The effect of *Gracilaria gracilis* (Rhodophyta) on growth and behaviour of farmed abalone *Haliotis midae* when included in the abalone diet

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Njabulo Madlala

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Abstract

Abalone feed naturally on algae, but commercial abalone farms rely on formulated feed. The inclusion of algae with the formulated feed in the diet improves growth, health and feed conversion ratio (FCR). However, the kelp that is currently included in the feed is wild harvested and this resource is limited and under increasing pressure. Thus, there is a need to develop an alternative sustainable source of algae.

The aim was to use *Gracilaria gracilis* algae (that was produced with mussel in an integrated multitrophic aquaculture system; IMTA) which was then included into abalone feeds and its influence on abalone growth, FCR and behaviour was evaluated.

Sea-based *G. gracilis* was harvested off an existing IMTA system, manufactured into Abfeed® and fed to abalone to determine growth efficiency. Abalone (30 - 40 g abalone^{-1}) originated from the same cohort at Whale Rock Abalone Farm (Pty) Ltd., Hermanus. Abalone were fed either: Abfeed® S34 only with no algae (G0, the control); Abfeed® with sea-based IMTA *G. gracilis* included in the formulated feed at 0.75 % (G0.75); 1.50 % (G1.50); 3.00 % (G3.00); 6.00 % (G6.00); and 12.00 % (G12.00) inclusion; and Abfeed® S34 with fresh-live *G. gracilis* (cultured at Whale Rock Abalone Farm (Pty) Ltd.; Gfresh + S34s).

Abalone shell length, whole body mass and FCR were measured at a four-months interval over eight months (two growth cycles). Differences in behaviour (i.e., level of activity and feeding activity) were also monitored and comparing between treatments.

There was significant difference in mean whole-body mass between treatments (RM-ANOVA, $F_{(6, 29)} = 3.71$, $p = 0.007$). Abalone fed the diet with the highest inclusion of dry *G. gracilis* (G12.00) had a lower mean whole-body mass value (74.13 ± 2.94 g abalone^{-1}) than abalone from all the other diets after eight months. There was a negative relationship between

the condition factor and dry *G. gracilis* inclusion rate for the first growth cycle ($y = -0.0044x + 1.222$, $R^2 = 0.24$, $p=0.009$) and second-growth cycle ($y = -0.0096x + 1.2233$, $R^2 = 0.51$, $p = 0.00002$). Abalone receiving fresh *G. gracilis* were less quiescent and more alert on the first hours after food distribution ($p < 0.05$). The same proportion of abalone with G0, G0.75, and G6.00 *G. gracilis* inclusion were feeding all along the 9 hours post-feeding.

This study will potentially reduce the dependence of abalone farms on wild-harvested kelp in formulated feed. This will improve their contribution to a more environmentally sustainable global production of sea food products, making them more competitive on international markets.

Key words: marine; aquaculture; abalone; diet; IMTA-cultivated seaweed; growth efficiency; behavioural response.

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List of abbreviations

ANOVA	Analysis of variance
CF	Condition factor
CP	Crude protein
DAFF	Department of Agriculture, Forestry and Fisheries
DISL	Daily increment in shell length
DMS	Dimethylsulfide
DMSP	Dimethylsulfoniopropionate
DO	Dissolved oxygen
EFC	Ethanol-from-cellulose
FAO	Food and Agriculture Organization
FCR	Feed conversion ratio
HDPE	High density polyethylene
HIPS	High-impact polystyrene
IMTA	Integrated multitrophic aquaculture
IUU	Illegal, unreported and unregulated
kJ	Kilojoules
LMM	Linear mixed model
mm	Millimetres
PVC	Polyvinyl chloride
RM-ANOVA	Repeated measures analysis of variance
SD	Standard deviation
SE	Standard error

SGR	Specific growth rate
SL	Standard length
SL_f	Final shell length
SL_i	Initial shell length
t	Tonnes
USA	United States of America
USD	United States Dollars
W_f	Final weight
W_i	Initial weight

Chapter 1 - Introduction

1.1. Problem identification

Haliotis midae abalone also referred to as *perlemoen* are herbivorous marine gastropod molluscs (Cotton 1959) that feed naturally on kelp (Tutschulte & Connell 1988) and widely distributed along coastal regions where cooler currents and brown algae predominate (Wood 1993). In South Africa *H. midae* is a lucrative mariculture product and a variety of information exists on its nutritional requirements (Britz *et al.* 1994; Britz 1996a; Britz & Hecht 1997; Robertson-Andersson 2006; Troell *et al*. 2006; Bautista-Teruel *et al.* 2011; Bullon *et al.* 2022). Diet significantly affects the growth of abalone and since algae is not freely available, commercial abalone farms in South Africa mainly rely on formulated feed (Britz *et al*. 1994; Cook 1998; Sales & Britz 2001; Bansemer *et al.* 2014). Formulated feed offers convenience and cost benefits to farm management compared to natural feed (Britz *et al.* 1994).

So far, the inclusion of macroalgae in formulated feeds promises to be a practical way of gaining the benefits of feeding macroalgae to abalone and there is literature that supports this assumption (Dlaza *et al.* 2008; O'Mahoney *et al.* 2014; Denti 2015; Nel *et al.* 2018). Japanese abalone (*Haliotis discus hannai*) fed a formulated diet with dietary inclusion of dried macroalgae meal exhibit superior feeding efficiency (5.61 ± 0.546 %) compared to Japanese abalone fed fresh kelp (*Laminaria digitata*; 3.22 ± 0.023 %; O'Mahoney *et al.* 2014). A study on *Haliotis tuberculate coccinea* observed similar results of high growth performance when abalone were fed algae-based diets that further promoted high survival, high protein utilization and natural pigmentation (Denti 2015).

Therefore, the inclusion of algae in *H. midae* abalone diet could support improved growth, health and feed conversion ratio (Nell & Numaguchi 1991; Nell & O'Connor 1992; Kemp *et al*. 2002; Nel *et al.* 2007; Dlaza *et al*. 2008; Tlakedi 2019; Wright 2019). However,

the kelp that is currently included in the feed is wild harvested and this resource is limited and under increasing exploitation pressure (Anderson *et al.* 2003; Troell *et al*. 2006).

The use of kelp is also related to various other limiting aspects such as, feed conversion ratio, price of feed, cost of handling and storage (Troell *et al.* 2006). Alternative sources of dietary algae are available. For example, some farms grow their own *Ulva* sp. in land-based ponds fed with seawater and organic fertilizer (Vandermeulen & Gordin 1990), whereas others use an integrated multitrophic aquaculture system (IMTA) where the waste from abalone is used to grow the *Ulva* sp., which is fed back to the abalone (Bolton *et al.* 2009), or the algae could be imported (Troell *et al.* 1999).

However, there are potential biosecurity risks associated with feeding the *Ulva* sp. back to the abalone stock; the practice of producing algae on one farm and incorporating it into a feed for other farms would be unacceptable. There is a need to develop an alternative source of algae, that will reduce the pressure on natural kelp resources with improved environmental sustainability and that is less likely to compromise the biosecurity of the abalone.

1.2. Literature review

1.2.1. General overview of abalone

Abalone are a group of small to large edible marine gastropod molluscs, belonging to the family *Haliotidae*, that are dioecious and slow growing (Cotton 1959). The largest genus is *Haliotis* which consists of more than 100 abalone species and is commonly found inhabiting intertidal and subtidal sea zones across temperate and tropical regions (Wood 1993). Abalone are broadcast spawners that release gametes through their respiratory pores into the water column (Visser-Roux 2011). This is where fertilization takes place, followed by the development of lecithotrophic larvae which survive on their yolk sac until they are ready to settle (Wood & Buxton 1996). Sex can be determined by the distinctive colour of the

reproductive organs. Males have cream-coloured gonads while females have green-coloured gonads (Wood 1993). When one abalone releases an egg or sperm, it coherently triggers all nearby abalone to spawn as well (Tarr 1991).

1.2.2. Description of *Haliotis midae* abalone

There are six endemic species of abalone (Figure 1.1) in South Africa. However, *H. midae* is the only farmed species within the region (Tarr 1995; DAFF 2018). *Haliotis midae* has a wide distributional range along the coastal line where cooler currents and brown algae predominate (Wood 1993). The species attains a maximum size of 230 mm standard length (SL) and is characterised by a flattened ear-shaped shell with a wide opening at the base (Figure 1.2). Beneath the shell exists a head, a large muscular foot and the visceral mass which are all attached to the shell via the adductor muscle (Muller 1986; Hahn 1988; Tarr 1995).

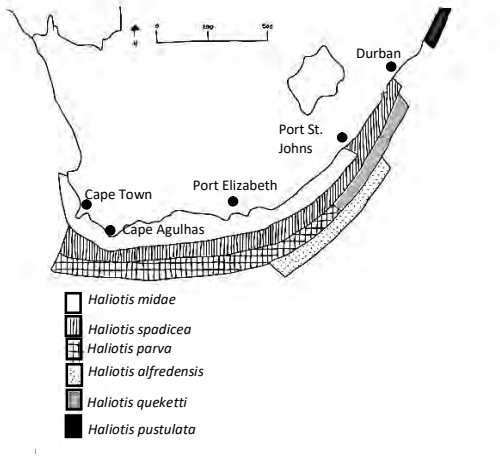

Figure 1.1: Distribution *of Haliotis* species along the South African coastal region (Lindberg 1992).

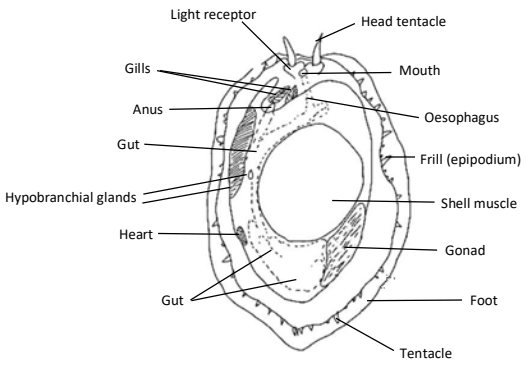

Figure 1.2: Ventral view diagram of the anatomy of abalone (Scott 2008).

1.2.3. History of abalone in South Africa

The abalone industry in South Africa began as early as 1949, as a commercial fishery located between Robben Island and Quoin Point, around the Cape southwest coast (Hauck 1999). The industry remained stable for almost half a century before overexploitation devastated wild abalone catches. This was mainly driven by the cumulative impact of political and socioeconomic complexities which are shared by most abalone fisheries (Hauck & Sweijd 1999; DAFF 2018). The scale of commercial illegal abalone fisheries was remarkably high by 2005 in the Eastern Cape (Serge & Britz 2009). A fleet of about 30 purposely built vessels existed with a capital investment of four million USD, employing 300 full-time crew, and harvesting 1000 - 2000 tonnes of abalone per year with an export value of 35 - 70 million USD. This illegal, unreported and unregulated (IUU) fishing activity had detrimental ecological impacts as the average size of abalone significantly declined as well as the

densities in sampling areas (Serge & Britz 2009). The decline in wild abalone density reduces the probability of sperm meeting eggs, which progresses until reproductive failure occurs due to individuals now spaced far apart (Allee effect; Branch & Branch 2017).

This paradigm fuelled the establishment of rapidly growing intensive shore-based abalone cultivation industries around the 1990s (Hauck & Swejid 1999). These industries now account for at least 40 % of the mass and 94 % of the value of mariculture, making *H. midae* abalone, by far, the most lucrative and important mariculture product in South Africa (Branch & Branch 2017).

1.2.4. Challenges of farming abalone

Rearing abalone through aquaculture has been challenging because establishing a farm requires high start-up and running costs and requires technical expertise (Robertson-Andersson *et al*. 2005). Seawater quality management becomes an important component as development and effluent disposal occur in the coastal area (Figure 1.3; Goldburg & Triplett 1997). This later affects the farms' environmental standards and production yield by influencing product quality and competitiveness (Britz *et al.* 1994). Other risks of food fish aquaculture include the nutrification of water bodies, the introduction of invasive species, user conflict and the addition of antibiotics and other chemicals to the ecosystem (Goldburg & Triplett 1997; Boyd 2003).

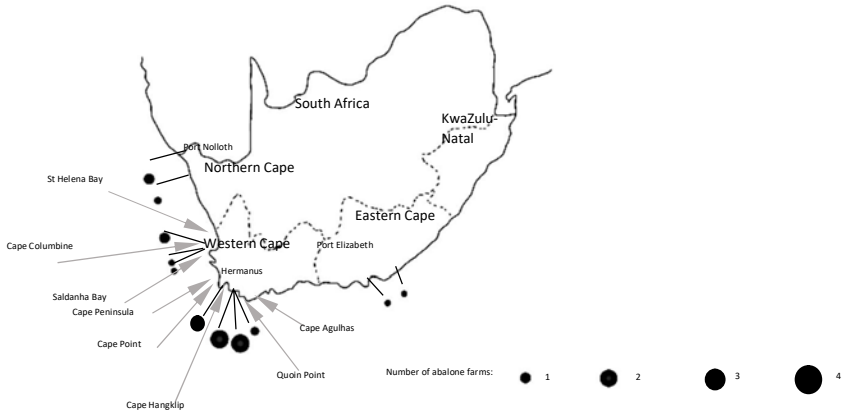

Figure 1.3: Established *Haliotis midae* farms around the South African coastline (Troell *et al*. 2006).

1.2.5. Abalone behaviour

The husbandry process along with abalone age, play an important role on behavioural changes of farmed organisms, and there is limited literature describing how abalone respond to biotic and abiotic stimulants in their natural and artificial environments (Steinarsson & Imsland 2003; Hecht 1994; Lloyd & Bates 2008; Buss *et al.* 2015). Therefore, in aquaculture where profits correspond with the number of animals produced and the period it takes to achieve marketable sizes, feeding efficiency and response becomes an important component (Sales & Britz 2001). The time of feeding and light intensity significantly affect the abalone feeding behaviour, with darkness stimulating higher foraging and growth compared to light exposure for post-larvae, six-day-old red abalone (*Haliotis rufescens*) in static conditions (Searcy-Bernal & Gorrostieta-Hurtado 2007). Two-year-old New Zealand blackfoot abalone (paua; *Haliotis iris*) fed formulated feed were also active during darkness (20h30 - 23h30; Allen *et*

al. 2006). Summer ingestion rates were higher than those in winter after assessing the feeding behaviour (Allen *et al*. 2006).

Stocking density was identified to have an impact on growth and behaviour of juvenile *Haliotis rubra* abalone (Huchette *et al*. 2003). The percentage of abalone that stack on top of others increased with density. Shading of the tanks completely changes the distribution of abalone and their behaviour during the daytime. And competition for shelter space reduced growth more than water quality (Huchette *et al.* 2003). The influence of density to feed consumption, forging and stacking behaviour on the growth rate of Northern abalone *Haliotis kamtschatkana,* was investigated (Lloyd & Bates 2008). At lower stocking densities, high growth rate (rationed feed = 0.04 and unlimited feed = 0.08 mm day^{-1}) took place versus at higher densities (rationed = 0.02 and unlimited = 0.03 mm day^{-1}). This was due to animals not being able to access the food item provided at high stocking densities. Foraging was limited to two hours after sunset and the amount of food consumed by each abalone was low (high: 0.95 g kelp $abalone^{-1}$) at high stocking densities versus low stocking densities (1.45 g kelp $abalone^{-1}$; Lloyd & Bates 2008). Aquaculture facilities commonly feed abalone "slightly in excess" to reduce intraspecific competition for food and the negative impacts of high stocking density (Steinarsson & Imsland 2003).

1.2.6. Artificial feed

Haliotids being over-exploited commercially and recreationally throughout their distributional range means the rapid expansion of *H. midae* cultivation in South Africa has led to the formulation of water-stable pelleted dry feed manufactured by the animal-feed industry (Britz *et al*. 1994). This development was made possible by the intensive collaboration between members of the farming industry and research institutions as a strategy for resource enhancement (Britz *et al.* 1994; Cook 1998). The adoption of formulated feed means that it needs to be nutritionally complete, have sufficient and readily digestible protein to yield faster

growth rates and provide benefits exceeding those of natural feed (Britz & Hecht 1997). An investigation was conducted on the suitability of locally available protein-rich abalone feed ingredients (casein, fishmeal, soya oil cake, *Spirulina* sp. and torula yeast), for inclusion in formulated practical diets (Britz 1996b). The abalone fed fishmeal and *Spirulina* spp. based diets exhibited higher growth rates than those fed on soya oil cake and torula yeast, casein and *E. maxima* based diets (Britz 1996b).

Inexpensive ingredients can be used to produce feed (Sales & Britz 2001). The resulting low-quality feed with insufficient or inundated protein and non-protein energy sources such as carbohydrates and lipids could cause issues relating to growth depression and fatty acid synthetase (Sales & Britz 2001). The effects of dietary protein and energy level on the growth rate, nutritional indices and body composition of two *H.midae* abalone size classes were investigated (Britz & Hecht 1997). The maximum growth rate was achieved at a higher protein level (44 %) in large abalone (7.0 - 14.0 g), whilst the condition factor (CF) was highest at 24 % protein diet for small abalone (0.2 - 1.0 g). The growth rate, protein deposition efficiency and feed conversion ratio (FCR) were also significantly lower at the highest level of dietary fat (10 %), for all size classes. Therefore, large abalone have a higher protein level requirement, and high levels of dietary lipid (10 %) will not achieve maximum abalone growth (Britz & Hecht 1997).

Moreover, low quality formulated feed could mean reduced water stability (Sales & Britz 2001). Since abalone are slow-feeding organisms, reduced feed stability means that feed will dissolve before abalone consume it (Britz *et al.* 1994). This further reduces water quality via enhanced decomposition (Britz *et al.* 1994). For abalone feed to ideally perform its function, the water-soluble nutrients should remain in the feed and the food particles should remain bound together for at least 2 days (Fleming *et al*. 1996). A 24 h study was conducted on the water stability of seven different binders (five alginates combined with two sequestrants, agar,

gelatine and a mixture of agar-gelatine; Britz *et al.* 1993). A 1: 3 agar: gelatine mixture retained 70.7 % of its dry matter after 24 h (Britz *et al.* 1993). However, these hydrocolloids have shown to be impractical and expensive in a commercial abalone diet thus a starch-bound dry pellet was developed (Britz *et al.* 1994).

Abalone, like most other animals require the same 10 essential amino acids which when supplied in optimal ratios with the appropriate level of digestible energy will maximize somatic growth (Sales & Britz 2001). Essential amino acids cannot be synthesised by abalone and thus must always be provided as part of the diet (Britz *et al.* 1994). The development of formulated feed means that issues relating to logistics and supply of fresh seaweed are eliminated as the industry becomes increasingly reliant on artificial dry feed (Britz 1996a).

1.2.7. Natural feed

Seaweed harvesting in South Africa focused mainly on red macroalgae (i.e., *Gracilaria* sp. and *Gelidium*) and began as early as the second world war (Anderson 2003). It was followed by the collection of dried beach-cast kelp (*Ecklonia maxima* and *Laminaria pallida*) which began around the 1950's where an average of about 1000 - 1500 t was harvested. Kelp are relatively large brown seaweeds (class Phaeophyceae) found in shallow coastal waters along the rocky coast from Cape Agulhas to the west coast in South Africa (Stegenga *et al.* 1997). Kelp forests predominate nutrient-rich waters and are highly productive. Most of the kelp material harvested was used mainly for alginate extraction (Anderson 2003; Branch & Branch 2017).

Aquaculture farming has been based on a monoculture system for many years which resulted in environmental degradation (Robertson-Andersson 2006). A major challenge was becoming sustainable based on a balanced ecosystem approach (Robertson-Andersson 2006). For example, seaweeds could potentially function as a biofilter which would promote economic feasibility for the resource. (Hampson 1998; Robertson-Andersson 2006). Kelp-fed

recirculatory systems do not favour parasites such as sabellids or other mobile macrofauna since they prefer organic matter less than 35 µm as feed (Robertson-Andersson 2006). The risk of spreading diseases or rising bacterial levels is considerably low (Robertson-Andersson 2006). However, quantities of natural kelp harvested are dependent on seasonal conditions (Troell *et al.* 2006). *Ulva* sp. has a high capacity of absorbing nutrients and improving the quality of the effluent water (Robertson- Andersson 2006). However, *Ulva* sp. produces significantly high levels of dimethylsulfoniopropionate (DMSP), which is an organosulfur compound. *Ulva* sp. DMSP levels can measure up to 6977 ± 1161 µg g^{-1} w wwt, while DMSP in *Gracilaria gracilis* and *E. maxima* kelp can range between 0.8 ± 0.3 µg g^{-1} w wwt and 26.8 ± 20.6 µg g^{-1} w wwt, respectively (Robertson-Andersson 2006). High accumulation of DMSP in tissue can result in an off-flavour in canned meat batches due to the break-down of DMSP to dimethylsulfide (DMS; Andreae 1990).

1.2.8. General overview of *Gracilaria* sp.

Gracilaria (Gigartinales, Rhodophyta) is a genus of red algae belonging to the family *Gracilariaceae* (Santelices and Doty 1989). It consists of more than 100 species globally, that inhabit temperate and tropical seawater, covering intertidal and subtidal regions as monospecific strands (Fredericq & Hommersand 1989). Originally, *Gracilaria* sp. were used in China as food and as a binding agent in the preparation of lime for painting walls (Santelices & Doty 1989). However, in many countries they have gained economic value and have shown to be an important source of income (Santelices & Doty 1989). The main *Gracilaria* sp. producing regions are currently Asia (China, Indonesia, Korea, Philippines and Vietnam), Africa (Namibia and South Africa), and America (Argentina, Brazil, Chile and Peru), all amounting to over 4 000 000 t of global *Gracilaria* sp. production by 2022 (Mantri *et al.* 2023).

1.2.9. Main applications for *Gracilaria* sp.

Many applications for *Gracilaria* sp. have been developed, each differing from the other based on the diverse bioactive compounds found within the genus (Bird *et al*. 1977; Santelices & Ugarte 1987; Amanullah *et al.* 2013). *Gracilaria tikvahiae* was used for fertilizer production and sewage treatment in the east coast of the United States due to its fast growth and wide distribution (Bird *et al.* 1977; Ryther *et al*. 1979). In South Africa, *G. gracilis* is used raw as abalone feed (Santelices and Doty 1989). Other examples include the use of agar extraction waste from *Gracilaria lemaneiformis* as a fibre source for papermaking or as a functional filter (Jicheng *et al*. 2013) and the use of field-cultivated *Gracilaria edulis* for ethanol production by fermenting its polysaccharides using *Saccharomyces cerevisie* (Amanullah *et al*. 2013).

1.2.10. Transition from wild crop harvesting to farming

The commercial value of *Gracilaria* sp. has mainly been driven by the discovery of its agar content which meant it possessed huge potential for the biotechnology and biochemical industries (Santelices & Ugarte 1987). Close to 5000 t of agar were processed per annum from *Gracilaria* sp. crops which contain 15 - 20 % of agar on a dry weight basis (Moss & Doty 1987). For example, between 1970 - 1990, *G. gracilis* was harvested in the Mediterranean Lesina Lagoon, Italy by local fisherman, dried and sold to private companies as raw material for agar extraction (D'Adamo *et al*. 2009). However drastic over-exploitation of this resource resulted in a decline in its natural biomass (D'Adamo *et al*. 2009). Similar developments took place in Chile where market demand for *G. gracilis* increased significantly between 1967 - 1983 until the lack of crop management led to the overharvesting of the natural strands of *G. gracilis* (Santelices & Ugarte 1987).

In South Africa, the commercial algae harvesting industry developed around the early 1950s in Saldanha Bay through the collection of beach-cast *G. gracilis* (Stackhouse) Steentoft

(Anderson *et al*. 1993). Annual yields varied from a maximum of 2000 t dry weight in 1967, until 1974 during the construction of, and dredging for an ore jetty (Anderson *et al*. 1993). Its breakwater caused the resource to collapse and to recover slowly (Anderson *et al.* 1989). At the end of 1988, the resource collapsed again mainly due to grazing by invertebrate herbivores (Anderson *et al.* 1993). This has resulted in shortages and higher costs for *G. gracilis* which coherently stimulated the demand for reliable *G. gracilis* quality and quantity (Anderson *et al.* 1993). The outcome has been a strong interest in *G. gracilis* farming which has permitted the development of a diversity of farming methods (Anderson *et al.* 1993).

1.2.11. Open water farming

Currently there are three approaches developed for planting *G. gracilis* crops in open waters: (a) bottom planting, (b) using nets or line and (c) on floating rafts. Either vegetative materials or spores can be used as planting material (Santelices & Doty 1989):

(a) Bottom planting - This labour-intensive method involves tying thalli to rocks and then transferring the rocks to the planting site. Another alternative procedure used on non-consolidated bottoms is to force the proximal ends of whole thalli into the planting site bottom. However, using this system means the *G. gracilis* will experience poor survival rates and will be susceptible to damage due to water movement or harvesting. Spore method consists of spores from selected fertile adult thalli that attach themselves to materials which are harvested after a certain time period. One disadvantage of this method is the expensive apparatus needed; however, it ensures control in *G. gracilis* productivity.

(b) Line farming - Vegetative material such as thalli or large *G. gracilis* cuttings are inserted or tied into ropes or other lines which are then suspended. When farming spores, nursery units are necessary for setting spores on lines. Preparations for the transferring of the seeded lines to the planting site should be carried out.

(c) Raft farming - This method was developed to cultivate *G. gracilis* in low fixed rafts. Long ropes with roughly 15 g of *G. gracilis* fragments are hung vertically or horizontally.

1.2.12. Pond and tank farming

Ponds can either be artificial or excavations in the ground that vary in length and depth (Chiang 1981). *G. gracilis* can be successfully cultivated in pond systems due to its high tolerance to low salinity and low water-motion environments (Chang & Xia 1976). Tank farming is more labour intensive and can permit more extensive control over the production process than other culture systems. This means greater productivity can be achieved per unit and more capital returns are gained (Chiang 1981).

1.2.13. Integrated farming

Intensive land-based aquaculture is a more sustainable farming approach than the capture fishery industry and single-species aquaculture (Edwards 1998). However, the discharge of poor-quality water (Porter *et al.* 1987) and the inefficient utilization of fishmeal and other natural resources for farmed species (Naylor *et al.* 1998) have led to many issues relating to the social acceptability of the practices (Ridler *et al*. 2007). “Sustainable aquaculture is a dynamic process, incorporating research, leaming and reassessment of methods, to practice and retain natural equilibrium in aquatic ecosystems. The objective is to sustainably produce and keep pace with society's food requirements without eroding natural capital” (Edwards 1998). To avoid public boycotts, litigation, lobbying and vandalism, a more positive social perception through industry-wide sustainability needs to be built (Ridler *et al.* 2007).

One concept that promises to resolve such issues for the aquaculture industry is integrated multitrophic aquaculture (IMTA). Integrated multitrophic aquaculture is the integrated rearing of fed species such as finfish, inorganic extractive species such as seaweed and organic extractive species such as deposit and suspension-feeders (Troell *et al*. 2009). This means that

the components operate in proximity and the biological and chemical processes that take place balance each other (Chopin 2006). An investigation was conducted on the sustainability of the integrated system for the intensive land-based culture of abalone (*Haliotis discus hannai*), fish (*Sparus aurata*) and seaweed (*Ulva lactuca* and *Gracilaria conferta*; Neori *et al*. 2000). This achieved nutrient recycling, reduced water-use, reduced nutrient discharge and high algal yields. The fish grew at 0.67 % day^{-1}, yielding 28 kg m^{-2} $year^{-1}$ and 80 % ammonia filtration, *U. lactuca* supported a total abalone yield of 9.4 kg $year^{-1}$, abalone growth rate of 0.9 % day^{-1}, length increase of 40-66 µm day^{-1} in abalone juveniles and 0.34 % day^{-1} and 59 µm day^{-1} in young abalone. Ammonia-N was lowered from 45 % - 10 % in post-seaweed discharge (Neori *et al*. 2000). Integrated Multitrophic Aquaculture can improve and diversify the supply of farmed aquatic organisms whilst eliminating the impacts that generate public concerns. Besides the reduction of the environmental impact, IMTA aims for long-term profitability, sustainability and reduced risk through economic diversification (Ridler *et al.* 2007). This could permit farmers to gain the ability to green-label products which is beneficial for social acceptability (Ridler *et al.* 2007; Troell *et al.* 2009).

1.3. Overall aim and objectives

The aim was to use *G. gracilis* algae in abalone feeds.

1. The growth of abalone fed *G. gracilis* based diets and a mixed diet was monitored over an eight-month growth cycle.
2. The feed conversion ratio of all dietary treatments was determined.
3. The behaviour of abalone fed the *G. gracilis* based diets and a mixed diet was evaluated.

Chapter 2 - Growth of abalone fed graded levels of *Gracilaria gracilis*

2.1. Introduction

South Africa is well known for producing unique and high-quality abalone in a niche market (Sales & Britz 2001; Huchette *et al.* 2003; Troell *et al.* 2006; Vosloo & Vosloo 2006). This reputation explains why it is the third-largest producer of abalone worldwide after Asia (i.e., China and South Korea; FAO 2018). Commercial abalone farms are mainly located in the Western Cape province due to optimum environmental conditions and established infrastructure (Troell *et al*. 2006). Western Cape is home to 13 out of 18 commercial abalone farms (DAFF 2018). The local industry is still small (1500 tonnes annum^{-1}) but rapidly growing and developing, thus considered a valuable and lucrative industry in the country (DAFF 2018). A few of the main challenges currently faced by land-based facilities are high running costs, specialized technical support, biosecurity risks and nutritional requirements (Troell *et al.* 2006).

Abalone feed accounts for a large portion of the production cost on South African abalone farms due to the high correlation between profitability and the time it takes to achieve market size (Sales & Britz 2001). The diet is either compound formulated feed, or macroalgae (Britz *et al*. 1994). Compound feeds are predominantly used over natural feed due to their cost benefits and superior growth results (Britz 1996a; Britz & Hecht 1997). However, the reliance on formulated feed by the abalone farming industry has meant that feed ingredients should be of high quality and the diet should be nutritionally complete (Britz & Hecht 1997; Sales & Britz 2001). When using low-quality feed with insufficient nutritional benefits, abalone may experience growth depression and deficiency issues that may increase susceptibility to diseases (Britz *et al.* 1994). Poor quality ingredients could also reduce water quality through accelerated decomposition (Britz *et al.* 1994).

Feeding seaweeds to abalone is accompanied by a range of benefits such as lowering production cost (Troell *et al.* 2006), acting as a feeding stimulant, improving water quality (Robertson-Andersson *et al.* 2006), product quality and marketability of abalone (Britz & Hecht 1997; Allen *et al.* 2006;). Some abalone naturally feed on brown algae/ kelp (McShane & Smith 1988) and in South Africa, *E. maxima* is commonly used as abalone feed (Robertson-Andersson 2006). However, kelp that is used is mainly harvested from the sea and not farm cultivated; as a result, this method contributed to the decline in its natural populations (Anderson *et al*. 2003). Fresh kelp has a low protein (5 - 15 %) and high water (68 - 83 %) content compared to formulated feed (Hahn 1989b), which suggest that kelp has a higher food conversion ratio and thus a lower feed conversion efficiency (Hahn 1989b; Britz 1996b). *Haliotis midae* abalone fed kelp with 10 % dry weight protein significantly grew less (0.257 % body weight day^{-1}) than abalone fed formulated feed (0.266 % body weight day^{-1}; Francis *et al*. 2008). Kelp relies on seasonal availability and legislation protects harvests against intense depletion (Robertson-Andersson *et al.* 2006). Therefore, formulated feed remain a superior option to promote abalone growth over macroalgae.

Red seaweeds are generally considered higher in nutritional value than other macroalgae and are a preferred diet by abalone (Gaillard *et al.* 2018). The crude protein level in red algae ranges between 20 - 31 % and is highest during the spring-summer period (Dawczvnski *et al.* 2007). Performance of abalone also relates directly to the nutritional value of algae (Gaillard *et al.* 2018). Previous studies indicate that the red and green algae account for 32 - 39 % of the food in the abalone gut although *H. midae* is largely restricted to areas where red and green algae are not common (Tarr 1992). This information suggests that red algae could be a viable alternative to kelp if supplied in large volumes.

Gracilaria gracilis is used raw as abalone feed in South Africa at present (Anderson *et al.* 1996). Limited beach-cast and farm-grown *G. gracilis* is currently used until an alternative supply source can be developed (Santelices & Doty 1989; Anderson *et al*. 1996; Troell *et al.* 2006; D'Adamo *et al.* 2009). Due to the dependence on natural stock and a high market demand for *Gracilaria* sp., the pressure for cultivating these species has also increased (Anderson *et al.* 1996).

Gracilaria gracilis can be cultivated to produce a high-quality crop at a reliable volume (Anderson *et al.* 1993). This characteristic is an advantage over kelp which is known to be relatively challenging to produce and harvest in the wild (Anderson *et al.* 1993). *Gracilaria gracilis* can be cultivated best in open waters through bottom planting, using nets or lines and on floating rafts or through tank farming (Santelices & Doty 1989). Open-water cultivation is limited by poor seedling survival due to damage from water movement and the cultivation process is associated with high labour and operation costs. However, the benefits of open-water cultivation are that it reduces dependence on water supply infrastructure and labour. In contrast, tank farming provides control over the production process due to tolerance of *G. gracilis* to low water movement. However, tank farming is labour intensive and costly (Santelices & Doty 1989).

Integrated multitrophic aquaculture (IMTA) is one approach that can be considered as an advanced cultivation method. However, producing algae using IMTA, where the waste from abalone is used in the growing process and algae is fed back to abalone stock has potential biosecurity risks associated with it (Porter e*t al.* 1987). Therefore, the IMTA approach should be environmentally sustainable and less compromising to the biosecurity of abalone. While some farms in South Africa currently rely on fresh seaweed, the quantities available are

limited by seasonal and logistics, as well as supply issues (Bansemer *et al.* 2014). If seaweeds are to be largely re-introduced into the abalone diet, their benefit on abalone growth and health could be recovered (Viera *et al.* 2011).

The use of dried macroalgae as a dietary ingredient promises to mitigate these challenges associated with macroalgae supply however, more research is still required to investigate the potential of this approach (Allen *et al.* 2006; O'Mahoney *et al.* 2014). Thus, a combination of various macroalgae inclusion levels needs to be tested to quantify the benefits on abalone growth.

Gracilaria gracilis algae produced with other organisms (i.e., mussels and not abalone) in a sea-based IMTA system was used in this study, whereafter it was included into abalone feeds. Its influence on abalone growth and food conversion ratio (FCR) were evaluated and compared. It was hypothesised that *G. gracilis* inclusion has no effect on the growth and FCR of farmed abalone. This study will potentially reduce the dependence on wild harvested kelp in formulated feed. The inclusion of natural feed will also permit farmers to green-label products, diversify the supply of farmed aquatic organisms and reduce their 'ecological footprint'. The main research questions are: Can *G. gracilis* macroalgae serve as a viable replacement to kelp, and at what inclusion level does *G. gracilis* allow for optimal abalone growth?

2.2. Materials and methods

2.2.1. Study site

This study was conducted in three locations: (1) Saldanha Bay on the Western Cape coast of South Africa where *Gracilaria gracilis* was cultivated at sea using a raft system; (2) Marifeed (Pty) Ltd (Hermanus, South Africa) where the experimental feeds were manufactured; and (3)

Aqunion (Pty) Ltd-Whale Rock farm (Hermanus, South Africa) where abalone growth trial and behavioural studies were carried out.

2.2.2. *Gracilaria gracilis* cultivation in Saldanha Bay

Vegetative *G. gracilis* thalli were harvested off the coast by SCUBA divers in Saldanha Bay at a depth of approximately three metres. The harvested *G. gracilis* (roughly 40 kg) was placed in tanks filled with seawater and transported on a boat to Blue Ocean Mussels' processing facility. Visible epiphytes were physically removed from the collection and the remaining *G. gracilis* was kept fresh within a tank supplied with running seawater and aeration. The wild harvested *G. gracilis* tufts were threaded through the lay of 300 x 5 m nylon split fibre ropes at 20 cm intervals. An additional 6 x 50 m culture lines were threaded with 215 seedlings at 20 cm intervals. In total 306 culture lines were seeded with *G. gracilis* thalli (Anderson *et al.* 1996). Four floating mussel raft systems 50 x 3 m comprised of twin 800 mm high density polyethylene (HDPE) pipes with timber crossbeams were used in this experiment. Each raft held 100 x 5 m ropes spaced 0.5 m apart. A one metre rope remained at the end of each culture line to securely tie to the raft and onto two-kilogram weights underwater. The six 50 m culture lines were all hung in one raft and spaced one metre apart in depth (Anderson *et al.* 1996).

Approximately 51.00 kg of algae was seeded for cultivation. Culture lines were harvested after 52 days of growth. During the harvest, each seeding site was partially harvested whereafter approximately 150 - 300 kg of the harvested algae was immediately dried, milled and processed into feed without storage. The algae cultivation period lasted one year and three months.

2.2.3. Non-IMTA *Gracilaria gracilis* cultivation at Aqunion (Pty) Ltd Whale Rock farm

At Aqunion (Pty) Ltd Whale Rock farm, another batch of farm-grown *G. gracilis* was cultivated in two aerated tanks (3627 L per tank) which utilise seawater pumped into the farm. The tanks were made of plastic material supported by a wood frame. Aeration was supplied in the form of compressed air via perforated 20 mm diameter polyvinyl chloride (PVC) pipes placed at the bottom of the tank. Water supply pipes were made of the same material as the aeration system and were the same diameter. The flow rate was maintained at 0.083 L s^{-1} on both the tanks. Culture tanks were drained and cleaned every week (Friedlander & Levy 1995; Troell *et al.* 2006). Epiphytes on *G. gracilis* were removed and after cleaning, fertilizers were added. Fertilizers used were mono-ammonium phosphate and mono-potassium phosphate. The rate of application was 200 g of each fertilizer per tank (Friedlander & Levy 1995; Troell *et al.* 2006). This farm grown, non-IMTA *G. gracilis* was used as a fresh macroalgae diet to supplement formulated feed.

2.2.4. Diet manufacturing and treatments

The IMTA *G. gracilis* cultured at Saldanha Bay was immediately processed into five compound feed diets through applying rigorous bio-secure measures. The feed was formulated to contain 34.00 % crude protein, 4.60 % lipid and 15.90 kJ kg^{-1} of energy (Table 2.1). A mixed diet treatment (i.e., macroalgae + formulated feed) was also designed where Abfeed© S34s was supplemented with fresh non-IMTA *G. gracilis* that was cultured at Aqunion Whalerock farm. Each macroalgae-based treatment included four replicate baskets. The control and mixed diet treatment had eight replicate baskets.

Table 2.1: The formulation of dietary treatments based on integrated multitrophic aquaculture *Gracilaria gracilis* macroalgae. Standard protein, lysine, lipid, and energy content were used to formulate treatments. CP- indicates crude protein. The manufactured Abfeed® based diets, non-IMTA *G. gracilis* and Abfeed® S34s established the following treatments: G0 (Abfeed® S34 only with no algae); G0.75 (Abfeed® with sea-based IMTA *G. gracilis*, at 0.75 % inclusion); G1.50 (Abfeed® with sea-based IMTA *G. gracilis* at 1.50 % inclusion); G3.00 (Abfeed® with sea-based IMTA *G. gracilis* at 3.00 % inclusion); G6.00 (Abfeed® with sea-based IMTA *G. gracilis* at 6.00 % inclusion); G12.00 (Abfeed® with sea-based IMTA *G. gracilis* at 12.00 % inclusion); and Gfresh + S34 (Abfeed® S34 + non-IMTA fresh farm-grown *G. gracilis*).

	G0 (Control)	G0.75	G1.50	G3.00	G6.00	G12.00
Target formulation						
IMTA *G. gracilis* meal (%)	0.00	0.75	1.50	3.00	6.00	12.00
Abfeed© S34 basal diet	100.00	99.25	98.50	97.00	94.00	88.00
Total	100.00	100.00	100.00	100.00	100.00	100.00
Total protein (%)	34.00	34.00	34.00	34.00	34.00	34.00
Lysine (%)	2.30	2.30	2.30	2.30	2.30	2.30
Total lipid (%)	4.60	4.60	4.60	4.60	4.60	4.60
Energy (kJ kg^{-1})	16.20	15.90	15.90	15.90	15.90	15.90

Energy was estimated using the equation: CP x 0.056 + Lipid x 0.094 + (100 - CP - Lipid - Ash) x 0.042, where CP stands for crude protein (Weiss & Tebbe 2019).

2.2.5. System design

Abalone baskets were made of a six mm plastic mesh securely attached to a frame using 14 PVC clamps (Figure 2.1). Each basket consisted of a feeder ring and inner rack fixed using cable ties. The inner rack was made of seven high-impact polystyrene (HIPS) plates separated by plastic spacers (Figure 2.2) and served the purpose of increasing the surface area within the basket for the abalone to attach (Riddin 2013). Inner rack plates had slots in the middle for a feeder ring with a diameter of 355 mm. The tanks that the baskets were placed into were identical to those described in section 2.2.3. Aeration was provided to each tank through three perforated 20 mm PVC tubing, lined along the bottom length of the tank (Naylor *et al.* 2011; Riddin 2013). Seawater was pumped from the sea into a reservoir dam fitted with microscreen drum filters (Riddin 2013; Wu *et al.* 2019). The flow rate was maintained above 0.25 L s^{-1}. The tanks were cleaned once a week to retain high water quality.

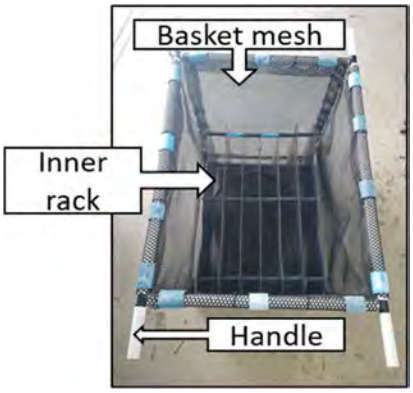

Figure 2.1: A complete abalone basket with inner rack fixed to it.

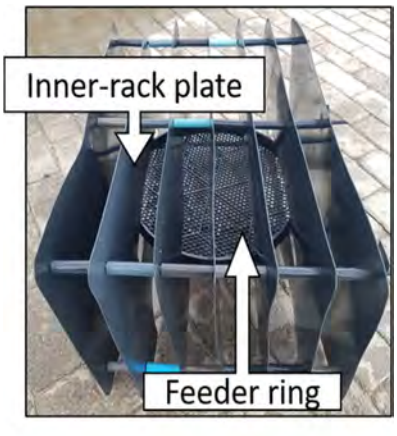

Figure 2.2: Inner rack made up of seven high-impact polystyrene plates and a feeder ring slotted in the middle.

2.2.6. Experimental animals, husbandry and growth period

Hatchery reared juvenile abalone (*Haliotis midae*) from Aqunion (Pty) Ltd Whale Rock farm were used in this study and were subject to farm standard husbandry procedures before the experiment. During the selection of the experimental animals, abalone from the same cohort were anaesthetized in carbon dioxide saturated seawater (pH between 4.9 - 4.5) in the holding tanks and then graded to a 30 - 40 g size range. Abalone with no shell or foot muscle damage were priority selected for the experiment.

The selected abalone were stocked into new designated baskets at a rate 17 % of the inner rack surface area. They were then fed the new diets "slightly in excess" from Monday to Saturday at 16h00 (Nel *et al.* 2017). This ensured feed availability did not limit growth (Naylor *et al.* 2011). Standard commercial abalone farm procedures for feeding were utilized.

The abalone were routine split for data collection purposes and to retain stocking density after four months of growth (Robertson-Andersson *et al*. 2011). A routine split involves anaesthetizing abalone using carbon dioxide for five minutes, de-plating the abalone off the

inner rack, counting all abalone per basket and then weighing the whole basket biomass. This routine split procedure was necessary for limiting intra-specific competition for food and space before baskets restocked with abalone were returned to tank systems (Robertson-Andersson *et al.* 2011). Whilst anaesthetized, 30 random abalone samples were selected per replicate basket. Excess water was allowed to drain from the selected samples before weight (to the nearest 0.01 g), sex (male/female) and shell length measurements (to the nearest millimetre) were recorded per animal (Robertson-Andersson *et al.* 2011). Examining abalone sex is not an invasive procedure, so no incisions or punctures were made to tissue. The abalone reproductive organs are tucked between the mantle and shell (Newman 1967). After measurements, the abalone were turned upside down, gonads exposed, and sex observed (Meusel *et al.* 2022). Females have green reproductive organs while male have cream reproductive organs (Newman 1967; Figure 2.3). All growth measurements were recorded, and abalone returned to their designated baskets for the next growth cycle.

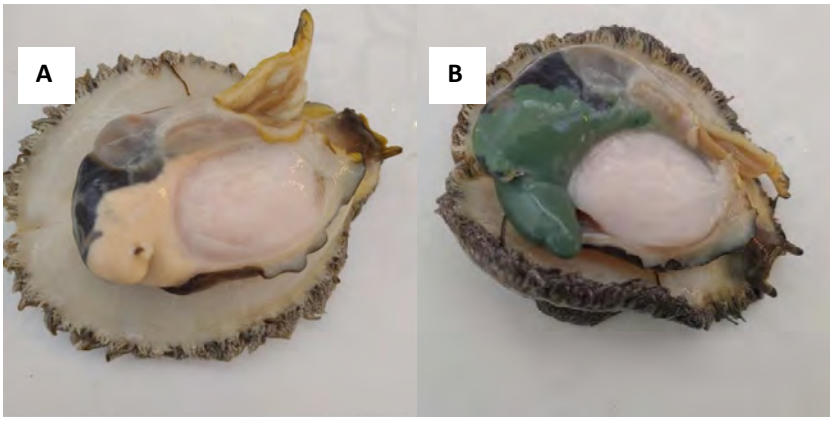

Figure 2.3: De-shelled abalone specimen indicating male (A) and female (B) gonads.

The growth trial consisted of two growth cycles and lasted for eight months (231 days). At the end of the trial the remaining abalone were returned to form part of the farm captive stock which eventually gets processed into sea food products. Biological waste (i.e., abalone shells,

dead tissue) was disposed of according to the Aqunion (Pty) Ltd-Whalerock farm waste disposal procedure.

At the end of each growth period, the following indices were calculated for all treatments (Britz 1996a):

Specific growth rate (% weight gain day^{-1}) = (ln (Wf) - ln (Wi))/t) × 100 (1)

where ln (Wf) is the natural log of the mean individual final weight of abalone, ln(Wi) is the natural log of the mean individual initial weight of abalone and t is the experimental period in days.

Feed-conversion ratio (FCR) = (dry feed consumed/wet weight gain) (2)

The FCR was not corrected for leaching and uneaten feed as the basket design did not permit collection of uneaten feed.

Daily increment in shell length (µm day^{-1}) = [(SL_f - SL_i)/t] x 1000 (3)

Daily increment in shell length (DISL) was calculated according to Tan & Mai (2001) where SL_f is the final mean shell length, SL_i is the initial mean shell length and t is the feeding trial period in days.

Condition factor (CF) = Weight (g)/Length $(mm)^{2.99}$ × 5575 (4)

The condition factor was calculated according to Britz (1996) using Eq. 3. Using a large data set, Britz (1996) calibrated the condition of an average size abalone as equal to 1. A condition factor > 1 indicates an above-average flesh-to-shell ratio, while <1 indicates a below-average flesh-to-shell ratio.

2.2.7. Calculating *Gracilaria gracilis* moisture content (%)

For diet treatments where fresh *G. gracilis* was used to supplement formulated feed, FCR was determined by combining the weights (g) of dry formulated feed and dry *G. gracilis* fed (Wakibia *et al.* 2001). To determine the moisture content, wet *G. gracilis* samples were weighed (i.e., after dripping) then oven-dried at 105 ℃ for 48 hours and left to cool for 10 minutes. The samples were weighed again and re-dried at 105 °C until a constant weight reading was determined (Wakibia *et al.* 2001). The drying temperature for *G. gracilis* was set to 105 °C due to frequent inconsistent final weight values after attempting to dry at 60 °C.

Nutrient leach test

A predetermined weight of 11.00 g feed pellets from each dietary treatment were immersed in seawater with aeration for 24 hours (Britz & Hecht 1997). After this period, the remaining feed pellets were collected in aluminium foil containers and oven dried at 60 °C for 6 hours (Sales *et al.* 2003). Percentage water loss was calculated as:

$$\text{Water stability (\% weight loss)} = 100 \times \frac{(B-A)(\%dry\ matter)}{A(\%\ dry\ matter)} \quad (5)$$

where B is the final feed weight and A is the initial feed weight.

2.2.8. Water quality

Water parameters including pH, temperature (℃), dissolved oxygen and oxygen saturation (%) were monitored for six days per week, using water quality meters (OxyGuard® Handy Polaris and OxyGuard® Handy pH, respectively; Figure 2.4). Both instruments were calibrated weekly. The OxyGuard® Handy Polaris had an automatic calibration adjustment feature where the probe was first rinsed with clean water, wiped and the temperature was allowed to stabilise before calibration. The OxyGuard® Handy pH was calibrated using pH

buffer solutions which measure the accuracy of the pH instrument before reconfiguration occurs.

Figure 2.4: Image of the portable Handy Polaris (left) and Handy pH (right) instruments used to determine water quality by measuring temperature, pH, dissolved oxygen, and saturation.

A spectrophotometer (Palintest Photometer 7100, United Kingdom) was used to monitor ammonia concentrations in the experiment seawater tanks. Firstly, acid-washed glassware was used to collect 50 ml water samples from each replicate tank. The water samples were kept in the dark for one hour after reagent alkaline salicylate was added. Water samples were then placed in a spectrophotometer for absorbance readings at a wavelength of 640 nm (Figure 2.5; Bower & Holm-Hansen 1980).

Figure 2.5: Image of the Palintest spectrophotometer along with the accessories used to determine ammonia concentration in the experiment tank system.

2.2.9. Proximate composition analysis

Abalone samples were collected before the growth trial commenced and after eight months. Animals (i.e., 15 abalone samples per replicate basket) were removed from the water prior to dissections. This is common as abalone live in the intertidal zones in the wild and are thus adapted to air exposure (Wood 1993). Samples were then taken and kept in a chilling room (± 10 °C) to slow down metabolism and normal bodily functions. Each animal was dissected by experienced staff at Whale Rock abalone farm (Pty) Ltd and kept frozen prior to analysis. Dissection procedure was adopted from Nel *et al.* (2017). Viscera were kept separate from the foot muscle before freezing. Samples were then analysed for proximate composition, including crude protein, lipid, gross energy, moisture, and ash (Knauer *et al.* 1994).

2.2.10. Statistical analysis

Statistical analyses were performed using Statistica™ 14.0.0.15 (TIBCO software Inc, USA). All data was expressed as means ± standard error and tested for homogeneity of variance

(Levene, 1960) and for the normal distribution of the residuals (Shapiro & Wilk 1965). A repeated measure analysis of variance (RM-ANOVA) was used to determine if there were interactions between factors within the experiment ($P < 0.05$). If there were no interactions between factors, a one-way ANOVA and a Tukey's multiple range analysis was used to compare the treatment means within each factor. Data that did not meet the assumptions of an ANOVA were analysed using either a Mann-Whitney U-test or a Kruskal-Wallis ANOVA. Multiple linear regression analysis was used to determine if there is a relationship between growth and treatments. All results were considered statistically significant at $P < 0.05$.

2.3. Results

2.3.1. Diet proximate analysis

The protein content of only compound feed ranged between 30.13 - 32.45 % with G0 (control) containing the lowest protein content and Abfeed® S34s containing the highest protein content (Table 2.2). Abfeed® S34s contained the lowest moisture (8.46 %), lowest ash (5.64 %) and highest lipid content (2.36 %) whilst G12.00 had the highest moisture content (10.33 %), highest ash content (10.58 %) and lowest lipid content (1.06 %) across compound diets. When comparing compound feed vs macroalgae, fresh macroalgae contained the highest moisture 85.86 %, lowest ash (3.44 %), lowest protein (4.39 %) and lowest lipid content (0.06 %; Table 2.2).

Table 2.2: Proximate analysis of formulated feed with *Gracilaria gracilis* inclusion levels 0 %; 0.75 %; 1.50 %; 3.00 %; 6.00 % and 12.00 %, corresponding to G0; G0.75; G1.50; G3.00; G6.00; G12.00, respectively. Abfeed® S34s along with fresh farm grown *Gracilaria gracilis* was also analysed.

Treatment	Dry matter (%)	Moisture (%)	Ash (%)	Protein (N x 6.25) (%)	Fat (ether extraction) (%)	Energy (Calculated) kJ 100 g^{-1}	Freeze drying (%)
Dry G0 (control) Feed	90.57	9.43	6.35	30.13	1.43	1460	-
Dry G0.75 feed	90.16	9.84	6.52	30.30	1.59	1454	-
Dry G1.50 feed	90.70	9.30	6.94	30.20	1.53	1455	-
Dry G3.00 feed	90.33	9.67	7.45	30.17	1.33	1436	-
Dry G6.00 feed	89.97	10.03	8.52	30.34	1.41	1413	-
Dry G12.00 feed	89.67	10.33	10.58	30.78	1.06	1366	-
Dry Abfeed® S34s	91.54	8.46	5.64	32.45	2.36	1508	-
Wet *G. gracilis*	14.14	85.86	3.44	4.39	0.06	183	15.31

2.3.2. Abalone proximate analysis

The protein content of abalone ranged between 57.77 - 67.24 % with abalone fed Gfresh + S34s containing the lowest protein content and abalone fed G12.00 containing the highest protein content (Table 2.3). The moisture content of abalone ranged between 9.63 - 11.53 % with abalone fed G1.50 containing the lowest moisture and abalone fed Gfresh + S34s containing the highest moisture content. The lipid content ranged between 0.51 - 0.67 % with abalone fed G3.00 having the lowest lipid content and abalone before the growth trial having the highest lipid content. Ash content ranged between 6.36 - 9.62 % with abalone fed

G0 having the lowest ash and abalone before the experiment having the highest ash content (Table 2.3).

Table 2.3: Proximate analysis of *Haliotis midae* abalone fed dietary treatments with *Gracilaria gracilis* inclusion levels 0 %; 0.75 %; 1.50 %; 3.00 %; 6.00 % and 12.00 %, corresponding to G0; G0.75; G1.50; G3.00; G6.00; G12.00, respectively. Abalone fed Abfeed® S34s along with fresh farm grown *Gracilaria gracilis* were also analysed.

Treatment	Dry matter (%)	Moisture (%)	Ash (%)	Protein (N x 6.25) (%)	Fat (ether extraction) (%)	Freeze drying (%)
Pre - Trial	88.79	11.21	9.62	60.83	0.67	22.42
G0 (control)	90.31	9.69	6.36	60.16	0.64	28.45
G0. 75	90.09	9.91	7.93	61.50	0.65	26.30
G1. 50	90.37	9.63	8.96	62.83	0.58	23.71
G3. 00	89.63	10.37	9.30	58.60	0.51	23.05
G6. 00	89.54	10.46	8.09	60.12	0.61	25.67
G12. 00	89.73	10.27	8.44	67.24	0.63	23.60
GFresh + S34s	88.47	11.53	8.27	57.77	0.60	24.77

2.3.3. Formulated feed water stability

There was a significant difference in the water stability of the formulated feed (ANOVA, $F_{(6, 14)} = 22.54$, $p = 0.000002$). G12.00 diet with the highest *G. gracilis* inclusion weighed significantly less than all the formulated feed (Table 2.4).

Table 2.4: Water stability of Abfeed® S34s along with *Gracilaria gracilis* based diets at inclusion levels 0 %; 0.75 %; 1.50 %; 3.00 %; 6.00 % and 12.00 %, corresponding to G0; G0.75; G1.50; G3.00; G6.00; G12.00, respectively. Abalone fed Abfeed® S34s along with fresh farm grown *Gracilaria gracilis* were also analysed. The formulated diets were immersed in seawater for 24 hours before oven-drying.

Diet	Weight retention (%)
G0	95.12 ± 1.01^{ab}
G0.75	88.10 ± 0.94^{ab}
G1.50	98.72 ± 1.05^{b}
G3.00	89.64 ± 0.96^{ab}
G6.00	68.27 ± 0.75^{a}
G12.00	15.38 ± 0.22^{c}
S34s	98.12 ± 1.04^{ab}

2.3.4. Whole-body mass

There was no interaction between time and dietary treatments (RM-ANOVA, $F_{(12, 58)}=1.80$, $p = 0.07$). However, there was an overall difference between treatments after eight months (RM-ANOVA, $F_{(6, 29)} = 3.71$, $p = 0.007$; Figure 2.6). Abalone fed the diet with the highest inclusion level of dry *G. gracilis* (G12.00) had the lowest whole-body mass (74.13 ± 2.94 g abalone^{-1}) than abalone from the control diet (85.06 ± 2.08 g abalone^{-1}), and abalone fed the G0.75 diet (84.17 ± 2.94 g abalone^{-1}), after eight months (Figure 2.6). Abalone fed diets

G1.50, G3.00, G6.00 and Gfresh + S34s were statistically the same after eight months (Figure 2.6).

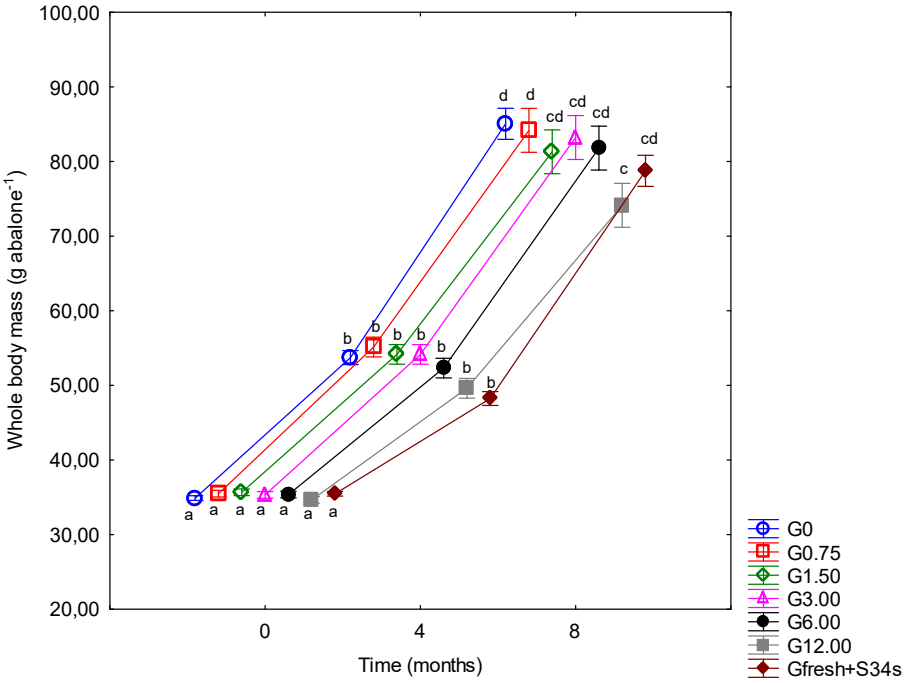

Figure 2.6: Mean (± SE) whole body mass (g abalone^{-1}) of *Haliotis midae* fed formulated diets with *Gracilaria gracilis* inclusion levels of 0 %, 0.75 %, 1.50 %, 3.00 %, 6.00 %, 12.00 %, and Abfeed S34 supplemented with fresh *G. gracilis* corresponding respectively to G0, G0.75, G1.50, G3.00, G6.00, G12.00, Gfresh + S34s. Comparative means with the same letter are not statistically different (RM-ANOVA, p = 0.007).

2.3.5. Relationship between whole-body mass and *Gracilaria gracilis* inclusion rate

There was a negative relationship between dry *G. gracilis* inclusion rate and whole-body mass, indicating that abalone were smaller as the inclusion rate increased during the first growth cycle ($y = - 0.3836x + 54.5322$, R^2=0.29, p=0.003) and the second growth cycle ($y = - 0.8191x + 84.8254$, $R^2 = 0.34$, $p = 0.0012$, Figure 2.7).

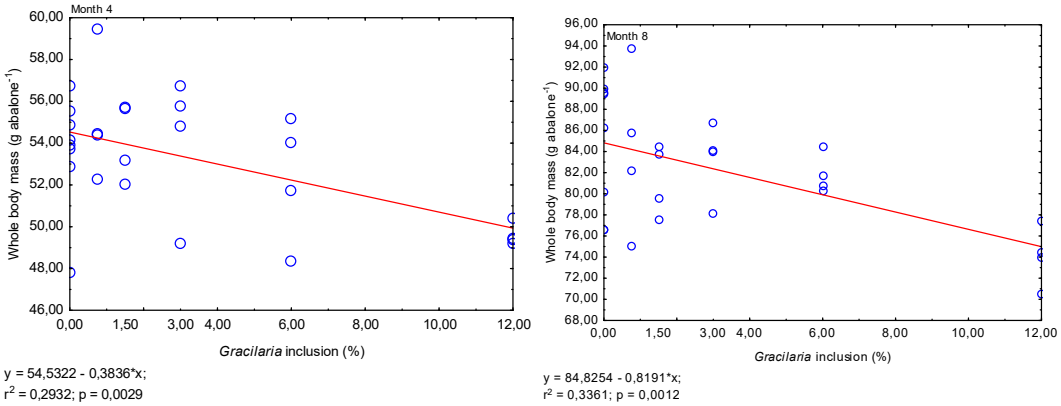

Figure 2.7: The relationship between dry *Gracilaria gracilis* inclusion level and whole body mass (g abalone^{-1}) in the first growth cycle and (b) the second growth cycle (Regression, $p < 0.05$).

2.3.6. Specific growth rate (SGR)

There was an interaction between time and dietary treatments (RM-ANOVA, $F_{(6, 29)} = 3.76$, $p = 0.0006$). The abalone fed Gfresh + S34s grew slower (0.27 ± 0.02 % weight gain day^{-1}) than abalone fed G0 (0.38 ± 0.02 % weight day^{-1}) and G0.75 diet (0.39 ± 0.02 % weight gain day^{-1}) in the first growth cycle (four months, Figure 2.8). However, abalone fed Gfresh + S34s grew significantly faster (0.41 ± 0.02 % weight gain day^{-1}) after eight months than in the previous growth cycle (Figure 2.8).

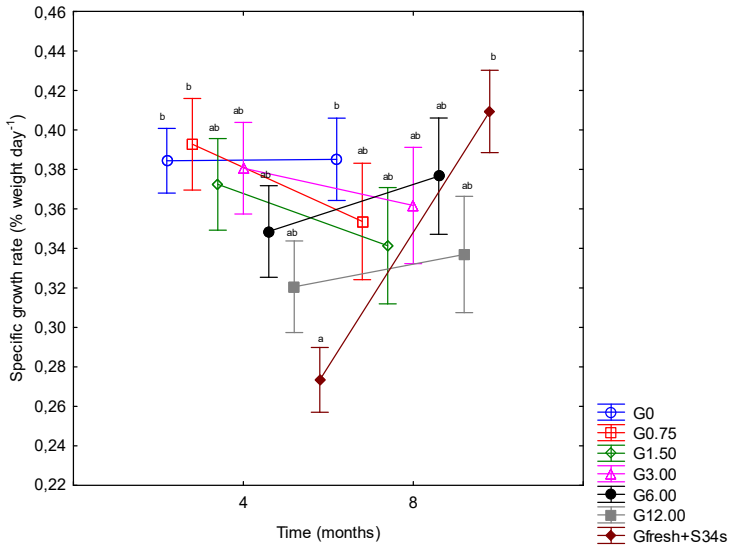

Figure 2.8: Mean (± SE) specific growth rate (% weight gain day^{-1}) of *Haliotis midae* fed formulated diets with *Gracilaria gracilis* inclusion levels of 0.75 %, 1.50 %, 3.00 %, 6.00 %, 12.00 %, and Abfeed S34 supplemented with fresh *G. gracilis* (RM-ANOVA, p = 0.00006). Inclusion levels correspond respectively to G0, G0.75, G1.50, G3.00, G6.00, G12.00, Gfresh + S34s. Comparative means with the same letter are not statistically different.

2.3.7. Relationship between specific growth rate and *Gracilaria gracilis* inclusion rate

There was a negative relationship between dry *G. gracilis* inclusion rate and specific growth rate, indicating that abalone grew slower as the *G. gracilis* inclusion rate increased during the first growth cycle ($y = - 0.0057x + 0.3879$, $R^2 = 0.27$, $p = 0.005$, Figure 2.9). No significant trend existed between the diets in the second growth cycle ($y = - 0.0057x + 0.381$, $R^2 = 0.03$, $p = 0.35$, Figure 2.9).

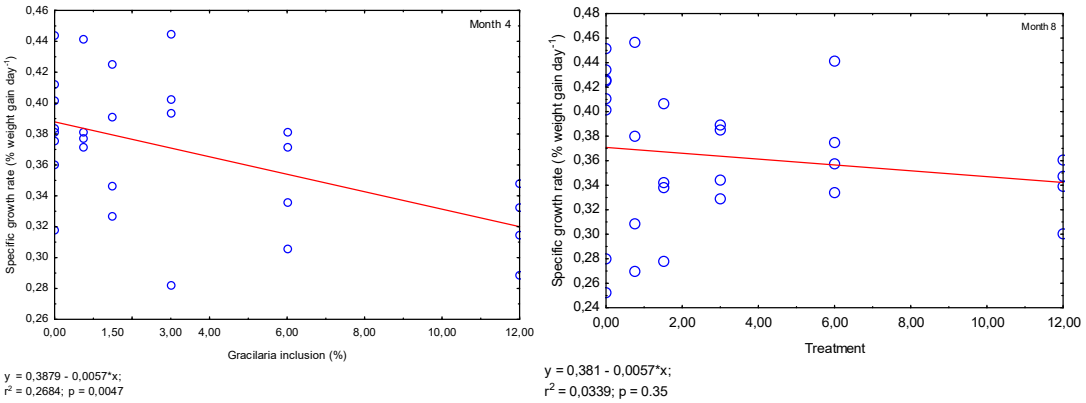

Figure 2.9: The relationship between dry *Gracilaria gracilis* inclusion level and specific growth rate (% weight gain day^{-1}) in the first growth cycle (Regression, $p < 0.05$).

2.3.8. Condition factor

There was no interaction between time and dietary treatments (RM-ANOVA, $F_{(12, 58)} = 1.40$, $p = 0.194$). However, there was a difference between treatments (RM-ANOVA, $F_{(6,29)} = 3.84$, $p = 0.006$). Abalone fed G12.00 had a significantly lower condition factor (1.11 ± 0.03 g mm^{-1}) than G0 fed abalone (1.23 ± 0.02 g mm^{-1}) and Gfresh + S34s (1.22 ± 0.02 g mm^{-1}) at the end of the eight months growth period (Figure 2.10).

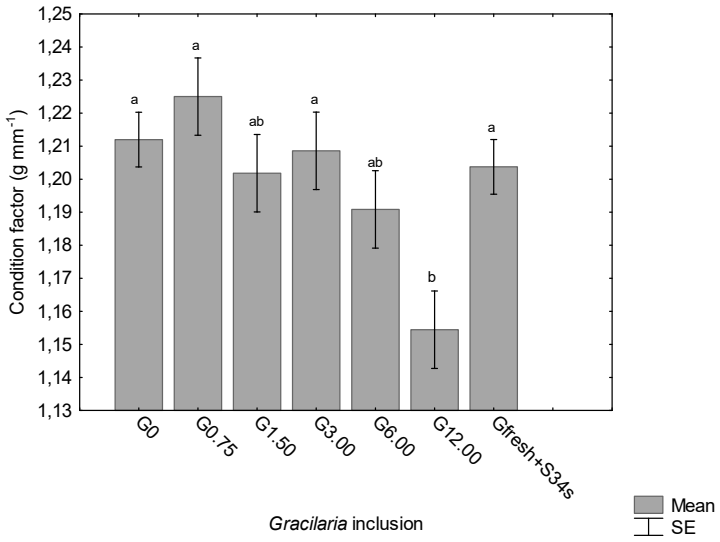

Figure 2.10: Mean (± SE) condition factor (g mm^{-1}) of *Haliotis midae* fed formulated diets with *Gracilaria gracilis* inclusion levels of 0.75 %, 1.50 %, 3.00 %, 6.00 %, 12.00 %, and Abfeed S34 supplemented with fresh *G. gracilis*. Inclusion levels correspond respectively to G0, G0.75, G1.50, G3.00, G6.00, G12.00, Gfresh + S34s. Comparative means with the same letter are not statistically different (RM-ANOVA, p = 0.006).

2.3.9. Relationship between condition factor and *Gracilaria gracilis* inclusion rate

There was a negative relationship between the condition factor and dry *G. gracilis* inclusion rate for the first growth cycle ($y = - 0.0044x + 1.222$, $R^2 = 0.24$, $p = 0.009$ and second-growth cycle ($y = - 0.0096x + 1.2233$, $R^2 = 0.51$, $p = 0.00002$), meaning abalone shell-to-flesh radio reduced with increasing inclusion level (Figure 2.11).

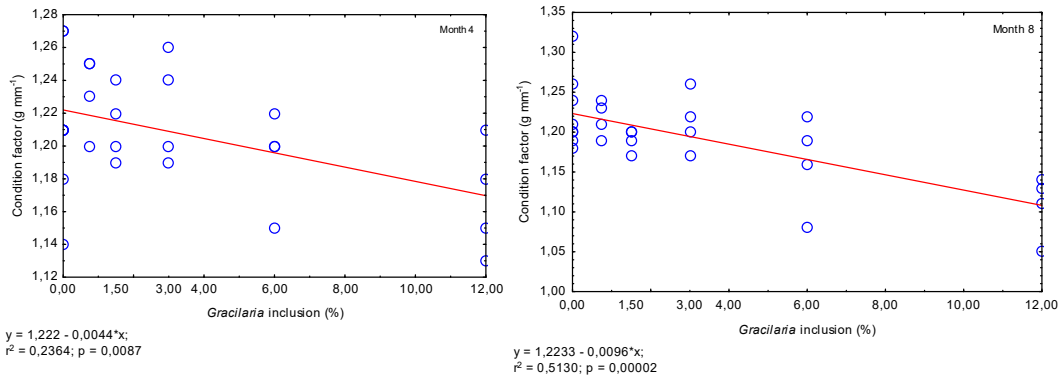

Figure 2.11: The relationship between dry *Gracilaria gracilis* inclusion level and condition factor (g mm^{-1}) in the first growth cycle and (b) the second growth cycle (Regression, $p < 0.05$).

2.3.10. Feed conversion ratio

There was no interaction between time and dietary treatments (RM-ANOVA, $F_{(6, 26)} = 2.47$, $p = 0.05$; Figure 2.12). However, there was a significant difference between treatments (RM-ANOVA, $F_{(6, 26)} = 4.90$, $p = 0.002$). The FCR for abalone fed G12.00 was significantly higher after four months than for the rest of the diets (Figure 2.12). The FCR was statistically the same for all the diets after eight months (Figure 2.12).

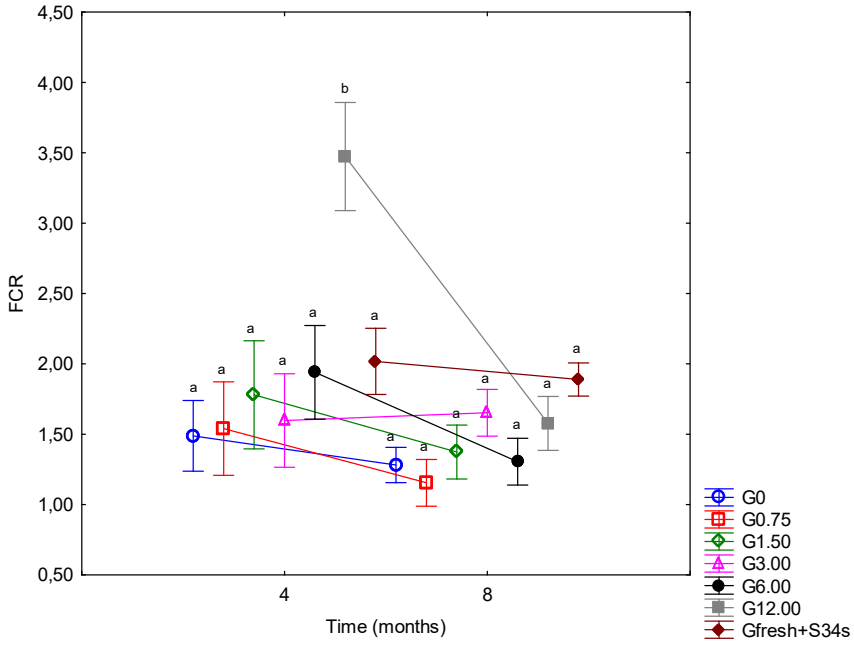

Figure 2.12: Mean (± SE) feed conversion ratio of *Haliotis midae* fed formulated diets with *Gracilaria gracilis* inclusion levels of 0.75 %, 1.50 %, 3.00 %, 6.00 %, 12.00 %, and Abfeed S34 supplemented with fresh *G. gracilis* (RM-ANOVA, p = 0.002). Inclusion levels correspond respectively to G0, G0.75, G1.50, G3.00, G6.00, G12.00, Gfresh + S34s. Comparative mean with the same letter are not statistically different.

2.3.11. Relationship between feed conversion ratio and *Gracilaria gracilis* inclusion rate

The positive relationship between FCR and dry *G. gracilis* inclusion in the first growth cycle indicates that FCR increased with inclusion level ($y = 0.1226x + 1.4225$, $R^2 = 0.37$, $p = 0.001$, Figure 2.13). There was no difference in the second growth cycle ($p = 0.09$, Figure 2.13).

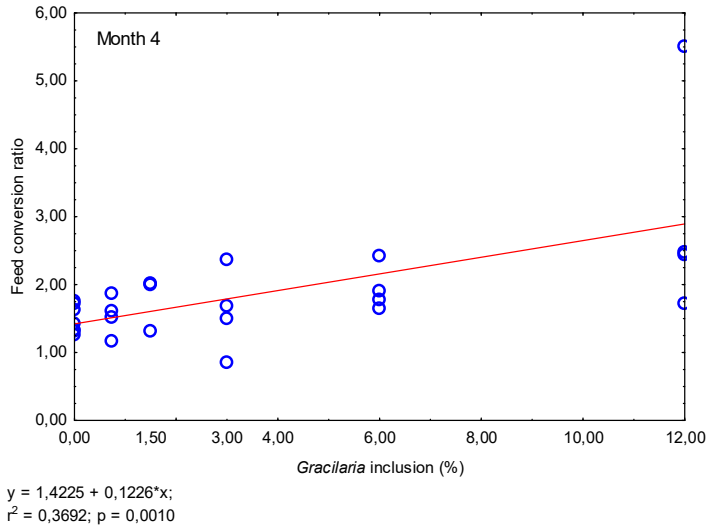

Figure 2.13: The relationship between *Gracilaria gracilis* inclusion level and feed conversion ratio in the first growth cycle (Regression, p = 0.001).

Anecdotal observations suggest that meat colour was affected by the inclusion of *G. gracilis* and colour intensity increased with algae inclusion level (Figure 2.14). The meat colour of the control diet was dark-brown with white discolouration (Figure 2.14a) whereas abalone fed Gfresh+S34s had a more yellow and brown meat (Figure 2.14c). Abalone fed G3.00 had a less apparent change in colour (Figure 2.14b).

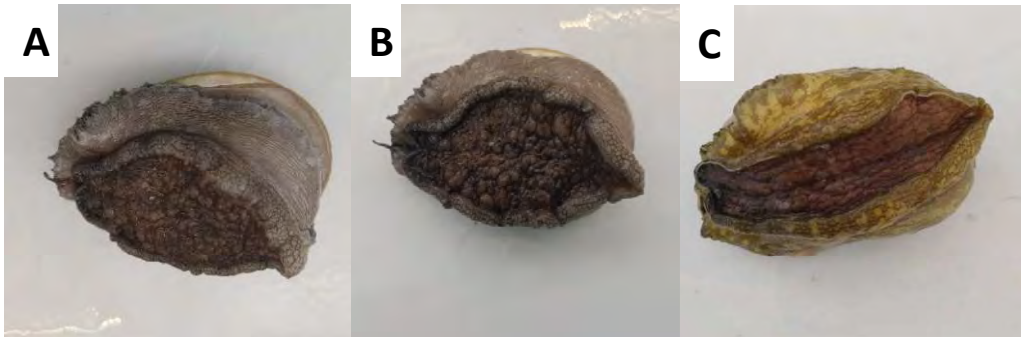

Figure 2.14: Details showing changes in meat colour of abalone *Haliotis midae* fed formulated diets with *Gracilaria gracilis* inclusion levels of 0 % (A), 3.00 % (B), and Abfeed S34 supplemented with fresh *Gracilaria gracilis* (C).

2.3.12. Water quality parameters

Table 2.5: Descriptive statistics of water quality parameters collected daily within culture tanks during the eight-month growth trial.

	Valid N	Mean	Median	Frequency of mode	Min.	Max.	Variance	SE
Flow rate (L s^{-1})	785	0.30	0.32	0.36	1.54	0.07	0.18	0.19
Temperature (°C)	787	16.61	15.9	34	12.5	24.3	5.29	0.08
pH	787	7.57	7.56	34	6.53	8.03	0.03	0.0061
DO (mg L^{-1})	787	6.66	6.64	10	4.86	8.33	0.36	0.021
Saturation (%)	787	83.68	84.1	10	7.55	103.2	56.78	0.27

2.4. Discussion

Feeding seaweed is a common practice by abalone farmers (Troell *et al.* 2006; Robertson-Andersson *et al.* 2007). However, mixing dry seaweed into formulated feed is still a novel approach towards developing sustainable diets. The aim was to use *G. gracilis gracilis* algae produced in a sea-based IMTA system to test its influence on abalone growth performance.

It is understood that the inclusion of dry seaweed into formulated feed impacts pellet water stability (Ighwela *et al.* 2013) due to the different physical properties (Sorenson & Phillips 1992) and dietary ingredients (Knauer *et al.* 1993). Reduced water stability means that feed pellets dissolve rapidly before being fully ingested (Ighwela *et al.* 2013). This arises from a low particle size homogeneity that increases dry matter leaching (Sales & Britz 2002; Sales & Janssens 2004; O'Mahoney *et al.* 2011). A stability of 90 % should be achieved during the time needed for consumption (Britz *et al.* 1994). Based on our variable water stability results, feeding a seaweed-based diet can be expected to affect feeding dynamics (McShane *et al.* 1994), digestion (Foale & Day 1992), absorption and eventually, the growth of abalone. In our study, the trend of reduction in abalone whole-body mass as *G. gracilis*

inclusion increased could indicate that the durability of the feed pellets with high macroalgae inclusion (between 3.00 % - 12.00 % inclusion) was compromised (Bansemer *et al.* 2014). Which means a high portion of pellets were going to waste and less remained for consumption. This explanation is also supported by G12.00 diet having the lowest retention (15.38 ± 0.22 %) of its dry matter than all formulated feeds. However, it must be noted that the stability of the pellet was calculated after 24 hours in water, and the abalone in this trial were fed "slightly in excess" where most feed was eaten within the first six to nine hours, so this retention rate was an over estimation (a retention rate after nine hours would have added additional value to this interpretation). Furthermore, it was not possible to collect uneaten food or account for leached nutrients in this trial because the experiment was carried out under commercial conditions on an abalone farm and not in a laboratory. Although the FCR measured in this trial was applicable to a real commercial situation, the interpretation of the FCR results, particularly when they are compared to laboratory studies, needs to be taken into consideration.

If pellets are less tough, the manipulation of feed particles by abalone during feeding could further impede the stability of the feed (Ighwela *et al.* 2013). In our study, the difference in the performance of the formulated feed may also be explained by the differences in physical properties of the diets associated with the varying seaweed inclusion levels (McShane *et al.* 1994). Naidoo *et al.* (2006) showed that fishmeal-based feeds produced significantly better growth and were more water-stable than seaweed-based feeds. McShane *et al.* (1994) demonstrated that the texture of a diet significantly influences the consumption rate of *Haliotis rubra* where the ingestion rate was lower for tougher pellets. In contrast, some studies predict that pellet toughness is of only minor importance (Fleming 1995).

Feeding a seaweed-based diet also changes the composition of dietary ingredients (Dawczynski *et al.* 2007; Gaillard *et al.* 2018; Shi *et al.* 2020). This means the necessary

carbohydrates, protein ratios and fat should be included to meet the nutritional requirements and achieve optimal abalone growth (Britz 1996a; Fleming *et al.* 1996; Sales & Britz 2001; Sales & Janssens 2004). To measure the influence of feed on growth, it is common practice to use growth indices such as body weight, growth rate and weight gain to quantify stock performance (Naidoo *et al.* 2006; Dlaza *et al*. 2008; O' Mahoney *et al.* 2014). However, it becomes a priority to also measure abalone health when developing a cost-effective commercial diet (Bansemer *et al.* 2014). Good abalone health is indicated by a high flesh-to-shell ratio, high shell quality, low physical damage during handling (Hooper *et al.* 2011), high survival rate (Stone *et al.* 2014). In our study, the high inclusion of dry *G. gracilis* as part of formulated feed resulted in lowered abalone health compared to the lowest *G. gracilis* inclusion diet (G0.75). The relatively lower condition factor values were abalone not meeting their nutritional requirements thus the poor growth rates and health. (Sales & Britz 2001).

Seaweeds have been recognized to have more benefits over abalone health than formulated feed due to bioactive molecules that reduce the vulnerability of abalone to biotic and abiotic stress (Hooper *et al*. 2007; Chojnacka *et al.* 2012; Bansemer *et al.* 2014). Dlaza *et al.* (2008) found that feeding a seaweed-based formulated diet resulted in negative change in condition factor values (0.864- final condition factor), whereas supplementing with fresh wild seaweed significantly improved the condition factor of all abalone (1.134). In our study, the increase in *G. gracilis* inclusion level resulted in a relative reduction in condition factor values which indicates that the health benefits from feeding seaweed could thus far be optimized by supplementing formulated feed with a fresh seaweed diet (Bullon *et al.* 2022). Although, the condition factor values lowered with increasing *G. gracilis* inclusion, they remained above 1.00 in both growth cycles.

It is crucial to maintain the benefits of formulated feed when developing a seaweed-based diet. However, risks exist from seasonal variations in the composition of the cultured

seaweed, which could limit controlled and consistent abalone growth (Qi *et al.* 2010; Bullon *et al.* 2022). *Gracilaria gracilis* cultured using a raft system yields the highest growth rate in summer (10 % per day). And in winter, there is an exceptionally low growth rate (below 7.5 % per day; Anderson *et al.* 1996). This also means the nutrient composition of seaweeds also varies seasonally, and differences could be affected by geographical area, species and water temperature (Jensen 1993; Marinho-Soriano *et al.* 2006; Rosen *et al.* 2000). Higher amounts of crude protein are usually present in seaweeds during spring and autumn (Gaillard *et al.* 2018). The poor performance of *G. gracilis* diets could be explained by the potential variations in the nutritional value of harvested sea-based *G. gracilis*. However, supplementing the formulated feeds with fresh farm-grown *G. gracilis* resulted in noteworthy, good outcomes.

The abalone fed Gfresh + S34s (supplemented diet) were growing slower than abalone fed the control and G0.75 diet in the first growth cycle (four months). However, the growth rate of abalone fed the supplemented diet was the same as abalone fed the control and G0.75 on the second growth cycle. The quantity of *G. gracilis* fed along with S34 abfeed in the first growth cycle was 7.18 kg dry weight whereas in the second growth cycle 36.16 kg (80.14 % more) dry *G. gracilis* was fed. The improvement in abalone growth is most likely attributed to the increase in fresh macroalgae volumes fed. Although the amount of fresh *G. gracilis* available to abalone changed over time, food scarcity was not a concern because the reduction in *G. gracilis* meant more S34s was available for abalone to meet the nutritional demand. Dlaza *et al*. (2008) observed that supplementing Abfeed® with fresh seaweed significantly improves abalone growth (1.05 ± 0.02 % weight gain day^{-1}; 63.61 ± 0.05 µm day^{-1}) and health (1.447 final condition factor). Abalone cultured under different macroalgae treatments had significantly higher growth rates and health compared to abalone fed formulated feeds (Mulvaney *et al.* 2013). Previous studies have found that the culture of macroalgae in

nutrient-rich water increases their protein content (Viera *et al.* 2005; Robertson-Andersson 2006). Viera *et al.* (2011) tested the growth performance of abalone fed enriched versus non-enriched macroalgae. The findings were that seaweed cultured in effluent had a higher protein content, and the weight gain and specific growth rate of abalone were positively correlated to the protein content of abalone along with seasonal variations in algae nutrient composition likely contributed to the improved growth rate of abalone fed the supplemented diet (Viera *et al.* 2011). The farm-grown *G. gracilis* seaweed in our study was nutrient enhanced through routine fertilization thus the benefit of this procedure could have translated to good abalone growth.

The changes in the nutritional profile of feed lead to a change in the appearance of abalone and this affects the product quality (Smit *et al.* 2007). Modifications in the foot muscle colour have been demonstrated in other studies (Cyrus *et al.* 2013). Denti (2015) determined that *Haliotis tuberculata coccinea* foot muscle became more yellow when fed formulated feed or IMTA produced fresh macroalgae, whereas wild abalone specimen developed a more orange colour. Our study demonstrated that macroalgae can influence the colour of the foot muscle and can in future be used as a tool to enhance the perception of farmed abalone depending on market acceptance (Sales & Britz 2001; Bullon *et al.* 2022).

2.5. Conclusion

The influence of IMTA *G. gracilis* based formulated feed and a mixed diet on abalone growth was evaluated. The growth performance of abalone fed the diet treatments was compared. There was a negative relationship between abalone whole body mass and *G. gracilis* inclusion. However, quantifying the benefits at high inclusion levels was potentially limited by the poor water stability and texture of the high inclusion feed. The condition factor values remained above 1.00 across all diets which indicates an above average flesh to shell ratio which is a proxy for good health.

Supplementing compound feed with *G. gracilis* however indicated noteworthy results. Less compound feed was used in the Gfresh + S34s dietary treatment but abalone CF and FCR was the same as the best performing diets (control and G0.75) after 8 months. The growth of abalone fed Gfresh + S34s seems to have been limited by the volume of fresh *G. gracilis* fed to abalone in each growth cycle. If feeding less compound feed and more fresh G. *gracilis* algae yields similar growth as that achieved from using compound feed only, then this provides the scope for further investigation of this diet. Evaluating the behaviour of abalone fed IMTA *G. gracilis* based diets along with a mixed diet would prove valuable in indicating the influence of *G. gracilis* on farmed abalone.

Chapter 3 - Behaviour of abalone fed graded levels of *Gracilaria gracilis*

3.1. Introduction

The high demand for abalone has resulted in a decline in wild stock while subsequently stimulating growth of global abalone aquaculture (Troell *et al.* 2006; FAO 2018). This growth has meant that understanding the life cycle and behaviour of abalone has become more important (Venter *et al.* 2018). When farming abalone, the broodstock are first spawned and reproductive cells fertilized (broodstock phase; Tutschulte & Connell 1988; Kawamura *et al.* 2001; Venter *et al.* 2018). Like in the natural environment, the process results in lecithotrophic larvae that eventually settle and begin to feed on microalgae (larvae phase). The larvae, now referred to as spat, are weaned off their natural diet into artificial feed (weaning phase). After this process, the abalone move to the grow-out phase, where they will remain until they reach marketable size (80 g 90 mm^{-1} shell length; Venter *et al.* 2018).

One of the main limiting factors for abalone culture is changes in normal abalone behaviour due to induced stress. According to Parker (2002), stress can be classified into four main groups, chemical stressors (water quality issues - low pH and dissolved oxygen, chemical treatment, metabolic waste), physical stressors (temperature & light), biological stressors (stocking density, food competition, predators, disease), and procedural stressors (handling, disease treatment and shipping). Properly managing and minimizing changes in normal abalone activity could mean the health and growth of the animals are maximised (Huchette *et al*. 2003).

The influence of stressors on intensively cultured abalone behaviour has been extensively studied (Pearse & Arch 1969; Breen & Adkins 1980; Shepard 1986; Buss *et al*. 2015). For instance, under intensive culture conditions, time of feeding and light intensity have been

identified to influence abalone behaviour, with darkness stimulating higher grazing and growth in relation to light exposure (Allen *et al.* 2006). Stocking density was also identified to influence abalone behaviour, with low densities yielding higher growth in comparison to higher densities (Huchette *et al.* 2003). Thermal stress has also influenced abalone *H. midae* behaviour (Hecht 1994). When abalone are subjected to various thermal regimes, the distribution (frequency of abalone occurrence) was highest at 24 ºC (Hecht 1994).

Abalone have been described largely as nocturnal feeders (Wood 1993; Wood & Buxton 1996), meaning they emerge at night to feed or migrate to a new site (Cotton 1959). Movement can also occur as escape locomotion when abalone are confronted with predators (Donovan & Carefoot 1997). Escape locomotion has been identified as a stress indicator because adhesive crawling is an energy-demanding activity requiring 44.1 J kg^{-1} m^{-1} of energy (Donovan & Carefoot 1998). The increase in velocity correlates with increased energy requirement and induced anaerobic metabolic processes. This means there is a high metabolic cost due to increased muscle activity and secretion of protein-rich mucus (Donovan & Carefoot 1998; Buss *et al.* 2015). Farmers address this concern by increasing food availability or ration, feeding before dusk, feeding "slightly in excess" and ensuring uniform dispersion of feed along the water column (Fleming *et al.* 1996; Allen *et al.* 2006; Buss *et al.* 2015).

Feed can also influence abalone behaviour (Barkai & Griffiths 1987; Fleming *et al*. 1996; Sales & Britz 2001; Allen *et al*. 2006). For example, *Haliotis laevigata* were observed to show homing behaviour and to reside on the same substrate for an extended period when food is available and only move to different substrates when food is scarce (Shepard 1973). In the wild abalone have been identified to feed particularly in two ways: by actively grazing and seeking food through locomotion, returning to inactivity during daytime (Bonnot 1948;

Tutschulte & Connell 1988; Cornwall *et al.* 2009), or by catching algae that drift within reach (Tutschulte & Connell 1988). The foraging behaviour is more plastic in response to the availability of drift algae (Cornwall *et al.* 2009). The natural feeding pattern of abalone also varies depending on the development stage of the radular morphology (Fleming *et al*. 1996; Kawamura *et al*. 2001) and ontogenetic changes in the digestive enzyme activities (Takami & Kawamura 2003).

Abalone farming facilities have adopted the use of nutritionally complete formulated feed as a food source (Britz *et al*. 1994; Cook 1998). This means that the response of abalone fed the same diet is mainly consistent (Allen *et al*. 2006). Artificial feeds require abalone to actively search for food which is energetically costly (Donovan & Carefoot 1998). However, feeding a mixed diet that constitutes live fresh or dried macroalgae and an artificial diet can prompt a feed preference driven response of abalone to the diet (Fleming *et al.* 1996).

When feeding a new or mixed diet, two components are realized: feed edibility and attractiveness (Fleming *et al*. 1996). Feed edibility is the rate at which the food item is handled and ingested (Nicotri 1980). Edibility can also correspond with food item toughness and the presence of antinutritional compounds (Fleming 1995). The ability of abalone to effectively ingest food items is also influenced by attractiveness (Nicotri 1980). Feed attractiveness is the action of chemosensory cues in stimulating or permitting abalone to choose between food items (Nicotri 1980). Abalone mainly rely on chemical and tactile cues to detect food particles for efficient ingestion and olfactory cues for larvae settlement (Donovan & Carefoot 1998). Therefore, a macroalgae inclusive diet could potentially cause abalone to behave differently than they would when feeding on formulated feed without macroalgae, due to feed edibility and attractiveness (Buss *et al.* 2015).

Therefore, it becomes crucial to determine the influence of a macroalgae inclusive diet on the behavioural response of abalone. The aim was to determine if the inclusion of macroalgae in formulated feeds, and fresh live macroalgae diets influence the activity of farmed abalone *Haliotis midae*. Changes in abalone activity among treatments with *G. gracilis* were monitored and compared with the control abalone treatment fed artificial pellets without *G. gracilis*. It is hypothesised that *G. gracilis* inclusion has no effect on the behaviour of abalone *Haliotis midae.* The main research questions are: Do abalone become active and stay alert when *G. gracilis* is included in their diet? Does the inclusion rate of *G. gracilis* mean abalone become more active and alert? Does *G. gracilis* inclusion and inclusion rate influence the feeding response of abalone?

3.2. Materials and methods

3.2.1. Experimental animals and treatments

The behavioural observations were conducted on the same system and animals as described previously (Chapter 2; Section 2). The animals were fed one of seven dietary treatments (Chapter 2; Section 2) for 155 days before behavioural observations were made.

3.2.2. System preparation

Tanks with the replicate baskets were cleaned 48 hours before the experiment to minimise the potential impacts of chemical and physical stressors from a reduction in water quality.

3.2.3. Quantifying activity

First, the abalone were observed, and their behaviour was described. Observed activities were grouped into three main categories (Table 3.1), and the behavioural descriptions were made unique to each activity. All activities were quantified by counting the abalone using the naked-eye and a red-light source for illumination. As abalone are nocturnal feeders all

observations were made at night (Muller 1984; Tutschulte & Connell, 1988; Wood & Buxton 1996).

Table 3.1: The predetermined specific activity states of abalone that are categorized under level of activity and feeding activity.

Category	Activity	Description
Level of activity	Quiescent	No locomotive movement; AND shell tightly held on aquarium; AND cephalic and mantle tentacles retracted.
	Alert	No locomotive movement; AND shell raised from aquarium surface; OR cephalic and mantle tentacles pronounced OR head raised from the surface.
	Moving	Actively locomoting.
Feeding activity	Feeding	Head in contact with feed item.
	Not feeding	Head not in contact with feed item.

3.2.4. Data collection

At 18h00 water quality parameters (pH, dissolved oxygen, water temperature, oxygen saturation, and water flow rate) were collected on all experimental tanks using the same instruments described previously (Chapter 2; Section 2). At 19h00, baskets from each treatment were randomly selected and observed. Observations on selected baskets were done before feeding and subsequently one hour, three hours, six hours and nine hours after feeding (Buss *et al.* 2015), corresponding with (T0 i.e., before feeding, T1h, T3h, T6h, T9h after feeding).

To make the observations, aeration was first switched off and the top plate removed from the water. The abalone on the top plate were counted in terms of their level of activity. The top plate was placed aside, and the level of activity (i.e., movement then alert and quiescent) was monitored on the whole basket from above the tank water surface. Level of activity was followed by feeding activity (Buss *et al*. 2015). Abalone not feeding were calculated based on the difference between the total abalone count in the basket and the count of abalone feeding (Figure 3.1).

When assessing activity, counting took place on observable abalone with shell fully exposed/visible (Lloyd & Bates 2008). Obscured abalone (i.e., under the feeder ring and inner rack) were neglected. Abalone stack under conditions where they compete for a limited resource which may include both space and food (Douros 1987). In this study, space and food were assumed not to be limiting; thus, abalone stacking were ignored (Day *et al.* 2004; Buss *et al.* 2015). Observations were made for no longer than two minutes per basket. No more than two baskets per tank were observed at a time to prevent prolonged disturbance from confounding the observed activity.

Feed was placed directly on top of the feeder ring before data collection for consistency. The same process was accomplished for the diet treatment where fresh *G. gracilis* was a supplement diet. Data (i.e., count, basket number, time) was captured on a recording sheet and used for further analysis.

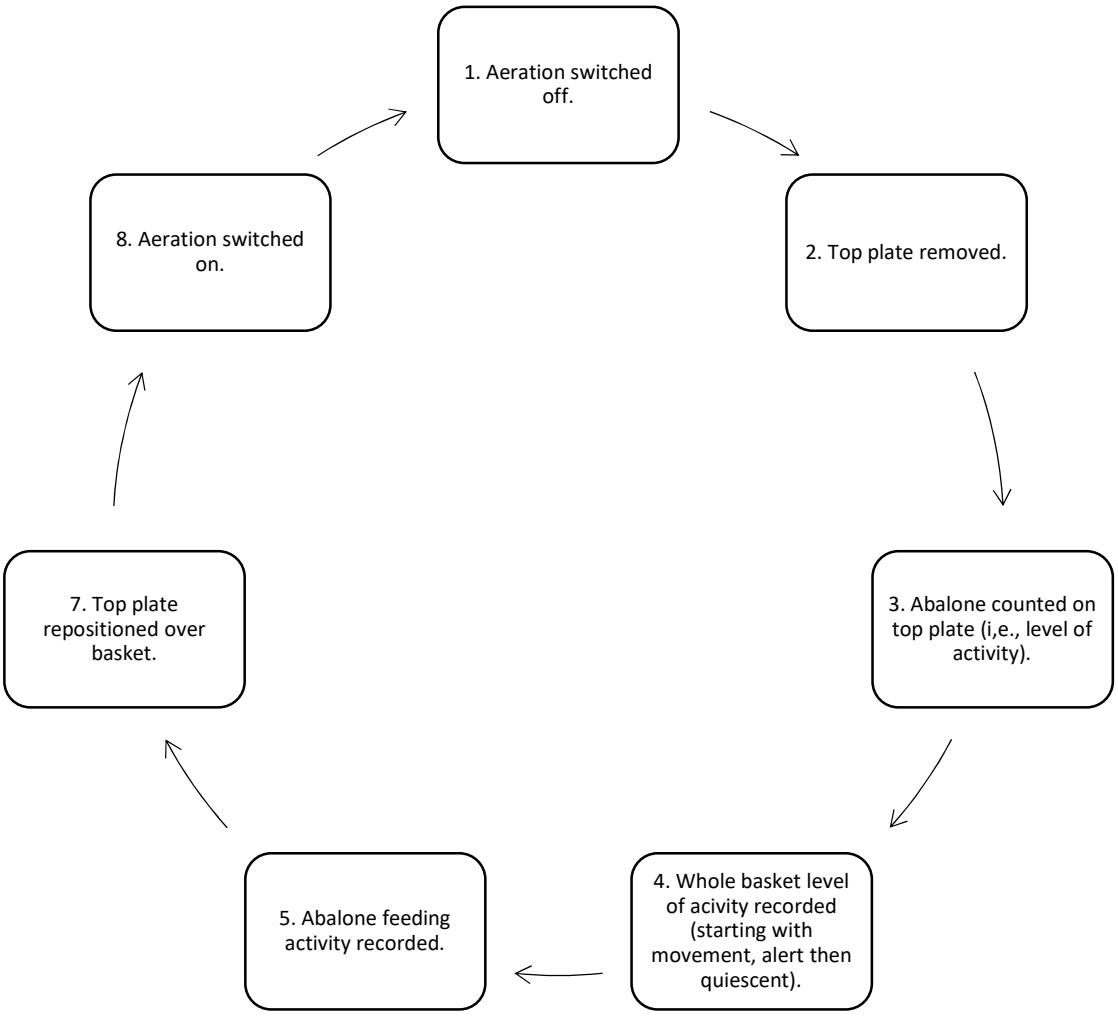

Figure 3.1: Diagram showing how data was collected during the study. The process was repeated according to the predetermined time sequence (0, 1, 3, 6, and 9 hours after feeding).

3.2.5. Statistical analysis

The effect of *G. gracilis* inclusion (G0, G0.75, G1.50, G3.00, G6.00, G12.00, Gfresh + S34s) on the behaviour was analyzed separately before feeding and after feeding using a one-way analysis of variance (ANOVA) after verifying the conditions of normality of residuals and homogeneity of variances (Levene 1960). The different behavioural variables obtained 1h, 3h, 6h and 9h after feeding were averaged across diet replicates to obtain the behaviour after

feeding. To compare the different treatments, post-hoc analysis was done using contrast analysis with emmeans R package (Lenth 2016) and the adjustment of Bonferroni.

Thereafter, a linear mixed model analysis (LMM) was used to study more precisely the interaction between *G. gracilis* inclusion and time of feeding using the package nlme (Pinheiro *et al.* 2018). The model included the treatment (G0, G0.75, G1.50, G3.00, G6.00, G12.00, Gfresh + S34s) and the time (T0 i.e., before feeding, T1h, T3h, T6h, T9h after feeding) as fixed effect and the basket number as random effect to take into account the repeated measures done on the same basket. To study the evolution of the behaviour in function of the time for each treatment, post-hoc analysis was done when the interaction was significant using contrast analysis with emmeans R package (Lenth 2016) with adjustment of Bonferroni.

3.3. Results

Table 3.2: Water quality parameters of tank systems holding the observed baskets during the nights of the behavioural study.

Diet treatment	Flow rate (L s^{-1})	Temperature (°C)	pH	Dissolved oxygen (mg L^{-1})	Oxygen saturation (%)
G0	0.29	15.30	7.63	6.38	79.20
G0.75	0.27	15.20	7.65	6.94	86.00
G1.50	0.14	15.40	7.48	6.34	78.80
G3.00	0.13	15.40	7.46	6.67	79.20
G6.00	0.24	15.20	7.46	6.43	78.40
G12.00	0.20	15.30	7.53	6.75	83.60
Gfresh + S34s	0.21	15.40	7.56	6.73	83.40

3.3.1. Behaviour before feeding

An effect of the *G. gracilis* inclusion was observed on the percentage of abalone quiescent ($p = 0.002$), alert ($p = 0.002$) and moving ($p < 0.001$) (Table 3.3). Before feeding, abalone from the lowest *G. gracilis* inclusion were the less quiescent, the more alert and moving more compared to abalone receiving a higher percentage of *G. gracilis* inclusion and fresh *G. gracilis* (see Table 3.3 for detailed comparison).

3.3.2. Behaviour after feeding

After feeding, an effect of the *G. gracilis* inclusion was observed on the percentage of moving abalone ($p = 0.003$) and feeding abalone ($p = 0.027$) (Table 3.3). The main finding was that abalone receiving fresh algae were moving less and feeding more than the abalone receiving pellets (see Table 3.3 for detailed comparison).

Table 3.3*:* Behaviour of abalone *Haliotis midae* before and after feeding in function of the percentage of inclusion of dry *Gracilaria gracilis* in pellets (no inclusion, 0.75 %, 1.50 %, 3.00 %, 6.00 %, 12.00 % inclusion and fresh *G. gracilis* corresponding respectively to G0, G0.75, G1.50, G3.00, G6.00, G12.00, Gfresh + S34s). ANOVA analysis with post-hoc analysis with Bonferroni adjustment Mean ± S.E. Different letters indicate significant difference between treatment ($p < 0.05$). δ indicate log transformation for analysis.

	G0	G0.75	G1.50	G3.00	G6.00	G12.00	Gfresh+S34s	S.E.	$F_{6,14}$	P
Before feeding										
Quiescent abalone (%)	55.7 ^{ab}	56.3 ^{ab}	37.6 ^{a}	90.0 ^{b}	63.7 ^{ab}	87.3 ^{b}	84.9 ^{b}	7.64	6.76	0.002
Alert abalone (%)	31.5 ^{ab}	38.4 ^{ab}	55.2 ^{a}	7.8 ^{b}	31.6 ^{ab}	11.8 ^{b}	11.1 ^{b}	6.96	6.26	0.002
Moving abalone (%)	12.8 ^{a}	5.3 ^{b}	7.2 ^{ab}	2.2 ^{b}	4.7 ^{b}	0.9 ^{b}	4.0 ^{b}	1.39	7.81	< 0.001
After feeding										
Quiescent abalone (%)	59.2	45.3	40.9	57.9	33.8	55.6	42.8	6.17	2.48	0.075
Alert abalone (%)	30.7	43.5	41.7	28.5	44.7	34.3	51.1	5.35	2.37	0.086
Moving abalone (%)	10.1 ^{ac}	11.2 ^{abc}	17.4 ^{ab}	13.5 ^{abc}	21.5 ^{b}	10.2 ^{ac}	6.1 ^{c}	2.15	5.76	0.003
Feeding abalone (%)	2.2 ^{ab}	1.9 ^{ab}	3.0 ^{ab}	1.9 ^{ab}	1.7 ^{a}	2.3 ^{ab}	8.0 ^{b}	0.98	3.42	0.027 $^{\delta}$

3.3.3. Interaction between dietary treatment and time - Quiescent abalone

For the percentage of quiescent abalone, a treatment, a time and an interaction effect between treatment and time was observed (respectively for treatment, time, and interaction effects: $F_{(6, 14)} = 3.10$, $p = 0.038$; $F_{(4, 56)} = 10.14$, $p < 0.001$, $F_{(24, 56)} = 1.81$, $p = 0.034$, LMM analysis). While no difference of the percentage of quiescence was observed before and after feeding for the lowest *G. gracilis* diet inclusion (G0, G0.75, G1.50), abalone with the highest *G. gracilis* inclusion were less quiescent between 3 and 6 hour post-distribution ($p < 0.05$, contrast analysis with Bonferonni correction, Figure 3.2). Abalone receiving fresh algae were less quiescent from the first hour post-distribution, and still more active 9 hours after ($p < 0.05$, contrast analysis with Bonferonni correction, Figure 3.2).

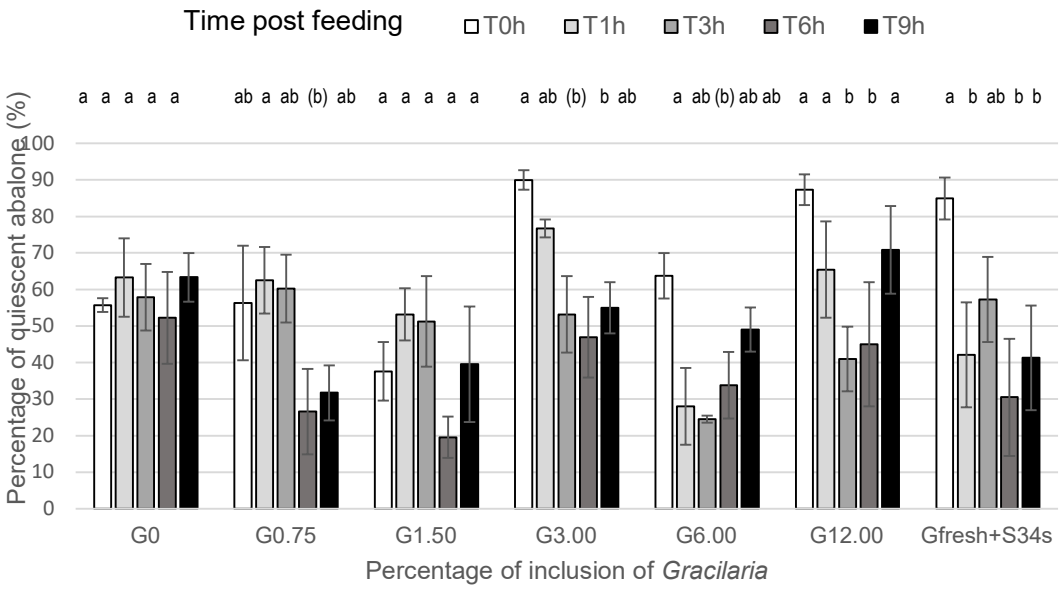

Figure 3.2: Percentage of (A) quiescent abalone during feeding session (before feeding and 1, 3, 6 and 9 h after feeding, respectively T0h, T1h, T3h, T6h and T9h) in function of the percentage of inclusion of dry *Gracilaria gracilis* in pellets (no inclusion, 0.75 %, 1.50 %, 3.00 %, 6.00 %, 12.00 % inclusion and fresh *G. gracilis* corresponding respectively to G0, G0.75, G1.50, G3.00, G6.00, G12.00, Gfresh+S34s). Different letters indicate significant difference intra diet ($p < 0.05$). Different letters within bracket indicate a trend intra diet ($p < 0.10$). Linear mixed model with contrast analysis for post-hoc analysis.

3.3.4. Interaction between dietary treatment and time - alert abalone

For the percentage of alert abalone, a time and an interaction between treatment and time were found, as well as a trend for a treatment effect (respectively for treatment, time, and interaction effects: $F_{(6, 14)} = 2.65$, $p = 0.063$; $F_{(4, 56)} = 6.91$, $p < 0.001$; $F_{(24, 56)} = 2.21$, $p = 0.008$; LMM analysis). Abalone from the lowest *G. gracilis* diet inclusion (G0, G0.75, G1.50) were characterized by only very few differences between before feeding and after feeding. In contrast, a higher percentage of alertness was observed the first hours after food

distribution when the fresh *G. gracilis* was distributed as well as 3.00 % and 12.00 % inclusion ($p < 0.05$, Figure 3.3).

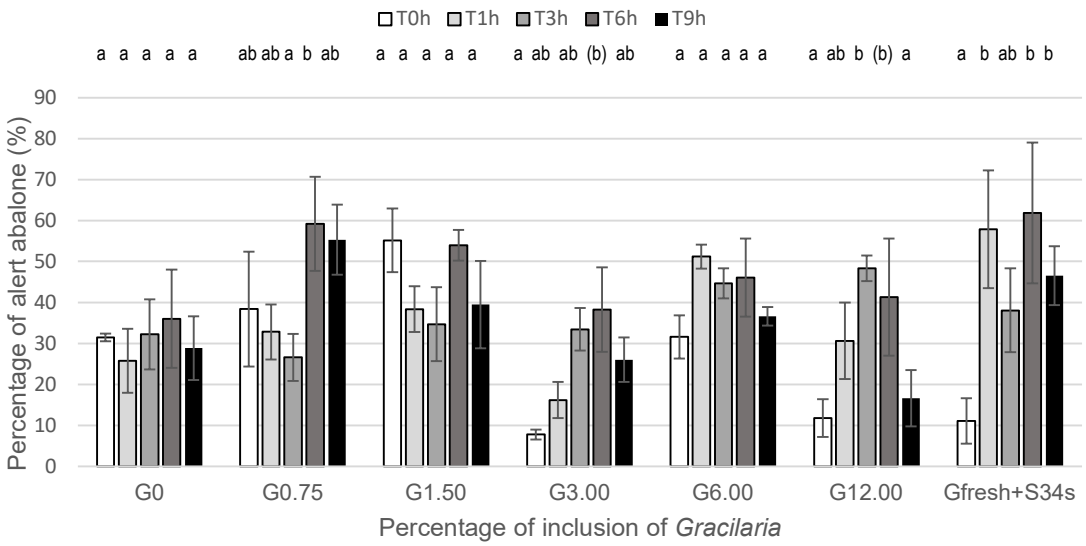

Figure 3.3: Percentage of alert abalone during feeding session (before feeding, 1 hour, 3 hours, 6 hours, 9 hours after feeding, respectively T0h, T1h, T3h, T6h, T9h) in function of the percentage of inclusion of dry *Gracilaria gracilis* in pellet (no inclusion, 0.75 %, 1.50 %, 3.00 %, 6.00 %, 12.00 % inclusion and fresh *G. gracilis* corresponding respectively to G0, G0.75, G1.50, G3.00, G6.00, G12.00, Gfresh + S34s). Different letters indicate significant difference intra diet ($p < 0.05$). Different letters within bracket indicate a trend intra diet ($p < 0.10$). Linear mixed model with contrast analysis for post-hoc analysis.

3.3.5. Interaction between dietary treatment and time - moving abalone

For the percentage of moving abalone, a treatment and a time effect were found, as well as a trend for an interaction (respectively for treatment, time, and interaction effects: $F_{(6, 14)} = 6.08$, $p = 0.003$; $F_{(4, 56)} = 9.80$, $p < 0.001$; $F_{(24, 56)} = 1.66$, $p = 0.061$; LMM analysis with log transformation). Abalone from the lowest *G. gracilis* diet inclusion were (G0, G0.75) and

abalone with fresh *G. gracilis* were active the same way before and after feeding ($p > 0.05$, Figure 3.4). In contrast, a higher percentage of abalone from the G1.50 up to G12.00 were characterized by increased movement up to 9 hours after feeding ($p < 0.05$, Figure 3.4).

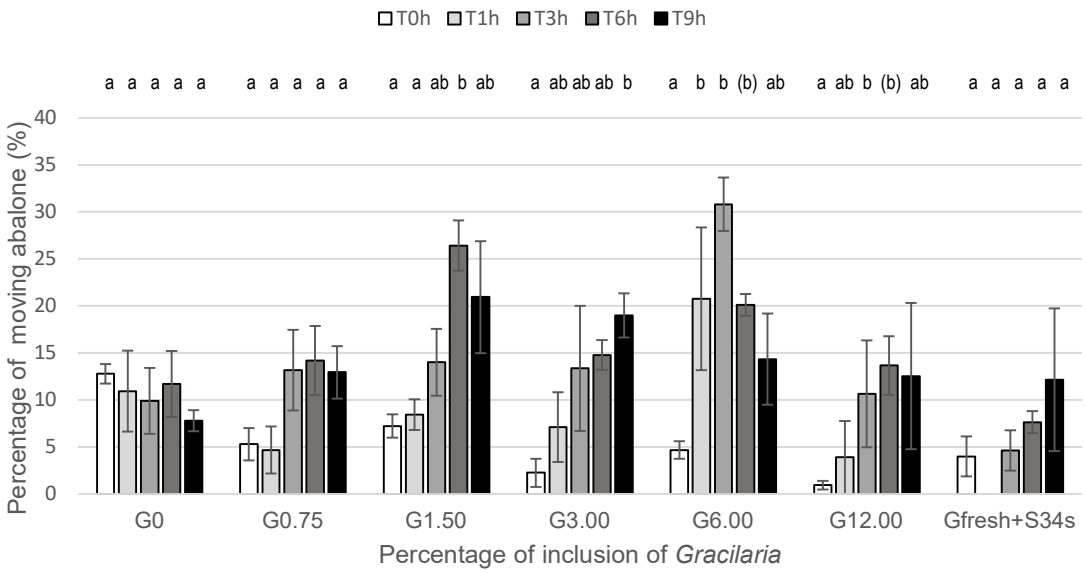

Figure 3.4: Percentage of moving abalone during feeding session (before feeding, 1 hour, 3 hours, 6 hours, 9 hours after feeding, respectively T0h, T1h, T3h, T6h, T9h) in function of the percentage of inclusion of dry *Gracilaria gracilis* in pellets (no inclusion, 0.75 %, 1.50 %, 3.00 %, 6.00 %, 12.00 % inclusion and fresh *G. gracilis* corresponding respectively to G0, G0.75, G1.50, G3.00, G6.00, G12.00, Gfresh + S34s). Different letters indicate significant difference intra diet ($p < 0.05$). Different letters within bracket indicate a trend intra diet ($p < 0.10$). Linear mixed model with contrast analysis for post-hoc analysis.

3.3.6. Interaction between dietary treatment and time - feeding abalone

A treatment, as well as a time and an interaction effect was found for the percentage of abalone feeding (respectively for treatment, time, and interaction effects: $F_{(6, 14)} = 5.10$, $p =$

0.006; $F_{(3, 42)} = 13.99$, $p < 0.001$; $F_{(18, 42)} = 2.66$, $p = 0.005$; LMM analysis). The same proportion of abalone with G0, G0.75, and G6.00 *G. gracilis* inclusion were feeding all along the 9 hours post-feeding. In contrast, a reduction of feeding was observed between T1h and T9h for abalone G3.00, G12.00, as well as for abalone receiving fresh algae ($p < 0.05$, Figure 3.5).

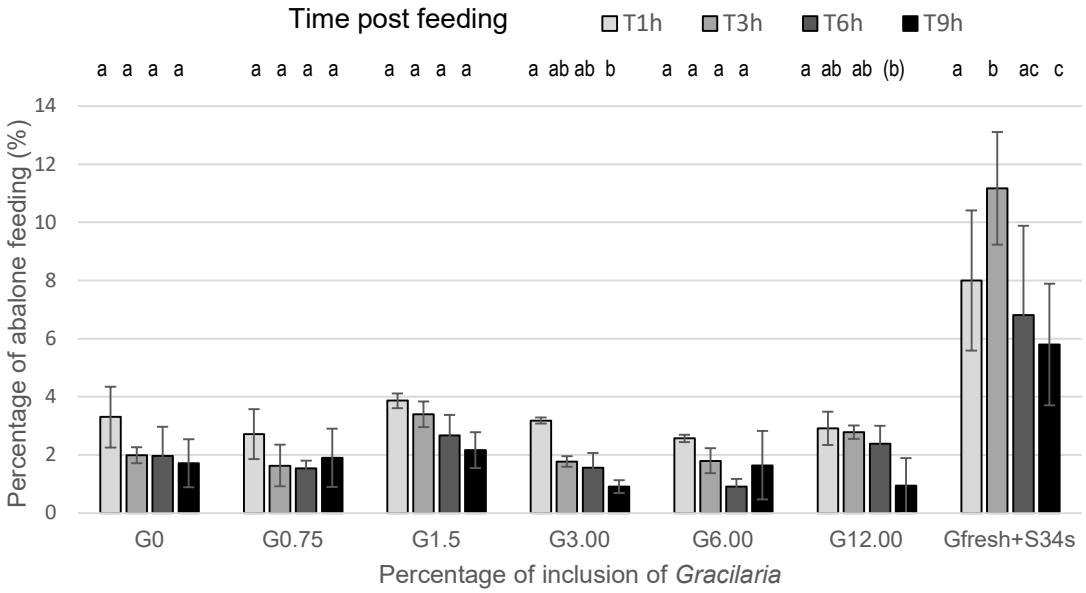

Figure 3.5: Percentage of abalone feeding (1 hour, 3 hours, 6 hours, 9 hours after feeding, respectively T1h, T3h, T6h, T9h) in function of the percentage of inclusion of dry *Gracilaria gracilis* in pellets (no inclusion, 0.75 %, 1.50 %, 3.00 %, 6.00 %, 12.00 % inclusion and fresh *G. gracilis* corresponding respectively to G0, G0.75, G1.50, G3.00, G6.00, G12.00, Gfresh + S34s). Different letters indicate significant difference intra diet ($p < 0.05$). Different letters within bracket indicate a trend intra diet ($p < 0.10$). Linear mixed model with contrast analysis for post-hoc analysis.

3.4. Discussion

The characteristics used to identify each level of abalone activity are more meaningful when examined under a controlled environment experiencing limited fluctuations in physical conditions (Allen *et al.* 2006). Literature suggests that changes in abalone quiescence are predominantly influenced by physical conditions more than a feeding response (Tutschulte & Connell 1988; Allen *et al.* 2006; Buss *et al.* 2015). Preliminary studies have investigated the behaviour of abalone and their appearance rate from their shelter at night (Knauer *et al.* 1995). It was found that the appearance rate increased from 18h00 and 23h00 whereafter the animals retreated to their shelter (Uki 1981; Knauer *et al*. 1995). In our study, the feeding response time was between 19h00 and 03h00 at night and the reduction in quiescence is more prominent in a few treatments with high *G. gracilis* inclusion levels (G3.00 to G12.00) between T0h and T3h (or between 19h00 to 22h00).

Many studies have attributed the trend of a reduction in quiescence during darkness as an evolutionary response to reduced vulnerability to predation on active abalone during the night (Shepard 1973, Hahn 1989a, Jenkins 2004). Other studies have established the change in quiescence as a thermal stress response (Sakai 1962; Yang 2019) which allows abalone to control their metabolic rate (Kang *et al*. 2019). Physiologically optimal temperature conditions for *H. midae* abalone range between 12 - 20 °C (Britz & Hecht 1997). During data collection for our study the temperature range did not exceed or fall below this range thus abalone were not considered thermally stressed. The response was potentially linked with feed type and feed condition. The softening of feed means palatability increases which allows abalone to easily access water soluble nutrients for digestion (Sales & Janssens 2004). This means water stability of the feed pellets potentially played a significant role on the activity of abalone after feeding (Mulvaney *et al*. 2013).

Although natural feeds have been reported to improve abalone health, marketability, product quality (Brown *et al.* 2008), it is understood that they can also act as a stimulant that is detected by abalone using not only chemosensory cues but also tactile cues (Allen *et al*. 2006). Acknowledging the influence of chemosensory and tactile cues on abalone behaviour could help in gaining an understanding on how abalone respond effectively to feed (Allen *et al.* 2006). Allen *et al.* (2006) investigated the effect of tactile stimulants on abalone behaviour. The findings indicated that abalone provided with formulated diet without dry *G. gracilis* (control) remained sedentary for most of the time (Allen *et al*. 2006). These findings also explain why the level of quiescence for abalone fed the control diet in our study was the same throughout the observation period.

Haliotis iris abalone exposed to *G. gracilis* particles spent 80% of their time engaging in feeding-related activities (Allen *et al.* 2006). When feeding a mixed diet consisting of fresh *G. gracilis*, abalone tend to demonstrate a distinct chemosensory preference based on algae attractiveness involving direct tactile interaction (Fleming 1995). In our study, the abalone exposed to low *G. gracilis* inclusion diets were less alert and moving less after introducing feed. But introducing fresh *G. gracilis* immediately increased alertness (between T0h and T1h). Introducing formulated feed with the highest inclusion level of dry *G. gracilis* only increased alertness between T0h and T3h. This high level of activity is energetically costly, high metabolism and could have an impact on energy allocation (Donovan & Carefoot 1998).

Understanding feeding preference to develop sustainable diets is highly essential due to the relationship between preference and performance (Fleming 1995; Lamire & Himmelman 1996). Feed preference can be influenced by a range of factors such as algae morphology, toughness and antinutritional chemicals such as phenolic compounds (Fleming 1995). A study investigating the feeding behaviour of Greenlip abalone, *Haliotis laevigata* fed live macroalgae (Ulva sp. and *Gracilaria cliftonii*) and formulated diets at different rations found

that macroalgae diets resulted in higher feed intakes. The results in our study suggest that the presence of fresh *G. gracilis* significantly improves the feeding response of abalone over time compared to the presence of dry *G. gracilis.* The movement of abalone had been identified to be associated with feeding behaviour (Donocan *et al.* 1999). Abalone have been recorded to increase forage activity under conditions of food scarcity (Shepard 1973).

3.5. Conclusion

The influence of *G. gracilis* on abalone behaviour was evaluated and compared over time and between treatments. The interaction between treatment and time indicated that there was a reduction in quiescence in G3.00 and G12.00 abalone between T0h and T3h. The change in the level of quiescence in abalone seems to be determined by changes in the physical environment such as light cycle and water quality and by feed condition and feed type. This means that the high feed stability could be associated with the absence of change in quiescence for abalone fed G0 control compared to abalone fed G12.00. Abalone fed Gfresh + S34s became less quiescent at T1h. Introducing fresh *G. gracilis* immediately increased abalone alertness. This high level of activity could be energetically costly. However, the high level of alertness could be an indication of chemosensory and tactile cues taking precedence. The presence of fresh *G. gracilis* appears to increase the feeding response of abalone since there was an increase in the number of feeding abalone immediately after introducing fresh *G. gracilis.*

Chapter 4 - Discussion

The aim was to use *G. gracilis* that was produced with mussel in an integrated multitrophic aquaculture system whereafter it was included into abalone feed. The effects of *G. gracilis* on abalone growth and behaviour were explored in chapters two and three, respectively. This research has demonstrated the potential of including seaweed particularly *G. gracilis* on the growth and behaviour of farmed abalone *H. midae*. The methods used included the following:

1. Cultivating wild harvested *G. gracilis* in an IMTA system (i.e., algae with mussel) under a 52-day growth cycle over one year and six months.
2. Harvesting the *G. gracilis* algae from the raft system and manufactured into Abfeed® at inclusion levels 0.75 %, 1.50 %, 3.00 %, 6.00 %, 12.00 % using biosecurity measures.
3. Conducting an experiment using the above dietary treatments along with a control diet (no algae) and a mixed diet where Abfeed® S34s was supplemented with fresh farm-grown *G. gracilis* algae.
4. Assessing abalone growth by weight and shell length measurements, condition factor and FCR every four months.
5. Assessing abalone behaviour by first identifying characteristic behaviours which were then categorized under level of activity (i.e., quiescence, alertness and movement) and feeding activity. The differences in behaviour were monitored by counting abalone exhibiting a particular behaviour over time (zero min. one hour, three hours, six hours, and nine hours after feeding) and compared between dietary treatments.

The incorporation of IMTA *G. gracilis* has some limitations. Anderson *et al.* (1996) investigated the mariculture of *G. gracilis* in Saldanha Bay, South Africa. The findings indicated that there are strong seasonal differences in the temperature and nutrient profiles in

the water column at Saldanha Bay. Due to south-westerly winds, there was a thermal stratification that took place in summer and there was a negative relationship between water temperature and nutrients. The best *G. gracilis* growth rates were obtained between September and December due to optimal environmental conditions (Anderson *et al.* 1996). Although the seaweed cultivation-harvesting period in our study was for over a year, the quality of the harvested IMTA macroalgae potentially varied over time. Our study was directly affected by limited production of IMTA *G. gracilis* which restricted the manufacturing and supply of large quantities of formulated feed with dry *G. gracilis* inclusion. Because of limited feed ingredients, the number of replicates in the experiment was reduced. Considering the poor seasonal growth patterns, a sustained production of *G. gracilis* in a sea-based IMTA system could be achieved by technical improvements, for example doubling the stocking yield. A more biological approach to mitigating poor *G. gracilis* growth yields would be the selection of faster growing strains (Anderson *et al.* 1996).

Sea-based raft system mariculture is prone to conditions associated with exceptional events or prolonged oligotrophic conditions that could potentially starve the *G. gracilis* of nutrients (Anderson *et al.* 1996). During *G. gracilis* harvest, several marine organisms grew as epiphytes on the *G. gracilis* particularly during summer months. These organisms included small mussels attaching within *G. gracilis* tufts and on the nylon ropes (culture lines). Anderson *et al.* (1996) found that epiphytes appear inside *G. gracilis* tufts and recommended that frequently harvesting or trimming the *G. gracilis* tuft could mitigate the epiphyte and fouling issue. This process will ensure good quality *G. gracilis* is included in abalone feed.

The growth experiment was conducted over eight months on a full commercial scale. Standard farm feeding practices were implemented. The results indicated that the incorporation of *G. gracilis* algae influences abalone growth. However, the negative impact of *G. gracilis* algae on growth performance increases as the proportion of the algae in the diet

exceeds 0.75 %. The reduction in abalone growth with increasing *G. gracilis* inclusion may be associated with abalone not feeding on the diets due to the low feed stability (i.e., particularly on high algae inclusion levels) which results in feed dissolving before being fed on. The level of quiescence was the same over time for abalone fed the control diet (G0) whereas high dry *G. gracilis* inclusion diet G12.00 experienced a significant decrease in the level of quiescence and an increase in alertness within the first three hours from feeding. Therefore, the significantly low pellet stability of G12.00 compared to G0 could mean the water with less stable feed was enriched with feed and macroalgae particles that stimulated a high behavioural activity response of abalone (Allen *et al.* 2006). However, the high alertness activity from high *G. gracilis* inclusion corresponded with a reduced growth and higher FCR. The reduced growth and higher FCR could be a result of lowered nutrient availability where the ingested feed was not meeting the nutritional requirements of the abalone (Allen *et al.* 2006; Mulvaney *et al.* 2013). It is crucial that water soluble nutrients in the feed remain bonded together as this could factor into feed acceptability, water quality and ultimately growth performance (Mai *et al.* 1996).

The particle size reduction process in feed manufacturing is considered the most time-consuming feed processing step which could account for at least 60 % of feed production cost (Sorenson & Philips 1992). Reducing soyabean meal particle size to 150 - 450 µm resulted in significantly lower dry matter loss and an increase in apparent digestibility when compared with a particle size above 450 µm which increased both (Sales & Britz 2002). Leaching could be mitigated by reducing the amount of air space between particles and increasing the contact surfaces of the ingredients to improve the bonding strength (Sales & Britz 2002).

Macroalgae has been extensively used as an inexpensive feed attractant and stimulant (Britz 1996b; Allen *et al.* 2006; Cornwall *et al.* 2009; Bansemer *et al.* 2014; Mau & Jha 2018). Supplementing Abfeed® S34s with fresh farm-grown *G. gracilis* could result in

higher cost benefits as the *G. gracilis* is not processed before being introduced as part of abalone diet. However, feeding a mixed diet could introduce an issue of feed preference where abalone choose feed items to consume while the rest go to waste. Alertness increased immediately after introducing fresh *G. gracilis.* The ability of abalone to rapidly detect macroalgae feed could stimulate increased ingestion (Allen *et al.* 2006). A significantly high feeding activity of abalone fed Gfresh + S34s was observed after one hour in our study. Although the water stability of Abfeed® S34s was higher than G12.00 feed, the variability in the structural properties of the mixed diet is the potential cause for the observed variations in abalone behavioural response. Thus, a new feeding regime would need to be designed to optimise feed intake.

Feeding live macroalgae to abalone has been extensively documented. However, it remains difficult to explain the effect of macroalgae. Naidoo *et al.* (2006) tested the growth of abalone fed a mixed macroalgae diet (*G. gracilis*, *Ulva lactuca* and *Ecklonia maxima*) and observed that the mixed diet leads to higher abalone growth than when feeding a formulated diet. In contrast, Bansemer *et al.* (2014) tested the growth of abalone fed nonenriched and enriched macroalgae (*Gracilaria cliftonii*, *Ulva lactuca*), and formulated feed. The findings indicated that abalone fed formulated feed was superior to abalone fed macroalgae diets, even when fed as a 1: 1 mix ratio. Mulvaney *et al.* (2013) observed that protein enhanced *Ulva australis* and *Grateloupia* sp. rendered the best abalone growth than two formulated feed diets. These disparities contribute to the slow adoption of macroalgae as feed in intensive culture environments. While our study demonstrated that abalone growth rates can be enhanced using macroalgae, the outcomes of dietary trials will depend on the feed formulations as well as the species and condition of the macroalgae (Mulvaney *et al.* 2013). The increase in fresh *G. gracilis* volumes fed to abalone resulted in improved abalone growth in the second growth cycle in our study. These findings further indicate the potential of a mixed diet to enhance

abalone growth. Conducting an experiment where *G. gracilis* is fed along with cultivable macroalgae and supplemented with formulated feed could yield interesting growth results.

Although *G. gracilis* included in the abalone diet induces a change in the behavioural response, physicochemical conditions (water quality) seem to extensively influence abalone activity. Anecdotal observations in our study were that baskets located next to the inflow seawater tap were less active but more responsive to the feed added. Whereas abalone at the outflow end of the tank were more active (i.e., moved above the top plate) and were clustered close to the water surface. However, the results were limited by the methods used in our behaviour experiment. There is a need to optimize the methods used to observe abalone behaviour.

A scientific approach to quantify abalone behaviour is by using a ventral video monitoring technique, dorsal view time-lapse cinematography (Tutschulte & Connell 1988; Buss *et al.* 2015). The data (i.e., video footage) can then be used to measure the activity of tagged abalone through determining the distance and velocity travelled by each abalone or by tracking movement over time. A scoring system based on the frequency of each observed difference can be developed to quantify how abalone behaviour varies between each treatment (Buss *et al.* 2015). This data could be paired with water quality readings for a more definitive outcome.

Abalone are considered a highly valuable seafood products with the prime demand in Asian countries where the abalone products are part of traditional cuisine and ceremony (Britz 1996b). This means meat quality characteristics are used to determine the level of acceptability (Sales *et al.* 1999). Most abalone consumers consider the lighter pigmentation as superior and thus command high market prices (Oakes & Ponte 1996). Since high *G. gracilis* inclusion affects abalone pigment or meat colour, such attributes could affect market

value and acceptability of sea food products. If changes in the foot muscle colour is a primary consideration in the market, then *G. gracilis* macroalgae should be used cautiously.

Preliminary studies attribute changes in abalone behaviour to biotic factors (Shephard 1986; Day *et al.* 2004; Allen *et al.* 2006; Lloyd & Bates 2008) while abiotic factors have been less studied. It is challenging to determine optimal conditions for abalone to exhibit behaviours that do not identify as a stress response thus it is proposed that future investigations should take into consideration how changes in water quality affect the behaviour of abalone and if the behavioural responses translate to good abalone growth.

Disease is a persistent concern in the aquaculture industry particularly in intensive systems as crowding of species favours more disease spread (Handlinger *et al.* 2005). Infectious diseases in abalone are commonly either viral, bacterial, or fungal. The absence of any disease during our experiment along with an above average abalone flesh to shell ratio indicates that feeding *G. gracilis* macroalgae does not compromise the biosecurity of abalone. Thus, future research should further explore the link between natural feed and abalone biosecurity.

In conclusion, integrated multitrophic aquaculture system was used to produce *G. gracilis* algae that was included into abalone feed at various inclusion level. This research has demonstrated the potential of including macroalgae into formulated feed. Feeding *G. gracilis* macroalgae could serve as a viable replacement to kelp. However, the quality of the commercial feed plays an important role in ensuring abalone growth (i.e., reach marketable size). The feeding regimes and labour cost need to be considered and should be beneficial (Mulvaney *et al.* 2013).

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