Assessment and Mitigation of Biosecurity Risks Associated with Macroalgae Inclusion in Farmed Abalone Diets in South Africa

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By

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ABSTRACT

The provision of biosecure diets for use in intensive aquaculture conditions requires attention, to reduce the risk of introducing potential pathogens to the farmed stock. Such introductions could lead to infections and disease outbreaks. Despite the benefits associated with macroalgae inclusion in abalone diets, several microbial hazard cases have been reported in the animal feed and human food industries. This has necessitated the application of biosecurity measures on fresh macroalgae to reduce or eliminate potential hazards and the risks of pathogen transfer to abalone stock when used as a feed or feed supplement. The present thesis assessed the efficacy of different processing treatments, including heat, ultra-violet irradiation (UVC), different pH levels, salinity concentrations, and povo-iodine on the inactivation of potential macroalgae-transmitted abalone pathogens. The effect of the processed macroalgae on the growth, health and gut microbial composition of abalone were also explored and compared with abalone fed non-processed diets.

The efficacy of the different processing treatments was initially assessed on pure cultures of the test pathogens, which included a bacterium (*Vibrio anguillarum*), an oomycete (*Halioticida noduliformans*) and the bacteriophage lambda. Data from the initial assessments were then used to select the most optimal treatments for further assessment on the test pathogens inoculated in a macroalgae matrix, to simulate a more natural scenario. The viability of the three test pathogens following exposure to different processing treatments was assessed using culture methods. The results indicated that a combination of three treatments; disinfection using povo-iodine solution (5000 mg/L) for 20 min, oven drying 40 ℃ for 8.0 h and UVC treatment for 10 min rendered all the test pathogens non-culturable.

Growth trials were conducted to assess the effect of the biosecure macroalgae *Ecklonia maxima*, *Ulva lacinulata* and *Gracilaria gracilis* on the growth performance of the abalone *Haliotis midae*. The macroalgae were subjected to the combination of three biosecurity processing treatments and experimental diets were then formulated to incorporate the macroalgae that had been subjected to the biosecurity processing treatments as well as macroalgae that were not. Growth parameters of the abalone after a 150-day feeding trial were compared between the biosecure and non-bioseeure macroalgae dietary treatments. Overall, the lowest growth was observed in the abalone fed with the control diet (Abfeed^{TM} S34®) compared to all the macroalgae diets. However, no significant differences in abalone weight and shell length were recorded between the dietary treatments after the 150-day growth trial with an overall mean final weight (± standard error) of 56.55 ± 0.78 g and a mean final length of 66.26 ± 0.344 mm (RM-ANOVA: $F_{(18,63)} = 0.706$; $p = 0.792$; : $F_{(18,63)} = 0.941$; $p = 0.535$ respectively). Similarly, the biosecurity process method (biosecure vs. non-biosecure) did not have an impact on abalone weights and shell length ($p > 0.05$). Moreover, specific growth rate, length gain and condition factor of abalone did not differ between the biosecure and non-biosecure dietary treatments with overall means (±standard error) of 0.27 ± 0.01 % bw/d, 1.79 ± 0.07 mm/month and 1.13 ± 0.01, respectively ($p > 0.05$).

Dietary macroalgae are known to contribute to the gut microflora of abalone. To determine if the biosecure process influenced this community complex, a next generation sequencing (NGS) approach was used to identify and compare the bacterial communities in abalone that were fed diets containing macroalgae that had been subjected to biosecurity treatment and those that were not. The NGS approach was also used to determine the gut microbiome profile of the abalone fed with a formulated diet supplemented with fresh *U. lacinulata* and *G. gracilis* to assess the potential modulatory effect seaweeds and their associated microbiota may have on the gut microbiome of *H. midae.* The bacterial alpha diversity did not differ significantly across all the diets at family, genus and species levels ($p > 0.05$). No significant differences in the

microbiome composition were detected indicating little or no dissimilarities of the bacterial communities between the diets for all the biosecure and non-biosecure macroalgae diets. A similar core microbiome was also observed in the digestive tracts of abalone fed with the biosecure and non-biosecure diets. It was concluded that the biosecure process did not influence the natural microbiota of abalone that were fed dietary ingredients that were subjected to the process.

The findings of this research have contributed to understanding the production of biosecure macroalgae formulated diets without compromising their benefits to the growth and health of farmed abalone. The combined treatment used in this study can be applied in the macroalgae and the feed industry to produce biosecure feeds.

DECLARATION

I declare that this is my original work and that has not been submitted to any University or institution for the award of a degree or any academic work. Any information incorporated here that is published or written by others has been acknowledged and references provided.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Problem identification

Feeds contaminated with pathogenic disease agents either at the time of harvest, processing, storage or transport become a risk pathway for the introduction of disease agents to cultured animals and systems (Tacon, 2017). Moreover, with the increase in international trade of feed ingredients, the biosecurity risk of disease introduction, especially of foreign origin, is anticipated to increase (AFIA, 2015). In aquaculture, tracing contamination to its ultimate source is regarded difficult since various factors influence the stable microbial community in culture water. This has contributed to aquafeeds given less consideration as carriers of pathogenic agents despite them representing a significant source of fish diseases in aquaculture production (Diyie *et al.* 2024). Although only a few cases that have linking both wet and dry animal feeds as the carrier of pathogens (Hasson *et al.* 2006; Maciorowski *et al*. 2007; Diyie *et al.* 2024), the prevention of entry and transmission of diseases is the goal for any biosecurity program (FAO, 2010).

Biosecurity is a collective term that refers to a set of measures aimed at preventing the introduction, spread and transmission of pathogenic agents, infectious diseases or infestations between people, animals and plants. This term is evolving and cuts across many disciplines hence its definition often varies among countries and sectors. In aquaculture, biosecurity is defined as practices put in place to minimize the risk of introduction and spread of infectious diseases within a facility or escape of the pathogenic agents to other sites and other susceptible species (Yanong and Erlacher-Reid, 2012).

One of the significant challenges that the aquaculture industry has encountered because of its expansion is an increase of disease outbreaks (Naylor *et al*. 2021). These have caused huge economic losses and to some extent have affected the sustainability of the industry (Tang and Bondad-Reantaso, 2019).

The aspect of biosecurity in aquaculture came to light in 1997 after its first introduction and discussion during a special session in the World Aquaculture Society annual conference (Pruder, 2004). This was necessitated by the global acute disease outbreaks in the shrimp industry, which resulted in massive mortalities and consequent income losses amounting to billions of dollars (Lightner, 2004). However, at that time biosecurity was not new to other industries, such as the poultry industry in the agriculture animal production systems. The poultry industry had fully developed and well-established biosecurity procedures in place and served as a point of reference for aquaculture. The shrimp farming industry was the first aquaculture industry to adopt biosecurity protocols and strategies from poultry since the two industries were perceived to have many similarities (Pruder, 2004). Biosecurity measures in the shrimp industry focused mainly on excluding pathogens and eliminating pathogens when present (Lee, 2002). This was achieved by ensuring the use of specific pathogen-free (SPF) certified stocks, quarantine, water treatment and disinfection of potential vectors, such as tools, footwear, and clothing using chlorine, ozone and iodine (Horowitz and Horowitz, 2003). To date, these measures and many others have also been adopted in other sectors in the aquaculture industry, including finfish and mollusk farming (Yoshimizu, 2003; Gavin *et al.* 2009).

There is published evidence of the benefits of macroalgae inclusion in abalone diets (Naidoo *et al.* 2006; O'Mahoney *et al.* 2014; Bansemer *et al.* 2016; Nel *et al.* 2017; Bullon *et al.* 2022). However, the biosecurity implications of this practice, to the abalone stock, are not fully understood. With the abalone being farmed in intensive culture conditions, health related risks

are inevitable. Fresh macroalgae are deemed to be a potential vector for parasites, pests, diseases and associated organisms and therefore presents a risk to cultured abalone stock (Bautista-Teruel *et al.* 2011; Dang *et al.* 2011). In addition, feeding abalone macroalgae grown in abalone effluent water, as in the case of farms utilizing recirculating system, or system where abalone farm effluent is treated using algae before being returned to the ocean, also poses a risk of reinfection to the stock. Standardized processing methods in the food/feed industries, vital to quality and safety of products, are still lacking in the macroalgae industry (Swinscoe *et al.* 2020). Furthermore, the effects of biosecurity processing treatments on the macroalgae as feed ingredient and on the growth performance and on the gut microbiome of abalone fed with the processed macroalgae diet remain unknown.

1.2 Literature review

1.2.1 Abalone aquaculture in South Africa

Abalone are marine gastropods belonging to the family Haliotidae of the phylum Mollusca and are found distributed along rocky shores and reefs of coastal temperate and tropical waters (Degnan *et al.* 2006). The exploitation of abalone by the coastal communities' dates to prehistoric days, but it's only after the early 1970s when they were exploited on a large-scale for commercial basis. Since then, there has been an increased demand for this resource, which has resulted in a decline of wild stocks, increased poaching and consequently a rapid growth of abalone farming to meet the market demands (Gordon and Cook, 2013). The global production of farmed abalone was recorded at approximately 180 000 mt in 2018 (Hernández-Casas *et al.* 2023).

South Africa is globally ranked as the third largest exporter of farmed abalone, with products exported predominantly to Asian markets and is the largest producer of cultured abalone outside Asia (FAO 2004, Robertson-Andersson *et al.* 2008). The fast expansion of the South African abalone industry is attributed to rapid decline of the wild abalone fishery due to poaching and high market prices. Other factors such as availability of cheap labour, coupled with the favourable coastal water quality and good infrastructure have also contributed to the rise of this industry (Troell *et al.* 2006). Six haliotid species occur in South African waters (*Haliotis midae L.*, *Haliotis parvum L.*, *Haliotis spadicea* (Donovan), *Haliotis queketti* (Smith), *Haliotis speciosa* (Reeve), *Haliotis pustulata* (Reeve)). Of all the six species, only *H. midae* (Linnaeus, 1758) locally known as 'perlemoen' is intensively cultured (Sales, 2001). The rest of the species are considered small and non-commercial species (Barkai and Griffiths, 1986). *Haliotis midae* grows at temperatures ranging between 9-24 °C with an optimum range of 12 °C-20 °C (Britz *et al*. 1997). It is a highly valued product in the world with its prime market being the South-East Asian countries (Sales, 2001).

1.2.2 Disease challenges facing the global abalone industry

The complex interplay between an animal's health, the environment, and the presence of a pathogen influences the progression of diseases. Factors such as overstocking, poor nutrition, and poor water quality are all associated with causing stress in cultured animals which in turn reduces their general health, compromises immune functions and increases their susceptibility to infection (Cheng *et al.* 2004). Pathogens including parasites, bacteria, viruses and fungi and associated diseases present serious biosecurity hazards in the aquaculture industry and are often a limiting factor to production. These pathogens can invade or be transmitted through multiple pathways, such as water, feeds, contaminated equipment, predators, newly introduced species, humans and waste among others.

Numerous disease outbreaks have been experienced globally for both cultured and wild abalone species owing to the intensification of the abalone aquaculture industry (Friedman *et al.* 2000; McGladdery, 2011). There are 29 aquatic animal diseases listed by the Office of International des Épizooties (OIE – World Organisation for Animal Health) as being of economic importance and nine of these are mollusk diseases (OIE, 2019). Since numerous microbial pathogens are natural occupants of the aquatic environment, infectious illnesses are one of the key obstacles to the effective growth and extension of the aquaculture sector (Subasinghe, 2005).

Viruses can significantly reduce stock and revenue in both wild and culture systems because they are easily transmissible and highly infectious (Renault and Novoa, 2004). The Abalone Herpesvirus (AbHV**)** from the family Malacoherpesviridae has been associated with acute disease mortalities in the abalone species *Haliotis rubra, H. diversicolor* (subspecies *aquatilis* and *supertexta*), *H. laevegata* and its hybrids. Infected species were characterized by an acute mortality process and necrotizing ganglioneuritis, which resulted in the assignment of the name Abalone Viral Ganglioneuritis (AVG). The virus was first reported in 2003 in Taiwan infecting the species *H. diversicolor supertexta* in both wild and farmed abalone (Chang *et al.* 2005). Similar cases were reported on several farms in Victoria, Australia in 2005 infecting *H. rubra* (blacklip abalone), *H. laevigata* (greenlip abalone) and their hybrids and killed 90% of the stock (Hooper *et al*. 2007; Lafferty *et al.* 2015). In Southern China, a devastating disease loss that wiped out the entire farmed abalone stock of the species *H. diversicolor supertext* occurred in the 1990s (Wu and Zang, 2016). Abalone Viral Ganglioneuritis is believed to have been the etiological agent responsible for the disease, since high amounts of the viral DNA particles were detected in the samples obtained from the diseased *H. diversicolor supertexta*, which had been collected during the outbreak (Bai *et al*. 2019). Infected abalone exhibited typical clinical

signs of AVG, such as protruding mouthparts, excessive production of mucus, reduced pedal adhesion to substrates and irregular peripheral concave elevation of the foot. A study designed to assess the stability of the virus in seawater indicated that the virus was infectious when animals were held in seawater for one day at 4 °C and 15 °C. The virus also was able to retain partial infectivity after five days of being held at 4 °C (Corbeil *et al.* 2012). This indicated that direct contact between healthy and infected abalone is not the only way the virus can be transmitted but can also be transmitted through water that has previously been inhabited by diseased abalone. Abalone Viral Ganglioneuritis is now an international and national trade barrier for the movement of live abalone. Inactivation of the virus has been demonstrated to be achieved after exposure to temperatures of 100 °C after 30 min or its equivalent (OIE, 2018).

Another viral disease of abalone caused by infectious amyotrophia (virus-like particles) has been reported in Japan infecting the species *H. discus hannai*. The virus is associated with a severe condition that causes muscular atrophy in the mantle and foot, impairing feeding and the adherence of the foot to substrates and eventually, mortality of the animals (Nakatsugawa *et al.* 1999). So far, no viral diseases have been reported in South Africa. This could probably be a case of resistance to the viruses to which they have exposed. Other possible reasons for this could be that either the environmental conditions may be unfavourable for disease development or due to the abalone not being exposed to pathogenic doses of the viruses (Corbeil *et al.* 2020). In addition the availability or absence of reports on viral diseases in South Africa is dependent on whether anyone is looking for these or other viruses and the obligation to report or document these findings.

Many aquatic bacteria ubiquitous in the environment but can become pathogenic when conditions favour their growth and the host is compromised and susceptible. Conditions such as deteriorated water quality, poor handling, high stocking density and inadequate nutrition among other stress factors that lead to compromise of the animal's immunity (Sobhana, 2009). Numerous groups of bacteria including *Vibrio spp., Francisella halioticida, Klebsiella oxytoca, Candidatus Xenohaliotis californiensis, Shewanella colwelliana, Clostridium lituseberense,* Flavobacterium-like bacteria, long Flexibacter/Cytophaga-like rod bacteria and *Pasteurella sp.* have been associated with abalone infections (Bower, 1987). Vibriosis is one of the major disease threats in marine fish and shellfish aquaculture worldwide and is caused by bacteria within the genus *Vibrio* (Nicolas *et al.* 2002). *Vibrios* are gram-negative rod-shaped bacteria that are ubiquitous in marine environments and estuarine ecosystems (Austin *et al.* 2007). Pathogenic species of *Vibrio,* such as *Vibrio vulnificus*, *Vibrio alginolyticus, Vibrio harveyi*, *Vibrio anguillarum* and *Vibrio parahaemolyticus,* have been isolated from diseased abalones in Japan, Taiwan and South Africa with signs of abscessing or ulceration in their mantle, white spots on the foot and general withering (Liu *et al.* 2000, Cai *et al.* 2007). Bacterial infection due to *V. harveyi,* which is now regarded as synonymous with *V. carchariae,* has been reported as the most problematic *Vibrio* species for many years in Tasmania and South Australia (Handlinger *et al.* 2006). The bacterium has also been reported to cause mortalities of abalone in Japan (Nishimori *et al.* 1998) and France (Nicolas *et al.* 2002). Although *Vibrios* are usually susceptible to a broad range of antimicrobials of veterinary and human significance, *V. vulnificus* and *V. parahaemolyticus* have been reported to show multiple antibiotic resistance due to misuse of antibiotics to control infections in aquaculture production. Also, both environmental and clinical isolates showed similar antibiotic resistance profiles. High temperature treatments of > 55 °C for 10 min have been reported to effectively inactivate or kill *V. parahaemolyticus* in oysters (Andrews *et al.* 2000).

Apart from vibriosis, another bacterial disease that has significantly affected the abalone industry is withering syndrome. This is a fatal disease affecting both wild and cultured abalone. The etiological agent for this disease is the bacterium *Candidatus Xenohaliotis californiensis* from the family *Rickettsiaceae* (Friedman *et al.* 2000). The bacterium is a gram-negative obligate intracellular organism, but studies have shown that it can survive outside the host and even be transmitted through water (Balseiro *et al.* 2006). The disease affects all sizes of abalone and a wide range of abalone species, including black abalone (*Haliotis cracherodii*), white abalone (*H. sorenseni*), red abalone (*Haliotis rufescens*), pink abalone (*Haliotis corrugata*), green (*H. fulgens*) and the small abalone (*H, diversicolor supertexta*) (Wetchateng, 2008). To date, this disease has not been recorded in the South African abalone *H. midae*. Withering syndrome was first reported in the 1990s and caused massive declines in populations of the black abalone *H. cracherodii* (Leach 1814) and *H. rufescens* in California, USA (Friedman *et al.* 1997, Moore *et al.* 2000). High water temperatures have been found to be a prerequisite for the development of clinical signs of this disease in infected individuals (Mouton, 2010). Infected abalone are characterized by the weakening of foot muscle, lethargy, retraction of the visceral tissues and reduced feeding (Day and Prince, 2007).

With regards to parasitic diseases of abalone, Perkinsus, caused by *Perkinsus olseni,* has had a devastating impact on abalone stocks, which led it to be listed by the WOAH as a reportable disease of international concern. The disease has mainly been reported from Australia and affects the species *H. rubra*, *H. laevigata* and *H. cylobates* (Bower *et al.* 1994). Another parasitic disease of abalone is abalone kidney coccidian disease, which has been reported in California, USA. The parasite responsible for this disease, *Labyrinthuloides haliotidi,* targets the kidney and the infected kidney becomes extremely hypertrophied. *Labyrinthuloides haliotidi* has been reported to infect juvenile *H. kamtschatkana* and *H. rufescens* in a Canadian mariculture facility. It is characterized by causing the destruction of head and foot tissues and

has been reported to cause up to 100 % mortality in juvenile *H. kamtschatkana* (Bower, 1987; Mouton, 2010). In South Africa, the sabellid worm *Terebrasabella heterouncinata* has been one of the main parasite problems in abalone culture (Ruck and Cook 1998). This parasite was also reported to cause numerous fatalities of the red abalone *H. rufescens* in California and is said to have accidentally been introduced from South Africa (Finley *et al.* 2000; Dlaza, 2006).

Pathogens causing fungal diseases in abalone include members of genera *Haliphthoros*, *Halioticida* and *Atkinsiella* (Hatai, 2012). In Japan, *Haliphthorus milfordensis* was reported in abalone *H. sieboldii* temporarily held in an aquarium (Hatai, 1982). The diseased abalone was characterised by flat or tubercle-like swelling formed on mantle, epipode and dorsal surface on foot. Another fungal infection with the causative agent identified as Atkinsiella awabi was later reported in the same abalone species *H. sieboldii*. Affected abalone showed external signs of infection of tubercle-like swelling on the mantle and melanized lesions on the peduncle (Kitancharoen *et al.* 1994).

In another case, mortalities were recorded in three species of abalone; *H. midae, H. sieboldii and H. rufescens.* Four strains belonging to Peronosporomycetes (formerly Oomycetes) were isolated from white nodules found on the mantle of diseased abalone. Although the four isolates formed fragments similar to those of genus *Haliphthoros* in artificial seawater, the protoplasm constriction was weaker, and fragments were longer, with smaller spaces between them. The causative agent was named as *Halioticida noduliformans* (Muraosa *et al.* 2009). In 2006, *H. midae* exhibiting typical clinical signs of tubercle mycosis were discovered in a South African abalone culture facilities the causative agent was identified as the *as H. noduliformans* (Macey *et al.* 2011). The fact that relatively few abalone diseases have been reported in South Africa does not mean that they are lacking. Handlinger *et al.* (2006) attributed the few recognized abalone diseases worldwide to the lack of examination (absence of proof, rather

than proof of absence) and that more diseases were likely to emerge with the rapid development of abalone aquaculture.

1.2.3 Biosecurity measures for pathogens control in abalone aquaculture

The control of diseases in aquatic environments is a complicated process due to the intimate relationship that exists between pathogens, the environment and their host (Olafsen, 2001). Good husbandry practices coupled with high levels of biosecurity and the development of specific biosecurity plans are recommended as effective ways to mitigate and reduce outbreaks of disease (Bower, 1987).

Over the years, broad-spectrum antimicrobials have been used extensively as a means of disease control on many aquaculture facilities worldwide and remain the method of choice for many farmers (Macey and Coyne, 2005). Few studies have attempted to describe the nature of the target pathogen and their susceptibility to different control measures (Table 1.1). This is crucial when deciding on what control method or agent should be applied.

Table 1.1. Susceptibility of microorganisms to chemical disinfectants and ultraviolet radiation.

Susceptibility to chemical disinfectants and UV radiation	**Microorganism**
Highly susceptible	Mycoplasmas
Susceptible	Gram-positive bacteria
	Enveloped viruses
	Gram-negative bacteria
	Fungal spores
Resistant	Non-enveloped viruses
	Mycobacteria

Highly resistant	Bacterial endospores
	Protozoal oocysts
	Protozoal-like spores
Extremely resistant	Prions

Source: Adapted from Quinn and Markey (2001)

For example, Abalone Viral Ganglioneuritis belongs to a group of viruses that have a lipid envelope and are of intermediate to large size. The lipid envelope of this type of virus makes it susceptible to a wide range of compounds, including soaps, detergents, and disinfectants (Gavin *et al*. 2009). Iodine-based disinfectants and chlorine-based disinfectants could effectively inactivate the virus based on their efficacy against other aquatic disease agents (Ellard, 2006).

In the control of parasites, it was observed that exposing infected abalone to temperatures near but below the upper thermal limits of the parasite, killed all life stages of sabellids. This was attributed to the fact that the life cycle and generation time of the sabellids are highly temperature dependent (Finley *et al.* 2000). As much as this treatment was effective, its disadvantage is that it is harmful to the host abalone. Culver and Kuris (1997) suggested the isolation of diseased stocks to limit the transmission of shell infection as a solution to this issue which is also the most often used strategy to stop the spread of these polychaetes.

In the case of the withering syndrome, oxytetracycline (OTC) has been suggested as an effective therapeutant for infected abalone (Friedman *et al.* 2000). An oral and injection administration of this chemical to withering syndrome infected abalone was found to increase abalone survival rates. However, the drawback of this treatment is that the residues of OTC were found to persist in the gut of treated abalone and could even affect the consumers of abalone (Friedman *et al.* 2003). For example, studies have reported that the residues of

antibiotics consumed could cause toxicity, allergic reactions, resistance development, mutagenic, anaphylactic shocks and digestive disorders (Mund *et al.* 2016; Jammoul and El Dara, 2019).

On the other hand, eradication of vibriosis causing bacteria in already infected abalone is seen to be almost impossible due to their ubiquitous nature. With all these cases it is therefore important to ensure optimal health of the animals by reducing physical and chemical stresses hence improving their ability to defend themselves against opportunistic infections (Elston and Lockwood, 1983).

As much as chemicals have vastly been used in aquaculture for the control of diseases, some chemical disinfectants have proved to be ineffective against certain organisms, particularly at high pH or against spore-forming microbes. In addition, the use of certain chemicals results in the production of harmful by-products, which not only affect the aquatic organism and water quality but may also be detrimental to humans (Karaca and Velioglu, 2007). For example, chlorine can react to form trihalomethanes, which pose a risk in both human diet and the environment. On the other hand, the indiscriminate use of antibiotics has been shown to lead to the development and spread of antibiotic-resistant bacteria, which may pose a risk to human health (Akinbowale *et al.* 2006; Alcaide *et al.* 2005). Chemotherapy has also been demonstrated to disturb the homeostasis of gut physiology and cause fish to be vulnerable and sensitive to infection (Kim and Austin, 2008).

Ozone has been used as a sanitizer and disinfectant for a wide range of products and its use has been suggested as a potential alternative to chemotherapeutic compounds in aquaculture; to achieve sustainable, economic and safer production (Hamad and Sana'a, 2018). Wang *et al.* (2004) further recommended the use of ozone as a viable substitute for food sterilization, due to its efficiency in killing microorganisms. A study by Hamad and Sana'a (2018) indicated that

ozone was an effective antifungal for controlling Saprolegnia infections in Common carp, *Cyprinus carpio*. In another study, ozone treatment showed a positive effect against viral pathologies of the pancreas in Atlantic salmon as well as in crustaceans (Chang *et al.* 1998). According to Dixon *et al.* (1991), treatment with broad-spectrum antibiotics and exposure to ozonated water were effective against bacterial infections by *Clostridium lituseberense* or *V. alginolyticus.*

1.2.4 Macroalgae as an abalone diet

Diet is another crucial aspect in the culture of abalone because it influences the health and growth of cultured animals. Just like all intensive animal production industries, aquaculture is heavily reliant on feed inputs to sustain its production (Glencross *et al.* 2019). Diet represents the major operational cost for most commercial farms with protein being the most expensive dietary component (Lovell, 2002; Güroy *et al.* 2007). Abalone are opportunistic herbivores, feeding predominantly on kelp in the wild, but also accept a wide range of diets in culture conditions (Troell *et al.* 2006). The wild harvested kelp (*Ecklonia maxima*) has been the primary diet for the farmed abalone in South Africa has recently been reported to reach a plateau of between 4000 and 5000 t fresh per annum (Rothman *et al.* 2020). However, with the growth of intensive abalone farms, there has been an increase in competition for these concession areas and a consequent reduction in allocations to abalone farmers (Troell *et al*. 2006). This coupled with rising demands due to increased production has led to the exploitation of alternative feeds, hence the use of formulated diets (Hahn, 1989; Sales and Britz, 2003). The use of formulated diets has also been critical to the development of an economically viable industry. Moreover, formulated diets have been reported to produce comparatively better growth than macroalgae (Troell *et al.* 2006).

Commercial formulated diets contain a variety of ingredients such as fish meal, cereal grains, oilseeds among others which are considered palatable, digestible and nutritionally balanced (Stone *et al*. 2013). Despite this, these ingredients are associated with potential ecological, economical and nutritional problems. Traditionally, the aquaculture sector heavily relied on the use of wild caught fishery products, like fishmeal and fish oil, in feeds for the cultured species (Naylor *et al.* 2009). These have been used as the main protein source in fish and shellfish diets as they are a rich source of essential amino acids, essential fatty acids, energy and minerals (Hertrampf and Piedad-Pascual, 2000). However, the increasing costs of these proteins for use in the aquaculture industry, together with the limited supply of fishmeal, questions the sustainability of this industry. For future sustainable development of the aquaculture industry, the reduction of fishmeal inclusion levels or its replacement with cost-effective, widely available and sustainable feedstuffs is crucial (Güroy *et al*. 2013). Conversely, plant protein sources such as soya bean meal are cost effective and have widely been used in the abalone diet. Replacement of fish meal with 50 % soybean resulted in significantly higher growth rate of abalone *H. discus hannai* but higher replacement level (≥75%) resulted to decreased anti-oxidative capacity of the abalone (Yu *et al.* 2022). Previous studies have also reported that soyabean meal contain some anti-nutritional factors that are detrimental to fish health and may also impair abalone health (Stone *et al.* 2013). Consequently, macroalgae and macroalgae meals have become interesting candidates for low inclusion levels in aquafeeds (Güroy *et al.*2013).

Numerous studies have shown that inclusion of macroalgae in fish and abalone diets results to improved health and growth (Naidoo *et al.* 2006; Viera *et al.* 2011; Bansermer *et al.* 2016; Nel *et al.* 2017). Macroalgae contain several biologically active compounds, including polysaccharides, proteins, polyunsaturated fatty acids, pigments, polyphenols and minerals. These have been documented to possess strong prebiotics, antimicrobial, antiviral, anti-

infection and antioxidant activities which are crucial for mitigating against biotic and abiotic stress as well as promoting growth (Chojnacka *et al.* 2012). This makes it a potential substitute for fish meal and can be used in the culture of many aquatic organisms such as fish, shrimp, and abalone (Naidoo *et al.* 2006; Ergün *et al.* 2009; Henry, 2012; Cyrus *et al.* 2015; Vatsos and Rebours, 2015).

1.2.5 Application of biosecurity measures to food and feed items

Several ways to manage risks associated with feeds have been recommended including sourcing feeds from disease free areas and by testing to ensure disease freedom (Ernst *et al*. 2016). However, this is extremely hard to do and can only be done for things that are known and where suitable diagnostics tests with proven sensitivity and specificity are available. Therefore, the only practical and feasible way to curb these risks would be by treatment to inactivate any potential pathogens that could be present in these feeds. Furthermore, the processing techniques applied to terrestrial plant crops, fresh fruits, vegetables and other fresh products to achieve microbiological safety could also be used on macroalgae.

The use of heat to kill pathogens has been used extensively since ancient times, especially in the food industry to destroy spoilage bacteria and to make food safe to eat. The key factors to consider when a thermal process is used is the determination of the most resistant microorganism with health concerns and understanding its levels of inactivation followed by an assessment of the thermal impact of the treatment on the quality attributes of the product. Heat destroys microorganisms by altering the physical and chemical characteristics of their proteins. Thermal processes are classified based on the heat intensity used and exposure time. Silva and Gibbs (2010) defined pasteurization as heat treatment usually below 100 °C to destroy microorganisms of public health significance. This process does not kill all organisms, like in sterilization, but only targets specific pathogens and lowers their levels to acceptable

standards. Sterilization involves heat temperatures above 110°C and destroys any form of life in products (Amir *et al.* 2013).

However, heat treatment has been reported to have detrimental effects on the quality of certain products. Turkmen *et al*. (2005), Rungapamestry *et al.* (2007), Cox *et al.* (2012) and Susanto et *al.* (2017) reported that heat treatment significantly influenced the concentrations of phytochemicals, antioxidants and the antimicrobial contents of macroalgae and vegetables. In addition, thermal processing methods have been shown to expose the phytochemicals such as phenols, flavonoids, antioxidants and vitamins to detrimental factors that may lead to alterations in concentrations and diminish their health-related qualities/affects. The loss of phytochemicals that are associated with health benefits is likely to be a consequence of drying and cooking characteristics including time, temperature and degree of wounding stress to the plant during these processes (Cox *et al.* 2012).

Nevertheless, inclusion of dried algae meal into formulated diets as opposed to feeding fresh macroalgae has been suggested as a solution to the biosecurity risks associated with macroalgae and also a practical option to gain the benefits of feeding macroalgae to abalone as long as the nutrient composition and quality is not compromised (O'Mahoney *et al.* 2014, Bansemer *et al.* 2016). However, other authors have reported inferior growth of abalone fed with dried macroalgae as compared to fresh macroalgae (Naidoo *et. al* 2006). It is therefore important that the methods used for processing should not only eliminate potential hazards (virus, bacteria, fungi, parasites) but also retain the nutritional and health properties of the products.

Besides heat treatments, non-thermal processes have been developed to minimize risks in the food industry and are considered to have minimal effect on the nutritional composition of products. These include high pressure processing (HPP), gases (chlorine dioxide, ozone, cold plasma), light (ultraviolet, pulsed light) and ionizing radiation (gamma irradiation, electron

beam). Ultraviolet (UV) light ranging from 200-280 nm is classified as UVC and is considered to have a germicidal effect on bacteria and viruses (Sastry *et al.* 2002). UVC works by altering the bond within the DNA double helix, which then results in either mutation or lethality to the cells (Morgan, 1989). UVC radiation has shown to effectively reduce the number of undesirable microorganisms in given environments and is recommended as one of the successful disinfection practices for water treatment and food products. Furthermore, it does not affect the sensory qualities of the treated products. Macroalgae extracts have also been suggested as a safer alternative to cultured animals (Chojnacka *et al.* 2012). Furthermore, the extracts have been reported to possess most of the benefits that could be obtained from using algal biomass (Chotigeat *et al.* 2004; Akbary and Aminikhoei, 2018; Wan *et al.* 2018).

1.3 Research aims and objectives

This study aimed to assess biosecurity risks associated with macroalgae inclusion in abalone diets and develop biosecurity measures to mitigate these risks.

The objectives were to:

1. Assess potential biosecurity measures on the inactivation of macroalgae-transmitted abalone pathogens;
2. Assess the effects of biosecure macroalgae on growth performance of abalone *Haliotis midae*; and
3. Assess the effects of biosecure macroalgae on the gut microbiome of abalone *Haliotis midae*

CHAPTER 2

ASSESSMENT OF POTENTIAL BIOSECURITY MEASURES ON THE INACTIVATION OF MACROALGAE-TRANSMITTED ABALONE PATHOGENS

2.1 INTRODUCTION

The provision of biosecure diets in intensive aquaculture facilities requires attention to reduce the risk of introducing potential pathogens to the farmed stock. Such introductions could result in the infection of susceptible stock and lead to disease outbreaks. Macroalgae are well recognized as an excellent source of bioactive compounds and when used as a feed or feed ingredient they have been associated with metabolic and immune regulation, resulting in improved health and growth of different cultured species (Chakraborty *et al.* 2014; Akbary and Aminikhoei, 2018). However, several microbial hazards and health/biosecurity related cases associated with the use of macroalgae as a feed or feed ingredient have been reported in the animal feed and human food industries (Mahmud *et al.* 2007; Quilliam *et al.* 2014; Banach *et al.* 2020). For example, pathogenic bacteria including *Vibrio parahaemolyticus*, enterohemorrhagic *Escherichia coli* O157:H7, and salmonella responsible for foodborne outbreaks were isolated from macroalgae (Mahmud *et al.* 2007; Nichols *et al.* 2017; Barberi *et al.* 2020). In the abalone farming industry, mortalities of cultured juvenile abalone *Haliotis asinina* caused by the gram-negative bacteria *Vibrio vulnificus* and *Vibrio alginolyticus* were confirmed to be transmitted by the macroalgae *Gracilaria changii* that had been fed to the abalone (Kua *et al.* 2011). In South Africa, abalone that were fed with kelp rather than artificial feed had a much higher prevalence of gut-associated parasites. This was linked to the kelp that was harvested from the wild, as well as less regular cleaning of the tanks where the kelp was fed (Mouton and Gummow, 2011). Uneaten kelp settling at the bottom of the tanks could lead to excess sludge where cleaning is not done frequently. Some studies have shown that

infectious diseases are frequently caused by sludge build-up in the culture unit (Khan 2018; Jasmin *et al.* 2020; Onomu *et al.* 2024).

This notwithstanding, abalone farms in South Africa still substantially feed their abalone with fresh *Ecklonia maxima*, *Ulva lacinulata* and *Gracillaria gracilis*. *Gracilaria gracilis* and *U. lacinulata* are grown in abalone farm as feed for the abalone with the latter being cultured in abalone effluent in an intergatted multi-tropic aquaculture (IMTA) systems (Robertson-Anderson *et al.* 2011). In one of the farms, the abalone are feed with *G. gracilis* once weekly and the effluent grown *U. lacinulata* two times weekly on alternative days (Onomu *et al.* 2024). A recent study was conducted in one of these farms to characterised the bacterial microbiome associated with the seawater and *Ulva* raceways receiving abalone effluent. The results showed that using effluent water as a means to fertilise *Ulva* does not introduce, nor increase the abundance of, harmful bacteria (de Jager *et al.* 2024). With some farms still reluctant to incorporate fresh macroalgae due to the aforementioned concerns, application of biosecurity processing treatments to eliminate any potential risk in the macroalgae would provide a great platform for their effective utilization.

One common attribute among all macroalgae is their high water content, ranging from 64.9% to 94% (Wan *et al.* 2018). To preserve this highly perishable biomass, drying offers a simplistic method for biomass preservation (Ratti, 2001). However, drying macroalgae at temperatures below 45 °C often achieves good chemical and microbiological stability but may not completely remove unwanted microbial contaminants, which may include harmful species. (Sieburth and Jensen, 1967; Del Olmo *et al.* 2018).

As the demand for safe food and products rises, non-thermal processing technologies that are an alternative to conventional thermal methods and do not use heat or enzymes to inactivate microorganisms are becoming more and more common in the fields of treatment, preservation,

and decontamination (Wang *et al.* 2016; Adebo *et al.* 2021). Ultraviolet light (UV) is a non-thermal process that has successfully been used as a disinfectant for surfaces, water and air, but is also gaining attention in the food industry as a fast, inexpensive method for sterilising of the surfaces of solid and liquid foods (Chiozzi *et al.* 2022). This technique has advantages over the thermal treatment method because it has causes minimum nutrients loss, does not leave toxic residues, does not alter the sensory quality of food and has low energy consumption. Moreover, this technique is effective in the inactivation of pathogens (Gayán, *et al.* 2014). For example, UVC doses of 0.6–6.0kJ/m^2 was able to reduce the populations of *E. coli* O157:H7 and *Salmonella* on the surface grape tomato by 2.3–3.5 and 2.15–3. log CFU per fruit, respectively. In addition, the UVC treatment had no effect on the firmness or colour of grape tomatoes during storage (Mukhopadhyay *et al.* 2014). However, the significant limitations of applying UVC light are the low penetration into products and the shade effect from complex surface properties of foods, which may shield the pathogens from the harmful wavelengths (Gayán, *et al.* 2014; Deng *et al.* 2020).

Other non-thermal techniques for achieving microbiological safety for various products and systems include the use of chemical disinfectants. Although there has been debate surrounding the use of chemicals citing toxic residues on products, some chemicals have been used successfully for the decontamination of different products. For example, chemical disinfectants such as povo-iodine formulations have been shown to limit the impact and spread of infectious diseases with potent antiviral, antibacterial and antifungal effects. Povo-iodine has several advantages, including a great safety record and a wide therapeutic range because of its multimodal activity (Eggers, 2019). Povo-iodine is an essential disinfectant or antiseptic agent, mainly used in the field of medicine and in the public health sector to prevent the spread of infectious agents (Eggers, 2019; Sauerbrei, 2020) and in the aquaculture sector for disinfection of aquaculture water or for disinfection of fish eggs (Cipriano *et al*. 2001;

Chen *et al.* 2018; Maapea *et al.* 2021). The best bactericidal and virucidal/high-level effect of povo-iodine was suggested to be present at a concentration range of approximately 0.08 %–0.9 %, depending on the free iodine concentration, with the maximum exposure times being 5.0 min for bacteria and 60 min for viruses (Sauerbrei, 2020). The efficacy of povo-iodine against the bacteria *Aeromonas hydrophila,* which was responsible for high mortalities in swamp eel (*Monopterus albus*) was tested in different types of water. A concentration of 25 ppm was effective against the bacteria after 12 h in outdoor aquaculture water whereas 10 ppm and 20 ppm of the povo-iodine could kill 99 % and 100 % respectively of 10^5 CFU/mL bacteria in indoor aquaculture water. In the same study, the minimal germicidal concentration of povo-iodine in Luria-Bertani broth was 4000 ppm, indicating that the organic substances present in the matrix had a negative impact on the effectiveness of povo-iodine (Chen *et al.* 2018).

Other techniques that have been suggested to be effective in decontaminating macroalgae from parasites and other pathogens, while still retaining their freshness, include soaking in freshwater, rapid pH change or high ammonia loads for a short period (Kirkendale *et al.* 2010). However, complete sterilization cannot be maintained using these techniques. Combining various techniques, such as drying, with methods like chemical disinfection or other non-thermal methods, has been recommended as the most effective strategy for overcoming the deficiencies of a single treatment in algae processing (Wang *et al.* 2019). The lethality against potential pathogens is maximized, while at the same time minimizing deterioration of product quality when combined treatments are used.

The current study aimed to improve the biosecurity of macroalgae by subjecting them to different processing treatments. Specifically, this study assessed the efficacy of different processing treatments, including heat, ultra-violet irradiation (UVC), different pH levels, salinity concentrations, and povo-iodine on the inactivation of potential macroalgae-

transmitted abalone pathogens. Three model 'pathogens' *Vibrio anguillarum*, *Halioticida noduliformans* and bacteriophage lambda, were selected for this study to represent the three major pathogenic groups associated with abalone diseases (bacteria, oomycetes/fungal, and viruses). The efficacy of the different processing treatments was assessed on pure cultures of the test pathogens and the test pathogens inoculated in macroalgae matrix. The results of this study will be helpful for the future establishment of feed safety procedures for inclusion of macroalgae and possibly other ingredients into aquaculture feeds.

2.2 MATERIALS AND METHODS

2.2.1 Test pathogens

The three test pathogens used in this study to test the various processing treatments included (1) the bacterium *Vibrio anguillarum,* which was previously isolated from diseased abalone *Haliotis midae* (Macey and Coyne, 2005); (2) an oomycete *Halioticida noduliformans,* the causative agent of abalone tubercle mycosis (Macey *et al.* 2011); and (3) bacteriophage lambda, which was used as a proxy for abalone herpesvirus (a double-stranded DNA virus and the aetiological agent of abalone viral ganglioneuritis (AVG) (Corbeil, 2020)). The *V. anguillarum* utilized in this study was also transfected with a stable *Vibrio*-derived plasmid pEVS146 coding for chloramphenicol (Cm) and kanamycin A (Kan) antibiotic resistance (Prof. Eric Stabb, University of Georgia), to facilitate specific detection of the pathogen, via selective plating following the various treatments. All the experiments were conducted at the Department of Forestry, Fisheries and the Environment (DFFE), Marine Research Aquarium (MRA) in Sea Point (Cape Town, South Africa).

2.2.2 Preparation of the test pathogen working stocks for the different processing treatments

Vibrio anguillarum working stock was prepared as described by Knapp *et al.* (2019), with slight modifications. Cells of *V. anguillarum* from a glycerol stock were aseptically streaked on freshly prepared sterile Tryptic Soy Agar (TSA) plates (40.0 g TSA, 2.0 % NaCl, 1000 mL deionized water) using a sterile metal loop. The media was supplemented with 50 µg/mL Cm (Sigma-Aldrich) and 200 µg/mL KAN for selective isolation of *V. anguillarum.* The culture plates were incubated for 48 h at 30 °C. Next, cells from the overnight culture of *V. anguillarum* were aseptically transferred using a sterile metal loop to 20 mL of sterile Tryptic Soy Broth (TSB) (15.0 g TSB, 2.0 % NaCl, 500 mL deionized water) in a 50 mL centrifuge tube. The contents were vortexed to resuspend the bacterial cells before determining the optical density at 540 nm using a spectrophotometer. The bacterial concentration was adjusted using sterile TBS or adding more bacterial cells to an optical density of 0.1 ± 0.005 at a wavelength 540 nm. This optical density was previously determined to be equivalent to a bacterial concentration of 4×10^7colony-forming units per mL (CFU/mL) (Mikulski *et al.* 2000).

Pure cultures of *H. noduliformans* were maintained on peptone-yeast-glucose-saline (PYGS) agar (1.25 g peptone, 1.25 g yeast extract, 3.0 g Glucose, 12.0 g agar, 1000 mL seawater). The culture media was supplemented with streptomycin sulphate and penicillin (0.5% w/v for each antibiotic) to prevent bacterial growth. A sterile No. 3 (5.5 mm in diameter) cork borer was used to make agar plugs at the advancing edges of the actively growing *H. noduliformans* colonies, which were aseptically transferred using a sterile metal loop to the centre of fresh PYGS agar plates (one plug per plate) and incubated at 21 °C. At the same time, agar plugs were also transferred to culture tubes containing 15 mL sterile PYGS broth (PYGS without agar). The *H. noduliformans* was sub-cultured once every three weeks. The cultures were incubated at 21°C for two weeks before being subjected to any processing treatment.

The double-layer technique described by Kropinski *et al.* (2009) was used to determine the phage titre for the bacteriophage lambda. A glycerol stock of the host phage bacterium *Escherichia coli* was aseptically streaked on Luria agar (LA) plates (10.0 g NaCl, 10.0 g Tryptone, 5.0 g yeast extract, 15.0 g agar, 1000 mL deionized water) and incubated overnight at 37 °C. A single colony of the overnight grown *E. coli* was transferred to 10 mL Luria broth (LB; 10.0 g NaCl, 10.0 g Tryptone, 5.0 g yeast extract, 1000 mL deionized water) and grown overnight at 37 °C in a shaking incubator set at 100 rpm. The overnight culture was diluted to 1:10 with LB and further incubated at 37 °C in the shaking incubator until an OD600 nm of 0.4-0.8 was reached. Sloppy LA media for the overlay was prepared using a lower concentration of agar, 7 g/L instead of 15 g/L. The sloppy agar facilitated the phage particles to diffuse through the media to infect neighboring bacterial cells. The sloppy agar was maintained in a water bath set at 50-55 °C to prevent it from solidifying until needed. Ten-fold serial dilutions (10^{-1}-10^{-9}) of the bacteriophage lambda stock, which was stored at 4° C, was made using peptone saline diluent (1.0 g peptone, 8.5 g NaCl and 1000 mL deionized water). The top agar overlay was prepared by adding 0.1 mL of each phage dilution and 0.1 mL of the host bacteria *E. coli* to 5.0 mL sloppy agar. The contents were mixed by gently rolling the test tube before pouring the contents onto a solid LA plate. This procedure was repeated for each dilution. All the plates were incubated for 24 h at 37 °C. Plaques-forming units (PFU) were counted thereafter and the plates with 30-300 plaques were selected and used to calculate the phage titer. The phage dilution that gave approximately 300 plaques per plate was used for the subsequent experiments.

Pure cultures of each test pathogen, prepared as described above, were subjected to the various processing treatments (different temperatures, salinity, and pH, as well as UVC and povo-iodine concentrations) for 15-60 min in 15 min increments (detailed procedures provided below). The viability of each test pathogen following treatment was assessed using culture

methods. The results obtained were used as the baseline for the inactivation of test pathogens inoculated into macroalgae.

2.2.3 Effect of different processing treatments on pure culture of the test pathogens

(a) Effect of heat treatment on inactivation of test pathogens

Experiments were carried out to determine the effect of the different temperatures (40, 50 and 60 °C) and exposure time on the survival of the bacteriophage lambda, *V. anguillarum* and *H. noduliformans.* One hundred microliter aliquots of *V. anguillarum* (4×10^4 CFU/mL) were transferred to microcentrifuge tubes and incubated on heating blocks set at 40, 50 and 60 °C. A total of 12 aliquots were incubated at each temperature treatment (three replicates for each exposure time). The same procedure was repeated for the bacteriophage lambda with a working stock of 1×10^6 PFU/mL. For the *H. noduliformans*, agar plugs of actively growing hyphae were transferred to microcentrifuge tubes containing 1.0 mL Phosphate-buffered saline (PBS) (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2PHO_4, 0.245 g KH_2HPO_4 and 1000 mL deionized water) solution. Thirty six agar plugs were placed in microcentrifuge tubes with each containing four agar plugs. Three sets of the microcentrifuge tubes were incubated at each of the set temperatures. The test pathogens were incubated for 15, 30, 45 and 60 min, respectively at each temperature tested in this study.

To assess the viability of the bacteriophage lambda following each treatment, three microcentrifuge tubes were removed from each heating block/respective temperature at each time point and cooled on ice for 1.0 min then briefly vortexed. Next, 100 µL aliquots of the treated samples were mixed with 100 µL of overnight-grown *E. coli*. The mixture was transferred to 5.0 mL molten LA and mixed by inversion several times before pouring the contents onto the surface of LA plates (three replicates). The plates were incubated at 37 °C for

24 h before quantifying the number of plaque-forming units. Similarly, for the *V. anguillarum,* three microcentrifuge tubes were removed from each heating block/respective temperature at each time point and the 100 µL aliquots of treated bacterial cells were spread-plated onto TSA plates. The plates containing bacteria were incubated at 30 ºC for 48 h before quantifying the number of colony-forming units per mL. To assess the viability of *H. noduliformans* after each treatment, three agar plugs were removed from each temperature treatment at each exposure time and blotted dry using sterile blotting paper before being transferred onto a sterile PYGS agar plate. Plates were incubated at 21 ºC and vegetative growth was recorded following two weeks of incubation. Four random measurements were taken from the center of the agar plug then averaged to determine the radial growth of the mycelia on each replicate plate.

The controls were prepared in the same manner as the treated samples except that they were plated immediately to determine the initial/ actual dose/concentration of the bacterial and viral pathogens and the radial growth of the oomycete that had not been exposed to any of the temperature treatments.

(b) Effect of different salt concentrations on the inactivation of test pathogens

To determine the effect of varying salt concentrations on the survival of the test pathogens, sterile, natural seawater was diluted with deionized (sterile) water to achieve salt concentrations of 5, 15 and 20 g/L. Pure deionised water and sterile seawater, 0 and 35 g/L respectively, were also tested as controls. The test pathogens were exposed to the different concentration for 15, 30, 45 and 60 min, respectively. A *V. anguillarum* suspension (4×10^7 CFU/mL) was prepared as described above and aliquots (15 µL) were added to each 15 mL salt concentration solution to achieve a final bacterial concentration of 4×10^4 CFU/mL. Three replicates were prepared for each salt concentration tested. An additional control was prepared by diluting the *V. anguillarum* stock suspension in TSB to a final concentration of 4×10^4 CFU/mL, instead of the salt solutions to determine the actual concentration/dose of the bacterium before the salinity

treatments. At each exposure time following re-suspension of the bacteria in the varying salt concentrations, a 100 µL aliquot from each replicate tube from each salinity treatment was spread plated onto a TSA plate and incubated for 48 h at 30 °C. A similar procedure was followed for the bacteriophage lambda, except that the stock lysate was diluted to 1×10^6 PFU/mL in each salt concentration treatment. The control was prepared to a similar dilution using the peptone saline diluent. At exposure time, a 100 µL aliquot was transferred from each replicate tube from each treatment (three replicates/treatment), and the double layer technique was used to assess the viability of the bacteriophage following treatment (Kropinski *et al.* 2009).

For the *H. noduliformans*, 12 agar plugs with actively growing hyphae were ascetically transferred to each replicate tube of the salt concentrations tested. A total of 180 agar plugs and 15 replicate tubes were used. The agar plugs were incubated in the different salt concentrations for 15-60 min. Three agar plugs were incubated in PBS solution as the control. After every 15 min, three agar plugs were removed from each replicate tube blotted dry using sterile blotting paper, then aseptically placed onto the surface of the fresh/sterile PYGS agar plate and incubated at 21 °C. Vegetative growth was assessed two weeks after treatment, as described above.

(c) Effect of pH levels on the inactivation of test pathogens

The pH of a phosphate-buffered saline solution was adjusted by adding either sodium hydroxide (NaOH) or hydrochloric acid (HCL) to create buffered solutions with a pH of 3, 4, 10, 11 and 12. Working stock suspension of bacteriophage lambda and *V. anguillarum* and the agar plugs of growing *H. noduliformans* were prepared by a similar procedure described above and were again exposed for 15, 30, 45 and 60 min, respectively.

(d) Effect of UVC light on the inactivation of test pathogens

Working stocks of bacteriophage lambda and *V. anguillarum* were prepared in a PBS solution and concentrations were adjusted to 1×10^6 PFU/mL and 4×10^4 CFU/mL, respectively. Two hundred microlitre aliquots of the phage lambda and *V. anguillarum* suspension were then aseptically transferred to wells of separate UV-transparent microplates, ensuring three replicates for each exposure time. The microplates were then placed in a UV transilluminator chamber under UV light of wavelength 312 nm and exposed for 15-60 min. Agar plugs of *H. noduliformans* were placed in microplate wells with 200 mL PBS solution and then placed in the transilluminator. Three replicate wells were used for each time point, with each well containing two agar plugs. The controls were not exposed to the UV light and were plated immediately. The viability of the test pathogens after the treatments was assessed as described above.

(e) Effects of Povo-iodine on inactivation of test pathogen

A povo-iodine (Polyvinylpyrrolidone-Iodine, complex PVP-I $(C_6H_9NO)n$; Sigma-Aldrich) stock solution (5 g/L) was prepared using autoclaved seawater. Two concentrations of povo-iodine were tested in this study; 5 g/L and 0.5 g/L were selected based on literature and from preliminary experiments of this study. Two hundred microliters of the stock lysate of bacteriophage lambda (1×10^8) were added to 18 mL of the povo-iodine treatments to a final concentration of 1×10^6 PFU/mL. Three replicates were prepared for each treatment. Similarly, 20 µL of the *V. anguillarum* suspension was added to 18 mL of each treatment solution to achieve a final concentration of 4×10^4 CFU/mL. A total of 12 agar plugs of *H. noduliformans* were transferred to 20 mL of each of the povo-iodine concentrations. This was done in three replicates. Phosphate-buffered saline solution was used instead of the povo-iodine solution for the controls. After every 15 min, a 100 µL aliquot was aseptically removed from the phage and bacterial replicate samples, respectively, and transferred to 900 µL of 0.5 % sodium

thiosulphate, pre-aliquoted into separate sterile 1.5 mL microcentrifuge tubes, to neutralize the iodine. Following mixing, the viability of the bacteria and phage was assessed as described previously. For the *H. noduliformans,* three agar plugs were removed from the treatment solution at each time point, washed in 0.5 % sodium thiosulphate to neutralize the iodine, blotted dry using sterile blotting papers, and then aseptically transferred onto the surface of fresh PYGS agar plates and incubated and assessed for growth as described above. The sodium thiosulphate was tested beforehand (data not included here) to ascertain that it does not affect the viability of the test pathogens.

2.2.4 Macroalgae-pathogen assays and inoculation of pathogens into macroalgae

Fresh *Gracilaria gracilis* was harvested from a commercial abalone farm and transported in a cooler box to the MRA laboratory, where it was stored at -80 ℃ until needed. Fresh *Ulva lacinulata* was harvested from culture tanks at the MRA. At the same time, kelp (*Ecklonia maxima*) was collected from the seashore in front of the MRA in Sea Point, Western Cape, South Africa and then cleaned with seawater. The *Ulva* and kelp were collected shortly before each treatment. Samples (ca. 50.0 g) of each macroalga were grounded in liquid nitrogen before the inoculation process.

Macroalgae were inoculated with test pathogens as follows: 2 g of the ground macroalgae were placed on sterile crucible bowls before being separately inoculated with either 1.0 mL of a 4×10^7 CFU/mL suspension of *V. anguillarum* or a 1×10^8 PFU/mL suspension of bacteriophage lambda. Each inoculated macroalgae was mixed thoroughly with a sterile glass rod to ensure even distribution of the test pathogens in the ground macroalgae. A total of three replicate samples were prepared for each treatment. The controls were macroalgae samples inoculated with the test pathogens but not subjected to any treatment. Culture-based viability assessments of test pathogens in a macroalgae matrix were done only for bacteriophage lambda

and *V. anguillarum*, as it was not possible to accurately assess the viability or growth of *H. noduliformans* following inoculation of hyphae in the macroalgae matrix.

(a) Effect of heat treatment on the viability of test pathogens in a macroalgae matrix

Three replicates of each macroalgae sample inoculated with both the *V. anguillarum* and bacteriophage lambda were placed in the oven at 40 °C for 8 h. The drying period was selected based on results from preliminary tests done in this study. At the end of the heat (drying) treatment, each sample was ground to a fine powder using a mortar and pestle before transferring 1.0 g of the sample to 9.0 mL sterile PBS solution in a 15 mL centrifuge tube. Samples were mixed thoroughly by vortexing before spread plating 100 µL aliquots onto TSA (three replicates per macroalgae) and incubated at 30 °C for 48 h to assess the number of culturable *V. anguillarum* remaining in the seaweed matrix. For the bacteriophage lambda, 100 µL aliquots were aseptically transferred from each 15 mL centrifuge tube to 5.0 mL sloppy agar containing 0.1 mL of *E. coli* to assess the number of PFU that remained viable in the seaweed matrix following treatment. Three replicate samples were tested for each macroalgae, and data were expressed as mean (±SE) CFU or PFU per gram macroalgae. Control samples for both test pathogens were plated immediately after inoculation.

(b) Effect of combined heat and UVC light treatments on the viability of test pathogens in a macroalgae matrix

Samples were first subjected to drying treatment as described above. The ground samples were then uniformly spread on the surface of a transparent sterile petri dish and exposed to UVC light of wavelength (254 nm) for 10 min. After the UVC treatment, 1.0 g of the samples were transferred to separate sterile 15 mL centrifuge tubes containing 10 mL PBS solution and mixed thoroughly by vortexing. Control samples for both test pathogens were plated immediately

after inoculation. The number of bacteria or phage particles remaining culturable or viable, respectively, following treatment was assessed as described above.

(c) Effect of combined treatments of heat and povo-iodine on the viability of test pathogens in a macroalgae matrix

Inoculated macroalgae samples (2.0 g) were transferred to separate 15 mL centrifuge tubes containing 10 mL of povo-iodine solution (5 g/L) and then incubated for 20 min. Following incubation, each sample was centrifuged at 10,000 × g for 10 min at 4 ℃ and the supernatant was carefully discarded (to avoid losing the pellet). Each sample/pellet was re-suspended in 10 mL of 0.5 % sodium thiosulphate solution to neutralize the iodine before centrifuging at 10,000 × g for 10 min at 4 ℃. The supernatant was discarded as before, and the pelleted samples were carefully transferred to separate sterile porcelain crucibles for heat (drying) treatment at 40 ℃ for 8.0 h. Following the drying treatment, 1.0 g of each sample was ground to a fine powder using a pestle then transferred to 10 mL PBS solution, vortexed briefly and prepared for colony and plaque counting as described above. For the control, the inoculated samples were suspended in 10 mL PBS, vortexed and 100 µL plated on the respective media for colony/plaque counting.

(d) Effect of combined treatments of povo-iodine, heat and UVC on the viability of test pathogens in a macroalgae matrix

The first steps of iodine and heat treatments were done as described above. After the heat (drying) treatment, the samples were ground to a fine powder using a mortar and pestle and uniformly spread on the surface of a transparent Petri dish. The Petri dishes containing ground samples were exposed to UVC light for 10 min. Following exposure, the samples were transferred to 15 mL centrifuge tubes containing 10 mL PBS solution and the number of bacteria or phage particles remaining culturable or viable, respectively, following treatment

was assessed as described above. The controls were inoculated with the test pathogens as the treatment samples, they were then suspended in 10 mL PBS, vortexed and 100 µL plated on the respective media for colony/plaque counting.

2.2.4 Data analysis

The mean values of the replicates were determined. Data from the treated samples of *V. anguillarum* and bacteriophage lambda were expressed as a percentage relative to the controls that were not treated. These data were tested for homoscedasticity using Levene's test (Levene, 1960) and, since all data were parametric, a one-way analysis of variance (ANOVA) was used to establish the effect of the treatments and exposure time on the test pathogens compared to their controls, at $p < 0.5$.

2.3 RESULTS

2.3.1 Effect of different temperature treatments on the culturability and growth of the pure culture of the test pathogens

A significant decrease in the number of culturable *V. anguillarum* was observed with exposure time when the temperature was increased to 50 ºC (One-way ANOVA: $F_{(4, 10)} = 6.9037$; $p = 0.006$; Figure 2.1). Conversely, bacteria were rendered non-culturable when incubated at 60 ℃ within 15 min. Similarly, a significant reduction in the plaque-forming units of the phage lambda was observed with an increase in temperature and exposure time (One-way ANOVA: $F_{(4, 10)} = 14.510$; $p = 0.003$; $F_{(4, 10)} = 84.755$; $p = 0.000$; $F_{(4, 10)} = 147.45$; $p = 0.000$ for 40, 50 and 60 ℃ respectively). The bacteriophage was rendered non-infective at 60 ℃ at 45 min (Figure 2.1). No vegetative growth was recorded for *H. noduliformans* after exposure to all the temperatures tested in this study except for the control that was not treated (Figure 2.1).

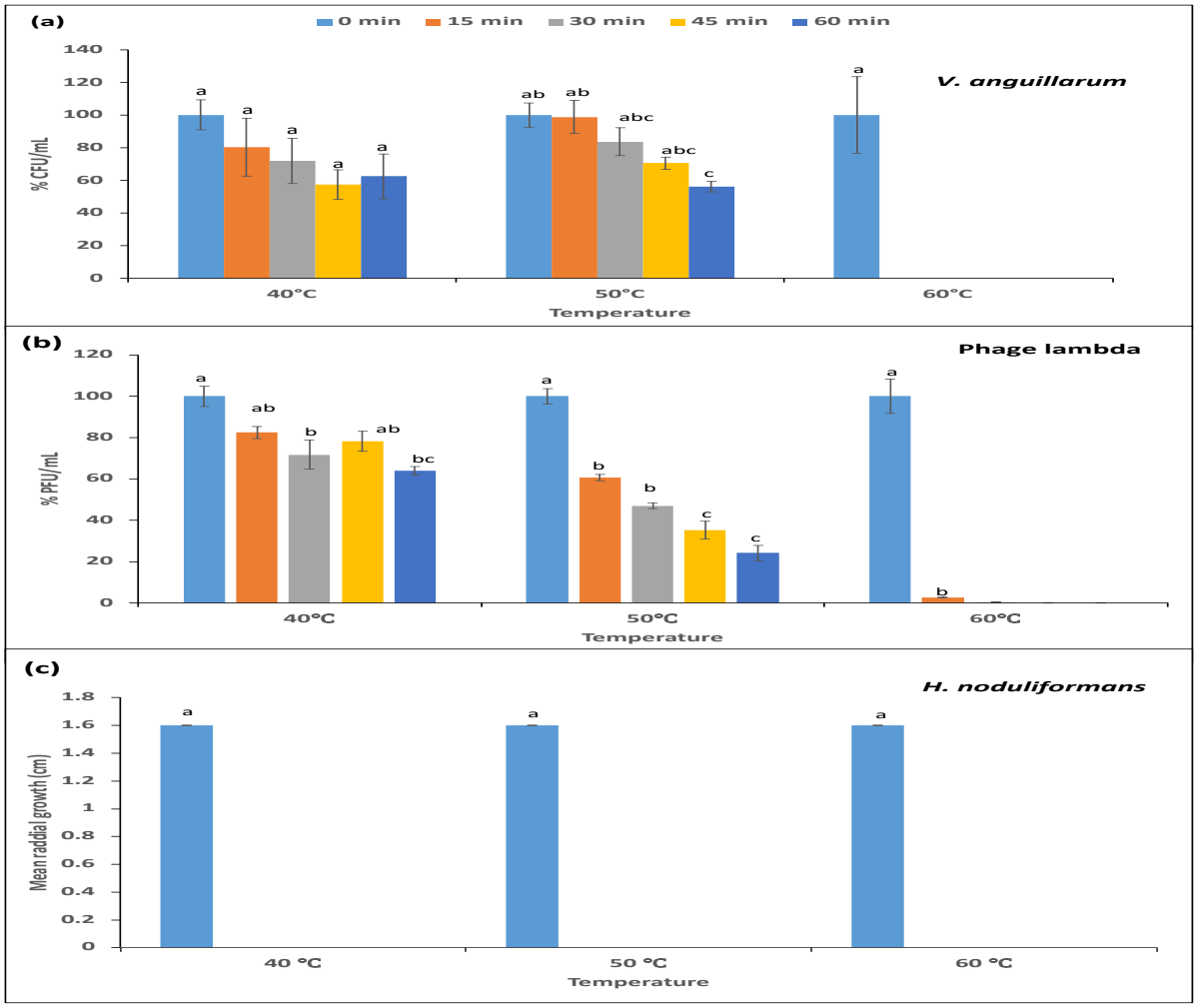

Figure 2.1. (a) Mean number of culturable V. anguillarum colony forming units, (b) plaque forming unit of phage lambda and (c) radial growth of H. noduliformans following heat treatment at temperatures 40, 50 and 60 °C after 0-60 min exposure time. The data represents the mean (± S.E.) of the samples in three replicates. Different superscript letters indicate statistically significant (P < 0.05) differences based on Tukey's post-hoc test, where no letters indicate no statistically significant differences between groups.

2.3.2 Effect of different pH treatments on the culturability and growth of the pure culture of the test pathogens

Exposure of the test pathogen *V. anguillarum* to varying pH solutions above and below neutral (pH 7) resulted in a significant decrease in the number of bacteria as the alkalinity or acidity of the solution increased (One-way ANOVA: $p < 0.05$; Figure 2.2). A pH of 3 reduced the colony-forming units of the bacteria *V. anguillarum* by 63 % after 60 min of exposure, while 100 % reduction was achieved at pH 11 after 15 min of exposure time.

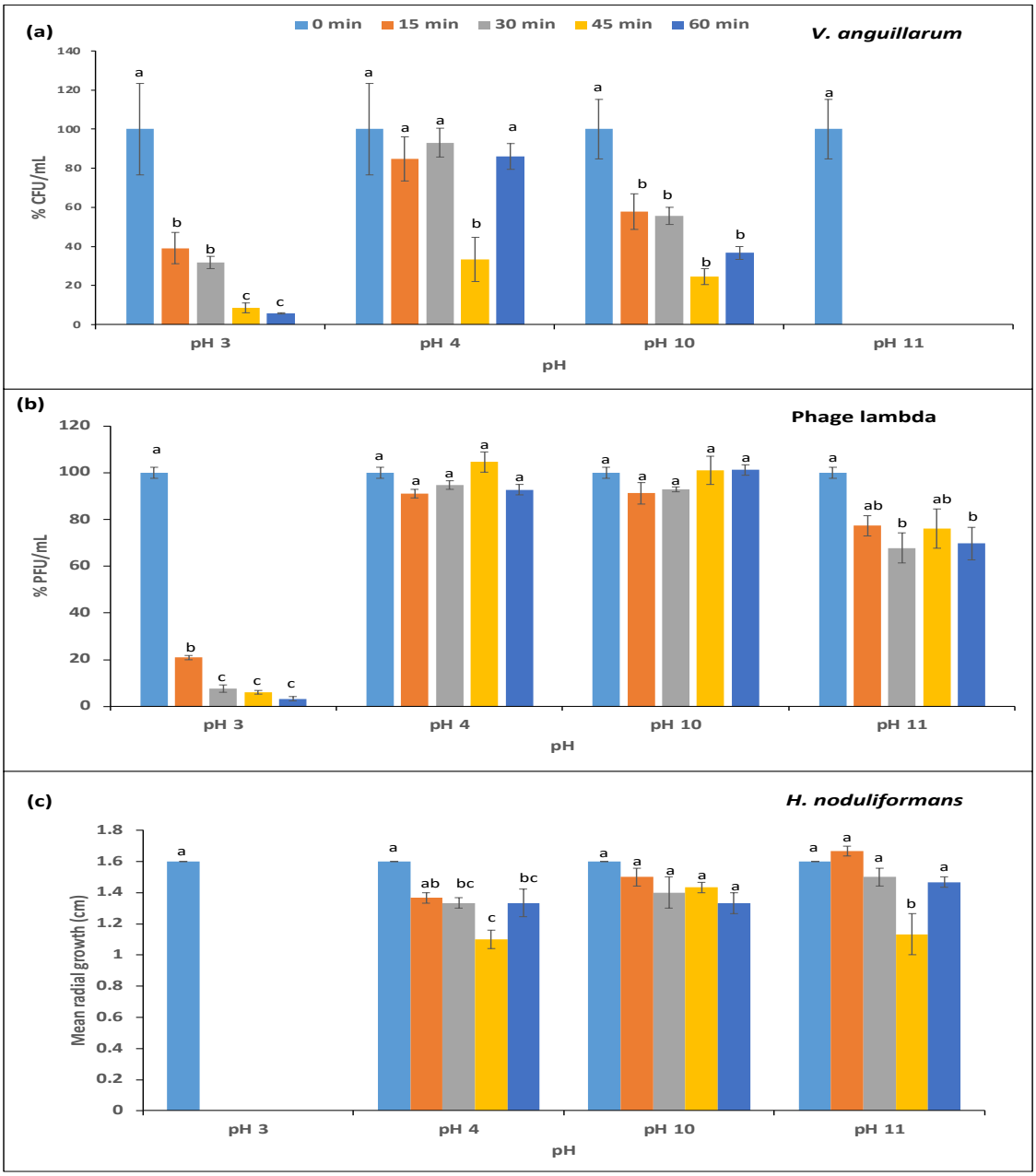

Figure 2.2. (a) Mean number of culturable V. anguillarum colony forming units, (b) plaque forming unit of phage lambda and (c) radial growth of H. noduliformans after exposure to different pH ranges, 3, 4,10 and 11 for 0-60 min exposure time. The data represents the mean (± S.E.) of the samples in three replicates. Different superscript letters indicate statistically significant ($p < 0.05$) differences based on Tukey's post-hoc test, where no letters indicate no statistically significant differences between groups.

For the phage lambda, a significant decrease of the plaque-forming units with an increase in exposure time was observed at pH 3 and 11 ($p < 0.05$). The optimal inhibition of the phage infectivity (97 %) was achieved following exposure to solutions with a pH of 3 for 60 min

(Figure 2.2). No growth of the *H. noduliformans* was observed following treatment with pH 3 after 15 min of exposure (Figure 2.2).

2.3.3. Effect of different salinity concentrations on the culturability and growth of the pure culture of the test pathogens

In this study, no salt concentrations tested resulted in a 100 % reduction of the test pathogens. However, a significant change was observed in the numbers of the colony-forming units of the *V. anguillarum* with increase in exposure time compared to the control for all the salt concentrations except at 35 g/L. For the *H. noduliformans*, no significant change in growth was observed between the control and all the treatments ($p > 0.05$).

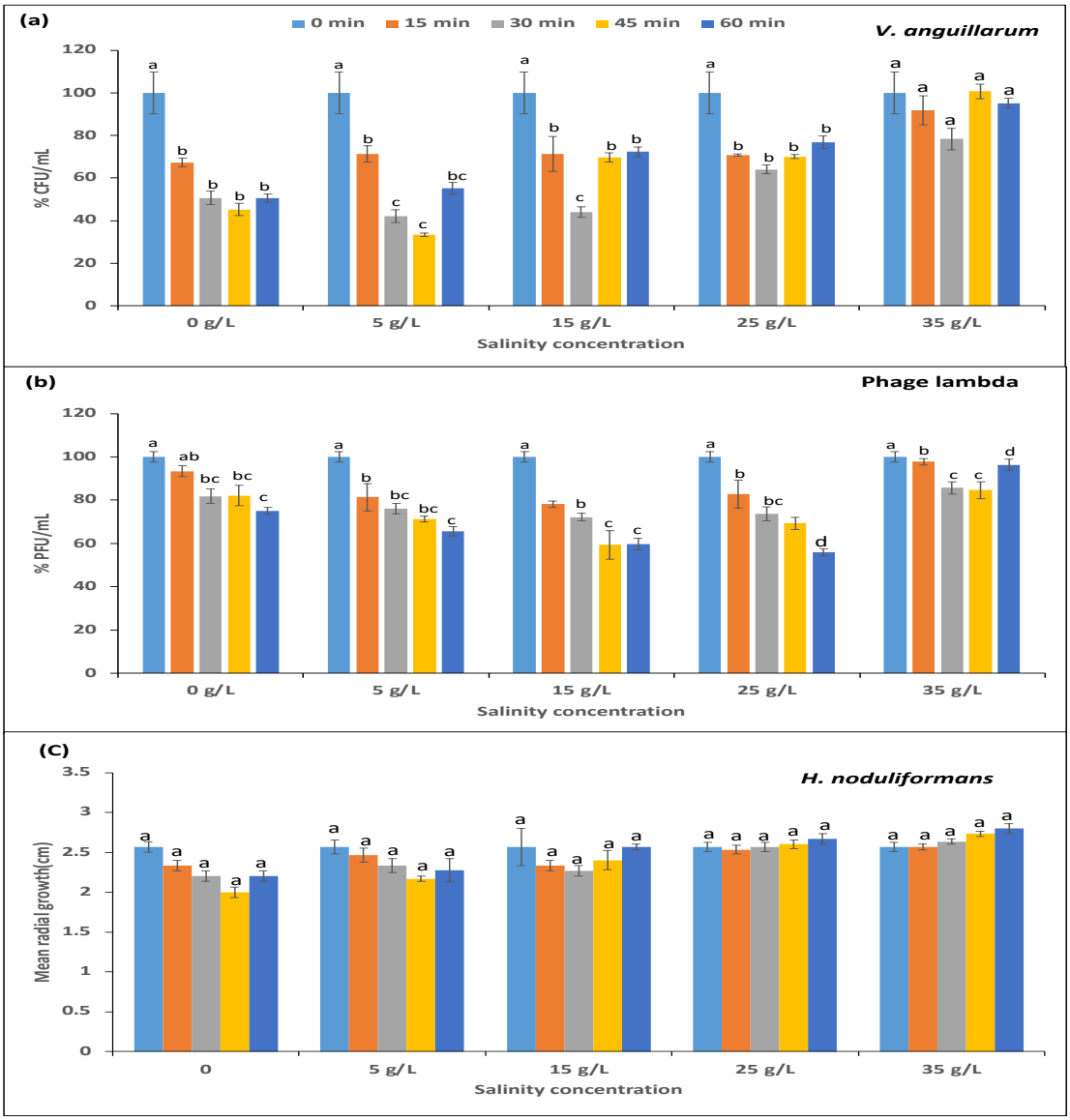

Figure 2.3. (a) Mean number of culturable V. anguillarum colony forming units, (b) plaque forming unit of phage lambda and (c) radial growth of H. noduliformans after exposure to different salinity concentrations 0, 5,15, 25, 35 g/L after 0-60 min exposure time. The data represents the mean (± S.E.) of the samples in three replicates. Different superscript letters indicate statistically significant ($p < 0.05$) differences based on Tukey's post-hoc test, where no letters indicate no statistically significant differences between groups.

2.3.4. Effect of different povo-iodine treatments on the culturability and growth of the pure culture of the test pathogens

Exposure to both concentrations of povo-iodine solution resulted in a significant decline in culturable *V. anguillarum* with increased exposure time. No colony-forming units were recorded following treatment with 5 g/L after 15 min exposure ($p < 0.05$; Figure 2.4). Similarly,

a significant decrease in plaque-forming units of the phage lambda was observed with 100% inhibition achieved following exposure to 5 g/L povo-iodine ($p < 0.05$). For the *H. noduliformans*, all the povo-iodine concentrations tested resulted in growth inhibition after 15 min of exposure.

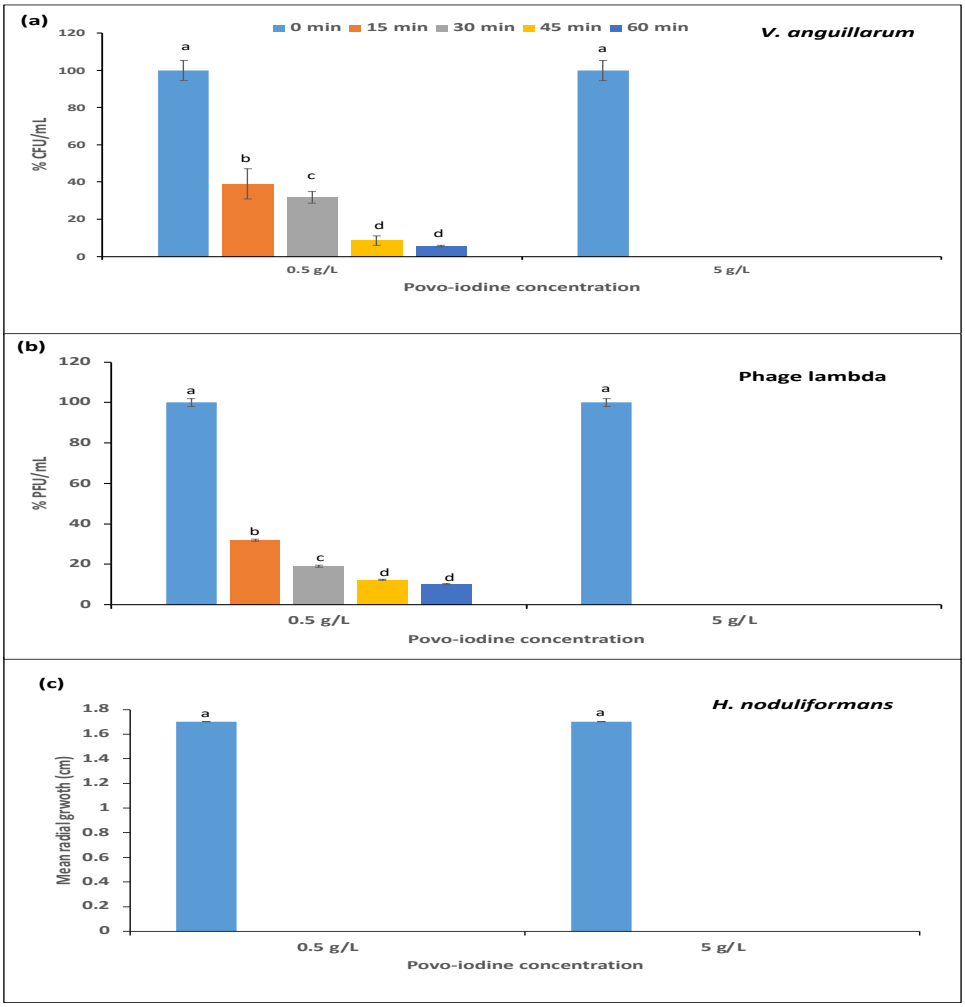

Figure 2.4. (a) Mean number of culturable colony forming units of V. anguillarum, (b) plaque forming unit of phage lambda and (c) radial growth of H. noduliformans after exposure to different povo-iodine 0.5 and 5 g/L after 0-60 min exposure time. The data represents the mean (± S.E.) of the samples in three replicates. Different superscript letters indicate statistically significant ($p < 0.05$) differences based on Tukey's post-hoc test, where no letters indicate no statistically significant differences between groups.

2.3.5. Effect of drying and combination of drying with other treatments on culturability and growth of test pathogens in a macroalgae matrix

Each test pathogen in a macroalgae matrix was treated with a single or combination of two or three treatments. These treatments were as follows: oven drying at 40 ℃ for 8 h; disinfection with povo-iodine solution (5 g/L) for 20 min; and exposure to UVC light for 10 min. Drying (heat) treatment resulted in a one-fold reduction of bacteriophage lambda and a five-fold reduction of *V. anguillarum*, respectively (Table 2.1). No colony-forming units were observed for the *V. anguillarum* after applying the combined treatments. On the other hand, the combined treatments of oven drying with UVC and oven drying with povo-iodine resulted in a reduction of the bacteriophage by 3-fold and 4-fold, respectively. Bacteriophage lambda was rendered non-infectious following a combination of all three treatments, namely povo-iodine oven drying and exposure to UVC light (Table 2.1).

Table 2.1. Effect of different methods; oven drying(D), the combination of oven drying+ UVC (D+UVC), oven drying + povo-iodine (D+PI) and oven drying + UVC + povo-iodine (D+UVC+PI) on the viability of phage lambda and culturability of V.anguillarum when inoculated into a macroalgae matrix. The data represents the mean concentrations of the V. anguillarum and phage lambda (±SE).

Test pathogens	Control	D	D + UVC	D + PI	D + UVC+ PI
Phage lambda	$(4.82\pm9.6)\times10^{8}$	$(3.6\pm2.6)\times10^{7}$	$(8.9\pm2.6)\times10^{5}$	$(5.4\pm2.04)\times10^{4}$	0
V. anguillarum	$(3.63\pm0.5)\times10^{7}$	$(5.7\pm3.3)\times10^{2}$	0	0	0

2.4 DISCUSSION

The development and optimization of processing methods for macroalgae is a necessary step to produce safe products. This study investigated the use of different treatments, viz. heat, pH, salinity, UVC light and povo-iodine to inactivate test pathogens used as model organisms for macroalgae-transmitted pathogens.

Generally, the bacteriophage lambda was the most difficult test pathogen to inactivate compared to the other test pathogens in this study, both in pure culture and the macroalgae matrix. The bacteriophage belongs to the family siphoviridae and members of this family are considered the most resistant to adverse conditions (Lasobras *et al.* 1997). A study on the use of the phage as a DNA vaccine delivery system revealed that the phage is highly stable when subjected to different temperatures ranging from -70 °C to 100 °C and pH ranges of pH 3-11 over different periods (Jepson and March, 2004). This test pathogen was therefore used as the reference point for the efficacy of the tested treatments.

The different salinity concentrations tested did not yield 100 % inactivation of any test pathogens. Some previous literature has shown that bacteria belonging to the genus *Vibrio* can survive in a wide range of salinity concentrations (Larsen *et al.* 2004). For example, *V. anguillarum* have been shown to cause vibriosis in a variety of fish species from both marine and brackish environments (Frans *et al.* 2011), providing support for the broad salinity tolerance of species within the genus *Vibrio*. The results of this study further indicated that the optimum growth of all the test pathogens was observed at a salinity of 35 g/L, which is full-strength seawater. Similar findings have been reported in a study that investigated the effect of external factors on the survival of bacteriophages, where the highest activity of the phages was found to be in salt concentrations roughly equivalent to full-strength seawater (Jończyk *et al.* 2011). In addition, the optimum growth of *H. noduliformans* has also been previously reported to occur in full-strength seawater (Macey *et al.* 2011). Since no complete inhibition of the test pathogens in pure culture was achieved with this treatment, it was therefore omitted from the subsequent experiments, which involved macroalgae-pathogen assays.

Inactivation of the test pathogens by varying pH indicated that a complete inactivation of the bacteria *V. anguillarum* was achieved at pH 11, while the growth of *H. noduliforman* was

inhibited by pH 3. A significant reduction of the bacterial colony-forming units and plaque-forming units was also observed at pH 3 with an increase in exposure time. The use of extremely acidic and alkaline solutions has been cautioned due to their corrosive and caustic nature, which can cause tissue damage and leave toxic by-products (Wirtanen and Salo, 2003). This treatment was therefore not pursued any further (i.e., for the macroalgae pathogen assays) because its application was deemed impractical and unsafe.

The findings of this study demonstrated that a drying temperature of 60 ºC was the most effective temperature to inactivate all the test pathogens. However, high temperatures such as this have been found to have a negative effect on heat-labile compounds (e.g., vitamins, proteins, unsaturated fatty acids, phenols and carotenoids) and consequently lead to their degradation (Gupta *et al.* 2011; Wan *et al.* 2018). Drying temperatures of (< 45 °C) are widely used in the macroalgae industry and have been demonstrated to preserve vitamins and pigments (Kadam *et al.* 2015). Published literature reported that drying seaweed at 40-50 ℃ permitted microbial development to a population of 43,000 bacteria/gram and 7,500 fungi/gram (Sieburth and Jensen, 1967). Similarly, in this study, treatment of the test pathogens in a macroalgae matrix at 40 ℃ did not result in 100 % inhibition of the test pathogens. Therefore, utilization of this treatment on its own proved not to be viable, hence it is necessary to combine this treatment with other treatments to achieve more effective inactivation of potential microorganisms of concern.

Povo-iodine was used in this study because of its broad antimicrobial activity against pathogens and because it causes minimum change in the nutritional quality of products. Iodine-based disinfectants have been reported to effectively inactivate pathogens in aquaculture based on their efficacy against several aquatic disease agents (Ellard, 2006). The optimum bactericidal and virucidal effects of povo-iodine have been demonstrated to be within the concentration

range of 0.08-0.9 % (Sauerbrei, 2020). A similar concentration of 0.5-1 % solution of iodinated polyvinyl pyrrolidine has also been frequently used in sterilization protocols (Kerrison *et al.* 2016). However, it should be noted that these studies were conducted *in vitro* without complex matrices such as organic matter. The concentration used in this study (5 g/L) was in the range of the cited studies and proved effective for the inactivation of the test pathogens in pure culture but not in the macroalgae matrix. A similar scenario has been reported for povo-iodine where the efficacy decreased in the presence of complex matrixes, such as environmental organic matter (Chen *et al.* 2018). This aspect should be taken into consideration when povo-iodine is used in aquaculture or other complex environments.

The findings of this study further indicated that under the UVC treatment, the conditions that could effectively inactivate the pure cultures of the test pathogens were not effective when the test pathogens were inoculated in macroalgae. This is most likely associated with the low penetration of UV light and shade effect from complex surfaces, hence reducing the efficacy of UVC as other studies have also reported (Gayán *et al.* 2014; Deng *et al.* 2020).

Heat treatment by drying offers better possibilities for combining with other physical treatments such as UVC (Pan *et al.* 2004). Recently, several sectors of the food manufacturing/processing industries including the fruit processing industry, have examined the prospect of combining both forms of physical treatments to decrease pathogen growth. For example, the combination of UVC and mild heat treatment of 45 °C applied on strawberry fruit resulted in reduced fungal infections and delayed *in vitro* germination of *Botrytis cinerea* conidia (Pan *et al.* 2004). In this study, the combination of heat treatment and UVC effectively inhibited the growth of *V. anguillarum* and significantly reduced the number of viable bacteriophage lambda particles. Another sterilization approach that has been suggested for consideration is the combination of povo-iodine with UVC (Eggers, 2019). Moreover, both

treatments have a broad antimicrobial spectrum with activity against a variety of pathogens and cause a minimum change in the nutritional quality of products. In this study, the combined treatment (povo-iodine, oven drying and UVC) was effective and resulted in 100 % inactivation of all the test pathogens.

2.5 CONCLUSION

The single treatments including UV light, povo-iodine, pH and heat treatments were effective on the pure cultures of the test pathogens, but not when the test pathogens were inoculated or seeded in the macroalgae matrix. Inactivation of the test pathogens by pH treatments was also only achieved at high acidic and alkalinity levels, hence these treatments were deemed impractical and/or unsafe and were not pursued further in the macroalgae-pathogen assays. The combination of different treatments increased the efficacy of the inactivation of test pathogens in this study compared to single treatments. Combined treatments of the povo-iodine solution (5 g/L) for 20 min, oven drying at 40 ℃ for 8 h and UVC exposure for 10 min inactivated all the test pathogens tested in this study. This combination was considered the most effective among all the other treatments tested in this study and was applied to produce biosecure macroalgae, which was used in the formulation of experimental diets. Future studies should look into optimizing different treatments based on the targeted pathogen and macroalga species. Although this study focused on treatment of macroalgae to be incorporated in the formulated diet in dried form, some abalone farms use fresh macroalgae as supplementary feed. Therefore, there is a need for further research to explore treatments that may be applied to achieve biosecure fresh macroalgae.

CHAPTER 3

EFFECTS OF PROCESSED (BIOSECURE) MACROALGAE ON GROWTH PERFORMANCE OF ABALONE *HALIOTIS MIDAE*

3.1 INTRODUCTION

Abalone are slow-growing marine gastropods taking an average of three years to reach a market size of 100 g. This slow growth (Britz, 1996) coupled with high feeding rates, cleaning and costs associated with pumping water throughout the production cycle requires a prolonged resource investment (Mohamed, 2020). One crucial aspect in the commercial production of abalone is ensuring the animals reach a marketable size within the economically viable time without compromising their health and quality (Naidoo *et al.* 2006). This has prompted more research into increasing production, with an emphasis on growth rates (Naylor *et al.* 2009; Mohamed, 2020). Optimization of feeds for faster growth rates is seen as an essential tool to shorten the growth period and consequently lower the resource investment into the production of the animals.

The existing research on feed formulation optimisation has focused on generating nutritionally balanced meals and cutting feed costs (Bullon *et al*. 2022). One of the strategies employed to achieve this is replacing the protein component in the abalone feed with a more cost-effective ingredient such as macroalgae (Sales *et al.* 2003; O'Mahoney *et al.* 2014; Bullon *et al*. 2022). With macroalgae and formulated feed being the main diets utilized in the commercial abalone culture, several studies have interrogated the effects of these types of diets on abalone growth (Mulvaney *et al.* 2013; Bansemer *et al.* 2015), health (Tanaka *et al.* 2003; Grandiosa *et al.* 2018) and nutritional quality (Bewick *et al.* 1997; Preece, 2006), among others. Since the abalone gut is biologically adapted to macroalgae diets, the use of combination diets of macroalgae and formulated feeds provides a mechanism to utilize formulated feed use without

completely abandoning macroalgae (Kemp *et al.* 2015). Furthermore, the incorporation of macroalgae in abalone feeds has been found to have significant positive effects on growth, feed utilization, physiological condition, stress response, and carcass quality of cultured animals (Naidoo *et al.* 2006; Dlaza *et al.* 2008; Viera *et al.* 2011; Bansemer *et al.* 2016; Nel *et al.* 2017).

In the South African abalone industry, macroalgae are often harvested from the sea (*Ecklonia maxima*) or grown in abalone farms in IMTA systems (*Ulva lacinulata*). Some farms also grows *Gracilaria gracilis* in fertilized seawater in tanks. The macroalgae is fed directly to the farmed abalone stocks as a supplementary feed or incorporated into formulated abalone feeds as a dry feed ingredient (Bolton *et al.* 2009). The use of fresh macroalgae as a supplementary feed for abalone stock has been associated with the potential to increase the risk of introduction of pathogenic agents competitors, predators, and pests (Bautista-Teruel *et al.* 2011). For example, mortalities of abalone *Haliotis asinina* were associated with the direct use of seaweed (*Gracilaria changii*) as food for the abalone. The outbreak was as a result of bacterial infections caused by *Vibrio* and *Pasteurella* introduced by the seaweed to the abalone stock (Kua *et al.* 2011). The inclusion of dried algae meal into formulated feeds as opposed to feeding fresh macroalgae has been suggested as a solution to reduce the perceived biosecurity risks associated with fresh macroalgae as feed and provide a practical solution to gain the benefits of feeding macroalgae to abalone (O'Mahoney *et al.* 2014; Bansemer *et al.* 2016).

Ensuring food safety while meeting the demands for retaining nutritional quality attributes has increased interest in emerging preservation techniques (Lima *et al*. 2018). There is a growing body of literature that suggests that processing properties of various methods can change the chemical composition and antioxidant properties of the macroalgae which could interfere with their intended purpose as functional bioproducts for food, feed and feed ingredients, pharmaceutical and other industrial applications (Gupta *et al.* 2011; Badmus *et al*. 2019;

Amorim *et al.* 2020). Drying processes such as sun-drying and oven-drying have been the most used methods for seaweed processing (Kadam *et al.* 2015). Drying suppresses microbiological activity and delays or prevents chemical processes (such as oxidation or reduction) that lead to food deterioration thus extending shelf life (Gupta and Abu-Ghannam 2010; Badmus *et al.* 2019). Both positive and negative impacts on the seaweeds have resulted from these processes depending on the temperature and duration of exposure, among other factors. For example, heat treatment at temperatures of 85-121 °C reduced the antimicrobial activity of brown Irish edible seaweeds, *Himanthalia elongata, Laminaria sachharina* and *L. digitata*, compared to the raw untreated seaweeds. The effect was more pronounced against Gram negative bacteria (Gupta *et al.* 2011). Conversely, Low drying temperatures on macroalgae, such as freeze-drying and oven-drying at 40 °C, yielded products with higher concentrations of nutritionally significant compounds and enhanced antioxidant activity (Badmus *et al.* 2019). Despite the extensive studies of the effect of different drying treatments of macroalgae, information of other processing treatments is still scanty.

Several unconventional preservation procedures are being developed to meet consumers' demands for nutritive and sensory quality food. Studies have shown that application of UVC radiation on harvested products can increase phenolic content and also antioxidant enzymatic activities to fight against oxidative stress in fruits and vegetables (Rivera-Pastrana *et al.* 2014; Sari *et al.* 2016; Lima *et al.* 2018). The limitation of UVC is that it has low penetration on products with complex surfaces due to the shading effect of the surfaces (Deng *et al.* 2020). Optimal processing methods should be designed and tailored for each macroalgal species based on the final products' quality standards (Stévant and Rebours, 2021). No study thus far has documented the effect of other macroalgae processing treatments or their combinations on abalone growth besides drying.

In this study, the macroalgae (*Ecklonia maxima*, *Ulva lacinulata* and *Gracilaria gracilis*) were subjected to a combination of biosecurity processes known to eliminate potential pathogens, and that were developed previously (Chapter 2). The effects of the processed macroalgae on the growth performance of abalone *Haliotis midae* were assessed. The growth indicators: shell length gain, mass gain, specific growth rate and condition factor of abalone fed with the diet containing biosecure and non-biosecure macroalgae were compared.

3.2 MATERIALS AND METHODS

3.2.1 Experimental system

The study was conducted at Wild Coast Abalone Farm (Pty) Ltd located the east coast region of South Africa (32° 45' 02.7" S, 28° 16' 29.0'' E), and data were collected under full commercial production conditions. The farm operates as a land-based, pump-ashore, flowthrough aquaculture system. All seawater for the production of abalone at this farm is pumped ashore into the abalone rearing tanks from the bordering Indian Ocean using eight pumps, which supply 6250 m^3 of seawater to the farm every hour. Water inflow to each experimental abalone rearing tank was maintained at 75.80 ± 0.53 L/min to allow seven full seawater exchanges per tank per day according to standard practices on the farm. These abalone rearing tanks, measuring 500 × 200 × 80 cm (length × breadth × height), were made of polyvinyl chloride (PVC), and were supported by wooden frames (Figure 3.1).

The abalone in each tank were stocked in oyster mesh baskets (94 × 54 × 46 cm; length × breadth × height) that were suspended in the tanks. Each basket contained five vertically orientated square high-density polyethylene plates that formed a rack to increase the surface area within the basket. The vertically orientated rack was covered with a rectangular corrugated fibre-crete feeder plate (60 × 45 cm; length × breadth), which was placed horizontally on top

of the vertical plates, approximately 10 cm below the surface of the water. These baskets were suspended in the rearing tanks and each tank contained eight abalone baskets arranged in series. The abalone baskets were moved to new tanks weekly to enable the tanks to be cleaned, according to the standard farm practice.

A B

Figure 3.1. (A) A commercial abalone production tank at the Wild Coast Abalone Farm (Pty) Ltd, similar to that used in this experiment. (B) Two oyster mesh baskets suspended in a production tank, showing a horizontal feeder plate with abalone attached, that had been removed from the water, similar to that used in this experiment.

3.2.2 Experimental animals and stocking

Sub-adult abalone (35-45 g and 55-65 mm) obtained from the same broodstock and same cohort were used as the experimental animals for this study. Approximately 10.5 kg of abalone were stocked in each basket per the farm's standard practice. The animals were acclimated for a period of two weeks and purged for 48 h before the feeding trial started.

3.2.3 Diets and experimental design

Ecklonia maxima was collected from the seashore in front of the Department of Forestry, Fisheries, and the Environment (DFFE) Marine Research Aquarium in Sea Point, Western Cape, South Africa, while *U. lacinulata* and *G. gracilis* were harvested from production tanks

on Wild Coast Abalone Farm Pty Ltd. In the Eastern Cape. The *U. lacinulata* and *G. gracilis* samples were collected on the same day while *E. maxima* was collected two days later since the sites are in different locations. Twenty kilograms fresh macroalgae were collected for each of the macroalage species. The macroalgae were first washed to remove any debris, then divided into two batches of 10 kg each. The first batches were subjected to a combination of three biosecurity processing treatments: disinfection using povo-iodine solution (5000 mg/L) for 20 min, oven drying at 40 ℃ for 48 h and UVC treatment for 10 min (Section 2.2.4 (b), Chapter 2). A separate batch of macroalgae was only dried at 40 ℃ for 48 h, which is the standard for existing commercial feed manufacture processes in diet ingredient preparation and that provided a comparison with the biosecurity-treated macroalgae.

Isoenergetic and isonitrogenous experimental diets were then formulated to incorporate 3 % of the dry mass of the macroalgae that had and had not been subjected to the biosecurity processing treatments. Samples of the macroalgae, with and without biosecurity treatment, and all the experimental diets were submitted for proximate analysis. The composition of all the formulated diets was identical to the control (Abfeed^{TM} S34®; Marifeed (Pty) Ltd, Hermanus South Africa (http://www.marifeed.com/abfeed/) except that the control was formulated without macroalgae. The experimental diets used in this study were as follows:

(T1) Abfeed^{TM} S34® (formulated diet/control, no macroalgae)

(T2) formulated diet + biosecure gracilaria (Abfeed_b_*Gracilaria*)

(T3) formulated diet + non-biosecure gracilaria (Abfeed_nb_*Gracilaria*)

(T4) formulated diet + biosecure ulva (Abfeed_b_*Ulva*)

(T5) formulated diet + non-biosecure ulva (Abfeed_nb_*Ulva*)

(T6) formulated diet + biosecure kelp (Abfeed_b_kelp) and

(T7) formulated diet + non-biosecure kelp Abfeed_nb_kelp).

The seven diet treatments were each fed to four replicate baskets of abalone using a randomized block design with each treatment represented once in each tank to ensure standardized conditions. All the experimental baskets were clearly labelled to indicate the treatment they belonged to and the feeding strategy to which they were subjected. Feeding was carried out daily between 15.00-17.00 using standard farm feeding procedures of 0.3-0.4 % of body weight per day (Nel, 2016). The rearing tanks were cleaned twice weekly. The trial was conducted for 150 days. Sampling was done at the start of the experiment, after one month, four months and at the end of the experiment (five months). Mortalities on the abalone baskets were monitored on daily basis during the entire experiment.

Table 3.1. The proximate composition of (A) the algae that were used in making the diets and (B) the dietary treatments that included the algae that were subjected to either biosecure process (b) nor not subject to a biosecure process (nb). The values displayed are expressed as a percentage of g/kg of total dry matter.

	Ash (%)	**Moisture (%)**	**Protein (%)**	**Fat (%)**	**Carbohydrates (%)**
(A) Algal ingredients					
Biosecure *Gracilaria*	35.74	6.57	18.36	0.53	38.80
Non-biosecure *Gracilaria*	42.68	5.39	17.86	0.36	33.71
Biosecure kelp	27.43	8.54	8.84	0.36	54.83
Non-biosecure kelp	25.24	8.63	8.79	0.80	56.54
Biosecure *Ulva*	32.82	8.03	14.69	1.13	43.33
Non-biosecure *Ulva*	40.87	7.60	11.86	1.04	38.63
(B) Dietary treatments					
Abfeed^{TM} S34®	5.77	12.38	28.00	2.81	51.04
Abfeed_b_*Gracilaria*	6.75	12.36	29.93	3.30	47.66
Abfeed_nb_*Gracilaria*	6.69	12.75	29.88	3.14	47.54
Abfeed_b_*Ulva*	6.45	12.77	29.99	2.99	47.80

Abfeed_nb_*Ulva*	6.37	12.62	29.46	3.35	48.20
Abfeed_b_kelp	6.16	12.26	30.09	3.61	47.88
Abfeed_nb_kelp	5.84	12.09	30.59	3.28	48.20

3.2.4 Growth parameters

The growth and condition of abalone within each treatment were assessed. The total combined weight of all the abalone in each of the baskets was measured using an electronic balance (Scalemass, East London, South Africa) and this total biomass per basket was recorded to the nearest one gram. These data were collected at the start of the trial, and again after 1, 4 and 5 months. Samples of thirty individual abalone were then taken randomly from each basket, excess water was allowed to drain off the abalone and the shell was blotted dry using a paper towel to remove excess water before recording individual shell lengths and weights. Individual abalone weight was recorded to the nearest 0.01 g using an electronic balance (Kern PLS 4200-2F, Balingen, Germany), while shell length was measured along the longest axis to the nearest 0.01 mm using Vernier calipers. These individual weight and length data were collected at the same time intervals presented above, and were used to calculate abalone shell length gain, mass gain, condition factor, feed conversion ratio and specific growth rate.

The condition factor (CF) was calculated according to the formulae of Britz (1996), where a CF of one is considered average and above one is considered above average. The CF was calculated from the mean length and weight of abalone in each treatment (n = 4 replicates) using the following equation:

$$\text{Condition factor (CF)} = 55575 \times (\text{weight (g)}/\ \text{length (mm)}^{2.99})$$

The specific growth rate (SGR) (% body weight increase per day) was calculated from the mean weight of abalone in each treatment using the following equation:

$$SGR = ((\ln (W_f) - \ln(W_i))/ t) \times 100$$

The feed conversion ratio (FCR) was calculated as the dry mass of feed applied/biomass gain per abalone basket (Naylor *et al*. 2011).

3.2.5 Water quality parameters

Water quality parameters: temperature, dissolved oxygen (DO) and pH were measured weekly from each of the abalone rearing tanks. Water temperature was measured using a pre-calibrated temperature logger (Ebro, Xylem Analytics, Germany). Dissolved oxygen was measured using a DO meter (Oxyguard handy Polaris, Denmark) while pH was measured using a pH meter (Thermo Scientific Eutech Expert, Singapore). Water quality parameters such as nitrates, nitrites and ammonia were not recorded in this study because the farm maintained the tank inflow flow rates at optimal range also there was no cause of alarm.

3.2.6 Data analysis

The mean length and weight data from each replicate for all dietary treatments (T1 to T7) were compared using one-way, repeated measures analysis of variance (RM-ANOVA). In addition, the interaction between macroalgae type (factor 1: *Ulva*, *Gracilarira* and kelp) and biosecurity process (factor 2: biosecure and not biosecure; T2 to T7 and excluding the Abfeed Reference diet) were tested using multifactor, repeated measures ANOVA (multi-factor RM-ANOVA). These data were tested for the assumptions of an ANOVA using a Sphericity test and, if necessary, adjusted using a univariate test for repeated measures. Mean specific growth rate and mean length increase per month were compared using one-way ANOVA (T1 to T7) and interaction between the same two factors were tested using multi-factor ANOVA. These data were tested for homogeneity of variance (Levene, 1960) and normal distribution of residuals (Shapiro-Wilk, 1965), and if the assumptions were not met, the data were natural log

transformed. If they still did not meet the assumptions, a nonparametric Kruskal-Wallis ANOVA was used to compare the treatments. Significance was assigned to p-values of < 0.05 for all analyses.

3.3 RESULTS

3.3.1 Growth parameters

Body weight and shell length

Abalone weights at the end of the 150-day feeding trial were compared between the seven dietary treatments, i.e., all treatments including the Abfeed® reference diet (Figure 3.2). For the entire experiment period, mortalities ranged between 0-3 per abalone basket. This was uniform in all the abalone baskets/treatments. No significant differences in the abalone weights were recorded between the dietary treatments (RM-ANOVA: $F_{(18,63)} = 0.706$; $p = 0.792$; Figure 3.2). For all these treatments combined, there was a significant increase in abalone weight over time (RM ANOVA: $F_{(3,63)} = 440.25$; $p < 0.001$). In the multifactorial analysis, where each algae type included either biosecure or non-biosecure algae in the diet, the biosecurity process method (biosecure vs. non-biosecure) did not have an impact on abalone weights, since no significant differences in mean weights were recorded between animals fed with the biosecure and non-biosecure macroalgae diets over time (multi-factor RM-ANOVA: $F_{(3,54)} = 0.140$; $p = 0.935$). Similarly, the type of macroalgae (kelp, *Gracilaria* and *Ulva*) included in the formulated feed did not significantly affect the weight of the abalone (multi-factor RM ANOVA: $F_{(6,54)} = 0.479$; $p = 0.821$). There was also no significant interactive effect of macroalgae type and process method (biosecure vs. non-biosecure) on abalone weights (multi-factor RM ANOVA: $F_{(6,54)} = 0.463$; $p = 0.832$).

A similar trend was observed in the abalone shell length over the growth period. Overall, the mean shell length of the abalone increased significantly over time irrespective of the macroalgae type and biosecurity process (multi-factor RM-ANOVA: $F_{(3,54)} = 507.933$; $p < 0.001$). However, no significant difference was observed between the seven diet treatments (RM-ANOVA: $F_{(18,63)} = 0.941$; $p = 0.535$). In the factorial analysis, both the macroalgae type and biosecurity process did not affect the shell length of the abalone (RM-ANOVA: $F_{(6,54)} = 0.775$; $p = 0.593$ and $F_{(3,54)} = 0.143$; $p = 0.934$, respectively). In addition, there was no significant interactive effect between the type of macroalgae and the biosecurity process on abalone shell length (multi-factor RM-ANOVA: $F_{(6,54)} = 1.196$; $p = 0.323$; Figure 3.3).

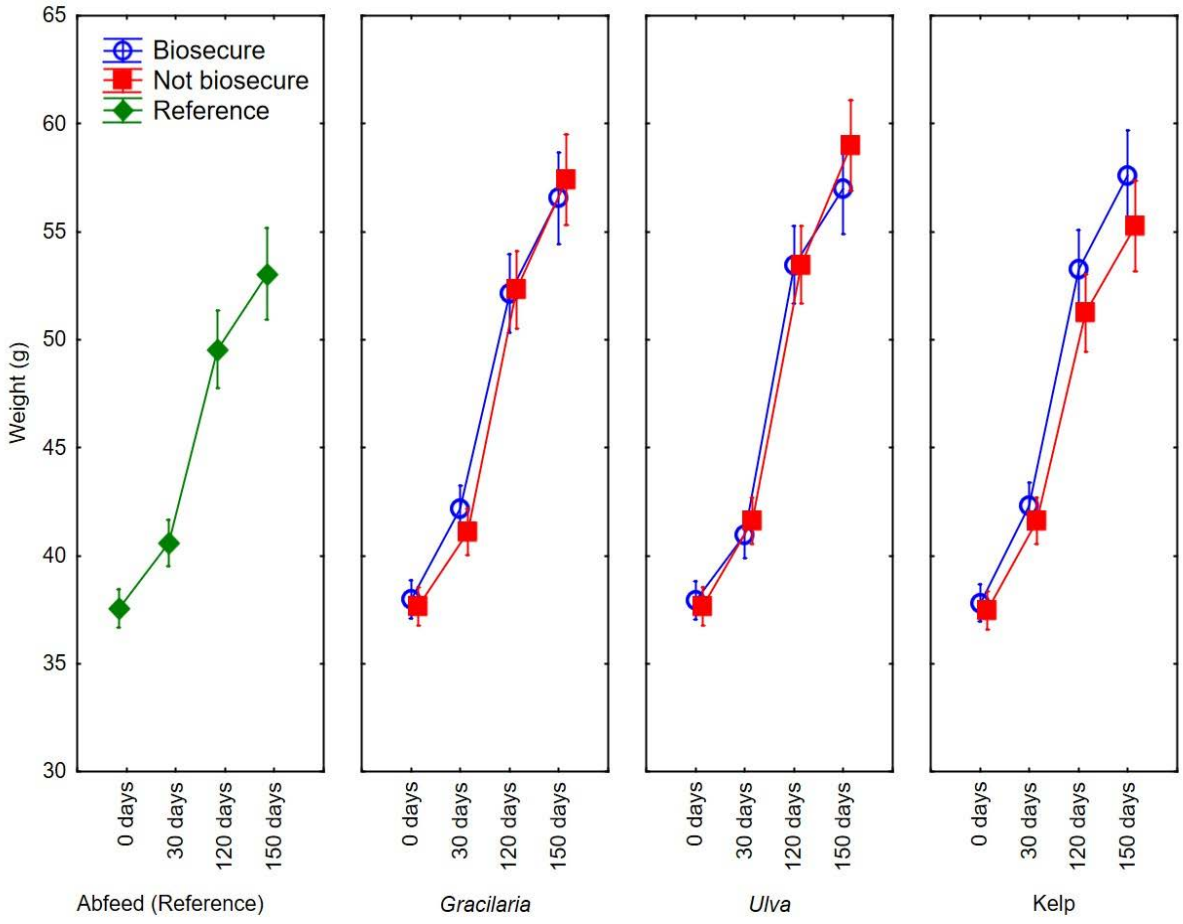

Figure 3.2. Mean body weight (± SE) of abalone H. midae fed with formulated diets containing biosecure and non-biosecure macroalgae (RM-ANOVA: $F_{(18,63)} = 0.706$; $p = 0.792$).

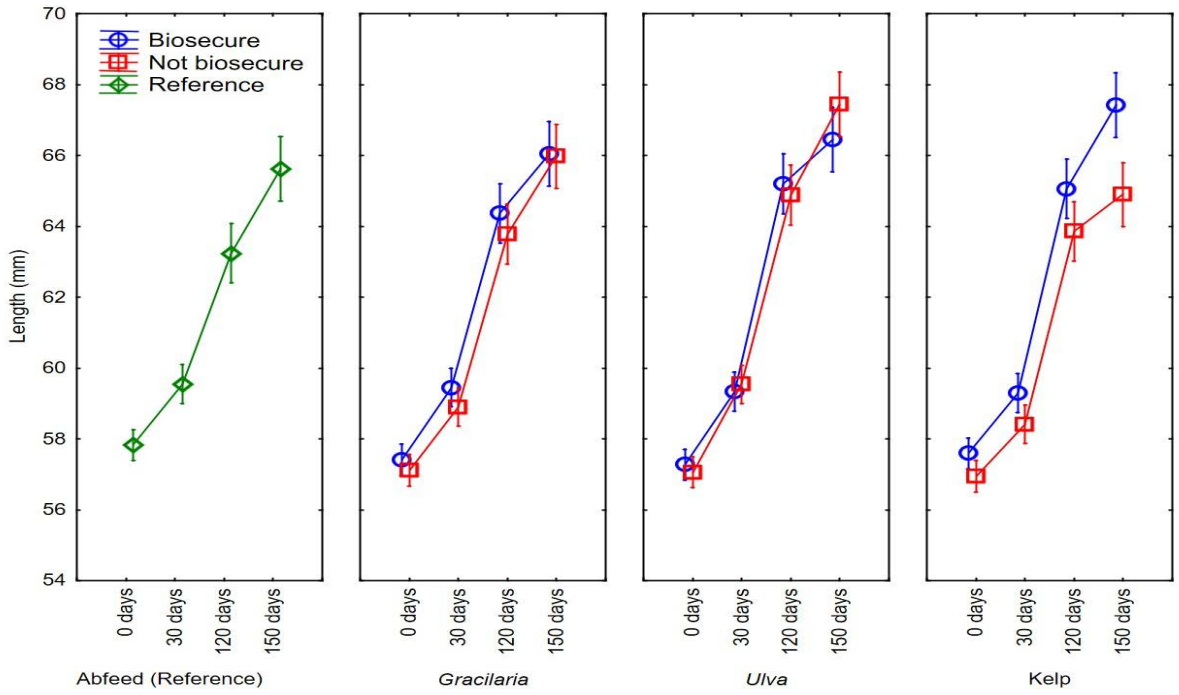

Figure 3.3. Mean shell length (± SE) of abalone H. midae fed with formulated diets containing biosecure and non-biosecure macroalgae (RM-ANOVA: $F_{(18,63)} = 0.941$; $p = 0.535$).

Specific growth rate and length gain

The mean specific growth rate (SGR; (% bw/d) was compared across all the diet treatments. No significant differences were detected between the diets (One-way ANOVA: $F_{(6,21)} = 0.731$; $p = 0.630$). Similarly, no significant differences in the growth rates were recorded between the formulated diets supplemented with the biosecure and non-biosecure macroalgae or between the different macroalgae types (multifactor ANOVA: $F_{(1,18)} = 0.190$; $p = 0.668$ and $F_{(2,18)} = 0.244$; $p = 0.786$ respectively). The interaction between macroalgae type and biosecurity process did not affect the mean specific growth rate of the abalone (multifactor ANOVA: $F_{(2,18)} = 0.465$; $p = 0.635$; Table 3.2). No significant differences in the shell length increase per month (mm/month) were detected between the diet treatments (Kruskal-Wallis test: $H_{(6, N=28)} = 5.209$; $p = 0.517$; Table 3.2). Likewise, the interaction between the macroalgae type and biosecurity treatment did not have a significant impact on the length increase per month (multi-factor ANOVA: $F_{(2,18)} = 2.272$; $p = 0.132$).

Condition factor and feed conversion ratio

The condition factor (CF) of the abalone was compared between the diets at the end of the 150-day feeding trial. Abalone from all the dietary treatments had above average condition factor at the end of the study ranging from 1.08 ± 0.010 to 1.18 ± 0.012 across the dietary treatments (Table 3.2). No statistically significant differences were however recorded between abalone fed the different diets. Additionally, the biosecurity process and macroalgae type did not significantly influence the condition factor of the abalone (multifactor ANOVA: $F_{(2,21)}$= 2.404; p = 0.138 and $F_{(2,21)}$ = 0.629; p = 0.545 respectively). On the other hand, the FCR of the abalone fed with the different diets ranged from 0.94 to 1.02.

Table 3.2. Mean (± SE) condition factor (CF), and feed conversion ratio (FCR), specific growth rate (SGR) and length increase (mm/month) of abalone H. midae fed with formulated diets containing biosecure (b) and non-biosecure (nb) macroalgae for five months.

Diet treatments	Length increase (mm/month)	SGR (% bw/d)	Initial CF	Final CF	FCR
Abfeed	1.560±0.28	0.228±0.03	1.127±0.01	1.081±0.01	1.02
Abfeed_b_*Gracilaria*	1.726±0.04	0.265±0.01	1.168±0.01	1.140±0.01	1.02
Abfeed_nb_*Gracilaria*	1.772±0.18	0.281±0.02	1.170±0.01	1.165±0.01	1.01
Abfeed_b_*Ulva*	1.835±0.15	0.271±0.02	1.179±0.01	1.157±0.04	1.07
Abfeed_nb_*Ulva*	2.078±0.12	0.299±0.02	1.180±0.01	1.117±0.01	0.94
Abfeed_b_kelp	1.966±0.18	0.2776±0.04	1.153±0.01	1.086±0.01	0.99
Abfeed_nb_kelp	1.590±0.18	0.260±0.02	1.198±0.01	1.176±0.01	1.08

3.3.2 Environmental variables

The water temperature in the abalone tanks ranged between 18.5 ± 0.17-22.5 ± 0.22 during the entire feeding trial. Dissolved oxygen and pH in the abalone tanks averaged 8.37 ± 0.14 mg/L and 8.47 ± 0.07 respectively.

3.4 DISCUSSION

Growth provides an indication of feed performance and how different feed ingredients impact the weight, length, and width of the animals, which is important in ensuring that cultured animals reach a marketable size and optimal condition within a time that is economically viable. Specific feed ingredients, such as seaweeds at inclusion level of as low as 0.44 % to 20 %, have been shown to improve the growth, health, physiology, and product quality of cultured abalone (Mulvaney *et al.* 2013; Kemp *et al.* 2015; Bansemer *et al.* 2016; Nel *et al.* 2017), as well as other invertebrates such as sea urchins (Cyrus *et al.* 2014, 2015ab) and shrimp (Schleder *et al.* 2018; Abbas *et al.* 2023;). However, biosecurity concerns around the use of seaweeds as supplementary feeds (Kua *et al.* 2011) or as ingredients in formulated feeds are preventing wider adoption of seaweeds as a functional ingredient for aquafeeds. A processing treatment for reducing the biosecurity risks of seaweeds added to formulated feed for abalone was previously developed (see Chapter 2), but the effect of the biosecure feeds and non-biosecure feeds on the growth and condition of abalone remained to be determined. In this study, it was demonstrated that the biosecurity process did not have a significant impact on the macroalgae and the subsequent growth performance and condition of the abalone. These results imply that macroalgae can be processed to achieve microbiological safety without losing their nutritional and health benefits.

In this study, the shell and weight gain, specific growth rate (SGR), and condition factor (CF) of the abalone fed with diets containing macroalgae did not differ significantly from those fed with the control diet that was not supplemented with macroalgae. On the contrary several published studies on abalone nutrition have reported significant growths of abalone fed with macroalgae supplemented diets. (Durazo-Beltrán *et al.* 2003; Naidoo *et al.* 2006; Dlaza *et al.* 2008; Mulvaney *et al.* 2013; Bansemer *et al.* 2016; Nel *et al.* 2017) For example, the inclusion of 0.4–3.54 % of kelp (*E. maxima*) in formulated feed promoted faster growth (65.7–74.5 % total mass gain) of *H. midae* when compared to animals fed on non-kelp supplemented feed (Nel *et al.* 2017). Similarly, post-weaning juvenile *H. midae* fed fishmeal-based formulated diets supplemented with fresh wild collected macroalgae (*E. maxima* and *U. lactuca*) were found to have improved specific growth rates, daily increases in shell length and condition factor (CF) compared to abalone fed diets without seaweed supplementation (Dlaza *et al.* 2008). A macroalgal-formulated pellet combination diet which consisted of 76.1 % macroalgae and 23.9 % pelleted diet as dry weight yielded a significantly higher specific growth rate than a formulated pellet diet (Kemp *et al.* 2015). The results of this study can be attributed to the low inclusion levels of the dried macroalgae (3%) and the experimental period which was not long enough to record significant changes.

The growth performance recorded for abalone fed the biosecure and non-biosecure macroalgal diets in the present study did not differ significantly. The biosecurity treatments applied to the seaweeds tested in this study, which included oven drying (40 °C), disinfection by povo-iodine and exposure to UVC light, have been used in processing macroalgae and other food products with success. However, these treatments have never been tested in combination. The drying temperature (40 °C) used in this study is widely used in the macroalgae industry and has been demonstrated to preserve bioactive compounds in macroalgae (Moreira *et al.* 2016; Badmus *et al.* 2019; Stévant and Rebours, 2021). For example, the total polyphenol (TP) content and

antioxidant activity of *Fucus vesiculosus* seaweed decreased when the drying temperature was raised. The significantly high TP content was achieved for seaweed dried at 35 °C, while increasing the temperature to 50 °C reduced the TP by 37 % and diminishes up to 54 % when a drying temperature of 75 °C was employed (Moreira *et al.* 2016).

On the other hand, UVC and povo-iodine have been used to achieve the microbiological safety of a variety of food products and have been demonstrated to cause minimum changes in the nutritional and sensory quality of foods (Chun *et al.* 2009; Kerrison *et al.* 2016). Several studies have shown that UVC radiation can increase phenolic content and antioxidant enzymatic activities as a defense mechanism against oxidative stress in fruits and vegetables (Rivera-Pastrana *et al.* 2014; Sari *et al.* 2016; Lima *et al.* 2018).

Despite the experimental diets being standardized and having similar macronutrients, the results obtained in this study suggest that the biosecurity treatments employed caused minimum changes to the quality of the macroalgae and hence, the final product quality of the feed as well as the growth of the cultured abalone. Macroalgae can therefore be processed to eliminate potential hazards and be incorporated into the abalone diet without compromising the functional potential of the ingredients and their growth benefits.

3.5 CONCLUSION

The biosecurity treatments used in this study had no significant effect on the growth-promoting factors of the macroalgae hence there were no significant differences in the growth performance of the fed abalone. The processing treatments employed in this study can be applied to the macroalgae and used in the feed industry to produce biosecure feed, without compromising the growth of the farmed abalone.

CHAPTER 4

EFFECTS OF BIOSECURE MACROALGAE ON THE GUT MICROBIOME OF ABALONE *HALIOTIS MIDAE*

4.1 INTRODUCTION

Most animals and plants depend on interactions with specific microorganisms to maintain their existence. A better understanding of these complex host-microbiome interactions across diverse hosts/environments such as the human gut, plant rhizosphere, sponges, seaweeds and various aquacultured organisms, amongst others, has been made possible by the recent emergence of high-throughput molecular methods, including next-generation sequencing (NGS) technologies (Turnbaugh *et al.* 2007; Singh and Reddy, 2016; Gobet *et al.* 2018). These technologies have vastly improved our ability to decipher the complex bacterial communities associated with the animal gut, as well as other host tissues and environments, to better understand the supportive role(s) of microorganisms in essential physiological activities such as growth, development, metabolism, food digestion through the provisioning of digestive enzymes, nutrient assimilation, and defense against invasion by foreign/opportunistic bacterial species, which in turn may prevent disease outbreaks (Hakim *et al*. 2015; Cicala *et al.* 2018; Danckert *et al.* 2020).

Studies conducted on abalone gut microbial flora have revealed important understanding into the role of these microorganisms in food assimilation, providing crucial information to the abalone farming industry (Erasmus *et al.* 1997; Guo, 2017), including aquafeed nutritionists and manufacturers. Furthermore, understanding the role of microorganisms resident in the gut of abalone is a crucial aspect for the improvement of growth rates of abalone considering their roles in digestive processes (Gobet *et al.* 2018). The gastrointestinal microbiota of abalone contains a very complex bacterial assemblage that is vital to the general health of these

gastropods (Cicala *et al.* 2018). However, this only occurs when the microbes and the host are in a symbiotic state (balanced state). Compromised immune status and auto-immune diseases have been associated with a dysbiotic (unbalanced) state of the gut microbiome (Kostic *et al*. 2013; Knight, 2017) that will impact the general health and growth of an organism. For example, the functional interactions between dietary fiber, the gut microbiota, and the colonic mucus barrier, which serves as a primary defense against enteric pathogens were studied using a gnotobiotic mouse model, colonized with a synthetic human gut microbiota (Desai *et al*. 2016). It was discovered that the gut microbiota turns to host-secreted mucus glycoproteins during chronic or intermittent dietary fiber deficiency as a source of nutrients, causing the colonic mucus barrier to erode and subsequently encouraging greater epithelial access and lethal colitis by the mucosal pathogen, *Citrobacter rodentium* (Desai *et al*. 2016).

The composition of the abalone gut microflora is influenced by a variety of factors such as diet, seasons, ontogenetic stages, and the bacteria in the surrounding aquatic environment, among others (Ingerslev *et al.* 2014). Several studies have examined bacteria isolated from the gut of abalone in relation to these factors and aquaculture production systems in general. For example, seasonal changes and abalone age were found to significantly impact the intestinal microbiomes of hybrid (*Haliotis laevigata x H. rubra*) and greenlip (*H. laevigata*) abalone grown over one year (Danckert *et al.* 2020). Similarly, the diversity and digestive microbiota profile of the European abalone *H. tuberculata* fed with different algal diets were shown to be linked to seasonal variations and not the diets administered to the animals (Gobet *et al.* 2018). In another study, the gut microbiota of juvenile abalone *H. discus hannai* changed when the abalone food sources were switched from microalgae to algal pellets. In addition, the gut microflora of juvenile abalone fed on microalgae matched the microflora cultured from seawater (Tanaka *et al.* 2003).

The inclusion of both fresh and dried macroalgae or their extracts in aquafeed has been found to have immunomodulatory effects on the fed animals (Peixoto *et al.* 2019; Gonçalves *et al.* 2022). The polysaccharides and other bioactive compounds in macroalgae have prebiotic effects and influence the intestinal microbiota by stimulating the growth of beneficial bacterial communities, thus exerting growth-promoting and health-improving effects on the host (Erasmus *et al.* 1997; Macey and Coyne, 2005; Nel *et al.* 2017; Gonçalves *et al.* 2022). In addition, fresh macroalgae have their own bacterial communities (Singh and Reddy, 2016) and when digested, many of these bacteria become resident within the digestive tract of the animals but can also be transient. Either way, they contribute towards the digestion of dietary ingredients, particularly the complex polysaccharides found within seaweeds, and facilitate the assimilation of feeds ingredients. In some cases, the supplementation of diets with macroalgae is considered the most promising method of controlling disease in aquatic animals because of the immunostimulants that they possess, beneficial microorganisms they introduce, and the potential modulatory effect seaweeds and their associated microbiota may have on the gut microbiome (Egan *et al.* 2013; Newaj-Fyzul and Austin, 2015; Hindu *et al*. 2019; Califano *et al.* 2020). For example, more stable gut bacterial communities were present in the gut of the South African abalone when fed with a kelp-supplemented formulated diet, whereas animals fed a control diet that was not supplemented with seaweed had more potential opportunistic bacteria (Nel *et al.* 2016). The latter observation could have important implications for animals, such as abalone, that are subjected to stress during their production cycle, something which is highly likely in intensive aquaculture. An improved understanding of the effects of various diets and dietary ingredients on the gut microbiome is therefore essential for improved management of stock health on aquafarms.

In most cases, macroalgae are supplemented in abalone diets either in fresh state or after some form of processing such as drying. However, no direct assessment of the impacts of these

processing treatments on the gut microbial composition of the animals fed with the processed macroalga diets has been made. Previous studies have shown that some processing treatments can change the nutritional compounds and phytochemical contents of seaweeds which in turn could compromise their functional benefits (Carrihlo *et al.* 2014; Susanto *et al.* 2017). For example, the microbial diversity and antimicrobial properties of Irish edible brown seaweeds changed following heat treatment at 95 ºC for 15 min, with a complete inactivation of surface microflora observed. In addition, heating the seaweed resulted in the reduction of antimicrobial activity of the macroalga when compared to raw, untreated seaweed (Gupta *et al.* 2011). Such findings suggests that the processing of macroalga could indirectly impact the gut microbiota of the animals fed with these diets.

To date, research on the impact of seaweeds as a supplementary diet or dietary ingredient on host-microbiome interactions in the South African abalone *H. midae* is limited. No study thus far has tested the effect of processed macroalgae on the gut microbial composition of this important commercial species. The overall aim of this study was to provide new knowledge on the gut microbiome composition of the South African abalone *H. midae* fed varying diets in order to support the production of biosecure macroalgae formulated diets that will not compromise their benefits to the growth and health of farmed abalone. The objectives of this study were to identify and compare the bacterial communities in abalone that were fed diets containing macroalgae (*Ulva lacinulata*, *Gracilaria gracilis,* or kelp (*Ecklonia maxima*)) that had been subjected to biosecurity treatment (including combination of disinfection using povo-iodine solution (5000 mg/L) for 20 min, oven drying at 40 ℃ for 48 h and UVC treatment for 10 min) and those that were not, using a NGS approach. The NGS approach was also used to determine the gut microbiome profile of the abalone fed with a formulated diet supplemented with fresh *U. lacinulata* and *G. gracilis* to assess the potential modulatory effect seaweeds and their associated microbiota may have on the gut microbiome of *H. midae.*

4.2 MATERIALS AND METHODS

4.2.1 Experimental animals, treatments and husbandry

Experimental diets were formulated to incorporate each macroalgae, including kelp, *Ulva lacinulata* or *Gracilaria gracilis*, with and without biosecurity treatment (Section 3.2; Chapter 3). Both the bio-secure and non-biosecure feeds contained macroalgae that was dried before incorporated in the diet. The feeding trial was conducted for 150 days, using the same abalone that were subjected to the experimental procedure and husbandry described previously (Sections 3.2.2 to 3.3.3; Chapter 3). An additional treatment of the standard farm diet was included to provide a comparison with the biosecure and non-biosecure macroalgae diet treatments. In this treatment, the animals were grown in the commercial standard farm set up and were fed the formulated diet Abfeed^{TM} S34® supplemented with fresh, macroalgae *U. lacinunata* and *G. gracilis* (Abfeed_fresh seaweed). The fresh macroalgae was supplemented at 0.29% body weight of the animals on alternative days as follows, *U. lacinunata* twice a weekly and *G. gracilis* once weekly.

Table 4.1. The experimental diets tested in this study. These diets included the algae that were subjected to either a biosecure process (b) or not subject to a biosecure process (nb) as well as a formulated diet that was supplemented with fresh macroalgae and fed to the abalone for 5.0 months.

Diet	Diet code
Abfeed^{TM} S34® (formulated diet/control)	FF1
Formulated diet + biosecure *Gracilaria* (Abfeed_b_*Gracilaria*)	FF1_b_G
Formulated diet + non-biosecure *Gracilaria* (Abfeed_nb_*Gracilaria*)	FF1_nb_G
Formulated diet + biosecure *Ulva* (Abfeed_b_*Ulva*	FF1_b_U
Formulated diet + non-biosecure *Ulva* (Abfeed_nb_*Ulva*)	FF1_nb_U
Formulated diet + biosecure kelp (Abfeed_b_kelp)	FF1_b_K
Formulated diet + non-biosecure kelp (Abfeed_nb_kelp);	FF1_nb_K
Formulated diet + fresh *Ulva* and *Gracilaria*	FF1_FS

4.2.2 Sampling and DNA extraction

At the end of the experiment, the animals were starved for 48 h before sampling to limit the presence of ingested food-associated (transient) bacteria in the gut (Nel *et al.* 2017). One healthy animal was sampled from each replicate resulting in a total of four abalone per treatment and a total of 32 animals (Ethics approval no: 2022-1027-6522). The sampled abalone were immediately euthanized by a complete transverse section with a scalpel blade just below the level of the cerebral ganglia and then shucked. To shuck an animal, a scalpel was used to cut the adductor muscle as close as possible to the shell without puncturing the digestive tract tissues (Harris *et al.* 1998). After the shell was removed, the abalone was mounted on the pre-sterilized surface of a dissection tray using sterilized pins and the entire digestive tract was carefully removed from the animal using a scalpel and tweezers. A scalpel was used to carefully dissect away the gills and other tissues and then the entire digestive tract was transferred to a sterile 15 mL centrifuge tube and immediately frozen at -20 °C then transported on ice to the South African Institute for Aquatic Biodiversity (SAIAB) laboratory for DNA extraction (within 12- hours of dissecting). The dissection surface and all equipment used were rinsed, dried and sterilized using 70 % ethanol after each animal dissection to prevent cross-contamination of bacteria between samples.

Once in the laboratory at SAIAB, whole gut samples were ground to a fine powder in liquid nitrogen. An aliquot (ca. 10 mg) of the powdered tissue from each abalone was used for the isolation of total genomic DNA. Total genomic DNA was extracted using a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany, Cat# 51304), following the manufacturer's protocols for the "Isolation of genomic DNA from tissues". The concentration and quality of the isolated DNA from each sample were confirmed using a NanoDrop spectrophotometer (GENOVA NANO, JENWAY, Bibby Scientific Ltd UK) and the DNA quality was assessed by determining the ratio 260/280 ratio, with a ratio of 1.8 indicative of DNA that is pure and free

of protein contaminants (Costa and Roberts, 2014). The extracted DNA from the whole gut tissue samples (n = 32) was then stored at -20℃ until needed for polymerase chain reaction (PCR) amplification and next-generation sequencing (NGS), as described below.

4.2.3 Polymerase Chain Reaction (PCR) amplification of the bacterial 16S rDNA V4 gene region

The V4 hypervariable region (360-370 base pairs) of the 16S rRNA gene was amplified using forward (515F) and reverse (806R) primers: 515F:5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA and 806R:5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT, where the underlined sequences are the adaptor overhang nucleotide sequences used in the Illumina 16S metagenomics workflow The initial PCR mixtures were prepared in a final volume of 25 μL with 1.0 μL genomic DNA, 200 nM of each primer (0.5 μL), 12.5 μL of 2 × KAPA HiFi HotStart Ready mix (Roche) and 10.5 μL PCR grade water. The reactions were run in duplicate to allow for the amplicons to be pooled before amplicon purification and DNA sequencing. The PCR thermal cycling conditions were optimized to a 'touchdown' PCR protocol to improve primer specificity. The PCR cycling conditions were as follows; initial denaturation at 95 °C for 5.0 min followed by 10 cycles of touchdown PCR (30 s at 95 °C, 30 s at 65 °C, with a 1 °C per cycle decrement, and 30 s at 72 °C), followed by an additional 25 cycles of PCR (30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C), and a final extension step for 10 min at 72 °C. A non-template control (PCR-grade H_2O instead of genomic DNA) was included during each PCR amplification to ensure that the reagents were not contaminated and there was only amplification of the bacterial genomic DNA in samples.

4.2.4 Purification of amplicons

The PCR products were purified using a PureLink PCR purification kit (Life Technologies Cat# K3100-01 and K3100-02). Binding buffer B2 (200 μL) was added to the combined (50 μL) amplified PCR products from each sample and mixed well by inverting the tubes several times. Samples were carefully transferred into separate PureLink spin columns that had been placed in a sterile collection tube, before centrifuging the columns at 10000 × g for 1.0 min. The flow-through from each sample was discarded following centrifugation before re-inserting each column into its respective collecting tube and adding 650 μL wash buffer containing ethanol to each column. Samples were centrifuged as before, the flow-through discarded, and each column returned to its collection tube. Columns were centrifuged at a maximum speed for 3.0 min, the collecting tubes were discarded and each column was then transferred to a clean 1.5 mL microcentrifuge (eluting) tube, and 50 μL eluting buffer was added at the center of the column. Columns were incubated at room temperature for 1.0 min before being centrifuged at a maximum speed for 2.0 min. The purified PCR products in each eluting tube were transferred to clean sterile 1.5 mL microcentrifuge tubes and stored at 4 °C. The quality and the size of the purified PCR products, including the negative control, were verified by performing agarose gel electrophoresis. The resultant PCR products, including the negative control, were electrophoresed and the PCR products were visualized using UV illumination.

4.2.5 Library preparation and next-generation sequencing (NGS)

Purified PCR products were used to amplify the 16S rDNA V4 hypervariable region before the addition of Illumina sequencing adaptors and dual-index barcodes, using the Nextera XT Index Kit (Illumina, Cat# FC-131-1001). Following the addition of the indices and sequencing adapters, the library was cleaned using the Mag-Bind Total Pure NGS magnetic bead

(OMEGA) kit and the final library was validated on a Bioanalyzer DNA 1000 chip. The DNA concentrations of the final libraries were determined using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Woldbronn, Germany), and the library concentrations were adjusted to 4 nM by diluting with 10 mM Tris (pH 8.5). Five microliter aliquots of the diluted DNA from each library that had a unique index were pooled. Pooled libraries were denatured with 0.2 N NaOH, diluted with hybridization buffer (HT1) and heat-denatured. Sequencing was conducted using paired-end (PE) Illumina MiSeq sequencing (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The Illumina MiSeq uses 2 × 300 bp PE v3 chemistry, which allows for the overlap and stitching together of paired amplicon reads into one full-length read of higher quality (Comeau *et al.* 2017). A minimum of 5 % PhiX was included in each run to serve as an internal control for lower diversity libraries. Sequences were generated using the 16S-based metagenomics workflow of MiSeq Reporter v2.3 (Illumina) and the resulting raw sequences were demultiplexed based on index sequences and converted to FASTQ files with corresponding Phred quality scores (Tawfiq *et al.* 2018).

4.2.6 Raw data processing and taxonomic assignment

The raw sequence reads were analyzed using available software Quantitative Insights into Microbial Ecology 2 (QIIME2; version 2022.2; http://qiime2.org/) software (Caporaso *et al.* 2010), where raw reads were imported using the Casava input format. This open-source software removes low-quality reads (Phred quality score < Q20) and reads with a low abundance (n < 10 counts) using the Divisive Amplicon Denoising Algorithm 2 (DADA2) plugin in QIIME (Callahan *et al.* 2016). This plugin was also used to merge forward and reverse reads, and screen for and remove chimeric sequences. Taxonomy was assigned to each Amplicon Sequence Variant (ASV) by mapping sequences using a naïve Bayes classifier trained on the SILVA database (release 132) (Quast *et al*. 2013; Glockner *et al*. 2017).

4.2.7. Data analysis

Microbiome Analyst, a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data was used to assess the taxonomic diversity and composition of the abalone gut samples in this study (Dhariwal *et al*. 2017) . The data were filtered to exclude low count reads (minimum 2 counts) and low variance reads, of 10 % based on standard deviation. This step aims to exclude features that are mostly low quality or uninformative to improve downstream statistical analysis (Dhariwal *et al*. 2017). The subsequent filtered data was used to generate rarefaction curves in Microbiome Analyst by randomly sampling a fixed number of reads and assessing the species diversity of each sampling event to show the depth of the sequencing. Alpha diversity (within sample) statistics, and the overall abundance of taxa was also assessed using this marginally filtered dataset. The data were normalized using relative log expression (RLE) transformation (Hawinkel, 2015) to correct the variability in sampling depth and the sparsity of the data to enable more biologically meaningful comparisons when performing multivariate beta diversity (between sample) tests and univariate differential abundance analysis.

The alpha (within sample) bacterial diversity was assessed using the R *phyloseq* (McMurdie and Holmes, 2013) and *vegan* packages (Dixon, 2003) at family, genus and species taxonomic levels, as implemented in Microbiome Analyst. These diversity measures included the Chao1 (Chao, 1984) and Shannon (Shannon, 1948) indices. The statistically significant differences between the samples were analyzed by the analysis of variance (ANOVA; statistical significance at $p < 0.05$).

Beta (between sample) diversity assessments were carried out using the same R packages as described above using the normalized data set. A non-metric multidimensional scaling (NMDS) analysis using a Bray-Curtis dissimilarity matrix, was performed using the non-rarefied dataset to visualize the bacterial community structure across the samples included in

this study. To test the statistical significance ($p < 0.05$) of differences observed between samples, a permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001), permutational analysis of multivariate dispersions (PERMDISP; Anderson *et al.* 2006) and an analysis of similarities (ANOSIM; Clarke, 1993) were performed.

Microbiome Analyst was also used to visualize the relative (%) taxonomic abundance by constructing abundance plots at family and genus level, where only the top 30 taxa were included and all the rest were merged as "Others". Differential abundance of ASVs across the samples collected from abalone fed different diets was assessed using the univariate method, DESeq2 (Love *et al.* 2014), with false discovery (FDR) corrected P-values to reduce the possibility of type I errors (Benjamini and Hochberg, 1995). The core microbiome analysis which shows the set of taxa detected in a high fraction of the population above a given abundance threshold was also performed.

4.3 RESULTS

4.3.1 Data processing

A total of 3,793,834 raw reads were generated across the 32 samples included in this study, with an average of 118,557 reads per sample. After the reads were mapped to a reference database, a total of 1198 ASVs were identified across the 32 samples. Six hundred and fifty two ASVs had less than two counts (>2). With subsequent filtering of data, a total of 248 low abundance features/ASVs were removed based on prevalence (10 %) and a total of 41 low-variance features were removed based on standard deviation (SD). A total of 363 ASVs remained after the data filtering step. The observed ASVs were assigned to 152 families, 249 genera and 272 species. The sampling depth within each sample appeared to be sufficient, with

the rarefaction curves reaching a plateau for all samples (Figure 4.1), demonstrating that only rare species remained to be sampled.

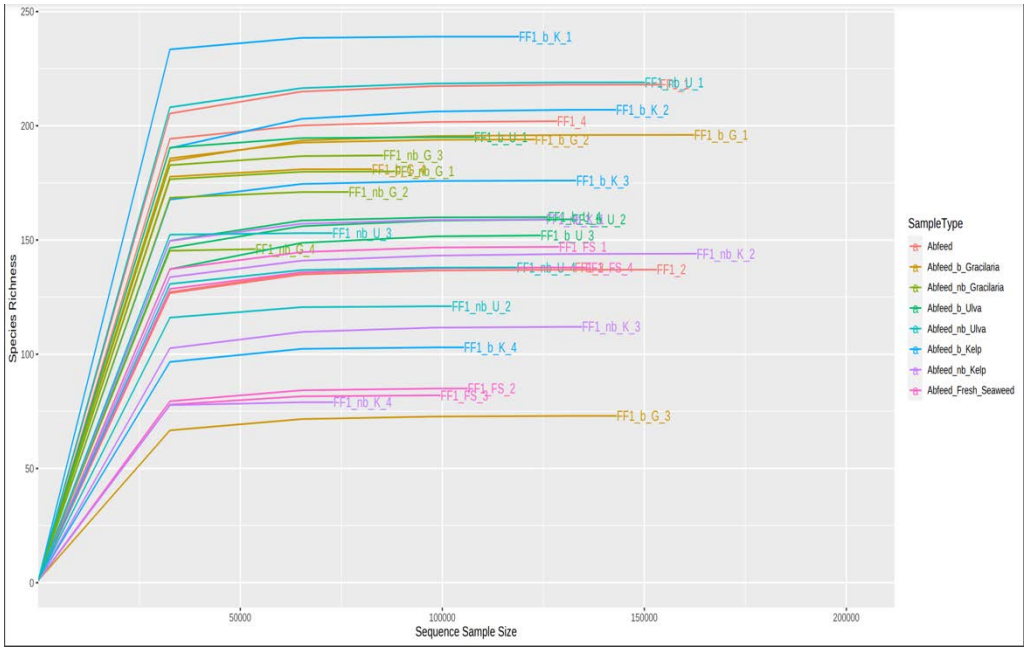

Figure 4.1. The rarefaction curves generated for the 16S rDNA V4 gene sequences for samples obtained from the digestive tract of abalone fed with the biosecure (b) and non-biosecure (nb) macroalgae diets, control (Abfeed) and the Abfeed diets supplemented with fresh macroalgae (Ulva lacinulata and Gracilaria gracilis). Each curve indicates the cumulative number of amplicon sequence variants (ASVs) (species richness) with the plateauing of species richness for each sample when a fixed number of reads are randomly sampled demonstrating that majority of the diversity in these libraries was detected.

4.3.2 Alpha diversity

The richness estimator Chao 1 (which provides an indication of the different kinds or species of organisms present), as well as the evenness indicator Shannon (which provides an indication of the uniformity of the population size of each species present in a sample) was generally higher for the formulated feed supplemented with either bio-secure or non-bio-secure macroalgae (with the exception of the bio-secure kelp treatment), when compared with the formulated feed supplemented with the fresh seaweeds, *Ulva* and *Gracilaria*. However, no significant difference in the two diversity measures were recorded across samples at the three taxonomic levels ($p > 0.05$).

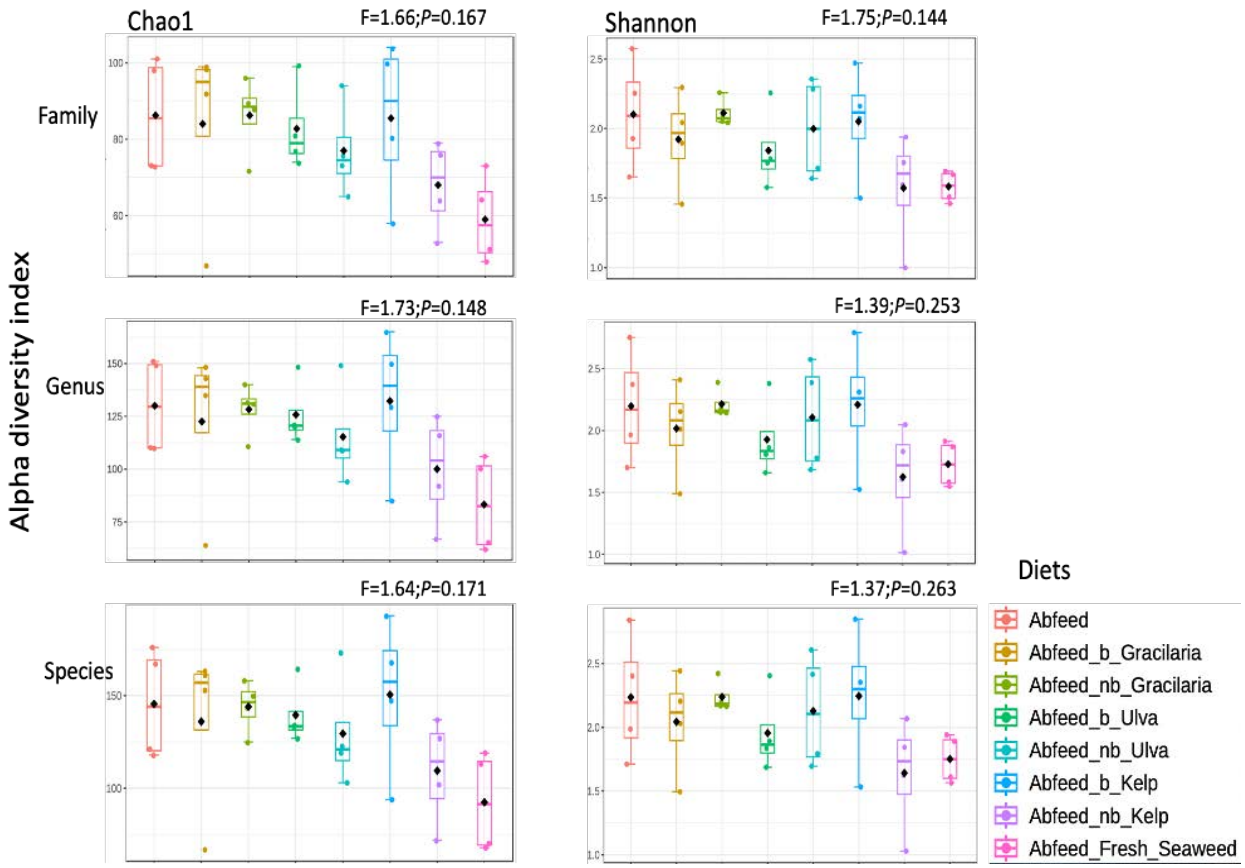

Figure 4.2. An overview of the alpha diversity (Chao1; Shannon) statistics for samples obtained from the digestive tract of abalone fed with the biosecure (b) and non-biosecure (nb) macroalgae diets, control (Abfeed) and the Abfeed diets supplemented with fresh macroalgae (Ulva lacinulata and Gracilaria gracilis) at the family, genus and species level. ANOVA F-values and P-values are indicated for each diversity index across each dietary treatment (n = 4 per dietary treatment).

4.3.3 Beta diversity

The variation in the composition of the microbiome observed between abalone fed the biosecure and non-biosecure macroalgae diets and those fed with mixed diet of formulated diet with fresh seaweed was determined using Bray-Curtis similarity indices and differences were visualized using non-metric multidimensional scaling (NMDS) plots at genus level. Venn diagrams were drawn to show the number of shared genera between the diets including varying macroalgae and the control diet samples. A large degree of overlap was observed for all abalone gut samples for all the diets combined (Figure 4.3). When the samples were analysed based on macroalgae type, a similar observation was made for the abalone gut samples associated biosecure and non-biosecure *Ulva* and kelp diets (Figure 4.4 A&B) indicative of a similar

microbial composition in samples collected from abalone fed with different diets. A more compact, and separated clustering pattern was observed in the abalone gut samples associated with Abfeed^{TM} S34® supplemented with non-biosecure *Gracilaria* and also in the gut of abalone fed with and the mixed diet of formulated diet and the fresh seaweed suggesting a further higher degree of similarity and less variability for samples in those dietary treatment groups (Figure 4.4 A, B, C & D). However, overall, limited differences between samples were observed as shown by PERMANOVA and ANOSIM statistical analyses ($p > 0.05$) (Table 4.2). The PERMDISP analyses, which tests for differences in spread variability among groups, further confirmed that there is no significant difference in the composition of the gut microbiome between treatments (p-values of 0.068-0.0986, Table 4.2). This was also confirmed by the high numbers of the shared genera between the biosecure and non-biosecure diets as shown by the venn diagrams (Figure 4.4 E,F,G &H). Conversely, gut samples of abalone fed with the biosecure macroalgae and the control diets had higher number of unique genera compared to those associated with non-bioseure macroalgae (Figure 4.4 E, F, & G).

Table 4.2. Beta diversity tests based on PERMANOVA, PERMDISP and ANOSIM statistical methods, where compositional differences were observed at the respective macroalga at genus level. Corresponding p-values are indicted in the brackets.

	Macroalgae			
Analysis	***Gracilaria***	***Ulva***	**Kelp**	**Fresh seaweed**
PERMANOVA; R^2	0.302 (0.059)	0.214 (0.293)	0.085 (0.904)	0.257 (0.127)
ANOSIM; R	0.219 (0.057)	0.049 (0.293)	0.206 (0.985)	0.197 (0.118)
PERMDISP; F	3.687 (0.068)	0.013 (0.986)	0.692 (0.525)	0.011 (<0.919)

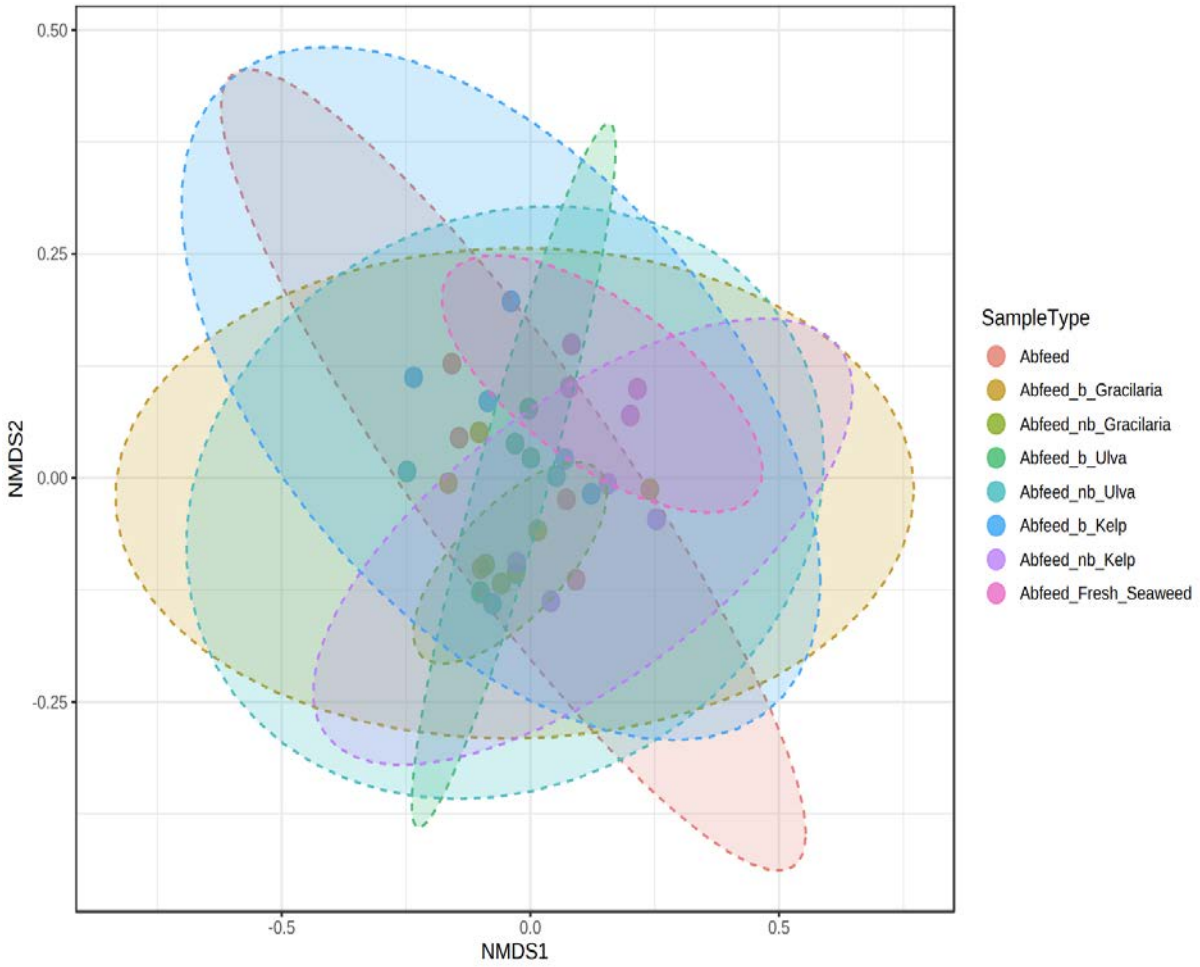

Figure 4.3. Beta diversity analysis of the microbial communities of abalone H. midae fed with formulated diets containing biosecure and non-biosecure macroalgae and formulated diets supplemented with fresh macroalgae. Non-metric multidimensional scaling (NMDS) analysis at the genus level, showed overlap of the bacterial communities across the samples.

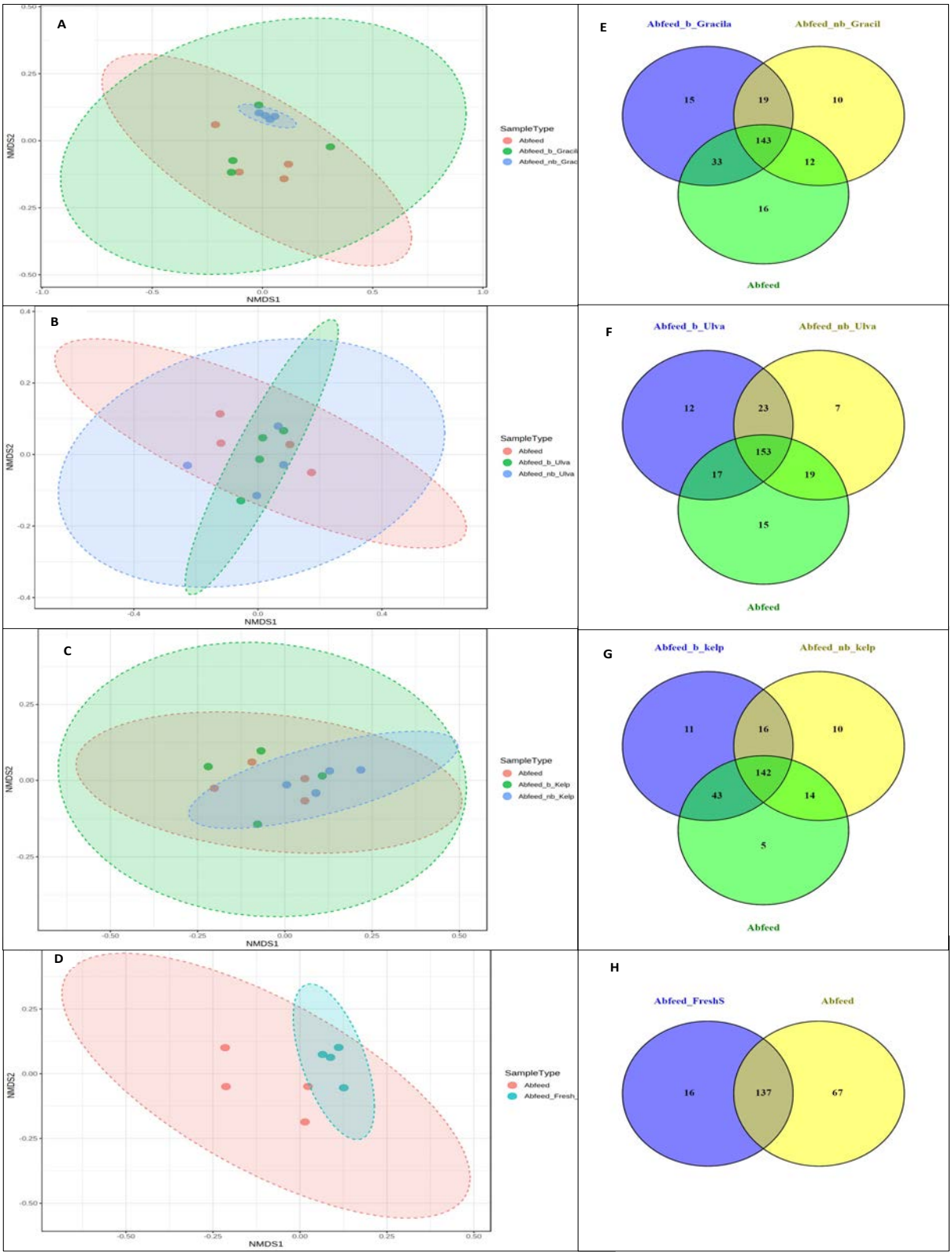

Figure 4.4. Beta diversity analysis of the microbial communities across abalone H. midae digestive tract samples obtained from animals fed with formulated diets containing biosecure (b) and non-biosecure (nb) macroalgae and mixed diet of the formulated diets with fresh macroalgae. (A,B,C and D) Non-metric multidimensional scaling (NMDS) analysis was performed to show sample cluster patterns based on the observed ASVs at the genus level. (E,F,G and H) Venn diagrams showing the total number ASVs and the number of shared or unique ASVs between the different macroalgae with the control Abfeed diet and Abfeed with the fresh seaweed diet.

4.3.4 Taxonomic profiling

For the taxonomic abundance, the biological replicates were merged, and the percentage abundance was calculated for the top 30 taxa. The dominant ASVs were shared between the guts of the abalone fed different macroalgae diets but differed in the relative abundance at all the taxonomic levels tested. Overall, differential abundance analysis by the univariate method DESeq2 identified nine differentially abundant ASVs at the family level, and 20 at the genus level (Appendix A) showing that there were fine-scale differences between the gut bacterial communities of abalone fed different diets.

At the family taxonomic level, SAR324 clade (Marine group B), Mycoplasmataceae, Fusobacteriaceae and Vibrionaceae were the most abundant ASVs across all the dietary treatments except for the abalone that were fed the Abfeed^{TM} S34® diet supplemented with the two fresh macroalgae, where bacteria belonging to the family Vibrionaceae had a lower relative abundance. Specifically, amongst the top four bacterial families identified in the various dietary treatment groups, the family Fusobacteriaceae was the most abundant in the digestive tract of abalone fed the biosecure and non-biosecure *Gracilaria* supplemented diets (27.5 and 30.3 % respectively; Table 4.3). Members of the deltaproteobacteria SAR324 clade (Marine group B) was the most abundant family-level classification observed in the gut of abalone fed with formulated diet supplemented with biosecure and non-biosecure kelp, non-biosecure *Ulva*, fresh macroalgae and Abfeed^{TM} S34®, followed by bacteria belonging to the families Mycoplasmataceae, Fusobacteriaceae, and Vibrionaceae (Table 4.3; Figure 4.5). The family Pirellulaceae (13 %) was more prevalent than Vibrionaceae (0.7 %) in the abalone fed with Abfeed^{TM} S34® supplemented with the fresh macroalgae and was the only differentially abundant family among the dominant families detected in this study (DESeq2; $p < 0.05$).

*Table 4.3. Most prevalent bacterial ASVs at family and genus level in each gut sample of abalone fed with biosecure (b) and non-biosecure (nb) macroalgae diets, formulated diet (*Abfeed™ S34®*) and formulated diet supplemented with fresh seaweed, where percentage abundance is indicated in brackets.*

Classification	**Sample**	**Most prevalent ASVs**			
Family	Abfeed™ S34®	SAR324 clade (Marine group B) (32%	Mycoplasmataceae (25%)	Fusobacteriaceae (15%)	Vibrionaceae (4%)
	Abfeed_b_Gracilaria	Fusobacteriaceae (28%)	SAR324_clade (Marine_group_B) (25%)	Mycoplasmataceae (24%)	Vibrionaceae (8%)
	Abfeed_nb_Gracilaria	Fusobacteriaceae (30%)	SAR324_clade (Marine_group_B) (28%)	Vibrionaceae (11%)	Mycoplasmataceae (10%)
	Abfeed_b_Ulva	Mycoplasmataceae (42%)	SAR324 clade (Marine group B) (17%)	Fusobacteriaceae (16%)	Vibrionaceae (6%)
	Abfeed_nb_Ulva	SAR324 clade (Marine group B) (28%)	Mycoplasmataceae (23%)	Fusobacteriaceae (21%)	Vibrionaceae (6%)
	Abfeed_b_kelp	SAR324 clade (Marine group B) (28%)	Mycoplasmataceae (26%)	Fusobacteriaceae (15%)	Vibrionaceae (4%)
	Abfeed_nb_kelp	SAR324 clade (Marine group B) (36%)	Mycoplasmataceae (23%)	Fusobacteriaceae (21%)	Vibrionaceae (8%)
	Abfeed_fresh seaweed	SAR324 clade (Marine group B) (36%)	Mycoplasmataceae (23%)	Pirellulaceae (13%)	Fusobacteriaceae (11%)
Genus	Abfeed	SAR324 clade (Marine group B) (32%)	*Mycoplasma* (22%)	*Psychrilyobacter* (15%)	*Vibrio (*4%)
	Abfeed_b_Gracilaria	*Psychrilyobacter* (27%)	*SAR324 clade (Marine group B)* (25%)	*Mycoplasma* (24%)	*Vibrio* (8%)
	Abfeed_nb_Gracilaria	*Psychrilyobacter* (30%)	SAR324 clade (Marine group B) (28%)	*Vibrio* (11%)	*Mycoplasma* (10%)
	Abfeed_b_Ulva	*Mycoplasma* (42%)	SAR324 clade (Marine group B) (17%)	*Psychrilyobacter* (16%)	*Vibrio* (6%)
	Abfeed_nb_Ulva	SAR324 clade (Marine group B) (28%)	*Mycoplasma* (23%)	*Psychrilyobacter* (21%)	*Vibrio* (6%)

	Abfeed_b_Kelp	SAR324 clade (Marine group B) (28%)	*Mycoplasma* (27%)	*Psychrilyobacter (14%)*	*Vibrio* (4%)
	Abfeed_nb_Kelp	SAR324 clade (Marine group B) (36%)	*Mycoplasma* (23%)	*Psychrilyobacter* (20%)	*Vibrio* (8%)
	Abfeed_fresh seaweed	*SAR324_clade(Marine_group_B)* (36%)	*Mycoplasma (21%)*	*Psychrilyobacter* (11%)	*Blastopirellula* (10%)

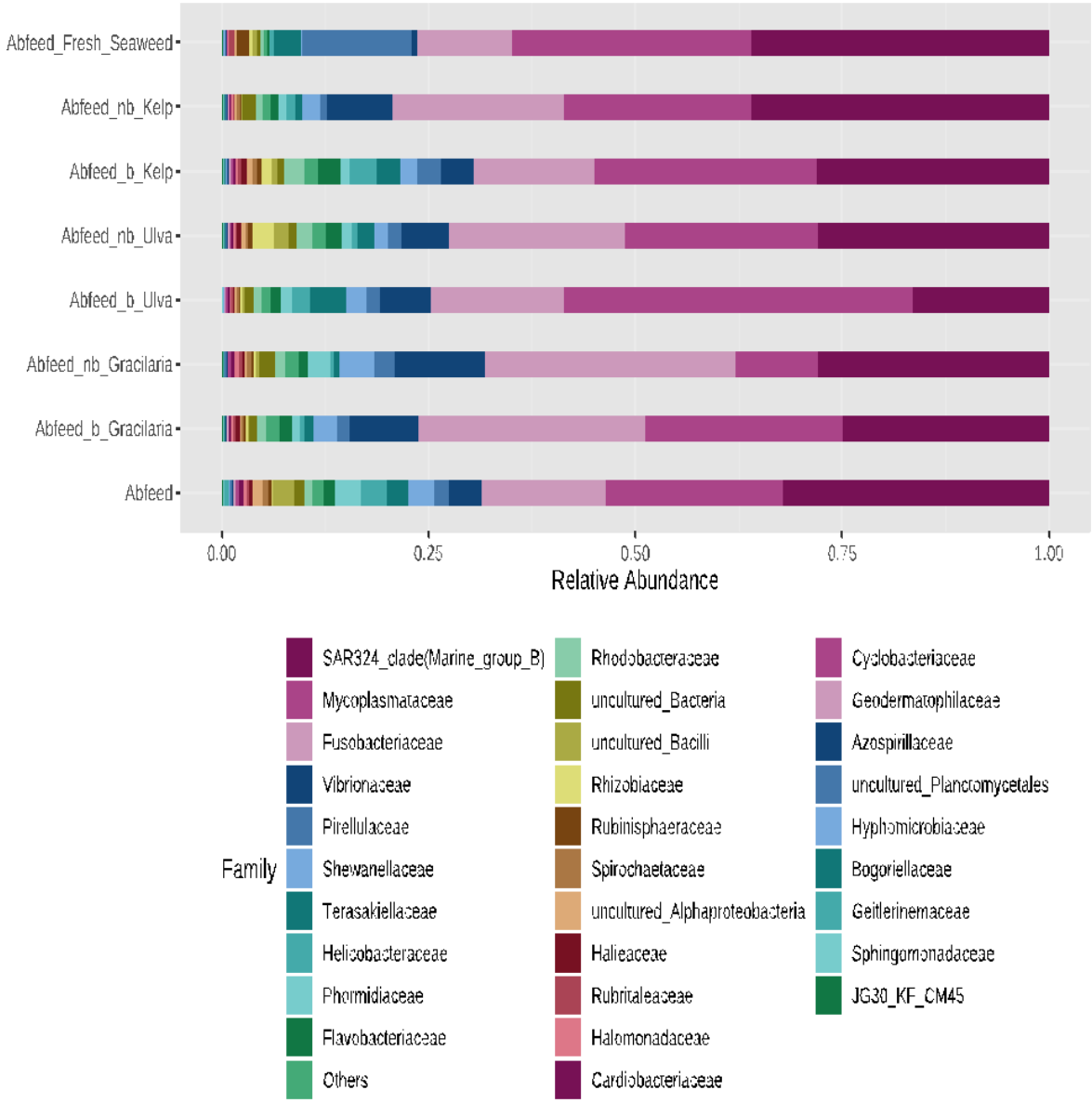

Figure 4.5. A stacked column bar graph of the top 30 most abundant taxa at the family level across abalone H. midae digestive tract samples (n = 4 per dietary treatment) obtained from animals fed with the formulated diets containing biosecure (b) and non-biosecure (nb) macroalgae and formulated diets supplemented with fresh macroalgae.

At the genus level, *Mycoplasma* had the highest relative abundance in the gut of abalone fed with biosecure *Ulva*. *Psychrilyobacter* dominated in the digestive tract of abalone fed the *Gracilaria* diets, while bacteria belonging to the SAR324 clade (Marine group B) was found in higher abundances in the gut of abalone fed the remaining diets tested in this study (Table 4.3, Figure 4.6). The genus *Blastopirellula*, which was among the dominant genera in the gut of abalone fed with Abfeed^{TM} S34® supplemented with fresh seaweeds (Abfeed_Fresh seaweed) was the only statistically significantly differentially abundant genus among the dominant genera (DESeq2; $p < 0.05$). The dominant genera described here also formed the core microbiome when all the samples were analysed together (Figure 4.7).

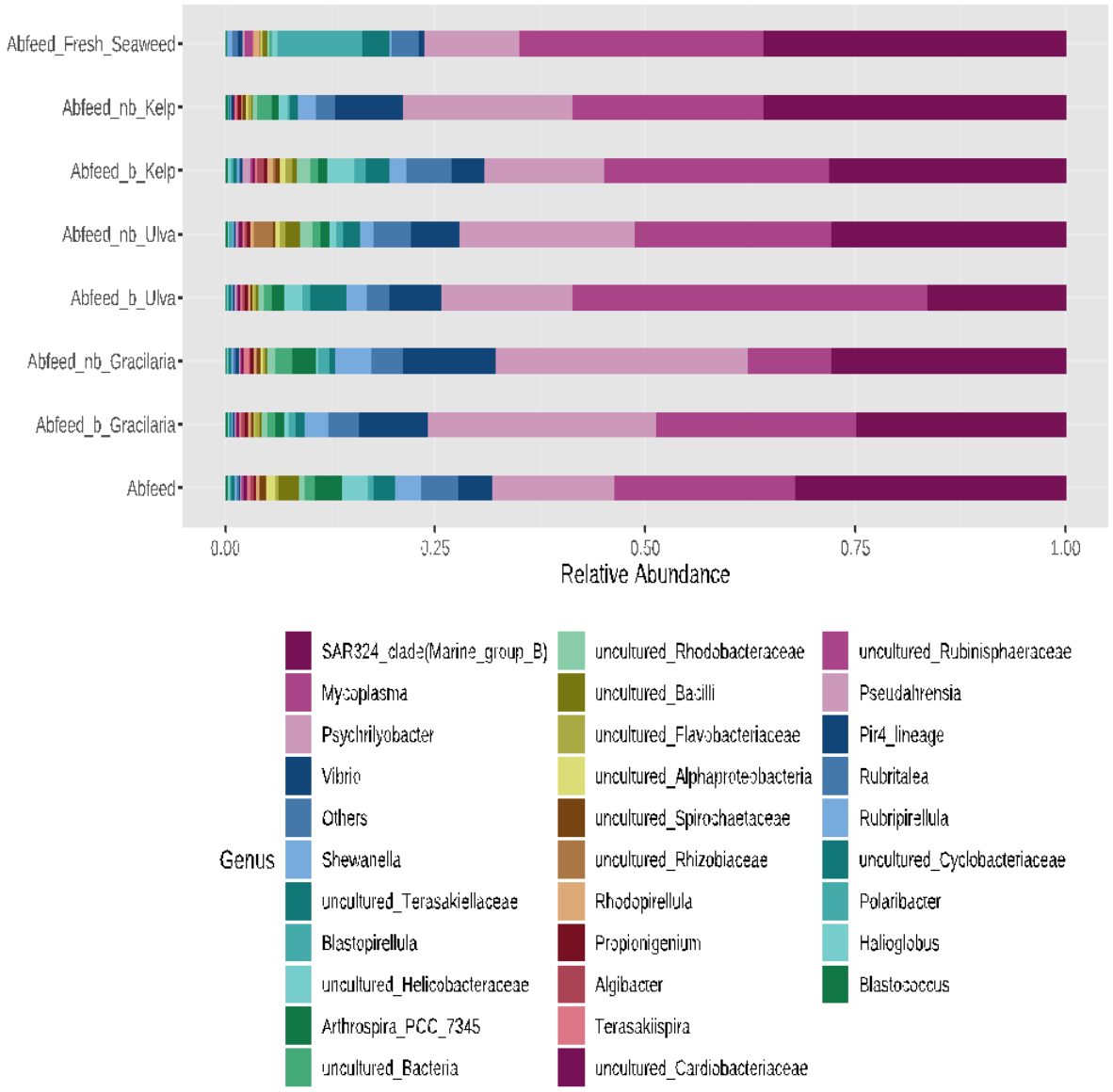

Figure 4.6. A stacked column bar graph of the top 30 most abundant taxa at the Genus level across abalone H. midae digestive tract samples (n = 4 per dietary treatment) obtained from animals fed with the formulated diets containing biosecure (b) and non-biosecure (nb) macroalgae and formulated diets supplemented with fresh macroalgae.

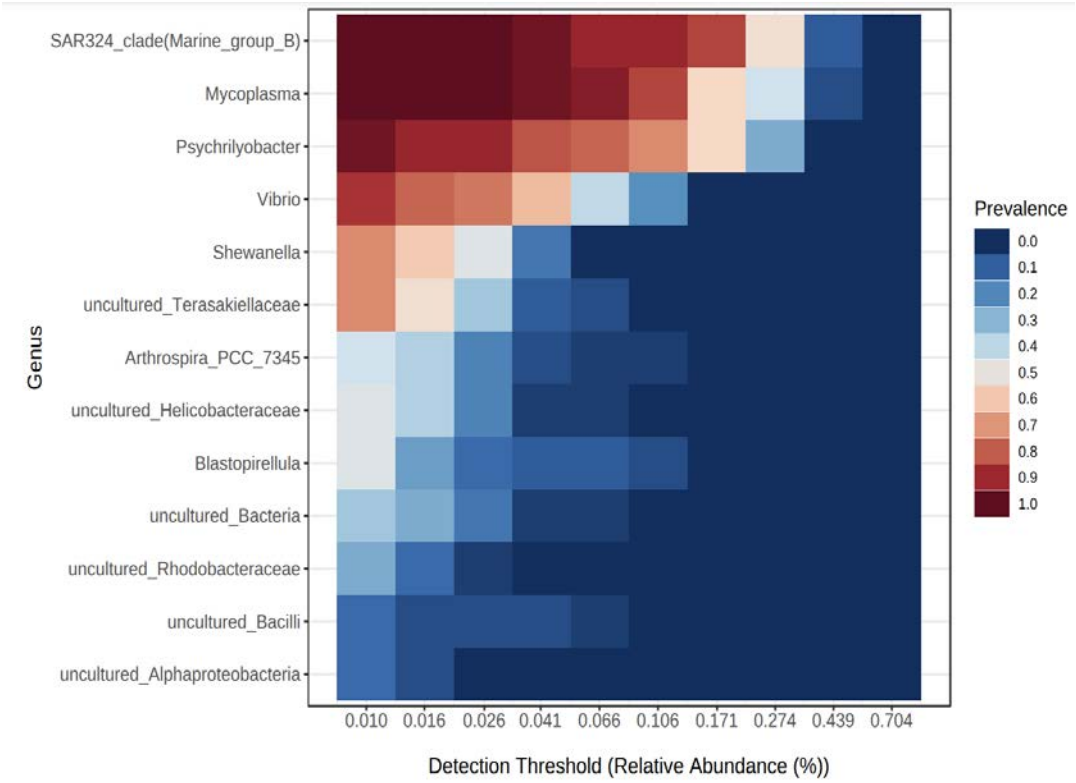

Figure 4.7. Composition of the core bacteria in abalone H. midae digestive tract samples obtained from animals fed with the formulated diets containing biosecure (b) and non-biosecure (nb) macroalgae and formulated diets supplemented with fresh macroalgae at the genus level.

4.4 DISCUSSION

Processing, as opposed to use of fresh ingredients or seaweeds, have a major impact on the microbiota associated with the ingredients/feeds that will have a major impact on the gut microbiome of the animals feeding on them (Del Olmo *et al.* 2018; Zang and Li, 2018). Processing of food or food ingredients often exerts a major effect on their constituents. Some studies reported heat treatment the altered the nutritional compounds and phytochemical contents of seaweed which in turn could compromise their functional benefits (Cox *et al.* 2012). This study investigated and compared the gut microbiome composition of the South African abalone *Haliotis midae* fed a formulated feed (Abfeed^{TM} S34®) supplemented with biosecure and non-biosecure macroalgae, and compared this with abalone fed the formulated diet alone or the formulated diet provided together with two fresh macroalgae species, *U. lacinulata* and

G. gracilis). It was observed that neither the biosecurity process nor the macroalgae type, when included as a dry ingredient (3%) in formulated feed or provided as an additional fresh feed supplement with the formulated feed, significantly changed the diversity or the composition of the gut microbiome of abalone fed the various diets. Similar findings have been reported for the European abalone *Haliotis tuberculata,* fed one or a combination of three macroalgae; the brown algae *Laminaria digitata* and *Saccharina latissimi,* the red alga *Palmaria palmata*, and the green alga *Ulva lactuca*. Variations in the composition of the microbiome were shown to be more linked to seasonal variations than the specific macroalgal diet (Gobet *et al.* 2018). In another study, commercial formulated feed supplementation with *G. gracilis* extracts did not alter the richness or diversity of the European seabass gut microbiome (Gonçalves *et al.* 2022). On the contrary, a higher richness of seabream intestinal communities was observed when fish were fed formulated feeds where the fish meal was partially substituted with dried *Gracilaria* or *Ulva* biomass (15 % w/w) (Rico *et al.* 2016). The discrepancy in results from different studies can be inferred that microbiome modulation by algae is a complex phenomenon and could be highly dependent on various factors such as the host and algae species, inclusion rate, duration of feeding, age of the host and season among other factors, reinforcing the complexity of the gut microbial community and the associated host-microbiome interactions (Danckert *et al*. 2020; Gonçalves *et al*. 2022). Assessing the impact of diet on the host-microbiome on a case-by-case basis is therefore imperative.

In the current study, the diets were formulated to be isoenergetic and isonitrogenous except for the macroalgal component which differed. Both the bio-secure and non-biosecure feeds contained macroalgae that was dried, with the exception of the Abfeed_fresh seaweed treatment. This would have attributed to the similarity in the gut microbiomes across treatments. Moreover, the biosecure or non-biosecure macroalgae were included at only 3% which might not make a significant change to the overall abalone gut microbiome. For

example, Cyrus *et al.* (2014, 2015ab) showed that *Ulva* needed to be included in a formulated feed at a level above 15 %, on a dry weight basis, before it had a significant impact on gonad quality, protein and energy efficiency, consumption etc. Although the latter authors did not assess impacts on the gut microbiome, the benefits they observed from dietary supplementation with seaweed may in part be attributed to impact of the seaweed on the gut microbiome. This notion is supported by the study of Brand (2023), who showed that when abalone *H. midae* are fed diets supplemented with 10 % dried *Ulva*, or lower levels of *Ulva* constituents (1 % Ulvan and 0.1 % glucoronic acid) or with fresh *Ulva*, there was a significant impact on the gut microbiome community; differing significantly from that of abalone fed Abfeed^{TM} S34® alone. More specifically, Nel *et al.* (2017) included kelp (< 4%) into Abfeed and showed that there was no overall significant impact of diet on the gut bacterial composition of *H. midae*. Collectively, these observations support the notion that dietary inclusion levels as well as macroalgae type may be important in shaping the microbiome. Interestingly, the lowest number of ASVs at family, and genus levels were observed in abalone fed with a diet supplemented with fresh macroalgae. Previous literature suggested that some macroalgae polysaccharides (i.e., agar and carrageenan) may have inhibitory effects on specific/certain microorganisms in the digestive tract associated with animals fed macroalgae (O'Sullivan *et al.* 2010; Nel *et al.* 2017; Bullon *et al.* 2022; Elizondo-González *et al.* 2020). Moreover, macroalgae have been documented to have the ability to defend themselves against other species in the environment by producing a variety of secondary metabolites including alkaloids, polyketides, cyclic peptides, quinines, and lipids with many biological activities (Al-Saif et *al.* 2014; Habbu et *al.* 2016), that can have an impact on the microorganisms colonising the seaweeds or the digestive tract of the animals digesting them. The low ASV numbers in the digestive tracts of abalone fed with fresh macroalgae can be attributed to macroalgae, or the microorganisms associated with them, inhibiting the growth of other bacteria in the environment. This could

explain the modulatory effect of the fresh macroalga and/or it's associated microbiome on the abalone gut microbiome.

In this study, the genera SAR324 clade (Marine group B), *Mycoplasma*, *Psychrilyobacter* and *Vibrio* dominated the gut bacterial community in almost all the diets, while *Blastopirellula* was also observed in higher abundances in the gut of animals fed with the Abfeed^{TM} S34® diets supplemented with fresh macroalgae. Notably, these genera formed the core microbiome of the abalone when samples were grouped together. Except for the *SAR324 clade (Marine group B)* and *Blastopirellula*, the other dominant genera; *Mycoplasma*, *Psychrilyobacter* and *Vibrio* have previously been reported to dominate the gut microbiome of abalone (*H. tuberculate*, *H. laevigata and H. midae*) and have also been identified as part of the core microbiome and associated with abalone fed a variety of diets and sampled from different environments (Tanaka *et al.* 2002; Huang *et al.* 2010; Iehata *et al.* 2014; Nel *et al.* 2017; Cicala *et al.* 2018; Gobet *et al.* 2018; Danckert *et al.* 2020).

The genus *Vibrio* was reported in high dominance for all the dietary treatments, except for abalone fed with the mixed diet of Abfeed^{TM} S34® diet and the two fresh macroalgal species. However, members of this genus were not classified to species level during taxonomic assignment. Although certain members of the genus *Vibrio* have been linked to high mortalities, especially during summer months, in several marine cultured species including oysters, shrimp, and various fish species and abalone (Liu *et al.* 2000; Austin and Zhang, 2006; Cai *et al.* 2007; Cardinaud *et al.* 2014; Dubief *et al.* 2017; Ina-Salwany *et al.* 2019), the high abundance of *Vibrio* spp. in the gut of several healthy abalone species (Nel *et al.* 2017; Danckert *et al*. 2020) suggests a strong association between this bacterium and the host. In addition, this association may confirm the important role of algal polysaccharide catabolism in symbiotic or commensal associations in their hosts because algae are the primary diet of

abalone species in the wild (Tanaka *et al*. 2003). For example, *Vibrios* produce exogenous digestive enzymes that aid in the fermentation of seaweed polysaccharides and they have been speculated to contribute to the digestion of both kelp and formulated feeds (Erasmus *et al.* 1997; Sawabe *et al*. 2003; Sawabe, 2006). In another case, specific species such as *V. halioticoli,* have been isolated from a variety of Haliotid species, including *H. midae*, and play an important role in the fermentation in the lower intestine and production of short chain fatty acids that are important for gut health, development, energy metabolism and immunity (Sawabe *et al.* 2003). This explains the high abundance of this group both in the macroalgae diets and the control (formulated diet) in this study. Interestingly, a clear reduction in the abundance of genus *Vibrio* and an increase in *Blastopirellula* of phylum Planctomycetota was observed in the animals that were fed the formulated diet supplemented with fresh macroalgae. These results are consistent with other studies that showed a lower abundance of *Vibrio* spp. when, for example, European seabass were fed with a commercial-like diet supplemented with dried algae *G. gracilis* or extract of the seaweed (Gonçalves *et al.* 2022). In another study, *Ulva clathrate* showed a significant inhibitory effect on *V. anguillarum*, in a culture system (Lu *et al*. 2008). A possible reason for the observation in the current study could be that the fresh macroalgae produce secondary metabolites that inhibit the proliferation of the *Vibrio*. Moreover, the bacteria resident on the fresh macroalgae have been shown to have antimicrobial action that could contribute to the modulation of the microbiome in the gut of animals fed the seaweed.

Conversely, the presence of the genus *Blastopirellula* as one of the dominating genera in this study suggests that these bacteria may be part of the bacterioplankton that was ingested along with the fresh alga. The *Blastopirellula* has also been isolated the gut microbiota of the sea urchin *Abatus agassizii* (Schwob *et al.* 2020). The group was associated with having a significant role in the breakdown of sulfated polymeric carbon by sulfatase enzyme activity

(Wegner *et al.* 2013). The sulphated compounds, found in marine photosynthetic organisms like microalgae and seaweeds were likely to have been deposited on the seafloor and got ingested by the host together with the sediments (Schwob *et al.* 2020).

The presence of SAR324 clade (Marine group B) as a dominant ASV in the abalone gut microbiome in this study appears to be a unique case since this has not been reported on in other abalone gut microbiome studies. Although the SAR324 clade (Marine group B) of the phyla deltaproteobacteria inhabits the entire water column, from the surface to the deep interior of the ocean, it has been found to make up a substantial part of the microbial community in the oxygen minimum zones (Boeuf *et al.* 2021). The higher abundance of this genus in this study can be associated with the seawater pumped into the abalone rearing tanks from the bordering Indian Ocean. In marine ecosystems, the seawater microbiome has been reported to colonize the gut of organisms. For example, the gut microflora of juvenile abalone fed on diatoms was almost identical to the supply of seawater (Tanaka *et al.* 2003). The fact that this group of bacteria survive in oxygen minimum environments may explain why it can survive, and appears to thrive, in the digestive tract of abalone, when oxygen levels are much lower than in the water column as observed in this study. A study by Harris *et al.* (1998) observed that the oxygen level in the gut of abalone *Haliotis laevigata* was below the limit of detection and suggested anaerobic conditions. Nonetheless, future studies should be conducted to establish the role of SAR324 clade (Marine group B) in the guts of abalone.

4.5 CONCLUSION

The gut microbiome composition of the South African abalone *Haliotis midae* fed varying macroalgae diets and formulated diet supplemented with fresh macroalgae were assessed in the current study. The bacterial diversity and composition of the abalone gut microbiome did not differ significantly between the abalone fed with the biosecure and non-bioseucre diets.

Similarly, the same dominant bacterial genera were observed across all the samples. However, supplementation of the formulated diets with fresh macroalgae resulted in a shift in the relative abundance of the dominant genera. This study shows that the biosecurity treatments to which the macroalgae were subjected before being included in the diet did not affect the gut microbiome of the abalone. These findings contribute to the knowledge of the gut microbiome of abalone *H. midae* and provide support for the production of biosecure macroalgae formulated diets without compromising their benefits to the growth and intestinal microbiome composition of farmed abalone.

CHAPTER 5

CONCLUDING DISCUSSION

The biosecurity concerns around the use of macroalgae as supplementary feeds or as ingredients in formulated feeds prevent wider adoption of macroalgae as a functional ingredient for aquafeeds. This study aimed to develop a processing treatment for mitigating the biosecurity risks of macroalgae added to formulated feeds for abalone. The thesis research approach consisted of three experiments designed to investigate the potential biosecure measures for macroalgae-transmitted pathogens and subsequent effects of the biosecure feeds on abalone growth and health. The efficacy of different processing treatments for inactivation of potential macroalgae-transmitted pathogens was assessed in Chapter 2. The three test pathogens used to assess the various processing treatments included the bacterium *Vibrio anguillarum*, an oomycete *Halioticida noduliformans* and the bacteriophage lambda (used as a proxy for abalone herpesvirus). Culture-based methods were used to determine the viability of the test pathogens following exposure to different processing treatments. Thereafter, the effects of the processed (biosecure) macroalgae on abalone growth performance were explored in Chapter 3. Growth performance indicators such as length and weight gain, feed conversion ratios and condition factor were assessed. Finally, a Next Generation Sequencing (NGS) approach was employed to identify and compare the bacterial communities in the digestive tracts of abalone fed diets containing biosecure and non-biosecure macroalgae in Chapter 4.

It was established in this study that single processing treatments were not effective for the inactivation of potential pathogens compared to combined treatments. Drying at 40 ℃, a treatment commonly used in the macroalgae feed manufacturing industry, did not result in 100 % inhibition of the test pathogens when exposed to this temperature for 8 h. Instead, the

combined processing treatment of disinfection by povo-iodine, followed by oven drying at 40 °C and UVC light treatment was shown to be effective and resulted in 100 % inactivation of all the test pathogens in the macroalga matrix. This combination has not been explored before in the processing of macroalgae as an aquafeed ingredient and this is also the first study to use povo-iodine for macroalga decontamination before their inclusion in abalone feed. Although the povo-iodine was combined with oven drying and UVC it can potentially be used for decontamination protocols where macroalgae are utilized in a fresh state. Future studies should work on optimizing the suitable concentrations if this treatment is to be used on its own, especially to achieve biosecure fresh macroalgae because the efficacy of povo-iodine was shown to decrease in the presence of complex matrixes (Chen *et al.* 2018). In this study, although thermal inactivation in combination with the other treatments was responsible for disinfection, it is possible that desiccation associated with the increased temperature might been responsible for the inactivation, and this needs to be established in future research.

This thesis research established that the efficacy of the various processing treatments depends on the pathogen species and the media/surfaces where they are applied. This implies that optimal processing strategies should be developed and adapted for each macroalgal species according to the quality requirements of the end products (Stévant and Rebours, 2021). Since the potential macroalgae-transmitted pathogen(s) is sometimes (and probably most frequently) unknown, this research emphasizes optimizing the treatments regarding the most resistant pathogen. However, the impact of the processing treatment(s) on the nutritional aspects of the macroalgae should also be considered. The viability of the test pathogens in this study was assessed using culture-based methods. Future studies should incorporate molecular techniques for viability testing to enable differentiation between viable and inactivated (viable but non-culturable) cells, which is crucial for correctly assessing microbial risk. Microbial culture methods are only able to detect viable and culturable cells. However, when subjected to

stressful conditions, microbial cells can often enter a temporary state of low metabolic activity (Truchado *et al.* 2020). These cells remain viable, but are non-culturable, a condition referred to as a viable but non-culturable (VBNC) state (Truchado *et al.* 2020). Cells can persist in this state for an extended period of time and the presence of these VBNC cells can only be detected using non-culture-based techniques. These include staining techniques or molecular techniques such as quantitative real-time polymerase chain reaction (qPCR) methodologies combined with the use of photoreactive dyes, including propidium monoazide (PMA) and ethidium monoazide (EMA) (Truchado *et al.* 2020).

Growth of an aquacultured organism provides an indication of feed performance and how different feed ingredients impact the weight, length, and width of the animals, which is essential in ensuring that cultured animals reach marketable size and optimal conditions within an economically viable time. In this study, growth performance of the the abalone fed with macroalgae diet did not differ significantly from the control diet without macroalgae. This could be attributed to the fact that the experiment was done for a shorter period and more time would be required to see any changes. Further the inclusion level of 3% of dried macroalgae could be low to cause significant differences. Future studies can test these diets for a longer growth period and also at a higher inclusion levels. Macroalgae contain biologically active compounds including polysaccharides, proteins, polyunsaturated fatty acids, pigments, polyphenols and minerals which have been documented to possess strong prebiotics, antimicrobial, antiviral, anti-infection and antioxidant activities that are crucial for mitigating against biotic and abiotic stress as well as promoting growth (Chojnacka *et al.* 2012). Although the effect of the processing treatments on the chemical compunds of macroalgae was not tested in this study, the lack of significant differences in the growth performance of the abalone fed with diets containing biosecure or non-biosecure macroalgae could indicate that biosecurity

treatments had no significant effect on the positive growth-promoting factors of the macroalgae. This needs to be tested in future studies.

To provide further insight into the role of microorganisms in food assimilation and the impact of biosecure and non-biosecure macroalgae as a dietary ingredient, this study investigated the impact of the various diets on the abalone gut microbiome. A growing body of literature indicates that microorganisms contribute to essential physiological activities such as growth, development, metabolism, food digestion (through the provisioning of digestive enzymes), nutrient assimilation, and defense against invasion by foreign/opportunistic bacterial species (Hakim *et al.* 2015; Cicala *et al.* 2018; Danckert *et al.* 2020). This study showed that overall, the bacterial diversity and composition of the abalone gut microbiome did not differ significantly between the abalone fed with the different diets. A similar core microbiome was also observed in the digestive tracts of abalone fed with the biosecure and non-biosecure diets. The latter findings are attributed to the diets being formulated with similar macronutrient composition. These findings may also be attributed to the inclusion level of the macroalgae (3 %) in the formulated feed. It is worth noting that, the experimental period in this research was relatively short (5 months), which may not have provided sufficient time for significant changes to occur in the morphology and gut microbiome of the abalone. Microbiome modulation by algae is a complex phenomenon and could be highly dependent on various factors such as the host and algae species, algae inclusion rate, duration of feeding, age of the host and season, among other factors, reinforcing the complexity of the gut microbial community and its relationships (Danckert *et al*. 2022; Gonçalves *et al*. 2022). Future studies should, therefore, consider combining different aspects besides the diets to get an accurate picture of the effect of diets on the abalone.

The scale-up and commercialisation of the work that has been developed here is currently being considered by industry. Abalone feed manufacturers acknowledge that biosecurity must be considered, particularly when including aquatic raw ingredients into aquafeeds. In South Africa, a responsible abalone feed manufacturer already subjects macroalgae to biosecurity procedures before it is included in commercial feeds; however, this process remains proprietary (Dirk Weich, Marifeed Pty Ltd. *pers. comm*. 2022). The work in this thesis was carried out in close collaboration with the abalone feed manufacturing industry and with abalone farmers, where the experimental diets were manufactured in the commercial feed factory and diets tested under farm conditions. When the biosecurity procedures were selected for testing, the viability of potentially up-scaling the procedure to commercial production was taken into account. After taking the various biosecurity procedures tested and developed in this thesis into consideration, and the positive results that were achieved in the farm growth trials and the gut microbiome work, it has been acknowledged by the feed manufacturing partner that part of this work is likely to be incorporated in the commercial production of abalone feeds in the future (Dirk Weich, Marifeed Pty Ltd. *pers. comm*. 2022). All that remains for commercialisation to take place, are practical changes to the feed manufacturing production line and associated investment.

In conclusion, this research has contributed to understanding the production of biosecure macroalgae formulated diets without compromising their benefits to the growth and health of farmed abalone. The combined treatment used in this study can be applied in the macroalgae and the feed industry to produce biosecure feed.

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APPENDIX A

SUPPLEMENTARY INFORMATION FOR CHAPTER 4

Table S1.1. Differentially abundant ASVs of the digestive tract of abalone fed with the biosecure and non-biosecure macroalgae diets, control (Abfeed) and the Abfeed diets supplemented with fresh macroalgae (Ulva lacinulata and Gracilaria gracilis) at the family, genus and species level at family level, where the log fold change (log2FC), p-values and false discovery rate (FDR) are indicated.

Family level differential abundance				**Genus level differential abundance**			
Family	**Log fold change**	**p-values**	**FDR**	**Genus**	**Log fold change**	**p-values**	**FDR**
Pirellulaceae	4.63	<0.001	<0.001	*Blastopirellula*	5.34	<0.001	<0.001
KD4_96	-18.87	<0.001	<0.001	*Rubinisphaera*	-22.87	<0.001	<0.001
Phormidiaceae	-10.07	<0.001	<0.001	*KD4_96*	-19.27	<0.001	<0.001
Rubinisphaeraceae	3.78	<0.001	<0.001	*Arthrospira_PCC_7345*	-10.22	<0.001	<0.001
uncultured_Firmicutes	-16.01	<0.001	<0.001	*uncultured_Firmicutes*	-16.17	<0.001	<0.001
uncultured_Alteromonadales	-7.17	<0.001	<0.05	*Fuerstia*	4.65	<0.001	<0.001
Spirochaetaceae	-2.79	<0.001	<0.05	*uncultured_Rubinisphaeraceae*	3.50	<0.001	<0.001
uncultured_Oligoflexales	5.38	<0.001	<0.05	*Algibacter*	-4.82	<0.001	<0.001
uncultured_Flavobacteriales	-6.31	<0.001	<0.05	*Persicobacter*	-6.83	<0.001	<0.001
				uncultured_Alteromonadales	-7.49	<0.001	<0.001
				Pir4_lineage	2.94	<0.001	<0.001
				Rubripirellula	2.70	<0.001	<0.001

	uncultured_Spirochaetaceae	-3.05	<0.001	<0.001
	uncultured_Flavobacteriales	-6.71	<0.001	<0.001
	Pirellula	4.62	<0.001	<0.001
	Parasphingopyxis	-20.22	<0.001	<0.001
	Shewanella	-2.95	<0.001	<0.05
	Bythopirellula	3.06	<0.001	<0.05
	Mesoflavibacter	-5.32	<0.001	<0.05
	uncultured_Oligoflexales	5.04	<0.001	<0.05

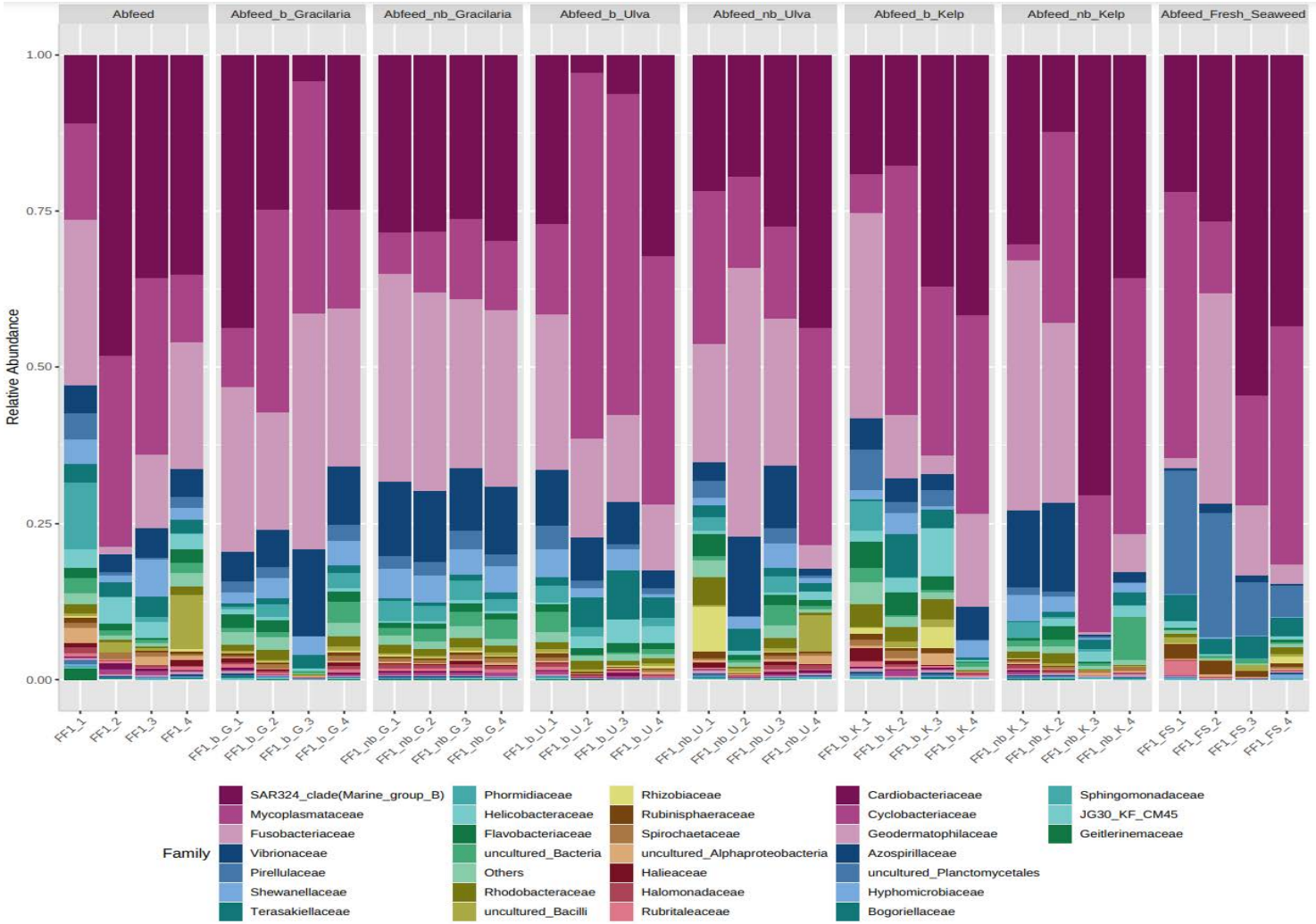

Figure S1.1. Sample-wise relative (%) of the top 30 most abundant taxa at the Family level across abalone H. midae digestive tract samples (n = 4 per dietary treatment) obtained from animals fed with the formulated diets containing biosecure (b) and non-biosecure (nb) macroalgae and formulated diets supplemented with fresh macroalgae.

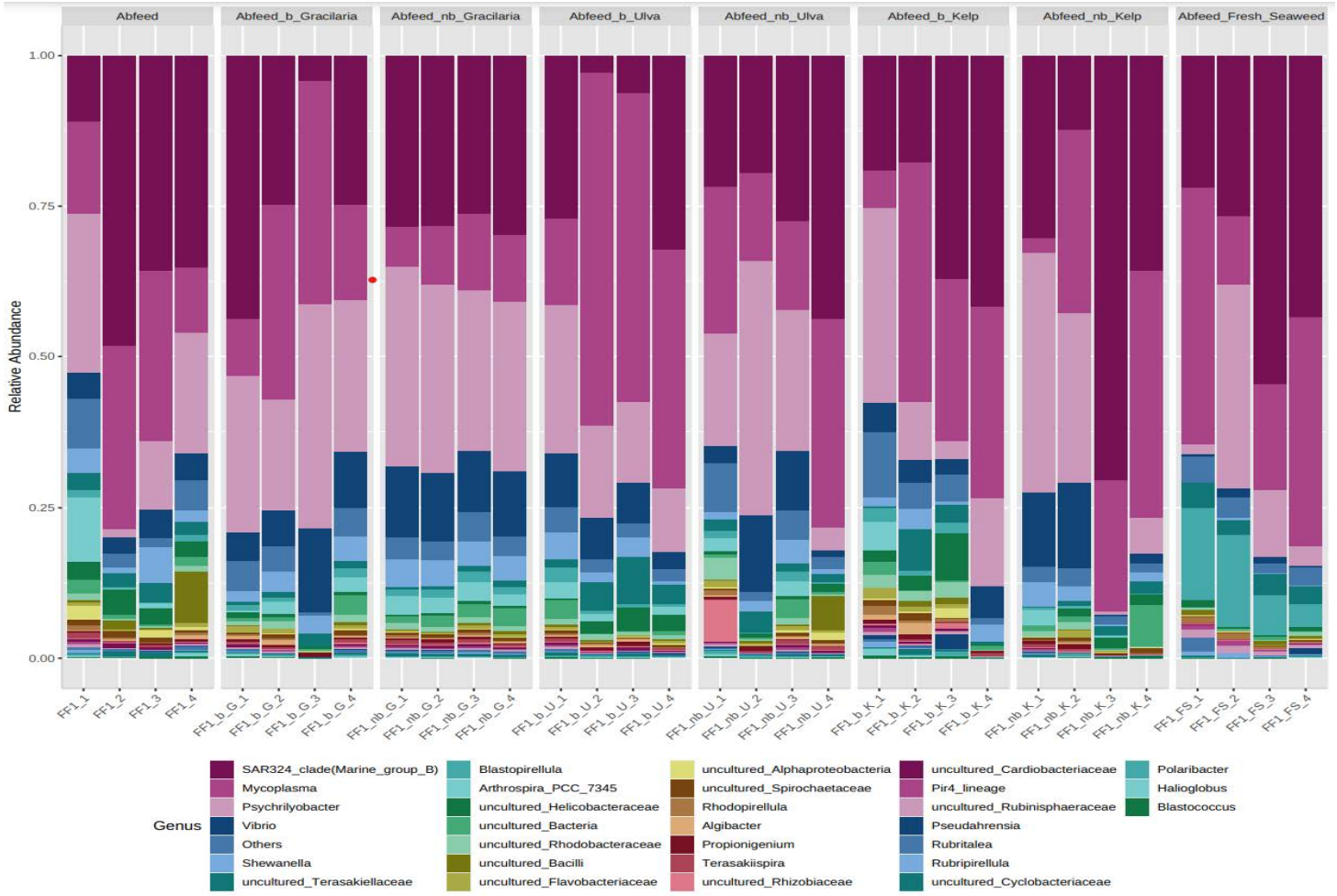

Figure S1.2. Sample-wise relative (%) of the top 30 most abundant taxa at the Genus level across abalone H. midae digestive tract samples (n = 4 per dietary treatment) obtained from animals fed with the formulated diets containing biosecure (b) and non-biosecure (nb) macroalgae and formulated diets supplemented with fresh macroalgae.