maquirang, 2020). In this study, low relative muscle glycogen levels were recorded from abalone fed a diet of fresh *Ulva*. Conversely, the muscle glycogen concentration of abalone fed a combination of *Ulva* and a formulated feed were similar to the levels of glycogen in abalone fed the formulated feed control. The land snail *Megalobulimus oblongus*, when fed a high carbohydrate diet, produced increased haemolymph glucose concentrations with significantly increased glycogen levels recorded in the muscle tissues (Rossi & Silva, 1993). These results are in line with

previous studies which have demonstrated that the deposition of glycogen is related to the level and digestibility of carbohydrates in the feed which may increase the circulating haemolymph glucose concentration (Kucharski & Silva, 1991; Rossi & Silva, 1993; Zhang, Zhou & Cheng, 2009).

The end-point analysis of glucose levels in haemolymph circulation can be an important indicator of the complex regulation of consumption, where elevated post-prandial glucose levels are associated negatively with appetite (Harvey-Anderson & Woodend, 2003; Schock et al., 2010; Johansson et al., 2013). Alania, Dyakonova & Sakharov (2004) investigated the involvement of D-glucose in the mechanisms that control feeding in the gastropod *Lymnaea stagnalis* and demonstrated that central glucoreceptors control feeding behaviour in *L. stagnalis* and that glucose is involved in the suppression of feeding related neurons. The glycaemic assay used in this study found the haemolymph glucose concentration in abalone maintained on formulated feeds were significantly elevated for an extended period when compared to abalone which had been maintained on a diet of *Ulva*. A review by Harvey-Anderson & Woodend (2003) presented evidence to support the case for maintaining satiety for extended periods when postprandial blood glucose concentrations are elevated. Harvey-Anderson & Woodend (2003) further identify that the main stimulator for feeding is in fact decreased glucose utilization, or decreased intracellular glucose concentrations, rather than the absolute concentration of glucose in the blood. Abalone in this study fed a high carbohydrate feed recorded significantly elevated resting haemolymph glucose concentrations for extended periods which may impact the feeding intervals and ultimately total consumption capacity by an animal. The enhanced consumption of feed by abalone in this study was reported when only a small amount of *Ulva* was offered and may be a result of the *Ulva* acting as a feeding stimulus and chemical que which stimulates feeding while also improving the glycaemic capacity of abalone.

Dietary manipulations that target specific biochemical pathways could be used to enhance/optimize the management and feeding programs in aquaculture facilities that may improve feeding efficiency of the abalone. Insulin is the primary hormone involved in the uptake of glucose from the haemolymph of abalone (Venter et al., 2018) and insulin resistance in humans correlates to the onset of a negative metabolic cascade termed the metabolic syndrome (Termizy & Mafauzy, 2009). Venter et al. (2018)

identified metabolic markers that were associated with fast growing *H. midae* where they could stimulate the release of insulin at a faster rate which may result in a greater metabolic response and increased glycogen synthesis to high carbohydrate diets, with an increased capacity for lipid deposition. When high carbohydrate diets (>45.55 %) were fed to the abalone *Haliotis discus hannai* the authors reported increased insulin secretion that contributed to the occurrence of insulin resistance (Guo et al., 2022). Insulin resistance in humans is associated with elevated serum triglycerides and this condition forms part of defining the metabolic disorder in humans (El-Baky, El-Baz & El-Baroty, 2009; Valente et al., 2016, Ludwig et al., 2021). Wang et al. (2009) reported an increase in serum triglycerides that correlated with the relative digestibility of carbohydrate sources fed to the abalone *Haliotis discus hannai.* Abfeed™ S34®, which was used in this study has a carbohydrate content of 41.7 % and is reported to be highly digestible for *H. midae* (Sales & Britz, 2002). The high concentration of triglycerides produced from the consumption of highly digestible carbohydrate feeds, such as Abfeed™ S34®, may indicate a metabolic state where the animal is 'starving in a land of plenty'. Such a state will result from insulin resistance, where the ineffective use of fuels by the tissues is starving them of nutrition (Moon, 1988; Flier, 2019). Ulvan, extracted from *Ulva pertusa,* has been shown to reduce circulating triglycerides in rats, where the high sulphur content was an important component in improving insulin sensitivity (Qi & Sheng, 2015). It would seem that if the appropriate biochemical pathways are available the increased energy derived from circulating triglycerides will create a positive energy balance which encourages the deposition of fatty tissue and may enhance growth rates of abalone fed on diets high in digestible carbohydrates (Venter et al., 2018; Ludwig et al, 2021).

The anaerobic hydrolysis of glycogen to glucose in the foot and adductor muscle of abalone during locomotion results in the production of tauropine (Wells et al., 1998; O'Omolo et al., 2003; Venter et al., 2018). The production of tauropine is an adaption that gastropods have acquired, one of the opine pathways, to allow tolerance to anoxic conditions by increasing the relative concentrations of tauropine (Livingstone, 1991). The build-up of tauropine provides abalone with the unique capacity to derive energy from anaerobic pathways with high efficiency and for prolonged periods. The differentiation of abalone into 'fast' (low tauropine) and 'slow' (high tauropine) growers with low and high tauropine levels by Venter et al. (2018), indicates that the

fast growing abalone are not just efficient at anaerobic metabolism, but more inclined to utilize aerobic pathways in the muscle where low levels of tauropine are detected. The specific ability that has allowed the abalone to tolerate prolonged anoxia, and remain energetically active, may also be an important feature in maintaining the tissue sensitivity for glucose uptake and assimilation in the abalone *Haliotis midae*. High carbohydrate diets may be able to produce high energy derivatives from feeding where abalone may be uniquely equipped with cellular metabolism making them able to deal with high loads of dietary carbohydrate that can lead to enhanced growth and feed efficiency in abalone (Guo et al., 2022).

Studies in which abalone were fed algae enhanced bacterial contribution to digestion of algal polysaccharides were they have demonstrated significant capacity to affect a range of physiological traits of the host (Sawabe, 2006; Gobet et al., 2018; Danckert et al., 2021). The molluscan gut can be separated into the foregut, midgut and hindgut housing a number of transient bacteria that are sourced from the immediate environment and/ or feeds (Erasmus, Cook & Coyne, 1997; Nam et al., 2018) that can form communities existing as a stable core microbiome (Gobet et al., 2018; Nam et al., 2018; Wang et al., 2020; Danckert et al., 2021). The midgut and hindgut contain a large range of endogenous enzymes and exogenous bacterial enzymes that are capable of digesting a variety of complex polysaccharides consumed in the diet of abalone (Erasmus, Cook & Coyne, 1997; Nam et al., 2018). There have been epiphytic polysaccharide degrading bacteria found in association with *Ulva* and are reported to be more abundant on healthy thallus of IMTA cultivated *Ulva*. The IMTA culture environment has also demonstrated the capacity to significantly shape the microbial community associated with the effluent-water of the aquaculture facility (Califano et al., 2020). Effluent-grown *Ulva*, that is intended to be used as a fresh feed for abalone, will likely have developed unique epiphytic microbial communities that are characteristic of the primary culture environment which has been demonstrated previously in culture systems with seabream (Califano et al., 2020). The consumption of *Ulva*, along with its epiphytic microbiome containing polysaccharide fermenters, is likely to impact the energy available for absorption by the abalone. The short chain fatty acids (SCFA) fermentation end-products of polysaccharide digestion by bacteria can provide functional changes for the abalone gut beyond nutrition (Danckert et al., 2021).

The abalone foot and gut are covered by a layer of mucus which is involved in several important functions including lubrication, locomotion, protection, and adhesion to the substrate (Portela et al., 2012) that accounts for up to 29.1 % of total energy expenditure in 50 g *Haliotis tuberculat* (Peck et al., 1987). A diet lacking in fermentable polysaccharides has been linked to a loss in production of beneficial SCFA while trigging the development of a gut microbiome which utilizes the colonic mucosal layer as a nutrient and can modify susceptibility to some diseases (Desai et al., 2016; Martin-Gallausiaux et al., 2020). Several reports have also highlighted the role of specific SCFAs in gut mucosal health through enhanced mucus production and elongation of ciliated intestinal epithelial cells that may impact the host appetite (Roberfroid et al., 2010; Harris et al., 2012; Byrne et al., 2015; Fernández et al., 2016; Hu et al., 2017; Wang et al., 2018; Venegas et al., 2019; Martin-Gallausiaux et al., 2020). Bacteria which have been identified as part of the core microbiome for other abalone demonstrate capacity for the fermentation of algal polysaccharides into SCFA (Gobet et al., 2018; Nel et al., 2018; Duong et al., 2021) where the assimilation of specific SCFAs may further provide the host with high energy molecules which are available for absorption (Sawabe, 2006; Roberfroid et al., 2010; Harris et al., 2012; Fernández et al., 2016; Hu et al., 2017; Wang et al., 2018; Venegas et al., 2019; Martin-Gallausiaux et al., 2020). Microenvironments within the mucus are home to a diverse microbial community that interacts with dietary ingredients and provides the abalone with its first line of defence to foreign particles (Ouwerkerk, Vos & Belzer, 2013; Geurts et al., 2014; Wells et al., 2016; Majee et al., 2018; Martin-Gallausiaux et al., 2020).

Hyaluronan, containing glucuronic acid, is an important component of the molluscan mucus that accounts for a significant proportion of the total energy budget for the abalone *Haliotis tuberculata* (Peck et al., 1987). Glucuronic acid, when included into a formulated feed at an inclusion level of 0.1 % for abalone, improved growth rates, increased relative shell weight contribution to the animal, lowered feed conversion ratios and a unique band/bacterium was shown to be associated with the gut of the abalone fed this diet. Glucuronic acid is an important component of; shell biomineralization that utilizes mucopolysaccharides (Arias & Fernández, 2008; O'Neill et al., 2013; Rao et al., 2014; Pellock & Redinbo, 2017), formation of the cell walls of bacteria (Yasuda et al., 2011), an important component of xenobiotic metabolism (Martínez-Leal, Ponce-García & Escalante-Aburto, 2020) and is a product of bacterial

fermentation where it has health promoting effects (Yavari et al., 2018). The epithelial cells lining the gut are sites of attachment for a consortium of microorganisms that will in turn form their own biofilms (Hansson, 2012; Portela et al., 2012; Ouwerkerk, Vos & Belzer, 2013; Desai et al., 2016) where the production of microbial exopolysaccharide substance comprised of glucuronic acid (Mojica, Elsey & Cooney, 2007) can contribute to the total capacity of the gut and immune function.

5.4 Limitations

Ulva can make a significant impact on important variables of value to the aquaculture industry through its inclusion into an integrated system with abalone and recirculating water; which can reduce pumping, save electricity, bio-remediate water (Nobre et al., 2010), increase farm water temperatures through recirculation and reduce the reliance on formulated feeds for the production of abalone. Culture environment and the availability of dissolved nutrients can impact the nutritional profile of seaweeds which should be considered when designing integrated systems. For example, effluent-grown *Ulva fasciata* demonstrated the capacity to increase carbohydrate content under high nitrogen environments (Prabhu et al., 2019; Shahar et al., 2020) which could form an important component of the bacterial contribution to the digestion and assimilation of nutrients for the abalone. The reductions in apparent costs would likely only be realised in areas where there is potential for increasing water temperatures in the culture systems and/ or the capacity for on-site production of effluent-grown algae. Areas where water temperatures exceed 22 °C, like Wild Coast Abalone where ambient water temperatures are already at the upper levels for optimal production, an increase in water temperatures may be detrimental to overall farm performance. The inclusion of *Ulva* into existing abalone production where it forms part of the feeding regime is recommended for improving the overall impact/sustainability of current production process and may enhance the profitability of specific marketable products.

DGGE demonstrated capacity for use as a diagnostic to monitor changes in the bacterial patterns associated with the gut of abalone following dietary interventions, identifying unique bands that would be useful to investigate further. The application of DGGE would likely be suited for the development of a platform for routine diagnostics, where standards can be used to better inform the results more appropriately or as part of a monitoring program that is part of identifying changes in bacterial communities under

specific environments or following specific managerial interventions, such as a change in diet. However, although DGGE provided valuable insight on the impact of *Ulva* and its components on the microbial patterns found in association with the gut of abalone in this study, next generation sequencing technologies would greatly enhance our understanding of the diversity and relative abundance of bacteria associated with the digestive tract of *H. midae* fed varying diets and provide crucial insights on the role of specific microorganism(s), and their metabolic potential, in the growth, health and physiology of abalone. Collectively, these findings support the notion that specific dietary ingredients and/ or algal polysaccharides can alter the microbiome of an organism, which may in turn impact the growth and health of the animal.

5.5 Recommendations

In this study a significantly improved total consumption was observed when the formulated feeds were partially supplemented with fresh *Ulva*. *H. asinina* demonstrated preference for *Ulva* when offered alongside other algae in a feeding choice experiment and consumed more *Ulva* when offered in a no choice feeding trial where each algae was presented individually. Feeding on fresh *Ulva* opposed to formulated feeds requires a change in the feeding behaviour of abalone due to the vastly different characteristics of the feed provisions. The design of the baskets should be further investigated if paired feeding regimes are adopted so that the abalone have equal access to each feed when it is presented to maximise efficiency. Dietary *Ulva* is likely contributing to the enhanced growth of abalone through adjusting the feeding behaviour of the abalone (Akakabe & Kajiwara, 2008; Cyrus et al., 2015; Currie et al., 2016) and modifications to the gut microbiome which can improve metabolic and immune homeostasis (Nel et al., 2017; Gobet et al., 2018; Danckert et al., 2021).

Marine macroalgae contain numerous bioactive compounds (Hafting et al., 2015; Sharifuddin et al., 2015; Wells et al., 2016; Doan et al., 2019) which could be harnessed as functional components of feeding regimes for the improvement of a specific aspect of animal husbandry. Hoang et al. (2016) reported beneficial effects associated with a 10 % dried *Ulva* inclusion in a diet for *H. laevigata* on shell and foot colour due to the accumulation of carotenoid compounds. *Ulva* has been included into a diet for Nile tilapia (*Oreochromis niloticus*) at 5 % and resulted in significantly higher levels of carotenoid pigments in tissues of fish fed this diet when compared to fish fed a standard

feed (Valente et al., 2016). *Ulva* has also been successfully used to improve gonad colouration in the sea urchin *Tripneustes gratilla* (Cyrus, Bolton & Macey, 2015). Fresh *Ulva* when fed alongside formulated feeds to the hybrid abalone *H. laevigata* × *H. rubra* consistently improved their tissue fatty acid profile and enhanced the perceived human health benefits (Mulvaney et al., 2015). The fortification of diets through supplementation with fresh seaweeds has led to improvements in the growth and health of cultured organisms which may have an impact on human health (Kwasek, Thorne-Lyman & Phillips, 2020). Although this research only looked at the inclusion of effluent-grown *Ulva*, it is likely that wild *Ulva* may illicit similar changes in bacterial association. The main difference would present in the nutritional composition with limited nitrogen available in wild harvested *Ulva* when compared to the higher protein content of effluent-grown *Ulva* (Robertson-Andersson et al., 2009).

5.6 Summary

The results from this thesis indicate that *Ulva* is able to modulate the gut microbiota when included as a partial replacement for formulated feeds, where it can improve the overall growth, health and consumption of abalone. It is possible that fresh *Ulva* and/ or its carbohydrate fraction is able to improve the digestive efficiency through its functional benefits to metabolism provided by the bacterial fermentation of algal polysaccharides which leads to an enhanced feed efficiency for the abalone. The denaturing gradient gel electrophoresis (DGGE) that was used to assess changes in gut microbial pattern and identify unique bacterial associations in the intestine of abalone fed various diets still requires further investigation to identify these species before any kind of metabolic capacity can be attributed to specific microorganism(s). Nonetheless, these unique bands identified would be an ideal starting point for the exploration of unknown microbiota that appear to have significant associations within the gut of an abalone.

In conclusion, IMTA is a partial solution to the problem of producing more food for the growing population. For the continued growth of aquaculture it will be important to diversify the production systems in an attempt to reduce the overall impact of the farming and the associated dependency on external inputs (Rosenzweig et al., 2020). The specific inclusion of *Ulva* and its components into formulated feeds has provided insights that are important to both operational and the feed development departments

of an existing industry. The results from this study add to the existing research which has identified *Ulva* as an ideal candidate for its use in IMTA processes, where it has the capacity to influence cultivation technologies across the world (Tenore, 1976; Taylor & Tsvetnenko, 2004; Robertson-Andersson et al., 2008; Bolton et al., 2009, 2016; Winberg, Ghosh & Tapsell, 2009; Macchiavello & Bulboa, 2014; Silva et al., 2014; Kemp, Britz & Agüero, 2015; Hoang et al., 2016; Guo, 2017; Prisco, 2019).

The general methods for abalone cultivation have been well understood but there remains a lack of knowledge on the abalone metabolome (Venter et al., 2016) which may account for the high variability in growth under commercial conditions. In a study that compared fast and slow growing abalone (*H. iris*) the authors reported increased muscle fatty acid content that was produced from glucose via de novo lipogenesis (Venter et al., 2022). The fact that fresh *Ulva* is able to provide such a diverse and significant impact to metabolic function (Tas et al., 2011; Sharifuddin et al., 2015; Majee et al., 2018; Wang et al., 2018; Guerreiro et al., 2019; Fumanal et al., 2020; Gómez-Zorita et al., 2020; Martin-Gallausiaux et al., 2020; Ponce et al., 2020; Duong et al., 2021) and the production of abalone, as observed in the present study, its inclusion as a fresh feed for maximum gains should be explored further. The various tissues that were preserved in this study would provide unique insights into the metabolic interactions and create a more complete picture of how *Ulva* is influencing the metabolic capacity of the highly valuable South African abalone *Haliotis midae*. In future research it would be worth taking a closer look at the role *Ulva* plays in the quality, structure and energetic cost of production of the abalone mucus. The mucosal energy budget has been found to be a significant contribution to the total energy budget of abalone and if its function and cost of production can be adjusted through diet the research can have an industrial application.

References

Referencing follows the styles provided by Read Cube for the University of Cape Town where lists of more than three authors is treated with *et al*.. If there is more than one publication for as a first author of three or more, the naming strategy is to use more authors to differentiate publications.

Abenavoli, L., Scarpellini, E., Colica, C., Boccuto, L., Salehi, B., Sharifi-Rad, J., Aiello, V., Romano, B., et al. 2019. Gut microbiota and obesity: a role for probiotics. *Nutrients*. 11(11):2690. DOI: 10.3390/nu11112690.

Adrien, A., Bonnet, A., Dufour, D., Baudouin, S., Maugard, T. & Bridiau, N. 2017. Pilot production of ulvans from *Ulva* sp. and their effects on hyaluronan and collagen production in cultured dermal fibroblasts. *Carbohydrate Polymers*. 157:1306–1314. DOI: 10.1016/j.carbpol.2016.11.014.

Aguilar-Briseño, J., Cruz-Suarez, L., Sassi, J.-F., Ricque-Marie, D., Zapata-Benavides, P., Mendoza-Gamboa, E., Rodríguez-Padilla, C. & Trejo-Avila, L. 2015. Sulphated polysaccharides from *Ulva clathrata* and *Cladosiphon okamuranus* seaweeds both inhibit viral attachment/entry and cell-cell fusion, in NDV infection. *Marine Drugs*. 13(2):697–712. DOI: 10.3390/md13020697.

Ai, C., Ma, N., Sun, X., Duan, M., Wu, S., Yang, J., Wen, C. & Song, S. 2017. Absorption and degradation of sulfated polysaccharide from pacific abalone in *in vitro* and *in vivo* models. *Journal of Functional Foods*. 35:127–133. DOI: 10.1016/j.jff.2017.05.022.

Akakabe, Y. & Kajiwara, T. 2008. Bioactive volatile compounds from marine algae: feeding attractants. *Journal of Applied Phycology*. 20(5):661–664. DOI: 10.1007/s10811-007-9309-x.

Akbary, P. & Aminikhoei, Z. 2017. Effect of water-soluble polysaccharide extract from the green alga *Ulva rigida* on growth performance, antioxidant enzyme activity, and immune stimulation of grey mullet *Mugil cephalus*. *Journal of Applied Phycology*. 1(5):87. DOI: 10.1007/s10811-017-1299-8.

References

Akbary, P. & Aminikhoei, Z. 2018. Effect of polysaccharides extracts of algae *Ulva rigida* on growth, antioxidant, immune response and resistance of shrimp, *Litopenaeus vannamei* against *Photobacterium damselae*. *Aquaculture Research*. 49(7):2503–2510. DOI: 10.1111/are.13710.

Alania, M., Dyakonova, V. & Sakharov, D.A. 2004. Hyperpolarization by glucose of feeding-related neurons in snail. *Acta Biologica Hungarica*. 55(1–4):195–200. DOI: 10.1556/abiol.55.2004.1-4.24.

Alberoni, D., Baffoni, L., Gaggìa, F., Ryan, P.M., Murphy, K., Ross, P.R., Stanton, C. & Gioia, D.D. 2018. Impact of beneficial bacteria supplementation on the gut microbiota, colony development and productivity of *Apis mellifera* L. *Beneficial Microbes*. 9(2):269–278. DOI: 10.3920/bm2017.0061.

Alstyne, K.L.V., Wolfe, G.V., Freidenburg, T.L., Neill, A., Hicken, C., 2001. Activated defense systems in marine macroalgae: evidence for an ecological role for DMSP cleavage. *Marine Ecology Progress Series* 213, 53–65. https://doi.org/10.3354/meps213053

Alstyne, K.V., Alstyne, K.L.V., Houser, L. & Houser, L.T. 2003. Dimethylsulfide release during macroinvertebrate grazing and its role as an activated chemical defense. *Marine Ecology Progress Series*. 250:175–181. DOI: 10.3354/meps250175.

Andrade, J.M., Besada, V., Schultze, F. & González, J.J. 2009. Heavy metals in edible seaweeds commercialised for human consumption. *Journal of Marine Systems*. 75(1–2):305–313. DOI: http://dx.doi.org/10.1016/j.jmarsys.2008.10.010.

Angell, A.R., Pirozzi, I., Nys, R. de & Paul, N.A. 2012. Feeding preferences and the nutritional value of tropical algae for the abalone *Haliotis asinina*. *PLoS ONE*. 7(6):e38857. DOI: 10.1371/journal.pone.0038857.

Ansorge, W.J. 2009. Next-generation DNA sequencing techniques. *New biotechnology*. 25(4):195–203. DOI: 10.1016/j.nbt.2008.12.009.

Arias, J.L. & Fernández, M.S. 2008. Polysaccharides and Proteoglycans in Calcium Carbonate-based Biomineralization. *Chemical Reviews*. 108(11):4475–4482. DOI: 10.1021/cr078269p.

References

Ariefdjohan, M.W., Savaiano, D. a & Nakatsu, C.H. 2010. Comparison of DNA extraction kits for PCR-DGGE analysis of human intestinal microbial communities from fecal specimens. *Nutrition Journal*. 9(1):23. DOI: 10.1186/1475-2891-9-23.

Ayres, D.W.P. 2013. Effect of diet and sex-sorting on growth and gonad development in farmed South African abalone, *Haliotis midae*.

Bachoo, T. 2021. Characterization of *Ulva* (*Ulvaceae*, Chlorophyta) species cultured in commercial abalone farms in South Africa, and comparison with closely related wild species, using morpho-anatomical and molecular methods. *University of Cape Town*. Available: http://hdl.handle.net/11427/35631.

Bansemer, M.S., Qin, J.G., Harris, J.O., Duong, D.N., Currie, K.-L., Howarth, G.S. & Stone, D.A.J. 2016. Dietary inclusions of dried macroalgae meal in formulated diets improve the growth of greenlip abalone (*Haliotis laevigata*). *Journal of Applied Phycology*. (April, 21):1–14. DOI: 10.1007/s10811-016-0829-0.

Bansemer, M.S., Qin, J.G., Harris, J.O., Duong, D.N., Hai, T., Howarth, G.S. & Stone, D.A.J. 2016. Growth and feed utilisation of greenlip abalone (*Haliotis laevigata*) fed nutrient enriched macroalgae. *Aquaculture*. 452:62–68. DOI: 10.1016/j.aquaculture.2015.10.025.

Barcelo, A., Claustre, J., Moro, F., Chayvialle, J.-A., Cuber, J.-C. & Plaisancié, P. 2000. Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. *Gut*. 46(2):218. DOI: 10.1136/gut.46.2.218.

Barkai, R. & Griffiths, C.L. 1986. Diet of the South African abalone *Haliotis midae*. *South African Journal of Marine Science*. 4(1):37–44. DOI: 10.2989/025776186784461891.

Barkai, R. & Griffiths, C.L. 1987. Consumption, absorption efficiency, respiration and excretion in the South African abalone *Haliotis midae*. *South African Journal of Marine Science*. 5(1):523–529. DOI: 10.2989/025776187784522441.

Barkai, R. & Griffiths, C.L. 1988. An energy budget for the South African abalone *Haliotis midae* Linnaeus. *Journal of Molluscan Studies*. 54(1):43–51. DOI: 10.1093/mollus/54.1.43.

References

Bashir-Tanoli, S. & Tinsley, M.C. 2014. Immune response costs are associated with changes in resource acquisition and not resource reallocation. *Functional Ecology*. 28(4):1011–1019. DOI: 10.1111/1365-2435.12236.

Bayne, B.L. & Newell, R.C. 1983. Physiological Energetics of Marine Molluscs. In *The Mollusca*. A.S.M. Saleuddin & K.M. Wilbur, Eds. *Academic Press*. 407–515. DOI: 10.1016/b978-0-12-751404-8.50017-7.

BelHadj, S., Hentati, O., Elfeki, A. & Hamden, K. 2013. Inhibitory activities of *Ulva lactuca* polysaccharides on digestive enzymes related to diabetes and obesity. *Archives of Physiology and Biochemistry*. 119(2):81–87. DOI: 10.3109/13813455.2013.775159.

Beltran, C.G.G. & Coyne, V.E. 2020. iTRAQ-based quantitative proteomic profiling of the immune response of the South African abalone, *Haliotis midae*. *Fish & Shellfish Immunology*. 99:130–143. DOI: 10.1016/j.fsi.2020.02.007.

Bennett, L.W., Keirs, R.W., Peebles, E.D. & Gerard, P.D. 2007. Methodologies of tissue preservation and analysis of the glycogen content of the broiler chick liver. *Poultry Science*. 86(12):2653–2665. DOI: 10.3382/ps.2007-00303.

Bobin-Dubigeon, C., Lahaye, M. & Barry, J. 1997. Human Colonic Bacterial Degradability of Dietary Fibres from Sea-Lettuce (*Ulva* sp). *Journal of the Science of Food and Agriculture*. 73(2):149–159. DOI: 10.1002/(sici)1097-0010(199702)73:2<149::aid-jsfa685>3.0.co;2-l.

Bolton, J.J. 2006. Do we have the vision to integrate our marine aquaculture? *South African Journal of Science*. 25(December):507–508. DOI: 10.1007/s10811-012-9902-5.

Bolton, J.J., Robertson-Andersson, D.V., Shuuluka, D. & Kandjengo, L. 2009. Growing *Ulva* (Chlorophyta) in integrated systems as a commercial crop for abalone feed in South Africa: a SWOT analysis. *Journal of Applied Phycology*. 21(5):575–583. DOI: 10.1007/s10811-008-9385-6.

Bolton, J.J., Macey, B.M., Cyrus, M.D., Joubert, M. & Brand, M.J. 2016. Why grow *Ulva*? Its potential role in the future of aquaculture. *Perspectives in Phycology*. (September, 8):1–1. DOI: 10.1127/pip/2016/0058.

References

Bossio, D.A., Girvan, M.S., Verchot, L., Bullimore, J., Borelli, T., Albrecht, A., Scow, K.M., Ball, A.S., et al. 2005. Soil microbial community response to land use change in an agricultural landscape of western Kenya. *Microbial Ecology*. 49(1):50–62. DOI: 10.1007/s00248-003-0209-6.

Boyd, C.E., McNevin, A.A. & Davis, R.P. 2022. The contribution of fisheries and aquaculture to the global protein supply. *Food Security*. 14(3):805–827. DOI: 10.1007/s12571-021-01246-9.

Braid, B.A., Moore, J.D., Robbins, T.T., Hedrick, R.P., Tjeerdema, R.S. & Friedman, C.S. 2005. Health and survival of red abalone, *Haliotis rufescens*, under varying temperature, food supply, and exposure to the agent of withering syndrome. *Journal of Invertebrate Pathology*. 89(3):219–231. DOI: 10.1016/j.jip.2005.06.004.

Brand, MJ., Bolton, JJ. & Macey, BM. 2013. POSTER: *Ulva* grown in abalone effluent water as a potential substitute for fishmeal-based feeds for the cultured abalone *Haliotis midae*. *World Aquaculture Conference 2013*, Nashville, United States of America

Britz, P.J. 1996. The suitability of selected protein sources for inclusion in formulated diets for the South African abalone, *Haliotis midae*. *Aquaculture*. 140(1–2):63–73. DOI: 10.1016/0044-8486(95)01197-8.

Britz, P.J. & Hecht, T. 1997. Effect of dietary protein and energy level on growth and body composition of South African abalone, *Haliotis midae*. *Aquaculture*. 156(3–4):195–210. DOI: 10.1016/s0044-8486(97)00090-2.

Britz, P.J., Hecht, T., Knauer, J. & Dixon, M.G. 1994. The Development of an artificial feed for abalone farming. *Suid-Afrikaanse Tydskrif vir Wetenskap*. 90:7–8.

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

Brown, G.K. 2000. Glucose transporters: Structure, function and consequences of deficiency. *Journal of inherited metabolic disease*. 23(3):237–246. DOI: 10.1023/a:1005632012591.

References

Brown, M.R., Sikes, A.L., Elliott, N.G. & Tume, R.K. 2008. Physicochemical factors of abalone quality: a review. *Journal of Shellfish Research*. 27(4):835–842. DOI: 10.2983/0730-8000(2008)27[835:pfoaqa]2.0.co;2.

Brownlee, M. 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature*. 414(6865):813–820. DOI: 10.1038/414813a.

Bullon, N., Seyfoddin, A. & Alfaro, A.C. 2022. The role of aquafeeds in abalone nutrition and health: A comprehensive review. *Journal of the World Aquaculture Society*. DOI: 10.1111/jwas.12883.

Burgents, J.E., Burgents, J.E., Burnett, K.G. & Burnett, L.E. 2004. Disease resistance of Pacific white shrimp, *Litopenaeus vannamei*, following the dietary administration of a yeast culture food supplement. *Aquaculture*. 231(1–4):1–8. DOI: 10.1016/j.aquaculture.2003.09.003.

Burgents, J.E., Burnett, L.E., Stabb, E.V. & Burnett, K.G. 2005. Localization and bacteriostasis of *Vibrio* introduced into the Pacific white shrimp, *Litopenaeus vannamei*. *Developmental & Comparative Immunology*. 29(8):681–691. DOI: 10.1016/j.dci.2005.01.004.

Burke, C., Thomas, T., Lewis, M., Steinberg, P. & Kjelleberg, S. 2011. Composition, uniqueness and variability of the epiphytic bacterial community of the green alga *Ulva australis*. *The ISME Journal*. 5(4):590–600. DOI: 10.1038/ismej.2010.164.

Butler, S.O., Btaiche, I.F. & Alaniz, C. 2005. Relationship between hyperglycemia and infection in critically ill patients. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*. 25(7):963–976. DOI: 10.1592/phco.2005.25.7.963.

Byrne, C.S., Chambers, E.S., Morrison, D.J. & Frost, G. 2015. The role of short chain fatty acids in appetite regulation and energy homeostasis. *International journal of obesity*. 39(9):1331–1338. DOI: 10.1038/ijo.2015.84.

Cáceres, M.D. & Legendre, P. 2009. Associations between species and groups of sites: indices and statistical inference. *Ecology*. 90(12):3566–3574. DOI: 10.1890/08-1823.1.

References

Califano, G., Kwantes, M., Abreu, M.H., Costa, R. & Wichard, T. 2020. Cultivating the macroalgal holobiont: effects of integrated multi-trophic aquaculture on the microbiome of *Ulva rigida* (Chlorophyta). *Frontiers in Marine Science*. 7:52. DOI: 10.3389/fmars.2020.00052.

Cameira, M. do R. & Pereira, L.S. 2019. Innovation issues in water, agriculture and food. *Water*. 11(6):1230. DOI: 10.3390/w11061230.

Campanati, C., Willer, D., Schubert, J. & Aldridge, D.C. 2021. Sustainable Intensification of Aquaculture through Nutrient Recycling and Circular Economies: More Fish, Less Waste, Blue Growth. *Reviews in Fisheries Science & Aquaculture*. 30(2):1–50. DOI: 10.1080/23308249.2021.1897520.

Cañedo-Castro, B., Piñón-Gimate, A., Carrillo, S., Ramos, D. & Casas-Valdez, M. 2019. Prebiotic effect of *Ulva rigida* meal on the intestinal integrity and serum cholesterol and triglyceride content in broilers. *Journal of Applied Phycology*. 31(5):3265–3273. DOI: 10.1007/s10811-019-01785-x.

Caon, I., Parnigoni, A., Viola, M., Karousou, E., Passi, A. & Vigetti, D. 2020. Cell Energy metabolism and hyaluronan synthesis. *Journal of Histochemistry & Cytochemistry*. 69(1):35–47. DOI: 10.1369/0022155420929772.

Carefoot, T.H. 1991. Blood-glucose levels in the sea hare *Aplysia dactylomela*: interrelationships of activity, diet choice and food quality. *Journal of Experimental Marine Biology and Ecology*. 154:1–14.

Carefoot, T.H., Taylor, B.E. & Land, S. 2000. Use of isolated digestive-gland cells in the study of biochemical and physiological processes in gastropod molluscs. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*. 125(4):497–502. DOI: 10.1016/s1095-6433(00)00181-1.

Castrillon, A.M., Navarro, M.P. & Garcia-Arias, M.T. 1996. Tuna protein nutritional quality changes after canning. *Journal of Food Science*. 61(6):1250–1253. DOI: 10.1111/j.1365-2621.1996.tb10972.x.

Castro, R., Zarra, I. & Lamas, J. 2004. Water-soluble seaweed extracts modulate the respiratory burst activity of turbot phagocytes. *Aquaculture*. 229(1–4):67–78. DOI: 10.1016/s0044-8486(03)00401-0.

Celikler, S., Tas, S., Vatan, O., Ziyanok-Ayvalik, S., Yildiz, G. & Bilaloglu, R. 2009. Anti-hyperglycemic and antigenotoxic potential of *Ulva rigida* ethanolic extract in the experimental diabetes mellitus. *Food and Chemical Toxicology*. 47(8):1837–1840. DOI: 10.1016/j.fct.2009.04.039.

Chen, Z.-H., Ren, X.-L. & Li, X.-D. 2012. The role of hyaluronic acid in biomineralization. *Frontiers of Materials Science*. 6(4):283–296. DOI: 10.1007/s11706-012-0182-4.

Chi, Y., Qiao, L., Zuo, S., Yan, H., Du, C., Hwang, H. & Yu, B. 2018. Submerged liquid fermentation of *Ulva prolifer*a Müller tissue and applications for improving growth performance in juvenile abalone (*Haliotis discus hannai* Ino). *Cell and Tissue Research*. 30:2639–2647. DOI: 10.1007/s10811-018-1413-6.

Chigumira, G. 2016. *Maximising niche markets: South African abalone*. Available: https://tips.org.za/policy-briefs/item/3128-maximising-niche-markets-south-african-abalone.

Chiou, T.-K., Lai, M.-M. & Shiau, C.-Y. 2001. Seasonal variations of chemical constituents in the muscle and viscera of small abalone fed different diets. *Fisheries Science*. 67(1):146–156. DOI: 10.1046/j.1444-2906.2001.00211.x.

Cho, C.Y. & Bureau, D.P. 2001. A review of diet formulation strategies and feeding systems to reduce excretory and feed wastes in aquaculture. *Aquaculture Research*. 32(s1):349–360. DOI: 10.1046/j.1355-557x.2001.00027.x.

Choi, M.-J., Oh, Y.D., Kim, Y.R., Lim, H.K. & Kim, J.-M. 2021. Intestinal microbial diversity is higher in Pacific abalone (*Haliotis discus hannai*) with slower growth rates. *Aquaculture*. 537:736500. DOI: 10.1016/j.aquaculture.2021.736500.

Chopin, T., Buschmann, A.H., Halling, C., Troell, M., Kautsky, N., Neori, A., Kraemer, G.P., Zertuche-González, J.A., et al. 2001. Integrating seaweeds into marine

aquaculture systems: a key toward sustainability. *Journal of Phycology*. 37(6):975–986. DOI: 10.1046/j.1529-8817.2001.01137.x.

Cicala, F., Cisterna-Céliz, J.A., Paolinelli, M., Moore, J.D., Sevigny, J. & Rocha-Olivares, A. 2022. The Role of Diversity in Mediating Microbiota Structural and Functional Differences in Two Sympatric Species of Abalone Under Stressed Withering Syndrome Conditions. *Microbial Ecology*. 1–11. DOI: 10.1007/s00248-022-01970-5.

Cochet, M., Brown, M., Kube, P., Fluckiger, M., Elliott, N. & Delahunty, C. 2013. Sensory and physicochemical assessment of wild and aquacultured green and black lip abalone (*Haliotis laevigata* and *Haliotis rubra*). *Journal of Shellfish Research*. 32(1):81–88. DOI: 10.2983/035.032.0114.

Cook, P.A. 2019. Worldwide Abalone Production Statistics. *Journal of Shellfish Research*. 38(2):401. DOI: 10.2983/035.038.0222.

Cornish, M.L. & Garbary, D.J. 2010. Antioxidants from macroalgae: potential applications in human health and nutrition. *Algae*. 25(4):155–171. DOI: 10.4490/algae.2010.25.4.155.

Costa, C., Alves, A., Pinto, P.R., Sousa, R. a, Borges, E.A., Reis, R.L. & Rodrigues, A. ’ı rio E. 2012. Characterization of ulvan extracts to assess the effect of different steps in the extraction procedure. *Carbohydrate Polymers*. 88(2):537–546. DOI: 10.1016/j.carbpol.2011.12.041.

Coyne, V.E. 2011. The importance of ATP in the immune system of molluscs. *Invertebrate Survival Journal*. 8(1):45–55. Available: http://www.isj.unimo.it/articoli/ISJ231.pdf.

Croll, R.P. 1983. Gastropod chemoreception. *Biological Reviews*. 58:293–319. DOI: 10.1111/j.1469-185x.1983.tb00391.x.

Currie, K.-L., Davidson, H., Bansemer, M.S., Harris, J.O. & Stone, D.A.J. 2016. Ventral videographic assessment of the feeding behavior of juvenile Greenlip [*Haliotis laevigata* (Donovan, 1808)] and Hybrid (*H. laevigata* × *Haliotis rubra*) abalone in

response to dietary and temperature manipulation. *Journal of Shellfish Research*. 35(3):641–651. DOI: 10.2983/035.035.0310.

Cyrus, M.D., Bolton, J.J., Scholtz, R. & Macey, B.M. 2015. The advantages of *Ulva* (Chlorophyta) as an additive in sea urchin formulated feeds: effects on palatability, consumption and digestibility. *Aquaculture Nutrition*. 21(5):578–591. DOI: 10.1111/anu.12182.

DAFF. 2016. *Aquaculture Yearbook 2016*. Cape Town: Aquaculture Technical Services.

Danckert, N.P., Wilson, N., Phan-Thien, K.-Y. & Stone, D.A.J. 2021. The intestinal microbiome of Australian abalone, *Haliotis laevigata* and *Haliotis laevigata* × *Haliotis rubra*, over a 1-year period in aquaculture. *Aquaculture*. 534:736245. DOI: 10.1016/j.aquaculture.2020.736245.

Davies, M.S. & Williams, G.A. 1995. Pedal mucus of a tropical limpet, *Cellana grata* (Gould): energetics, production and fate. *Journal of Experimental Marine Biology and Ecology*. 186(1):77–87. DOI: 10.1016/0022-0981(94)00156-8.

Dawood, M., Koshio, S., El-Sabagh, M., Billah, M., Zaineldin, A., Zayed, M.M. & Omar, A.A.E.-D. 2017. Changes in the growth, humoral and mucosal immune responses following β-glucan and vitamin C administration in red sea bream, *Pagrus major*. *Aquaculture*. 470:214–222. DOI: 10.1016/j.aquaculture.2016.12.036.

De Jager, K. 2021. Microorganisms associated with *Ulva* grown in abalone effluent water: Implications for biosecurity. *University of Cape Town*.

De Prisco, J.A. 2019. An investigation of some key physico-chemical water quality parameters of an Integrated Multi-Trophic Aquaculture (IMTA) system operating recirculation methodology in the Western Cape of South Africa. *University of Cape Town*. Available: https://open.uct.ac.za/handle/11427/32653.

Desai, M.S., Seekatz, A.M., Koropatkin, N.M., Kamada, N., Hickey, C.A., Wolter, M., Pudlo, N.A., Kitamoto, S., et al. 2016. A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. *Cell*. 167(5):1339-1353.e21. DOI: 10.1016/j.cell.2016.10.043.

Devaki, T., Sathivel, A. & BalajiRaghavendran, H.R. 2009. Stabilization of mitochondrial and microsomal function by polysaccharide of *Ulva lactuca* on D-Galactosamine induced hepatitis in rats. *Chemico-Biological Interactions*. 177(2):83–88. DOI: 10.1016/j.cbi.2008.09.036.

Díaz, G.G. & Nakagawa, H. 1990. Effects of dietary carbohydrates on growth and body components of the giant freshwater prawn, *Macrobrachium rosenbergii*. *Aquatic Living Resources*. 3(2):99–105. DOI: 10.1051/alr:1990009.

Dierking, K. & Pita, L. 2020. Receptors Mediating Host-Microbiota Communication in the Metaorganism: The Invertebrate Perspective. *Frontiers in Immunology*. 11:1251. DOI: 10.3389/fimmu.2020.01251.

Dignam, B.E.A., O'Callaghan, M., Condron, L.M., Kowalchuk, G.A., Nostrand, J.D.V., Zhou, J. & Wakelin, S.A. 2018. Effect of land use and soil organic matter quality on the structure and function of microbial communities in pastoral soils: Implications for disease suppression. *PLOS ONE*. 13(5):e0196581. DOI: 10.1371/journal.pone.0196581.

Dlaza, T.S., Maneveldt, G.W. & 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(1):199–203. DOI: 10.2989/ajms.2008.30.1.22.472.

Doan, H.V., Hoseinifar, S.H., Esteban, M.Á., Dadar, M. & Thu, T.T.N. 2019. Chapter 2 Mushrooms, seaweed, and their derivatives as functional feed additives for aquaculture: an updated view. *Studies in Natural Products Chemistry*. 62:41–90. DOI: 10.1016/b978-0-444-64185-4.00002-2.

Donaghy, L., Hong, H.-K., Lambert, C., Park, H.-S., Shim, W.J. & Choi, K.-S. 2010. First characterisation of the populations and immune-related activities of hemocytes from two edible gastropod species, the disk abalone, *Haliotis discus discus* and the spiny top shell, *Turbo cornutus*. *Fish & Shellfish Immunology*. 28(1):87–97. DOI: 10.1016/j.fsi.2009.10.006.

References

Duong, D.N., Stone, D.A.J., Qin, J.G., Hoang, T.H., Bansemer, M.S. & Harris, J.O. 2021. Improvement in energy allocation in Greenlip Abalone (*Haliotis laevigata* Donovan) fed diets substituted with different types and levels of macroalgal meal. *Aquaculture*. 531:735816. DOI: 10.1016/j.aquaculture.2020.735816.

Durand, M., Beaumatin, P., Bulman, B., Bemalier, A., Grivet, J., Serezat, M., Gramet, G. & Lahaye, M. 1997. Fermentation of green alga sea-lettuce (*Ulva* sp) and metabolism of its sulphate by human colonic microbiota in a semi-continuous culture system. *Reproduction Nutrition Development*. 37(3):267–283. DOI: 10.1051/rnd:19970303.

Dworjanyn, S., Pirozzi, I. & Liu, W. 2007. The effect of the addition of algae feeding stimulants to artificial diets for the sea urchin *Tripneustes gratilla*. *Aquaculture*. 273(4):624–633. DOI: 10.1016/j.aquaculture.2007.08.023.

Egan, S., Harder, T., Burke, C., Steinberg, P., Kjelleberg, S. & Thomas, T. 2013. The seaweed holobiont: understanding seaweed–bacteria interactions. *FEMS Microbiology Reviews*. 37(3):462–476. DOI: 10.1111/1574-6976.12011.

El-Baky, H.H., El-Baz, F.K. & El-Baroty, G.S. 2009. Natural preservative ingredient from marine alga *Ulva lactuca* L. *International Journal of Food Science & Technology*. 44(9):1688–1695. DOI: 10.1111/j.1365-2621.2009.01926.x.

Erasmus, J.H., Cook, P.A. & Coyne, V.E. 1997. The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. *Aquaculture*. 155(1–4):377–386. DOI: 10.1016/s0044-8486(97)00112-9.

Falkenmark, M. 2013. Growing water scarcity in agriculture: future challenge to global water security. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*. 371(2002):20120410. DOI: 10.1098/rsta.2012.0410.

FAO. 2009. *Fish as feed inputs for aquaculture*. (518). FAO.

FAO. 2020. *The State of World Fisheries and Aquaculture 2020*. Rome, Italy: FAO. Available: http://www.fao.org/documents/card/en/c/ca9229en.

Fernández, J., Redondo-Blanco, S., Gutiérrez-del-Río, I., Miguélez, E.M., Villar, C.J. & Lombó, F. 2016. Colon microbiota fermentation of dietary prebiotics towards short-chain fatty acids and their roles as anti-inflammatory and antitumour agents: A review. *Journal of Functional Foods*. 25:511–522. DOI: 10.1016/j.jff.2016.06.032.

Fernández-Elías, V.E., Ortega, J.F., Nelson, R.K. & Mora-Rodriguez, R. 2015. Relationship between muscle water and glycogen recovery after prolonged exercise in the heat in humans. *European Journal of Applied Physiology*. 115(9):1919–1926. DOI: 10.1007/s00421-015-3175-z.

Fleming, A.E., Barneveld, R.J.V. & Hone, P.W. 1996. The development of artificial diets for abalone: a review and future directions. *Aquaculture*. 140(1996):5–53.

Flier, J.S. 2019. Starvation in the midst of plenty: reflections on the history and biology of insulin and leptin. *Endocrine Reviews*. 40(1):1–16. DOI: 10.1210/er.2018-00179.

Foster-Powell, K., Holt, S.H.A. & Brand-Miller, J.C. 2002. International table of glycemic index and glycemic load values: 2002. *The American Journal of Clinical Nutrition*. 76(1):5–56. DOI: 10.1001/jama.1997.03540300040031.

Francis, T.L., Maneveldt, G.W. & Venter, J. 2008. Determining the most appropriate feeding regime for the South African abalone *Haliotis midae* Linnaeus grown on kelp. *Journal of Applied Phycology*. 20:597–602. DOI: 10.1007/s10811-007-9266-4.

Freitas, A.C., Rodrigues, D., Rocha-Santos, T. a P., Gomes, A.M.P. & Duarte, A.C. 2012. Marine biotechnology advances towards applications in new functional foods. *Biotechnology Advances*. 30(6):1506–1515. DOI: 10.1016/j.biotechadv.2012.03.006.

Fry, J.C., Webster, G., Cragg, B.A., Weightman, A.J. & Parkes, R.J. 2006. Analysis of DGGE profiles to explore the relationship between prokaryotic community composition and biogeochemical processes in deep subseafloor sediments from the Peru Margin. *FEMS Microbiology Ecology*. 58(1):86–98. DOI: 10.1111/j.1574-6941.2006.00144.x.

Fry, J.P., Love, D.C., MacDonald, G.K., West, P.C., Engstrom, P.M., Nachman, K.E. & Lawrence, R.S. 2016. Environmental health impacts of feeding crops to farmed fish. *Environment International*. 91:201–214.

Fumanal, M., Zeo, D.E.D., Anguís, V., Fernández-Diaz, C., Alarcón, F.J., Piñera, R., Albaladejo-Riad, N., Esteban, M.A., et al. 2020. Inclusion of dietary *Ulva ohnoi* 5% modulates *Solea senegalensis* immune response during *Photobacterium damselae* subsp. *piscicida* infection. *Fish & Shellfish Immunology*. 100:186–197. DOI: 10.1016/j.fsi.2020.03.007.

Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., et al. 2004. Increased oxidative stress in obesity and its impact on metabolic syndrome. *Journal of Clinical Investigation*. 114(12):1752–1761. DOI: 10.1172/jci200421625.

Gao, G., Clare, A.S., Rose, C. & Caldwell, G.S. 2018. *Ulva rigida* in the future ocean: potential for carbon capture, bioremediation and biomethane production. *GCB Bioenergy*. 10(1):39–51. DOI: 10.1111/gcbb.12465.

Garcia-Esquivel, Z. & Felbeck, H. 2006. Activity of digestive enzymes along the gut of juvenile red abalone, *Haliotis rufescens*, fed natural and balanced diets. *Aquaculture*. DOI: 10.1016/j.aquaculture.2006.08.022.

Gaspard, D., Marin, F., Guichard, N., Morel, S., Alcaraz, G. & Luquet, G. 2007. Shell matrices of Recent rhynchonelliform brachiopods: microstructures and glycosylation studies. *Cambridge Core*. 98(3–4):415–424. DOI: 10.1017/s1755691007078401.

Ge, H., Ni, Q., Chen, Z., Li, J. & Zhao, F. 2019. Effects of short period feeding polysaccharides from marine macroalga, *Ulva prolifera* on growth and resistance of *Litopenaeus vannamei* against *Vibrio parahaemolyticus* infection. *Journal of Applied Phycology*. 31(3):2085–2092. DOI: 10.1007/s10811-018-1663-3.

Geurts, L., Neyrinck, A.M., Delzenne, N.M., Knauf, C. & Cani, P.D. 2014. Gut microbiota controls adipose tissue expansion, gut barrier and glucose metabolism: novel insights into molecular targets and interventions using prebiotics. *Beneficial Microbes*. 5(1):3–17. DOI: 10.3920/bm2012.0065.

Glencross, B.D., Booth, M. & Allan, G.L. 2007. A feed is only as good as its ingredients – a review of ingredient evaluation strategies for aquaculture feeds. *Aquaculture Nutrition*. 13(1):17–34. DOI: 10.1111/j.1365-2095.2007.00450.x.

Gobet, A., Mest, L., Perennou, M., Dittami, S.M., Caralp, C., Coulombet, C., Huchette, S., Roussel, S., et al. 2018. Seasonal and algal diet-driven patterns of the digestive microbiota of the European abalone *Haliotis tuberculata* , a generalist marine herbivore. *Microbiome*. 6(1):60. DOI: 10.1186/s40168-018-0430-7.

Gómez-Zorita, S., González-Arceo, M., Trepiana, J., Eseberri, I., Fernández-Quintela, A., Milton-Laskibar, I., Aguirre, L., González, M., et al. 2020. Anti-obesity effects of macroalgae. *Nutrients*. 12(8):2378. DOI: 10.3390/nu12082378.

Gotteland, M., Riveros, K., Gasaly, N., Carcamo, C., Magne, F., Liabeuf, G., Beattie, A. & Rosenfeld, S. 2020. The pros and cons of using algal polysaccharides as prebiotics. *Frontiers in Nutrition*. 7:163. DOI: 10.3389/fnut.2020.00163.

Green, A.J., Jones, C.L.W. & Britz, P.J. 2011a. The protein and energy requirements of farmed South African abalone *Haliotis midae* L. cultured at optimal and elevated water temperatures. *Aquaculture Research*. 42(11):1653–1663. Available: https://doi.org/10.1111/j.1365-2109.2010.02759.x.

Green, A.J., Jones, C.L.W. & Britz, P.J. 2011b. Effect of dietary lipid level on growth and feed utilization in cultured South African abalone *Haliotis midae* L. fed diets with a constant protein-to-energy ratio. *Aquaculture Research*. 42(10):1501–1508. DOI: 10.1111/j.1365-2109.2010.02742.x.

Gricourt, L., Bonnec, G., Boujard, D., Mathieu, M. & Kellner, K. 2003. Insulin-like system and growth regulation in the Pacific oyster *Crassostrea gigas*: hrIGF-1 effect on protein synthesis of mantle edge cells and expression of an homologous insulin receptor-related receptor. *General and Comparative Endocrinology*. 134(1):44–56. DOI: 10.1016/s0016-6480(03)00217-x.

Guerreiro, I., Oliva-Teles, A. & Enes, P. 2018. Prebiotics as functional ingredients: focus on Mediterranean fish aquaculture. *Reviews in Aquaculture*. 10(4):800–832. DOI: 10.1111/raq.12201.

Guerreiro, I., Magalhães, R., Coutinho, F., Couto, A., Sousa, S., Delerue-Matos, C., Domingues, V.F., Oliva-Teles, A., et al. 2019. Evaluation of the seaweeds *Chondrus crispus* and *Ulva lactuca* as functional ingredients in gilthead seabream (*Sparus*

aurata). *Journal of Applied Phycology*. 31(3):2115–2124. DOI: 10.1007/s10811-018-1708-7.

Guerrero, Salvador & Cremades, Javier. (2012). Integrated Multi-trophic Aquaculture (IMTA): A sustainable, pioneering alternative for marine cultures in Galicia.. Guo, J. 2017. Metabarcoding analyses of gut microbiome compositions in red abalone (*Haliotis Rufescens*, Swainson, 1822) fed different macroalgal diets.

Guo, J., Fu, Y., Wu, Z., Yu, X., Guo, Y., Liu, J., Zhang, W. & Mai, K. 2022. Effects of dietary carbohydrate levels on growth performance, body composition, glucose/lipid metabolism and insulin signaling pathway in abalone *Haliotis discus hannai*. *Aquaculture*. 557:738284. DOI: 10.1016/j.aquaculture.2022.738284.

Gurney, W.S.C. & Nisbet, R.M. 2004. Resource allocation, hyperphagia and compensatory growth. *Bulletin of Mathematical Biology*. 66(6):1731–1753. DOI: 10.1016/j.bulm.2004.03.008.

Hafting, J.T., Critchley, A.T., Cornish, M.L., Hubley, S.A. & Archibald, A.F. 2012. On-land cultivation of functional seaweed products for human usage. *Cell and Tissue Research*. 24:385–392. DOI: 10.1007/s10811-011-9720-1.

Hafting, J.T., Craigie, J.S., Stengel, D.B., Loureiro, R.R., Buschmann, A.H., Yarish, C., Edwards, M.D. & Critchley, A.T. 2015. Prospects and challenges for industrial production of seaweed bioactives. *Journal of Phycology*. 51(5):821–837. DOI: 10.1111/jpy.12326.

Hansson, G.C. 2012. Role of mucus layers in gut infection and inflammation. *Current Opinion in Microbiology*. 15(1):57–62. DOI: 10.1016/j.mib.2011.11.002.

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

Harris, J.O., Bruke, C.M. & Maguire, G.B. 1998. Characterisation of the digestive tract of greenlip abalone, *Haliotis laevigata* Donovan. I. Morphology and Histology. *Journal of Slwllfish Research*. 4:474–98^.

Harris, K., Kassis, A., Major, G. & Chou, C.J. 2012. Is the gut microbiota a new factor contributing to obesity and its metabolic disorders? *Journal of Obesity*. 2012(Figure 1):879151. DOI: 10.1155/2012/879151.

Harvey-Anderson, G. & Woodend, D. 2003. Effect of glycemic carbohydrates on short-term satiety and food intake. *Nutrition Reviews*. 61(5):S17–S26. DOI: 10.1301/nr.2003.may.s17-s26.

Hecht, T. 1994. Behavioural thermoregulation of the abalone, *Haliotis midae*, and the implications for intensive culture. *Aquaculture*. 126(1–2):171–181. DOI: 10.1016/0044-8486(94)90258-5.

Hecht, T. & Britz, P.J. 1992. The current status, future prospects and environmental implications of mariculture in South Africa. *Suid-Afrikaanse Tydskrif vir Wetenskap. 88.*

Hehemann, J.-H., Boraston, A.B. & Czjzek, M. 2014. A sweet new wave: structures and mechanisms of enzymes that digest polysaccharides from marine algae. *Current Opinion in Structural Biology*. 28:77–86. DOI: 10.1016/j.sbi.2014.07.009.

Hindu, S.V., Chandrasekaran, N., Mukherjee, A. & Thomas, J. 2019. A review on the impact of seaweed polysaccharide on the growth of probiotic bacteria and its application in aquaculture. *Aquaculture International*. 27(1):227–238. DOI: 10.1007/s10499-018-0318-3.

Helbert, W. 2017. Marine Polysaccharide Sulfatases. *Frontiers in Marine Science*. 4:6. DOI: 10.3389/fmars.2017.00006.

Hemre, G.-I., Lambertsen, G. & Lie, Ø. 1991. The effect of dietary carbohydrate on the stress response in cod (*Gadus morhua*). *Aquaculture*. 95(3–4):319–328. DOI: 10.1016/0044-8486(91)90097-q.

Heuzé, V., Tran, G., Kaushik, S. 2020. Soybean meal. Feedipedia, a programme by INRAE, CIRAD, AFZ and FAO. *https://www.feedipedia.org/node/674* Last updated on March 4, 2020, 18:25

Hoang, T.H., Qin, J.G., Stone, D.A.J., Harris, J.O., Duong, D.N. & Bansemer, M.S. 2016. Colour changes of greenlip abalone (*Haliotis laevigata* Donovan) fed fresh macroalgae and dried algal supplement. *Aquaculture*. 456:16–23. DOI: 10.1016/j.aquaculture.2016.01.022.

Hoang, T.H., Stone, D.A.J., Duong, D.N., Harris, J.O. & Qin, J.G. 2020. Changes of body colour and tissue pigments in greenlip abalone (*Haliotis laevigata* Donovan) fed macroalgal diets at different temperatures. *Aquaculture Research*. 51(12):5175–5183. DOI: 10.1111/are.14855.

Holman, J.D., Burnett, K.G. & Burnett, L.E. 2004. Effects of hypercapnic hypoxia on the clearance of *Vibrio campbellii* in the Atlantic blue crab, *Callinectes sapidus* Rathbun. *The Biological bulletin*. 206(3):188–196. DOI: 10.2307/1543642.

Hooper, C., Day, R., Slocombe, R., Handlinger, J. & Benkendorff, K. 2007. Stress and immune responses in abalone: limitations in current knowledge and investigative methods based on other models. *Fish & Shellfish Immunology*. 22(4):363–379. DOI: 10.1016/j.fsi.2006.06.009.

Hooper, C., Day, R., Slocombe, R., Benkendorff, K. & Handlinger, J. 2011. Effect of movement stress on immune function in farmed Australian abalone (hybrid *Haliotis laevigata* and *Haliotis rubra*). *Aquaculture*. 315(3–4):348–354. DOI: 10.1016/j.aquaculture.2011.02.012.

Hu, J., Lin, S., Zheng, B. & Cheung, P.C.K. 2017. Short-chain fatty acids in control of energy metabolism. *Critical Reviews in Food Science and Nutrition*. 58(8):00–00. DOI: 10.1080/10408398.2016.1245650.

Ikerd, J.L., Burnett, K.G. & Burnett, L.E. 2015. Effects of salinity on the accumulation of hemocyte aggregates and bacteria in the gills of *Callinectes sapidus*, the Atlantic blue crab, injected with *Vibrio campbellii*. *Comparative Biochemistry and Physiology Part A.* 183:97–106. DOI: 10.1016/j.cbpa.2014.12.030.

Imanikia, S. & Stürzenbaum, S.R. 2013. Invertebrates in obesity research. (Animal Models for the Study of Human Disease). Elsevier. 265–275. DOI: 10.1016/b978-0-12-415894-8.00012-9.

Irungu, F.G., Mutungi, C.M., Faraj, A.K., Affognon, H., Kibet, N., Tanga, C., Ekesi, S., Nakimbugwe, D., et al. 2018. Physico-chemical properties of extruded aquafeed pellets containing black soldier fly (*Hermetia illucens*) larvae and adult cricket (*Acheta domesticus*) meals. *Journal of Insects as Food and Feed*. 4(1):19–30. DOI: 10.3920/jiff2017.0008.

Jagannathan, R., Neves, J.S., Dorcely, B., Chung, S.T., Tamura, K., Rhee, M. & Bergman, M. 2020. The Oral Glucose Tolerance Test: 100 Years Later. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*. 13:3787–3805. DOI: 10.2147/dmso.s246062.

Jenkins, D.J., Wolever, T.M., Taylor, R.H., Barker, H., Fielden, H., Baldwin, J.M., Bowling, A.C., Newman, H.C., et al. 1981. Glycemic index of foods: a physiological basis for carbohydrate exchange. *The American Journal of Clinical Nutrition*. 34(3):362–366.

Johansson, E.V., Nilsson, A.C., Östman, E.M. & Björck, I.M.E. 2013. Effects of indigestible carbohydrates in barley on glucose metabolism, appetite and voluntary food intake over 16 h in healthy adults. *Nutrition Journal*. 12(1):46. DOI: 10.1186/1475-2891-12-46.

Jorgensen, D.D., Ware, S.K. & Redmond, J.R. 1984. Cardiac output and tissue blood flow in the abalone, *Haliotis cracherodii* (Mollusca, Gastropoda). *Journal of Experimental Zoology*. 231:309–324. DOI: 10.1002/jez.1402310303.

Kemp, J.O.G. 2018. Aspects of the nutritional physiology of the perlemoen *Haliotis midae* (L.) and red abalone H. rufescens (Swainson). *Rhodes University*.

Kemp, J.O.G., Britz, P.J. & Agüero, P.H.T. 2015. The effect of macroalgal, formulated and combination diets on growth, survival and feed utilisation in the red abalone *Haliotis rufescens*. *Aquaculture*. 448(C):306–314. DOI: 10.1016/j.aquaculture.2015.06.016.

Kennedy, D.A., Kurath, G., Brito, I.L., Purcell, M.K., Read, A.F., Winton, J.R. & Wargo, A.R. 2016. Potential drivers of virulence evolution in aquaculture. *Evolutionary Applications*. 9(2):344–354. DOI: 10.1111/eva.12342.

Kessler, R.W., Weiss, A., Kuegler, S., Hermes, C. & Wichard, T. 2018. Macroalgal–bacterial interactions: Role of dimethylsulfoniopropionate in microbial gardening by *Ulva* (Chlorophyta). *Molecular Ecology*. 27(8):1808–1819. DOI: 10.1111/mec.14472.

Kidgell, J.T., Magnusson, M., Nys, R. de & Glasson, C.R.K. 2019. Ulvan: A systematic review of extraction, composition and function. *Algal Research*. 39:101422. DOI: 10.1016/j.algal.2019.101422.

Kim, S.S. & Lee, K.J. 2008. Effects of dietary kelp (*Ecklonia cava*) on growth and innate immunity in juvenile olive flounder *Paralichthys olivaceus* (Temminck et Schlegel). *Aquaculture Research*. 39(15):1687–1690. DOI: 10.1111/j.1365-2109.2008.02046.x.

Klinger, D. & Naylor, R. 2012. Searching for solutions in aquaculture: charting a sustainable course. *Annual Review of Environment and Resources*. 37(1):247–276. DOI: 10.1146/annurev-environ-021111-161531.

Knapp, J.L., Hoffman, L.C., Auerswald, L. & Macey, B.M. 2019. Effects of chronic hypercapnia and elevated temperature on the immune response of the spiny lobster, *Jasus lalandii*. *Fish & Shellfish Immunology*. 93:752–762. DOI: 10.1016/j.fsi.2019.05.063.

Knauer, J., Brady, D., Duncan, J.R. & Hecht, T. 1995. Amino acid, fatty acid and mineral element profile of juvenile South African abalone, *Haliotis midae* L. *Aquaculture Research*. 26(4):283–288. DOI: 10.1111/j.1365-2109.1995.tb00913.x.

Knauer, J., Hecht, T. & Duncan, J.R. 1994. Proximate composition of the South African abalone, *Haliotis midae* (Haliotidae: Gastropoda). *Aquaculture Research*. 25(3):351–354. DOI: 10.1111/j.1365-2109.1994.tb00699.x.

Kohn, A.J. 1961. Chemoreception in gastropod molluscs. *American Zoologist*. 1(2):291–308.

Kucharski, L.C.R. & Da Silva, R.S.M. 1991. Effect of diet composition on the carbohydrate and lipid metabolism in an estuarine crab, Chasmagnathus granulata (Dana, 1851). Comparative biochemistry and physiology. Part A, Physiology. 99(1–2):215–218. DOI: 10.1016/0300-9629(91)90261-a.z

Kumar, S., Magnusson, M., Ward, L., Paul, N. & Brown, L. 2015. Seaweed supplements normalise metabolic, cardiovascular and liver responses in high-carbohydrate, high-fat fed rats. *Marine Drugs*. 13(2):788–805. DOI: 10.3390/md13020788.

Kurtz, J. 2002. Phagocytosis by invertebrate hemocytes: Causes of individual variation in *Panorpa vulgaris* scorpionflies. *Microscopy Research and Technique*. 57(6):456–468. DOI: 10.1002/jemt.10099.

Kwasek, K., Thorne-Lyman, A.L. & Phillips, M. 2020. Can human nutrition be improved through better fish feeding practices? a review paper. *Critical Reviews in Food Science and Nutrition*. (January, 25):1–14. DOI: 10.1080/10408398.2019.1708698.

Laas, A. & Vosloo, A. 2010. Exploring basic biochemical constituents in the body tissues of South African abalone *Haliotis midae* reared in shore-based mariculture systems. *African Journal of Marine Science*. 32(1):55–63. DOI: 10.2989/18142321003714302.

Lachnit, T., Thomas, T. & Steinberg, P. 2015. Expanding our understanding of the seaweed holobiont: RNA viruses of the red alga *Delisea pulchra*. *Frontiers in Microbiology*. 6(e368):1489. DOI: 10.3389/fmicb.2015.01489.

Lam, K.-L. & Cheung, P.C.-K. 2019. Carbohydrate-Based Prebiotics in Targeted Modulation of Gut Microbiome. *Journal of Agricultural and Food Chemistry*. 67(45):12335–12340. DOI: 10.1021/acs.jafc.9b04811.

Lange, B., Currie, K.-L., Howarth, G.S., Stone, D.A.J., 2014. Grape seed extract and dried macroalgae, *Ulva lactuca* Linnaeus, improve survival of greenlip abalone, *Haliotis laevigata* Donovan, at high water temperature. *Aquaculture* 433, 348–360. https://doi.org/10.1016/j.aquaculture.2014.06.028

Laramore, S.E., Wills, P.S. & Hanisak, M.D. 2022. Seasonal variation in the nutritional profile of *Ulva lactuca* produced in a land-based IMTA system. *Aquaculture International*. 1–13. DOI: 10.1007/s10499-022-00950-3.

References

Largo, D.B., Diola, A.G. & Marababol, M.S. 2016. Development of an integrated multi-trophic aquaculture (IMTA) system for tropical marine species in southern cebu, Central Philippines. *Aquaculture Reports*. 3:67–76. DOI: 10.1016/j.aqrep.2015.12.006.

Lee, S.M. 2004. Utilization of dietary protein, lipid, and carbohydrate by abalone *Haliotis discus hannai*: a review. *Journal of Shellfish Research*. 23(4):1027–1030.

Legendre, P. & Gallagher, E.D. 2001. Ecologically meaningful transformations for ordination of species data. *Oecologia*. 129(2):271–280. DOI: 10.1007/s004420100716.

Leiro, J.M., Castro, R., Arranz, J.A. & Lamas, J. 2007. Immunomodulating activities of acidic sulphated polysaccharides obtained from the seaweed *Ulva rigida* C.

Lewis, D. & Cerrato, R. 1997. Growth uncoupling and the relationship between shell growth and metabolism in the soft shell clam *Mya arenaria. Marine Ecology Progress Series*. 158:177–189. DOI: 10.3354/meps158177.

Li, Q., Luo, J., Wang, C., Tai, W., Wang, H., Zhang, X., Liu, K., Jia, Y., et al. 2018. Ulvan extracted from green seaweeds as new natural additives in diets for laying hens. *Journal of Applied Phycology*. 30(3):2017–2027. DOI: 10.1007/s10811-017-1365-2.

Li, Y., Qin, J., Cheng, Y., Lv, D., Li, M., Qi, Y., Lan, J., Zhao, Q., et al. 2021. Marine Sulfated Polysaccharides: Preventive and Therapeutic Effects on Metabolic Syndrome: A Review. *Marine Drugs*. 19(11):608. DOI: 10.3390/md19110608.

Lin, S.-M., Shi, C.-M., Mu, M.-M., Chen, Y.-J. & Luo, L. 2018. Effect of high dietary starch levels on growth, hepatic glucose metabolism, oxidative status and immune response of juvenile largemouth bass, *Micropterus salmoides*. *Fish & Shellfish Immunology*. 78:121–126. DOI: 10.1016/j.fsi.2018.04.046.

Liu, S. 2002. Intake of refined carbohydrates and whole grain foods in relation to risk of type 2 diabetes mellitus and coronary heart disease. *Journal of the American College of Nutrition*. 21(4):298–306. DOI: 10.1080/07315724.2002.10719227.

Livingstone, D.R. 1991. Origins and evolution of pathways of anaerobic metabolism in the animal kingdom. *Integrative and Comparative Biology*. 31(3):522–534. DOI: 10.1093/icb/31.3.522.

Loker, E.S. 2010. Gastropod Immunobiology. *Invertebrate Immunity*. 708:17–43. DOI: 10.1007/978-1-4419-8059-5_2.

Lopes-Lima, M., Ribeiro, I., Pinto, R.A. & Machado, J. 2005. Isolation, purification and characterization of glycosaminoglycans in the fluids of the mollusc Anodonta cygnea. *Comparative Biochemistry and Physiology Part A*. 141(3):319–326. DOI: 10.1016/j.cbpb.2005.06.007.

Lopez-Santamarina, A., Miranda, J.M., Mondragon, A. del C., Lamas, A., Cardelle-Cobas, A., Franco, C.M. & Cepeda, A. 2020. Potential use of marine seaweeds as prebiotics: A review. *Molecules*. 25(4):1004. DOI: 10.3390/molecules25041004.

Ludwig, D.S., Aronne, L.J., Astrup, A., de Cabo, R., Cantley, L.C., Friedman, M.I., Heymsfield, S.B., Johnson, J.D., et al. 2021. The carbohydrate-insulin model: a physiological perspective on the obesity pandemic. *The American Journal of Clinical Nutrition*. nqab270-. DOI: 10.1093/ajcn/nqab270.

Macchiavello, J. & Bulboa, C. 2014. Nutrient uptake efficiency of *Gracilaria chilensis* and *Ulva lactuca* in an IMTA system with the red abalone *Haliotis rufescens*. *Latin american journal of aquatic research*. (July, 1). Available: https://www.biodiversitylibrary.org/part/140954.

Macey, B.M. 2005. Probiotic effect of *Vibrio midae* SY9 , *Cryptococcus sp* . SS1 and *Debaryomyces hansenii* AY1 on the growth and disease resistance of farmed *Haliotis midae*.

Macey, B.M. & Coyne, V.E. 2005. Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment. *Aquaculture*. 245(1–4):249–261. DOI: 10.1016/j.aquaculture.2004.11.031.

Macey, B.M., Rathburn, C.K., Thibodeaux, L.K., Burnett, L.E. & Burnett, K.G. 2008. Clearance of *Vibrio campbellii* injected into the hemolymph of *Callinectes sapidus*, the Atlantic blue crab: the effects of prior exposure to bacteria and environmental hypoxia. *Fish & Shellfish Immunology*. 25(6):718–730. DOI: 10.1016/j.fsi.2008.02.009.

Macey, B.M., Achilihu, I.O., Burnett, K.G. & Burnett, L.E. 2008. Effects of hypercapnic hypoxia on inactivation and elimination of *Vibrio campbellii* in the Eastern

oyster, *Crassostrea virginica*. *Applied and Environmental Microbiology*. 74(19):6077–6084. DOI: 10.1128/aem.00317-08.

Majee, S.B., Avlani, D., Ghosh, P. & Biswas, G.R. 2018. Absorption, distribution, metabolism and elimination (ADME) and toxicity profile of marine sulfated polysaccharides used in bionanotechnology. *African Journal of Pharmacy and Pharmacology*. 12(1):1–10. DOI: 10.5897/ajpp2017.4869.

Makhande, E.D. 2008. Growth of the South African Abalone (*Haliotis midae*) on three diets, under commercial conditions. *M.Sc.,* Nelson Mandela Metropolitan University, Nelson Mandela Bay.

Marta, M., Marta, L. & Marco, G. 2017. Macroalgae for Functional feed development: applications in aquaculture, ruminant and swine feed industries. P. Newton, Ed. (Seaweeds Biodiversity, Environmental Chemistry and Ecological Impacts). NOVA science publishers. Available: http://hdl.handle.net/10197/9045.

Martínez-Leal, J., Ponce-García, N. & Escalante-Aburto, A. 2020. Recent evidence of the beneficial effects associated with glucuronic acid contained in kombucha beverages. *Current Nutrition Reports*. 9(3):163–170. DOI: 10.1007/s13668-020-00312-6.

Martin-Gallausiaux, C., Marinelli, L., Blottière, H.M., Larraufie, P. & Lapaque, N. 2020. SCFA: mechanisms and functional importance in the gut. *Cambridge Core*. (January, 1):1–13. DOI: 10.1017/s0029665120006916.

Mayer, J. 1953. Glucostatic mechanism of regulation of food intake. *The New England Journal of Medicine*. 249(1):13–16. DOI: 10.1056/nejm195307022490104.

McCauley, J.I., Winberg, P.C., Meyer, B.J. & Skropeta, D. 2018. Effects of nutrients and processing on the nutritionally important metabolites of *Ulva* sp. (Chlorophyta). *Algal Research*. 35:586–594. DOI: 10.1016/j.algal.2018.09.016.

McKeown, N.M., Meigs, J.B., Liu, S., Saltzman, E., Wilson, P.W.F. & Jacques, P.F. 2004. Carbohydrate nutrition, insulin resistance, and the prevalence of the metabolic syndrome in the Framingham Offspring Cohort. *Diabetes Care*. 27(2):538–546.

McLenon, A.L. & DiTullio, G.R. 2012. Effects of increased temperature on dimethylsulfoniopropionate (DMSP) concentration and methionine synthase activity in *Symbiodinium microadriaticum*. *Biogeochemistry*. 110(1–3):17–29. DOI: 10.1007/s10533-012-9733-0.

Meseguer, R.C.J. 2011. Current knowledge in synbiotic use for fish aquaculture: a review. *Journal of Aquaculture Research & Development*. S1(008):1–7. DOI: 10.4172/2155-9546.s1-008.

Meusel, E., Menanteau-Ledouble, S., Naylor, M., Kaiser, H. & El-Matbouli, M. 2021. Gonad development in farmed male and female South African abalone, *Haliotis midae*, fed artificial and natural diets under a range of husbandry conditions. *Aquaculture International*. 30:1279–1293. DOI: 10.21203/rs.3.rs-735927/v1.

Mohan, K., Ravichandran, S., Muralisankar, T., Uthayakumar, V., Chandirasekar, R., Seedevi, P., Abirami, R.G. & Rajan, D.K. 2018. Application of marine-derived polysaccharides as immunostimulants in aquaculture: A review of current knowledge and further perspectives. *Fish & Shellfish Immunology*. 86:1177–1193. DOI: 10.1016/j.fsi.2018.12.072.

Mojica, K., Elsey, D. & Cooney, M.J. 2007. Quantitative analysis of biofilm EPS uronic acid content. *Journal of Microbiological Methods*. 71(1):61–65. DOI: 10.1016/j.mimet.2007.07.010.

Molden, D., Lautze, J., Shah, T., Bin, D., Giordano, M. & Sanford, L. 2010. Governing to grow enough food without enough water—second best solutions show the way. *International Journal of Water Resources Development*. 26(2):249–263. DOI: 10.1080/07900621003655643.

Mo'o, F.R.C., Wilar, G., Devkota, H.P. & Wathoni, N. 2020. Ulvan, a polysaccharide from macroalga ulva sp.: a review of chemistry, biological activities and potential for food and biomedical applications. *Applied Sciences*. 10(16):5488. DOI: 10.3390/app10165488.

Moon, T.W. 1988. Adaptation, constraint, and the function of the gluconeogenic pathway. *Canadian journal of zoology*. 66:1059–1068.

Moon, T.W. 2001. Glucose intolerance in teleost fish: fact or fiction? *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 129(2–3):243–249.

Mora-Rodríguez, R., Roncero, A.S., Fernández-Elías, V.E., Guadalupe-Grau, A., Ortega, J.F., Dela, F. & Helge, J.W. 2016. Aerobic exercise training increases muscle water content in obese middle-age men. *Medicine and Science in Sport Exersice*. 2:474–480.

Morash, A.J. & Alter, K. 2015. Effects of environmental and farm stress on abalone physiology: perspectives for abalone aquaculture in the face of global climate change. *Reviews in Aquaculture*. 7:1–27. DOI: 10.1111/raq.12097.

Motard-Côté, J. & Kiene, R.P. 2015. Osmoprotective role of dimethylsulfoniopropionate (DMSP) for estuarine bacterioplankton. *Aquatic Microbial Ecology*. 76(2):133–147. DOI: 10.3354/ame01772.

Msuya, F.E. & Neori, A. 2008. Effect of water aeration and nutrient load level on biomass yield, N uptake and protein content of the seaweed *Ulva lactuca* cultured in seawater tanks. *Journal of Applied Phycology*. 20(6):1021–1031. DOI: 10.1007/s10811-007-9300-6.

Mulvaney, W.J. 2016. Effect of various seaweed diets on the growth performance and fatty acid profiles of abalone (Haliotis laevigata x H. rubra) in a trial offshore culture in South-Eastern Australia. University of Wollongong.

Mulvaney, W.J., Winberg, P.C. & Adams, L. 2013. Comparison of macroalgal (*Ulva* and *Grateloupia* spp.) and formulated terrestrial feed on the growth and condition of juvenile abalone. *Journal of Applied Phycology*. 25(3):815–824. DOI: 10.1007/s10811-013-9998-2.

Mulvaney, W.J., Winberg, P.C., Turchini, G.M., Jahangard, S. & Ingram, B. a. 2015. Recovery of omega-3 profiles of cultivated abalone by dietary macroalgae supplementation. *Journal of Applied Phycology*. 27(5):2163–2171. DOI: 10.1007/s10811-015-0530-8.

Naidoo, K., Maneveldt, G., Ruck, K. & Bolton, J.J. 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(3–5):437–443. DOI: 10.1007/s10811-006-9045-7.

Nam, B.-H., Jang, J., Caetano-Anolles, K., Kim, Y.-O., Park, J.Y., Sohn, H., Yoon, S.H., Kim, H., et al. 2018. Microbial community and functions associated with digestion of algal polysaccharides in the visceral tract of *Haliotis discus hannai*: Insights from metagenome and metatranscriptome analysis. *PLOS ONE*. 13(10):e0205594. DOI: 10.1371/journal.pone.0205594.

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., et al. 2009. Feeding aquaculture in an era of finite resources. *Proceedings of the National Academy of Sciences of the United States of America*. 106(36):15103–15110. DOI: 10.1073/pnas.0910597106.

Naylor, R.L., Hardy, R.W., Buschmann, A.H., Bush, S.R., Cao, L., Klinger, D.H., Little, D.C., Lubchenco, J., et al. 2021. A 20-year retrospective review of global aquaculture. *Nature*. 591(7851):551–563. DOI: 10.1038/s41586-021-03308-6.

Nel, A., Pletschke, B., Jones, C., Kemp, J., Robinson, G. & Britz, P. 2017. 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(2):183–192. DOI: 10.2989/1814232x.2017.1338203.

Nelson, M.M., Leighton, D.L., Phleger, C.F. & Nichols, P.D. 2002. Comparison of growth and lipid composition in the green abalone, *Haliotis fulgens*, provided specific macroalgal diets. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 131(4):695–712. DOI: 10.1016/s1096-4959(02)00042-8.

Neto, R.T., Marçal, C., Queirós, A.S., Abreu, H., Silva, A.M.S. & Cardoso, S.M. 2018. Screening of *Ulva rigida*, *Gracilaria* sp., *Fucus vesiculosus* and *Saccharina latissima* as functional ingredients. *International Journal of Molecular Sciences*. 19(10):2987. DOI: 10.3390/ijms19102987.

Neveux, N., Bolton, J.J., Bruhn, A., Roberts, D.A. & Ras, M. 2018. The Bioremediation Potential of Seaweeds: Recycling Nitrogen, Phosphorus, and Other Waste Products. *In Blue Biotechnology*. S.L. Barre & S.S. Bates, Eds. 217–239. DOI: 10.1002/9783527801718.ch7.

Nobre, A.M., Robertson-Andersson, D., Neori, A. & Sankar, K. 2010. Ecological–economic assessment of aquaculture options: comparison between abalone monoculture and integrated multi-trophic aquaculture of abalone and seaweeds. *Aquaculture*. 306(1–4):116–126. DOI: 10.1016/j.aquaculture.2010.06.002.

Ogier, J.C., Lafarge, V., Girard, V., Rault, A., Maladen, V., Gruss, A., Leveau, J.Y. & Delacroix-Buchet, A. 2004. Molecular fingerprinting of dairy microbial ecosystems by use of temporal temperature and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*. 70(9):5628–5643. DOI: 10.1128/aem.70.9.5628-5643.2004.

Øiseth, S.K., Delahunty, C., Cochet, M. & Lundin, L. 2013. Why is Abalone So Chewy? Structural Characterization and Relationship to Textural Attributes. *Journal of Shellfish Research*. 32(1):73–79. DOI: 10.2983/035.032.0113.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., et al. 2020. vegan: community ecology package. *R*. (February, 1). Available: https://CRAN.R-project.org/package=vegan.

Olsen, Y. 2011. Resources for fish feed in future mariculture. *Aquaculture Environment Interactions*. 1(3):187–200. DOI: 10.3354/aei00019.

O'Neill, M., Gaume, B., Denis, F. & Auzoux-Bordenave, S. 2013. Expression of biomineralisation genes in tissues and cultured cells of the abalone *Haliotis tuberculata*. *Cytotechnology*. 65(5):737–747. DOI: 10.1007/s10616-013-9576-0.

O'Omolo, S., Taylor, P., Cook, P.A., Omolo, S.O., Gäde, G., Brown, A.C. & Brown, A.C. 2003. Can the end products of anaerobic metabolism, tauropine and D-lactate, be used as metabolic stress indicators during transport of live South African abalone *Haliotis midae*? *African Journal of Marine Science*. 25(1):301–309. DOI: 10.2989/18142320309504019.

Osimani, A., Milanović, V., Roncolini, A., Riolo, P., Ruschioni, S., Isidoro, N., Loreto, N., Franciosi, E., et al. 2019. *Hermetia illucens* in diets for zebrafish (*Danio rerio*): A study of bacterial diversity by using PCR-DGGE and metagenomic sequencing. *PLOS ONE*. 14(12):e0225956. DOI: 10.1371/journal.pone.0225956.

O'Sullivan, L., Murphy, B., McLoughlin, P., Duggan, P., Lawlor, P.G., Hughes, H. & Gardiner, G.E. 2010. Prebiotics from marine macroalgae for human and animal health applications. *Marine Drugs*. 8(7):2038–2064. DOI: 10.3390/md8072038.

Ouwerkerk, J.P., Vos, W.M. de & Belzer, C. 2013. Glycobiome: Bacteria and mucus at the epithelial interface. *Best Practice & Research Clinical Gastroenterology*. 27(1):25–38. DOI: 10.1016/j.bpg.2013.03.001.

Park, J.-W. & Crowley, D.E. 2010. Nested PCR bias: a case study of *Pseudomonas* spp. in soil microcosms. *Journal of environmental monitoring : JEM*. 12(4):985–988. DOI: 10.1039/b924160d.

Park, H., Lee, K.W. & Shin, D. 2022. Association of Seaweed Consumption with Metabolic Syndrome and Its Components: Findings from the Korean Genome and Epidemiology Study. *Foods*. 11(11):1635. DOI: 10.3390/foods11111635.

Peck, L.S., Peck, L., Culley, M.B. & Helm, M.M. 1987. A laboratory energy budget for the ormer *Haliotis tuberculata* L. *Journal of Experimental Marine Biology and Ecology*. 106(2):103–123. DOI: 10.1016/0022-0981(87)90150-x.

Peckol, P. & Putnam, A.B. 2017. Differential toxic effects of *Ulva lactuca* (Chlorophyta) on the herbivorous gastropods, *Littorina littorea* and *L. obtusata* (Mollusca). *Journal of Phycology*. 53(2):361–367. DOI: 10.1111/jpy.12507.

Peixoto, M.J., Svendsen, J.C., Malte, H., Pereira, L.F., Carvalho, P., Pereira, R., Gonçalves, J.F.M. & Ozório, R.O.A. 2015. Diets supplemented with seaweed affect metabolic rate, innate immune, and antioxidant responses, but not individual growth rate in European seabass (*Dicentrarchus labrax*). *Journal of Applied Phycology*. 28(3):2061–2071. DOI: 10.1007/s10811-015-0736-9.

Pellock, S.J. & Redinbo, M.R. 2017. Glucuronides in the gut: Sugar-driven symbioses between microbe and host. *Journal of Biological Chemistry*. 292(21):8569–8576. DOI: 10.1074/jbc.r116.767434.

Perry, W.B., Lindsay, E., Payne, C.J., Brodie, C. & Kazlauskaite, R. 2020. The role of the gut microbiome in sustainable teleost aquaculture. *Proceedings of the Royal Society B.* 287(1926):20200184. DOI: 10.1098/rspb.2020.0184.

Pimentel, T., Marcelino, J., Ricardo, F., Soares, A.M.V.M. & Calado, R. 2017. Bacterial communities 16S rDNA fingerprinting as a potential tracing tool for cultured seabass *Dicentrarchus labrax*. *Scientific Reports*. 7(1):11862. DOI: 10.1038/s41598-017-11552-y.

Pinheiro, J., Bates, D. & R-core. 2016. nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-127

Plisetskaya, E. & Joosse, J. 1985. Hormonal regulation of carbohydrate metabolism in molluscs. *Current Trends in Comparative Endocrinology*. 2:1077–1079.

Polakof, S., Panserat, S., Soengas, J.L. & Moon, T.W. 2012. Glucose metabolism in fish: a review. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*. 182(8):1015–1045. DOI: 10.1007/s00360-012-0658-7.

Ponce, M., Zuasti, E., Anguís, V. & Fernández-Díaz, C. 2020. Effects of the sulfated polysaccharide ulvan from *Ulva ohnoi* on the modulation of the immune response in Senegalese sole (*Solea senegalensis*). *Fish & Shellfish Immunology*. 100:27–40. DOI: 10.1016/j.fsi.2020.02.054.

Portela, I.B., Martinez-Zorzano, V.S., Molist-Perez, I. & García, P.M. 2012. Ultrastructure and glycoconjugate pattern of the foot epithelium of the abalone *Haliotis tuberculata* (Linnaeus, 1758) (gastropoda, Haliotidae). *The Scientific World Journal*. 2012(9):1–12. DOI: 10.1100/2012/960159.

Powell, J.W.B., Burge, E.J., Browdy, C.L. & Shepard, E.F. 2006. efficiency and sensitivity determination of Shrimple® an immunochromatographic assay for white spot syndrome virus (WSSV) using quantitative real time PCR. *Aquaculture*. (1–4). DOI: 10.1016/j.aquaculture.2006.03.010.

Prabhu, M., Chemodanov, A., Gottlieb, R., Kazir, M., Nahor, O., Gozin, M., Israel, A., Livney, Y.D., et al. 2019. Starch from the sea: The green macroalga *Ulva ohnoi* as a potential source for sustainable starch production in the marine biorefinery. *Algal Research*. 37:215–227. DOI: 10.1016/j.algal.2018.11.007.

Prisco, J.A.D. 2019. *An investigation of some key physico-chemical water quality parameters of an Integrated Multi-Trophic Aquaculture (IMTA) system operating recirculation methodology in the Western Cape of South Africa*. *M.Sc.*, University of Cape Town. Available: https://open.uct.ac.za/handle/11427/32653.

Probyn, T.A., Pretorius, M., Seanego, K. & Bernatzeder, A. 2017. Characterisation of water quality in effluents of land-based abalone farms in the Western Cape, South Africa. *Aquaculture Environment Interactions*. 9:87–102. DOI: 10.3354/aei00217.

Pulkkinen, K., Suomalainen, L.R., Read, A.F., Ebert, D., Rintamaki, P. & Valtonen, E.T. 2010. Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. *Proceedings. Biological sciences*. 277(1681):593–600. DOI: 10.1098/rspb.2009.1659.

Qi, H. & Sheng, J. 2015. The antihyperlipidemic mechanism of high sulfate content ulvan in rats. *Marine Drugs*. 13(6):3407–3421. DOI: 10.3390/md13063407.

Qi, Z., Liu, H., Li, B., Mao, Y., Jiang, Z., Zhang, J. & Fang, J. 2010. Suitability of two seaweeds, *Gracilaria lemaneiformis* and *Sargassum pallidum*, as feed for the abalone *Haliotis discus hannai* Ino. *Aquaculture*. 300(1–4). DOI: 10.1016/j.aquaculture.2010.01.019.

Queirós, A.S., Circuncisão, A.R., Pereira, E., Válega, M., Abreu, M.H., Silva, A.M.S. & Cardoso, S.M. 2021. Valuable Nutrients from *Ulva rigida*: Modulation by Seasonal and Cultivation Factors. *Applied Sciences*. 11(13):6137. DOI: 10.3390/app11136137.

Radford, C.A., Marsden, I.D., Davison, W. & Taylor, H.H. 2005. Haemolymph glucose concentrations of juvenile rock lobsters, *Jasus edwardsii*, feeding on different carbohydrate diets. *Comparative Biochemistry and Physiology Part A*. 140(2):241–249. DOI: 10.1016/j.cbpb.2005.01.002.

Rajauria, G. 2015. Seaweeds: a sustainable feed source for livestock and aquaculture. B.K. Tiwari & D.J. Troy, Eds. San Diego: *Academic Press*. 389–420. DOI: 10.1016/b978-0-12-418697-2.00015-5.

Rajeev, R., Adithya, K.K., Kiran, G.S. & Selvin, J. 2021. Healthy microbiome: a key to successful and sustainable shrimp aquaculture. *Reviews in Aquaculture*. 13(1):238–258. DOI: 10.1111/raq.12471.

Ramirez-Higuera, A., Quevedo-Corona, L., Paniagua-Castro, N., Chamorro-Ceballos, G., Milliar-Garcia, A. & Jaramillo-Flores, M.E. 2014. Antioxidant enzymes gene expression and antihypertensive effects of seaweeds *Ulva linza* and *Lessonia trabeculata* in rats fed a high-fat and high-sucrose diet. *Journal of Applied Phycology*. 26(1):597–605. DOI: 10.1007/s10811-013-0134-0.

Rana, K.J., Siriwardena, S. & Hasan, M.R. 2009. *Impact of rising feed ingredient prices on aquafeeds and aquaculture production*. (541). FAO.

Rao, A., Berg, J.K., Kellermeier, M. & Gebauer, D. 2014. Sweet on biomineralization: effects of carbohydrates on the early stages of calcium carbonate crystallization. *European Journal of Mineralogy*. 26(4):537–552. DOI: 10.1127/0935-1221/2014/0026-2379.

Rasouli, M., Ostovar-Ravari, A. & Shokri-Afra, H. 2014. Characterization and improvement of phenol-sulfuric acid microassay for glucose-based glycogen. *European review for medical and pharmacological sciences*. 18(14):2020–4.

Ratcliff, J.J., Wan, A.H.L., Edwards, M.D., Soler-Vila, A., Johnson, M.P., Abreu, M.H. & Morrison, L. 2016. Metal content of kelp (*Laminaria digitata*) co-cultivated with Atlantic salmon in an Integrated Multi-Trophic Aquaculture system. *Aquaculture*. 450(C):234–243. DOI: 10.1016/j.aquaculture.2015.07.032.

Ray, B. & Lahaye, M. 1995. Cell-wall polysaccharides from the marine green alga *Ulva* "*rigida*" (Ulvales, Chlorophyta). Chemical structure of ulvan. *Carbohydrate Research*. 274:313–318. DOI: 10.1016/0008-6215(95)00059-3.

Rensburg, M.J. van & Coyne, V.E. 2009. The role of electron transport in the defence response of the South African abalone, *Haliotis midae*. *Fish & Shellfish Immunology*. 26(1):171–176. DOI: 10.1016/j.fsi.2008.09.016.

Ringø, E., Zhou, Z., Vecino, J.L.G., Wadsworth, S., Romero, J., Krogdahl, Å., Olsen, R.E., Dimitroglou, A., et al. 2016. Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquaculture Nutrition*. 22(2):219–282. DOI: 10.1111/anu.12346.

Ritchie, H. & Roser, M., 2017. Meat and Dairy Production. Published online at OurWorldInData.org. Retrieved from: 'https://ourworldindata.org/meat-production' [Online Resource]

Roberfroid, M. 2002. Functional food concept and its application to prebiotics. *Digestive and Liver Disease*. 34:S105–S110. DOI: 10.1016/s1590-8658(02)80176-1.

Roberfroid, M., Gibson, G.R., Hoyles, L., McCartney, A.L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., et al. 2010. Prebiotic effects: metabolic and health benefits. *The British journal of nutrition*. 104 Suppl 2(S2):S1-63. DOI: 10.1017/s0007114510003363.

Roberts, S.B. 2000. High–glycemic index foods, hunger, and obesity: is there a connection? *Nutrition Reviews*. 58(6):163–169. DOI: 10.1111/j.1753-4887.2000.tb01855.x.

Roberts, R.D., Kawamura, T. & Nicholson, C.M. 1999. Growth and survival of postlarval abalone (*Haliotis iris*) in relation to development and diatom diet. *Journal of Shellfish Research*. 18(1):243–250.

Robertson-Andersson, D.V., Potgieter, M., Hansen, J., Bolton, J.J., Troell, M., Anderson, R.J., Halling, C. & Probyn, T. 2008. Integrated seaweed cultivation on an abalone farm in South Africa. *Journal of Applied Phycology*. 20(5):579–595. DOI: 10.1007/s10811-007-9239-7.

Robertson-Andersson, D.V., Wilson, D.T., Bolton, J., Anderson, R. & Maneveldt, G. 2009. Rapid assessment of tissue nitrogen in cultivated *Gracilaria gracilis*

(Rhodophyta) and *Ulva lactuca* (Chlorophyta). *African Journal of Aquatic Science*. 32(2):169–172. DOI: 10.2989/ajas.2009.34.2.7.894.

Robertson-Andersson, D.V., Maneveldt, G.W. & Naidoo, K. 2011. Effects of wild and farm-grown macroalgae on the growth of juvenile South African abalone *Haliotis midae* Linnaeus. *African Journal of Aquatic Science*. 36(3):331–337. DOI: 10.2989/16085914.2011.636910.

Rosenzweig, C., Mbow, C., Barioni, L.G., Benton, T.G., Herrero, M., Krishnapillai, M., Liwenga, E.T., Pradhan, P., et al. 2020. Climate change responses benefit from a global food system approach. *Nature Food*. 1(2):94–97. DOI: 10.1038/s43016-020-0031-z.

Rossi, I.C. & Da Silva, R.S.M. 1993. Effects of starvation and a carbohydrate-rich diet on glycogen metabolism in a gastropod mollusc, *Megalobulimus oblongus*. *Comparative Biochemistry and Physiology Part A: Physiology*. 106(4):831–836. DOI: 10.1016/0300-9629(93)90404-r.

Ruiz-Núñez, B., Pruimboom, L., Dijck-Brouwer, D.A.J. & Muskiet, F.A.J. 2013. Lifestyle and nutritional imbalances associated with Western diseases: causes and consequences of chronic systemic low-grade inflammation in an evolutionary context. *The Journal of Nutritional Biochemistry*. 24(7):1183–1201.

Safavi, S.V., Kenari, A.A., Tabarsa, M. & Esmaeili, M. 2019. Effect of sulfated polysaccharides extracted from marine macroalgae (*Ulva intestinalis* and *Gracilariopsis persica*) on growth performance, fatty acid profile, and immune response of rainbow trout (*Oncorhynchus mykiss*). *Journal of Applied Phycology*. 31(6):4021–4035. DOI: 10.1007/s10811-019-01902-w.

Sajilata, M.G., Singhal, R.S. & Kulkarni, P.R. 2006. Resistant starch - A review. *Comprehensive reviews in Food Science and Food Saftey*. 5(1):1–17. DOI: 10.1111/j.1541-4337.2006.tb00076.x.

Sales, J. 2001. Nutrient digestibility in South African abalone (*Haliotis midae* L.). *Rhodes University*.

Sales, J. & Britz, P.J. 2002. Influence of ingredient particle size and inclusion level of pre-gelatinised maize starch on apparent digestibility coefficients of diets in South African abalone (*Haliotis midae* L.). *Aquaculture*. 212(1–4):299–309. DOI: 10.1016/s0044-8486(01)00895-x.

Sales, J. & Britz, P.J. 2003. Apparent and true availability of amino acids from common feed ingredients for South African abalone (*Haliotis midae* L.). *Aquaculture Nutrition*. 9(1):55–64. DOI: 10.1046/j.1365-2095.2003.02371.x.

Sales, J. & Janssens, G.P.J. 2004. Use of feed ingredients in artificial diets for abalone: a brief update. *Nutrition Abstracts and Reviews*. 7(74):13N-21N.

Sales, J., Truter, P.J. & Britz, P.J. 2003. Optimum dietary crude protein level for growth in South African abalone (*Haliotis midae* L.). *Aquaculture Nutrition*. 9(2):85–89. DOI: 10.1046/j.1365-2095.2003.00229.x.

Sandström, V., Chrysafi, A., Lamminen, M., Troell, M., Jalava, M., Piipponen, J., Siebert, S., Hal, O. van, Virkki, V., & Kummu, M. 2022. Food system by-products upcycled in livestock and aquaculture feeds can increase global food supply. *Nature Food*, 3(9), 729–740. https://doi.org/10.1038/s43016-022-00589-6

Santizo-Taan, R., Bautista-Teruel, M. & Maquirang, J.R.H. 2020. Enriched *Ulva pertusa* as partial replacement of the combined fish and soybean meals in juvenile abalone *Haliotis asinina* (Linnaeus) diet. *Journal of Applied Phycology*. 32(1):741–749. DOI: 10.1007/s10811-019-01977-5.

Sathivel, A., Raghavendran, H.R.B., Srinivasan, P. & Devaki, T. 2008. Anti-peroxidative and anti-hyperlipidemic nature of *Ulva lactuca* crude polysaccharide on D-galactosamine induced hepatitis in rats. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*. 46(10):3262–3267. DOI: 10.1016/j.fct.2008.07.016.

Sawabe, T. 2006. The mutual partnership between *Vibrio halioticoli* and abalones. *The Biology of Vibrios*. 219–230. Available: http://hdl.handle.net/2115/48579.

Sawabe, T., Setoguchi, N., Inoue, S., Tanaka, R., Ootsubo, M., Yoshimizu, M. & Ezura, Y. 2003. Acetic acid production of *Vibrio halioticoli* from alginate: a possible role for

establishment of abalone–*V. halioticoli* association. *Aquaculture*. 219(1–4):671–679. DOI: 10.1016/s0044-8486(02)00618-x.

Schiraldi, Chiara & La Gatta, Annalisa & Rosa, Mario. (2010). Biotechnological Production and Application of Hyaluronan. 10.5772/10271.

Schlegel, A. & Stainier, D.Y.R. 2007. Lessons from "lower" organisms: what worms, flies, and zebrafish can teach us about human energy metabolism. *PLOS Genetics*. 3(11):e199. DOI: 10.1371/journal.pgen.0030199.

Schock, T.B., Stancyk, D.A., Thibodeaux, L., Burnett, K.G., Burnett, L.E., Boroujerdi, A.F.B. & Bearden, D.W. 2010. Metabolomic analysis of Atlantic blue crab, *Callinectes sapidus*, hemolymph following oxidative stress. *Metabolomics*. 6(2):250–262. DOI: 10.1007/s11306-009-0194-y.

Scholz, U., Diaz, G.G., Ricque, D., Cruz-Suárez, L.E., Vargas-Albores, F. & Latchford, J. 1999. Enhancement of vibriosis resistance in juvenile *Penaeus vannamei* by supplementation of diets with different yeast products. *Aquaculture*. 176(3–4):271–283. DOI: 10.1016/s0044-8486(99)00030-7.

Schroeder, G., Bates, S.S. & Barre, S.L. 2018. bioactive marine molecules and derivatives with biopharmaceutical potential. *Blue Biotechnology* 96:611–641. DOI: 10.1002/9783527801718.ch19.

Seong, H., Bae, J.-H., Seo, J.S., Kim, S.-A., Kim, T.-J. & Han, N.S. 2019. Comparative analysis of prebiotic effects of seaweed polysaccharides laminaran, porphyran, and ulvan using in vitro human fecal fermentation. *Journal of Functional Foods*. 57:408–416. DOI: 10.1016/j.jff.2019.04.014.

Shahar, B., Shpigel, M., Barkan, R., Masasa, M., Neori, A., Chernov, H., Salomon, E., Kiflawi, M., et al. 2020. Changes in metabolism, growth and nutrient uptake of *Ulva fasciata* (Chlorophyta) in response to nitrogen source. *Algal Research*. 46:101781. DOI: 10.1016/j.algal.2019.101781.

Sharifuddin, Y., Chin, Y.-X., Lim, P.-E. & Phang, S.M. 2015. Potential bioactive compounds from seaweed for diabetes management. *Marine Drugs*. 13(8):5447–5491. DOI: 10.3390/md13085447.

Sheedy, J.R., Lachambre, S., Gardner, D.K. & Day, R.W. 2015. 1H-NMR metabolite profiling of abalone digestive gland in response to short-term starvation. *Aquaculture International*. (24):503–521. DOI: 10.1007/s10499-015-9941-4.

Shimano, S., Sambe, M. & Kasahara, Y. 2012. Application of nested PCR-DGGE (denaturing gradient gel electrophoresis) for the analysis of ciliate communities in soils. *Microbes and Environments*. 27(2):136–141.

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

Shpigel, M., Guttman, L., Ben-Ezra, D., Yu, J. & Chen, S. 2019. Is *Ulva* sp. able to be an efficient biofilter for mariculture effluents? Journal of Applied Phycology. 31(4):2449–2459. DOI: 10.1007/s10811-019-1748-7.

Shuuluka, D., Bolton, J.J. & Anderson, R.J. 2013. Protein content, amino acid composition and nitrogen-to-protein conversion factors of *Ulva rigida* and *Ulva capensis* from natural populations and *Ulva lactuca* from an aquaculture system, in South Africa. *Journal of Applied Phycology*. 25(December):677–685. DOI: 10.1007/s10811-012-9902-5.

Silva, A.F.R., Abreu, H., Silva, A.M.S. & Cardoso, S.M. 2019. Effect of Oven-Drying on the recovery of valuable compounds from *Ulva rigida*, *Gracilaria* sp. and *Fucus vesiculosus*. *Marine Drugs*. 17(2):90. DOI: 10.3390/md17020090.

Silva, D.M., Valente, L.M.P., Sousa-Pinto, I., Pereira, R., Pires, M.A., Seixas, F. & Rema, P. 2014. Evaluation of IMTA-produced seaweeds (*Gracilaria*, *Porphyra*, and *Ulva*) as dietary ingredients in Nile tilapia, *Oreochromis niloticus* L., juveniles. Effects on growth performance and gut histology. *Journal of Applied Phycology*. 27(4):1671–1680. DOI: 10.1007/s10811-014-0453-9.

Singh, R.P. & Reddy, C.R.K. 2016. Unraveling the functions of the macroalgal microbiome. *Frontiers in Microbiology*. 6. DOI: 10.3389/fmicb.2015.01488.

Singh, J., Dartois, A. & Kaur, L. 2010. Starch digestibility in food matrix: a review. *Trends in Food Science & Technology*. 21(4):168–180. DOI: 10.1016/j.tifs.2009.12.001.

Smit, A., Robertson-Andersson, D.V., Peall, S. & Bolton, J.J. 2007. Dimethylsulfoniopropionate (DMSP) accumulation in abalone *Haliotis midae* (Mollusca: Prosobranchia) after consumption of various diets, and consequences for aquaculture. *Aquaculture*. 269:377–389. DOI: 10.1016/j.aquaculture.2007.03.034.

Smit, A., Robertson-Andersson, D.V. & Bolton, J.J. 2010. The effect of macroalgal and compound feeds on the sensory quality of cultivated South African abalone, *Haliotis midae* Linnaeus (Mollusca, Gastropoda). *Aquaculture Nutrition*. 16(6):590–603. DOI: 10.1111/j.1365-2095.2009.00696.x.

Smith, M.J. 2007. Seasonal variation in nutritional content of the kelp *Ecklonia maxima* on the west and south west coasts of South Africa, with reference to its use as abalone feed. *University of Cape Town*.

Sokolova, I.M. 2009. Apoptosis in molluscan immune defence. *Invertebrate Survival Journal*. 6:49–58. Available: http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.518.1647&rep=rep1&type=pdf.

Souza, D. da S., Tahan, S., Weber, T.K., Araujo-Filho, H.B. de & Morais, M.B. de. 2018. Randomized, double-blind, placebo-controlled parallel clinical trial assessing the effect of fructooligosaccharides in infants with constipation. *Nutrients*. 10(11):1602. DOI: 10.3390/nu10111602.

Stentiford, G.D., Sritunyalucksana, K., Flegel, T.W., Williams, B.A.P., Withyachumnarnkul, B., Itsathitphaisarn, O. & Bass, D. 2017. New paradigms to help solve the global aquaculture disease crisis. *PLoS Pathogens*. 13(2):e1006160. DOI: 10.1371/journal.ppat.1006160.

Styskal, J., Remmen, H.V., Richardson, A. & Salmon, A.B. 2012. Oxidative stress and diabetes: What can we learn about insulin resistance from antioxidant mutant mouse models? *Free Radical Biology and Medicine*. 52(1):46–58. DOI: 10.1016/j.freeradbiomed.2011.10.441.

Tacon, A.G.J., Tacon, a, Metian, M. & Metian, M. 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture*. 285(1–4):146–158. DOI: 10.1016/j.aquaculture.2008.08.015.

Tarnecki, A.M., Burgos, F.A., Ray, C.L. & Arias, C.R. 2017. Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. *Journal of Applied Microbiology*. (February, 1). DOI: 10.1111/jam.13415.

Tarr, R. 1995. Growth and movement of the South African abalone *Haliotis midae*: A reassessment. *Marine and Freshwater Research*. 46(3):583. DOI: 10.1071/mf9950583.

Van Der Merwe, M., Franchini, P. & Roodt-Wilding, R. 2011. Differential growth-related gene expression in abalone (*Haliotis midae*). *Marine biotechnology*. 13(6):1125–1139. DOI: 10.1007/s10126-011-9376-5.

Tas, S., Celikler, S., Ziyanok-Ayvalik, S., Sarandol, E. & Dirican, M. 2011. *Ulva rigida* improves carbohydrate metabolism, hyperlipidemia and oxidative stress in streptozotocin-induced diabetic rats. *Cell biochemistry and function*. 29(2):108–113. DOI: 10.1002/cbf.1729.

Taylor, M.H. & Tsvetnenko, E. 2004. A growth assessment of juvenile abalone *Haliotis laevigata* fed enriched macroalgae *Ulva rigida*. *Aquaculture International*. 12(4/5):467–480. DOI: 10.1023/b:aqui.0000042138.14961.c2.

Team, R.C. 2016. *R: A Language and Environment for Statistical Computing*.

Tenore, K.R. 1976. Food chain dynamics of abalone in a polyculture system. *Aquaculture*. 8(1):23–27. DOI: 10.1016/0044-8486(76)90016-8.

Termizy, H.M. & Mafauzy, M. 2009. Metabolic syndrome and its characteristics among obese patients attending an obesity clinic. *Singapore medical journal*. 50(4):390–394.

Thilsted, S.H., Thorne-Lyman, A., Webb, P., Bogard, J.R., Subasinghe, R., Phillips, M.J. & Allison, E.H. 2016. Sustaining healthy diets: The role of capture fisheries and aquaculture for improving nutrition in the post-2015 era. *Food Policy*. 61:126–131. DOI: 10.1016/j.foodpol.2016.02.005.

Trabal, N., Mazón-Suástegui, J.M., Vázquez-Juárez, R., Asencio-Valle, F., Morales-Bojórquez, E. & Romero, J. 2012. Molecular analysis of bacterial microbiota associated with oysters (*Crassostrea gigas* and *Crassostrea corteziensis*) in different growth phases at two cultivation sites. *Microbial Ecology*. 64(2):555–569. DOI: 10.1007/s00248-012-0039-5.

Trentin, R., Custódio, L., Rodrigues, M.J., Moschin, E., Sciuto, K., Silva, J.P. da & Moro, I. 2020. Exploring *Ulva australis* Areschoug for possible biotechnological applications: *In vitro* antioxidant and enzymatic inhibitory properties, and fatty acids contents. *Algal Research*. 50:101980. DOI: 10.1016/j.algal.2020.101980.

Troell, M., Bolton, J.J., Robertson-Andersson, D., Halling, C., Anderson, R.J., Probyn, T. & Maneveldt, G. 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(1–4):266–281. Available: http://www.sciencedirect.com/science/article/B6T4D-4JKRH4M-1/2/6accbd43e552a3786e17fc7324a74843.

Tujula, N.A, Crocetti, G.R., Burke, C., Thomas, T., Holmstrom, C. & Kjelleberg, S. 2010. Variability and abundance of the epiphytic bacterial community associated with a green marine *Ulvacean* alga. The ISME Journal. 4(2):301–311. DOI: 10.1038/ismej.2009.107.

Valente, L.M.P., Araújo, M., Batista, S., Peixoto, M.J., Sousa-Pinto, I., Brotas, V., Cunha, L.M. & Rema, P. 2016. Carotenoid deposition, flesh quality and immunological response of Nile tilapia fed increasing levels of IMTA-cultivated *Ulva* spp. *Journal of Applied Phycology*. 28(1):691–701. DOI: 10.1007/s10811-015-0590-9.

Velasco-Muñoz, J.F., Mendoza, J.M.F., Aznar-Sánchez, J.A. & Gallego-Schmid, A. 2021. Circular economy implementation in the agricultural sector: Definition, strategies and indicators. *Resources, Conservation and Recycling*. 170:105618. DOI: 10.1016/j.resconrec.2021.105618.

Veldhuijzen, J.P. 1974. Glucose-Tolerance in the Pond Snail *Lymnaea Stagnalis* as Affected By Temperature and Starvation. *Netherlands Journal of Zoology*. 25(2):206–218. DOI: 10.1163/002829675x00227.

Venegas, D.P., Fuente, M.K.D. la, Landskron, G., González, M.J., Quera, R., Dijkstra, G., Harmsen, H.J.M., Faber, K.N., et al. 2019. short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Frontiers in Immunology*. 10:277. DOI: 10.3389/fimmu.2019.00277.

Venter, L., Loots, D.T., Vosloo, A., Rensburg, P.J. van & Lindeque, J.Z. 2016. Abalone growth and associated aspects: now from a metabolic perspective. *Reviews in Aquaculture*. 0:1–23. DOI: 10.1111/raq.12181.

Venter, L., Vosloo, A., Loots, D.T., Mienie, L.J., Rensburg, P.J.J. van & Lindeque, J.Z. 2018. Characterising the metabolic differences related to growth variation in farmed *Haliotis midae*. *Aquaculture*. 493:144–152. DOI: 10.1016/j.aquaculture.2018.04.052.

Viana, M.T., D'Abramo, L.R. & Gonzalez, M.A. 2007. Energy and nutrient utilization of juvenile green abalone (*Haliotis fulgens*) during starvation. *Aquaculture*. 264(1–4):323–329. DOI: 10.1016/j.aquaculture.2007.01.004.

Vosloo, A. & Vosloo, D. 2006. Routes of water loss in South African abalone (Haliotis midae) during aerial exposure. *Aquaculture*. 261(2):670–677. DOI: 10.1016/j.aquaculture.2006.06.015.

Wang, L., Wang, X., Wu, H. & Liu, R. 2014. Overview on Biological Activities and Molecular Characteristics of Sulfated Polysaccharides from Marine Green Algae in Recent Years. *Marine Drugs*. 12(9):4984–5020. DOI: 10.3390/md12094984.

Wang, L.C., Di, L.Q., Li, J.S., Hu, L.H., Cheng, J.M. & Wu, H. 2017. Elaboration in type, primary structure, and bioactivity of polysaccharides derived from molluscs. *Critical Reviews in Food Science and Nutrition*. 17:1–24. DOI: 10.1080/10408398.2017.1392289.

Wang, W., Mai, K., Zhang, W., Xu, W., Ai, Q., Yao, C. & Li, H. 2009. Effects of dietary carbohydrates sources on lipids compositions in abalone, *Haliotis discus hannai* Ino. *Journal of Ocean University of China (Oceanic and Coastal Sea Research*. 8(3):254–258. DOI: 10.1007/s11802-009-0254-y.

Wang, X., Wang, X., Jiang, H., Cai, C., Li, G., Hao, J. & Yu, G. 2018. Marine polysaccharides attenuate metabolic syndrome by fermentation products and altering

gut microbiota: An overview. *Carbohydrate Polymers*. 195:601–612. DOI: 10.1016/j.carbpol.2018.05.003.

Wang, X., Tang, B., Luo, X., Ke, C., Huang, M., You, W. & Wang, Y. 2020. Effects of temperature, diet and genotype-induced variations on the gut microbiota of abalone. *Aquaculture*. 524:735269. DOI: 10.1016/j.aquaculture.2020.735269.

Wang, Y., Pyecroft, S. & Barton, M. 2020. Seasonal microbial profiling of left kidney and stomach of farmed adult greenlip abalone (*Haliotis laevigata*). *Aquaculture Research*. DOI: 10.1111/are.15060.

Watanabe, H., Yamanaka, H. & Yamakawa, H. 1992. Seasonal variations of extractive components in the muscle of disk abalone. *Nippon Suisan Gakkaishi*. 58(5):921–925. DOI: 10.2331/suisan.58.921.

Wells, M.L., Potin, P., Craigie, J.S., Raven, J.A., Merchant, S.S., Helliwell, K.E., Smith, A.G., Camire, M.E., et al. 2016. Algae as nutritional and functional food sources: revisiting our understanding. *Journal of Applied Phycology*. 29(2):949–982. DOI: 10.1007/s10811-016-0974-5.

Wells, R.M.G., McShane, P.E., Ling, N., Wong, R.J., Lee, T.O.C. & Baldwin, J. 1998. Effect of wave action on muscle composition, metabolites and growth indices in the New Zealand abalone, Paua (*Haliotis iris*), with implications for harvesting and aquaculture. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 119(1):129–136. DOI: 10.1016/s0305-0491(97)00295-2.

Wijesekara, I., Pangestuti, R. & Kim, S.-K. 2011. Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. *Carbohydrate Polymers*. 84(1):14–21. DOI: 10.1016/j.carbpol.2010.10.062.

Williams, H.R., Macey, B.M., Burnett, L.E. & Burnett, K.G. 2009. Differential localization and bacteriostasis of *Vibrio campbellii* among tissues of the Eastern oyster, *Crassostrea virginica*. *Developmental & Comparative Immunology*. 33(4):592–600. DOI: 10.1016/j.dci.2008.10.008.

Winberg, P.C., Ghosh, D. & Tapsell, L. 2009. *Seaweed Culture in Integrated Multi-Trophic Aquaculture-Nutritional Benefits and Systems for Australia*. Rural Industries Research and Development Corporation (09/005), Australian Government

Wu, Y., Kaiser, H. & Jones, C.L.W. 2019. A first study on the effect of dietary soya levels and crystalline isoflavones on growth, gonad development and gonad histology of farmed abalone, *Haliotis midae*. *Aquaculture International*. 27(1):167–193. DOI: 10.1007/s10499-018-0315-6.

Xu, X., Xu, P., Ma, C., Tang, J. & Zhang, X. 2013. Gut microbiota, host health, and polysaccharides. *Biotechnology Advances*. 31(2):318–337. DOI: 10.1016/j.biotechadv.2012.12.009.

Yang, J., Wang, H.-P., Zhou, L. & Xu, C.-F. 2012. Effect of dietary fiber on constipation: A meta-analysis. *World Journal of Gastroenterology*. 18(48):7378–7383. DOI: 10.3748/wjg.v18.i48.7378.

Yasuda, E., Tateno, H., Hirabayashi, J., Hirabarashi, J., Iino, T. & Sako, T. 2011. Lectin microarray reveals binding profiles of *Lactobacillus casei* strains in a comprehensive analysis of bacterial cell wall polysaccharides †. *Applied and Environmental Microbiology*. 77(13):4539–4546. DOI: 10.1128/aem.00240-11.

Yavari, N., Mazaheri-Assadi, M., Mazhari, Z.H., Moghadam, M.B. & Larijani, K. 2018. Glucuronic acid rich kombucha-fermented pomegranate juice. *Journal of Food Research*. 7(1):61–69. DOI: 10.5539/jfr.v7n1p61.

Yu, W., Gong, S., Lu, Y., Shen, Y., Liu, J., Huang, Z., Luo, X., You, W., et al. 2022. Genome sequence-based genome-wide association study of feed efficiency in Pacific abalone. *Aquaculture*. 561:738630. DOI: 10.1016/j.aquaculture.2022.738630.

Yu, W., Lu, Y., Shen, Y., Liu, J., Gong, S., Yu, F., Huang, Z., Zou, W., Zhou, M., Luo, X., You, W., Ke, C. 2022. Exploring the Intestinal Microbiota and Metabolome Profiles Associated With Feed Efficiency in Pacific Abalone (*Haliotis discus hannai*). *Frontiers in Microbiology*. 13:852460. DOI: 10.3389/fmicb.2022.852460.

Yu, L.Q.J., Mu, Y., Zhao, Z., Lam, V.W.Y. & Sumaila, U.R. 2017. Economic challenges to the generalization of integrated multi-trophic aquaculture: An empirical

comparative study on kelp monoculture and kelp-mollusc polyculture in Weihai, China. *Aquaculture*. 471:130–139. DOI: 10.1016/j.aquaculture.2017.01.015.

Yuan, D., Li, C., You, L., Dong, H. & Fu, X. 2020. Changes of digestive and fermentation properties of *Sargassum pallidum* polysaccharide after ultrasonic degradation and its impacts on gut microbiota. *International Journal of Biological Macromolecules*. 164:1443–1450. DOI: 10.1016/j.ijbiomac.2020.07.198.

Zarei, I., Koistinen, V.M., Kokla, M., Klåvus, A., Babu, A.F., Lehtonen, M., Auriola, S. & Hanhineva, K. 2022. Tissue-wide metabolomics reveals wide impact of gut microbiota on mice metabolite composition. *Scientific Reports*. 12(1):15018. DOI: 10.1038/s41598-022-19327-w.

Zhang, Y., Hedo, R., Rivera, A., Rull, R., Richardson, S. & Tu, X.M. 2019. Post hoc power analysis: is it an informative and meaningful analysis? General Psychiatry. 32(4):e100069. DOI: 10.1136/gpsych-2019-100069.

Zhang, L.-L., Zhou, Q.-C. & Cheng, Y.-Q. 2009. Effect of dietary carbohydrate level on growth performance of juvenile spotted Babylon (*Babylonia areolata* Link 1807). *Aquaculture*. 295:238–242. DOI: 10.1016/j.aquaculture.2009.06.045.

Zhao, J., Wu, X., Nie, C., Wu, T., Dai, W., Liu, H. & Yang, R. 2012. Analysis of unculturable bacterial communities in tea orchard soils based on nested PCR-DGGE. *World Journal of Microbiology and Biotechnology*. 28(5):1967–1979. DOI: 10.1007/s11274-011-0999-5.

Zheng, L.-X., Chen, X.-Q. & Cheong, K.-L. 2020. Current trends in marine algae polysaccharides: The digestive tract, microbial catabolism, and prebiotic potential. *International Journal of Biological Macromolecules*. 151:344–354. DOI: 10.1016/j.ijbiomac.2020.02.168.

Supplementary information

S1 - Phenol-sulphuric method for the determination of glucose

Methods adopted from with modifications (Bennett et al., 2007; Rasouli, Ostovar-Ravari & Shokri-Afra, 2014)

Tissue processing and preparation

Preparation for processing

- Label 12 5 mL washed Eppendorf tubes (12 at a time if processing in duplicate
- Pipet 2 mL of 10 % ice cold PERC acid into each Eppendorf
- Collect samples stored at -80C

Processing the tissue

1. Zero the electronic scale (0.000g) with a labelled 5 mL Eppendorf that has 2 mL of ice cold PERC
2. Carefully cut off tissue (180 > mg <250) and add it to the Eppendorf
3. Record the *actual* weight of the tissue
4. "Mince" the tissue with sharp nose dissecting scissors for 10-15sec so that there are no large 'chunks' as these will get stuck in the homogenizer
5. Process all samples
6. Homogenize at highest RPM for 15 - 30 sec
7. Leave the samples on ice for 1h

an hour later...

8. Vortex each sample for 15 sec
9. Centrifuge at 280 ×G for 17 min
10. This is done on the large bench top centrifuge, briefly;
    a. fit the 15 mL inserts
    b. used old 15 mL centrifuge tubes with no lid as an insert
    c. place the 5 mL tubes within these
11. Carefully pipet 500 uL of supernatant into new 1.5 mL micro centrifuge tubes
12. This should be done in at least duplicate

13. Precipitate the glycogen using 96 % ethanol
14. Add 600 µL of EtOH to each sample and vortex for 15 sec

Leave on ice for 1h

15. Centrifuge at 1700 ×G for 10 min
16. Remove the supernatant by carefully inverting the 1.5 mL micro centrifuge tubes
17. Once all the supernatant has been discarded, use a 200 µL pipet to carefully remove any excess
18. Re dissolve the pellet in 1 mL distilled water (sample)
19. Vortex until dissolved
20. Incubate at 50 ºC for 5min and then vortex for 15 sec

Dilute the samples 1:20 before continuing the assays protocol

- 50 µL of sample
- 950 µL of diH_2O

Assay determination of glycogen in a 96-well plate

1. Add 10 µL of 1:20 working stock into 96-well microplate in triplicate
2. Add 30 µL of diH_2O to each sample
3. Add 40 µL of 6.5 % phenol
    a. mix via pipetting n=3
4. Rapidly add 200 µL of H2SO4 to the surface of each sample
    b. mix via pipetting n=3

Creation of a Glucose Standard Curve

Use premade frozen stocks (2 mL aliquots) of 2 mg/mL glucose. The sensitivity of this assay means that the sample glucose must fall between 0 - 20 µg

For the standard curve it is important to keep the absorbance value between 0.1 and 0.9 to ensure accuracy. The tissue samples have been diluted by a factor of 20 to ensure the reading fall within the above-mentioned range.

Ensure the curve and the sample tissue range is a similar profile and calculate the unknown samples form the standard caurve for glucose.

Mixing the curve

1) 0 ug glucose
    a) Just add 40 µL distilled water
2) 1 ug glucose
    a) add 0.5 µl stock solution and make up to 40 µL by adding distilled water
3) 2 ug glucose
    a) add 1 µl stock solution and make up to 40 µL by adding distilled water
4) 3 ug glucose
    a) add 1.5 µl stock solution and make up to 40 µL by adding distilled water
5) 4 ug glucose
    a) add 2 µl stock solution and make up to 40 µL by adding distilled water
6) 5 ug glucose
    a) add 2.5 µl stock solution and make up to 40 µL by adding distilled water

S2 - Extraction of the water-soluble polysaccharide ulvan

Things to consider:

- Approximately 10% yield of ulvan from dry Ulva
- Ulva has 85 – 90 % moisture
- At least 2-3 volumes of ethanol are required for precipitation

Extraction protocol to be followed for the crude extraction of ulvan from dried ulva

1) Dry Ulva at 45 ºC for 24h or until constant weight is reached
2) Measure out a desired amount of *Ulva* for ethanolic extraction
   a) Place a stirring bar in the beaker and then put it on the magnetic stirrer
   b) Place dry flaked Ulva into a beaker on the magnetic stirrer
   c) Pour in 96 % ethanol until flakes are all covered. Approximately 10 g *Ulva* to 100 mL ethanol but can be reduced if this fraction is not required at full potency.
   d) Switch on the heating element and try get it to 35 – 40 ºC
   e) Leave to extract for 12 – 24h
3) Remove all *Ulva* from ethanolic extract using a sieve and briefly rinse with fresh diH2O
4) Place rinsed *Ulva* into an autoclavable beaker and add 1:5 – 1:10 diH_2O (10 g *Ulva*, 50 – 100mL diH_2O)
5) Autoclave the *Ulva* water mix for 15 min and let it cool

Figure 2-a Dried Ulva (50 g) was ground to pieces of approximately 1 cm. The course powder was then added to 450 mL diH2O in a 5000 mL glass beaker which was then place in the autoclave at 120 ºC for 15min.

6) It is recommended that this should be concentrated down to decrease the amount of ethanol required but for small amounts it is not necessary.
7) Centrifuge the mixture at 5000 g for 5 min to get rid of any particulate

Figure 2-b The suspension was filtered through a strainer (0.25 mm) and the remaining liquid was centrifuged at 5000 RCF for 5 minutes.

8) To precipitate (ppt) the ulvan, add a pinch of salt and 3 volumes of 70 – 96 % ethanol which will induce a cloudy ppt to start forming.

Figure 2-c The pellets were discarded and 2.5 volumes (1125 mL) of 70 % ethanol were added which precipitated out the Ulvan. After 1h the doe-like precipitate was removed and washed with 96.9 % ethanol before being centrifuged for five minutes at 5000 rpm.

9) Harvesting the ppt can be different depending on scale of extraction. For small volumes it can be collected using sieve and 'washed' with 96 % ethanl in 50 mL centrifuge tubes to be spun down.
   a) Discard the supernatant and place the ppt into 1.5 mL – 5 mL centrifuge tubes depending on amount and chop up using a pair of dissecting scissors in 96 % ethanol as a second wash to further decrease the water content
10) The ppt must then be dried completely using the evaporative centrifuge at 60 ºC until a soft light beautiful white product is achieved.

Figure 2-d From the initial 50g, 4g of ulvan was successfully extracted giving a yield of 8 %

a) To re-dissolve this in diH_2O it may be necessary to autoclave.
b) To purify further it can be extracted again using ethanol precipitation after being reconstituted in diH_2O

S3 - R-code for data handling, plotting and analysis

This thesis has been written in Microsoft Word 2016 (Mac) while all the analysis and visualizations have been done in R. RStudio has been a critical component of this Thesis and is the core component of moving toward reproducible research. Unfortunately, the lack of a formalized workflow with dedicated channels for collaborative input leaves the reproducibility of this system in an unreproducible state. At the onset, a solid workflow for data capture, analysis, processing and collaboration should be outlined that will significantly improve the overall process of compiling a thesis.

An attempt has been made to extract the working chunks of code that form part of this Thesis. The data required for the work in this thesis have been compiled into a repository with data objects saved with .Rdata and .rds extensions that can be shared to recreate the analysis and visualization on request. The code is organized into chapters that correspond to the three experimental chapters within this thesis. To include them as txt would create an unnecessarily long document.

The code is organized in the following format and can be found in my public repository (brand_2021) on GitHub:

- chpt2.Rmd
- chpt3.Rmd
- chpt4.Rmd
- setup.Rmd
- /data
- PhD_supplementary_information-r-code.Rproj

The code chunks in the .Rmd files call for the relevant data from the /data folder for the analysis and visualizations. In order to reproduce these analysis it will be necessary to open the "PHD_supplementary_information-r-code.Rproj" in RStudio, identify the code chucks required and run the necessary lines of code. This is my attempt to provide a foundation for recreating the results of my analysis and adding to the building of a more streamlined mechanism of reproducibility.