ALTERNATIVE MACROALGAL DIETS FOR JUVENILE GREENLIP ABALONE (*HALIOTIS LAEVIGATA*) IN THE LATER NURSERY PHASE

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DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work, which has not previously been submitted for a degree at any tertiary education institution.

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ABSTRACT

Greenlip abalone (*Haliotis laevigata*) are a highly valued fishery resource, grown in aquaculture facilities around the southern states of Australia. These commercial farms have a nursery system that utilises natural algal diets adhered to vertical plates for rearing postlarval and juvenile abalone. However, as the juvenile abalone grow (5 – 15 mm shell length), the current commercial nursery diet of the green alga *Ulvella lens* plus the diatom *Navicula* cf. *jeffreyi* does not supply adequate algal biomass to maintain commercially viable abalone growth rates at the required stocking densities. The focus of this research was to identify, develop and evaluate alternative macroalgal diets to overcome the restrictions in algal biomass during the later nursery phase.

Macroalgae as an alternative natural diet for juvenile abalone can increase the algal biomass supplied on the vertical plates given its fast, 3-dimensional growth; while also considered a suitable nutrition source, as it is the primary feed of wild adult abalone. Australian abalone species have a preference for red macroalgae and to accommodate this, propagation methods including carpospore liberation, protoplast production and vegetative propagation; were assessed to determine the fitness of Rhodophyta species as an alternative diet for juvenile abalone in the later nursery phase. Protoplasts were readily isolated from red macroalgal species, however due to their limited regeneration, high cost of production and significant expertise required, the method was deemed unsuitable for utilisation in a commercial abalone nursery.

Vegetative propagation successful established fragment culture of several red macroalgal species. The development of an artificial adhesion protocol, utilising active immobilisation processes through gel entrapment by the natural polysaccharide agar; enabled macroalgal fragments to be presented to juvenile abalone on the vertical plates in the nursery system. A diet of *Laurencia* sp. fragments adhered to the plates with agar produced juvenile abalone growth rates (50 $\mu m.day^{-1}$) comparable to the current commercial nursery diet of *U. lens* and *N. jeffreyi*. The grazing resistance of the *Laurencia*/agar diet was low and fragments did not regenerate; so regular re-application

was required, making artificial adhesion protocols unsuitable for use in the development of juvenile abalone diets within the nursery system. Instead of integrating alternative macroalgal diets in the nursery system, a different abalone management (weaner) system utilising an artificial diet, was able to produce significantly greater juvenile abalone growth rates and weight gain for abalone larger than 8 mm shell length.

Macroalgal sporelings were incorporated as an alternative diet to remove the need for artificial adhesion protocols, as they can be seeded directly onto the plates whilst still presenting high algal biomass to the juvenile abalone. The morphology and life cycle of the green alga, *Ulva* allows for the high spore production and sporeling densities required to create a juvenile abalone diet. An *Ulva* spp. sporeling diet on the nursery plates produced abalone growth rates of nearly 100 $\mu m.day^{-1}$ and was comparable to the current commercial nursery diet (*U. lens*/*N. jeffreyi*). However, the *Ulva* sporeling diet was unable to maintain suitable growth rates for abalone greater than 8 – 9 mm shell length and consequently, did not overcome the biomass limitation of natural algal diets in the nursery system.

Given the *Ulva* sporeling diets ability to produce commercially viable growth rates for juvenile abalone less than 8 – 9 mm shell length and Australian abalone preference for red macroalgae, a composite green and red macroalgal sporeling diet was identified as an alternative diet for juvenile abalone in the later nursery phase. To incorporate Rhodophyta species into the diet, propagation via carpospore liberation was achieved for several red macroalgal species by temperature, dark and osmotic pressure induction treatments, with *Hypnea* sp. liberating the greatest number of carpospores ($67.23 \pm 10.19 \times 10^3$ carpospores.g^{-1}). Therefore, the combination *Hypnea* and *Ulva* sporeling diet was developed, which also reduced the biomass of red macroalgal carposporophyte required compared to that needed for creating a monospecies diet. This composite sporeling diet produced larger juvenile abalone (15 mm shell length), faster growth rates (87 $\mu m.day^{-1}$) and weight gain (2.5 $\mu g.day^{-1}$), when compared to the current commercial diets in the nursery (*U. lens*/*N. jeffreyi*) and weaner (artificial feed) systems. The addition of new seeded plates for all nursery diets during the trial allowed the composite sporeling diet to provide sufficient algal biomass. The *Hypnea*/*Ulva*

sporeling diet was able to overcome the biomass limitations of algal diets and accommodate the juvenile abalone (<15 mm shell length) high grazing pressure, while producing commercial viable growth rates throughout the entire later nursery phase. This composite sporeling diet has been incorporated into a detailed feeding regime for Australian commercial abalone nursery practices, to help improve juvenile *Haliotis laevigata* culture and increase overall farm production of this highly valued resource.

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PUBLICATIONS

The published material listed below forms the basis for parts of this thesis.

Peer Reviewed Scientific Manuscripts

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Research Reports

Strain, L.W.S., Borowitzka, M.A. & Daume, S. Chapter 6. Growth and survival of juvenile greenlip abalone (*Haliotis laevigata*) feeding on germlings of the macroalgae

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CHAPTER 1
GENERAL INTRODUCTION

Abalone are marine molluscs within the class Gastropoda, family Haliotidae, and typified by a flattened, calcareous shell covering a large muscular foot that adheres the animal to the substrata. Species of the genus *Haliotis* are widely distributed throughout the exposed coastal waters of the Pacific, Atlantic and Indian Oceans, from the tropics to cold temperate regions (Nash 1991, Castell 2003). Abalone are found along these diverse coastlines, inhabiting the intertidal zone from just below the low-tide mark to depths of approximately 40 metres (Hahn 1989a). The habitat's exposure to human activity has allowed extensive harvesting of abalone species for their muscular foot, which are considered a delicacy around the world (Landau 1992, Castell 2003). However, only a small percentage of abalone species are currently commercially exploited, with Australia's coastal waters home to three major, commercially harvested abalone: *Haliotis laevigata* (Greenlip), *Haliotis rubra* (Blacklip) and *Haliotis roei* (Roe's) (Shepherd 1973, Castell 2003).

As an economically important genus, abalone have a slow and heterogeneous growth rate of approximately 2 – 3 $cm.y^{-1}$ (Hahn 1989b). This growth rate has generally been described by the von Bertalanffy growth curve (e.g. Shepherd & Hearn 1983, Keesing & Wells 1989), however due to variation in *Haliotis* species growth between life stages, alternative growth models such as the Gompertz growth curve can be fitted (Troynikov *et al.* 1998, Bardos 2005). Juvenile abalone have been shown to exhibit linear growth trends (Shepherd *et al.* 1992), even when there was a distinct seasonal effect present (Helidoniotis & Haddon 2012). Recently, an inverse logistic growth model has been recommended, which caters for the linear growth in juveniles while also modelling growth across the entire size range of abalone (Haddon *et al.* 2008, Helidoniotis *et al.* 2011). The variation in growth rates over the life span of an abalone can not solely be attributed to the different stages of abalone development, as the weather, season, localised areas, abalone density and food availability can all vary an individual animals growth (Day & Fleming 1992, Troynikov *et al.* 1998).

1.1 ABALONE NUTRITION

Abalone are generalist, unselective herbivores that graze on hard surfaces for algae as juveniles and capture drift algae as adults (Landau 1992). The algal diet an abalone consumes can be influenced by a variety of factors, including food availability, avoidance of chemical deterrents and digestibility of foods (Fleming 1995a). Given these factors impact the food intake of abalone and the algal diet itself has a different nutritional profile to their animal tissue, nutrient limitations can occur (Fleming 1992) The energy content and biochemical nature of consumable nutrients in algae, coupled with the herbivores ability to obtain these nutrients from algae can be of important dietary consequence and may contribute to the slow growth rate present in natural abalone populations.

Examining the diet of early life stage abalone (settled, postlarvae and juvenile) shows they tend to consume epiphytic and epilithic diatoms, extra cellular products, crustose coralline algae, turf algae, mucus and bacteria as major dietary components, until the dietary intake becomes dominated by macroalgae (e.g. Garland *et al.* 1985, McShane *et al.* 1994, Kawamura *et al.* 1995, Kawamura & Takami 1995, Kawamura 1996, Takami *et al.* 1998, Dunstan *et al.* 2002). The feeding and growth of postlarval and juvenile abalone can be categorised into three distinct dietary transitions.

1) Lecithotrophy and dissolved organic matter absorption from seawater to particulate feeding.

2) Change in digestibility of diatom diets for postlarval abalone at 600 to 800 μm shell length.

3) Biofilm-dominated diet to macroalgae-dominated diet at approximately 5 to 10 mm shell length.

(Kawamura *et al.* 1998b, Takami & Kawamura 2003)

1.1.1 Lecithotrophy and dissolved organic matter absorption from seawater to particulate feeding.

Abalone have a lecithotrophic, planktonic larval stage (days) and do not possess a functional mouth or digestive tract (Levinton 1995). The larvae's primary energy source of yolk reserves can be supplemented through the uptake of dissolved organic matter (DOM) from ambient seawater (Manahan & Jaeckle 1992, Kawamura *et al.* 1998b, Takami *et al.* 2000, Roberts *et al.* 2001). Once the larvae have settled and metamorphosed, the postlarvae transition from the energy sources of yolk and DOM to particulate food at around 400 – 500 μm shell length (Takami *et al.* 2000, Roberts *et al.* 2001). Particulate feeding generally includes the ingestion of diatom extra cellular products, mucus, bacteria and other components of the biofilm (Kawamura 1996). This transition does not necessarily occur immediately after metamorphosis as the digestive system of postlarval abalone may not yet be completely developed and therefore, unable to fully utilise different feeds (Manahan & Jaeckle 1992, Martínez-Ponce & Searcy-Bernal 1998, Roberts *et al.* 1999a).

In the natural environment abalone species are reported to preferentially settle and metamorphose on crustose coralline algae (CCA) (McShane 1992, Roberts 2001). Different species and growth forms of CCA can influence the settlement of abalone larvae, particularly through the variation in CCA surface characteristics (McShane 1996, Daume *et al.* 1999a, Roberts *et al.* 2004). It's understood that CCA itself, and not the surface biofilm of associated diatoms and bacteria, are considered the source of settlement cues for temperate abalone larvae (Roberts *et al.* 2010). Although, diatom species can cue rapid larval settlement, few induce consistently strong metamorphosis; therefore diatom films generally produce considerable variation in the density of abalone post settlement (Roberts *et al.* 2007).

Crustose coralline algae can also be a suitable particulate feed, especially the cuticle and epithelial cell contents (McShane 1992, Kawamura *et al.* 1995, Takami *et al.* 1997a, Daume *et al.* 1999b). The exact dietary value of CCA may differ between species but in general, if grazing has been heavy, the cytoplasmic content of the

epithallium can become the nutritional basis for postlarval abalone (Garland *et al.* 1985, Shepherd & Daume 1996, Takami *et al.* 1997a). The presence of biofilm components, specifically diatoms and their extra cellular products, on the surface of CCA tend to produce significantly higher growth rates than just the cuticle and epithelial cell contents of CCA (Takami *et al.* 1997a, Daume *et al.* 2000).

The trail of mucus left by abalone during grazing has been shown to produce elevated levels of postlarval survival and may indicate gastropod mucus could be nutritionally important (Slattery 1992, Takami *et al.* 1997b). Takami *et al.* (1997b) showed that postlarvae feeding on the mucus trail of abalone were able to grow up to 700 µm in shell length, which was compatible with another study on the growth obtained by postlarvae consuming the extra cellular mucus of the diatoms *Navicula ramosissima* and *Stauroneis constricta* (Kawamura & Takami 1995). However, for postlarval abalone greater than 700 µm shell length, a mucus diet consisting of protein and polysaccharides with low C:N ratio was not sufficient to support high growth rates (Peduzzi & Herndl 1991, Takami *et al.* 1997b). Therefore, mucus as a nutrition source may be become inadequate as the postlarvae grows, but does provide an excellent medium for bacterial growth (Takami *et al.* 1997b). These bacteria colonies can be used as a nutritional food source forming a minor component of the postlarval diet, but are also thought to perform metabolic functions in the gut aiding in the digestion process (Garland *et al.* 1985, Kawamura *et al.* 1998b). Most of these ingestible bacteria are cocco-bacilli from the genus *Moraxella* and generally reflect the bacterial biota of the CCA surface (Garland *et al.* 1985).

1.1.2 Change in digestibility of diatom diets for postlarval abalone at 600 to 800 µm shell length.

Rapid growth rates are achievable on yolk and DOM uptake, as well as particulate feeding of biofilm components including, CCA, trail mucus and bacteria, until the postlarvae are approximately 700 µm in shell length (Roberts *et al.* 2001). Diatoms and particularly their extra cellular products, are an important part of the particulate feed and can be consumed from the second day after metamorphosis

(Martínez-Ponce & Searcy-Bernal 1998). However, the species and digestibility of the diatoms ingested does not significantly affect the growth rates obtained during this early postlarval stage as long as there are adequate supplies of biofilm material (Kawamura & Takami 1995, Kawamura *et al.* 1998a). Conditions and diatom species that have been shown to induce good settlement rates often support good early postlarval survival and growth (Daume *et al.* 1999b, Gordon *et al.* 2006).

The efficiency at which an abalone can digest diatoms has two key components, ingestibility and digestibility once ingested. Ingestibility can be associated with the ease at which diatoms are removed from the substrata and swallowed, based on characteristics such as cell morphology, attachment and structural strength (Roberts *et al.* 1999a, 2001). Diatom cell morphology includes cell size and growth form, which can both limit diatoms digestion by precluding ingestion altogether (Kawamura *et al.* 1998a). Cell size can be considered an important factor as diatoms smaller than the postlarval mouth are likely to have high ingestion rates while large diatom species will have very inefficient ingestion (Fleming *et al.* 1996, Roberts *et al.* 1999a).

Attachment strength conveys how firmly the diatoms are connected with the substratum. Diatom species with high attachment strength require force to be removed and are usually broken if dislodged, while diatoms with low attachment strength tend to be ingested without any deformation or rupture (Kawamura *et al.* 1995, 1998a, 1998b). The majority of ingested cells pass unbroken (low attachment strength) and these particular diatoms provide very little nutritional benefit compared with the high attachment strength diatoms (Kawamura *et al.* 1995, 1998a).

Structural strength of diatoms relates to the strength of the frustules. Some diatom species with low attachment strengths are still able to produce high digestion efficiencies and good growth rates, through the ability of postlarval abalone to rupture their weak silica frustules (Kawamura *et al.* 1995, 1998a, 1998b). Postlarvae cannot digest diatom cell contents in their alimentary canal without first physically rupturing

the frustule and/or girdle elements (Kawamura *et al.* 1995). Rupture of the frustules frequently results from contact with the radula or from rotation within the stomach. This process of rupturing the frustules on grazing can be considered one of the most important factors in determining a diatom's dietary value and whether the cells contents can be metabolised (Kawamura *et al.* 1995). These two diatom strength characteristics directly affect the ability of postlarval abalone to digest the diatom cell contents and subsequently produce variation in the nutritional value of different diatom species.

Dietary benefits of diatoms tend to depend on the size of the abalone, as the differences between the nutritional value of different diatom species become apparent when postlarvae grow to over 800 μm shell length (Daume *et al.* 2000, Gordon *et al.* 2006). To maintain the growth rates associated with smaller postlarvae on diatoms with variable digestibility, the larger postlarval abalone require high levels of digested diatom cell contents (Kawamura *et al.* 1998b). Once the postlarvae reach a size (1 – 2 mm shell length) where they can efficiently ingest diatoms and digest the cell contents, growth rates of 40 – 60 $\mu m.day^{-1}$ can be supported (Kawamura *et al.* 1998a, 1998b). Therefore, diatom selection during grazing by postlarval abalone can be highly important in securing the correct nutritional requirements to improve growth and survival (Viana *et al.* 2007).

1.1.3 Biofilm-dominated diet to macroalgae-dominated diet at approximately 5 to 10 mm shell length.

Prior to the consumption of macroalgae, the dominant components in the natural benthic biofilm of diatoms and their extra cellular secretions (mucus) are considered the primary food source for juvenile abalone (Kawamura *et al.* 1995, Kawamura 1996, Kawamura *et al.* 1998b). Once the abalone reach 4 to 5 mm shell length (juveniles), diatoms alone are no longer sufficient to maintain the nutrition of postlarval abalone (Daume & Ryan 2002, Takami *et al.* 2003). The nutritional requirements of juvenile abalone therefore, begin to shift from a biofilm-dominated diet to a macroalgae-dominated diet at around 5 – 10 mm shell length (McShane *et al.* 1994, Kawamura *et al.* 1995, Dunstan *et al.* 2002). Crustose coralline algae have been shown to be the principle

feed for juveniles of 5 – 10 mm shell length, with the transition to erect macroalgae and drift algae occurring as they reach 20 mm shell length (Shepherd & Cannon 1988). However, this dietary transition can occur across a highly varied juvenile abalone size range and may be dependent on food availability and composition (Kawamura *et al.* 1998b, Takami & Kawamura 2003).

These three dietary transitions between nutrition sources, particularly the shift from diatoms to macroalgae, are considered closely related to the ontogenetic changes in digestive enzyme activity and radula morphology of postlarval and juvenile abalone (Takami & Kawamura 2003). Abalone species such as *Haliotis discus hannai* that prefer to feed on brown algae as adults, have detectable polysaccharide-degrading enzyme activity by 17 d post settlement and exhibit a rapid increase in enzyme activity with growth after 37 d, indicating that even during the postlarval stage (settlement – 2 mm shell length) enzymes for brown macroalgae feeding have been produced in the digestive tract (Takami *et al.* 1998). The structural and functional changes in the radula that occur by 4 mm shell length for this abalone species, suggest that the transition to macroalgae could be partly due to this feeding apparatus development, in particular the addition of the lateral teeth L3 – L5 when the abalone are between 0.9 and 1.9 mm shell length (Kawamura *et al.* 2001). *Haliotis iris* also add lateral teeth (L3 – L5) at a similar size of between 1 and 1.7 mm shell length, with the radula of postlarvae this size or larger considered more suitable for collecting large particles and gouging feeding substrata, compared to postlarvae smaller than 1 mm shell length who's less developed radula can only collect small diatoms and loose particles (Roberts *et al.* 1999b). Not all abalone species exhibit these radula developmental changes at a similar shell length, with *Haliotis diversicolor aquatilis* adding lateral teeth L3 and L4 at 1.5 to 2 mm shell length, while L5 teeth are not added until 6 mm shell length (Onitsuka *et al.* 2004). The Australian abalone species *H. rubra* develops L5 teeth completing the adult compliment of teeth on the radula, exhibits greater enzyme production and increased digestive efficiency at between 80 and 102 d post settlement, indicating the preparation for feeding on macroalgae and the beginning of the third nutritional transition (Johnston *et al.* 2005). This all demonstrates the dietary transitions, particularly the third from a diatom to macroalgae-dominated diet, may occur at various size ranges for abalone species. Given these ontogenetic changes in digestive enzyme activity and radula

morphology occur at a shell length of less than 5 mm for many *Haliotis* species, postlarval and juvenile abalone could ingest macroalgae earlier than the transition between diatoms and adult macroalgal thalli suggests at 5 – 10 mm shell length.

Palatable, juvenile macroalgae (sporelings) have been identified as an important form of food that provides the nutritional requirements for the transition from a diatom-dominated to a macroalgae-dominated diet as the ontogenetic changes of juvenile abalone occur (Kawamura *et al.* 1998b, Takami & Kawamura 2003). Herbivores generally play a considerable role in the mortality of early post settlement stages (sporelings) of macroalgae (Vadas *et al.* 1992, Van Alstyne *et al.* 1999), while the structural properties of sporelings differ from adult thalli by being thinner, smaller and more delicate (Van Alstyne *et al.* 2001). Factors such as these suggest that juvenile macroalgae could be the key component in the dietary transition of juvenile abalone from diatoms to macroalgae, however only limited research has occurred into the dietary value of macroalgal sporelings for juvenile abalone. Takami *et al.* (2003) has shown that postlarval *H. discus hannai* of greater than 1.8 mm shell length, actively ingested gametophytes and juvenile sporophytes of the brown macroalga *Laminaria japonica*. *Haliotis diversicolor* of greater than 3 mm shell length also effectively consumed juvenile gametophytes of the brown macroalgal species *Eisenia bicyclis*, *Ecklonia cava* and *Undaria pinnatifida* (Onitsuka *et al.* 2010, 2011). Juvenile macroalgae of other brown algal species such as *Colpomenia sinuosa*, *Ectocarpus siliculosus* and *Endarachne binghamiae*, as well as the green alga *Enteromorpha* spp. have been shown to support moderate to rapid growth in 3 – 4 mm *Haliotis discus discus* (Maesako *et al.* 1984). The dietary shift from diatoms to macroalgal sporelings of *Ulva rigida* mixed with *Ulvella lens*, has been observed for postlarval *Haliotis tuberculata coccinea* by the sudden increase in growth rate when the juveniles reached 2 mm shell length (Courtois de Vicose *et al.* 2012). All of these studies demonstrate that abalone at a small size (2 – 3 mm shell length) can consume and digest macroalgae, with the transition from a diatom-dominated to a macroalgae-dominated diet being facilitated by the consumption of juvenile macroalgae.

Once the transition in dietary profiles to macroalgae has occurred, adult abalone can acquire their nutritional requirements from the adult thalli of a variety of different macroalgal species (Leighton & Boolootian 1963, Poore 1972a, Shepherd 1973, Shepherd & Cannon 1988). The abalone can locate and consume the macroalgae either by foraging locally, catching drift algae, or searching for food across the substratum (Tutschulte & Connell 1988). Australian and New Zealand abalone species tend to feed on drift algae with only limited localised foraging, which can be influenced by the macroalgal composition available (Poore 1972b, Shepherd 1973).

Preference for macroalgal species differs between *Haliotis* species with adult abalone requiring large amounts of fresh material (Dunstan *et al.* 1996, Corazani & Illanes 1998). The macroalgal species consumed varies seasonally and can reflect the flora where the abalone resides (Leighton & Boolootian 1963, Wells & Keesing 1989). Australian and New Zealand abalone species prefer red algae, unlike their counterparts elsewhere in the world that feed predominately on brown algae (Poore 1972a, Shepherd & Cannon 1988, Shepherd & Steinberg 1992, Fleming 1995b). A bias towards brown macroalgae such as *Ecklonia* and *Laminaria* has been shown in gut content analysis in a variety of abalone species, as these macroalgae become unrecognizable relatively slowly during the digestion process (Foale & Day 1992, Day & Cook 1995). Cultures of abalone faecal matter have indicated that less preferred macroalgal species remain viable through digestion, increasing identification, while preferred species are digested more efficiently and do not survive (Foale & Day 1992, Westphalen & Cheshire 1995). Food preference trials can be influenced by the macroalgal diet to which the abalone was originally acclimatised to (Stepto & Cook 1996), leading to a bias for more commonly consumed macroalgal species.

Given that abalone are sedentary and feed on drift algae, the preference shown by Australian and New Zealand abalone species for red algae could be due to their high abundance in the natural environment, while the consumption of brown algae tends to occur when more suitable algae are absent (Shepherd & Steinberg 1992). The presence of high concentrations of unpalatable polyphenolic compounds in Australian brown

algae could also increase the deterrent of predation by abalone species (Steinberg 1988, 1989, Steinberg & van Altena 1992). In the Australian abalone *H. rubra*, the growth rate was shown to be related to the hierarchy of chemosensory preference for macroalgal diets (Fleming 1995b), which strongly corresponds to the digestible nitrogen content of the algae (Fleming 1995a). Conversely, food toughness has been identified as a primary factor when compared to chemical deterrence, in the feeding preference of *H. rubra* (McShane *et al.* 1994).

1.2 ABALONE AQUACULTURE

The popularity in economically important abalone species has resulted in over-harvesting in many countries, while the prospect of high prices and world demand has opened a major market opportunity for cultured abalone (Mgaya & Mercer 1994, Oakes & Ponte 1996). The decline in wild harvest yields since the 1970's through the combination of over fishing, disease, habitat loss and illegal fishing, has coincided with the substantial increase in development of abalone cultivation facilities around the world (Gordon & Cook 2004). In fact, aquaculture production worldwide increased by over 350 % during the 6 years before 2008 (Cook & Gordon 2010). Over this time, aquaculture has made significant advances in growth and survival of rearing abalone from egg to adult. However, further improvement to the current culture protocols, in particular juvenile nutrition, would reduce the time for culturing high quality abalone product to commercial harvest size, consequently increasing the economic performance of abalone aquaculture.

Feeding early life stages of abalone in aquaculture are dependent on cultured algae and the control of this feed can be critical in their development (Hahn 1989b). Cultured algae are initially utilised to induce larval settlement and then provide a suitable food source for postlarval and juvenile abalone (Daume 2006). As the abalone transition from a biofilm-dominated diet to a macroalgae-dominated diet at around 5 – 10 mm shell length, the type of feed provided in aquaculture must adjust accordingly. To advance this area of production, new juvenile diets need to be explored

that supply the appropriate type of feed at a sufficient biomass and provide greater nutritional benefits over a longer time period.

Abalone aquaculture within many countries accommodates this dietary change by altering the type and developmental stage of the cultured algal fed in the nursery and growout systems. A wide diversity of whole, live red, brown and green macroalgal species have been examined around the world for many different juvenile abalone species (>10 mm shell length) in a variety of culture systems including tanks, baskets, cages and containers (e.g. Stuart & Brown 1994, Corazani & Illanes 1998, Simpson & Cook 1998, Boarder & Shpigel 2001, Viera *et al.* 2005, Qi *et al.* 2010). For instance, South African abalone farms have generally preferred artificial feeds due to convenience and reduced costs (Sales & Britz 2001), but more recently they are also utilising whole macroalgal species in specially designed growout systems (Naidoo *et al.* 2006, Robertson-Andersson *et al.* 2011). Wild harvest kelp (*Ecklonia maxima*) has dominated this feed composition, however, the resource nears sustainable harvest limits and consequently, farms are researching other macroalgal species (red and green) as alternatives (Troell *et al.* 2006).

1.3 AUSTRALIAN ABALONE AQUACULTURE

In Australia, two of the commercially harvested abalone species *H. laevigata* and *H. rubra*, are considered suitable for aquaculture due to their relatively fast growth rates (Hahn 1989a). Wild or conditioned captive and farmed broodstock of these species are induced to spawn, and after fertilisation and the free-swimming larval period, the larvae are settled in the nursery system which consists of coffin tanks with PVC plates arranged vertically in baskets (Section 2.4.1). The crustose, green alga, *U. lens* pre-seeded onto the PVC plates has been shown to induce higher settlement rates of abalone larvae compared to monospecies benthic diatom films (Daume *et al.* 2000, Krsinich *et al.* 2000). However, by itself *U. lens* only supports moderate growth rates for postlarvae less than 2 – 3 mm shell length, due to its high grazing resistance (Kawamura 1996, Daume *et al.* 2000, Huggett *et al.* 2005). Therefore, easily digestible diatoms such as *Navicula* cf. *jeffreyi* are incorporated into the system, and the combined *U. lens*/diatom

diet can sustain high growth rates for postlarval abalone (Kawamura *et al.* 1998b, Daume *et al.* 2004, Daume & Ryan 2004).

Australian abalone nurseries consequently use *N. jeffreyi* and/or any naturally occurring diatoms for postlarval nutrition and as the abalone develops, *U. lens* becomes available for consumption (e.g. Daume & Ryan 2004, Daume 2006). By utilising both types of algae the diet can provide a biofilm consisting of diatoms and their extra cellular products required for the first two dietary transitions in early postlarval feeding and growth. Then the *U. lens* can accommodate the juvenile abalone dietary transition from a biofilm-dominated to a macroalgae-dominated diet. Daume & Ryan (2002) have shown that *H. laevigata* growth rates towards the end of a feeding trial (58 d) remained high, as the juveniles were able to access the *U. lens* as a food source and therefore, transitioned smoothly through the third nutritional phase. This has also been observed for *H. tuberculata coccinea* where at 2 mm shell length, juvenile growth rates on a variety of benthic diatom diets all increased simultaneously, indicating a nutritional shift from diatoms to *U. lens* and *U. rigida* germlings (Courtois de Vicose *et al.* 2012). The use of two forms of algae allows the nursery system to maintain a supply of readily ingestible and digestible food, specifically tailored to the nutritional transitions in postlarval and juvenile feeding. Therefore, abalone nurseries try and present a series of algal species with different grazing resistances that closely follow the ontogenetic changes in digestive enzyme activity and radula morphology of the juvenile abalone. This reduces starvation periods due to feed inaccessibility and can increase commercial growth rates and survival, consequently improving overall production in the nursery system.

The current commercial nursery diet utilised in Australian abalone farms of *U. lens* and *N. jeffrey*i provides adequate nutrition and can achieve growth rates of 70 $\mu m.day^{-1}$ for juvenile *H. laevigata* over extended nursery feeding trials (Daume & Ryan 2004, Daume *et al.* 2007). However, once abalone exceed approximately 5 mm shell length the commercial nursery diet becomes unable to support the high abalone biomass per plate during the later stages of the nursery phase (Daume & Ryan 2004,

Daume *et al.* 2007). The inadequate feed biomass caused by the high abalone grazing pressure, jeopardises growth rates and consequently affects the future growth and survival of the abalone. Therefore, maintenance of adequate food has been seen as a major limiting factor in the intensification of abalone hatcheries (Krsinich *et al.* 2000, Daume 2006).

Australian commercial abalone farms wean juvenile abalone from the nursery system onto an artificial diet in specifically designed tanks in the growout system. This can be done at anytime during the nursery stage and depends on growth rates, food availability and tank space. However, the transition between juvenile abalone (5 – 15 mm shell length) feeding on natural algal diets in the nursery system and weaning onto artificial feed in the growout system can be an extremely variable process (Daume 2003). Many studies around the world have examined the advantages or disadvantages of artificial diets with varying results. Some studies have observed greater growth rates of juvenile abalone consuming various algal species (Daume & Ryan 2004, Naidoo *et al.* 2006), whereas others show an increase in growth rates utilising artificial diets (Viana *et al.* 1993, Britz 1996a, Corazani & Illanes 1998), while no difference between artificial and natural diets have also been recorded (Knauer *et al.* 1996, Boarder & Shpigel 2001, Serviere-Zaragoza *et al.* 2001). In Australia however, directly after the transition onto artificial diets, juvenile abalone growth rates are generally lower than those on natural algal diets, while survival can be considerably lower due to the stress of harvesting and weaning (Daume & Ryan 2004, Daume *et al.* 2007).

A positive alternative to address the deficiencies of weaning too early would be to maintain the juvenile abalone in the nursery system for an extended period of time (up to 15 mm shell length), by either reducing the density of juvenile abalone on the settlement plates or utilising different algal diets that can withstand the intense abalone grazing pressure (Daume 2006). Both of these management strategies would maintain growth rates and remove the husbandry mortality, subsequently producing high quality juvenile abalone through the later nursery phase.

The density of postlarval abalone in the nursery system can affect the algal biomass attached to the plates, consequently varying their growth rates and survival. Manipulation of food supply (diatom) in a small-scale experiment has shown that the effect of postlarval *H. rubra* density on mortality was mostly independent of food, whereas abalone growth was strongly related to food supply and significantly reduced at high initial densities (Day *et al.* 2004). On a commercial scale, growth rates of juvenile *H. laevigata* (≈7 mm shell length) stocked at both low (40 juveniles per plate) and high (80 juveniles per plate) densities fluctuated with food density (*U. lens*/*N. jeffrey*i), however overall the growth rates, weight gain and survival were all higher at the lower stocking density (Daume *et al.* 2007). Reducing the stocking density on the settlement plates further would have considerable ramifications on the infrastructure requirements (number of tanks/plates required), labour and abalone biomass production of the nursery system. Given abalone growth can be highly dependent on food biomass over a range of densities, the composition of the diet consumed was considered important for juvenile abalone growth. In order to critically evaluate the ability of different algal forms and species to improve juvenile abalone growth and survival in the later nursery phase, stocking densities were standardised at current commercial levels.

To integrate different algae into the current nursery system consisting of vertical PVC plates, which are designed for encrusting algae and diatom diets, a variety of species and techniques need to be identified and examined. Several species of the encrusting Ulvophyceae have been tested to improve nursery culture of juvenile *H. iris*, with these green algal species easily grazed by juvenile abalone of 2 – 3 mm shell length, indicating an appropriate food source for facilitating the transition between diatoms and macroalgae, but were not sufficiently resistant to grazing as to provide an alternative diet for juvenile abalone greater than 7 mm shell length (Dyck *et al.* 2011). Given it has been demonstrated that Australian abalone species exhibit a preference for red algae (Section 1.1.3), Rhodophyta species could be incorporated into the nursery system to provide an algal diet closer to what abalone consume in wild populations. Algal species that can accommodate the ontogenetic changes in postlarval abalone of 1 – 3 mm shell length, while also providing significant biomass to overcome the high grazing pressure of 5 – 15 mm shell length juvenile abalone, may be a rare commodity

and very important to improving juvenile abalone nutrition in the nursery system. Macroalgal species with a soft, erect 3-dimensional thalli form could have the ability to aid the nutritional transition from a diatom to macroalgae-dominated diet, while also accommodating the intense grazing pressure of 5 – 15 mm shell length juvenile abalone in the later nursery phase.

1.4 RESEARCH AIMS IN THE THESIS

The central aim of this research was to identify, develop and evaluate alternative macroalgal diets that supply sufficient biomass to maintain commercially viable growth rates of the juvenile greenlip abalone (*Haliotis laevigata*) throughout the later nursery phase. This was achieved by:

1) Assessing the viability of three Rhodophyta propagation methods (carpospore liberation, protoplast production and vegetative propagation) in developing red macroalgal diets for juvenile greenlip abalone.

2) Determining the effectiveness of artificial adhesion protocols for incorporating red macroalgal propagules in the current Australian abalone nursery system.

3) Examining the growth and survival of juvenile greenlip abalone (5 – 15 mm shell length) feeding on alternative macroalgal diets in the later nursery phase.

4) Comparing abalone management strategies, including different harvest sizes and system designs.

The research in this thesis consists of a series of interrelated, fine-scale laboratory experiments and large-scale commercial abalone feeding trials structured to address these objectives. The experimental work focused on the algal diet limitations currently experienced in the nursery system utilised within Australian abalone farms. Subsequently all algal species and propagation techniques were evaluated with the specification of being utilised on a commercial production scale.

In Chapter 3 the complex triphasic life history of Rhodophyta species was identified, with the propagation techniques, carpospore liberation, protoplast production and vegetative propagation examined in detail. Propagule production was the primary assessment of the methods' viability, however, the requirement for algal biomass and the ease of incorporation into a commercial operation were also considered, with preliminary experimental results published in Strain *et al.* (2007). From these three propagation methods, suitable approaches were developed to create alternative macroalgal diets for juvenile abalone in the later nursery phase. In Chapter 4 the artificial adhesion protocols for incorporating red macroalgal propagules into the current commercial abalone nursery system were developed. Fragment culture of several red macroalgal species was successfully established utilising vegetative propagation, and through the artificial adhesion protocol a unique red macroalgal fragment diet was able to present substantial algal biomass to juvenile greenlip abalone in the nursery system (Strain *et al.* 2005, Strain *et al.* 2007).

The natural recruitment processes of spore liberation and attachment, prompted macroalgal sporelings to be developed as an alternative diet. This was the first study (Chapter 5) to investigate sporelings as a diet for 5 – 15 mm shell length juvenile abalone on a semi-commercial scale (Strain *et al.* 2006). Spore liberation from red macroalgal species allowed a multi-species sporeling diet to provide greater algal biomass and diversity for juvenile abalone. In Chapter 5 a composite macroalgal sporeling diet was evaluated against an easy culture diet and the current commercial diet in the nursery system. A different abalone management system utilising an artificial diet was also compared to the alternative macroalgal diets in the nursery system for rearing juvenile greenlip abalone during the later nursery phase.

The outcomes of this research will significantly contribute to the management of juvenile abalone aquaculture by:

1) Reducing the effect on growth and survival of juvenile abalone during the transition in nutritional requirements from a biofilm-dominated to a macroalgae-dominated diet.

2) Extending the time juvenile abalone can be maintained in the later nursery phase, therefore removing the survival and growth restrictions around the lack of algal biomass and early weaning.

3) Provide commercial farms with a variety of diet and management strategies to produce fast growing, high quality juvenile abalone.

The protocols evaluated and developed in this thesis are transferable throughout the commercial abalone and macroalgal aquaculture industries. The investigation into the macroalgal propagation methods can be incorporated into various aspects of the economically important algal cultivation industry. These methods will also aid the development of integrated aquaculture systems in commercial abalone farms through the culture of macroalgal species in nutrient enriched outflow for abalone feed. On a broader level, this study provides insight into juvenile abalone nutritional requirements and expands the limited knowledge associated with the role juvenile macroalgae plays in the growth and development of abalone.

CHAPTER 2
GENERAL MATERIAL AND METHODS

2.1 ALGAL SPECIES

Numerous algal species have been recognised as food sources for Greenlip Abalone, *Haliotis laevigata* Donovan. However, eight species were selected from the Rhodophyta, Chlorophyta and Cyanophyta divisions, to examine their potential in forming diets for juvenile greenlip abalone on the PVC plates during the later nursery phase. The current commercial nursery diet consisting of the green alga *Ulvella lens* plus the diatom *Navicula* cf. *jeffreyi* was used as a positive control in the juvenile abalone feeding trials.

Gracilaria chilensis Bird, McLachlan & Oliveira

Gracilaria chilensis was obtained from the culture collection at the School of Botany, University of Melbourne in Victoria. The *G. chilensis* culture was established by Professor John West and isolated in Victoria during 1999.

Gracilaria flagelliformis (Sonder) Womersley

Gracilaria flagelliformis was collected from next to the Leeuwin boat ramp in the Swan River Estuary, Western Australia (32°01'50 S 115°45'45 E) (Figure 1). The area consisted of a small, shallow (0 – 1 m depth) sand bay broken up by rock walls. The *G. flagelliformis* formed large mats and was partially submerged in the inter-tidal sand/mud flat located on the southern side of the boat ramp.

Gracilaria sp.

Gracilaria sp. was collected from around the eastern end of Mistaken Island in King George Sound, Western Australia (35°03'40 S 117°56'50 E) (Figure 1). The

Gracilaria was found at a depth of 4 to 12 m attached to the limestone bottom, which sloped down on a 45^{o} angle from the low tide mark.

***Hypnea* sp.**

Hypnea sp. was collected from Waylen Bay in the Swan River Estuary, Western Australia (32^{o}00'15 S 115^{o}50'25 E) (Figure 1). The *Hypnea* was attached to small rocks and shells scattered around the eastern side of the bay (<5 m from shore) in less than 1 m of water.

***Laurencia* sp.**

Laurencia sp. was collected from around the eastern end of Mistaken Island in King George Sound, Western Australia (35^{o}03'40 S 117^{o}56'50 E) (Figure 1). The *Laurencia* was attached to rocks at 8 to 12 m depth, just before the sea floor levelled off to sand with seagrass present.

***Plocamium mertensii* (Greville) Harvey**

Plocamium mertensii was collected from around the eastern end of Breaksea Island in King George Sound, Western Australia (35^{o}03'45 S 118^{o}04'10 E) (Figure 1). The *P. mertensii* was found among a variety of kelp species attached to limestone rocks at 12 to 20 m depth.

***Phormidium* sp.**

Phormidium sp. was identified as an epiphyte on the *Gracilaria* collected from around Mistaken Island in King George Sound, Western Australia (35^{o}03'40 S 117^{o}56'50 E) (Figure 1). The cyanobacterium was isolated from *Gracilaria* thallium by removing a few trichomes (filaments) and placing them on a microscope slide, then culturing in 250 mL Petri dishes with 200 mL of f/2 medium (Section 2.2, DFSW).

Ulva spp.

The *Ulva* spp. thalli collected consisted predominately of *Ulva lactuca*, however a small percentage of *Ulva fasciata* was also present in some samples. *Ulva* was collected from next to the East Street Jetty (32°02'28 S 115°45'25 E) and along the South Mole (32°03'19 S 115°43'59 E) in the Swan River Estuary, Western Australia (Figure 1). East Street Jetty has large limestone boulder/rock walls running adjacent to the jetty, which angle down into the water where at a depth of 1 to 5 m the substratum becomes sand or mud. South Mole is a groyne (rock wall) at the entrance of the Swan River Estuary consisting of large limestone and granite boulders. The *Ulva* thalli removed from both locations were attached to the rock substrata just below (<1 m) the mean seawater level.

Ulvella lens P.L. Crouan & H.M. Crouan

Ulvella lens was acquired from the stock culture at the commercial abalone farm, Great Southern Marine Hatcheries in Albany, Western Australia. PVC plates with large mature patches of *U. lens* attached were wiped down to remove contaminating algal species and placed in a 400 L nursery tank with 1 µm filtered flow through seawater (2 $L.min^{-1}$) and kept in complete darkness.

Navicula cf. *jeffreyi*

The diatom *Navicula* cf. *jeffreyi* (CS-514) was obtained from the culture collection at CSIRO Marine Research, Microalgae Research Branch in Hobart, Tasmania. It was maintained as a stock culture in 50 mL cell culture flasks with 25 mL of f/2 medium (Section 2.2, DFSW). This particular strain of *Navicula jeffreyi* was isolated from Port Fairy, Victoria by Dr Sabine Daume in 1999.

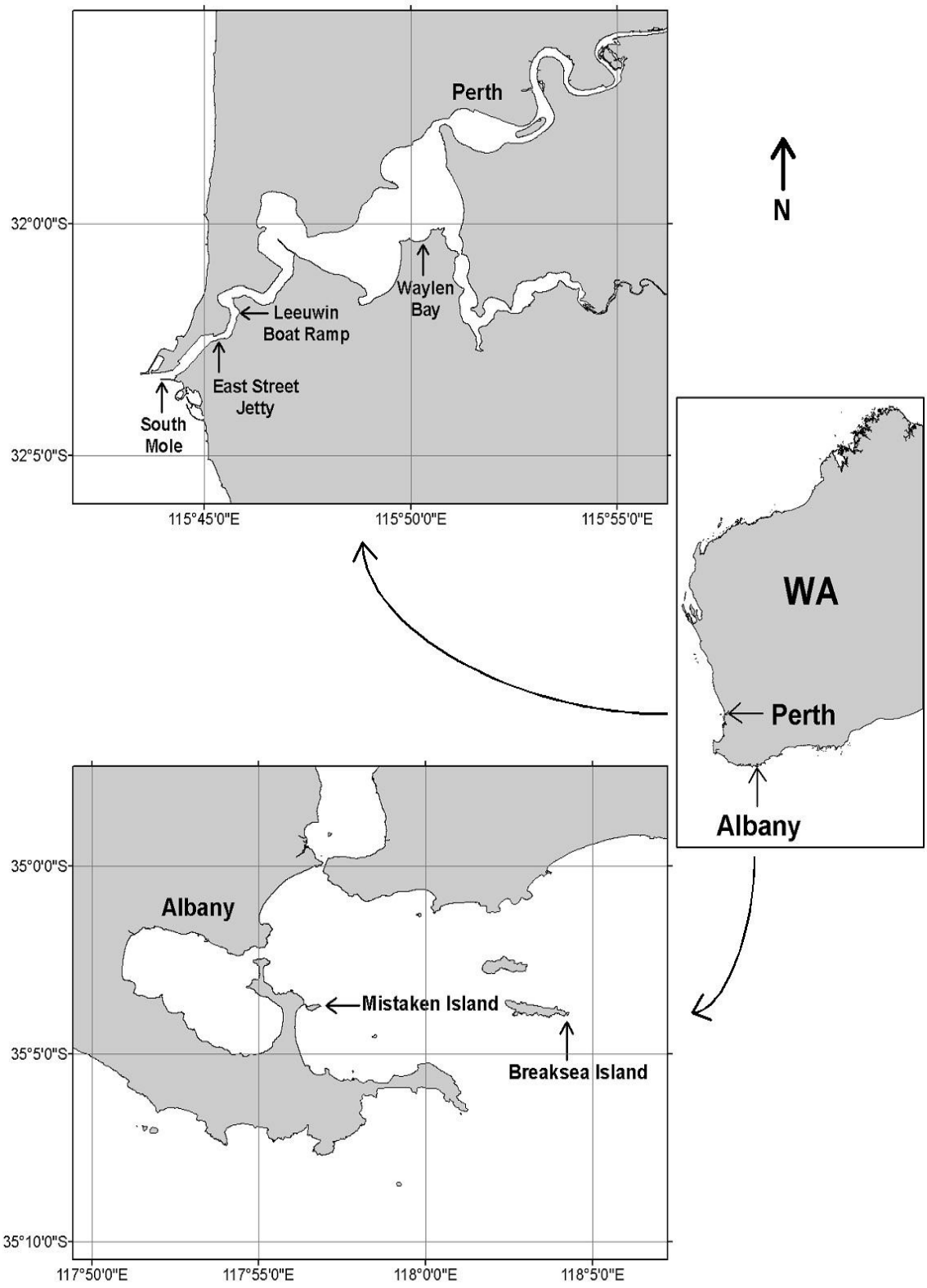

Figure 1: Map of Western Australia with the Perth and Albany regions enlarged, to show the locations where the algal species were collected.

2.1.1 Macroalgal Collection

The macroalgal material collected from the environment was carefully removed by hand at the holdfast to maintain the maximum amount of thalli. After collection, the algal thalli were placed in between moist paper towel within insulated containers, which had cool packs to maintain a constant and even temperature gradient (Chen & Shih 2000). The macroalgal species were then transported to the various locations depending on the experimentation (Murdoch University, Fremantle TAFE, Great Southern Marine Hatcheries and the Western Australian Fisheries Marine Research Laboratories).

2.2 ALGAL CULTURING

2.2.1 Seawater Filtration

The seawater utilised came from a variety of sources and depending on the location of the experiments, facilities available and volume of seawater required, different sterilisation methods were used to remove any biological contaminates. The salinity of the seawater was measured using a Hand Refractometer (Atago N1, Japan) to check that a consistent level of 35 – 36 PSU was maintained.

Double Filtered Seawater (DFSW)

The seawater was treated with activated charcoal (1 $g.L^{-1}$) for 2 h and then left overnight. The charcoal treated seawater was then filtered twice, once through two Whatman No. 1 filter papers and then again through a Whatman nitrocellulose membrane 0.45 µm filter (Advantec, MFS) (Buttery 2000). The double filtered seawater (DFSW) was then left to cure at 18 ± 2^{o}C, in polycarbonate containers in the dark and when required, autoclaved at 121^{o}C for 20 min before use.

Charcoal Column Filtered Seawater (CCFSW)

A Charcoal Column Filter (Eheim, Germany) packed with activated charcoal beads and glass cotton wool was connected directly to the seawater storage tank's

outlet. The required volume of seawater was filtered through the Charcoal Column Filter into polycarbonate containers.

Inline Column Filtration (ICF)

The seawater was filtered directly through an inline column filtration system (Puretec Water Filters) utilising either a 1 or 5 μm poly-micro filter cartridge. For flow through experiments the cartridge filters were cleaned twice a week by high-pressure hose and then exposed to the Chlorination process weekly. This enabled the seawater to be effectively filtered and the desired flow rate maintained throughout the various experiments.

Chlorination

The amount of seawater required for culturing was filtered through a 1 μm inline column filtration system and measured into the appropriate experimental vessel (flask, polycarbonate containers, tank, etc). Chlorine at 10 ppm was added to the seawater and left overnight in darkness for sterilisation. Liquid pool chlorine has 125 $g.L^{-1}$ available chlorine as sodium hypochlorine (12.5 % active ingredient), which equates to an application rate in this process of 0.08 mL per litre of seawater. To neutralise the active chlorine the dechlorination process utilises 0.025 $mL.L^{-1}$ of 2 M sodium thiosulphate under heavy aeration for several hours.

2.2.2 Culture Media

All chemicals and solvents used throughout this research were analytical grade (A.R.). The deionised water required for media stock solutions and other experimental procedures was attained from the laboratory in-house unit, Permutit Water Generation System. Different culture media were utilised during experimentation depending on the methodology, volume required and algal species being cultured. f/2 and modified Provasoli enriched seawater medium were used for stock cultures and fine-scale laboratory procedures, while Micro Algae Food with silicate and Abasol both water-

soluble fertilisers, were incorporated into large-scale algal culture for the juvenile greenlip abalone feeding trials.

f/2 Medium

f/2 culture medium (Guillard & Ryther 1962) was used as a growth medium for microalgal cultures and macroalgal sporeling cultures to provide nutrient supplemented seawater for faster growth (Steffensen 1976, Stratmann *et al.* 1996, Gómez-Pinchetti *et al.* 1998). The f/2 culture medium was prepared according to Table 1 and followed the procedure of Guillard & Ryther (1962) as modified by Buttery (2000).

Table 1: f/2 Medium (Guillard & Ryther 1962).

Compound	**Stock Soln.** ($g.L^{-1}$)	**Vol. Stock Soln. Added** ($mL.L^{-1}$)
$NaNO_3$	150	0.5
$NaH_2PO_4.H_2O$	10	0.5
$FeCl_3.6H_2O$	1.22	0.5
Na_2EDTA	0.945	0.5
Vitamin Mix		0.5
Cyanocobalamin	1.0×10^{-3}	
Biotin	1.0×10^{-3}	
Thiamine HCL	2	
PII Metal Solution		0.5
$CuSO_4.5H_2O$	0.0196	
$ZnSO_4.7H_2O$	0.04	
$CoCl_2.6H_2O$	0.02	
$MnCl_2.4H_2O$	0.072	
$Na_2MoO_4.2H_2O$	0.013	

Modified Provasoli Enriched Seawater Medium

Provasoli enriched seawater culture medium (Provasoli 1968) was modified by 25 % more nitrate, 50 % less iron (West & McBride 1999) and used as a general purpose, marine growth medium for axenic, macroalgal cultures. The modified Provasoli enriched seawater medium was prepared according to Table 2, with a final 10 mL inoculation volume poured into 1 L of sterilised seawater and the addition of a Germanium dioxide solution (GeO_2, 1 $g.L^{-1}$, pH 8) at 2 $mL.L^{-1}$, to inhibit diatom growth.

Table 2: Modified Provasoli Enriched Seawater Medium (West & McBride 1999).

Compound	**Stock Soln.** ($g.L^{-1}$)	**Vol. Stock Soln. Added** ($mL.L^{-1}$)
$NaNO_3$	35	110
Na_2glycerophosphate.$5H_2O$	50	8
Tris buffer (Trizma 7.7 pH crystals)	0.05	80
Vitamin B_{12}	0.025	3.5
Thiamine	0.5	8
Biotin	0.05	8
Iron EDTA		100
$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.7	
Na_2EDTA	0.66	
PII Trace Metal		200
Na_2EDTA	1.0	
H_3BO_3	1.12	
$FeCl_3.6H_2O$	0.048	
$MnSO_4.H_2O$	0.12	
$ZnSO_4.7H_2O$	0.022	
$CoSO_4.7H_2O$	0.005	

Micro Algae Food with silicate (MAF)

Micro algae food with silicate (MAF, Manutec PTY LTD) a water-soluble fertiliser for the aquaculture industry, incorporates similar compound contents (Table 3) to f/2 medium and was used for large-scale diatom culture at an application rate of 0.06 $g.L^{-1}$.

Table 3: Micro Algae Food with silicate (MAF) composition.

Compound	Amount
Nutrients	(w/w %)
NO_3	12
$NaNO_3$	21
SiO_2	3.1
PO_4	1.3
Ferric Citrate	4.5
Trace Elements	($g.L^{-1}$)
$CuSO_4$	0.025
$ZnSO_4$	0.044
$CoCl_2$	0.028
$MnCl_2$	0.5
MoO_4	0.025
Vitamin	($g.L^{-1}$)
Vitamin B_1 (Thiamine Hydrochloride)	1.0

Abasol

Abasol (Manutec PTY LTD), another water-soluble fertiliser used in aquaculture was incorporated as an alternative, complete f-mix medium in experiments requiring large volumes of nutrient enriched seawater. Abasol contains nutrients at the proportions shown in Table 4 and was utilised at an application rate of 0.06 $g.L^{-1}$.

Table 4: Abasol composition.

Compound	**Amount** (w/w %)
Total Nitrogen (N)	23.0
N as Urea	18.6
N as NO_3	2.6
N as NH_4	1.8
Total Phosphorus (P)	4.0
Total Potassium (K)	18.0
KNO_3	7.8
KCl	10.2
Fe as Chelate	0.06
$CuSO_4$	0.06
$MnSO_4$	0.15
$ZnSO_4$	0.05
B_2O_3	0.011
MoO_4	0.0013
Maximum Biuret	0.4

2.2.3 Equipment Sterilisation

All equipment utilised for the small-scale algal culture and laboratory experiments was first washed 3 times with the biodegradable, phosphate free, non flammable Decon 90 (2 – 5 % solution). The equipment was then rinsed 12 times in deionised water to remove the biological detergent and air-dried. Sterilisation was achieved via a commercial autoclave set at 121^{o}C for 20 min. Once sterilised the equipment was dried in an oven (70^{o}C) overnight and then placed in a laminar flow unit under UV light until required. The high volume equipment (tanks, aquaria, etc) required for the large-scale experiments and algal culturing were scrubbed clean, then the Chlorination process (Section 2.2.1) administered to sterilise the equipment and seawater simultaneously.

2.2.4 Culture Isolation and Maintenance

Stock cultures of all the algal species were created to maintain axenic, single species culture as reserve during experimentation. For the macroalgal species of *Gracilaria chilensis*, *Gracilaria flagelliformis*, *Gracilaria* sp., *Hypnea* sp., *Laurencia* sp., *Plocamium mertensii* and *Ulva* spp., the thalli was first cleaned of epiphytes using sterile seawater, deionised water and sterile cotton wool. The macroalgae was then surface sterilised using a 1 % (v/v) Betadine solution (Lawlor *et al.* 1991) and rinsed with sterile seawater, before being placed in 250 mL Erlenmeyer flasks with 150 mL of modified Provasoli enriched seawater medium (Section 2.2, DFSW). The epiphytic cyanobacterium, *Phormidium* sp. and the diatom *Navicula* cf. *jeffreyi* were isolated and cultured as described in Section 2.1. All algal stock cultures were in replicates of three and maintained at 18 ± 2^{o}C on a 12 h:12 h, light:dark cycle at 35 µmol photons.m^{-2}.s^{-1} until required.

The macroalgal species essential for large-scale experiments were not grown in sufficient biomass from the stock cultures, hence fresh material was collected as required. The harvested macroalgae were maintained in conical tanks (200 L) with 1 µm flow through seawater (ICF) at 2 L.min^{-1} and received strong aeration, outdoors under natural irradiance.

2.3 BIOCHEMICAL ANALYSIS

Algal samples were taken by scraping a known area of the PVC plate's surface and collecting the algal biomass that was removed. Samples were then stored at -20^{o}C until needed.

2.3.1 Ash Weight by Deduction

Samples were filtered through Whatman GF/C (2.5 cm) glass microfibre filters that had been washed, pre-combusted (450^{o}C) and pre-weighed. The filtrate was then washed with 10 mL ammonium formate solution (0.65 M) to remove excess salts, dried

in an oven for 12 h (80^{o}C) and placed in a vacuum desiccator over silica gel for 24 h. The resulting dried filtrate was weighed to 4 decimal places on an analytical balance and the initial filter weight subtracted to determine the sample's dry weight. The ash weight was calculated by deducting the biochemical components, lipid, protein and carbohydrate from the sample's dry weight. All biochemical components were presented on a dry matter basis and expressed as g/100 g dry weight of the filtered sample.

2.3.2 Lipid Determination

The lipid content of algal samples were determined by the method of Bligh & Dyer (1959), incorporating modifications by Kates & Volcani (1966) and adaptations by Mercz (1994). Samples were filtered through Whatman GF/C (2.5 cm) glass microfibre filters, rinsed with 10 mL ammonium formate (0.65 M) and stored at -20^{o}C for approximately 2 months.

Once thawed, the filtered samples were homogenised in a glass mortar and pestle with 5 mL methanol:chloroform:deionised water solution (2:1:0.8 v/v/v). The extract was centrifuged at 3000 rpm for 5 min and the supernatant transferred to a second, 10 mL graduated glass centrifuge tube. The volume was made up to 5.7 mL with fresh methanol:chloroform:deionised water, then 1.5 mL chloroform and 1.5 mL deionised water were added while mixing well. The tubes were re-centrifuged (3000 rpm for 5 min), after which phase separation was complete and the lower green chloroform layer containing the lipids was carefully transferred into dry, pre-weighted 4 mL glass vials. A few drops of toluene were added and the extract dried under ultra pure nitrogen. The vials containing lipid were placed in a vacuum desiccator over KOH pellets for 24 h and then weighed to 4 decimal places. The weight of lipid in the samples was determined by subtracting the pre-weight of the vials (no lipid present) from the final weight of the vials, containing lipid.

2.3.3 Protein Determination

The protein content of the algal samples was determined utilising a modification of the Lowry *et al.* (1951) method by Dorsey *et al.* (1978) and Mercz (1994). Samples were prepared as in the Lipid Determination procedure above.

The filtered samples were homogenised with 5 mL Biuret reagent in a glass mortar and pestle, then transferred into 10 mL graduated glass centrifuge tubes and 0.14 mL deionised water added. Protein standards (Bovine Serum Albumin) of 0, 10, 20, 30, 40, 50, 60, 70 µg were made up to 0.14 mL with deionised water and 5 mL Biuret reagent added. All tubes were incubated at 100^{o}C for 60 min and immediately after 0.5 mL Folin Phenol reagent was added while mixing on a Vortex stirrer. The tubes were cooled for 15 min at 10 – 15^{o}C and 15 min at room temperature then centrifuged (3000 rpm for 5 min). The absorbance of the supernatant was read at 660 nm and the protein content determined from the standard curve.

2.3.4 Carbohydrate Determination

The carbohydrate content of the algal samples was determined using the method of Kochert (1978) with modifications by Ben-Amotz *et al.* (1985) and Mercz (1994). Samples were prepared as in the Lipid Determination procedure above.

Five millilitre of H_2SO_4 (1 M) was used to homogenise the filtered samples in a glass mortar and pestle before being transferred into 10 mL graduated glass centrifuge tubes and incubated at 100^{o}C for 60 min. After cooling to room temperature and centrifuging (3000 rpm for 5 min), a known volume of supernatant (<50 µg total carbohydrate, which was between 0.1 to 0.5 mL, depending on initial algal concentration) was taken and made up to 2 mL with deionised water in 10 mL graduated glass centrifuge tubes. Carbohydrate standards (Glucose) of 0, 10, 20, 30, 40, 50 µg were made up to 2 mL with deionised water. One millilitre of 5 % (w/v) phenol solution was added and mixed well on a Vortex stirrer. Five millilitre of concentrated

H_2SO_4 (98 %, 18 M) was added rapidly and then the tubes left for 30 min to cool. Absorbance was measured at 485 nm and the carbohydrate content determined from the standard curve.

2.4 ABALONE FEEDING TRIAL

2.4.1 System Design

The set up, configuration and maintenance of the nursery and weaner systems used for the juvenile greenlip abalone feeding trials, endeavoured to replicate what's currently used at Australian commercial abalone nurseries.

Nursery System

The nursery system consisted of 400 L fibreglass coffin tanks connected in banks (Figure 2a). Each tank contained three metal baskets of 20 vertically arranged PVC settlement plates (60 x 30 cm). Inline column filtration (Section 2.2.1) supplied 1 µm filtered seawater at 10 $L.min^{-1}$ via a spray bar above the water surface and a standpipe (50 mm PVC pipe) was used as a drain to maintain water level. The filtered seawater was aerated by three weighted airlines (seepage hose) spaced evenly along the bottom of the tank. All tanks were shaded with 70 % shade cloth when stocked with abalone. For each of the experimental algal diets and the current commercial nursery diet of *U. lens* and *N. jeffreyi* tested in the feeding trial, three replicate and randomly positioned, 400 L nursery tanks were stocked with abalone.

Weaner System

The weaner tank was a shallow (160 L) round tank with cascading flat levels to a centre standpipe or drain (Figure 2b). The inner level of the tank at the drain was 20 cm deep while the depth of the outer level was only 2 cm. Each tank had artificial grass attached to the side to prevent abalone from crawling out. Inline column filtration (Section 2.2.1) supplied 1 µm filtered seawater at 5 $L.min^{-1}$ via a spray bar with holes in opposite directions on either side of the drain, to create greater water movement

(whirlpool effect), as there was no aeration. All tanks were covered with 70 % shade cloth and each diet (treatment) was replicated in three, randomly assigned tanks.

a)

b)

Figure 2: Examples of the juvenile *Haliotis laevigata* rearing systems, including the nursery system utilising vertical PVC plates with natural algal diets attached (a) and the weaner system incorporating artificial diets (b).

2.4.2 System Maintenance

Nursery System

The water and material at the bottom of all nursery tanks were siphoned through a 50 µm mesh weekly, to remove any abalone mortalities, abalone faeces and dead algal matter. This enabled the tanks to remain clean and reduce the growth of any algal contamination, while also making only the algal material attached to the PVC plates be consumed by the abalone. All nursery tanks had the PVC plate's rotated 180^{o} about the horizontal and Abasol added, at regular intervals to maintain an even coverage of the algal diet. The commercial nursery diet (*U. lens* and *N. jeffreyi*) tanks were inoculated with *N. jeffreyi* and MAF added, at regular intervals throughout the trial to maintain the diatoms' density.

Weaner System

The weaner system utilised commercially produced artificial feeds (50 % coarse crumb and 50 % 3 mm noodle, Adam and Amos, Mt. Barker, South Australia) at a rate of 2 % body weight per day (dry food, live abalone). The feed was delivered by hand, left overnight and the tank cleaned with increased water flow to reduce bacteria build up. This procedure was repeated every second day throughout the trial.

2.4.3 Juvenile Greenlip Abalone

Juvenile greenlip abalone (*H. laevigata*) of the appropriate size, were taken off an *U. lens* and naturally occurring diatoms diet at a commercial abalone farm. They were then transported on PVC plates seeded with *U. lens*, between wet sponge sheets in insulated containers. The PVC plates with juveniles attached were placed either across the top of the baskets in nursery tanks or on the bottom of weaner tanks and left for a week to allow the juveniles to migrate off. Juvenile abalone were stocked at commercially appropriate densities of approximately 40 juveniles per 60 x 30 cm plate (110 abalone.m^{-2}) in each of the nursery tanks and between 1100 – 1900 juvenile abalone.m^{-2} in the weaner tanks, depending on the number of juveniles obtained and the size of the tank available.

2.4.4 Commercial Nursery Diet

Ulvella lens

The nursery tanks and all contents (plates, baskets and airlines) were scrubbed clean, filled with 1 µm filtered seawater (ICF) and sterilised using the Chlorination process (Section 2.2.1). The *U. lens* spore collection methods were adapted from Takahashi & Koganezawa (1988), with twenty seed plates (60 x 30 cm PVC) that had been dark adapted for 2 weeks placed in each nursery tank at regular intervals between clean 60 x 30 cm PVC plates and exposed to sunlight. The tanks received no water flow (static) and Abasol was added to the sterilised seawater to provide nutrients, while the aeration was low to allow the released *U. lens* spores to attach onto the new clean plates. Once sporulation had occurred (5 – 7 d) the *U. lens* seed plates were removed and the filtered seawater and Abasol exchanged weekly.

Navicula* cf. *jeffreyi

The diatom, *N. jeffreyi* was incorporated as part of the control diet and when required for nursery tank inoculation was scaled up according to Table 5. The various sized, horizontally laid algal bags were clear plastic bags of approximately 5 cm depth with a tap cut into one corner. Stock and bag cultures were all maintained in a controlled temperature room at 18 ± 2°C on a 12 h: 12 h, light:dark cycle at 35 µmol photons.m^{-2}.s^{-1}. The Mexican hat was a shallow (≈10 cm deep), round tank placed outside, receiving natural irradiance. The seawater sterilisation process and medium source differed depending on the volume required, with 1 µm filtered (ICF), autoclaved f/2 medium used for the stock culture, small and medium bags, and 1 µm filtered (ICF), Chlorination treated with MAF used in the large bag, Mexican hat and nursery tank cultures (Section 2.2).

To harvest the bag and Mexican hat cultures the diatom cells were detached from the bottom by gentle agitation and drained into the next size culture vessel. The *N. jeffreyi* density in the Mexican hat was determined (≈ x10^5 – 10^6 diatom.mL^{-1}) and the inoculums (60 L initially then 20 L thereafter) were then poured into the nursery

tank along with the addition of MAF, at regular intervals. The nursery tank had no water flow, with the static seawater receiving light aeration for 24 h to allow the *N. jeffreyi* to attach onto the PVC plates. All culture stages were harvested during the exponential phase, 5 to 7 d after inoculation and conducted in replicates of three in order to increase the volume and decrease contamination while maintaining healthy *N. jeffreyi* cultures.

Table 5: *Navicula* cf. *jeffreyi* culture scale indicating vessel, inoculation volume, media volume and source.

Scale	Inoculation Vol.	Media Vol.	Source
Stock culture	1 mL	25 mL	Stock culture
Small bag	8 mL	200 mL	Stock culture
Medium bag	200 mL	1.5 L	Small bag
Large bag	1.5 L	60 L	Medium bag
Mexican hat	10 L	100 L	Large bag
Nursery tank	20 L	400 L	Mexican hat

2.4.5 Measurements

Abalone shell length as measured across the longest axis of the shell (mm) and whole weight (g) were recorded at the beginning of the nursery feeding trial and then periodically throughout the trial, by collecting a sub sample of juveniles from every nursery and weaner tank. To compare the growth performance of abalone between the experimental diets and the current commercial diets (both nursery and weaner systems) in the feeding trial; growth rate, specific growth rate and weight gain were determined. Abalone shell length (mm) was utilised for growth rate ($\mu m.day^{-1}$) and specific growth rate (% $length.day^{-1}$), which were calculated by Equation 1 and Equation 2 respectively; while whole, live abalone weight (g) was used in Equation 3 for computing weight gain ($\mu g.day^{-1}$). After the juvenile abalone had been measured, the contents of each tank were siphoned and the mortalities (dead abalone) counted. The number of dead abalone was subtracted from the stocking density to give a survival estimate or progressive abalone density at each measurement time period.

$$\text{Growth rate} = \frac{\left(SL_{(i)} - SL_{(i-1)}\right)\times 1000}{t} \qquad \text{Equation 1}$$

where *SL* was the abalone shell length (mm), *i* was a measurement interval and *t* was the time between the measurement intervals (d).

$$\text{Specific growth rate} = \left\{\frac{\left[\ln\left(SL_{(i)}\times 1000\right)\right] - \left[\ln\left(SL_{(i-1)}\times 1000\right)\right]}{t}\right\}\times 100 \qquad \text{Equation 2}$$

where *SL* was the abalone shell length (mm), *i* was a measurement interval and *t* was the time between the measurement intervals (d).

$$\text{Weight gain} = \frac{\left(W_{(i)} - W_{(i-1)}\right)\times 1000}{t} \qquad \text{Equation 3}$$

where *W* was the abalone whole weight (g), *i* was a measurement interval and *t* was the time between the measurement intervals (d).

Counting the number of algae per cm^2 or estimating the percentage cover of algae on the settlement plates at regular time intervals determined the algal density of the experimental diets. Every fifth plate was sampled with the algal cover measured diagonally across the plate in 5 randomly selected fields of view through a dissecting microscope (0.785 cm^2). The density of *U. lens* was determined by estimating percentage cover within a field of view, using the same sampling procedure as the experimental diet's algae. The density of *N. jeffreyi* was measured on two removable, sample plates attached at the top and bottom of every sixth settlement plate. The number of diatoms present on the sample plates was counted along a microscope graticule (x40 magnification) in 20 randomly chosen fields of view and the number of diatom.cm^{-2} calculated.

For each of the algal species utilised as a diet within the nursery system, its change in abundance or density over time (d) was converted into a consumption rate. This "consumption rate" was actually a balance between the algal species consumption

by abalone and its reproduction/settlement. Given only a few of the algal species showed evidence of reproduction at various times during the feeding trials and the consumption by juvenile abalone was the major cause of the algal species change in density, consumption rate was used to illustrate the change in algal biomass during the abalone feeding trial. Therefore, if "negative" consumption rates occurred, it indicated that the algal species were reproducing at a faster rate than the juvenile abalone could consume them. The consumption rates for algae measured as a density (algae.cm^{-2}) were calculated by Equation 4 and presented as algae.abalone^{-1}.day^{-1}, while the consumption rates for the algae recorded as percentage cover of the settlement plate were determined by Equation 5 and had units of % cover.abalone^{-1}.day^{-1}.

$$\text{Consumption rate} = \left\{ \frac{\left[\left(AD_{(i)} - AD_{(i+1)} \right) \times SA(plates) \right]}{Abalone} \right\} \Big/ t \qquad \text{Equation 4}$$

where *AD* was the algal species density, *i* was a measurement interval, *SA(plates)* was the surface area (cm^2) of all the settlement plates in the nursery tank, *Abalone* was the abalone stocking density in the nursery tank at $i + 1$ and *t* was the time between measurement intervals (d).

$$\text{Consumption rate} = \left\{ \frac{\left(\%\,\text{cover}_{(i)} - \%\,\text{cover}_{(i+1)} \right)}{Abalone} \right\} \Big/ t \qquad \text{Equation 5}$$

where *% cover* was the algal species percentage cover of the settlement plate, *i* was a measurement interval, *Abalone* was the abalone stocking density in the nursery tank at $i + 1$ and *t* was the time between measurement intervals (d).

CHAPTER 3
PROPAGATION OF RHODOPHYTA SPECIES FOR NUTRITION OF JUVENILE GREENLIP ABALONE

3.1 INTRODUCTION

To incorporate different macroalgae, particularly species of Rhodophyta, in a diet for juvenile greenlip abalone on the PVC settlement plates utilised within the nursery system, appropriate techniques need to be identified and developed. Three cell culture techniques; (1) spore liberation, (2) protoplast production and (3) vegetative propagation are examined here, as they facilitate propagation and development of macroalgal genotypes (Reddy *et al.* 2008b) suitable for juvenile abalone nutrition.

3.1.1 Carpospores of Red Macroalgae

Algae of the Phylum Rhodophyta (red algae), Class Florideophycidae have a triphasic life history with an alternation of haploid and diploid phases (Figure 3), including a stage unique to this Phylum; the carposporophyte, a diploid phase (cystocarp) parasitic on the gametophyte (Hommersand & Fredericq 1990, Kain & Destombe 1995). Therefore, this triphasic life history consists of the gametophyte (haploid), carposporophyte (diploid) and tetrasporophyte (diploid) stages (Guimarães *et al.* 1999, Engel *et al.* 2001). Red macroalgal species produce two types of non-motile spores; the tetraspores (meiospores) and the carpospores (zygospores), that once released will settle out of the water column, attach to substratum and grow into small plantlets (Destombe *et al.* 1989, Destombe *et al.* 1992) (Figure 3).

Different phenotype stages, spore mortality and recruitment all play vital roles in the population structure of red macroalgal communities (Mantri *et al.* 2010). Plants of each of the 3 life history stages are not usually found in equal abundance in natural red algal communities and dominance of one phase over the others can often occur (Kong & Ang 2004). This causes variation in the seasonality of material, as sexual phenotypes,

sexual maturity and the vegetative phase change over a range of spatial and temporal scales (Kain 1982, Destombe *et al.* 1989, Guillemin *et al.* 2008). Therefore, the production of spores can be extremely variable and depends on numerous factors including species, season, temperature, irradiance, photoperiod, desiccation and osmotic pressure (Friedlander & Dawes 1984, Guzmán-Urióstegui & Robledo 1999, Orduña-Rojas & Robledo 1999, Garza-Sánchez *et al.* 2000). The number of carpospores released from 1 g of fertile female gametophyte can be up to 8 x 10^4 carpospores.g^{-1} (*Gracilaria arcuata*, *Gracilaria asiatica*, *Gracilaria corticata* and *Gracilaria pacifica*), while tetraspore releases of up to 1 x10^5 tetraspores.g^{-1} (*G. arcuata*, *G. corticata*, *G. pacifica*) have been achieved (Umamaheswara Rao 1976, Kaliaperumal *et al.* 1986, Xiuliang & Meizhen 1989, Garza-Sánchez *et al.* 2000). These are maximum reported yields and variability in spore release and the relationship between spore and sporeling density (Azanza & Ask 2003), will directly affect the plantlet density achieved and the quantity of fertile red macroalgal biomass required.

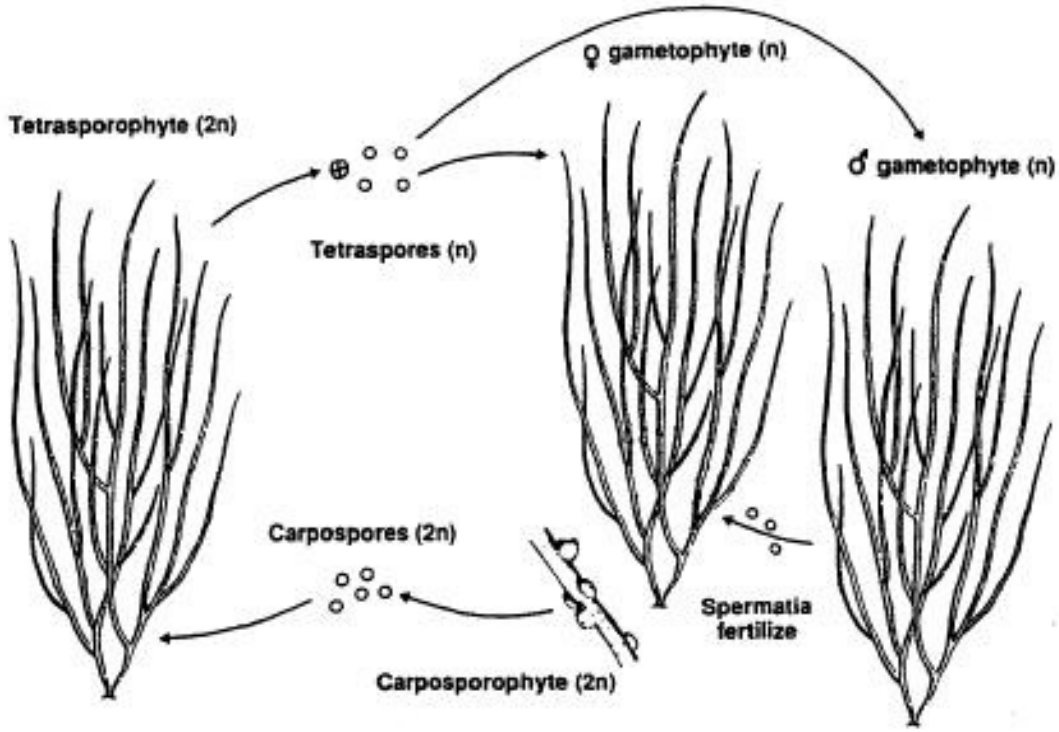

Figure 3: The triphasic life history of *Gracilaria*, a representation of Rhodophyta species reproductive propagation through spore liberation (Sourced from Kain & Destombe (1995) as reproduced from Kain (1991)).

Macroalgal spore recruitment also influences the density of tetraspores and carpospores germinating into plantlets and can range from 0 to 85 % (Oza *et al.* 1994, Buschmann *et al.* 1999, Garza-Sánchez *et al.* 2000). The variations in germination rates are dictated by two factors: the dispersal of spores and the attachment to the substratum. The dispersal rates of the non-motile red macroalgal spores are primarily dependent on spore size and settlement rates, which directly affect spore viability (Coon *et al.* 1972, Clayton 1992, Destombe *et al.* 1992). Locating suitable substrata through settlement cues and subsequent attachment also plays an important role in the successful germination of red macroalgal spores (Fletcher & Callow 1992, Santelices & Aedo 1999). *Palmaria palmata* sporelings with a settlement survival of 35 %, produced an average density of approximately 200 sporelings.cm^{-2} after 2 months of culturing on plates for juvenile abalone consumption (Le Gall *et al.* 2004). Spore densities in this order of magnitude (200 spores.cm^{-2}) have been identified for multiple algal species as producing adequate sporeling cover (density) on substrata (Alveal *et al.* 1997, Le Gall *et al.* 2004). The green algae, *Ulva* spp. can readily produce sporeling densities of 160 – 180 sporeling.cm^{-2} on artificial substrata from a single induced, spore release event (Section 5.1.3).

Spore release is the fundamental means of propagation for marine benthic macroalgae. The successful incorporation of this propagation method into macroalgal cultivation protocols can be determined by two critical factors, spore liberation potential and early sporeling development (Mantri *et al.* 2009). Once these two factors have been examined, the amount and source of the reproductive phenotype also plays a role in the ability of commercial abalone farms' to incorporate spore-based macroalgal propagation into culture protocols designed for juvenile abalone diet development.

The red macroalgal species, *Gracilaria chilensis*, *Hypnea* sp. and *Plocamium mertensii* were exposed to three treatments – temperature, dark and osmotic pressure – to induce the release of carpospores and determine the alga's capacity for naturally seeding the vertical PVC plates in the abalone nursery system.

3.1.2 Red Macroalgal Protoplast Production

Protoplasts have been isolated and cultured for decades from a diverse range of higher plants including herbaceous and tree species, for use in physiological, cytological and molecular studies. Their use has substantially expanded in genetic research including hybridisation, gene manipulation and mutation isolation (Carlson 1973, Galun 1981, Ahuia 1982). These applications for protoplast isolation of terrestrial plants have also been applied to marine algae for use in commercial seaweed cultivation (Reddy *et al.* 2008a).

Initially the majority of protoplast research was on morphologically simple macroalgae, such as *Ulva*, *Enteromorpha* and *Porphyra* (Polne-Fuller & Gibor 1984, Chen 1986, Polne-Fuller & Gibor 1986, Reddy *et al.* 1989, Björk *et al.* 1992). By the use of cell wall lytic enzymes, protoplast isolation protocols have improved allowing the more complex Rhodophyta (red) and Phaeophyta (brown) species to readily produce viable protoplasts (Reddy *et al.* 2008a). This improvement in reliability of protoplast procedures has instigated a focus shift to the commercially important culture of the agarophyte algae, in particular the *Gracilaria* species (see Table 4 in Gupta *et al.* 2011). However, protoplasts have also been successfully isolated from numerous other red macroalgae genera, for example *Bangia*, *Grateloupia* and *Palmaria* (Liu *et al.* 1992, Araki *et al.* 1994, Chen & Chiang 1994, Lafontaine *et al.* 2011). In fact, over 40 species of Rhodophyta have been successfully used for protoplast isolation, while more than 20 species of both Chlorophyta and Phaeophyta have also been studied (see Table 2 in Reddy *et al.* 2008a).

A protoplast can be generally defined as a plant cell devoid of its cell wall (Galun 1981) and their production allows single cells to be isolated from whole sections of algal thallus. The connective tissue between cells must be broken down, allowing the cells to separate and subsequently the cell wall digested, creating a protoplast. Enzyme extracts from the gut of sea snails, including abalone have been used as the cell wall digestants (Liu *et al.* 1984, Cheney *et al.* 1986, Inoue *et al.* 2008), while numerous enzymes isolated from Phycophages and micro-organisms have also been tested (see

Table 1 in Reddy *et al.* 2008a). Over time this method has evolved and now technical procedures utilising agarase and commercial enzymes (Cellulase Onozuka RS and Macerozyme R-10) have been developed for the isolation of protoplasts from *Gracilaria* species (Araki *et al.* 1998, Gupta *et al.* 2011).

Once protoplasts have been produced from the macroalgal thallus, cell wall regeneration and subsequently cell differentiation can occur. Protoplasts can be maintained in culture directly after isolation, before cell wall regeneration has begun (Björk *et al.* 1990, Mollet *et al.* 1995). In some cases *Gracilaria* protoplasts in culture develop into callus formations (Cheney *et al.* 1986). This callus can also form whole plantlets, but only a small percentage of protoplasts isolated from various species have been shown to do this (Yan & Wang 1993, Yeong *et al.* 2008). As callus formation occurs and filaments develop into plantlets, the isolated macroalgae can directly attach to substrata.

Protoplast production requires very small amounts of macroalgal material, for example, 1 g fresh weight of *Gracilaria verrucosa* can produce up to 1.03×10^8 protoplasts.g^{-1} (Araki *et al.* 1998). This small amount of red macroalgal material does not need to come from any specific phenotype and subsequently the variation in the seasonality of material in terms of sexual phenotypes, sexual maturity and vegetative phase becomes irrelevant. The isolation of a large number of protoplasts from suitable red macroalgae eliminates the issues associated with the triphasic life history (carpospore production) and has the potential to produce large quantities of single-celled biomass, which can potentially be used as seed material to grow plantlets for use in aquaculture. This has been achieved under laboratory conditions using green macroalgae, by seeding threads with isolated protoplasts, and after 3 – 4 weeks tiny plantlets were present (Reddy *et al.* 2006).

The ability of protoplasts to be used as a seed material for macroalgal culture indicated it could be a possible propagation method to develop red macroalgal diets for

juvenile abalone in the nursery system. Red macroalgal species *Gracilaria flagelliformis*, *Gracilaria* sp., *Hypnea* sp. and *Laurencia* sp. were examined as to whether this propagation method can produce commercial quantities of protoplasts and subsequently seed the vertical PVC plates with whole plantlets of sufficient biomass, to accommodate the grazing pressure of juvenile greenlip abalone.

3.1.3 Vegetative Propagation of Red Macroalgae

The application of algal thalli fragments to produce plantlets by vegetative growth has been successfully used in macroalgal propagation for the mass culturing of seaweeds. Over 200 macroalgal species are in commercial production around the world (Zemke-White & Ohno 1999) and many of these are cultured by vegetative propagation in both sea/pond culture and onshore facilities (McLachlan 1991, Oliveira *et al.* 2000). This method of propagation has been particularly profitable in the agarophyte industry, in the commercial cultivation of red algae genera such as *Gelidium*, *Gracilaria* and *Gigartina* (Ajisaka & Chiang 1993, Buschmann *et al.* 1995, Buschmann *et al.* 2001). Commercial cultivation occurs through a variety of methods with a common practice being to splice/braid the algal thalli on ropes, and either suspending them or bottom plant them, in the sea or in shallow ponds or tank systems (Santelices & Doty 1989, Oliveira *et al.* 2000). Commercial growth rates of *Gracilaria* species using this suspended rope cultivation system can average 3.5 $\%.day^{-1}$ (Dawes 1995), while vegetative fragments (10 cm long) stocked at low density (20 cm intervals) on ropes suspended in floating cages have reported growth rates up to 10.5 $\%.day^{-1}$ (Hurtado-Ponce 1990). Maximum growth rates ranging from 0.86 to 4.45 $\%.day^{-1}$ have also been attained using 5 – 10 cm fragments for a variety of *Gracilaria* species (Chaoyuan *et al.* 1993, Chirapart & Ohno 1993).

The use of vegetative fragments in the agarophyte industry by artificially attaching the macroalgae to substrata rather than by spore production and subsequent germination; has removed the issues associated with the complex triphasic life history of the Rhodophyta, including seasonal availability of the sexual phenotypes and sexual maturity. There then becomes no need to rely on spore liberation for algal production, as

vegetative propagation utilises the regenerative capacity of the algae with any thallus section of any phenotype suitable (Dixon 1973, Santelices & Varela 1995). Young branches are generally selected for their fast growth meristem, which can be found in the apex of the thallus; however, this does not always relate to faster growth of fragments (Santelices & Varela 1995). *Gracilaria chilensis* has shown bud differentiation and callus formation when cut into 5 mm apical and medial fragments, then cultured for a month (Collantes *et al.* 2004). Smaller fragments (2 mm) of *Eucheuma denticulatum* produced new cells from the cut surface after 2 – 3 d, while growth rates ranged from 5.8 – 7.2 %.day^{-1} when the fragments were out-planted at 2 – 5 cm (Hurtado & Cheney 2003). Fragments of *Kappaphycus alvarezii* (2 mm) also took 2 – 3 d for new cells to be present at the cut surfaces with callus formation after 25 – 30 d (Hurtado & Biter 2007), while the earliest shoot primordia of *K. alvarezii* fragments (2 mm) have been produced in under 3 weeks (Yunque *et al.* 2011).

During culture of small macroalgal fragments, wound healing of the cut surfaces can have significant implications for survival and growth. The wound healing process occurs by new cortex sealing the excised edge and can be completed within 3 weeks for *Eucheuma alvarezii* (Azanza-Corrales & Dawes 1989). Fragments of *Gigartina skottsbergii* can achieve complete wound healing by day 20 in culture conditions of 5 μmol photons.m^{-2}.s^{-1} at 10 and 15^{o}C (Buschmann *et al.* 1999, Correa *et al.* 1999), while the excised edges of *Gelidium* sp. fragments (1 – 2 mm) were covered by crust cells and growth had appeared in the first 2 weeks (Titlyanov & Titlyanova 2006). To enable red macroalgal fragments to be utilised in juvenile abalone diets, it was important for wound healing to occur and that appropriate survival rates are achieved, so that the fragments can grow and increase the algal biomass available for abalone consumption.

Vegetative propagation of thallus fragments can be a simple means of determining culture conditions for macroalgae that are not suited to the more advanced propagation methods of protoplast and tissue culture (Reddy *et al.* 2008b). Given the ease of macroalgal cultivation by fragmentation compared to other propagation

methods, it's a common commercial propagation method that has the potential to produce significant macroalgal biomass as an alternative diet for juvenile abalone. Therefore, the vegetative growth and survival of fragments from the red macroalgal species *Gracilaria* sp., *Hypnea* sp. and *Laurencia* sp. were assessed for their suitability for use in a commercial abalone nursery diet.

3.2 MATERIAL AND METHODS

3.2.1 Carpospores of Red Macroalgae

A preliminary, small-scale experiment was carried out using *Plocamium mertensii* harvested from the Breaksea Island collection site (Section 2.1), with a combined temperature and dark treatment to induce the release of spores. Fertile thalli of *P. mertensii* were selected and an induction treatment of 4 – 6^{o}C in darkness for 24 h applied by refrigerating the thalli in plastic trays covered with moist newspaper. Ten gram of blotted algal thalli was then placed in a container with 200 mL of 1 µm filtered seawater (ICF) at 16 ± 2^{o}C, exposed to a 12 h:12 h, light:dark cycle at 100 µmol photons.m^{-2}.s^{-1} with spore release monitored over a 4 h period.

For the larger carpospore production experiment, female gametophytes with mature cystocarps (carposporophyte stage) of *Gracilaria chilensis*, *Hypnea* sp. and *P. mertensii* were selected from either the algal material cultured on site or harvested at various wild collection locations (Section 2.1). Fertile thalli branches with the highest density of cystocarps were removed and cleaned of epiphytes with sterile seawater, deionised water and sterilised cotton wool, then surface sterilised using a 1 % (v/v) Betadine solution and rinsed with sterile seawater (Lawlor *et al.* 1991). The thalli were then placed into Erlenmeyer flasks with 1 L sterile seawater (DFSW) at 18 ± 2^{o}C on a 12 h:12 h, light:dark cycle at 35 µmol photons.m^{-2}.s^{-1} for 1 d.

The fertile branches were blotted, weighed and then exposed to three different spore induction treatments including temperature, dark and osmotic pressure. In the

temperature treatment the algae were exposed to 4°C for 40 h by refrigerating the thalli in plastic trays covered with moist newspaper. In the dark treatment the algae were kept within the Erlenmeyer flask cultures and placed in complete darkness for 40 h. For the osmotic pressure treatment the algal thalli were hand rinsed for 20 min under flowing deionised water. After the respective treatments, the fertile branches were placed in Petri dishes with 50 mL sterile seawater (DFSW) and a microscope slide on the bottom. The 3 macroalgal species examined had 3 replicate Petri dishes apiece for each of the induction treatments, all cultured at two temperatures of 18 ± 2°C and 25 ± 2°C with two irradiances of 10 and 100 μmol photons.m^{-2}.s^{-1} for both temperatures. The fertile branches were left for 24 h to allow the cystocarp time to release the carpospores.

The number of carpospores settled within a field of view (40x magnification) was then counted on the microscope slide from the bottom of the Petri dish to determine how many spores had been released per gram of alga. Spore release from the three macroalgae, *G. chilensis*, *Hypnea* and *P. mertensii* was compared by a three-way analysis of variance (ANOVA) with a Tukey Post-Hoc test taking the treatment, temperature and irradiance as fixed factors.

3.2.2 Red Macroalgal Protoplast Production

Initial Enzyme Experiment

Gracilaria flagelliformis was harvested from the Leeuwin collection site (Section 2.1) and the young branches cleaned of epiphytes with sterile seawater, deionised water and cotton wool. The branches were then placed in Erlenmeyer flasks with 1 L of f/2 medium (Section 2.2, DFSW) at 18 ± 2°C and exposed to a 12 h:12 h, light:dark cycle at 50 μmol photons.m^{-2}.s^{-1}, 1 d prior to protoplast production.

The protocol for isolating a large biomass of protoplasts from the agarophyte algae genera *Gracilaria*, using agarase and commercial enzymes was modified from the procedure of Araki *et al.* (1998). The enzymes Cellulase Onozuka RS, Macerozyme

R-10 (Yakult Pharmaceutical Ind. Co, Ltd), agarase, papain and mannitol (Sigma-Aldrich) were dissolved separately in two different base treatments, sterile seawater (DFSW) and deionised water (osmotic treatment), then filter-sterilised through a 0.2 µm membrane filter. Young *G. flagelliformis* branches (1 g blotted wet weight of branches, 3 – 5 cm in length) were pre-treated with 30 mL papain solution (20 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer, pH 6.5 – 7.5, containing 5 % (w/v) papain and 0.7 M mannitol) in a 100 mL beaker and incubated at 25 ± 2°C for 30 min with agitation on a reciprocal shaker (30 rev.min^{-1}). Treated branches were then washed three times with f/2 medium containing 0.7 M mannitol (osmotic stabiliser) and cut into 1 – 2 mm fragments. Fragments totalling 0.5 g were treated with 10 mL of the agarase enzyme mixture (Table 6) in a 50 mL Erlenmeyer flask at 25 ± 2°C and agitated in the dark for 150 min (30 rev.min^{-1}).

The reaction mixture consisting of the *G. flagelliformis* fragments treated by the agarase enzyme mixture was then filtered through a 45 µm nylon mesh and the protoplasts collected by centrifugation at 190 g for 5 min. The pellet was gently re-suspended in 2 mL of f/2 medium containing 0.7 M mannitol and the number of protoplasts counted using a haemocytometer. The protoplasts isolated from *G. flagelliformis* by the two enzyme base treatments (3 replicates each), sterile seawater and deionised water (osmotic treatment), directly following isolation and their survival after 33 h in culture were both compared by analysis of variance (one-way ANOVA).

Table 6: The enzymes and chemicals utilised in the agarase enzyme mixture for the isolation of protoplasts from red macroalgal species (Araki *et al.* 1998).

Chemical / Enzyme	Amount	Unit
Agarase	4	units
Cellulase Onozuka RS	4	% (w/v)
Macerozyme R-10	2	% (w/v)
Mannitol	0.7	M
MES buffer (pH 6.5)	20	mM

Protoplast Isolation from 3 Red Macroalgal Species

Three red macroalgal species, *Gracilaria* sp., *Hypnea* sp. and *Laurencia* sp. were harvested from the various collection sites (Section 2.1) and cleaned of epiphytes with sterile seawater, deionised water, cotton wool and a Betadine solution (1 %, v/v). Young branches were then placed in Erlenmeyer flasks with 1 L modified Provasoli enriched seawater medium plus GeO_2 (Section 2.2, DFSW), to provide a balanced marine growth medium specifically designed for macroalgal cultures rather than the universal f/2 medium used in the Initial Enzyme Experiment (Section 3.2.2). The cultures remained at 18 ± 2°C and were exposed to a 12 h:12 h, light:dark cycle at 50 μmol photons.m^{-2}.s^{-1} for 1 week, then dark adapted for 1 d prior to protoplast production.

The protocol for isolating protoplasts from the three red macroalgal species followed that of the Initial Enzyme Experiment (Section 3.2.2) with minor modifications. The enzymes were all dissolved separately in deionised water, then filter-sterilised through a 0.45 μm membrane filter. Young algal branches 1 – 2 cm long (1.5 g blotted wet weight of each alga) were pre-treated with the papain solution and incubated in the dark at 22 ± 2°C for 30 min. Treated branches were washed three times with modified Provasoli enriched seawater medium containing 0.7 M mannitol and fragments of each algae (0.3 g) immersed in the agarase enzyme mixture (Table 6) at 22 ± 2°C. The reaction mixture of treated algal fragments in the agarase enzyme mixture was filtered through a 100 μm nylon mesh and after centrifuging, the pellet was washed and re-centrifuged twice more before being gently re-suspended in 1 mL modified Provasoli enriched seawater medium containing 0.7 M mannitol. The numbers of isolated protoplasts were then counted immediately and after 14 h using a haemocytometer.

Once the re-suspended protoplasts (1 mL modified Provasoli enriched seawater medium containing 0.7 mM mannitol) had been counted for the second time, the protoplast culture was then transferred into 50 mL Erlenmeyer flasks with fresh 10 mL modified Provasoli enriched seawater medium containing 0.7 M mannitol and cultured

at 22 ± 2°C on a 12 h:12 h light:dark cycle at 20 µmol photons.m^{-2}.s^{-1}. The mannitol was gradually removed from the medium during subculturing by replacing the medium with new medium at lower concentrations of mannitol, so as to not impair cell division. The initial protoplast yields (time = 0) from the 3 macroalgal species (3 replicates each) were compared by analysis of variance (one-way ANOVA) and the survival of protoplasts in culture over time assessed by a Pearson Product Moment Correlation analysis.

3.2.3 Vegetative Propagation of Red Macroalgae

Preliminary Vegetative Fragment Experiment

Gracilaria sp. was harvested from the Mistaken Island collection site (Section 2.1) and placed in Erlenmeyer flasks with 1 L, f/2 medium (Section 2.2, DFSW) under aeration at 18 ± 2°C and exposed to a 12 h:12 h, light:dark cycle at 35 µmol photons.m^{-2}.s^{-1}, for 2 weeks. Thalli pieces of approximately 5 cm in length were cleaned of epiphytes with sterile cotton wool, surface sterilised with a 1 % (v/v) Betadine solution and then rinsed with sterile seawater.

The cleaned thalli pieces were cut by razor blade into 5 – 7 mm fragments and placed in Erlenmeyer flasks with 150 mL f/2 medium. The growth of *Gracilaria* fragments was examined over 2 weeks at two temperatures (15 ± 2°C and 18 ± 2°C) in the natural range of juvenile greenlip abalone (Section 5.2.3). The effect of irradiance was also tested by using two irradiances, 75 and 350 µmol photons.m^{-2}.s^{-1} on 12 h:12h, light:dark cycle that reflect the conditions in abalone nursery tanks (Section 5.2.3).

Vegetative Fragment Experiment

Young branches of the red macroalgae *Gracilaria* sp., *Hypnea* sp. and *Laurencia* sp. were harvested from various collection sites (Section 2.1). The culturing, cleaning/sterilising and fragment preparation procedures followed that of the Preliminary Vegetative Fragment Experiment (Section 3.2.3), except that modified

Provasoli enriched seawater medium plus GeO_2 (Section 2.2, DFSW) was used instead of f/2 medium and exchanged on a weekly basis. The algal fragments were cultured (12 replicate cultures for each specie) for 11 weeks at 15 ± 2°C and under 75 µmol photons.m^{-2}.s^{-1} on a 12 h:12h, light:dark cycle (Section 3.3.3). A Friedman Repeated Measures Analysis of Variance on Ranks was carried out to compare both the growth rate and survival of the 3 macroalgal species fragment cultures over the 11-week period. If there were significant differences ($p<0.05$) between species a Pairwise Multiple Comparison Procedure (q values) was completed.

3.3 RESULTS

3.3.1 Carpospores of Red Macroalgae

In the preliminary experiment only 8.69 % of the fresh *P. mertensii* thalli collected from the field had fertile fronds. A spore yield of 4.05 x10^3 spores.g^{-1} of thallus was recorded after 2 h, increasing to 4.75 x10^3 spores.g^{-1} after 4 h.

Carpospores of 20 – 50 µm diameter (Figure 4) were successfully liberated from the three red macroalgal species by all of the induction treatments, temperature, dark and osmotic pressure (Figure 5). The mean carpospore production was significantly different between species with *Hypnea* producing 67.23 ± 10.19 x10^3 carpospores.g^{-1}, while P. *mertensii* released 43.61 ± 4.38 x10^3 carpospores.g^{-1} and *G. chilensis* liberated 17.09 ± 2.46 x10^3 carpospores.g^{-1} ($F_{(df\ 2,105)}$=30.904, $p<0.05$). The mean number of carpospores released for each alga was significantly different from both of the other two macroalgal species (Tukey, $p<0.05$). The variation in carpospore release between each macroalgae was clearly demonstrated by the change of scale (y axis) in Figure 5. The temperature treatment and culture conditions of 25 ± 2°C and 10 µmol photons.m^{-2}.s^{-1} liberated the most carpospores (*Hypnea*, 140.21 ± 19.73 x10^3 carpospores.g^{-1}), while the osmotic pressure treatment and culture conditions of 18 ± 2°C and 10 µmol photons.m^{-2}.s^{-1} resulted in the least number of carpospores released (*G. chilensis*, 9.08 ± 0.89 x10^3 carpospores.g^{-1}) (Figure 5).

On a species level the osmotic pressure treatment resulted in the lowest level of carpospore liberation of the three induction treatments for both *G. chilensis* and *Hypnea*, however for *P. mertensii* it produced the highest number of released carpospores (Figure 5). In fact, the osmotic pressure treatment resulted in significantly less *Hypnea* carpospores being released ($F_{(df\ 2,33)}$=14.903, p<0.05 (Tukey, p<0.05)), but was able to liberate a significantly greater number of *P. mertensii* carpospores ($F_{(df\ 2,33)}$=7.314, p<0.05 (Tukey, p<0.05)) when compared with the other two induction treatments. On the other hand *G. chilensis* carpospore release induced by the dark treatment was significantly greater than both the temperature and osmotic pressure treatments ($F_{(df\ 2,33)}$=13.021, p<0.05 (Tukey, p<0.05)).

Culture temperature and irradiance affected carpospore release differently for each species (Figure 5). However, the carpospore release from the three species, over all three of the induction treatments were not significantly different between the two culture temperatures of 18 and 25°C ($F_{(df\ 1,106)}$=0.71, p=0.401), or the two irradiances of 10 and 100 µmol photons.m^{-2}.s^{-1} ($F_{(df\ 1,106)}$=1.631, p=0.204).

Figure 4: An example of the carpospores liberated from red macroalgal species (*Plocamium mertensii*) induced by three different treatments, temperature, dark and osmotic pressure. Scale bar = 50 µm.

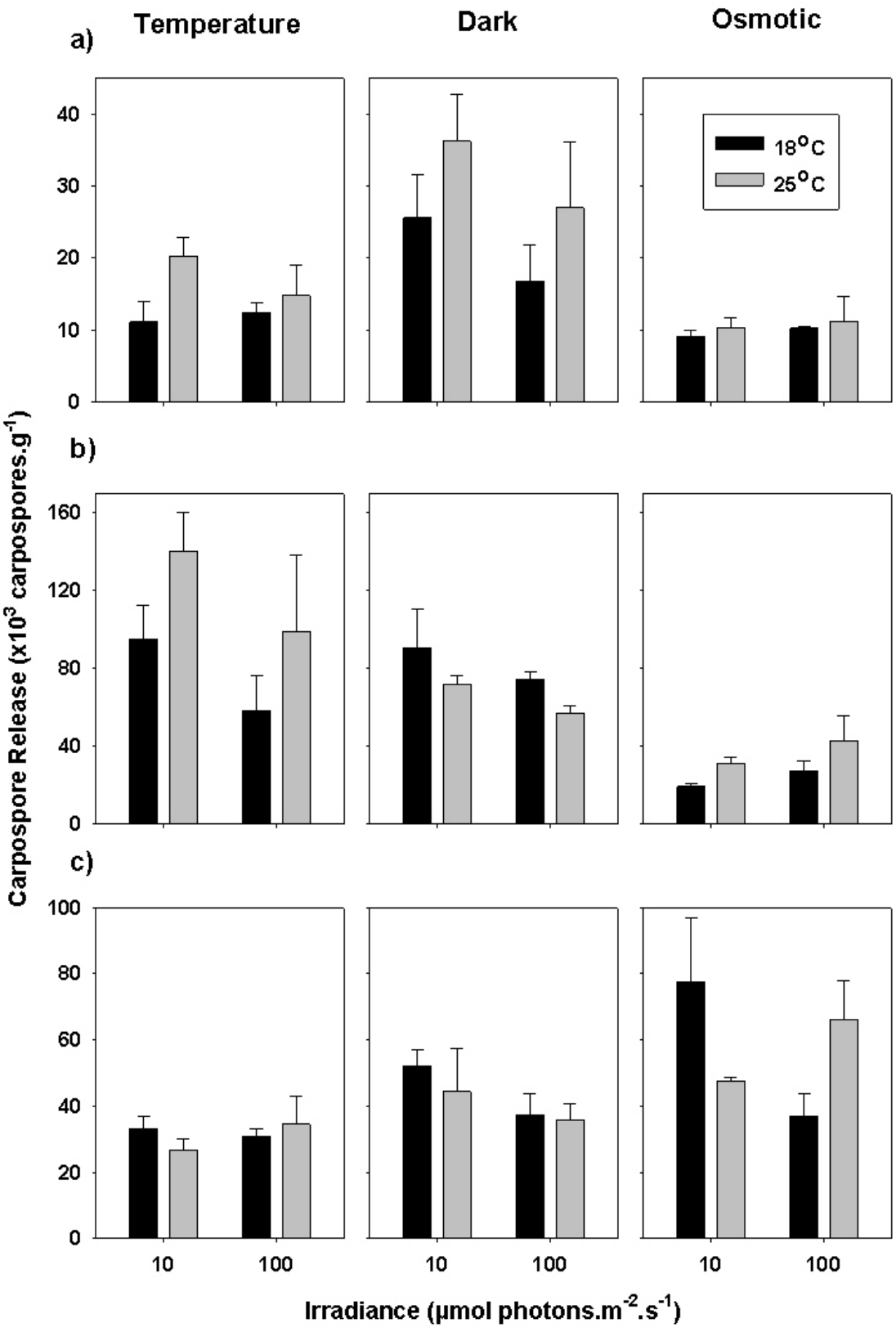

Figure 5: Carpospore release (x10^3 carpospores.g^{-1} alga) from three red macroalgal species; *Gracilaria chilensis* (a), *Hypnea* sp. (b) and *Plocamium mertensii* (c) induced by three treatments; temperature, dark and osmotic pressure. The experiment was conducted at two culture temperatures of 18 and 25 ± 2°C and at two irradiances of 10 and 100 µmol photons.m^{-2}.s^{-1}. Mean ± std. error (n=3).

3.3.2 Red Macroalgal Protoplast Production

Initial Enzyme Experiment

A yield of 9.40 ± 1.03 x10^5 protoplasts was obtained from 1 g blotted wet weight of *G. flagelliformis* fragments digested using the enzyme mixture with the osmotic treatment base, deionised water (Table 7). This was significantly greater than the number of protoplasts isolated utilising the enzyme mixture dissolved in sterile seawater. The survival of protoplasts at 33 h obtained using the osmotic treatment base was not significantly greater than for the sterile seawater treatment base (Table 7).

Table 7: Protoplast yield (x10^5 protoplasts.g^{-1} alga) from *Gracilaria flagelliformis* and the survival (%) of protoplasts intact after 33 h for the two agarase enzyme treatment bases, sterile seawater and deionised water (osmotic). Mean ± std. error (n=3).

Enzyme Base	Yield (x10^5 protoplasts.g^{-1})	Survival (%)
Seawater	3.27 ± 0.44	76.7 ± 5.7
Deionised Water	9.40 ± 1.03	95.2 ± 10.6
df	1, 4	1, 4
F	29.996	2.340
p value	<0.05	0.201

Protoplast Isolation from 3 Red Macroalgal Species

Protoplasts such as that shown in Figure 6 were successfully produced from all three of the red macroalgae tested. *Gracilaria* yielded the greatest number of protoplasts at 28.83 ± 8.69 x10^5 protoplasts.g^{-1}, closely followed by *Hypnea* with 26.16 ± 18.84 x10^5 protoplasts.g^{-1} (Figure 7). *Laurencia* produced 5.52 ± 0.64 x10^5 protoplasts.g^{-1}, which was significantly less than that isolated from both *Gracilaria* ($F_{(df\ 1,4)}$=464.53, p<0.05) and *Hypnea* ($F_{(df\ 1,4)}$=107.40, p<0.05) thalli.

Once the re-suspended protoplasts were placed in culture, cell wall regeneration and division were visually monitored during the counts of intact protoplasts, with no

cell division recorded over the 142 h period. Protoplast survival for all three red macroalgal species decreased at each subculture time interval (Figure 7), with the protoplast survival curves all significantly, positively correlated (*G.* versus *H.* (R=0.937, p<0.05), *G.* versus *L.* (R=0.937, p<0.05) and *H.* versus *L.* (R=0.964, p<0.05)).

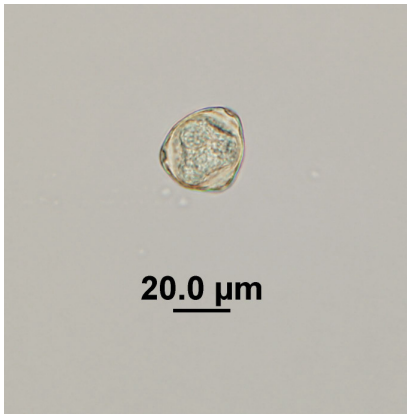

Figure 6: An example of the protoplasts produced from red macroalgal species (*Laurencia* sp.) using an agarase enzyme treatment. Scale bar = 20 µm.

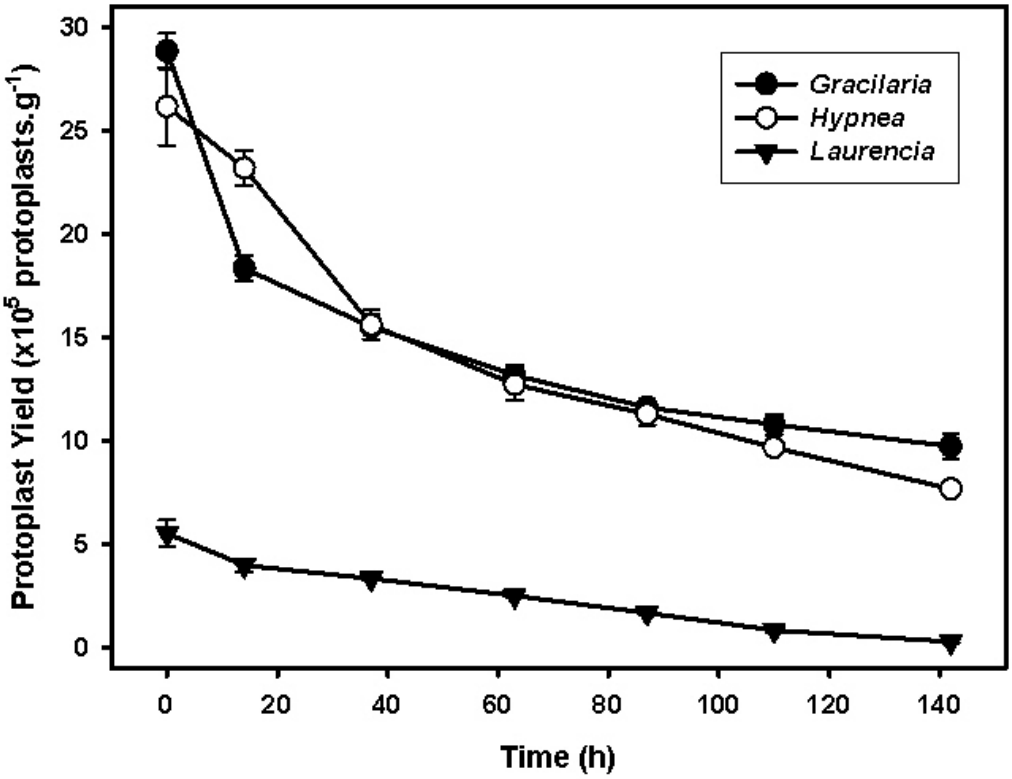

Figure 7: Protoplast yield (x10^5 protoplasts.g^{-1} alga) and survival in culture over 140 h for the three red macroalgal species, *Gracilaria* sp., *Hypnea* sp. and *Laurencia* sp. Mean ± std. error (n=3).

3.3.3 Vegetative Propagation of Red Macroalgae

Preliminary Vegetative Fragment Experiment

During the first week of culture the *Gracilaria* fragments lost biomass, whereas in the second week the biomass increased in all treatments (Table 8). New bud formation was also evident on the *Gracilaria* fragments as shown in Figure 8. The highest specific growth rate of *Gracilaria* fragments over the 2-week period was 4.42 %.day^{-1} in the 15 ± 2^{o}C, 75 µmol photons.m^{-2}.s^{-1} treatment (Table 8).

Vegetative Fragment Experiment

Fragment culture was successfully established and maintained over 11 weeks for both *Gracilaria* and *Hypnea*. The *Gracilaria* fragments exhibited a very slow first week of growth but then grew at 0.035 ± 0.003 µg.day^{-1} during the trial (Figure 9a). The *Hypnea* fragments had the highest growth rates during week 3 to 8 and averaged a growth rate of 0.041 ± 0.003 µg.day^{-1} over the 11-week culture period (Figure 9b). A few *Gracilaria* and *Hypnea* fragments were able to adhere to the bottom of the culture flasks throughout the trial.

The *Laurencia* fragments displayed substantial variation in growth with only 2 weeks of positive growth and an overall trial growth rate of -0.084 ± 0.051 µg.day^{-1} (Figure 9c). Growth rates of the macroalgal fragments over the 11-week culture period were significantly different ($F_{(df\ 2,33)}$=9.556, $p<0.05$), with the *Laurencia* fragments exhibiting a significantly slower growth rate than both the *Gracilaria* ($q=3.77$, $p<0.05$) and the *Hypnea* ($q=4.33$, $p<0.05$) fragments. The growth rate, or in the *Laurencia* fragments case the decay rate in the first week of culture was -0.446 ± 0.007 µg.day^{-1}, while an extremely low fragment survival of 12.5 % was recorded. The *Laurencia* fragment survival was also highly variable, reaching 75 % at week 6 and averaging 53.3 % over the culture period (Figure 9c). The survival rates of the macroalgal fragments over the 11-week culture period were significantly different ($F_{(df\ 2,33)}$=15.943, $p<0.05$), with the *Laurencia* fragments producing significantly lower survival than both

the *Gracilaria* ($q=3.5$, $p<0.05$) and the *Hypnea* ($q=5.5$, $p<0.05$), which both had close to 100 % fragment survival.

Table 8: The specific growth rate (%.day^{-1}) of *Gracilaria* fragments (5 – 7 mm) grown at two temperatures of 15 and 18 ± 2°C and at two irradiances of 75 and 350 µmol photons.m^{-2}.s^{-1} over a 2-week (wk) period.

Temperature	**Irradiance**	**Specific Growth Rate** (%.day^{-1})		
(°C)	(µmol photons.m^{-2}.s^{-1})	**Wk 1**	**Wk 2**	**Total**
15 ± 2	75	-4.42	19.2	4.42
	350	-6.56	14.41	0.06
18 ± 2	75	-4.09	8.18	1.44
	350	-7.48	6.59	-1.5

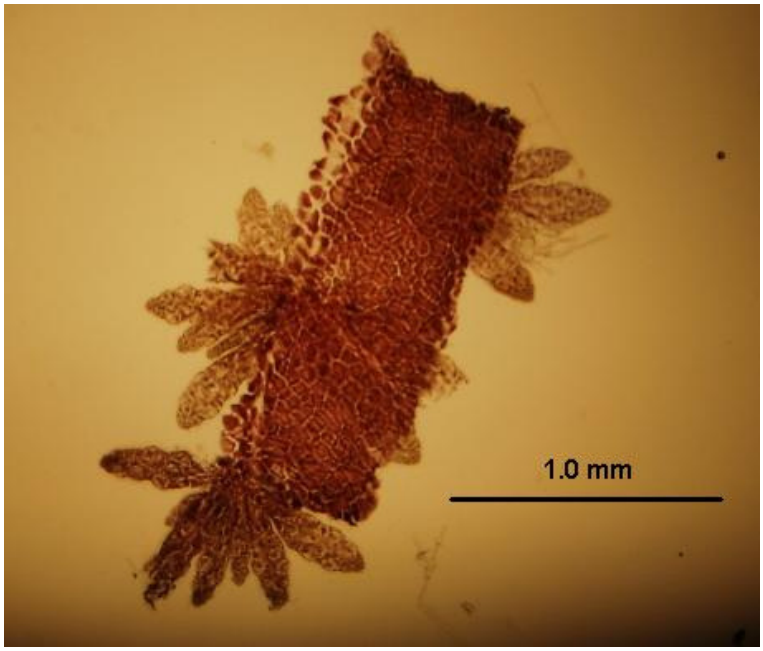

Figure 8: A fragment of the red macroalga *Gracilaria* sp. produced for vegetative propagation, sectioned to show new bud formation. Scale bar = 1.0 mm (Photo by Andreas Isdepsky).

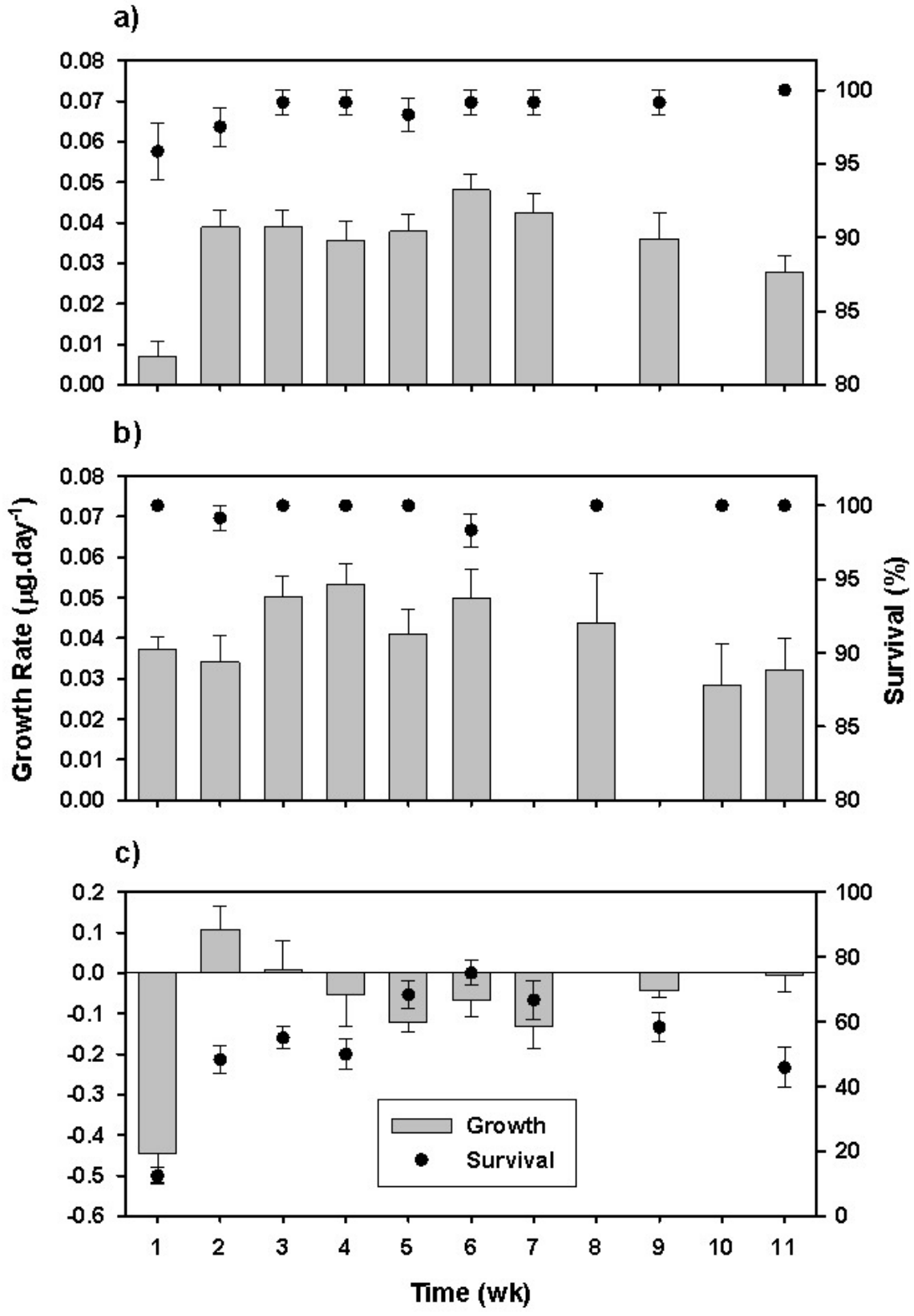

Figure 9: Growth rate (µg.day^{-1}) and survival (%) of thalli fragments from three red macroalgal species, *Gracilaria* sp. (a), *Hypnea* sp. (b) and *Laurencia* sp. (c) in culture over an 11-week (wk) period. Mean ± std. error (n=12).

3.4 DISCUSSION

3.4.1 Carpospores of Red Macroalgae

Only a small percentage of the fresh *P. mertensii* material (8.69 %) harvested for the preliminary spore experiment (Section 3.3.1) consisted of thalli with reproductive structures, indicating that the majority of thalli were vegetative or in the gametophyte phase (Destombe *et al.* 1989). Fertility of *Plocamium cartilagineum* has been shown to be less than 10 % at some times of the year, varying considerably with season, depth and plant size, while also being dominated by tetrasporophyte (Kain 1982, 1986). The *G. chilensis*, *Hypnea* and *P. mertensii* collection for the large carpospore production experiment (Section 3.2.1) targeted carposporophyte thalli and had variable amounts of cystocarp present. *Hypnea charoides* populations have shown a major dominance of vegetative and tetrasporophytic plants with very few carposporophytes (Kong & Ang 2004). Le Gall *et al.* (2004) found that of the total amount of *P. palmata* harvested from the field, only 10 % was represented by fertile pieces. The abundance of reproductive plants can be highly variable to the point where tetrasporophytes and/or carposporophytes are absent in the natural population at certain times of the year (Orduña-Rojas & Robledo 2002, Martín *et al.* 2011), therefore field collection of macroalgae for spore liberation experiments needs to be time and target specific.

The combined temperature and dark treatment used in the preliminary spore experiment yielded 4.75 $x10^3$ spores.g^{-1} from *P. mertensii*, however greater carpospore liberation was possible using the different induction treatments in the large carpospore production experiment (Section 3.3.1). Temperature and dark treatments applied separately yielded 10 fold the number of spores released, but over an extended period of time. Osmotic pressure induction produced the highest yield of carpospores for *P. mertensii* across both experiments. This was the opposite to *G. chilensis* and *Hypnea*, which liberated higher carpospore numbers using the temperature and dark treatments than the osmotic pressure induction treatment. *Hypnea* yielded the greatest number of carpospores of the three species when induced by temperature. Mshigeni (1976) also used a combination of temperature and irradiance to liberate spores from *Hypnea cervicornis* and *Hypnea chordacea*. Even though the Polysiphonia-type, triphasic life history of these red macroalgae allows the use of either carposporophytes or

tetrasporophytes to release spores for the production of sporelings, carpospores were used because of their robust vigour, growth rate and diploid nature of the derived tetrasporophyte sporelings (Polifrone *et al.* 2006, Mantri *et al.* 2009).

The lower numbers of carpospores released by *G. chilensis* in the osmotic pressure treatment compared to the dark treatment, may be due to a high resistance to osmotic changes (Alveal *et al.* 1997). Carpospore shedding can be induced by salinity changes, but culture temperature and irradiance have been shown to affect carpospore liberation in *Gracilaria cornea* and *G. pacifica* (Orduña-Rojas & Robledo 1999, Garza-Sánchez *et al.* 2000), while temperature and photoperiod, rather than the induction through osmotic shock and drying significantly affected *G. cornea* carpospore release (Guzmán-Urióstegui & Robledo 1999). Dark treatment induced the greatest carpospore liberation from *G. chilensis* in this study compared to the temperature and osmotic pressure treatments. Photoperiod has been shown to affect *G. corticata* carpospore release, with the greatest liberation occurring in periods of 20 to 24 h darkness, while *Gracilaria foliifera* carpospore release was optimal in dark or low light conditions (Umamaheswara Rao 1976, Friedlander & Dawes 1984).

Gracilaria chilensis and *Hypnea* thalli remained intact throughout the experiment and were placed directly back into culture, whereas the *P. mertensii* thalli degraded during culture and this may have impacted on the release of carpospores. The degradation of *P. mertensii* in culture made it difficult to use this species in the protoplast production (Section 3.2.2) and fragment propagation (Section 3.2.3) experiments. It also has implications for repetitive carpospore releases as the other two macroalgal species could be placed back in culture and then utilised to induce the release of carpospores at a later point in time. Carpospore liberation can occur over several days after induction, with some *Gracilaria* species having a 3 to 4 d delay until maximum release (Guzmán-Urióstegui & Robledo 1999, Garza-Sánchez *et al.* 2000, Mantri *et al.* 2009). The delay in spore release and the ability to utilise cultured macroalgae would allow carpospore release to be induced over multiple days, at various times throughout the year without the need for wild, fertile macroalgal thalli harvest.

The variation in spore liberation between the three species and the three induction treatments create issues when large amounts of spore biomass are required to seed the vertical PVC plates in the abalone nursery system. Spore densities of 200 spore.cm^{-2} are deemed appropriate to reduce over crowding and competition for light, while also supplying appropriate densities of macroalgal sporelings within an abalone hatchery (Alveal *et al.* 1997, Le Gall *et al.* 2004). Based on the result that 3 kg of fresh alga (*P. palmata*) seeded 1 m^2 in the Le Gall *et al.* (2004) study, 21.6 kg of alga biomass would be required to seed one of the nursery tanks used in the abalone feeding trial (Section 2.4.1), let alone the tens of tonnes to seed a commercial nursery system. Utilising the mean number of carpospores isolated from *Hypnea* (67.23 ± 10.19 x10^3 carpospores.g^{-1}) and with a 35 % germination rate, only 1.84 kg of carposporophyte material would be required to seed sporelings at 200 sporeling.cm^{-2} in an abalone feeding trial nursery tank.

Acquiring a regular, large supply of fertile algal material may be difficult given the high percentage (91.4 %) of vegetative *P. mertensii* harvested for the preliminary spore experiment and the targeted collection for the large carpospore production experiment (Section 3.3.1). To overcome this, cultures could be developed and maintained to produce the required fertile biomass. However, the lower overall carpospore liberation from *G. chilensis* across the three treatments could have been due to the macroalga developing cystocarp in culture rather than in the wild as with the *P. mertensii* and *Hypnea* collected. If this were the case, then there would be ramifications for utilising cultured algae for carpospore production. However, complete life histories of red macroalgae such as *Gracilaria* species have been cultured under laboratory conditions, indicating cultured algae do have potential for carpospore production (Guimarães *et al.* 1999, Avila *et al.* 2011).

If enough fertile algal material can be attained and carpospores liberated, the vertical PVC plates used in the nursery system could affect spore settlement rates. Red algal propagules are non-motile and the majority of germination experiments are conducted on horizontal surfaces (Fletcher & Callow 1992, Buschmann *et al.* 1999,

Orduña-Rojas & Robledo 1999), which would most likely produce higher settlement rates compared to settlement on the vertical PVC plates. To overcome this settlement problem, gentle aeration of the water could maintain the spores in the water column for a sufficient period of time to allow attachment to the PVC plates. Even with a lower settlement rate, the ability of carpospores to settle and attach to the PVC plates unassisted in the nursery system would allow red macroalgae to be provided to juvenile abalone. This process would require very few consumables, while equipment for spore cultivation is relatively simple (Alveal *et al.* 1997), making it a cheap and easy method for commercial abalone farms to seed red macroalgae onto the PVC plates in the nursery system.

The spore liberation from *Hypnea* would be sufficient to produce a sporeling diet, and to guarantee this level of release multiple induction treatments could be performed over an extended period of time. Overall, with the required amount of suitable fertile red macroalga, appropriate induction treatments and culture conditions, carpospores can be used to create an alternative diet for juvenile abalone in the later nursery phase.

3.4.2 Red Macroalgal Protoplast Production

Protoplast production as a propagation method can be used on a variety of Rhodophyta species as indicated by the isolation of protoplasts from all four macroalgae used in this study (Section 3.3.2). However, *G. flagelliformis*, *Gracilaria*, *Hypnea* and *Laurencia* yielded protoplasts with varying degrees of success. For example, immediately after isolation the number of protoplasts obtained ranged from 5.52 $x10^5$ protoplasts.g^{-1} for *Laurencia* up to 2.88 $x10^6$ protoplasts.g^{-1} for *Gracilaria*. The protoplast yields from the two *Gracilaria* species tested are comparable to that of other *Gracilaria* species studied; including *G. asiatica*, which has produced 4 – 8 $x10^5$ protoplasts.g^{-1} (Yan & Wang 1993), while *G. lemanaeformis* and *G. tikvahiae* yielded 3 – 10 $x10^5$ protoplasts.g^{-1} (Cheney *et al.* 1986), and *G. lemanaeformis*, *G. sordida*, *G. tenuistipitata* and *G. verrucosa* yielded from 10^5 to 10^7 protoplasts.g^{-1} (Björk *et al.* 1990, Mollet *et al.* 1995). The range in protoplast

yields for a variety of different *Gracilaria* species, across numerous studies all using slightly different enzyme mixtures to digest the thalli has been summarised in Gupta *et al.* (2011). Some macroalgal species from other red algal Families have been shown to produce slightly higher numbers of viable protoplasts, with *Bangia atropurpurea* yielding 2.85 $x10^7$ protoplasts.g^{-1} (Araki *et al.* 1994), *P. palmata* 4 – 6 $x10^7$ protoplasts.g^{-1} (Liu *et al.* 1992) and *Grateloupia sparsa* and *Grateloupia turuturu* yielding up to 1 and 1.5 $x10^7$ protoplasts.g^{-1}, respectively (Chen & Chiang 1994, Lafontaine *et al.* 2011). Morphologically simpler algae can produce a very wide range in the numbers of protoplasts isolated, starting from similar values to that recorded for the four red macroalgae species used in this study. For example, protoplast yields from species of the green alga *Monostroma* can range from 10 – 100 $x10^6$ protoplasts.g^{-1}, while *Enteromorpha* species have produced 4 – 95 $x10^6$ protoplasts.g^{-1} and *Ulva* species 4.5 – 88 $x10^6$ protoplasts.g^{-1} (Rusig & Cosson 2001, Uppalapati & Fujita 2002, Reddy *et al.* 2006). Other research on *Ulva* species has reported 3.23 $x10^7$ protoplasts.g^{-1} for *U. fasciata* (Chen & Shih 2000), up to 10 $x10^6$ protoplasts.g^{-1} for *U. conglobata*, 6 $x10^6$ protoplasts.g^{-1} for *U. pertusa* (Reddy *et al.* 1989) and more than $x10^7$ protoplasts.g^{-1} for *U. rigida* (Björk *et al.* 1992).

In the Initial Enzyme Experiment (Section 3.2.2) dissolving all the enzymes in deionised water as an osmotic treatment enhanced protoplast isolation, producing significantly greater numbers of protoplasts than using sterile seawater as an enzyme base. The two enzyme solution bases, sterile seawater and deionised water, were tested to determine if individually they could increase protoplast yields compared with the commonly used enzyme base of 60 % seawater, 40 % deionised water (Cheney *et al.* 1986, Björk *et al.* 1990, Yan & Wang 1993, Yeong *et al.* 2008). Due to the increase in protoplast yield, the osmotic treatment base was used in the Protoplast Isolation from 3 Red Macroalgal Species experiment (Section 3.2.2).

Despite all four macroalgae tested producing comparable numbers of protoplasts to the literature, no protoplast division was evident in any of the cultures. Even though deionised water as the enzyme base produced a greater number of protoplasts, this

treatment could have affected cell division and plantlet development. To overcome this, either more work needs to be done to determine the optimum level of osmotic stabiliser, as it has been found that protoplasts are unable to be isolated without the addition of an osmotic stabilizer (Araki *et al.* 1998), or by simply reverting back to the common enzyme base of seawater and deionised water in a ratio of 60:40.

Protoplast production as a propagation method only requires small amounts of red macroalgal biomass to produce plantlets, as 2.88 ± 0.87 $x10^6$ protoplasts were produced from 1 g of *Gracilaria*. This removes the limitations associated with collection of reproductive plants for spore production, as fertile thalli were not required to produce protoplasts. Substituting the density of 200 sporelings.cm^{-2} (Alveal *et al.* 1997, Le Gall *et al.* 2004) identified in Section 3.1.1 for protoplasts, the amount of red macroalgal material required for protoplast isolation to seed one of the abalone feeding trial nursery tanks (Section 2.4.1) can be determined. Given the number of protoplasts isolated from 1 g of *Gracilaria* and a hypothetical, low regeneration rate of 1 % to take into account the high variability of plantlet development, only 1.5 kg of fresh material would be required. This amount of red macroalgal thalli was less than the 1.84 kg of carposporophyte material (Section 3.4.1) needed to seed an abalone feeding trial nursery tank with plantlets at the same density, through spore liberation.

Given the lack of protoplast cell division and subsequent formation of whole plantlets, protoplast production appears significantly limited for use in culturing a diet for juvenile abalone; specifically when compared to carpospore production, as carpospores can readily attach and form sporelings on substrata. For protoplast isolation to be useful in abalone aquaculture at least a small percentage of protoplasts need to exhibit cell division, form into callus-like cell masses and subsequently produce whole plantlets. Even though plantlet formation has been shown to occur in other studies, it can be highly variable and in more complex macroalgae this process can take considerable time, with species such as *Gracilaria changii* and *G. asiatica* requiring up to 2 – 3 months to produce plantlets (Yan & Wang 1993, Yeong *et al.* 2008). This would require an extensive culturing procedure to be developed at an abalone farm with

protoplasts produced and cultured in separate systems to the abalone, until they are developed enough to self-attach to the PVC plates in the abalone nursery system. If the protoplasts cannot self-attach through callus formations and develop into plantlets, artificial adhesion protocols would need to be developed to aid the attachment process. Procedures such as immobilising the protoplasts in beads of calcium alginate to create a seed stock facilitating macroalgal culture (Rusig & Cosson 2001); or immobilising the plant material in alginate beads before being treated with the protoplast isolation enzyme, to form the protoplasts inside the alginate (Aoyagi & Tanaka 1999), could be adapted to artificially adhere the macroalgal protoplasts onto the PVC plates in the nursery system.

The propagation method of protoplast production remains very specialised and with the high costs of enzymes, it's both an extremely time consuming and costly procedure, even without having to incorporate additional artificial adhesion protocols. Given that specialised culture equipment, laboratory facilities and skilled technicians are required to produce protoplasts, it would be difficult to incorporate protoplasts into a macroalgal diet for juvenile abalone in the nursery system. Even though protoplast isolation can be extremely useful for creating singled celled cultures of macroalgae for laboratory studies on physiological, developmental, genetic improvement and metabolic engineering (Reddy *et al.* 2008a); the variability in the number of protoplasts isolated, inadequate regeneration rate into plantlets, time and costs associated, makes it commercially unviable to use protoplast propagation methods at an abalone farm. Therefore, protoplast production was not explored further to create an alternative diet for juvenile abalone in the later nursery phase.

3.4.3 Vegetative Propagation of Red Macroalgae

In the Preliminary Vegetative Fragment Experiment, *Gracilaria* was only able to produce positive growth over the 2 weeks in the 15 ± 2^{o}C, 75 µmol photons.m^{-2}.s^{-1} culture treatment and the 18 ± 2^{o}C, 75 µmol photons.m^{-2}.s^{-1} culture treatment (Section 3.3.3). This indicates an irradiance of 350 µmol photons.m^{-2}.s^{-1} was unsuitable for *Gracilaria* fragment culture. Given the culture treatment of 15 ± 2^{o}C and 75 µmol

photons.m^{-2}.s^{-1} produced the highest *Gracilaria* fragment growth rate of 4.42 %.day^{-1}, these conditions were used for the Vegetative Fragment Experiment (Section 3.2.3). These results compare with the indoor cultivation conditions of 10 – 14°C and an irradiance of 50 µmol photons.m^{-2}.s^{-1} recommended for *G. skottsbergii* fragments (Buschmann *et al.* 2004).

Gracilaria produced negative growth rates in all treatments during the first week of the Preliminary Vegetative Fragment Experiment, while slow growth rates were recorded during the first week of the Vegetative Fragment Experiment (Section 3.3.3). This was due to degradation of the excised section on the macroalga fragments edge while wound healing occurred. A similar decrease in biomass has also been recorded for *G. skottsbergii* fragments, where the exposed margin of the fragments degraded during the first 2 weeks, before specific growth rates of up to 1 %.day^{-1} were attained (Hernández-González *et al.* 2007). *Gelidium* sp. has also exhibited slower growth rates for 1 – 2 mm fragments during the first 2 weeks of culture compared to the following 4 weeks, indicating a period of wound healing (Titlyanov & Titlyanova 2006). *Hypnea* however, recovered quickly from being cut into fragments in the Vegetative Fragment Experiment (Section 3.3.3), as the first week of growth was not substantially lower than other weekly growth rates.

Gracilaria and *Hypnea* fragments were both able to maintain growth rates of over 0.035 µg.day^{-1} throughout the 11-week culture period. Comparable growth rates for *G. chilensis* fragments can theoretically be achieved when the specific growth versus fragment length curve presented by Santelices & Varela (1995) was extrapolated to include an initial fragment size of 5 – 7 mm. The ability of the *Gracilaria* and *Hypnea* fragments to produce viable cultures was further highlighted by the high fragment survival (>98.5 %) throughout the experiment.

Laurencia had variable survival, ranging from a maximum of 75 % during the culture period to 12.5 % in the first week. The extremely low survival in the first week

indicated *Laurencia* had limited ability to heal the wound created by fragmentation, while the increase in survival to 40 – 75 % for the remainder of the culture period showed the extended time frame wound healing can take. This has also been shown in *Laurencia* sp. explants (fragments 10 mm) that developed callus formations in the wounds and then buds after 30 d (Robaina *et al.* 1990). The constant mortality of *Laurencia* fragments had a detrimental effect on growth rates and resulted in the high variability and multiple, negative weekly growth rates.

One potential cause of the *Laurencia* fragment mortality could be secondary metabolites, which are natural compounds produced by marine algae that are not necessary for the survival of cells themselves but responsible for a wide range of properties (e.g. Cabrita *et al.* 2010). Rhodophyta species produce suites of halogenated secondary metabolites with *Laurencia* particularly well know for their production (e.g. Fenical 1975, Erickson 1983). In fact, specific *Laurencia* species have been reported to produce such a diversity of secondary metabolites that it causes geographic variations, where morphologically similar but chemically distinct populations can be found (e.g. Fenical 1976, Masuda *et al.* 1997). This diversity also extends to potentially toxic, bioactive secondary metabolites that accumulate in refractile inclusions and when the fragments are cut, causing the cells to rupture, the toxic secondary metabolites are released into the culture medium. This process could have implications for the growth and survival of fragments within media and subsequently explain the performance of the *Laurencia* fragments in culture.

The culture temperature of 15 ± 2^{o}C used in this vegetative fragment experiment (Section 3.2.3) could be another possible reason for the limited growth rates of fragments cut from *Laurencia* thalli. Various *Laurencia* species have shown growth and bud regeneration from small fragments (<10 mm) in culture at temperatures ranging from 10 to 32^{o}C. However, these studies all produced very limited *Laurencia* fragment growth at culture temperatures (10 to 16^{o}C) similar to that of the 15^{o}C used in this experiment, while maximum fragment growth rates where achieved at much higher temperatures of between 25 and 30^{o}C (Kuwano *et al.* 1998, Nishihara *et al.* 2004, Shen

et al. 2010, Sudatti *et al.* 2011). This indicates that higher culture temperatures should be utilised to optimise fragment culture of *Laurencia*, however, this may not be possible when the fragments are incorporated as a diet for the temperate water species (<18^{o}C) of greenlip abalone.

Fragments of both *Hypnea* and *Gracilaria* were able to attach to the bottom of the culture flask through the production of new cells at the cut edge during the wound healing process. This natural attachment process has considerable benefits, as the ability for self-adhesion to a substratum allows the red macroalgal fragments to attach directly to the vertical PVC plates in the nursery system, creating a simple low maintenance diet for juvenile abalone. However, the natural attachment only occurred in a few fragments throughout the 11-week culture period. Given it can take at least 2 – 3 d to produce cells at the cut margins, let alone 2 to 3 weeks to complete wound healing and show signs of growth (Titlyanov & Titlyanova 2006, Hurtado & Biter 2007), this would be an extremely inefficient means of adhering fragments to the vertical plates. Even with this natural attachment to substrata in culture, the process needs to be artificially accelerated so that all of the fragments used to seed the vertical PVC plates in the abalone nursery system are able to attach and grow. Otherwise, substantial biomass of the algal fragments will not attach to the plates and the percentage that does, would take an extremely long time to produce a patchy algal distribution over the plates. Therefore, appropriate adherence protocols need to be developed to enable any red macroalgal fragments to be utilised as a juvenile abalone diet in the nursery system.

Given fragments can be excised from any part of any phenotype it removes the problems associated with Rhodophyta triphasic life history and allows cultured algal biomass to be utilised. An important benefit of fragment regeneration and growth was that macroalgal biomass can be easily cultured and this would reduce the reliance on collection from wild populations. It could also allow the biochemical composition of the algae to be manipulated by nutrient enrichment in culture, potentially providing juvenile abalone with a consistent, nutritionally superior red macroalgal diet. Abalone farms within Asia, South America and South Africa, that have access to large, reliable

quantities of algae (Zemke-White & Ohno 1999, Lüning & Pang 2003) could either culture on site or use wild harvest material to culture fragments. In Australia the use of integrated polyculture systems within the abalone farm would allow sufficient nutrient enriched, appropriate red macroalgal species to be cultured on site, subsequently reducing reliance on wild harvest while also helping to treat effluent discharge (Neori *et al.* 2004).

Based on the survival and growth rates obtained in the Vegetative Fragment Experiment (Section 3.3.3), *Hypnea* and *Gracilaria* were both propagated successfully by vegetative fragmentation and are considered suitable for utilisation in juvenile abalone nutrition. By developing an appropriate artificial adhesion protocol that can facilitate the attachment of algae to the PVC plates in the nursery system, vegetative fragments of adult red macroalgal thalli could be used as an alternative diet for juvenile abalone in the later nursery phase.

CHAPTER 4
MACROALGAL FRAGMENTS AS A DIET FOR JUVENILE GREENLIP ABALONE

4.1 MACROALGAL FRAGMENT ARTIFICIAL ADHESION PROTOCOLS

4.1.1 Introduction

Of the three algal propagation methods examined, carpospore liberation and vegetative propagation were identified as suitable methods for incorporating red macroalgae into a diet for juvenile greenlip abalone in the nursery system (Section 3.4). However unlike carpospore liberation, which allows a large, consistent biomass of macroalgae to naturally attach to a substratum, vegetative propagation through fragmentation of macroalgal thalli requires some form of artificial adhesion to attach the fragments onto the vertical PVC plates used in the commercial abalone nursery system.

Immobilisation techniques used for microorganisms are artificial fixing/adhesion processes that could be adapted to attach the small, red macroalgal fragments onto the PVC plates. Active immobilisation includes the use of flocculent agents, chemical attachment or gel entrapment; with the latter the most extensively used technique for algal immobilisation (Moreno-Garrido 2008). Natural polysaccharides are the most appropriate gel entrapment, algal immobilisation technique, when compared to synthetic polymers and proteins, given the compounds are derived from algae and this artificial adhesion process was being adapted to produce a diet consumable by juvenile abalone. Of the three natural polysaccharides, two (agar and carrageenan) are obtained from Rhodophyta species, while the third (alginate) can be extracted from Phaeophyceae species (Moreno-Garrido 2008).

Two of these algae-derived polysaccharides, alginate and agar are common gelling agents used to bind dietary nutrients in artificial feeds for abalone consumption. Combinations of these two binders can be used, while a mixture of agar and gelatine has

been shown to provide the best water stability of seven different binders over a 24 h period (Knauer *et al.* 1993). Concentrations of the two binders (alginate and agar) can affect the dietary intake of nutrients by abalone from artificial diets, and when used as the primary binder they form an expensive component of the aquaculture feed, which can be prohibitive to low cost feed production (Fleming *et al.* 1996, O'Mahoney *et al.* 2011). Both of these gelling agents have also been used to adhere artificial diets onto vertical plates, to try and improve settlement and growth rates of postlarval abalone (Stott *et al.* 2002, 2004b). These artificial adhesion protocols can therefore, be adapted to seed fragments of cultured red macroalgae onto the PVC plates in the abalone nursery system.

Substantial work has already been done on the immobilisation of living cells, with the calcium alginate gel matrix one of the most widely used techniques (Smidsrød & Skjåk-Braek 1990). Generally the immobilisation process consists of mixing the plant material with sodium alginate and then calcium chloride to form beads of an insoluble calcium alginate gel matrix. This method has been successfully used to immobilise a range of microalgae, which can then be cultured for extended periods in the alginate matrix (Hertzberg & Jensen 1989, Faafeng *et al.* 1994, Romo & Pérez-Martínez 1997). Given the algal cells retain their respiratory and photosynthetic activities after immobilisation, the long-term storage of algal cells can greatly improve stock culture management (Romo & Pérez-Martínez 1997, Chen 2001). This has significant applications for the aquaculture industry as the immobilisation technique could allow facilities to easily maintain large-volume algal cultures for extended periods of time, before inoculating tanks with the immobilised algal beads that slowly leak/release the algal material to supply food for the farmed specie. This methodology has already been trialled for use in oyster and clam aquaculture (Lebeau *et al.* 1998, Chen 2003), and could aid in the culture and presentation of the red macroalgal fragments as a juvenile abalone diet on the PVC plates in the nursery system.

To create the alginate beads that immobilise fragments of red macroalgae, the fragments are gently mixed with a sodium alginate solution (2 – 3 % w/v) at room

temperature and then the alginate/algae mixture gelled by contacting with the calcium chloride solution (1 % w/v). The gelled alginate would then be left for 30 min to 1 h so that solidification can occur and the immobilised macroalgal fragments then transferred into culture medium and monitored to detect regeneration. However, to adhere the fragments of red macroalgae onto the PVC plates in the nursery system, the gelling methodology has to be altered to incorporate the plates when the alginate/algae mixture and the calcium chloride solution bond together. This would allow the insoluble calcium alginate gel matrix with the fragments suspended inside, to cover the surface of the plates and remained attached (Stott *et al.* 2002, 2003a, 2003b). Various physical methods could achieve this before the alginate was exposed to the calcium chloride solution; including painting and dipping the sodium alginate/algae mixture onto the plates or via mechanical means such as Chromatography plate spreaders. The concentration of sodium alginate and calcium chloride, and their effects on wound healing of the fragments cut edges are an important component of the adherence method. Particularly, given that when protoplasts are immobilised in alginate the prevention of cell wall regeneration can be important for long term processes (Tanaka *et al.* 1996, Aoyagi *et al.* 1998). Fragment regeneration in the alginate matrix would provide a substantial biomass of red macroalgal material on the plates in the abalone nursery system.

The second alternative adherence method would be to suspend the fragments of cultured macroalgae within the gelling agent, agar. Agar a sulphated galactan, without a protein source can be consumed by juvenile abalone, however it leads to reduced growth and survival compared with other standard artificial diets (Ismail *et al.* 2009). The agar containing the suspended macroalgal fragments can be adhered to the PVC plates by either, dipping the plates in the agar/fragment mixture or by spraying the agar/fragment mixture onto the plates. A method of spraying an agar/artificial diet mixture, utilising an agar concentration of approximately 1 %, could be adapted to suit fragments of cultured macroalgae (Stott *et al.* 2004a, 2004b, 2004c, 2004d). This spray method has been used to test the effect of various refinements in artificial diets on the development and nutrition of postlarval abalone (Chao *et al.* 2010).

The adhesion of macroalgal fragments to an artificial substratum was examined by utilising the two algae-derived polysaccharide gel entrapment agents, calcium alginate gel matrix and agar. Protocols were then developed to incorporate the preferred gelling agent into a commercial application method to create a diet for juvenile greenlip abalone, by adhering fragments of cultured red macroalgae onto the PVC plates currently used in the nursery system.

4.1.2 Material and Methods

Comparison of Two Gelling Agents Adhesion

Algal Material

The unicellular red alga, *Porhyridium purpureum* was used as a test algal substitute with both the alginate and agar gelling agents to determine which agent was most suitable for adhering algal biomass onto the PVC plates used in the abalone nursery system. *Porhyridium purpureum* was cultured in Erlenmeyer flasks with 1 L of f/2 medium (Section 2.2, DFSW) at 18 ± 2°C on a 12 h:12 h, light:dark cycle at 35 µmol photons.m^{-2}.s^{-1}, then harvested and concentrated by filtration to 9.1 x 10^5 cell.mL^{-1}.

Alginate Gelling Agent

Intermediate sized PVC plates (20 x 15 cm) were immersed in 1 % (w/v, DFSW) calcium chloride solution for 30 min. Two hundred and fifty millilitre of *P. purpureum* cell suspension was gently mixed with a sodium alginate solution (250 mL, 6 % w/v, DFSW), once it had cooled to room temperature (25 – 27°C). The alginate/algae mixture with a sodium alginate concentration of 3 % was then sprayed using a Canyon hand held sprayer onto the PVC plates pre-soaked in the $CaCl_2$ solution. The plates with the adhering alginate/algae mixture were then left to dry for 30 min and the procedure repeated on the same plates, giving them 2 coats of the mixture.

Agar Gelling Agent

An agar/algae mixture was created by combining 250 mL of 2 % agar solution (DFSW) with 250 mL of the *P. purpureum* cell suspension, producing a mixture with 1 % agar concentration. The agar solution was first allowed to cool to room temperature (25 – 27^{o}C) and once the algal culture had been added to the agar the resultant mixture was sprayed using a Canyon hand held sprayer onto the intermediate PVC plates (20 x 15 cm). The plates with the agar/algae mixture adhering were then allowed to harden for 30 min and the procedure repeated on the same plates, giving them 2 coats of the mixture.

Gelling Agent/Algae Mixture Culture

The PVC plates seeded with the alginate/algae mixture and the agar/algae mixture were placed vertically in a 50 L aquarium with f/2 medium (Section 2.2, CCFSW) and no aeration, at 18 ± 2^{o}C on a 12 h:12 h, light:dark cycle at 35 µmol photons.m^{-2}.s^{-1}. The two sets of plates were monitored for 6 d to determine the rate of gelling agent/algae deterioration, as measured by the percentage cover of agent and the density of *P. purpureum* (cells.cm^{-2}) adhered to the plate, both counted within a microscope field of view (40x magnification). Directly after application the two gelling agent percentage covers and algal densities were both compared by an Independent t-test; paired two samples for means, while a Repeated Measures Analysis of Variance examined the reduction in gelling agent cover over time.

Development of an Agar Adhesion Protocol

Application by Dipping

Gracilaria flagelliformis thalli were harvested from the Leeuwin collection site (Section 2.1) and used as a test algal substitute in developing the agar artificial application protocols for adhering macroalgal fragments onto the PVC plates. Adult thalli of *G. flagelliformis* were blended in a commercial food processor to produce fragments of less than 1 mm in size. The amount of agar powder (Becton Dickinson, U.S.A.) dissolved in 1 µm filtered seawater (ICF) was dictated by the volume ratio of

agar to algae fragments (i.e. 1:1 or 2:1). One litre of macroalgal fragments was prepared from 800 g blotted wet weight *G. flagelliformis* and added to 1 L of 2 % agar solution at 38^{o}C, giving the combined mixture a 1 % agar concentration. A dipping method was utilised as the Canyon hand held sprayer's nozzle was too fine to accommodate the *G. flagelliformis* fragments. Therefore, the resultant agar/*G. flagelliformis* fragment mixture was placed in a tray and the intermediate PVC plates (20 x 15 cm) hand dipped into the mixture, then the mixture allowed to set on the plates for 2 h.

Ten intermediate plates with the agar/*G. flagelliformis* fragment mixture adhered, were stood vertically inside a glass aquarium (50 L) with flow through seawater (2 L.min^{-1}, ICF) being aerated by two airlines at the bottom. Measuring both the density of algal fragments and the percentage cover of agar after a week assessed the mixtures stability. The presence and absence of juvenile abalone (50 *Haliotis laevigata* of ≈16 mm shell length per aquarium) were examined as to the effect of abalone on the removal of the agar/*G. flagelliformis* mixture, in 12 replicate aquariums as described above. A Pearson Product Moment Correlation was conducted to compare the rate of the agar/*G. flagelliformis* mixture removal from the plates between treatments.

Application by Spraying

For the spray application a 3.5 mm gravity fed, pot spray gun powered by compressed air was used, as it had a larger nozzle size and a faster application rate than the Canyon hand held sprayer. A 400 mL volume of 1.5 % agar solution at 35^{o}C had 200 mL of *G. flagelliformis* fragments added (2:1 ratio agar:algae), to give an agar/algae mixture with a 1 % agar concentration. The agar/*G. flagelliformis* mixture was then applied to the intermediate PVC plates (20 x 15 cm) using the 3.5 mm pot spray gun.

Given the results of spraying the agar/*G. flagelliformis* mixture with a 1 % agar concentration (Section 4.1.3), different agar concentrations were trialled. Two concentrations of pure agar solution (0.5 % and 0.75 %) at 35^{o}C were sprayed onto intermediate PVC plates with the 3.5 mm pot spray gun. Agar and *G. flagelliformis*

fragments were mixed together to produce two separate agar/*G. flagelliformis* mixtures with 0.5 % and 0.75 % agar concentrations (2:1 ratio agar:algae), then both sprayed onto intermediate PVC plates. An abalone feeding trial using the aquarium system described above (Section 4.1.2) was set up to test the effectiveness of the spraying application in adhering the agar/*G. flagelliformis* fragment mixture to the PVC plates. An agar/*G. flagelliformis* mixture at the most effective agar concentration (0.75 % with a 2:1 ratio at 35^{o}C, Section 4.1.3) was prepared and sprayed using the 3.5 mm pot spray gun onto the intermediate PVC plates.

Ambient temperature affected the spraying procedure over time; so three concentrations of pure agar solution (0.5 %, 0.75 % and 1 %) were examined at two air temperatures. Spraying occurred at an ambient air temperature of 23^{o}C and an increased air temperature of 28^{o}C. Placing the air compressor within a controlled temperature room allowed the air temperature to be increased. Agar solutions and *G. flagelliformis* fragments were combined at a 2:1 ratio, producing agar/*G. flagelliformis* mixtures with agar concentrations of 0.5 %, 0.75 % and 1 %. These three mixtures were then sprayed at the ambient (23^{o}C) and increased (28^{o}C) air temperatures onto the intermediate PVC plates.

A 5 mm undercoat (direct fed) spray gun was also tested to spray larger macroalgal fragments and reduce solidification of the agar at lower temperatures. Three mixtures of agar and *G. flagelliformis* fragments (approximately 2 – 3 mm) were produced at a 2:1 ratio, with the mixtures having agar concentrations of 1 %, 1.25 % and 1.5 %. The spraying was conducted at an air temperature of 24^{o}C with a compressed air temperature of 20^{o}C.

4.1.3 Results

Comparison of Two Gelling Agents Adhesion

The initial percentage cover of agar on the intermediate PVC plates was over 50 % greater than the initial percentage cover of alginate on the plates (Figure 10). The

agar percentage cover was significantly higher than the alginate cover ($t_{(df\ 19)}$=2.09, p<0.05) and in turn produced a significantly higher algal density adhered to the plates directly after application ($t_{(df\ 19)}$=2.09, p<0.05). The decrease in cover of the agar and alginate gelling agents over the 6 d trial without abalone present was approximately 15 % and 12 %, respectively. This decrease recorded in the cover of both gelling agents was positively correlated (R=0.902, p<0.05), with Figure 10 showing the mixtures deteriorated at a similar rate over time. Consequently, the percentage cover of agar was significantly higher than the percentage cover of alginate throughout the 6 d trial ($F_{(df\ 1,5)}$=3760, p<0.05). Given agar's greater adhesion properties, it was utilised as the preferred gelling agent to establish commercial application protocols to adhere fragments of red macroalgae to the PVC settlement plates in the abalone nursery system.

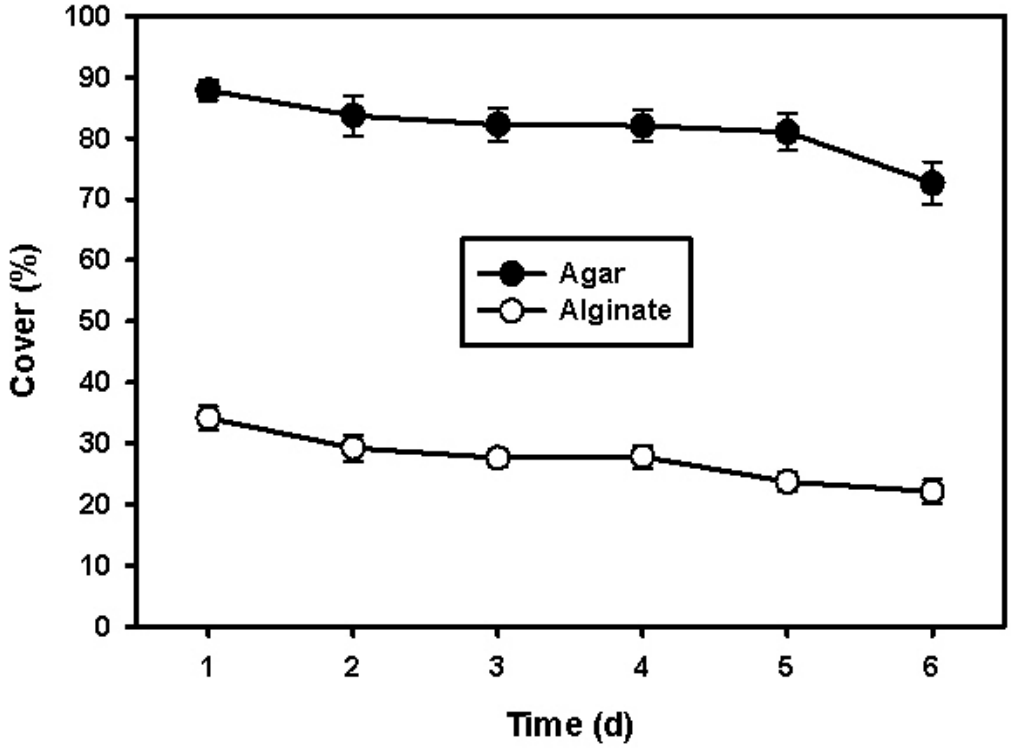

Figure 10: Removal rate of the alginate/algae mixture and the agar/algae mixture from the intermediate PVC plates (20 x 15 cm) during 6 d in culture, as determined by the gelling agent percentage cover (% cover) on the plates. Mean ± std. error (n=20).

Development of an Agar Adhesion Protocol

Application by Dipping

The agar/*G. flagelliformis* fragment mixture was easily applied to the intermediate PVC plates by manual dipping, however the mixture tended to clump and did not spread evenly across the plates. The uneven horizontal coverage also showed considerable variation in the thickness of the mixture and contributed to its rapid removal (Table 9), whereby large sections "peeled" off the plates (Figure 11). Grazing by abalone on the agar/*G. flagelliformis* mixture only marginally increased the rate of removal (Table 9), but importantly the mixture was still consumed and produced an abalone growth rate of 25.96 ± 4.15 $\mu m.day^{-1}$. There were significant, positive correlations between the treatments with abalone and without abalone (control) for the removal of both agar ($R=0.999$, $p<0.05$) and algae ($R=0.986$, $p<0.05$) from the plates. This may suggest that the presence of abalone had only a minor effect on the deterioration of the agar/*G. flagelliformis* mixture, which could also be implied by the removal of the *G. flagelliformis* fragments significant, positive correlation with the removal of agar regardless of the treatment (control ($R=0.982$, $p<0.05$) and abalone ($R=0.999$, $p<0.05$)).

Table 9: Density of *Gracilaria flagelliformis* fragments ($fragment.cm^{-2}$) and agar cover (% cover) on the intermediate PVC plates (20 x 15 cm), for both the control (without abalone) and abalone treatments during the 6 d juvenile *Haliotis laevigata* feeding trial. Mean ± std. error (n=6).

	Treatment	Start	Day 3	Day 6
Algae	Control	12.62 ± 0.45	6.84 ± 0.02	3.7 ± 0.07
($fragment.cm^{-2}$)	Abalone	18.19 ± 3.29	5.92 ± 3.42	2.92 ± 2.01
Agar	Control	47.86 ± 2.97	17.42 ± 0.36	10.97 ± 0.08
(% cover)	Abalone	40.22 ± 8.05	14.53 ± 7.47	7.72 ± 4.5

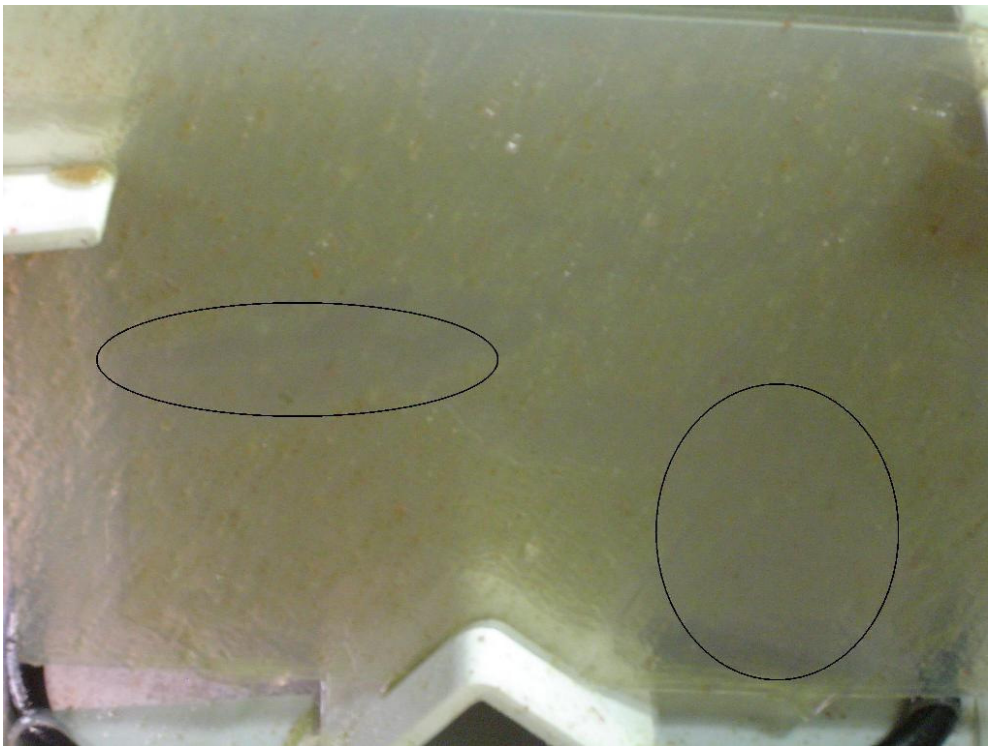

Figure 11: The agar/*Gracilaria flagelliformis* mixture adhered to the intermediate PVC plates (20 x 15 cm) by the dipping application. The circles identify areas of the plate where the mixture has "peeled off".

Application by Spraying

The agar concentration in the agar/*G. flagelliformis* mixture used in the dipping application (1 %) was maintained, but the ratio of agar to algae was altered to 2:1 in an attempt to improve adhesion of the mixture. This mixture solidified inside the chamber of the 3.5 mm pot spray gun and application was not possible (Table 10). Therefore, by reducing the mixtures agar concentration to 0.5 % and 0.75 %, it allowed the mixtures to be sprayed onto the plates (Table 10). As the mixture of agar and *G. flagelliformis* fragments (2:1 ratio) with an agar concentration of 0.75 % could be sprayed, it was to be used in an abalone feeding trial in the glass aquarium system. However, after spraying half of the 120 intermediate PVC plates the mixture began to solidify in the 3.5 mm pot spray gun as the ambient temperature decreased. Therefore, the abalone feeding trial was not conducted, as the agar/*G. flagelliformis* mixture could not be adhered to the required number of plates.

To overcome the mixture solidification in the 3.5 mm pot spray gun, three concentrations of pure agar solution (0.5, 0.75 and 1 %) were sprayed at two air temperatures (23 and 28°C), resulting in all three-agar solutions able to be sprayed (Table 11). Three agar/*G. flagelliformis* mixtures (2:1 ratio) with agar concentrations of 0.5 %, 0.75 % and 1 % were also sprayed, however the 0.75 % and 1 % solidified at the ambient (23°C) temperature (Table 11). As the concentration of agar within the agar/*G. flagelliformis* mixture increased, the air temperature coming out of the compressor only had to decrease to around 20 – 21°C to cause solidification.

Maintaining an air temperature of 28°C around the compressor was difficult, when using the 3.5 mm pot spray gun to spray mixtures at higher agar concentrations without solidification of the mixture occurring. Therefore, a 5 mm undercoat spray gun was tested as an alternative application device to reduce blockages while spraying. The larger diameter nozzle on the 5 mm undercoat spray gun also allowed larger macroalgal fragments to be sprayed, as evident when comparing the fragments in Figure 12 a and b. Three agar/*G. flagelliformis* fragment mixtures (2:1 ratio) with agar concentrations of 1 %, 1.25 % and 1.5 % were successfully sprayed using the 5 mm undercoat spray gun at 24°C ambient temperature (20°C air compressor temperature) (Table 12). However, the agar/*G. flagelliformis* mixture with a 1.5 % agar concentration did result in a few blockages that could be removed, but slowed the spraying process considerably.

Table 10: Agar concentration experiment, spraying 0.5 and 0.75 % pure agar solutions as well as agar/*Gracilaria flagelliformis* fragment mixtures (2:1 agar:algae) with agar concentrations of 0.5, 0.75 and 1%.

Mixture Agar (% agar conc.)	**Fragments** (+ / -)	**Ratio** agar:algae	**Agar** (%)	**Sprayed / Solidified**
0.5	-		0.5	Sprayed
0.5	+	2:1	0.75	Sprayed
0.75	-		0.75	Sprayed
0.75	+	2:1	1.25	Sprayed
1	+	2:1	1.5	Solidified

Table 11: Effects of temperature on pure agar solutions and mixtures of agar and *Gracilaria flagelliformis* fragments (2:1), both over 3 agar concentrations (% agar) sprayed at two different air temperatures (^{o}C).

Mixture Agar (% agar conc.)	**Fragments** (+ / -)	**Ratio** agar:algae	**Agar** (%)	**Ambient** (^{o}C)	**Compressor** (^{o}C)	**Sprayed / Solidified**
0.5	-		0.5	23	20.5	Sprayed
0.5	-		0.5	28	23.5	Sprayed
0.5	+	2:1	0.75	23	20.5	Sprayed
0.5	+	2:1	0.75	28	23.5	Sprayed
0.75	-		0.75	23	20.5	Sprayed
0.75	-		0.75	28	23.5	Sprayed
0.75	+	2:1	1.25	23	20.5	Solidified
0.75	+	2:1	1.25	28	23.5	Sprayed
1	-		1	23	20.5	Sprayed
1	-		1	28	23.5	Sprayed
1	+	2:1	1.5	23	20.5	Solidified
1	+	2:1	1.5	28	23.5	Sprayed

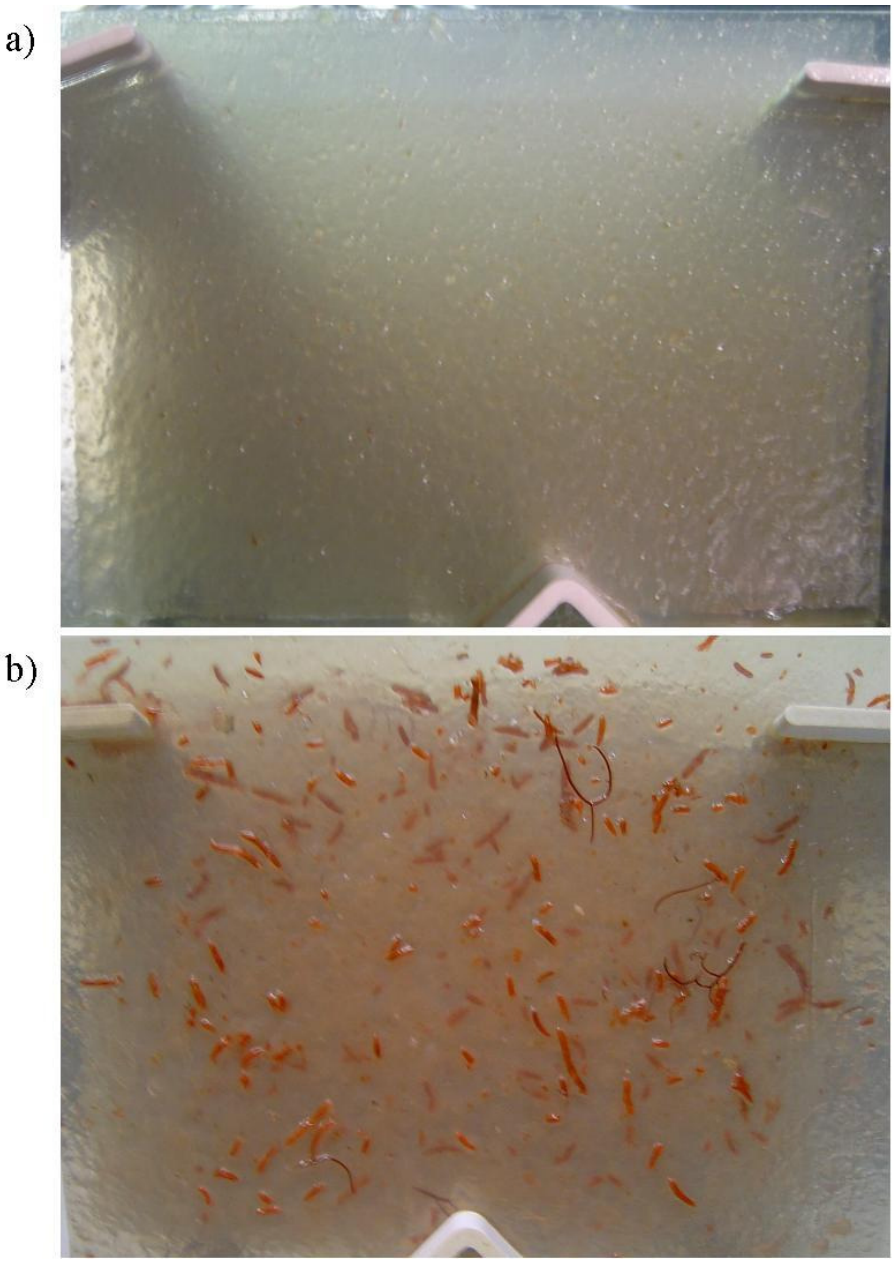

Figure 12: The agar/*Gracilaria flagelliformis* mixture adhered to the intermediate PVC plates (20 x 15 cm) by the spraying application, utilising the 3.5 mm pot spray gun (a) and the 5 mm undercoat spray gun (b).

Table 12: 5 mm undercoat spray gun applying an agar/*Gracilaria flagelliformis* fragment mixture (2:1 agar:algae) at three mixture agar concentrations of 1 %, 1.25 % and 1.5 %.

Mixture Agar (% agar conc.)	**Fragments** (+ / -)	**Ratio** agar:algae	**Agar** (%)	**Sprayed / Solidified**
1	+	2:1	1.5	Sprayed
1.25	+	2:1	1.875	Sprayed
1.5	+	2:1	2.25	Solidified

4.1.4 Discussion

Agar was shown to be the most appropriate gelling agent compared to a calcium alginate gel matrix for adhering a test algal culture to artificial substrata. There was over 50 % greater cover of agar than alginate on the intermediate PVC plates at application and this was maintained over a 6 d period. The greater agar cover meant that approximately 3 times the amount of algal biomass could be adhered to the plates during application. The agar/algae mixture was also considerably easier to apply to the PVC plates, as the application process only required one solution to be prepared and once this agar solution had cooled, the algal culture could be added and the resultant mixture applied directly to the plates. The original application process for the alginate/algae mixture was to combine the sodium alginate solution with the algal suspension then spray or brush this onto the plates. The plates were to then be dipped in the calcium chloride solution to form the insoluble calcium alginate gel matrix, which would adhere the algal culture to the plates (Stott *et al.* 2002, 2003a, 2003b). This method proved difficult as the sodium alginate and algal mixture (3 % alginate) "ran off" the plates and could not be exposed to the calcium chloride solution. To overcome this "run off" the method utilised in the Comparison of Two Gelling Agents Adhesion experiment (Section 4.1.2), of pre-soaking the PVC plates in the $CaCl_2$ solution before spraying the sodium alginate/algae mixture onto the plates, allowed the alginate/algae mixture to bond with the $CaCl_2$ solution, adhering the algae onto the plates. Stott *et al.* (2002) found that a 2 % sodium alginate/artificial diet mixture painted onto corrugated plates and dipped into a 5 % calcium chloride solution maintained adhesion for 2 d in seawater. The viscosity produced by the artificial diet combined with sodium alginate may have reduced the "run off" before the plates were dipped into the calcium chloride, when compared to the highly fluid sodium alginate/algae mixture used in this particular experiment. However, one of the main limitations for the use of calcium alginate matrixes has been the loss of stability in seawater (Moreno-Garrido 2008), which would contribute to the short time period it can adhere algae to the plates when placed in abalone culture. The ease of application combined with the higher gelling agent percentage cover and algal abundance, indicated the adherence of algal biomass to PVC plates was best achieved with agar as the gelling agent.

Suitable artificial adhesion protocols were developed using the agar gelling agent with live macroalgal fragments; given fragment culture was identified as an appropriate propagation method for incorporating red macroalgae into the abalone nursery system (Section 3.4.3). The application method of hand dipping the intermediate PVC plates into the agar/*G. flagelliformis* fragment mixture was very simple to use, while also easily scaled up to allow mass application of the mixture. Due to the uneven cover produced, the combination of aeration and water flow in the aquariums caused large sheets of the mixture to "peel off" the plates. Abalone grazing did not really affect the rate of removal, as the agar/*G. flagelliformis* mixture was removed too quickly for the abalone to consume the macroalgal fragments while attached to the plates. Importantly, the abalone did consume the macroalgal fragments and agar when it was on the bottom of the tank, producing a growth rate of 25.96 $\mu m.day^{-1}$. Research by Stott *et al.* (2003b) has demonstrated that a combination of artificial diet and alginate binder produced significantly larger postlarval abalone when the same amount was placed on the bottom of a culture vessel, compared to when the alginate binder attached the artificial diet to plates. Agar by itself can be consumed by *Haliotis midae* (4 – 6 mm shell length) when fed as a control diet for testing artificial diets and produced an abalone growth rate of 7 $\mu m.day^{-1}$, indicating agar was not detrimental but must have a protein source added to produce commercially viable abalone growth (Ismail *et al.* 2009).

To reduce the amount of agar/*G. flagelliformis* mixture peeling off the plates, a compressed air, gravity fed spray gun (3.5 mm) was used to provide a more even coverage. The force at which the diet was sprayed also helped in producing a stronger adherence onto the plates. Stott *et al.* (2004a, 2004b, 2004d) had to regularly re-apply (2 d) an agar/artificial diet mixture when using a hand held fine mist sprayer, to maintain sufficient artificial diet biomass on the plates for postlarval abalone. This re-application rate would not be feasible on a large-scale, hence the use of the compressed air spray gun to cover greater surface areas, more efficiently, while producing a stronger adhesion.

The agar/*G. flagelliformis* mixture (2:1 ratio) with a 1 % agar concentration used in the dipping experiment blocked the nozzle of the spray gun. Pure agar solution could be sprayed easily and the agar/algae mixture with the lower agar concentration of 0.75 % (2:1 ratio) could also be sprayed effectively. When the mixture was sprayed over a long period of time both the mixture and the ambient temperatures decreased, resulting in the mixture solidifying in the spray gun. Given agar is a thermo-reversible gel the application temperature was critical in the adhesion protocol, as the mixture has to be sprayed before the agar solidifies at temperatures of between 35 and 40^{o}C (Moreno-Garrido 2008). However, the temperature of the agar cannot be too warm, as the live macroalgal fragments could be damaged during the short thermal shock before application. To compensate for this, the ambient air temperature was raised to produce a warmer air compressor temperature rather than using agar at a higher temperature, which allowed an agar/*G. flagelliformis* mixture with up to a 1 % agar concentration to be sprayed. Maintaining an air compressor temperature of around 28^{o}C was difficult and furthermore, the *G. flagelliformis* fragments were not regenerating in the agar, most likely due to the small size of the fragments (1 mm) needed when using the 3.5 mm pot spray gun. To overcome both of these limitations a 5 mm undercoat (direct feed) spray gun was utilised. The bigger diameter nozzle allowed larger fragments (2 – 3 mm) and mixtures with higher agar concentrations (1.25 %) to be sprayed at lower temperatures.

The method for adhering red macroalgal fragments to PVC plates with agar was refined from a dipping to a spraying application procedure. For the spraying method, the agar/algae mixtures agar concentration, macroalgal fragment size and the temperature at which it was sprayed were critical. The final protocol required fragments of macroalgae to be combined with a pure agar solution of 1.875 % at a 2:1 ratio of agar to algae, producing a mixture with 1.25 % agar concentration. This agar/macroalgal fragment mixture could then be sprayed using a 5 mm undercoat spray gun onto the PVC plates, allowing an alternative diet of high biomass red macroalgal fragments to be trialled in the nursery system for juvenile greenlip abalone.

4.2 *LAURENCIA* FRAGMENT / AGAR DIET FEEDING TRIAL

4.2.1 Introduction

Vegetative propagation by fragmentation was identified (Section 3.4.3) as a possible method for producing high red macroalgal plantlet biomass to accommodate the intense grazing pressure of juvenile *Haliotis laevigata* (>5 mm shell length). Due to the plate design of the nursery system currently used by Australian abalone farms, protocols were developed to facilitate the artificial adhesion of red macroalgal fragments onto the PVC plates (Section 4.1), generating an alternative juvenile abalone diet for the later nursery phase.

The use of macroalgal thalli fragments to produce plantlets by vegetative growth removes the need for spore production and therefore the variability in collection of fertile macroalgal material. Vegetative propagation has been a common method of commercial macroalgal production and the vertical PVC nursery plates provided a unique substratum on which to seed the macroalgal fragments. A protocol utilising an agar solution (1.875 %) combined with macroalgal fragments at a 2:1 ratio, applied using a 5 mm undercoat (direct fed) compressed air spray gun, allowed thalli of adult red macroalgae to be seeded onto the vertical plates on which the juvenile abalone reside. Small thalli fragments (2 – 3 mm) could then attach and grow into plantlets, creating a 3-dimensional red macroalgal diet with greater algal biomass than the current 2-dimensional, commercial nursery diet consisting of the green alga *Ulvella lens* plus the diatom *Navicula* cf. *jeffreyi*.

In the Vegetative Fragment Experiment (Section 3.3.3), *Gracilaria* sp. and *Hypnea* sp. were both successfully established as fragment cultures. However, on a large-scale neither of these macroalgae could be processed via mechanical means into the small fragment size required, and therefore had to be cut by hand, which significantly reduced the efficiency of producing the diet. The increased fragment size of *Gracilaria* and *Hypnea* due to manual cutting, caused extensive problems for the artificial adhesion protocol developed (Section 4.1), as the fragments would continually

block the 5 mm undercoat spray gun and very little agar/algae mixture could be applied effectively to the PVC plates. Even though both *Gracilaria* and *Hypnea* fragments were able to produce growth rates of over 0.035 $\mu g.day^{-1}$ and survival rates greater than 98.5 % in the Vegetative Fragment Experiment (Section 3.3.3), this did not translate into suitability for the agar artificial adherence protocols. Subsequently, these two red macroalgae were unable to be adhered onto the vertical PVC plates used in the abalone nursery system. Neither macroalga could be harvested in sufficient biomass near the abalone feeding trial location, further limiting the ability of the algae to be utilised in the experimental juvenile abalone nursery diet.

To integrate red macroalgae by vegetative propagation into the current nursery system, *Laurencia* sp. was utilised. Even though it had variable growth rates and an average survival of only 53.3 % (Section 3.3.3), the *Laurencia* thallus was extremely suited to large-scale production of fragments. The artificial adherence protocols developed also suited *Laurencia* thalli, just as *Laurencia okamurai* has shown bud and callus regeneration when fragments (<5 mm) where cultured on solid medium with greater than 0.5 % agar (Robaina *et al.* 1990, Shen *et al.* 2010). Furthermore, *Laurencia* could also be harvested in the biomass required for the juvenile abalone feeding trial from a collection site close to the trials location (Mistaken Island, Section 2.1). To test the vegetative propagation method by fragmentation and the artificial adherence protocols developed, fragments of *Laurencia* adhered to the PVC plates with agar were used as an alternative red macroalgal diet for juvenile abalone in the later nursery phase.

Different system designs are another management strategy currently employed by abalone aquaculture, where juvenile abalone are weaned early onto an artificial diet in a growout system. This strategy of utilising an artificial diet was compared to the dietary value of two nursery system diets, the fragmented red macroalga, *Laurencia* adhered (sprayed) to the vertical PVC plates with agar, and the current commercial nursery (*U. lens* plus *N. jeffreyi*). All three of these diet treatments were evaluated for their ability to maintain juvenile greenlip abalone (*H. laevigata*) growth and survival throughout the later nursery stage.

4.2.2 Feeding Trial Experimental Design

Location

The juvenile abalone feeding trial was conducted at the commercial abalone farm Great Southern Marine Hatcheries in Albany, Western Australia between May and August 2005. Greenlip abalone (*Haliotis laevigata*) were spawned in October 2004 and the juveniles grown in Great Southern Marine Hatcheries' nursery system until required.

Algal Culture – Diets

Laurencia sp. Fragments Adhered with Agar Diet

Laurencia sp. thalli were harvested from the Mistaken Island collection site (Section 2.1) and placed in a conical tank with flow through seawater under strong aeration, outdoors exposed to natural irradiance. To prepare the diet, *Laurencia* thalli were cut into 2 – 3 mm fragments using a commercial food processor, with 1.8 kg blotted wet weight of thalli needed to produce 2 L of fragments (Figure 13). Agar was used as the adhesion substance (Section 4.1.3) and 75 g was dissolved in 4 L of 1 µm filtered seawater (1.875 %, ICF). Once the agar had cooled to less than 40^{o}C, 2 L of fragmented *Laurencia* was added (2:1 agar:algae) resulting in a mixture with an agar concentration of 1.25 %. The mixture was then sprayed onto 60 clean PVC settlement plates (60 x 26 cm) using the 5 mm undercoat compressed air spray gun (Figure 13). The plates were allowed to set for 1 h and then distributed into a nursery tank filled with 5 µm filtered seawater (ICF) non-flow through, receiving no aeration for the first 24 h. This process was performed for the 3 replicate nursery tanks and the *Laurencia*/agar diet was re-applied during the trial to maintain sufficient algal biomass. The re-applications of the *Laurencia*/agar diet were performed as described above, with the diet sprayed onto clean PVC plates with no abalone present and then placed into the nursery tank, while at the same time the old plates were removed.

Commercial Nursery Diet (*Ulvella lens* plus *Navicula* cf. *jeffreyi*)

The construction of the commercial nursery diet followed the protocol discussed in the General Materials and Methods Section 2.4.4.

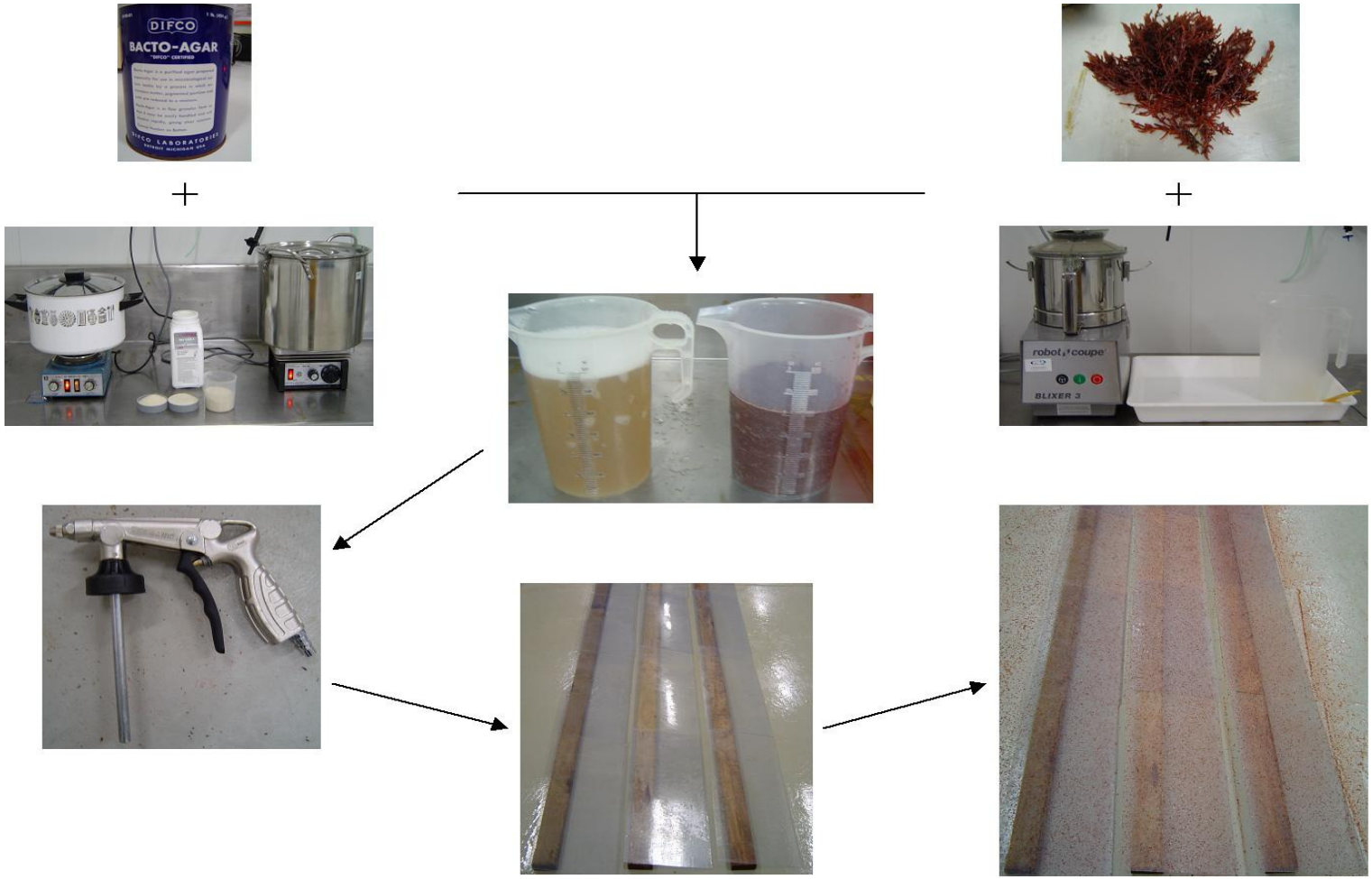

Figure 13: The artificial adhesion protocol in a schematic diagram to demonstrate the combination of the *Laurencia* sp. fragments and agar, to produce the *Laurencia* fragment/agar diet adhered to the PVC settlement plates in the nursery system.

System Design

The nursery and weaner systems complied with the set up and maintenance outlined in General Material and Methods Section 2.4 with some minor alterations. All tanks were flow-through with 5 µm filtered seawater (ICF) and 60 x 26 cm PVC plates used in the nursery system. Each of the 6 nursery tanks were stocked with 2400 juvenile greenlip abalone (8 mm shell length) averaging 40 juveniles per plate (128 abalone.m^{-2}), while each weaner tank (3 replicates) was stocked with 6000 juvenile abalone (8 mm shell length, 1910 abalone.m^{-2}) fed the artificial diet (Section 2.4.2). The feeding trial was for a period of 12 weeks with the baskets containing the PVC plates in the nursery system rotated 180^{o} about the horizontal fortnightly, and the three commercial nursery diet tanks re-inoculated with *N. jeffreyi* and MAF (Section 2.2.2) added, weekly.

Measurements

Measurement of the feeding trial parameters followed those outlined in General Material and Methods Section 2.4.5. Abalone were measured by shell length, fortnightly and weight, monthly using a sub-sample of 50 juveniles per tank in the nursery system and 100 juveniles per tank in the weaner system. The *Laurencia* fragments were counted as the number of fragments per cm^2 at the beginning and end of each application, while the *U. lens* and *N. jeffreyi* were counted fortnightly during the trial.

Data Analysis

Repeated Measures Analysis of Variance compared abalone growth rates throughout the 12-week feeding trial. The abalone initial shell length, final shell length, total trial growth rate, specific growth rate, weight gain and survival were all examined by analysis of variance (one-way ANOVA). If significant differences between diets were observed ($p<0.05$) the Tukey Post-Hoc test for multiple comparisons of means was applied. The variation between each application of the *Laurencia*/agar diet expressed by both the *Laurencia* fragment removal and agar removal were also assessed by analysis of variance (one-way ANOVA). The relationship between abalone growth rates and survival, as well as the relationship between the density of *Laurencia* fragments and

agar removed from the plates were both explored through a Pearson Product Moment Correlation analysis.

4.2.3 Results

Abalone Growth

The juvenile *H. laevigata* had an initial shell length of 8.13 ± 0.04 mm (Figure 14), with no significant difference between the shell lengths of abalone stocked onto the three diets ($F_{(df\ 2,6)}$=2.14, p=0.199). The juvenile abalone increased in size on all diets as they actively fed, with the *Laurencia*/agar diet producing abalone of 11.29 ± 0.10 mm shell length, compared to 11.80 ± 0.20 mm for the commercial nursery diet and 12.80 ± 0.05 mm for the artificial diet (Figure 14). At the conclusion of the growth trial the juveniles feeding on the artificial diet were significantly larger than the abalone consuming the *Laurencia*/agar diet and the commercial nursery diet ($F_{(df\ 2,6)}$=35.327, p<0.05 (Tukey, p<0.05).

Monthly growth rates (shell length) of juvenile abalone feeding on the two algal nursery diets followed a similar profile, increasing to a maximum of 51.47 μm.day^{-1} in the second month on the *Laurencia*/agar diet and 59.89 μm.day^{-1} on the commercial nursery diet, then decreasing during the last month (Table 13). The abalone consuming the artificial diet had a significantly slower growth rate than those on the commercial nursery diet for the first month of the trial. The growth rate then increased rapidly during the second month, resulting in a significantly faster growth rate for the last month of the trial than that achieved by the abalone on the *Laurencia*/agar diet and the commercial nursery diet (Table 13). The ability of the artificial diet to produce larger and faster growing abalone was also demonstrated by the significantly faster total feeding trial growth rate of 57.23 ± 1.47 μm.day^{-1}, compared to 36.68 ± 0.94 and 42.82 ± 2.44 μm.day^{-1} obtained on the two nursery algal diets over the 12-week trial (Table 13). The specific growth rates for the abalone consuming the two nursery algal diets also exhibited similar profiles, with the commercial nursery diet peaking at 0.51 % length.day^{-1} during week 8 and finishing at 0.44 % length.day^{-1} (Figure 15). Both the maximum and final specific growth rates of the abalone on the commercial nursery diet

were only slightly higher than those on the *Laurencia*/agar diet. The artificial diet initially produced a lower specific growth rate than the nursery algal diets, but by the end of the trial the abalone specific growth rate was significantly faster than on both the *Laurencia*/agar diet and the commercial nursery diet ($F_{(df\ 2,6)}$=29.762, $p<0.05$ (Tukey, $p<0.05$)) (Figure 15).

The average daily weight gain per individual abalone feeding on the artificial diet was nearly 2 times that of the abalone on the commercial nursery diet and approximately 2.5 times greater than the abalone on the *Laurencia*/agar diet (Table 14). Subsequently, the abalone weight gain on the artificial diet was significantly greater than on both the commercial nursery and *Laurencia*/agar diets. The commercial nursery diet was also able to produce significantly greater weight gain in juvenile abalone than the *Laurencia*/agar diet (Table 14).

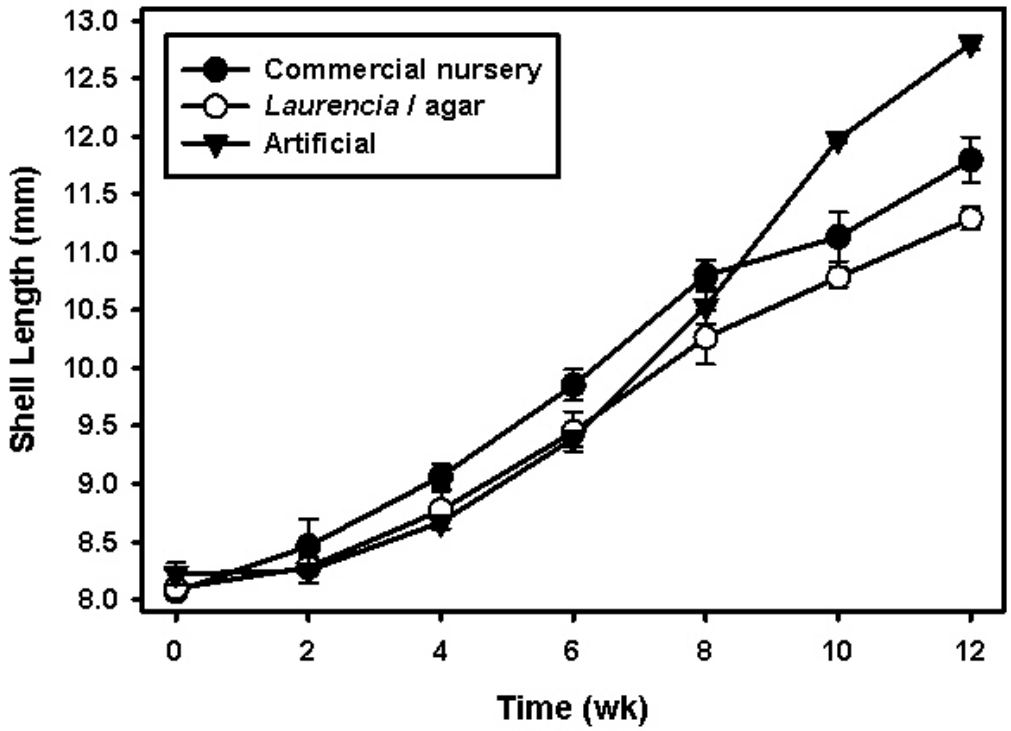

Figure 14: Growth in shell length (mm) of juvenile *Haliotis laevigata* during the 12-week (wk) feeding trial, on the *Laurencia* fragment/agar diet, the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet and an artificial diet. Mean ± std. error (n=3).

Table 13: Mean monthly growth rates and total trial growth rate (μm.day^{-1}) for juvenile *Haliotis laevigata* during the 12-week (wk) feeding trial, on the *Laurencia* fragment/agar diet, the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet and an artificial diet. Repeated Measures Analysis of Variance (RM-ANOVA) shows significant differences ($p<0.05$) in mean abalone growth rates between diets over the trial (rows). One-way ANOVA indicated significant differences ($p<0.05$) in mean abalone growth rates between diets at each growth rate period as illustrated by different superscripts (columns). Mean ± std. error (n=3).

Diet	**Wk 0 – 4**	**Wk 4 – 8**	**Wk 8 – 12**	**RM-ANOVA**	**Total Trial Growth Rate**
Commercial nursery	35.42 ± 3.26^{a}	59.89 ± 2.19	33.22 ± 6.09^{b}	2	42.82 ± 2.44^{b}
Laurencia fragment/agar	23.99 ± 5.39^{ab}	51.47 ± 4.92	34.22 ± 4.99^{b}	2	36.68 ± 0.94^{b}
Artificial	23.40 ± 1.86^{b}	64.38 ± 5.15	70.84 ± 6.42^{a}	1	57.23 ± 1.47^{a}
df	2, 6	2, 6	2, 6	2, 6	2, 6
F	10.252	2.321	13.353	10.308	37.102
p value	<0.05	0.179	<0.05	<0.05	<0.05

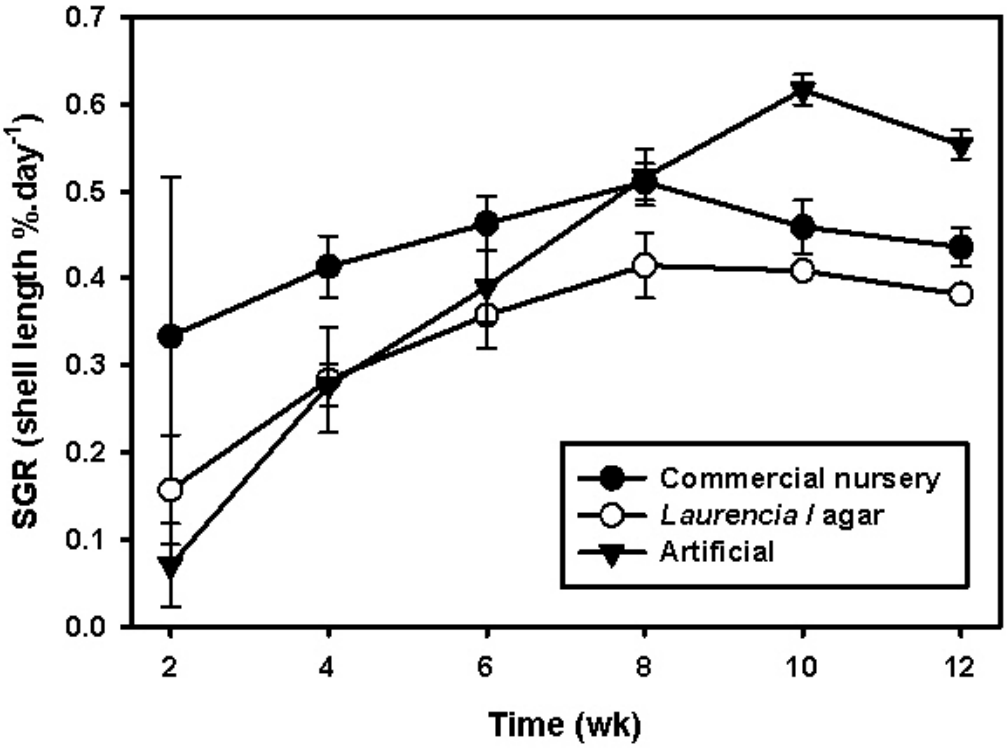

Figure 15: Specific growth rate (% length.day^{-1}) of juvenile *Haliotis laevigata* during the 12-week (wk) feeding trial, on the *Laurencia* fragment/agar diet, the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet and an artificial diet. Mean ± std. error (n=3).

Table 14: Weight gain per individual (µg.day^{-1}) and survival (%) of juvenile *Haliotis laevigata* over the 12-week feeding trial, on the *Laurencia* fragment/agar diet, the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet and an artificial diet. One-way ANOVA indicated significant differences ($p<0.05$) between diet means for each abalone measurement as illustrated by different superscripts (columns). Mean ± std. error (n=3).

Diet	Weight Gain	Survival
Commercial nursery	1.62 ± 0.09^{b}	77.5 ± 3.5
Laurencia fragment/agar	1.19 ± 0.09^{c}	67.1 ± 3.5
Artificial	3.15 ± 0.08^{a}	73.6 ± 0.8
df	2, 6	2, 6
F	133.548	3.376
p value	<0.05	0.104

Abalone Survival

The juvenile abalone had a survival of 77.5 % on the commercial nursery diet, while the survival on the artificial diet was approximately 4 % lower and the survival of abalone on the *Laurencia*/agar diet was 6.5 % lower again (Table 14). Even though the survival of juvenile abalone on the *Laurencia*/agar diet was lower than the survival on the other two diets, it was not significantly different. The change in survival of abalone during the trial was significantly, negatively correlated with the monthly abalone growth rate, particularly for the abalone feeding on the artificial diet ($R=-0.99$, $p<0.05$) and to a lesser extent the abalone on the commercial nursery diet ($R=-0.65$, $p<0.05$) and the *Laurencia*/agar diet ($R=-0.62$, $p<0.05$).

Algal Consumption

The density of *Laurencia* fragments removed was positively correlated with the percentage cover of agar removed throughout the feeding trial ($R=0.92$, $p<0.05$). At the beginning of the trial the diet "peeled" of the plates due to problems with adhesion rather than the abalone grazing pressure, and therefore regular re-application was required. The dramatic decrease in the amount of agar removed after the 3rd application was evident by the significant difference between the first 3 applications and all the remaining applications apart from the 6th application (Figure 16). This decrease in removal was associated with the agar no longer "peeling" off the plates but staying attached for the required application period and consequently, allowing the *Laurencia* fragments to be consumed by juvenile abalone on the plates (Figure 17). The change in adhesion at application 4 with the diet no longer "peeling" off the plates, was attributed to the plates being sprayed from a greater distance, which reduced clumping and increased the evenness of the diets cover. Given the variation in adhesion strength of the diet during the trial, the agar cover removal and consequently the density of *Laurencia* fragments removed were both significantly different between individual applications (($F_{(df\ 10,22)}=15.55$, $p<0.05$) and ($F_{(df\ 10,22)}=25.451$, $p<0.05$)) (Figure 16). Even after the improved adherence from the 4th application on, the resistance to grazing of the *Laurencia*/agar diet was still low, so regular applications were required to maintain sufficient algal biomass.

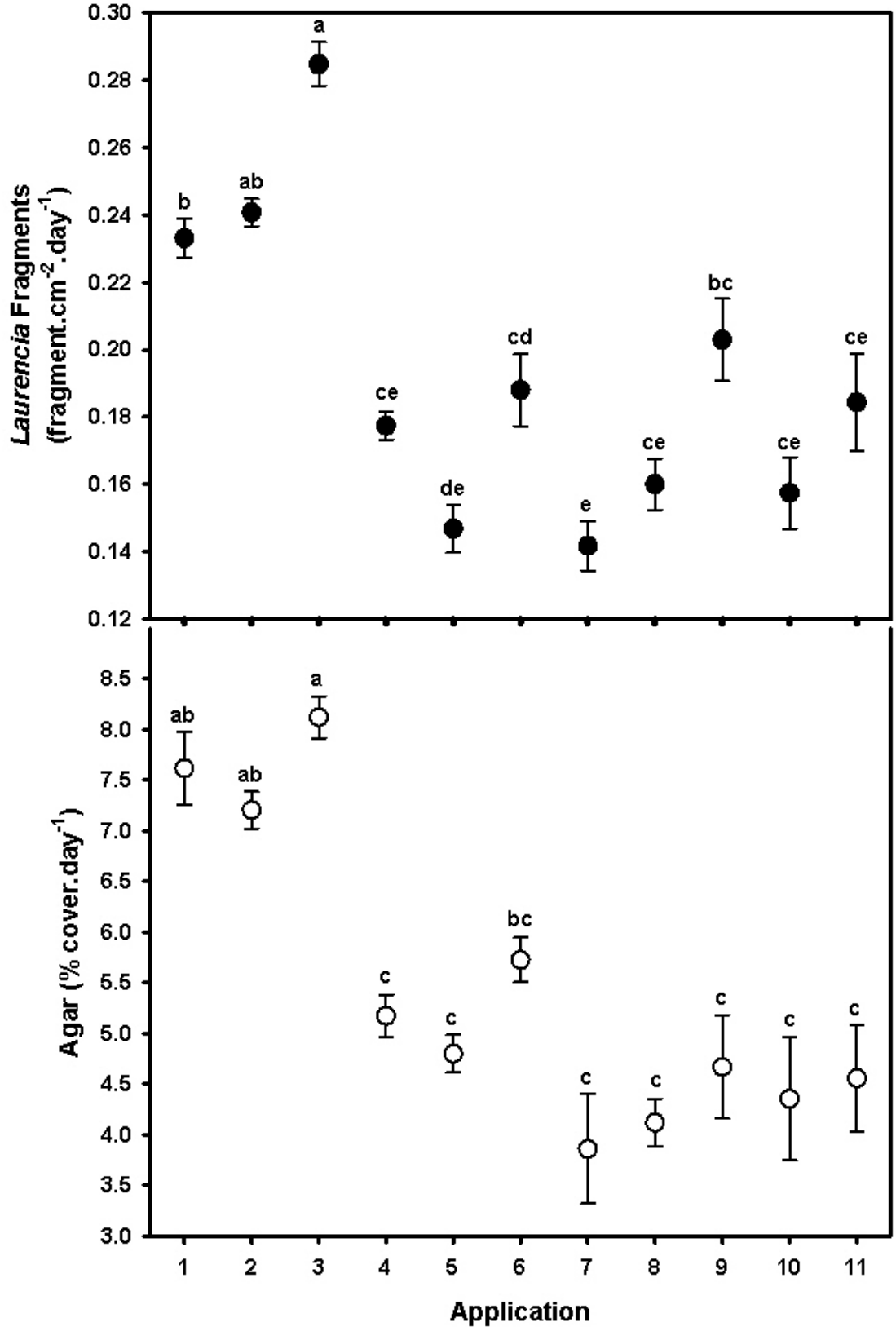

Figure 16: Removal rate of the *Laurencia* sp. fragments (fragment.cm^{-2}.day^{-1}) and the agar cover (% cover.day^{-1}) from the PVC plates during the 12-week, juvenile *Haliotis laevigata* feeding trial. Significant differences ($p<0.05$) in means denoted by letters were derived from a one-way ANOVA (Tukey Post-Hoc test). Mean ± std. error (n=3).

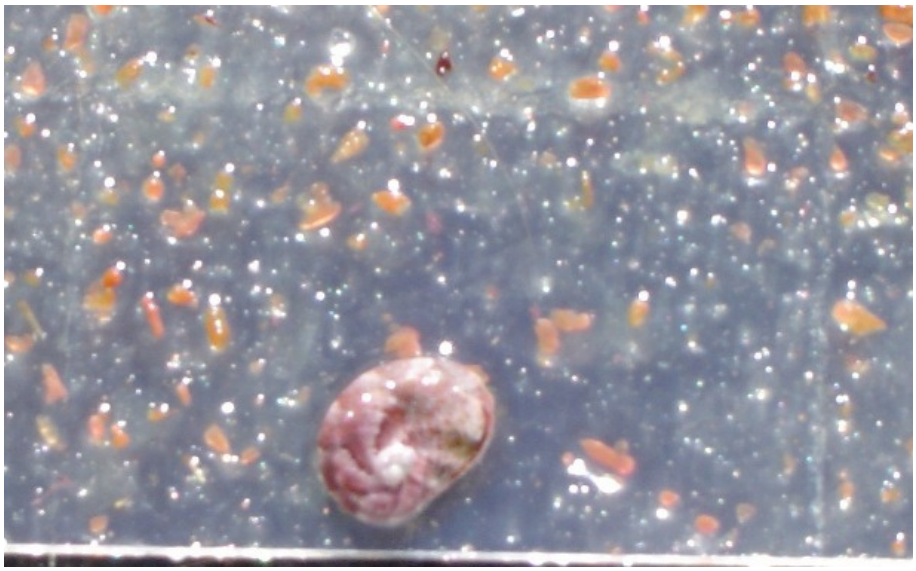

Figure 17: The *Laurencia*/agar diet being consumed by a juvenile *Haliotis laevigata* (10 mm shell length). Note the cleared area around the abalone indicating both fragments and agar were being ingested.

Juvenile abalone actively grazed on the *U. lens*, as evident by the grazing tracks through the alga cover on the settlement plate in Figure 18. The percentage cover of *U. lens* decreased by over 25 % during week 2 to 4 and then by 17 % between week 6 to 8 (Figure 19), indicating substantial juvenile abalone grazing pressure. The time periods following these decreases had reduced rates of decline and between week 4 and 6 there was actually a slight increase in *U. lens* cover. During these two periods *U. lens* spore release events occurred, which was demonstrated in Figure 20 at week 6 of the trial by the "negative" consumption rate, due to the rate of *U. lens* production being greater than it's consumption by abalone resulting in an increase in *U. lens* biomass. Overall the commercial nursery diet had approximately 70 % of the *U. lens* cover grazed by the juvenile abalone during the 12-week feeding trial, even though two spore release events occurred. *Navicula* cf. *jeffreyi* density showed a regular decrease over the 12 weeks and was reduced to less than 20 $x10^4$ diatom.cm^{-2} by the end of the trial, which was only 15 % of the starting density (Figure 21). After week 2 the consumption rate of *N. jeffreyi* fluctuated between 80 and 145 $x10^3$ diatom.$abalone^{-1}$.day^{-1}, indicating that even with a steady decline in overall density the consumption rate of diatoms by juvenile *H. laevigata* can still vary (Figure 22).

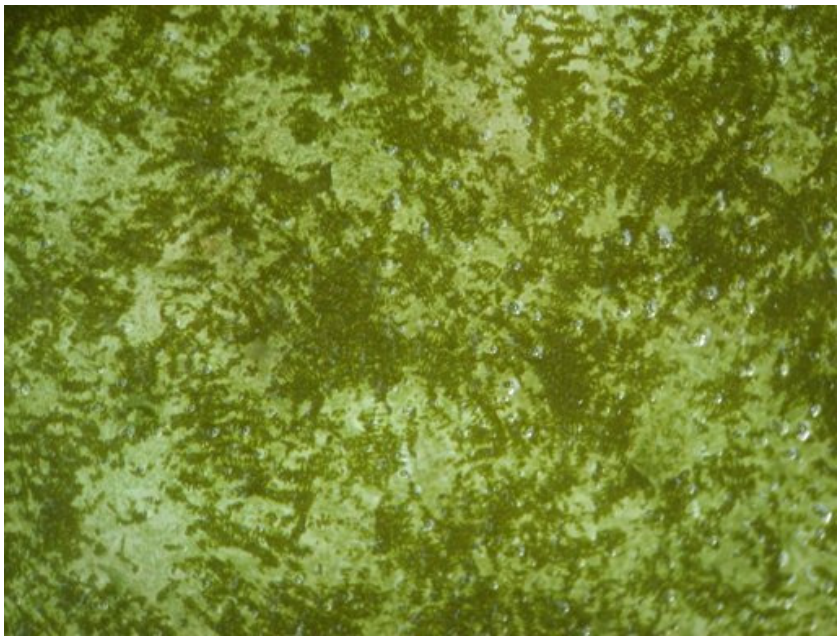

Figure 18: The commercial nursery diet's green alga *Ulvella lens*, showing extensive grazing marks by juvenile *Haliotis laevigata*.

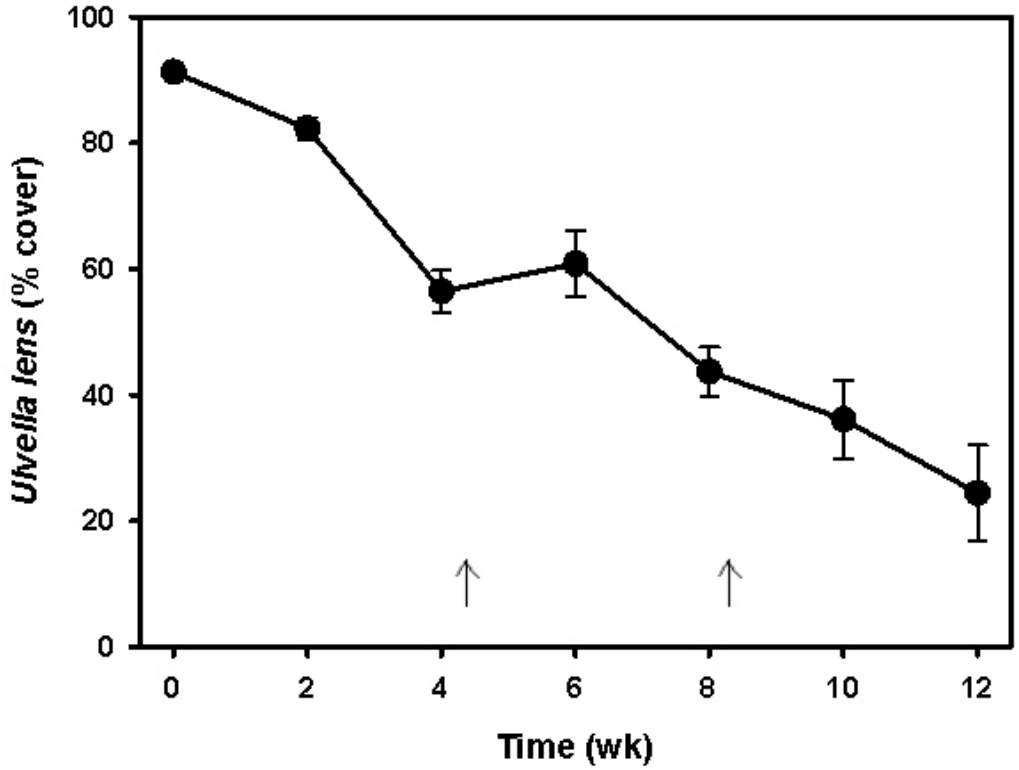

Figure 19: Percentage cover (% cover) of *Ulvella lens* during the 12-week (wk) juvenile *Haliotis laevigata* feeding trial. Mean ± std. error (n=3). The arrows indicate when *U. lens* sporulation occurred.

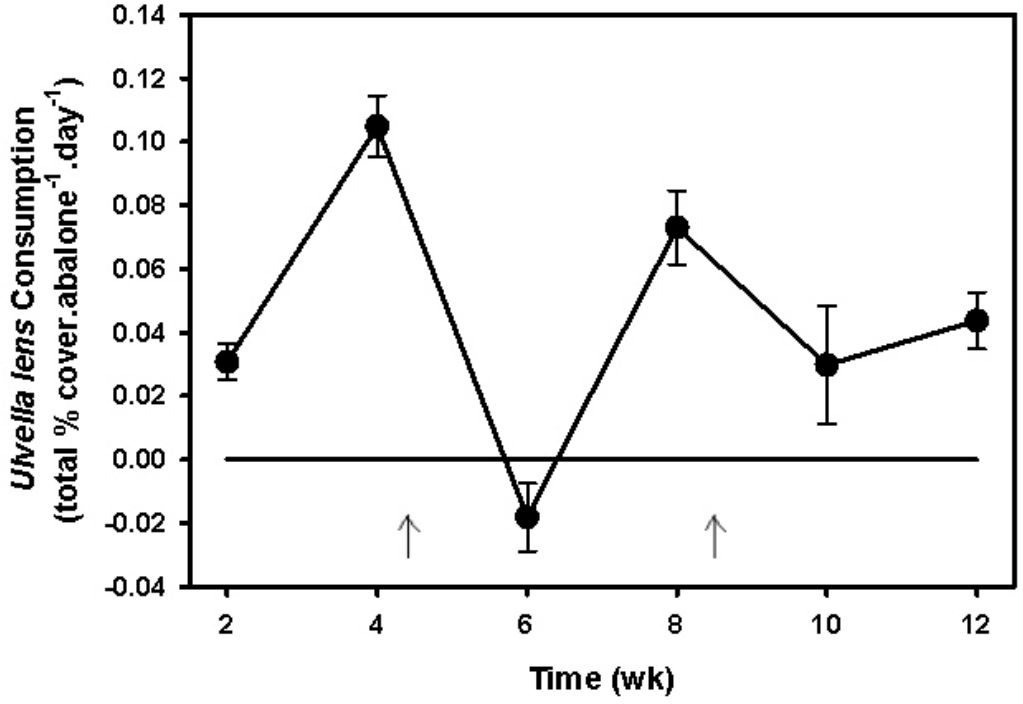

Figure 20: The consumption rate (% cover.abalone^{-1}.day^{-1}) of *Ulvella lens* by juvenile *Haliotis laevigata* during the 12-week (wk) feeding trial. Mean ± std. error (n=3). The arrows indicate when *U. lens* sporulation occurred.

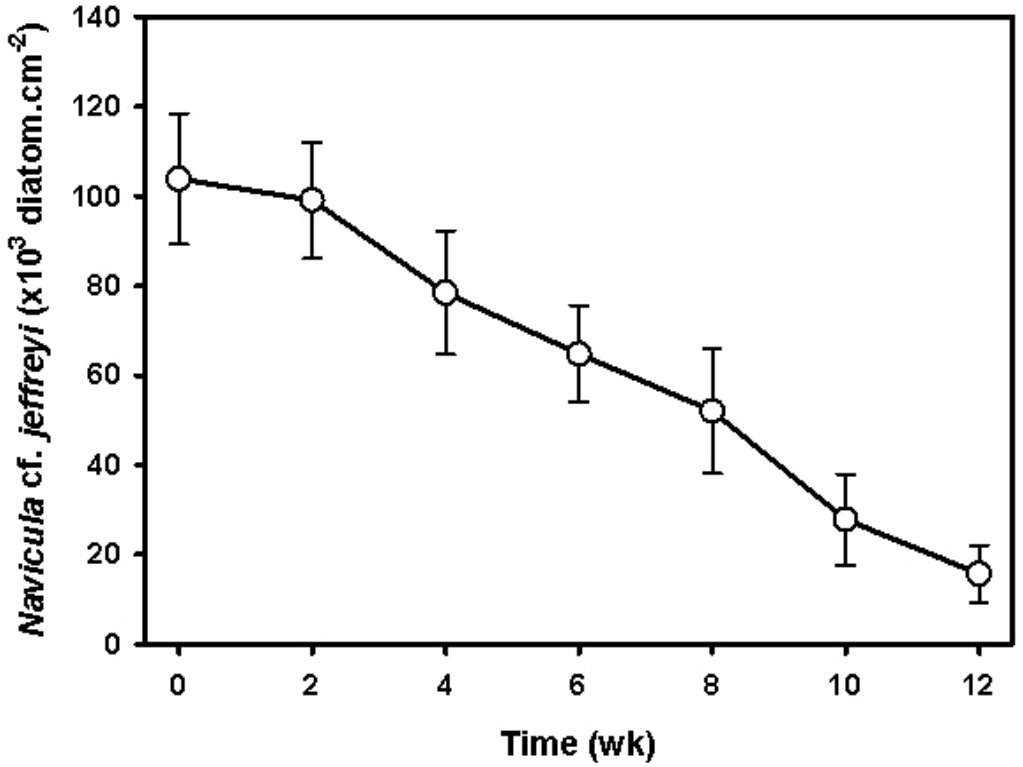

Figure 21: The density (x10^3 diatom.cm^{-2}) of *Navicula* cf. *jeffreyi* during the 12-week (wk) juvenile *Haliotis laevigata* feeding trial. Mean ± std. error (n=3).

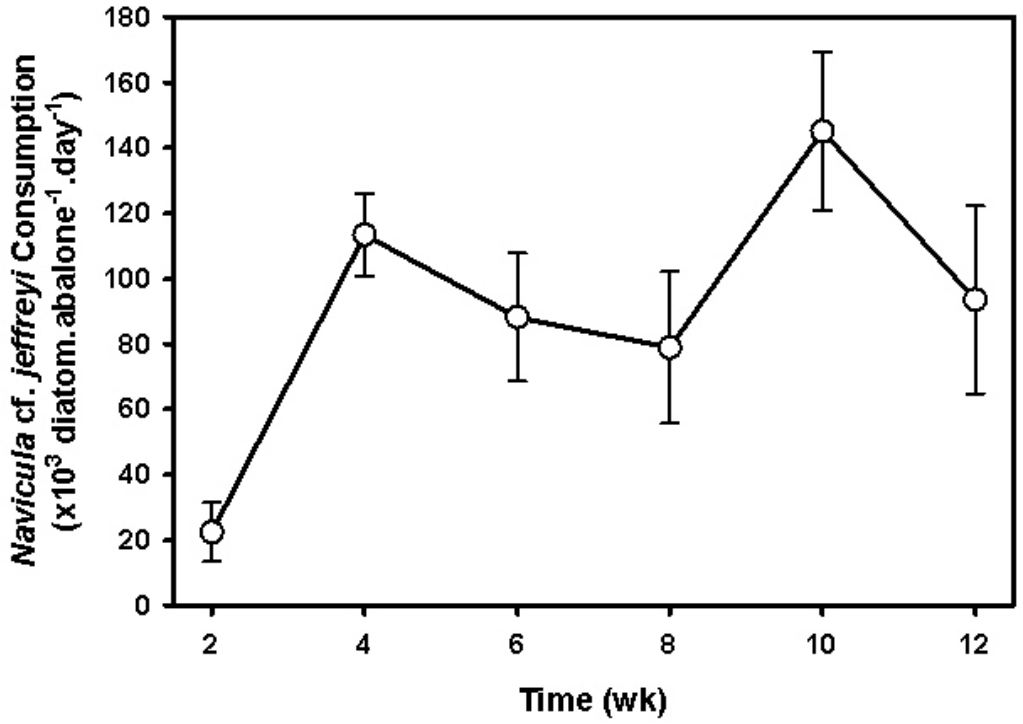

Figure 22: The consumption rate (x10^3 diatom.abalone^{-1}.day^{-1}) of *Navicula* cf. *jeffreyi* by juvenile *Haliotis laevigata* during the 12-week (wk) feeding trial. Mean ± std. error (n=3).

4.2.4 Discussion

The growth in shell length and survival of juvenile *H. laevigata* on the experimental macroalgal diet of *Laurencia* fragments adhered to PVC plates using agar, was comparable to the current commercial nursery diet consisting of *U. lens* and *N. jeffreyi*. The abalone feeding on the *Laurencia*/agar diet had similar, but slightly slower growth rate and specific growth rate profiles throughout the trial. This resulted in slightly smaller abalone (0.5 mm shell length difference) at the end of the 12-week trial, but there was no significant difference between the two nursery diets for growth parameters based on shell length. Both diets exhibited a decrease in growth rates and specific growth rates after week 8 of the trial. This could indicate that, at an appropriate abalone stocking density (≈128 abalone.m^{-2}), neither of the nursery algal diets can supply sufficient biomass to sustain adequate abalone growth once the juveniles reach over 10 mm shell length.

The commercial nursery diet was able to produce significantly greater abalone weight gain (1.62 μg.day^{-1}) than the *Laurencia*/agar diet (1.19 μg.day^{-1}). The greater abalone weight gain combined with a 10 % higher survival rate on the commercial nursery diet compared to the *Laurencia*/agar diet has implications regarding the biomass of juvenile abalone able to be produced in the nursery system. These implications will subsequently affect the abalone biomass harvested and weaned onto artificial diets in the growout system and therefore, the carrying capacity and overall production of the abalone farm.

Harvesting abalone that are greater than 8 mm shell length from the nursery system and weaning them onto artificial diets in a growout system, could be an appropriate strategy to improve growth in juvenile greenlip abalone. The artificial diet utilised in the weaner system was able to produce abalone of significantly greater size, growth rate, specific growth rate and weight gain at the end of the 12-week feeding trial, compared to either of the algal diets in the nursery system. The abalone growth rate on the artificial diet increased rapidly throughout the trial, reaching 70.84 μm.day^{-1} during the last month. Juvenile abalone (3 to 18 mm shell length) of different species have regularly achieved growth rates of up to 85 μm.day^{-1} on various artificial diets during long-term feeding trials (>3 months) (Fleming *et al.* 1996, Daume *et al.* 2007, Dlaza *et al.* 2008).

The commercial nursery diet in this trial produced a juvenile abalone growth rate of 59.89 μm.day^{-1} at week 8, with an overall trial growth rate of 42.82 μm.day^{-1}. These growth rates are comparable to those attained in two feeding trials using the same commercial nursery diet, similar size juvenile abalone and stocking density (Daume *et al.* 2007). However, juvenile *H. laevigata* growth rates of up to 70 – 80 μm.day^{-1} have been recorded over a 16-week feeding trial (Daume & Ryan 2004). Even though the maximum specific growth rate (0.51 % length.day^{-1} at week 8) achieved by the abalone consuming the commercial nursery diet in this trial was comparable to that (0.48 % length.day^{-1}) recorded by Daume *et al.* (2007), the weight gained by the abalone in this trial was only slightly less but achieved in about half the time.

The juvenile abalone in this feeding trial were harvested from an *U. lens* and *N. jeffreyi* diet in the source nursery system at Great Southern Marine Hatcheries, consequently producing a significantly faster growth rate on the commercial nursery diet than the artificial diet during the initial stage of the trial. The first month of abalone growth rates were low on the *Laurencia*/agar diet and the artificial diet, which can be accounted for by a weaning period between the source diet and the experimental diets (Fleming *et al.* 1996). Taking this weaning period into account, the *Laurencia*/agar diet was still able to produce juvenile abalone growth rates comparable to the current commercial nursery diet, while the artificial diet produced significantly higher growth rates.

The weaning period also had implications on abalone survival between the diet treatments, with the survival of the abalone on the commercial nursery diet (77.5 %) higher than those on the *Laurencia*/agar diet (67.1 %) and the artificial diet (73.6 %). This relationship between survival of juvenile abalone on *U. lens* diets in the nursery system (70 – 80 %) and artificial diets in the weaner system (60 – 70 %) has also been identified by other juvenile *H. laevigata* (3 – 15 mm shell length) feeding trials (Daume & Ryan 2004, Daume *et al.* 2007). The mortality for juvenile abalone fed the artificial diet in this trial predominately occurred in the initial stages due to the husbandry stress of harvesting and weaning, which also corresponded to a period of slow growth. The mortality for juvenile abalone on the commercial nursery diet was low at the start, but as growth rates decreased when food became a limiting factor towards the end of the trial, the mortality increased. Juvenile abalone growth rates are negatively correlated against survival, with the predominate mortality periods occurring at either end of the later nursery phase.

The consumption of algae in the commercial nursery diet was extensive, with the *U. lens* and *N. jeffreyi* reduced to 30 % and 15 % of their original cover and density, respectively. The two *U. lens* spore release events were critical in the algal cover not decreasing even further during the trial. The *N. jeffreyi* consumption rate was maintained ($\approx$100 x10^3 diatom.abalone^{-1}.day^{-1}) throughout the entire trial and did not

decrease as the abalone grew to larger sizes, which are not usually associated with the consumption of diatoms (Kawamura *et al.* 1995, Kawamura 1996). Despite the *U. lens* spore release events providing additional algal cover and the constant consumption of *N. jeffreyi*, food availability still became limited and the algal biomass could not support the faster growth rates (week 8) for the duration of the 12-week feeding trial. To overcome this, new seeded plates of *U. lens* could be introduced to accommodate the intensive grazing pressure of the juvenile abalone and maintain the abalone in the nursery system for the entire later nursery phase (Fleming 1995b, Daume *et al.* 2004).

The uniqueness of the *Laurencia*/agar diet makes it difficult to compare with other juvenile abalone nursery feeding trials. Although, a procedure utilising an artificial diet attached to plates has been developed for postlarval settlement and growth (Stott *et al.* 2004a, 2004b, 2004c, 2004d). A study incorporating alginate as the gelling agent for adhering artificial diets to plates recorded growth rates of approximately 25 $\mu m.day^{-1}$ for *Haliotis discus discus* postlarvae (≈400 µm shell length), while the same artificial diet and alginate gelling agent not attached to the plates, increased the growth rate by a further 10 $\mu m.day^{-1}$ (Stott *et al.* 2003b). Higher postlarval growth rates of 40 – 50 $\mu m.day^{-1}$ have been recorded using the agar (1 %) adhesion methodology with artificial diets as the food source (Stott *et al.* 2004b, 2004d). A recent study using artificial diets supplemented with emulsified fish oil and adhered to plates with a 1.5 % solution of agar has shown *Haliotis diversicolor supertexta* growth rates of over 70 $\mu m.day^{-1}$ are achievable in shaded conditions, while unshaded conditions produced abalone growth rates reaching 90 $\mu m.day^{-1}$ (Chao *et al.* 2010). All of these studies utilised artificial diets instead of live macroalgae, which allowed the artificial diet/agar mixtures to be sprayed at much higher temperatures. This makes the application process significantly easier than with the *Laurencia*/agar diet, as the agar temperature while spraying can be further from its solidification point. However, these artificial diet/agar mixtures still required re-application onto the plates at least every 2 to 7 d. Until the Chao *et al.* (2010) study, all of the abalone growth rates recoded were similar to that attained on the *Laurencia*/agar diet. Substantial points of difference occur between all of those studies and this feeding trial, as the growth rates recorded were for postlarval

abalone consuming artificial diets and not the consumption of a macroalgal fragment diet by juvenile abalone greater than 8 mm shell length.

The removal of the juvenile abalone feed (*Laurencia* fragments) from the plates positively correlated with the removal of agar. The first few weeks saw a majority of the diet "peel" off the plates, but as the trial continued the agar removal and subsequently the macroalgal removal decreased dramatically. From application 4 on, the removal of both agar and macroalgal fragments was a representation of abalone consumption rather than the diet "peeling" off the plates. Visible grazing tracks through the agar and observations of the juveniles eating the fragments indicated the abalone were able to readily consume the diet.

Even though the adhesion of the *Laurencia*/agar diet to the PVC plates improved after the 3rd application, the inability of the diet to remain on the plates for the duration of the trial meant that re-application at regular intervals was still required. During the nursery feeding trial 5.4 kg of *Laurencia* was used in each application to inoculate 180 PVC settlement plates (0.03 kg.plate^{-1}). This translated into 59.4 kg of *Laurencia* and 2.475 kg of agar costing US$195 being consumed over the entire 12-week trial. To use this diet on a commercial scale for the production of juveniles from 5 to 15 mm shell length, the amount of *Laurencia* and agar required would be considerably greater. If the juvenile abalone are able to maintain the maximum growth rate of 51.47 µm.day^{-1} over the 10 mm increase in shell length, then the number of *Laurencia*/agar applications (A) can be calculated by Equation 6.

$$\text{Applications (A)} = \left[\frac{GI}{GR}\right] / t \qquad \text{Equation 6}$$

where *GI* was the growth interval (µm shell length), *GR* was the growth rate (µm.day^{-1}) and *t* was the time between applications (d).

The *Laurencia*/agar would need to be applied 27.6 times to produce the 10 mm of shell length growth, assuming that the diet will be re-applied on a weekly basis. If a commercial abalone farm can produce 500,000 juvenile abalone to 15 mm in shell length while stocking at 128 abalone.m^{-2}, the farm would require at least 35 nursery tanks with 360 plates per tank. If the plates are applied with 0.03 kg.plate^{-1} of *Laurencia*, then the biomass of red macroalga required by the abalone nursery can be determined using Equation 7.

$$\text{Algae Biomass} = A(plate) \times Pl \times A \qquad \text{Equation 7}$$

where *A(plate)* was the amount of algae or agar per plate (kg.plate^{-1}), *Pl* was the number of plates used and *A* was the number of applications.

Over 10 t of *Laurencia* thalli would have to be cultured or harvested to produce the 10 mm of juvenile abalone shell length growth in the commercial farm. To adhere the 10.43 t of macroalgae over the 6 months, 435 kg of agar would be required at a cost of US$34,269 (Equation 7). This biomass of algae, quantity of agar and cost associated; would be completely unattainable and uneconomical for a commercial abalone farm given the juvenile abalone growth and survival recorded consuming the *Laurencia*/agar diet.

These preliminary calculations do not even factor in the substantial labour time and associated expenses required in creating the *Laurencia*/agar diet. The *Laurencia*/agar diet took a considerable amount of time (8 h.application^{-1}) to produce, given the fragments had to be adhered to the PVC plates. The commercial nursery diet was a fairly self-sufficient natural diet, as the release of *U. lens* spores can be induced regularly via temperature, light and nutrient enhancement (Takahashi & Koganezawa 1988), therefore only routine maintenance during the nursery phase was required (Section 2.4.2). The juvenile abalone weaned onto an artificial diet had different time allocations and costs associated. Labour requirements for the growout system can be

highly variable and depend on growth rates, stocking densities, feeding rate and weather season.

Even though the *Laurencia*/agar diet was able to produce comparable shell length growth to the current commercial nursery diet, the regular re-application makes this diet extremely costly and labour-intensive, therefore commercially unviable. If the protocols developed to adhere macroalgal fragments onto the plates (Section 4.1.3) could be refined to utilise the successful fragment cultures of *Gracilaria* and *Hypnea* (Section 3.3.3), the regeneration of macroalgal fragments to plantlets on the plates could be achieved. This would reduce the need for re-application, as the initial application could be sufficient to create a lasting diet for juvenile abalone. However, given the scale of a commercial abalone farm and expertise required to construct the diet; the labour, algal biomass and material costs may not reduce sufficiently, for macroalgal fragment cultures to be used as an alternative algal diet for juvenile abalone during the later nursery phase.

Given the faster growth rates (shell length) and substantial weight gain of abalone feeding on the artificial diet in a weaner system, compared to both algal diets in the nursery system, it can be regarded as an important management strategy to rear juvenile greenlip abalone of over 8 – 10 mm shell length. Even though the *Laurencia*/agar diet was able to produce comparable juvenile abalone growth (shell length) and survival to the current commercial nursery diet, it would be an uneconomical diet to use in a commercial abalone farm. The inability of the artificial adhesion protocol to maintain algal cover on the PVC plates for extended periods of time, means the use of red macroalgal fragments to overcome the intense grazing pressure of juvenile abalone was extremely limited. Therefore, alternative algal diets will not be feasible if artificial adhesion protocols are required to seed the plates currently used in Australian abalone nurseries.

CHAPTER 5
MACROALGAL SPORELINGS AS A DIET FOR JUVENILE GREENLIP ABALONE

Providing adequate algal diets to support the substantial grazing pressure and nutritional requirements of juvenile abalone greater than 5 mm shell length has become a major bottleneck in the intensification of abalone nurseries (e.g. Krsinich *et al.* 2000, Daume 2006). The transition of juvenile abalone (5 – 15 mm shell length) feeding on natural algal diets in the nursery system onto artificial diets in the growout (weaner) system, results in highly variable outcomes with respect to growth and mortality of abalone (Daume 2003). To overcome this, macroalgal sporelings have been identified as a potential alternative algal diet to maintain abalone in the nursery system throughout the later phase of juvenile production.

Sporelings can supply a large biomass of a variety of suitable macroalgal species to juvenile abalone by harnessing the algal recruitment processes through spore propagation. Large spore releases can occur from fertile thalli and after the algal spores settle out of the water column, they can attach to substrata (PVC plates) and germinate, creating sporelings. This natural propagation process would allow macroalgae to be utilised in the nursery system without any physical intervention. Subsequently, there would be no need for the time consuming and expensive artificial adhesion protocols utilised for macroalgal fragments, as in the *Laurencia* Fragment/Agar Diet Feeding Trial (Section 4.2). Juvenile macroalgae (sporelings) have the potential to provide a much greater algal biomass per plate due to their 3-dimensional morphology, compared to the 2-dimensional encrusting green alga *Ulvella lens* and the diatom *Navicula* cf. *jeffreyi* currently used in commercial nurseries. This 3-dimensional growth reduces the settlement plate surface area required and has the potential to provide the juvenile abalone with sufficient algal biomass at a time when they can consume 10 – 30 % of their body weight in algae each day (Hahn 1989b). By utilising spore production a

relatively cost effective, high algal biomass diet can be developed for juvenile abalone in the nursery system.

In natural algal communities, herbivores appear to be a major source of mortality for early post settlement stages (sporelings) of macroalgae and can have a significant affect on the recruitment of macroalgal species (Vadas *et al.* 1992). This could be because juvenile macroalgae have different structural and nutritional properties to adult macroalgal thalli of the same species (Van Alstyne *et al.* 1999). Juvenile macroalgae structural properties are smaller, thinner and more delicate (Van Alstyne *et al.* 2001), making them at greater risk to grazing by juvenile abalone with small feeding apparatus, than the more robust, larger adult macroalgal thalli. Macroalgal sporelings may also contain more nutrients (i.e. proteins, lipids and carbohydrates) on a weight or volume basis, however; an increase in nitrogen concentration does not necessarily determine herbivorous predators food choice between juvenile and adult macroalgae (Van Alstyne *et al.* 2001). These properties indicate that juvenile macroalgae could be a key food source for herbivores and consequently, macroalgal sporelings are considered important to meet the nutritional requirements of juvenile abalone as they transition from a diatom-based diet to a macroalgae-based diet at approximately 5 – 10 mm shell length (Kawamura *et al.* 1998b, Takami & Kawamura 2003).

This chapter describes the results of two large-scale juvenile greenlip abalone (*Haliotis laevigata*) feeding trials utilising macroalgal sporelings. The first trial compares sporelings of *Ulva* spp. against the current commercial diet of *U. lens* plus the diatom *N. jeffreyi* (Section 5.1). The second trial compares two abalone rearing system designs, nursery and weaner, while also examining five different diets including; (1) a combination of *Hypnea* sp. and *Ulva* spp. sporelings, (2) the cyanobacterium *Phormidium* sp., (3) the current commercial diet (*U. lens* plus *N. jeffreyi*), all in the nursery system, and (4) a whole macroalgal thalli, (5) an artificial diet, both within the weaner system (Section 5.2).

5.1 *ULVA* SPORELING DIET FEEDING TRIAL

5.1.1 Introduction

The green alga *Ulva* may be one such macroalgae that could produce sporelings in sufficient biomass to accommodate the grazing pressure of juvenile greenlip abalone. *Ulva* has an isomorphic, haplodiplontic life cycle and the adult thallium can reproduce both sexually and asexually, reducing the importance of fertilisation in propagation (Hoxmark 1975). The spore producing parts of the plant are not limited to specific reproductive structures and any blade cell can expel spores, resulting in a large spore production capacity (Stratmann *et al.* 1996, Hiraoka *et al.* 2003). The relatively simple means to induce large spore release events through a variety of physical changes, are another advantage for utilising *Ulva* in commercial cultivation (Nordby 1977, Han *et al.* 2003, Mantri *et al.* 2011).

The combination of these factors has led to the development of *Ulva* sporelings as a diet for juvenile abalone. A small-scale pilot study using *Ulva* sporelings was able to produce abalone growth rates of approximately 33 $\mu m.day^{-1}$ (Strain 2003). Given the reproductive processes of *Ulva* can be conducive to easy culture and juvenile abalone actively feed on the sporelings, *Ulva* sporelings were considered as an alternative diet for juvenile abalone in the later nursery phase. The preliminary sporeling diet was subsequently converted from small, horizontal plates into a fully working diet for the vertical settlement plates in the nursery system. The protocols developed during this *Ulva* sporeling feeding trial have since been utilised to test different rearing systems for juvenile greenlip abalone (Daume *et al.* 2007).

A large-scale feeding trial was conducted to determine if the experimental sporeling diet could accommodate the high grazing pressure of juvenile greenlip abalone through the entire (5 – 15 mm shell length) later nursery phase. The *Ulva* sporeling diet was compared to the current commercial nursery diet consisting of the green alga *U. lens* plus the diatom *N. jeffreyi* on the growth and survival of juvenile *H. laevigata*.

5.1.2 Feeding Trial Experimental Design

Location

The juvenile abalone feeding trial was conducted in a greenhouse at the Aquaculture Development Unit, Challenger TAFE in Fremantle, Western Australia between March and August 2004. Greenlip abalone (*Haliotis laevigata*) were spawned in December 2003 and the juveniles produced by Great Southern Marine Hatcheries in Albany, Western Australia.

Algal Culture – Diets

Ulva spp. Sporeling Diet

Ulva spp. thalli were harvested from the South Mole collection site (Section 2.1) and exposed to a temperature and dark treatment to induce gametogenesis (Strain 2003). The *Ulva* thalli were arranged in layers between moist newspapers on trays and then refrigerated at 4^{o}C in complete darkness for 24 h. After a 7 d treatment, 10 kg blotted wet weight of *Ulva* thalli were placed into each of the five, 400 L nursery tanks filled with f/2 culture medium modified by removing the PII metals, sodium metasilicate and vitamin stock solutions (Section 2.2, 1 µm filtered, ICF). Each tank held three baskets of twelve, 60 x 30 cm PVC settlement plates orientated horizontally. Only light aeration of the non-flow through seawater was used within the tanks to reduce water motion and allow maximum spore attachment.

After 6 d the *Ulva* thalli were removed from the tanks and the sporeling seeded PVC plates redistributed into three, 400 L nursery tanks each containing three baskets of 20 plates, now orientated vertically. The sporelings were then cultured over 5 weeks in the modified f/2 medium (1 µm filtered, ICF) as described above, which was exchanged twice weekly.

Commercial Nursery Diet (*Ulvella lens* plus *Navicula* cf. *jeffreyi*)

The commercial nursery diet of *U. lens* and *N. jeffreyi* followed the protocol discussed in General Materials and Methods Section 2.4.4 with some minor adjustments. The *U. lens* seed plates were left exposed to sunlight for 5 d to induce the release of spores and the same modified f/2 medium (1 µm filtered, ICF) used for the *Ulva* sporeling diet was exchanged twice weekly. The diatom *N. jeffreyi* was cultured in 4 Petri dishes rather than the small bags (200 mL) and no large bags were used, instead the cultures went from 4 medium bags to a Mexican hat tank.

System Design

The nursery system was as outlined in the General Material and Methods Section 2.4.1 with the system maintenance described in Section 2.4.2. However, 1700 juvenile abalone of 3.5 to 4 mm shell length were stocked in each of the six tanks, giving approximately 28 juveniles per 60 x 30 cm plate (79 abalone.m^{-2}). The feeding trial was for a period of 14 weeks and the three *U. lens* tanks were re-inoculated with *N. jeffreyi* during week 2, 4 and 8, while the plates in all tanks were rotated 180^{o} about the horizontal in week 3 and 10. The water temperature over the trial started at 20.8 ± 0.13^{o}C (May), then reduced to 19.7 ± 0.18^{o}C (June) and finished at 19 ± 0.08^{o}C (July).

Measurements

Measurement of the feeding trial parameters followed those outlined in General Material and Methods Section 2.4.5. A sub sample of 50 juvenile abalone from 10 randomly selected settlement plates in each tank, were measured weekly for shell length (mm). *Ulva* sporeling abundance was determined by counting the number of sporeling blades per cm^2 on the settlement plates and the density of *N. jeffreyi* was measured on 2 removable 16 cm^2 sample plates, positioned 3 cm from the top and bottom of 9 settlement plates in each tank. Both algal diets were measured at weekly intervals during the feeding trial.

Biochemical Analysis

The biochemical analysis of the *Ulva* sporeling and commercial nursery diets was detailed in General Material and Methods Section 2.3. Samples were taken by scraping diagonally across the settlement plates that were used for determining weekly algal abundance. Five millilitre of the commercial nursery diet samples were required for each of the dry weight, lipid, protein and carbohydrate analyses. The amount of *Ulva* sporeling diet sample required for each analysis was, 0.05 g for dry weight, 0.025 g for lipid, 0.0125 g for protein and 0.012 g for carbohydrate.

Data Analysis

Juvenile abalone growth and density for the two dietary treatments, *Ulva* sporeling and commercial nursery were compared by analysis of variance (one-way ANOVA). A Univariate Analysis of Variance (Tukey Post-Hoc) was applied to test for differences between mean weekly abalone sizes (shell length) on the two diets. Comparison of the algal species consumption was achieved through Bivariate Correlation, while analysis of variance (one-way ANOVA) was used for examining the biochemical composition. An Independent t-test was utilised to compare the diatom consumption at the top and bottom of the settlement plates during the plate rotation (180^{o} about the horizontal) sequence.

5.1.3 Results

Abalone Growth

At the commencement of the feeding trial there was no significant difference between the average shell lengths of the abalone stocked on the *Ulva* sporeling diet and the commercial nursery diet ($F_{(df\ 1,4)}$=0.317, p=0.604). The juvenile abalone grew on both diets with the *Ulva* sporeling diet producing significantly larger abalone (shell length) throughout the first 5 weeks ($F_{(df\ 1,28)}$=6.779, p<0.05). During the first 4 weeks the mean weekly increase in shell length of the abalone on the *Ulva* sporeling diet was 0.51 ± 0.1 mm, with each increase shown to be significant (Table 15). The abalone on the commercial nursery diet only averaged a weekly increase in shell length of

0.41 ± 0.1 mm for the 4 weeks, but were able to maintain significant increases in shell length until week 7 (Table 15). This extended period of significantly greater growth resulted in the abalone on the commercial nursery diet surpassing the size (shell length) of the abalone on the *Ulva* sporeling diet. This transition can be seen in Figure 23 where the two growth profiles intersect between week 5 and 6. The commercial nursery diet then proceeded to yield significantly larger abalone (shell length) from week 6 to the end of the feeding trial ($F_{(df\ 1,46)}$=24.671, $p<0.05$).

The growth rates of juvenile abalone were fastest (reaching nearly 100 μm.day^{-1}) on both the *Ulva* sporeling diet and the commercial nursery diet during week 3 to 6 of the trial (Table 16). The abalone growth rates for the first 2 weeks of the feeding trail have been excluded to account for a weaning period. The *Ulva* sporeling diet achieved a greater abalone specific growth rate during the initial stages of the trial, reaching a maximum of 1.5 % length.day^{-1} at week 4 (Figure 24). After week 8 of the feeding trial abalone shell length was not significantly different between adjacent weeks on either diet, apart from week 10 for the abalone on the commercial nursery diet (Table 15), which produced a reduction in growth rate (Table 16) and specific growth rate (Figure 24). From Table 16 it was evident that the *Ulva* sporeling diet resulted in slightly slower growth rates during the entire feeding trial ($F_{(df\ 1,82)}$=0.583, p=0.448). Subsequently, the specific growth rate of the juvenile abalone throughout the 14-week trial was not significantly affected by diet ($F_{(df\ 1,82)}$=1.968, p=0.164), with both diets producing abalone specific growth rates of approximately 1 % length.day^{-1} by the end of the trial (Figure 24). The juveniles consuming the *Ulva* sporeling diet were smaller in shell length at the completion of the trial with an average of 9.61 ± 0.1 mm, compared to the 10.29 ± 0.1 mm the abalone on the commercial nursery diet achieved (Figure 23). The final abalone shell lengths were significantly different, indicating that the commercial nursery diet produced significantly larger abalone over the 14-week feeding trial ($F_{(df\ 1,4)}$=8.218, $p<0.05$).

Table 15: Weekly growth in shell length (mm) of juvenile *Haliotis laevigata* during the 14-week (wk) feeding trial, on an *Ulva* sporeling diet and the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet, shown by the mean difference (mm shell length) and significance (Univariate Analysis of Variance ($F_{(df\ 1,82)}$=2.212, p<0.05 (Tukey, p-value))) (n=3).

Wk Interval	**Commercial Nursery Mean Difference** (mm)	**Significance** (p-value)	***Ulva* Sporeling Mean Difference** (mm)	**Significance** (p-value)
Start – 1	0.00	1.000	0.24	0.904
1 – 2	0.40	0.018	0.40	0.019
2 – 3	0.67	0.000	0.67	0.000
3 – 4	0.56	0.019	0.72	0.000
4 – 5	0.80	0.000	0.52	0.052
5 – 6	0.60	0.009	0.44	0.212
6 – 7	0.70	0.000	0.59	0.010
7 – 8	0.44	0.219	0.34	0.636
8 – 9	0.46	0.151	0.41	0.331
9 – 10	0.39	0.663	0.50	0.085
10 – 11	0.66	0.002	0.41	0.322
11 – 12	0.38	0.461	0.47	0.143
12 – 13	0.23	0.972	0.08	1.000
13 – 14	0.25	0.949	0.08	1.000

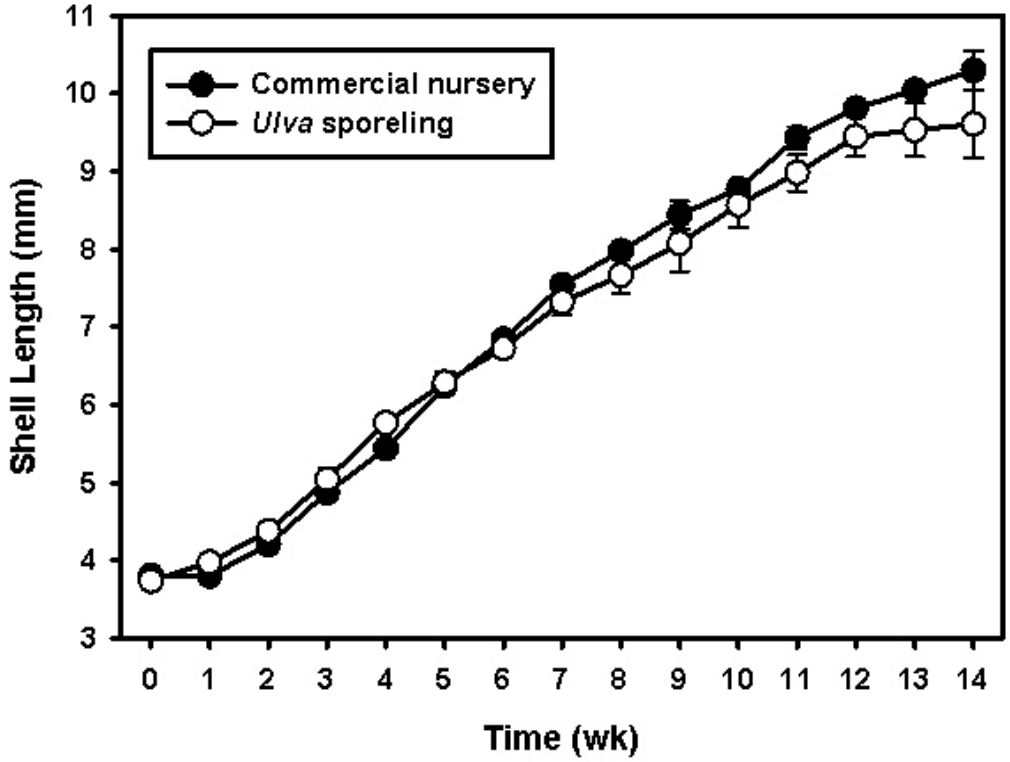

Figure 23: Growth in shell length (mm) of juvenile *Haliotis laevigata* during the 14-week (wk) feeding trial, on the *Ulva* sporeling diet and the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet. Mean ± std. error (n=3).

Table 16: Growth rates (µm.day^{-1}) of juvenile *Haliotis laevigata* for 3 months during the 14-week (wk) feeding trial, on the *Ulva* sporeling diet and the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet. Mean ± std. error (n=3).

Diet	Wk 3 – 6	Wk 7 – 10	Wk 11 – 14
Commercial nursery	94.1 ± 5.0	68.2 ± 7.5	52.9 ± 11.7
Ulva sporeling	84.1 ± 9.5	66.1 ± 8.7	35.7 ± 11.8

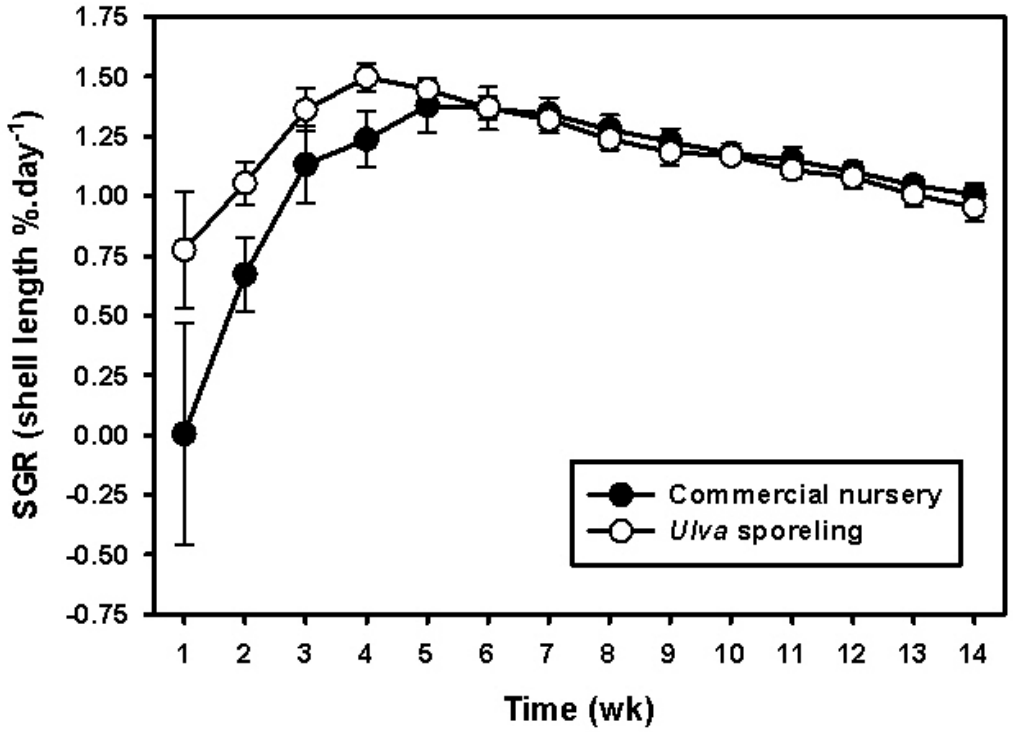

Figure 24: Specific growth rate (% length.day^{-1}) of juvenile *Haliotis laevigata* during the 14-week (wk) feeding trial, on the *Ulva* sporeling diet and the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet. Mean ± std. error (n=3).

Abalone Survival

In conjunction with growth, mortality and subsequently abalone density are important in comparing the two diets' effectiveness as a nutrition source for juvenile abalone. Weekly mortality on both diets exhibited similar profiles with the commercial nursery diet producing an average of 91 mortalities in week 3, but thereafter the *Ulva* sporeling diet recorded slightly higher mortalities until week 10 (Table 17). Calculating the progressive abalone density from the weekly mortality, it was found that there was no significant difference in abalone density between the two diets ($F_{(df\ 1,82)}$=0.569, p=0.453). Even though the survival of abalone at the end of the 14-week trial was lower on the *Ulva* sporeling diet (38.2 %) compared to the commercial nursery diet (46.9 %), the difference was not significant ($F_{(df\ 1,4)}$=3.911, p=0.119).

Table 17: Weekly mortality (individual number) of juvenile *Haliotis laevigata* during the 14-week (wk) feeding trial, on the *Ulva* sporeling diet and the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet. Mean ± std. error (n=3).

Wk	Commercial Nursery	*Ulva* Sporeling
1	63 ± 5.7	71 ± 5.8
2	81 ± 23.2	85 ± 22.1
3	91 ± 22.4	56 ± 6.4
4	64 ± 14.2	72 ± 12.0
5	53 ± 3.2	57 ± 3.5
6	27 ± 2.1	59 ± 22.2
7	16 ± 3.0	45 ± 11.3
8	18 ± 2.5	28 ± 2.3
10	24 ± 1.7	25 ± 1.7
12	17 ± 4.6	12 ± 2.0
14	15 ± 1.8	12 ± 0.9

Algal Consumption

The juvenile abalone consumed entire *Ulva* sporelings (i.e. both blade and attachment regions) as demonstrated by the sections of cleared settlement plate in Figure 25. During the first month of the feeding trial consumption of sporelings peaked at 500 sporeling blade.abalone^{-1}.day^{-1}, but by week 6 the consumption had decreased to 100 sporeling blade.abalone^{-1}.day^{-1}. Consumption gradually increased after that (week 6), doubling by the end of the feeding trial (Figure 26). During the last 2 months there was a significant, positive correlation between the increase in *Ulva* sporeling consumption and the increase in abalone shell length ($R=0.583$, $p<0.05$).

The consumption of *U. lens* followed a similar trend and was significantly, positively correlated ($R=0.422$, $p<0.05$) to the *Ulva* sporeling consumption, but with a reduced rate of decline after the initial period of high consumption (Figure 27). Consumption of *U. lens* also correlated significantly with the growth rate ($R=0.544$, $p<0.05$) and subsequently the specific growth rate ($R=0.618$, $p<0.05$) of the juvenile abalone.

Diatom consumption showed a similar profile to that of the *Ulva* sporelings and *U. lens* consumption, including the slow increase after week 7 (Figure 28). However, the diatom consumption was "negative" or zero diatom.abalone^{-1}.day^{-1} for week 7 to 12, because the diatoms divided (reproduced) faster than they were consumed by the abalone. Diatom consumption did not correlate with either the *Ulva* sporeling or the *U. lens* consumption, nor did it relate to abalone growth. During the feeding trial *N. jeffreyi* was re-inoculated and the PVC plates rotated as illustrated in Figure 28 and Table 18, with the consumption of diatoms corresponding to the plate rotation rather than the re-inoculation. The diatom consumption by abalone on the intermediate sample plates at the top and bottom of the settlement plates were statistically different ($t_{(df\ 41)}$=-2.986, $p<0.05$), indicating that rotating the settlement plates had a considerable effect on the consumption of *N. jeffreyi*.

Two other species of diatom, *Melosira* cf. *moniliformis* and *Synedra* sp. contaminated the *Ulva* sporeling diet treatment at various stages (Table 18). These diatoms were present throughout the 14-week period, however they only bloomed at the top of the plates on three separate occasions (start, week 11 and week 13). The contamination was quickly removed by physical detachment (abrasion) from the substratum and then siphoning the nursery tank contents.

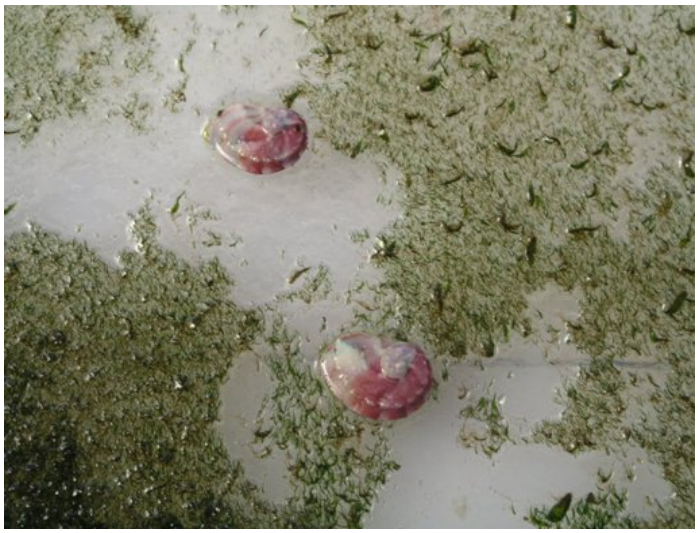

Figure 25: The *Ulva* sporeling diet being consumed by juvenile *Haliotis laevigata* (5 mm shell length). Note the cleared area of settlement plate, indicating the abalone ingest both the sporeling blade and attachment regions.

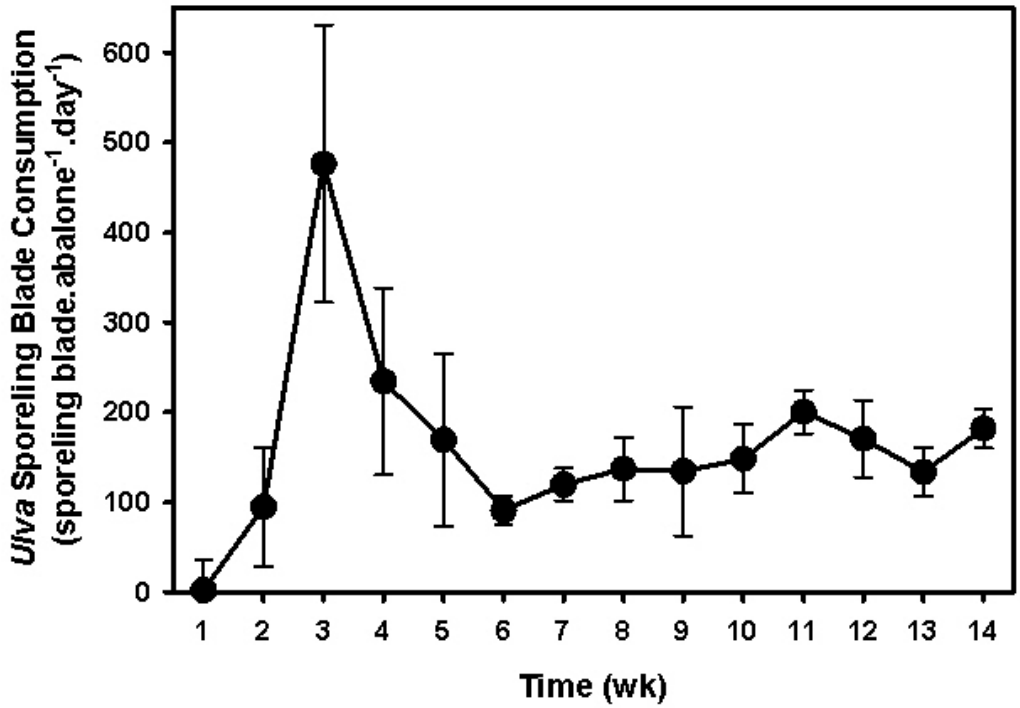

Figure 26: The consumption rate (sporeling blade.abalone^{-1}.day^{-1}) of *Ulva* sporeling blades by juvenile *Haliotis laevigata* during the 14-week (wk) feeding trial. Mean ± std. error (n=3).

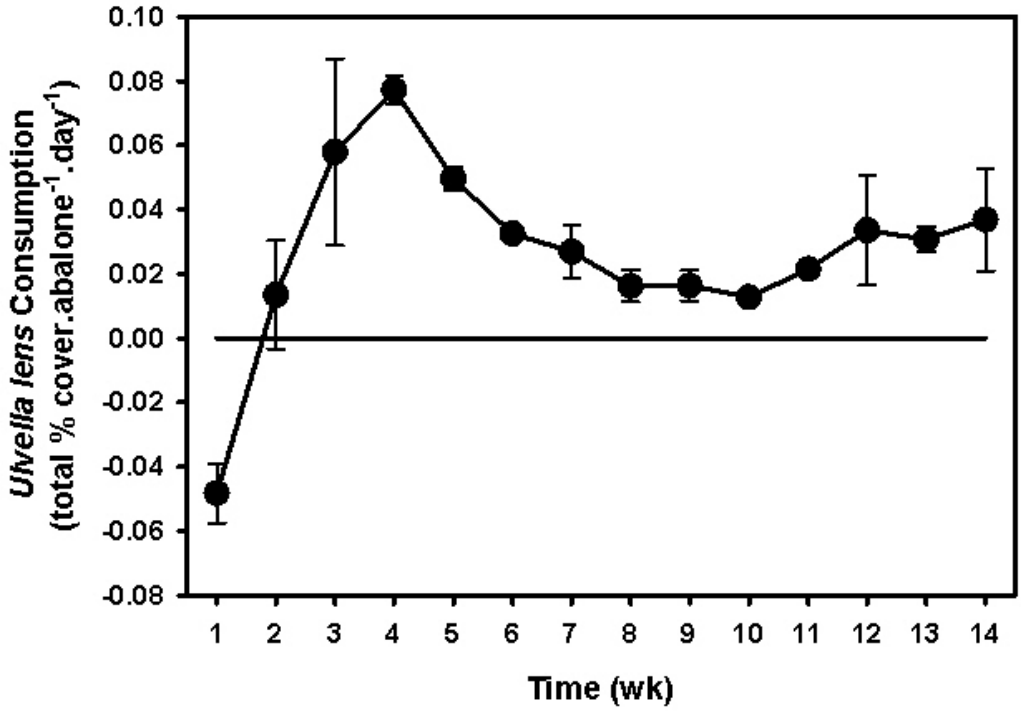

Figure 27: The consumption rate (% cover.abalone^{-1}.day^{-1}) of *Ulvella lens* by juvenile *Haliotis laevigata* during the 14-week (wk) feeding trial. Mean ± std. error (n=3).

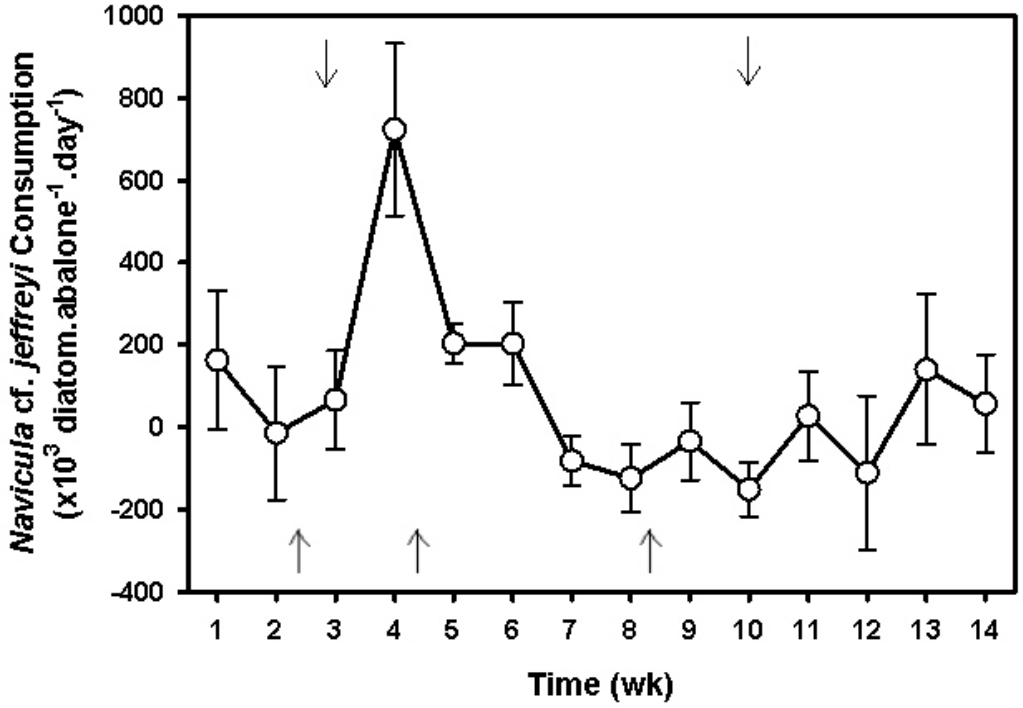

Figure 28: The consumption rate (x10^3 diatom.abalone^{-1}.day^{-1}) of *Navicula* cf. *jeffreyi* by juvenile *Haliotis laevigata* during the 14-week (wk) feeding trial. Mean ± std. error (n=3). The arrows at the top indicate when plate rotation occurred and the arrows at the bottom indicate inoculation.

Biochemical Composition of Algal Diets

The approximate biochemical composition of the *Ulva* sporeling diet and the commercial nursery diet has been shown in Table 19. Even though the lipid, protein and carbohydrate contents were slightly higher in the *Ulva* sporeling diet, these were not significantly different to the commercial nursery diet biochemical composition. However, the *Ulva* sporeling diet's ash weight by deduction (15.64 %) was significantly lower than the commercial nursery diet's ash weight by deduction (35.5 %) (Table 19).

Table 18: The occasions (week) when inoculation of *Navicula* cf. *jeffreyi* occurred, the plates were rotated and contaminant diatom species were observed, including the contaminants relative size (µm).

	1st	2nd	3rd
Inoculation	Week 2	Week 4	Week 8
Plates Rotated	Week 3	Week 10	
Contamination Present			
Melosira cf. *moniliformis*	Before Start	Week 11	Week 13
Synedra sp.	Before Start	Week 11	Week 13
Size of Contaminating Algae	Cell Length	Cell Width	(µm ± SE)
Melosira cf. *moniliformis*	109.5 ± 2.09	8.9 ± 0.29	
Synedra sp.	21.5 ± 0.81	19.7 ± 1.42	

Table 19: The biochemical composition of the *Ulva* sporeling diet and the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet. Values are on dry matter basis and expressed as g/100 g dry weight. Ash weight was determined by deduction of the other biochemical components. One-way ANOVA on means ± std. error (n=3).

Diet	Ash	Lipid	Protein	Carbohydrate
Commercial nursery	35.50 ± 3.03	4.48 ± 0.98	24.17 ± 1.93	35.85 ± 1.74
Ulva sporeling	15.64 ± 2.72	7.12 ± 1.21	32.30 ± 2.42	44.95 ± 4.30
df	1, 4	1, 4	1, 4	1, 4
F	23.775	2.862	6.917	3.850
p value	<0.05	0.166	0.058	0.121

5.1.4 Discussion

The alternative juvenile macroalgal diet of *Ulva* sporelings was comparable to the current commercial nursery diet consisting of *U. lens* and *N. jeffreyi* for the growth and survival of juvenile *H. laevigata*. The two diets produced similar abalone growth rates and specific growth rates, but the commercial nursery diet did result in significantly larger abalone at the completion of the 14-week feeding trial. Juveniles feeding on the commercial nursery diet reached 10 mm shell length in less than 13 weeks, whereas on the *Ulva* sporeling diet the juveniles only reached 9.61 mm shell length at week 14. Daume & Ryan (2004) found that for *H. laevigata* of a similar initial size (4 mm shell length) it took less than 15 weeks to reach 10 mm shell length on just *U. lens* with a stocking density of approximately 50 animals per plate.

The transition at week 5, between the *Ulva* sporeling diet and commercial nursery diet producing significant larger abalone, indicates that *Ulva* sporelings were a better diet for *H. laevigata* in the size range of 3.5 to 6.5 mm shell length. The failure to sustain a growth advantage through to week 14 signifies better performance of the commercial nursery diet for *H. laevigata* in the size range of 6.5 to 10 mm shell length. The *Ulva* sporeling diet can therefore, be considered an acceptable commercial diet for juvenile greenlip abalone (<6.5 mm shell length) and employed as either an alternative or in conjunction with the current commercial nursery diet. The use of *Ulva* sporelings for smaller juvenile abalone could allow the diet to be incorporated from the start of the nursery phase (settlement). Green macroalgal species including *Ulva australis* and *Ulva compressa* have been shown to induce good settlement rates for *Haliotis rubra* (Huggett *et al.* 2005), while settlement and early growth of *Haliotis tuberculata coccinea* can be possible on a combination of diatom species inoculated with *Ulva rigida* sporelings and *U. lens* (Courtois de Vicose *et al.* 2012).

Growth rates of *H. laevigata* fed the *Ulva* sporeling diet were not significantly different from those produced on the commercial nursery diet. The growth rate profile was similar to that obtained by Daume & Ryan (2004) utilising *U. lens*, where once the first 2 weeks were removed the next 6 weeks gave abalone growth rates of 84 $\mu m.day^{-1}$

and the final 6 weeks, 63 μm.day^{-1}. This decline in growth rate over time was also present, all be it at a slightly faster rate between the first 2 months of a trial using 6 mm shell length juvenile *H. laevigata* at the same stocking density, feeding on *U. lens* (Daume *et al.* 2007). *Haliotis varia* of 3 – 4 mm shell length have also shown a decreasing trend in growth rates from 88 to 25 μm.day^{-1} over 129 d when fed small pieces of *Ulva lactuca* (Najmudeen & Victor 2004).

The growth rates during the first 2 weeks of this trial were excluded from the analysis in Table 16 as any nutrient deficiency in a diet may be masked by the ability of the abalone to utilise its own stores (Fleming *et al.* 1996). As the juvenile abalone were taken off an *U. lens*/naturally occurring diatom diet, the weaning process was considered minimal compared to the recommendation of approximately 50 d (Day & Fleming 1992). However, it was important to run the subsequent trial (Multi Diet and System Feeding Trial, Section 5.2) for as long as possible to detect any effects of nutrient limitation, and determine the capacity of an algae diet to maintain acceptable abalone growth throughout the later nursery phase.

The *Ulva* sporeling diet was able to produce abalone growth rates of nearly 100 μm.day^{-1} during week 3 to 6 of the feeding trail. This was comparable to the growth rates of 3 – 4 mm shell length *Haliotis discus discus* attained over 4 weeks on a variety of macroalgal species sporelings, including the brown algae *Colpomenia sinuosa* and *Ectocarpus siliculosus* as well as the green alga *Enteromorpha* spp. (Maesako *et al.* 1984). Takami *et al.* (2003) also showed that *Haliotis discus hannai* of approximately 1.8 – 2.2 mm and 2.8 – 2.9 mm shell length could reach growth rates of 80 and 100 μm.day^{-1} on juvenile sporophytes of the kelp *Laminaria japonica*.

The juvenile abalone specific growth rates reached over 1.3 % length.day^{-1} and finished at 1 % length.day^{-1} on both the *Ulva* sporeling and commercial nursery diets, with no significant difference between them. The *Ulva* sporeling diet did produce a specific growth rate of 1.5 % length.day^{-1} at week 4 but then slowly declined. Other

studies have also found that *Ulva* spp. can produce adequate abalone specific growth rates. For example, Corazani & Illanes (1998) reported that *H. discus hannai* had a higher specific growth rate (0.69 % length.day^{-1}) utilising adult *U. rigida* than other macroalgal diets, while *Haliotis rufescens* achieved the lowest specific growth rate. This was comparable to the 0.71 % length.day^{-1} achieved by *H. discus hannai* on an *Ulva* diet (Uki & Watanabe 1992). *Ulva lactuca* has been found to have reasonable dietary value for *Haliotis tuberculata* (1.16 % length.day^{-1}) but was significantly lower for *H. discus hannai* (0.75 % length.day^{-1}) (Mai *et al.* 1996). *Haliotis iris* was only able to achieve specific growth rates of 0.1 % length.day^{-1} on *U. lactuca* (Stuart & Brown 1994), while Simpson & Cook (1998) found that the suitability of *Ulva* as a feed was dependent on the abalone species.

The nutritional quality of the *Ulva* being used in the present study was not manipulated by nutrient enrichment using a nitrogen source to increase the *Ulva* protein content during the 14-week feeding trial. Enriched *U. rigida* has been shown to produce growth rates of juvenile *Haliotis roei* comparable to those achieved on the best performing artificial diets (Boarder & Shpigel 2001). Taylor & Tsvetnenko (2004) showed that only 15 mg N.L^{-1} enriched *U. rigida* resulted in significantly higher specific growth rates than non enriched *U. rigida*. Shpigel *et al.* (1999) reported growth rates of 44.47 and 121.47 μm.day^{-1} for *H. discus hannai* and *H. tuberculata* respectively, on a high ammonia-N enriched *U. lactuca* compared with 31.7 and 80.72 μm.day^{-1} on low ammonia-N enriched *U. lactuca*. The growth rate for *H. tuberculata* produced on the high-ammonia-enriched *U. lactuca* (Shpigel *et al.* 1999) was the only abalone growth rate achieved on an *Ulva* species in the reviewed literature, which exceed that obtained on the *Ulva* sporeling diet in the present study.

During the algal culturing process before the feeding trial began, the *Ulva* sporelings were grown in a modified f/2 medium containing only the $NaNO_3$, $NaH_2PO_4.H_2O$ and $FeCl_3.6H_2O$ stock components (Section 2.2.2). The elevation in nutrients (most likely protein) at the start may have led to the significantly larger abalone size during the first 5 weeks and in turn, the maximum specific growth rate of

1.5 % length.day^{-1} at week 4. After this point of the trial the growth rate decreased, which could be related to the *Ulva* sporelings no longer providing adequate nutrients to the juvenile abalone. By not supplying the *Ulva* sporelings during the trial with nutrients like the modified f/2 medium used before the trial, the diet may have become deficient in a particular nutrient (most likely protein) important to abalone growth. Therefore, potential benefits may exist in culturing nutrient enriched *Ulva* sporelings to achieve the best juvenile abalone growth rates, a protocol that was subsequently integrated into the Multi Diet and System Feeding Trial (Section 5.2).

The reduction in abalone weekly growth to only 0.08 mm on the *Ulva* sporeling diet and 0.24 mm on the commercial nursery diet during the last 2 weeks, may indicate that the two nursery diets were no longer able to supply the juvenile abalone with the specific nutrients required for adequate performance. *Haliotis rubra* has been shown to achieve steady growth for 100 d and then fail to grow further on some mono-species algal diets (Day & Fleming 1992). Lack of algal biomass could also have became a problem towards the end of the feeding trial as the *Ulva* sporeling density and the *U. lens* cover were reduced to 43 % and 25 % of their original biomass, respectively. At this point of the feeding trial (week 12) when the required nutrients or algal biomass may have become inadequate, both diets had produced abalone of approximately 9.5 mm shell length and could be weaned onto artificial diets (Fleming *et al.* 1996, Dunstan *et al.* 2002). An alternative to weaning could be to incorporate new seeded plates into the system to reduce competition for food as well as the stress caused by handling (Fleming 1995b, Daume *et al.* 2004). Daume *et al.* (2004) utilised this procedure for *U. lens*, which enabled the high, initial growth rates of newly settled *H. rubra* to be maintained for 114 d. The introduction of new seeded plates when the juvenile abalone are 9 –10 mm shell length should allow the animals to maintain adequate commercial growth rates throughout the entire later nursery phase.

To address both the nutrient limitation associated with a monospecies diet and the lack of biomass caused by the high grazing pressure of 9 – 10 mm shell length abalone, it would be beneficial to incorporate the *Ulva* sporeling diet as part of a mixed

or rotational diet. The incorporation of a second suitable algal species may account for the nutrient deficiencies in the monospecies diet, while also increasing the algal biomass available (Day & Fleming 1992, Fleming 1995b, Simpson & Cook 1998). The plate design of the nursery system for feeding juvenile abalone would be well suited to diet rotation or a mixed diet regime, whereby plates seeded with different diets can be interspersed throughout the tanks. Stuart & Brown (1994) and Simpson & Cook (1998) demonstrated that *Ulva* as a monospecies diet produced the slowest abalone growth rates, but when presented in a rotational/mixed diet it sustained excellent growth rates. Dang *et al.* (2011) also found that *H. laevigata* fed single macroalga species (e.g. *U. lactuca*) had the slowest growth rates when compared to combination diets.

The consumption rate of *Ulva* sporelings was extremely high during the first month and so the abalone grew rapidly. However, once the consumption rate declined so did the growth rates. Hone (1992) showed that *U. australis* was rapidly consumed by abalone, while Simpson & Cook (1998) found that *Ulva* had the lowest consumption in $g.abalone^{-1}.day^{-1}$ compared with five other macroalgae and subsequently, produced the slowest growth rates for *Haliotis midae*. Boarder & Shpigel (2001) also reported that N-enriched *U. rigida* had the lowest consumption rate, but was still able to produce growth rates of *H. roei* comparable to those achieved on some of the best artificial diets. As mentioned before, ammonia-N enriched *U. lactuca* produced the highest growth rates for both *H. discus hannai* and *H. tuberculata* but these rates were recorded while consuming significantly less biomass (Shpigel *et al.* 1999). This indicates the rate of *Ulva* sporeling consumption by abalone can affect the abalone growth rate, while nutrient-enriched *Ulva* produces faster growth rates with less biomass required to do so.

The juvenile abalone may have had a similar preference for these two species of green algae, given the consumption of *Ulva* sporelings correlated significantly with the consumption of *U. lens*. This could be understandable given the biochemical components (lipid, protein, carbohydrate) of the two diets were not statistically different. It was difficult to compare these two diets with the approximate composition of algal species in other studies as they are expressed as ash weight by deduction and

not ash-free dry weight (Lewin & Guillard 1963). The algal samples were washed with 10 mL ammonium formate solution (0.65 M) to reduce the problems associated with dry weight comparison. Even with this alteration, the *Ulva* sporeling diet composition was nearly identical to that of *U. rigida* when enriched from 11.4 % to 32.2 % protein by using high nutrient water (5 $gN.m^{-2}.day^{-1}$; 0.6 $gP.m^{-2}.day^{-1}$), which as a diet recorded a specific growth rate of approximately 0.14 % $length.day^{-1}$ for *H. roei* of 32 mm shell length (Boarder & Shpigel 2001). The *Ulva* sporelings being 3-dimensional juvenile macroalgae are in a phase of high growth and may be able to utilise the limited nutrient supply in the water more effectively than mature *Ulva* thalli.

The high abundance of the diatom *N. jeffreyi* resulted in the commercial nursery diet having a significantly higher ash content by deduction than the *Ulva* sporeling diet. The biochemical composition of diatoms as a monospecies diet (>50 % DW) has been shown to have considerably higher ash weights but lower lipid, protein and carbohydrate components, when compared to composite diets of diatom species mixed with *U. rigida* sporelings and *U. lens* (<40 % DW) (Courtois de Vicose *et al.* 2012). Diatoms have high ash content because of their silica cell walls and due to differences in thickness of the silicified cell walls can vary both inter and intra-specifically (Lewin & Guillard 1963). Three diatom species shown to induce settlement (Gordon *et al.* 2004), produce good growth and survival of postlarval *H. discus hannai* ranged in ash content from 28.36 % to 41.56 % DW (Gordon *et al.* 2006), which was similar to the commercial nursery diet ash content by deduction in this trial. The biochemical composition of *N. jeffreyi* has been shown to be 18 % lipid, 33 % protein and 4.9 % carbohydrate dry weight (Brown & Jeffrey 1995). However, biochemical composition can vary considerably between diatom species, even when grown in the same medium and harvested at the same phase of growth (Brown 1991, Brown *et al.* 1997). Therefore, an abalone nursery diet containing *U. lens*, diatoms and biofilm components could have even greater variation in biochemical composition.

The commercial nursery diet with lower levels of lipid, protein and carbohydrate than the *Ulva* sporeling diet produced significantly larger abalone at the end of the

14-week feeding trial. This may suggest that higher amounts of biochemical components do not necessarily increase growth, but rather an optimal level of these components may be responsible. Lipid levels of 3 – 5 % have been shown to be optimal for abalone, which corresponds with the commercial nursery diet while the *Ulva* sporeling diet had a greater lipid content (Uki & Watanabe 1992, Mercer *et al.* 1993, Mai *et al.* 1995b, Dunstan *et al.* 2000). Higher levels of dietary lipid (>5 %) can be detrimental to abalone growth and are thought to depress the digestibility of components such as fatty acids and amino acids (Uki & Watanabe 1992, Britz & Hecht 1997, Van Barneveld *et al.* 1998, Bautista-Teruel *et al.* 2011). Therefore, the high lipid level present in the *Ulva* sporeling diet (7.12 %) may have inhibited the maximum potential growth rates achievable by abalone during the trial.

The protein content of the commercial nursery (24.17 %) and *Ulva* sporeling (32.3 %) diet were both slightly outside the optimal protein levels of 27 – 28 % for abalone, however these levels can range from 20 to 35 % depending on abalone species (Uki & Watanabe 1992, Mai *et al.* 1995a, Bautista-Teruel & Millamena 1999, Coote *et al.* 2000, Vandepeer & Van Barneveld 2002). *Haliotis midae* and *Haliotis fulgens* have shown increases in growth with an increase in protein up to 47 and 44 % respectively (Britz 1996b, Gómez-Montes *et al.* 2003), while similar levels have corresponded to greater shell production (wider and heavier) in juvenile *H. iris* (Tung & Alfaro 2011). The range in crude protein requirements for abalone reported in the literature may vary with a number of factors including abalone size, feeding trial length, digestible protein to energy ratios, amino acid pattern, culture surroundings or the statistical analysis methods used (Lee 2004). However, to maximise protein utilisation the diet should not only contain sufficient, readily digestible protein but also a well-balanced mixture of essential and non–essential amino acids (Mai *et al.* 1994, Britz & Hecht 1997, Daume *et al.* 2003).

The commercial nursery diet had a carbohydrate component (35.85 %) much closer to the carbohydrate levels of 20 – 30 % that are considered suitable for abalone growth (Mercer *et al.* 1993), than the *Ulva* sporeling diet (44.95 %). However, the

required carbohydrate levels can vary depending on species, for example artificial diets containing 40 and 47.8 % carbohydrate are recommended for *Haliotis asinina* (Bautista-Teruel & Millamena 1999, Thongrod *et al.* 2003). Carbohydrate serves as the preferential energy source for abalone along with a consistent amount of dietary protein, while lipids levels need to be minimised to allow digestibility of other nutrients (Montaño-Vargas *et al.* 2005). Overall the commercial nursery diet may have had a biochemical composition closer to the optimum requirements for juvenile *H. laevigata* than the *Ulva* sporeling diet and subsequently, produced larger animals at the conclusion of the feeding trial.

The *N. jeffreyi* consumption did not correlate with either of the other 2 algal species or any of the abalone growth results. Week 7 to 10 and 12 all showed "negative" consumption, indicating the *N. jeffreyi* cells were dividing faster than the abalone could consume them. The consumption rate of *N. jeffreyi* did not correspond with the re-inoculation, but rather the rotation of the settlement plate's 180^{o} about the horizontal. There are two reasons for this; firstly the light gradient through the tanks allowed *N. jeffreyi* situated at the top of the plates to receive more light, consequently dividing faster and secondly, changes in grazing pressure due to the light sensitivity/migration of abalone. These hypotheses were supported by visual observation during the trial as diatom counts increased on the sample plates at the top and juvenile abalone where found on the bottom of the tanks during the day. Daume *et al.* (2004) also indicated that the light intensity tended to be higher at the top of plates and migration to the bottom of the tank by abalone was evident, while positive relationships between feed intake and the duration of darkness have also been shown (Dixon 1992). Settlement plate rotation allowed the high densities of diatoms to be closer to the abalone, reducing the effort expended by abalone to graze. When the plates were rotated at week 3 the high *N. jeffreyi* densities at the top were transferred to the bottom of the tank. This caused the high diatom densities to be closer to the majority of abalone and subsequently a spike in consumption occurred. *Navicula* cf. *jeffreyi* consumption then dropped as the density at the bottom declined, while the density increased at the top due to greater light intensity and less grazing pressure. At week 7 the consumption of *N. jeffreyi* became "negative" because the growth at the top exceeded consumption at the bottom of the plates. Once the second rotation at week 10 was performed the consumption began to increase again

as the high *N. jeffreyi* density was available to the juvenile abalone at the bottom of the tank. Given the benefits of rotating the diatom-cultured plates to maintain high diatom densities close to where the majority of juvenile abalone reside, this practice was integrated into subsequent juvenile abalone feeding trials.

The contaminating diatom species only occurred in the *Ulva* sporeling diet tanks and were present from the creation of the diet. It was not determined if the juvenile abalone utilised these contaminating diatoms as a food source, although *Synedra* was probably of a suitable size. It would have been difficult for the juveniles to deal with the *M. moniliformis*, because along with its large cell size it formed into dense mats with chains exceeding 5 cm in length. The large blooms of *M. moniliformis* that occurred at week 11 and 13 may have had some impact on the declining abalone growth rates, as juvenile abalone are susceptible to smothering and entanglement (Daume *et al.* 2004).

The cooling in seawater temperatures from 20.8^{o}C to 19^{o}C during the trial correlated with the decrease in abalone growth rate on both diets. The colder water temperatures may have resulted in reduced metabolic activity of the juvenile abalone, contributing to slower growth rates. Daume *et al.* (2007) also found that growth rates correlated with mean seawater temperatures, with juvenile *H. laevigata* (6 mm shell length) growth rates highest at the start of a feeding trial when seawater temperature averaged 20.1^{o}C, then decreased after the first month coinciding with a decline in seawater temperature.

Overall, *Ulva* sporelings are a suitable diet for juvenile *H. laevigata* as they produced comparable growth rates and specific growth rates to the commercial nursery diet of *U. lens* and *N. jeffreyi* currently used in Australian abalone aquaculture. However, the diet was not able to overcome the substantial grazing pressure of juvenile abalone in the later nursery phase and further investigations into the theoretical and procedural principles behind the development of the *Ulva* sporeling diet are required. By utilising *Ulva* sporelings in a mixed sporeling diet the nutrient limitation associated

with monospecies diets may be avoided. Incorporating new seeded plates into the nursery system could be a protocol to combat the grazing pressure of abalone greater than 5 mm shell length and allow the juvenile greenlip abalone to remain in the nursery system past the 9 – 10 mm shell length achieved in this trial.

5.2 MULTI DIET AND SYSTEM FEEDING TRIAL

5.2.1 Introduction

The use of macroalgal sporelings (*Ulva* sporelings) as a diet has already demonstrated the ability to produce juvenile abalone growth rates of nearly 100 $\mu m.day^{-1}$ and was considered comparable to the current commercial nursery diet of *U. lens* and *N. jeffreyi* (Section 5.1.3). However, the diet was unable to maintain commercially viable juvenile abalone growth rates in the nursery system for animals greater than 9 – 10 mm shell length. A combination of nutrient limitation due to the monospecies nature of the diet and an inability to support the substantial algal biomass requirements of juvenile abalone greater than 5 mm shell length restricted the effectiveness of the diet.

To maintain juvenile abalone throughout the entire later nursery phase (5 – 15 mm shell length) a mixed sporeling diet of *Ulva* and other macroalgal species was identified, along with the incorporation of new seeded plates as algal biomass becomes inadequate (Section 5.14). A combination diet of *Ulva* sporelings, *U. lens* and *N. jeffreyi* has produced significantly larger juvenile *H. laevigata* than the current commercial nursery diet (Daume *et al.* 2007). Juvenile brown macroalgae have exhibited potential as an alternative nursery diet for several abalone species including *Haliotis diversicolor* and *H. discus hannai*, but have not yet maintained suitable juvenile abalone growth rates for extended periods of time (Takami *et al.* 2003, Onitsuka *et al.* 2010, 2011). Given the preference of Australian abalone for red macroalgae (Rhodophyta) over brown macroalgae (Phaeophyceae), it would be beneficial to utilise red macroalgal sporelings in the nursery system.

Research into the propagation methods of Rhodophyta species identified carpospore release of *Hypnea* through temperature and dark induction treatments (Section 3.3.1), as a suitable macroalgae and protocol for developing a sporeling diet on the PVC settlement plates in the nursery system. The establishment of *Hypnea* fragment culture by vegetative propagation produced fast growth rates and high survival (Section 3.3.3), indicating the species robust nature and suitability for use in aquaculture. Adult *Hypnea* species thallium's grown in flow-through integrated systems (nutrient enriched) have been shown to produce growth rates of 80 to 126 $\mu m.day^{-1}$ for juvenile *H. tuberculata coccinea* (11 mm shell length) (Viera *et al.* 2005). To reduce the amount of *Hypnea* carposporophytic biomass required to produce adequate carpospore numbers and subsequently sporeling densities, incorporating *Ulva* sporelings as a combination diet could render red macroalgal sporelings suitable for commercial use. Given that sporelings may have different nutritional properties to adult macroalgae and with the inclusion of nutrient enrichment, the incorporation of *Hypnea* sporelings could provide the juvenile abalone with a greater range of dietary nutrition, consequently producing faster growth rates with greater survival during the later nursery phase.

The use of non-toxic, encrusting, cyanobacterium as an alternative algal diet may address the inadequate feed biomass due to the high grazing pressure of juvenile abalone in the later nursery phase. Cyanobacteria can act as a food source for molluscs and as production of algae can be costly (Sharma *et al.* 2011), genera such as *Phormidium* could be suitable for juvenile abalone nutrition. *Phormidium* are a red-pigmented, epiphytic, Cyanophyceae that can be found inhabiting deeper waters (Pintner & Provasoli 1958) where wild greenlip abalone reside (≈20 m). These "blue-green algae" are very fast growing, have simple growth needs, rapidly absorb nutrients and are a good source of proteins (Fujimoto *et al.* 1997, Sharma *et al.* 2011). Their ease of culture would reduce the expertise and labour required by the abalone nursery to utilise *Phormidium* as a diet. As the algae are fast growing it may have greater recovery post grazing than the current commercial nursery diet and consequently, be able to maintain sufficient algal biomass under the intense grazing pressure of juvenile abalone in the later nursery phase.

A whole macroalgal thalli diet was developed for the weaner system to examine the juvenile abalone nutritional transition from a diatom-based diet to a macroalgae-based diet. This whole seaweed diet in the weaner system may be a more suitable method of presenting the desired macroalgae, than seeding sporelings into the nursery system. The diet also allows a direct comparison of an adult macroalgal diet to an artificial diet in the weaner system, rather than relying on macroalgal fragments adhered to the vertical plates in nursery tanks (Section 4.2).

The alternative management strategy of weaning juvenile abalone greater than 8 mm shell length onto an artificial diet has been shown to produce significantly larger and faster growing abalone than the commercial nursery diet (*Laurencia* Fragment/Agar Diet Feeding Trial, Section 4.2.3). Even though there was a high initial mortality, utilising the weaner system with an artificial diet was an effective means of rearing juvenile abalone. However, to compare the nursery and weaner systems throughout the later nursery phase (5 – 15 mm shell length) weaning at a smaller size needs to occur as it may affect the juvenile abalone performance on the artificial diet.

To culture juvenile abalone from 5 to 15 mm shell length through the later nursery phase, various potential diets and system designs have been identified and tested in this multi diet and system feeding trial, as to their effect on *H. laevigata* growth and survival. A combination red (*Hypnea*) and green (*Ulva*) macroalgal sporeling diet, as well as a separate Cyanobacteria (*Phormidium*) diet, were both compared to the current commercial nursery diet (*U. lens* plus *N. jeffreyi*) for their suitability as alternative algal diets in the nursery system. These diets' ability to supply adequate algal biomass to accommodate the intensive grazing pressure of juvenile abalone during the later nursery phase, were also evaluated against the management strategy of rearing juvenile *H. laevigata* in a weaner system on two separate diets of whole macroalgal thalli and an artificial feed.

5.2.2 Feeding Trial Experimental Design

Location

The juvenile abalone feeding trial was conducted in the aquaculture facilities at Western Australian Fisheries and Marine Research Laboratories (WAFMRL) in Hillarys, Perth, Western Australia between June and December 2006. Greenlip abalone (*Haliotis laevigata*) were spawned in December 2005 and the juveniles produced by Bay Side Abalone Pty Ltd in Bremer Bay, Western Australia.

Algal Culture – Diets

Mixed Sporeling Diet (*Hypnea* sp. and *Ulva* spp. sporelings)

Hypnea sp. carposporophytes were harvested from the Waylen Bay collection site and transported to the aquaculture facilities at WAFMRL (Section 2.1). Fertile *Hypnea* thalli were placed into plastic trays and covered with moist newspaper, then exposed to three spore release induction treatments as described in the large carpospore production experiment (Section 3.2.1). The temperature and dark treatments were applied simultaneously by storing the trays of *Hypnea* at 4^{o}C in complete darkness for 24 h, while the osmotic pressure treatment was conducted after by hand rinsing the thalli with deionised water for 10 min. Once the three treatments had been administered, 430 g of *Hypnea* carposporophyte thalli were placed on plastic mesh submerged 5 cm under the water surface in a nursery tank. Ninety, 60 x 26 cm PVC plates were arranged vertically below the mesh and light aeration was supplied to the non-flow through seawater (1 µm filtered, ICF). The fertile *Hypnea* thalli were then left overnight (24 h) in the nursery tank to allow carpospore release.

After 24 h the thalli were removed from the nursery tank and the entire spore release induction procedure described above repeated twice more. The *Hypnea* carposporophytes were induced for carpospore release on three consecutive occasions, each with a 24 h release period in the nursery tank, giving a total of 3 d for carpospore release. This multiple induction procedure allowed the carpospore yield attained in the large carpospore production experiment (Section 3.3.1) to be replicated three times

within the nursery tank using the same macroalgal thalli. Before the *Hypnea* thalli were added to the tank on each spore release occasion the PVC plates were rotated 180^{o} about the horizontal to achieve even spore settlement.

Ulva spp. thalli were collected from the East Street Jetty site (Section 2.1) and exposed to the same temperature and dark treatment as in the *Ulva* Sporeling Diet Feeding Trial (Section 5.1.2). Approximately 10 kg of *Ulva* thalli were then placed into each of the three nursery tanks. Each tank had three baskets of ten, 60 x 26 cm PVC plates orientated horizontally with the non-flow through seawater (1 µm filtered, ICF) lightly aerated. The *Ulva* thalli were removed from each tank after a week and the baskets of 10 plates then stood upright in the vertical position.

The *Hypnea* sporeling seeded plates and the *Ulva* sporeling seeded plates were cultured separately for 6 weeks in nursery tanks, with non-flow through seawater (1 µm filtered, ICF) exchanged twice weekly and the algal fertiliser Abasol added at each exchange (Section 2.2). The entire spore release, inoculation and culture procedure described above for both *Hypnea* and *Ulva* was repeated twice, so that the plates in the feeding trial could be replaced with newly seeded sporeling plates every 2 months. Just prior to the commencement of the trial (addition of the juvenile abalone) the separate *Hypnea* and *Ulva* sporeling covered plates were combined into three nursery tanks of 60 plates each. The sporeling-covered plates were alternated, so that it was *Hypnea* sporeling plate, then *Ulva* sporeling plate and so on throughout each nursery tank.

Phormidium sp. Diet

The cyanobacterium (blue-green alga) *Phormidium* sp. was grown on glass microscope slides in 250 mL Petri dishes with f/2 medium (Section 2.2, DFSW) at 18 ± 2^{o}C on a 12 h:12 h, light:dark cycle at 35 µmol photons.m^{-2}.s^{-1}. The *Phormidium* culture was scaled up by placing the microscope slides with the alga attached on the top of nine, intermediate 20 x 15 cm PVC plates orientated vertically in two non-flow through, 6 L glass aquaria with f/2 medium (CFSW) exchanged twice weekly. Once the

Phormidium had attached to these intermediate plates, the plates were then distributed into three nursery tanks with one plate placed across the top of each basket containing twenty, 60 x 26 cm PVC settlement plates (9 baskets total). Placing the stock culture microscope slides and the intermediate PVC plates at the top of the next culture size plates allowed the *Phormidium* to colonise the new plates and therefore, the culture could be scaled up to the nursery system. Three, non-flow through nursery tanks with *Phormidium* culture attached to the plates had the seawater (1 µm filtered, ICF) exchanged twice weekly with the addition of Abasol and light aeration. This inoculation and culture procedure for *Phormidium* was repeated 4 times, to ensure there were new seeded plates available for replacement every month of the trial, commencing after the 2nd month.

Commercial Nursery Diet (*Ulvella lens* plus *Navicula* cf. *jeffreyi*)

The preparation of the commercial nursery diet followed the protocol in General Materials and Methods Section 2.4.4 with only minor alterations. Culture of *N. jeffreyi* began with 3 Petri dishes instead of 1 small bag and were scaled up to 2, 1.5 L medium bags then 1, 60 L large culture bag and finally, 2 Mexican hats. *Navicula* cf. *jeffreyi* cell culture and MAF (Section 2.2.2) were added to the commercial nursery diet tanks on a weekly basis, while the induction protocol to release *U. lens* spores was performed every 2 months.

Whole Macroalgal Thalli Diet (WMT)

Whole thalli of *Gracilaria flagelliformis*, *Hypnea* and *Ulva* were harvested from the various collection sites and transported to WAMRL as described in General Material and Methods Section 2.1. Thalli of the 3-macroalgal species were mixed together and presented in three mesh pouches (40 x 15 cm, 10 mm gauge mesh) within each weaner tank (Figure 29) and fed weekly at a rate of 5 % body weight per day (blotted wet weight macroalgae, live abalone). The tanks were cleaned every second day and the pouches moved around the tank to provide the juvenile abalone greater access to the macroalgal thalli.

System Design

The nursery and weaner systems were set up and maintained as detailed in General Material and Methods Section 2.4, with some alterations outlined below.

Nursery System

The nursery tanks were arranged in 3 banks of 3 tanks with one replicate of each diet treatment assigned to one of the banks using a randomised block design. The nursery tanks housed 60 x 26 cm PVC settlement plates and each of the 9 nursery tanks were stocked with 2400 juvenile greenlip abalone (6 mm shell length), averaging 40 juveniles per plate (128 abalone.m^{-2}). All the nursery system diets had their plates replaced with new algal seeded plates at regular intervals during the feeding trial to maintain algal biomass. The water-soluble fertiliser Abasol (Section 2.2.2) was added to all the nursery tanks on a fortnightly basis to improve the nutritional composition of the algae throughout the trial. The baskets containing the PVC plates were rotated 180^{o} about the horizontal every fortnight to maintain a balance between algal biomass growth at the top of the plates and the grazing pressure from the photoperiod sensitive feeding of juvenile abalone at the bottom of the plates.

Weaner System

Weaner tanks were the 100 L Mexican hat tanks (1.3 m diameter) normally used for diatom culture (Section 2.4.4) (Figure 29). Each weaner tank was stocked with 1500 juvenile greenlip abalone of 6 mm shell length (1130 abalone.m^{-2}). The WMT diet and an artificial diet (Section 2.4.2) were tested in the weaner system in separate tanks with 3 replicate tanks per diet.

Measurements

To measure the shell length and weight of the juvenile *H. laevigata*, a sub-sample of 100 abalone per tank was collected every 2 weeks from all tanks in both the nursery and weaner systems.

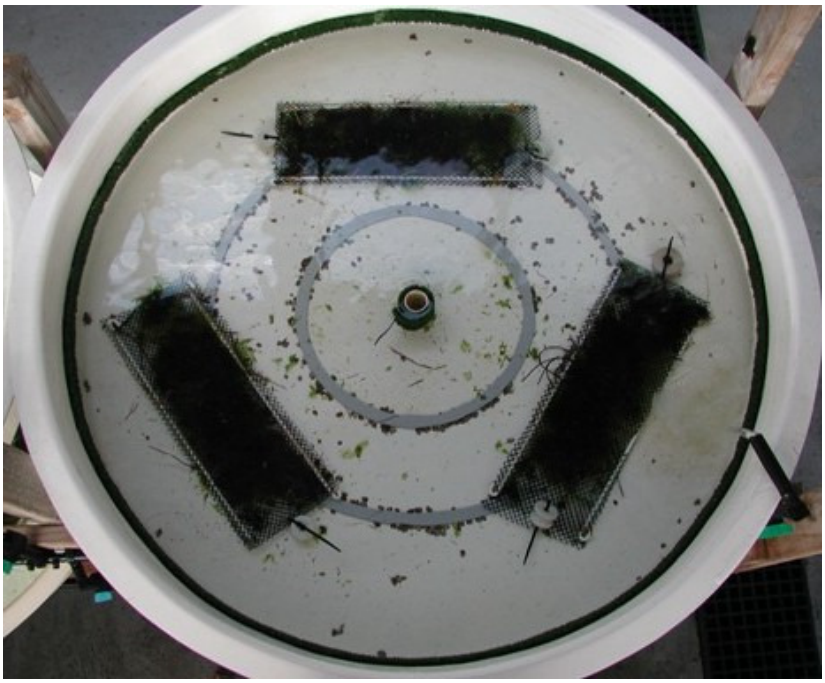

Figure 29: The Mexican hat tank used as a substitute weaner tank, with the Whole Macroalgal Thallus (WMT) diet presented in the mesh pouches.

Abundance of the algal species in the nursery system diets were determined as described in General Material and Methods Section 2.4.5. The *Hypnea* and *Ulva* sporelings were measured as the number of sporeling blades per cm^2, while the *Phormidium* was recoded as an estimate of percentage cover on the settlement plate. The algal diets were all measured at the start and then fortnightly during the trial to determine the algal consumption by juvenile abalone. When new plates seeded with algae were introduced, the algal density remaining on the old plates was measured first and used to calculate the consumption rate for the previous time period (fortnight). The algal density on the new seeded plates was then measured before they were introduced to the tank and used as the initial algal density to determine the consumption rate for the following time period (fortnight).

Irradiance and mean seawater temperature were measured throughout the trial to monitor the change in environmental conditions. The irradiance was measured for all diet replicates (tanks) by a Digitech light meter at the seawater surface while the tank was full, both when exposed to natural irradiance and covered with shade cloth. To compare irradiance across the three tank banks each containing one replicate tank of the

nursery system diets, the abalone parameters were standardised. The abalone growth and survival in each replicate nursery tank were weighted according to the proportion they differed from the diet treatments mean. Thermocron General Purpose Loggers (OnSolution) placed in three tanks on separate tank banks recorded the seawater temperature throughout the trial.

Data Analysis

Initial, week 2, week 16 and final shell length, total trial growth rate, specific growth rate, weight gain, initial mortality and total survival of the abalone on the diets were all compared separately using analysis of variance (one-way ANOVA). A Repeated Measures Analysis of Variance examined abalone growth rates throughout the trial. If significant differences ($p<0.05$) between diets were observed the Tukey Post-Hoc test for multiple comparisons of means was applied.

Fortnightly algal consumption rates and the density of *Hypnea* and *Ulva* sporelings on the new seeded plates at the start, week 8 and 16 were assessed by analysis of variance (one-way ANOVA). Algal consumption rates, mean fortnightly seawater temperature and irradiance were compared with juvenile abalone growth and survival through a Pearson Moment Correlation Analysis. Irradiance was also examined between systems, diets and nursery tank banks by analysis of variance (one-way ANOVA) with a Tukey Post-Hoc test identifying significant differences ($p<0.05$) between means.

5.2.3 Results

Abalone Growth, Survival and Algal Consumption

At the beginning of the feeding trial the juvenile *H. laevigata* had a mean initial shell length of 5.82 ± 0.08 mm. The initial shell length of abalone stocked in each of the diet treatments was significantly different between only two treatments; the commercial nursery system diet and the WMT diet in the weaner system (Table 20). During the first

week of the feeding trial the abalone exhibited extremely high mortality, after being harvested and transported from the commercial farm Bay Side Abalone in Bremer Bay to the WAFMRL aquaculture facilities in Perth. A mean juvenile abalone mortality of 29.7 ± 2.7 % in the nursery and 27 ± 1.9 % in the weaner system was recorded after the dead animals were collected during regular maintenance. However, the high mortality rates in the first week were not influenced by either the system or diet the juvenile abalone were stocked into (Table 21). Additional juvenile abalone were harvested from Bay Side Abalone and transported to WAFMRL, then stocked into all replicate tanks in order to maintain the initial trial stocking densities. Week 2 was considered the official start point of the trial, because of the high mortality and subsequent restocking during the first 2 weeks, and the significant difference between the initial shell lengths of abalone distributed into two diet treatments. The mean juvenile abalone shell length at week 2 was 5.98 ± 0.02 mm, with no significant difference between the animals stocked into each diet treatment (Table 20).

The juvenile abalone actively consumed the various feeds and increased in size on all diets over the 26-week trial. The increase in shell length was similar across all diets for the first 14 weeks, until the juvenile abalone reached approximately 9 mm shell length (Figure 30). Growth rates of juvenile abalone fluctuated between 27 and 40 $\mu m.day^{-1}$ on all diets during this time period and were not significantly different (Table 22). At week 16 the abalone shell length was significantly smaller on the WMT diet compared to both the mixed sporeling and the commercial nursery diets (Tukey, $p<0.05$). This was the only significant difference recorded in any growth parameter up to this point of the feeding trial. Therefore, the two experimental juvenile abalone nursery diets, mixed sporelings and *Phormidium* can be considered comparable to the current commercial nursery system diet and the artificial diet in the weaner system for growth of juvenile abalone less than 9 mm shell length.

Consumption rates of the algal biomass in each diet treatment played an important role in producing the similarity between juvenile abalone growth rates during the first 14 weeks of the feeding trial. *Ulva* sporeling consumption decreased from

150 to 0 sporeling blade.abalone^{-1}.day^{-1} over the first 10 weeks. The inclusion of new seeded plates after week 8 caused the consumption rate to increase back up to 150 sporeling blade.abalone^{-1}.day^{-1}, before it gradually declined again until week 16 of the trial (Figure 31). The rate of *Hypnea* sporeling consumption during the first half of the trial was approximately half that of the *Ulva* sporelings, but did follow a similar trend by reducing to nearly 0 sporeling blade.abalone^{-1}.day^{-1} by week 18 (Figure 31). The difference in the sporeling consumption of the two species was due to the density of *Hypnea* sporelings being only 48.2 % of the *Ulva* sporelings at the start of the trial. The *U. lens* consumption varied between 0.06 and 0.02 % cover.abalone^{-1}.day^{-1} during the first 10 weeks of the trial (Figure 32). The "negative" consumption rate at week 12 was due to an *U. lens* spore release event (week 10). This subsequently provided additional *U. lens* cover and therefore greater algal biomass on the plates for the juvenile abalone, which in turn allowed the consumption rate to return to the range mentioned above. *Phormidium* was readily consumed by the juvenile greenlip abalone and in the first 2 weeks 77 % of the 87.1 % cover was removed (Figure 33). The consumption during the next 6 weeks was minimal as very little *Phormidium* biomass was available on the plates. The new seeded plates incorporated at week 8 and 12 presented the abalone with feed and subsequently the consumption rates increased until week 16.

The consumption rates of algal biomass by juvenile abalone indicated a range in grazing tolerances of the various algal species, with *U. lens* exhibiting the highest grazing resistance, followed by the mixed sporelings then the *Phormidium*. This was particularly evident for the *Phormidium* as the abalone removed nearly all of the algal cover in a short period of time and therefore, algal regeneration on the plates was unable to occur. *Ulvella lens* had visible grazing tracks in its cover but the algae was not completely removed, which allowed regeneration. These grazing tolerances dictated the diets' algal cover, rate of removal and ability to maintain suitable biomass through to week 16 when new seeded plates were introduced to all diets. The *Phormidium* diet had new seeded plates introduced twice at week 8 and 12, the mixed sporelings diet at week 8, while the commercial nursery diet had an induced release of spores at week 10. This maintenance of algal biomass in the nursery system enabled the growth rates of juvenile abalone for the first 14 weeks to be similar across the nursery and weaner diets (Table 22).

After week 14 the abalone growth rates began to vary between diets resulting in different mean shell lengths (Figure 30). During week 16 to 20 the juvenile abalone on the mixed sporeling diet grew significantly faster at 64.13 ± 3.82 $\mu m.day^{-1}$ than the other diets, apart from those abalone feeding on the commercial nursery diet (45.42 ± 5.94 $\mu m.day^{-1}$) (Table 22). Abalone consuming the other three diets had similar growth rates of around 40 $\mu m.day^{-1}$, which were only slightly higher than those achieved during the first 14 weeks of the feeding trial. In the last 6 weeks of the feeding trial there was a further increase in abalone growth rates on three diets, with the artificial diet producing 74.17 ± 4.12 $\mu m.day^{-1}$, the commercial nursery diet 78.06 ± 7.80 $\mu m.day^{-1}$ and the mixed sporeling diet sustaining the maximum growth rate recorded during the trial of 87.70 ± 4.50 $\mu m.day^{-1}$ (Table 22). These three abalone growth rates were significantly faster than those produced on both the *Phormidium* and WMT diets. Even though the juvenile abalone growth rates varied between diets in the different 6-week intervals during the trial (Table 22), overall they still demonstrated the same statistical relationship between diets as the total trial growth rate calculated utilising the week 2 and final shell lengths (Table 20).

The variation in growth rates after week 16 was related to the amount of biomass the new seeded plates could provide to the juvenile abalone in each nursery diet. The new *Ulva* sporeling seeded plates introduced after week 16 had a substantial effect on consumption rate by reducing it to -450 sporeling $blade.abalone^{-1}.day^{-1}$ at week 18 (Figure 31), which was significantly different than any other fortnightly consumption rate (Tukey, $p<0.05$). The extremely high density of sporelings on the new plates were able to reproduce faster than the juvenile abalone could consume them, resulting in the "negative" consumption rate. The density of *Ulva* sporelings peaked at 185 sporeling $blade.cm^{-2}$ during week 18, which was significantly greater than the density on both the initial and week 8 new seeded plates ($F_{(df\ 2,6)}=61.168$, $p<0.05$ (Tukey, $p<0.05$)). The greater sporeling density and therefore, higher algal biomass per plate led to the increase in consumption rate (Figure 31) by the juvenile abalone, and the subsequent significantly faster abalone growth rates during the last 12 weeks of the trial (Table 22). The introduction of new seeded *Hypnea* sporeling plates at week 16 also allowed the *Hypnea* sporeling consumption rate by abalone to increase for the rest of the trial

(Figure 31). The density of *Hypnea* sporelings on the new seeded plates at week 16 was significantly greater than that on the plates at the start of the trial, but not the week 8 plates ($F_{(df\ 2,6)}$=6.435, p<0.05 (Tukey, p<0.05)). Sporeling consumption rates of these two macroalgae were significantly, positively correlated throughout the 26-week feeding trial (R=0.847, p<0.05) (Figure 31).

New seeded plates of *U. lens* were introduced to the tanks at week 16 because the biomass of the green alga had been grazed to only 16.2 % cover. The introduction of new seeded plates contributed to a "negative" consumption rate as the algal density was increasing faster than the abalone could consume it. After this, the consumption rate then increased and even with another spore release at week 20, the rate remained positive for the remainder of the trial (Figure 32). The consumption of *N. jeffreyi* by the juvenile abalone was significantly, positively correlated with that of *U. lens* cover consumption during the trial (R=0.576, p<0.05). After an initial consumption of 100 $x10^3$ diatom.abalone^{-1}.day^{-1} at week 2, the consumption rate of *N. jeffreyi* remained on or under 0 diatom.abalone^{-1}.day^{-1} until the last week of the trial (Figure 32). Inoculations of the diatom into the nursery tanks were conducted on a weekly basis and this constant supply caused the density (diatom.cm^{-2}) in the tanks to increase faster than the juvenile abalone could consume them. Consequently, *N. jeffreyi* was the only algal feed that did not exhibit significant differences between fortnightly consumption rates ($F_{(df\ 12,26)}$=1.46, p=0.202).

New seeded plates of the grazing conducive *Phormidium*, had to be added into the tanks at week 16 and 20 to maintain algal biomass to support the juvenile abalone. This resulted in fluctuating food availability and subsequently, variable algal consumption rates (Figure 33) and lower abalone growth rates than the mixed sporeling and commercial nursery diets. Overall, none of the algal consumption rates statistically correlated with the growth of juvenile abalone consuming that particular diet.

At the end of the trial the mixed sporeling diet had produced the largest mean juvenile abalone shell length of 15.04 ± 0.26 mm, which was significantly bigger than that achieved on both the *Phormidium* and WMT diets. The WMT diet in the weaner system produced the smallest abalone at 11.30 ± 0.03 mm shell length, which was similar to the abalone feeding on the *Phormidium* diet, while the other 3 diets produced significantly larger abalone (Table 20). The growth rate, specific growth rate and weight gain of juvenile abalone over the entire feeding trial exhibited the same statistical relationship between diets as the final shell length, with the mixed sporeling diet producing the fastest and heaviest abalone growth (Table 20). This relationship between growth parameters was exemplified by the abalone feeding on the commercial nursery diet producing an identical final mean shell length (13.9 mm) to the juveniles consuming the artificial diet in the weaner system. This resulted in an almost identical total abalone growth rate (47 $\mu m.day^{-1}$), specific growth rate (0.50 % $length.day^{-1}$) and weight gain (2 $\mu g.day^{-1}$) on both diets (Table 20). Even though the mixed sporeling diet was able to produce the greatest juvenile abalone growth (both as shell length and as weight gain) over the 26-week feeding trial, it was not significantly superior to that recorded by abalone consuming the commercial nursery diet or the artificial diet in the weaner system (Table 20). This indicates that all three diets, the mixed sporeling and commercial nursery diet in the nursery system, and the artificial diet in the weaner system would be suitable to sustain growth of juvenile abalone from 5 to 15 mm shell length throughout the later nursery phase.

Another vital parameter in assessing the suitability of the various diets was the survival of abalone over the entire trial. The survival on the various diets ranged from 35.2 ± 2.2 % on the WMT diet to 62.8 ± 1.5 % on the artificial diet, with the artificial diet having a significantly higher juvenile abalone survival than the other four diets (Table 21). When the abalone survival on each diet was combined for its respective system, the survival in the nursery system of 44.8 ± 2.2 % was not significantly different to the survival of 49.0 ± 6.3 % in the weaner system. All analyses of growth and survival parameters did not show any significant difference between the nursery and weaner systems, which could possibly indicate that the differences in abalone growth and survival were due to the diet consumed rather than the system utilised.

Table 20: The initial shell length, 2nd week (wk) shell length and final shell length (mm) with the growth rate (µm.day^{-1}), specific growth rate (% length.day^{-1}) and weight gain (µg.day^{-1}) of juvenile *Haliotis laevigata* during the 26-week feeding trial. The abalone were fed three separate algal diets of mixed sporelings (*Hypnea*/*Ulva*), *Phormidium* and the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet in the nursery system, while the WMT (Whole Macroalgal Thallus) diet and an artificial diet were used in the weaner system. One-way ANOVA indicated significant differences ($p<0.05$) between diet means in each abalone growth measurement as illustrated by different superscripts (columns). Mean ± std. error (n=3).

Diet	**Initial Length**	**Wk 2 Length**	**Final Length**	**Growth Rate**	**SGR**	**Weight Gain**
	mm ± SE	mm ± SE	mm ± SE	µm.day^{-1} ± SE	% length.day^{-1} ± SE	µg.day^{-1} ± SE
Commercial nursery	5.67 ± 0.09^{a}	6.04 ± 0.07	13.90 ± 0.61^{ab}	47.13 ± 3.90^{ab}	0.50 ± 0.03^{ab}	2.04 ± 0.30^{ab}
Mixed sporelings	5.70 ± 0.05^{ab}	6.01 ± 0.05	15.04 ± 0.26^{a}	53.81 ± 1.72^{a}	0.55 ± 0.01^{a}	2.50 ± 0.16^{a}
Phormidium	5.69 ± 0.09^{ab}	5.94 ± 0.08	12.33 ± 0.43^{bc}	38.38 ± 2.97^{bc}	0.44 ± 0.03^{bc}	1.30 ± 0.17^{bc}
WMT	6.03 ± 0.09^{b}	5.93 ± 0.10	11.30 ± 0.03^{c}	32.16 ± 0.47^{c}	0.39 ± 0.01^{c}	0.86 ± 0.01^{c}
Artificial	6.01 ± 0.06^{ab}	5.99 ± 0.09	13.90 ± 0.20^{ab}	47.39 ± 0.10^{ab}	0.50 ± 0.01^{ab}	2.05 ± 0.09^{ab}
df	4, 10	4, 10	4, 10	4, 10	4, 10	4, 10
F	5.762	0.369	16.408	12.792	9.499	14.043
p value	<0.05	0.825	<0.05	<0.05	<0.05	<0.05

Table 21: Initial mortality (%) during the first 2 weeks and the total survival (%) of juvenile *Haliotis laevigata* over the remaining 24 weeks of the 26-week feeding trial. The abalone were fed three separate algal diets of mixed sporelings (*Hypnea*/*Ulva*), *Phormidium* and the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet in the nursery system, while the WMT (Whole Macroalgal Thallus) diet and an artificial diet were used in the weaner system. One-way ANOVA indicated significant differences ($p<0.05$) between diet means in each abalone survival measurement as illustrated by different superscripts (columns). Mean ± std. error (n=3).

Diet	Initial Mortality	Total Survival
Commercial nursery	30.0 ± 3.1	47.0 ± 3.8^{b}
Mixed sporelings	27.7 ± 7.3	47.7 ± 2.3^{b}
Phormidium	31.4 ± 4.5	39.6 ± 4.2^{b}
WMT	24.5 ± 2.8	35.2 ± 2.2^{b}
Artificial	29.2 ± 2.2	62.8 ± 1.5^{a}
df	4, 10	4, 10
F	0.367	12.879
p value	0.837	<0.05

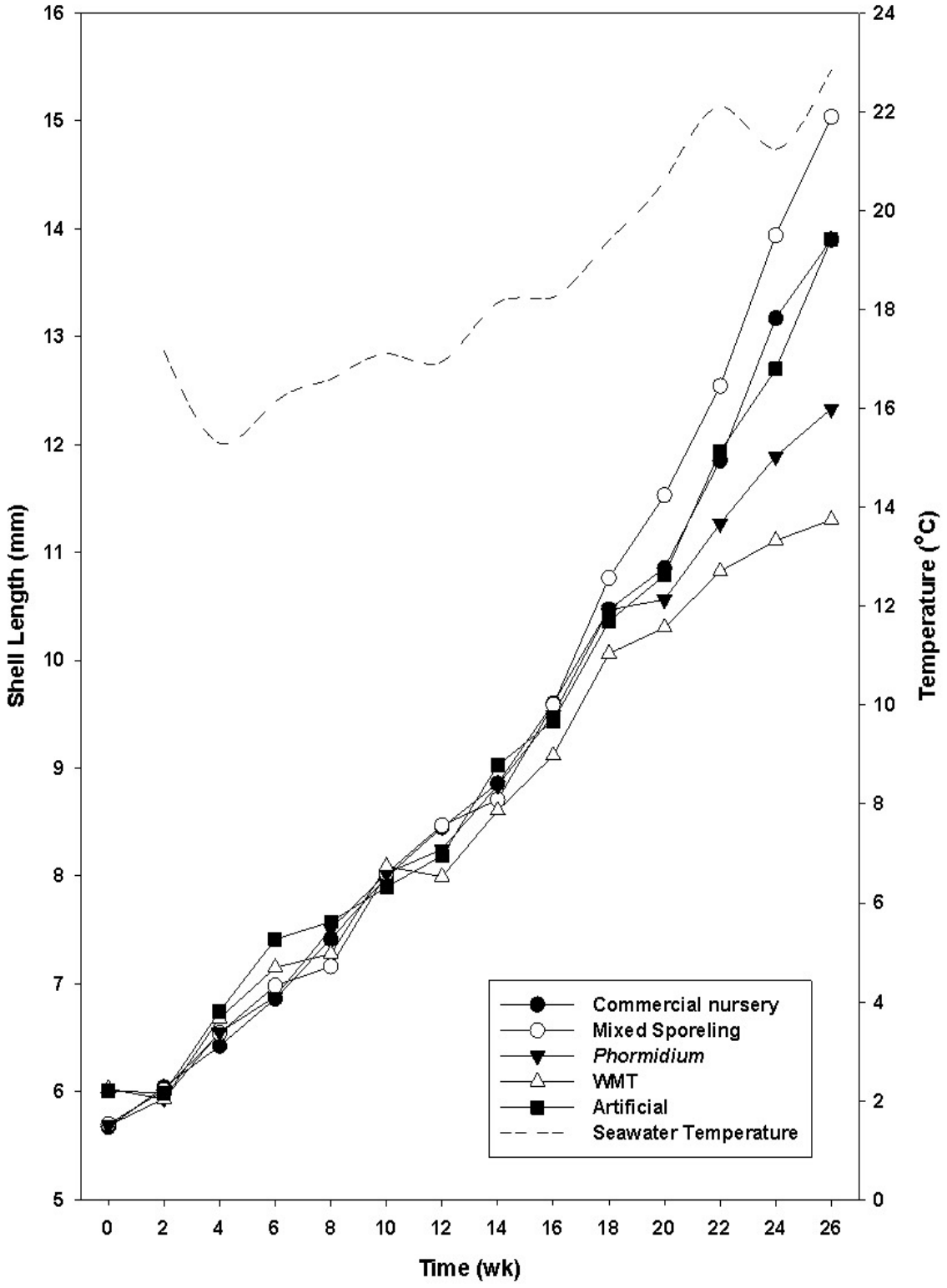

Figure 30: Growth in shell length (mm) of juvenile *Haliotis laevigata* and mean seawater temperature (°C) during the 26-week (wk) feeding trial (n=3). The abalone were fed three separate algal diets of mixed sporelings (*Hypnea*/*Ulva*), *Phormidium* and the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet in the nursery system, while the WMT (Whole Macroalgal Thallus) diet and an artificial diet were used in the weaner system.

Table 22: The growth rate (μm.day^{-1}) of juvenile *Haliotis laevigata* in 6-week (wk) intervals during the 26-week feeding trial. The abalone were fed three separate algal diets of mixed sporelings (*Hypnea*/*Ulva*), *Phormidium* and the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet in the nursery system, while the WMT (Whole Macroalgal Thallus) diet and an artificial diet were used in the weaner system. Repeated Measures Analysis of Variance (RM-ANOVA) showed significant differences ($p<0.05$) in mean abalone growth rates between diets over the trial (rows). One-way ANOVA indicated significant differences ($p<0.05$) in mean abalone growth rates between diets at each growth rate period as illustrated by different superscripts (columns). Mean ± std. error (n=3).

Diet	**Wk 4 – 8**	**Wk 10 – 14**	**Wk 16 – 20**	**Wk 22 – 26**	**RM-ANOVA**
Commercial nursery	33.00 ± 1.70	34.31 ± 3.54	45.42 ± 5.94^{ab}	78.06 ± 7.80^{a}	1,2
Mixed sporelings	27.54 ± 2.01	36.79 ± 1.15	64.13 ± 3.82^{a}	87.70 ± 4.50^{a}	1
Phormidium	37.72 ± 3.92	31.68 ± 3.42	39.18 ± 4.80^{b}	45.39 ± 7.05^{b}	2,3
WMT	33.74 ± 5.48	30.85 ± 3.51	40.40 ± 1.28^{b}	23.76 ± 2.33^{b}	3
Artificial	39.70 ± 0.99	33.77 ± 2.90	41.90 ± 5.12^{b}	74.17 ± 4.12^{a}	1,2
df	4, 10	4, 10	4, 10	4, 10	4, 10
F	2.075	0.590	5.250	22.907	27.483
p value	0.159	0.677	<0.05	<0.05	<0.05

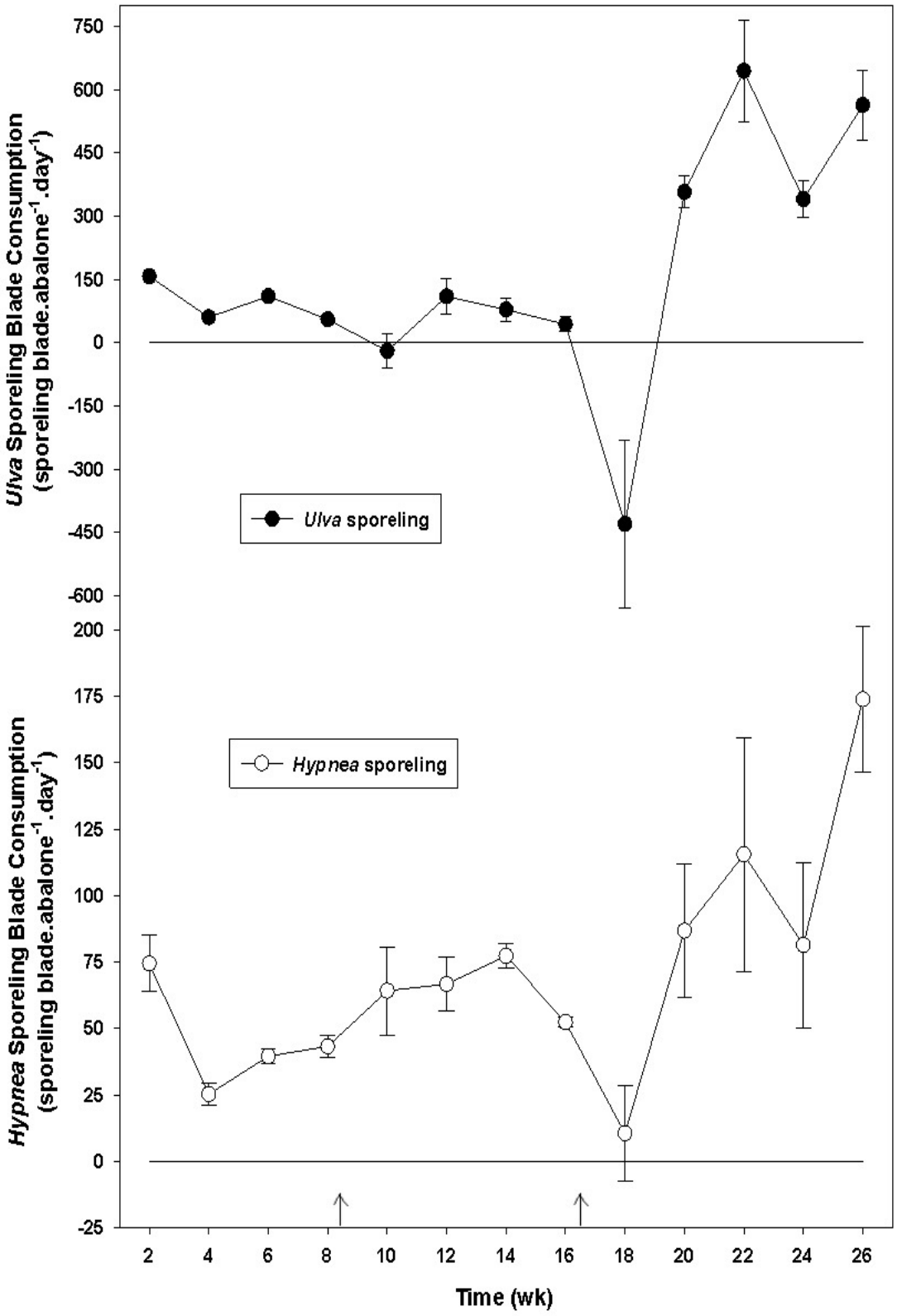

Figure 31: The consumption rate (sporeling blade.abalone^{-1}.day^{-1}) of the mixed sporeling (*Hypnea* and *Ulva*) diet by juvenile *Haliotis laevigata* during the 26-week (wk) feeding trial. Mean ± std. error (n=3). The arrows indicate when new seeded plates of *Hypnea* and *Ulva* sporelings were introduced.

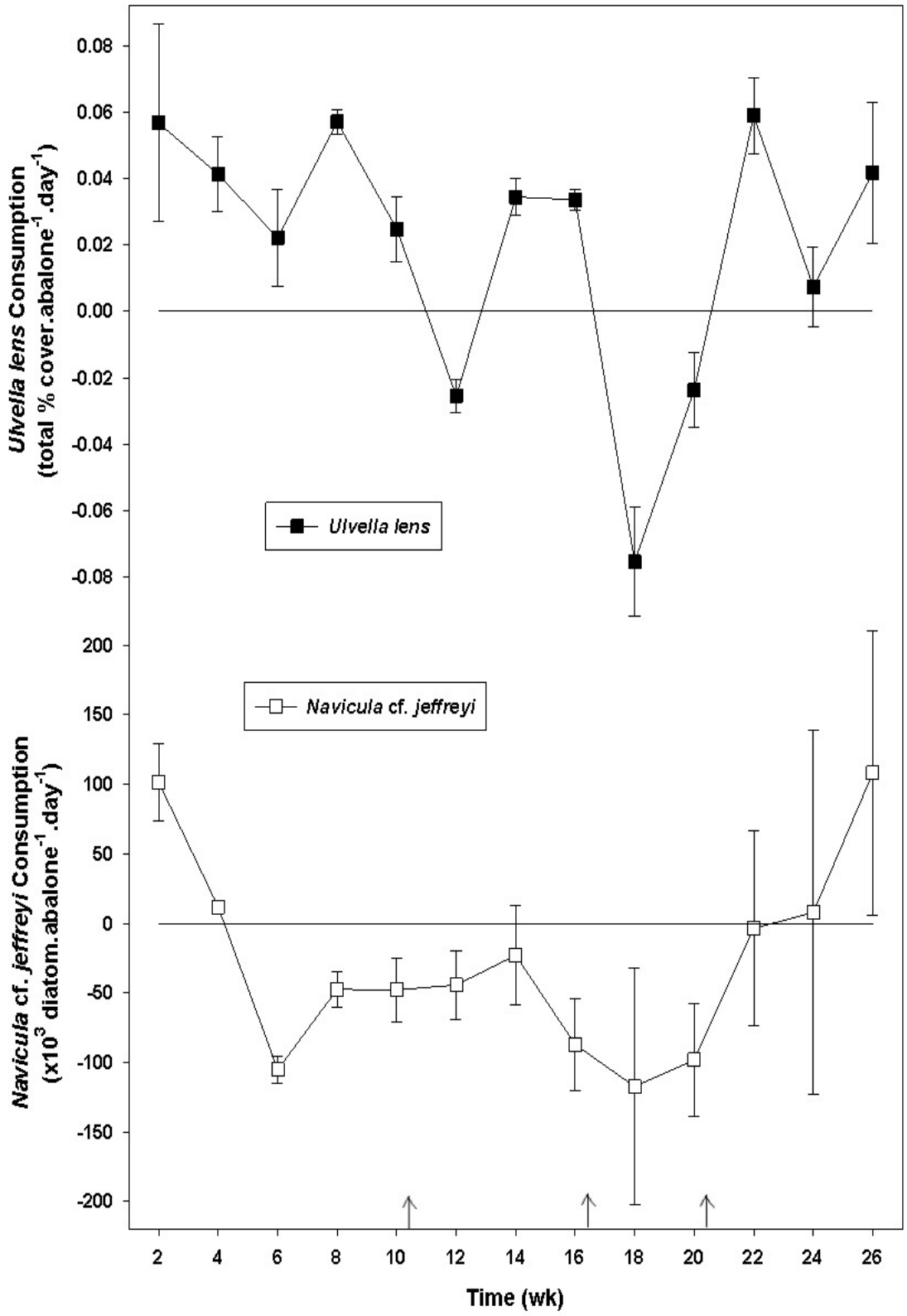

Figure 32: The consumption rate (% cover.abalone^{-1}.day^{-1} and x10^3 diatom.abalone^{-1} .day^{-1}) of the commercial nursery (*Ulvella lens*/*Navicula* cf. *jeffreyi*) diet by juvenile *Haliotis laevigata* during the 26-week (wk) feeding trial. Mean ± std. error (n=3). The arrows indicate when visible *Ulvella lens* spore release occurred and new seeded plates were introduced at week 16.

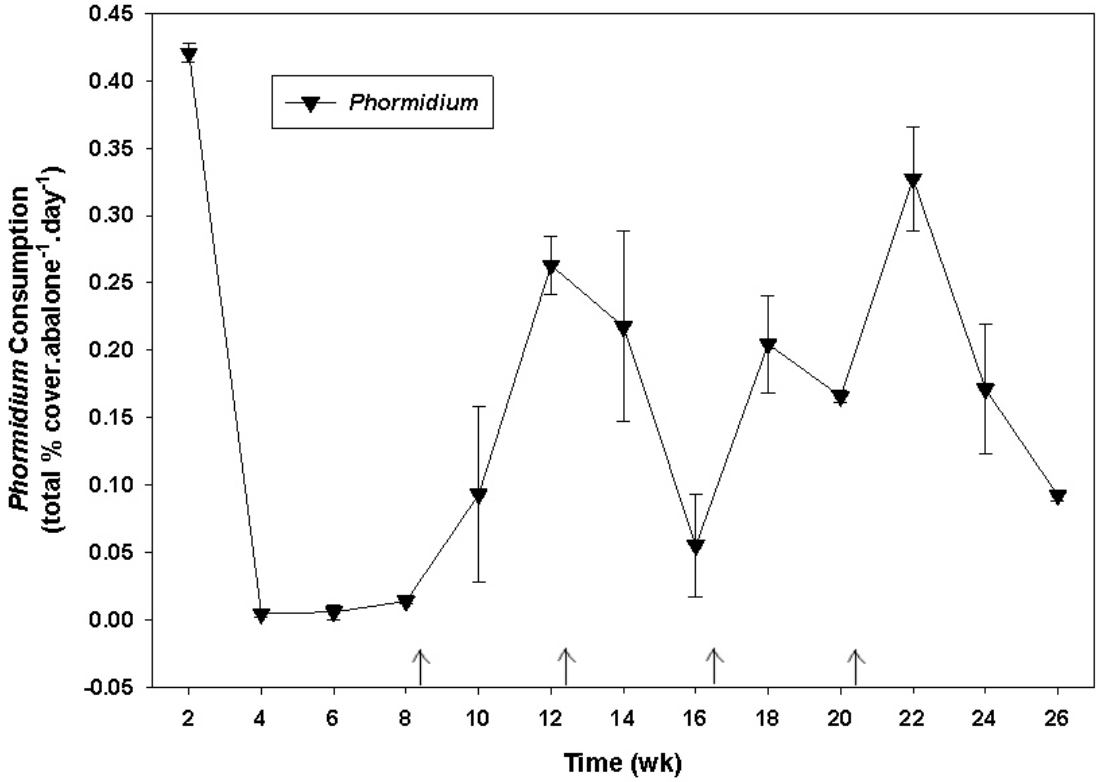

Figure 33: The consumption rate (% cover.abalone^{-1}.day^{-1}) of the *Phormidium* diet by juvenile *Haliotis laevigata* during the 26-week (wk) feeding trial. Mean ± std. error (n=3). The arrows indicate when new seeded plates of *Phormidium* were introduced.

Environmental Parameters

Temperature

During the feeding trial the mean seawater temperature increased from 15.3°C in week 4 to 22.8°C at the conclusion (Figure 30). The mean seawater temperature was colder than 18°C for the first 14 weeks and corresponded to abalone growth rates slower than 40 µm.day^{-1} on all diets. Once the temperature had surpassed 18°C there were significant differences in abalone growth rates between diets, specifically the increase recorded on the mixed sporeling, commercial nursery and artificial diets (Table 22). In fact, the juvenile abalone growth rates on the mixed sporeling diet ($R=0.753$, $p<0.05$), the commercial nursery diet ($R=0.655$, $p<0.05$) and the artificial diet ($R=0.605$, $p<0.05$) showed significant, positive correlation with the rise in mean seawater temperature throughout the trial. However, the mean seawater temperature was only significantly, positively correlated with the consumption rates of *Hypnea* and *Ulva* sporelings

(R=0.746, p<0.05 and R=0.618, p<0.05). Mean seawater temperature could not be related to the algal biomass of the diets in the nursery system as the new seeded plates introduced at specific time intervals changed the density of the algal species. The continued inoculation of *N. jeffreyi* was the one exception and the diatoms abundance was significantly, positively correlated with temperature (R=0.904, p<0.05).

Irradiance

The irradiance the two systems received varied throughout the day, as well as over the entire feeding trial with a maximum, natural, un-shaded irradiance of 518 µmol photons.m^{-2}.s^{-1}. All irradiance measurements used for comparison were taken at the water surface underneath the tank covers, to provide a representation of what the algae and abalone were exposed to. The weaner system was deliberately constructed to be darker as it did not require sunlight to maintain an algal diet and consequently, the irradiance recorded was significantly lower than that of the nursery system ($F_{(df\ 1,8)}$=16.580, p<0.05). In the weaner system there was no significant difference between the irradiances the WMT and artificial diet each received ($F_{(df\ 1,8)}$=0.836, p=0.387).

The nursery system required natural irradiance throughout the trial to maintain suitable algal biomass of each diet. The irradiance received had an effect on the amount of algae present within the tanks of each diet, which subsequently affected the growth and survival of the juvenile abalone. The nursery system design of diet replicates (tanks), randomised between 3-nursery tank banks had significant differences between the irradiance the banks received (Table 23). The western bank was exposed to a mean irradiance of 36.26 ± 8.69 µmol photons.$m^{-2}s^{-1}$ throughout the trial, which was significantly greater than the 12.63 ± 2.77 µmol photons.$m^{-2}s^{-1}$ the eastern bank received. This variation in irradiance from east to west across the nursery tank banks coincided with a significant increase in the standardised growth and survival of the juvenile abalone in the western bank (Table 23). Given the randomisation of diet replicate tanks, none of the abalone growth rates produced on the 3 nursery algal diet treatments correlated with irradiance during the trial.

Table 23: The irradiance (μmol photons.m^{-2}.s^{-1}), standardised juvenile *Haliotis laevigata* growth (mm shell length) and survival (%) for the 3 banks of nursery tanks during the 26-week feeding trial. One-way ANOVA indicated significant differences ($p<0.05$) between nursery tank bank means of irradiance, standardised abalone growth and survival as illustrated by different superscripts (columns). Mean ± std. error (n=3).

Tank Bank	Irradiance	Growth	Survival
West	36.26 ± 8.69^{a}	1.12 ± 0.04^{a}	1.14 ± 0.03^{a}
Middle	16.01 ± 3.43^{ab}	0.98 ± 0.03^{b}	0.95 ± 0.02^{b}
East	12.63 ± 2.77^{b}	0.90 ± 0.02^{b}	0.90 ± 0.05^{b}
df	2, 12	2, 6	2, 6
F	5.156	13.350	13.249
p value	<0.05	<0.05	<0.05

5.2.4 Discussion

Juvenile abalone growth rates on all five diets were remarkably similar (27 – 40 μm.day^{-1}) during the first 14 weeks of the multi diet and system feeding trial. This was further emphasised by only the WMT diet having a significant difference in shell length at week 16 to the mixed sporeling and the commercial nursery diets, which could be attributed to sufficient algal biomass being supplied to the juvenile abalone in the nursery diets. However, this similarity in growth for the first 14 – 16 weeks was unusual as variation in growth on different diets generally occurs earlier in feeding trials (demonstrated below), even though delayed growth and extended weaning periods have been reported (Day & Fleming 1992, Garcia-Esquivel & Felbeck 2009). The *Ulva* Sporeling Diet Feeding Trial (Section 5.1.3) showed that *Ulva* sporelings were an appropriate diet for juveniles of 3.5 – 6 mm shell length, while the commercial nursery diet was acceptable for larger juveniles (>6 mm shell length). *Haliotis laevigata* of the same size range had significantly faster growth rates during the first 4 weeks of a trial on an *U. lens* and *N. jeffreyi* diet, then once they reached 9 mm shell length the juveniles on an artificial diet had significantly faster growth rates (Daume *et al.* 2007). This was the same outcome as the abalone growth differences between the commercial nursery and the artificial diet in the *Laurencia* Fragment/Agar Diet Feeding Trial (Section 4.2.3). These studies highlight the rare, extended period of similar abalone growth exhibited in this trial, particularly between algal nursery diets and artificial diets in the

weaner system, as artificial diets are generally less suitable feeds for juveniles 5 – 10 mm shell length and typically result in slower growth and higher mortalities (Daume & Ryan 2004).

After week 16 of the feeding trial abalone growth began to vary between diets, as evident in the divergences of shell length profiles (Figure 30) and the significant difference in growth rates during the last two 6-week intervals (Table 22). Importantly, this variation in growth occurred when the juvenile abalone reached approximately 9 – 10 mm shell length. Up until this size range, all diets other than the WMT diet were able to sustain acceptable juvenile abalone growth rates. Thus indicating a wide variety of diets in both the nursery and weaner systems can be utilised to rear juvenile greenlip abalone of less than 9 – 10 mm shell length. The growth of juvenile abalone on the nursery macroalgal diets in both the *Ulva* Sporeling and *Laurencia* Fragment/Agar Diet Feeding Trials (Section 5.1.3 and 4.2.3) also exhibited this transition at 9 – 10 mm shell length. Daume *et al.* (2007) confirmed this transitional size range in two separate feeding trials using the current commercial nursery and weaner diets, as well as a composite diet of *Ulva* sporelings, *U. lens* and *N. jeffreyi*. This transitional size range can be considered a key point in the later nursery phase, as it's when commercially viable growth rates decline due to insufficient algal biomass, requiring either the addition of algal biomass into the nursery system or weaning onto artificial diets (Fleming *et al.* 1996, Dunstan *et al.* 2002, Daume *et al.* 2004). This multi diet and system feeding trial incorporated both scenarios to allow a detailed comparison of which system and diet best suits the rearing of juvenile greenlip abalone.

The growth rates of juvenile abalone on the commercial nursery diet and artificial diet in the weaner system both increased after week 16 to over 70 $\mu m.day^{-1}$. Different species of juvenile abalone have attained these growth rates on various artificial diets in the later part of extended feeding trials (Fleming *et al.* 1996, Daume *et al.* 2007, Dlaza *et al.* 2008, Garcia-Esquivel & Felbeck 2009). However, the commercial nursery diet generally produced these abalone growth rates at the earlier size ranges (5 mm shell length) when food availability was high, but rarely when the

juveniles were over 10 mm shell length and feed limitation has become an issue (Daume & Ryan 2004, Daume *et al.* 2007). Dyck *et al.* (2010) utilised a combination diet of *U. lens* and an artificial feed in specifically designed V nursery tanks to achieve growth rates of well over 100 μm.day^{-1} for juvenile *H. iris* (≈5 to 15 mm shell length) during the later part of two trials (88 and 122 d). The decrease in consumption of *U. lens* within the combination diet also indicated the transition from the algal to artificial feed when the abalone were 8 – 9 mm shell length. Therefore, the early stage growth rates of approximately 70 μm.day^{-1} in the Dyck *et al.* (2010) trial are more likely a representation of *U. lens* consumption, while the high abalone growth rates of over 100 μm.day^{-1} in the later stages of the trial were produced by artificial feed consumption. Given there was no significant difference in abalone growth rates or survival between a diatom (*Nitzschia longissima*)/artificial feed diet where the diatoms were consumed in 4 – 5 d, and an *U. lens*/artificial feed diet which had at least 20 % *U. lens* cover towards the end of the trial (88 d) (Dyck *et al.* 2010), it could be suggested that the growth rates of juvenile abalone were solely dependent on the artificial feed throughout the trial and not either of the algal species.

The mixed sporeling diet in the present feeding trial actually produced the fastest growth rate of 87.7 ± 4.5 μm.day^{-1} during the last 4 weeks. In fact, this composite red and green macroalgal sporeling diet recorded the largest, fastest growing and heaviest juvenile abalone over the entire trial. It can therefore, be considered a suitable alternative macroalgal diet when compared to the current commercial nursery and weaner system diets for the later nursery phase of juvenile abalone production. The abalone growth rate obtained during the last 4 weeks on the mixed sporeling diet was similar to that recorded on the monospecies *Ulva* sporeling diet (Section 5.1.3). However, that growth rate was only produced during the first 6 weeks of the trial with animals of less than 6.5 mm shell length, while the mixed sporeling diet produced the fast growth rate during week 22 – 26 with juveniles of greater than 12 mm shell length. This may suggest the inclusion of *Hypnea* sporelings was able to improve the overall performance and addressed the deficiencies in the monospecies, *Ulva* sporeling diet. The mixed sporeling diet could have achieved this by creating a more balanced diet with

the essential nutrients required by juvenile abalone (Day & Fleming 1992, Fleming 1995b, Brown *et al.* 1997, Simpson & Cook 1998).

Abalone consuming mixed algal diets generally perform better than those feeding on monospecies diets, as shown by the mixed sporeling diet in this feeding trial producing the fastest growth rates. *Ulva* thalli as part of combination diets has been shown to improve growth rates in various juvenile abalone species (Stuart & Brown 1994, Simpson & Cook 1998), while a mixture of *L. japonica* and *Gracilaria lemaneiformis* was one of the best performing diets for *H. discus hannai* (75 mm shell length) (Qi *et al.* 2010). A red, green and brown composite macroalgal diet as well as a combination diet of *Ecklonia maxima* and farmed *U. lactuca*, produced the best growth for *H. midae* in two separate trials of 35 mm and 20 mm shell length animals, respectively (Naidoo *et al.* 2006, Robertson-Andersson *et al.* 2011). Enriched *U. rigida*, *Hypnea spinella* and *Gracilaria cornea* combined, also performed significantly better than when fed to *H. tuberculata coccinea* (12.5 mm shell length) as single macroalga diets (Viera *et al.* 2011). Even though these mixed diets produced good abalone growth rates, they utilised whole adult thalli of the macroalgae fed to larger juvenile abalone (>12.5 mm shell length). To accommodate this, different culture systems including baskets, cages and containers, rather than the PVC settlement plates in the nursery system were used to present the mixed macroalgal diets to abalone. In this trial the incorporation of the WMT diet (*G. flagelliformis*, *Hypnea* and *Ulva*) into the weaner system using mesh pouches allowed the growth of juvenile abalone consuming adult macroalgal thalli to be compared with abalone fed macroalgal sporelings.

The WMT diet produced the smallest, slowest growth rate and lowest weight gain abalone of all the diets in the multi diet and system feeding trial. This was the complete opposite to the mixed sporeling diet and may be because two forms of the same macroalgal species can have different nutritional and structural properties (Van Alstyne *et al.* 1999). However, the more direct cause of the significantly slower growth on the WMT diet was the mesh gauge (10 mm) of the pouches the algae were presented in, as the juvenile abalone had difficulty accessing the macroalgal thalli once they

reached around 9 mm shell length. The pouches were designed for ease of feeding and cleaning, however it may be more appropriate to enclose the abalone in baskets, cages or containers as in the mixed, adult thalli macroalgal diet feeding trials mentioned above, but specifically constructed to accommodate juvenile abalone of 5 – 15 mm shell length. The use of the WMT diet in Australian nurseries would require completely new abalone production systems and macroalgal culture facilities to be created. Therefore, macroalgal sporelings seeded on settlement plates in the nursery system has been shown to be the most appropriate method of exposing a mixed diet of macroalgae to juvenile abalone within Australian abalone farms.

There are only a small number of studies utilising macroalgal sporelings for juvenile abalone nutrition and this feeding trial was the only study using a red macroalga specie (*Hypnea*). In the *Ulva* Sporeling Diet Feeding Trial (Section 5.1.4) it was mentioned that growth rates of 100 μm.day^{-1} have been obtained by 3 – 4 mm shell length *H. discus discus* when fed on a variety of monospecies sporelings (Maesako *et al.* 1984). While Takami *et al.* (2003) has shown that the brown macroalga *L. japonica* can be first utilised by *H. discus hannai* at 1.8 mm in shell length, with growth rates up to 80 μm.day^{-1} achievable on the juvenile sporophytes, however considerable variation in juvenile growth between algae and developmental stages of abalone was present. Recent small scale studies have indicated that juvenile *H. diversicolor* of greater than 3 mm shell length can achieve growth rates of more than 60 μm.day^{-1} when feeding on gametophytes of the brown alga *Eisenia bicyclis*, while animals greater than 5 mm shell length fed on juvenile sporophytes (Onitsuka *et al.* 2010). A similar study utilising juvenile gametophytes of the brown algae *E. bicyclis*, *Ecklonia cava* and *Undaria pinnatifida* showed growth rates of 100 μm.day^{-1} were easily attainable for juvenile *H. diversicolor* of 3 – 8 mm shell length (Onitsuka *et al.* 2011). A mixed diet of *U. rigida* sporelings and *U. lens* used to settle *H. tuberculata coccinea* and test four diatom species as post-larval feed, showed a sudden increase in growth rates to 94 μm.day^{-1} on all diatom diets for juveniles of 2 mm shell length, signifying a dietary shift from the diatom species to the green macroalgae (Courtois de Vicose *et al.* 2012).

The only use of macroalgal sporelings to the semi-commercial scale as in this multi diet and system feeding trial, was the *Ulva* sporeling diet used in Section 5.1 and a mixed *Ulva* sporeling, *U. lens* and *N. jeffreyi* diet which produced juvenile *H. laevigata* growth rates of 105 μm.day^{-1} (Daume *et al.* 2007). The incorporation of three algal species covering the juvenile abalone dietary range (diatoms – crustose algae – macroalgae) may have improved the overall nutritional profile of the diet. The addition of red macroalgal sporelings into this tri-species diet would further increase the algal biomass across the abalone dietary range and could maintain higher abalone growth rates for an extended later nursery phase.

The very fast growing, simple to culture, *Phormidium* diet was only able to produce a total feeding trial juvenile abalone growth rate of 38.38 ± 2.97 μm.day^{-1}, which remained relatively constant throughout with a maximum of 45.39 ± 7.05 μm.day^{-1} between week 22 – 26. This was only slightly slower than the 60.3 μm.day^{-1} *H. discus discus* juveniles of 3.6 mm shell length produced on *Phormidium fragile*, while other cyanobacteria (blue-green algae) species including *Entophysalis deusta* and *Plectonema golenkiniatum* have been able to achieve considerably faster growth rates of 96.7 and 86.7 μm.day^{-1} over 30 d trials (Maesako *et al.* 1984). The savings in time and resources for a commercial nursery due to the ease of *Phormidium* culture would not offset the relatively slow juvenile abalone growth rate it produced in this trial. The use of the cyanobacterium *E. deusta* and *P. golenkiniatum* may be feasible due to the faster abalone growth rates achieved, depending on what duration (month) these species can provide adequate biomass throughout the later nursery phase.

Over the multi diet and system feeding trial, the final shell length, growth rate, specific growth rate and weight gain showed the same statistical trend across all diets, making these four measurements of abalone growth interchangeable when identifying the most suitable juvenile abalone diet. The mixed sporeling diet produced the highest growth parameters; followed by the commercial nursery diet and the artificial diet,

while the *Phormidium* diet recorded lower growth parameters, with the WMT diet resulting in the smallest juvenile abalone.

Abalone survival over the 26-week feeding trial did not exhibit the same relationship between diets as all the growth parameters identified. The artificial diet in the weaner system had a significantly higher abalone survival of 62.8 ± 1.5 % than all the other diets. This was lower than the 73.6 % abalone survival on the artificial diet in the *Laurencia* Fragment/Agar Diet Feeding Trial (Section 4.2.3), however it did occur over a three times longer time period. The abalone survival in this trial was very similar to the 65 and 62 % on artificial diets shown in two shorter feeding trials using similar size juvenile *H. laevigata* (Daume *et al.* 2007). Survival on the mixed sporeling and the commercial nursery diets were just under 50 %, which was higher than the abalone on the monospecies diet of *Ulva* sporelings and similar to the commercial nursery diet survival in Section 5.1.3. However, it was considerably lower than the 68 to 82 % abalone survival on the *U. lens*/*N. jeffreyi* diet and the *Ulva* sporeling/*U. lens*/*N. jeffreyi* diet in Daume *et al.* (2007). The lower survival on natural diets was unusual given the animals came from a natural (*U. lens*/diatom) diet, which generally reduces the weaning process when compared to artificial diets (Fleming *et al.* 1996). The weaning process from the juvenile abalone onto all diets may have been masked by the high mortality (28.5 %) that occurred in the first week of the trial. The mortality was not significantly different between systems or diets and can be attributed to the source abalone nurseries husbandry and harvesting, as well as transporting the animals which can impose great stress where juvenile mortalities in excess of 50 % have been reported (Pang *et al.* 2006). To overcome the husbandry, harvest and transport mortality all the nursery and weaner tanks were restocked to initial trial densities.

Juvenile abalone growth and survival did not significantly correlate to any of the nursery algal diet consumption rates. There were relationships within diets as the consumption of *Hypnea* sporelings correlated with *Ulva* sporelings, indicating there may have been no preference for either species. This correlation was also present between the consumption rate of *U. lens* and *N. jeffreyi*. Rather than the growth and

survival of juvenile abalone correlating with algal consumption rates, the incorporation of new seeded algal plates had a substantial impact on growth. The correlation between the monospecies *Ulva* sporeling diet consumption and abalone growth occurred because it was a single inoculation diet and had no increase in biomass during its trial (Section 5.1.3). The regular input of algal material in this feeding trial caused fluctuations in consumption rates. However, this was not instantly translated into changes in growth rates because of juvenile abalone predisposition to go long periods without food, subsequently reducing metabolic activity while utilising their own energy stores (Fleming *et al.* 1996, Durazo-Beltrán *et al.* 2004).

In the mixed sporeling diet, consumption of both algae showed a decrease just as the new seeded plates were incorporated and a sharp increase in the following sample period. The initial decrease in consumption was caused by the sporelings growing faster than the juveniles could consume them, while the subsequent rapid increase in consumption was directly dependent on the higher algal biomass provided on the new plates. The substantial increase in consumption rates of both macroalgae after week 18 was due to the significantly greater density of sporelings on the new seeded plates, compared to the plates at the start for *Hypnea* and at the start and week 8 for *Ulva*. The incorporation of these new seeded plates at week 16 and the subsequent increase in consumption, translated into the significantly faster growth rate of the juvenile abalone on the mixed sporeling diet.

The consumption of *U. lens* generally fluctuated between 0.02 and 0.06 % cover.abalone^{-1}.day^{-1}, except when it became "negative" after a spore release event occurred. Even *U. lens* with a higher grazing resistance than the sporelings and several spore release events, had its cover reduced to 16.2 % at week 18 and subsequently, new seeded plates were introduced. The increase in algal biomass by the introduction of new plates corresponded to the increase in growth rates during the second half of the trial. Regular inoculation of the diatom *N. jeffreyi*, caused the consumption rate by juvenile abalone to be "negative" for almost all of the trial and was subsequently, the only feed that did not show significant fluctuations in consumption rate. This may also be

attributed to the juvenile abalone size, as the transition from a diatom-based diet to macroalgae-based diet occurs during the earlier part (5 – 10 mm shell length) of this feeding trial (Kawamura *et al.* 1998b, Takami & Kawamura 2003).

The *Phormidium* diet was the most heavily grazed, as shown by the 77 % decrease in cover at the start of the trial and the subsequent drop in consumption rate over the following 6 weeks due to a lack of biomass present. The high consumption rate required new seeded plates to be incorporated more often than the other nursery diets. Therefore, the fast growing cyanobacteria could not accommodate the feeding pressure of the juvenile abalone and the growth rate in the last 6 weeks was significantly slower than the other two nursery diets.

The fluctuations in consumption rates and the reduction in algal biomass during this feeding trial indicated the algal species tolerance to grazing could play an important role in the ability of a diet to support juvenile abalone. Generally abalone nurseries seek nutritionally adequate algal species that have sufficient grazing tolerance to support the pressure of juvenile abalone (Parker *et al.* 2007). The green alga *U. lens* currently used in Australian nursery systems has a high attachment strength onto the PVC plates, which can be problematic for consumption by juveniles less than 3 mm shell length (Kawamura 1996, Daume *et al.* 2000, Huggett *et al.* 2005). Other Ulvophyceae species have shown to be easily grazed by abalone less than 3 mm shell length and have greater recovery rates following grazing (Dyck *et al.* 2011). However, it was the intensive grazing pressure applied by juvenile abalone of up to 9 – 10 mm shell length, which reduced all diets tested in this feeding trial to an insufficient biomass regardless of the algal species attachment strength.

All three algal nursery diets required new seeded plates to be incorporated and without their introduction the diets would have had insufficient biomass to support juvenile abalone throughout the trial. For the *Phormidium* diet this occurred very early in the feeding trial and the diet would not have sustained the animals much past the first

2 months (≈7.5 mm shell length). The mixed sporeling diet biomass would have become inadequate around week 12 to 14 (≈9 mm shell length) of the feeding trial. This corresponded to similar time periods for the monospecies *Ulva* sporelings diet to reach insufficient algal biomass in the *Ulva* Sporeling Diet Feeding Trial (Section 5.1.3), as well as when the density of *Ulva* sporelings reduced to minimal numbers in the Daume *et al.* (2007) nursery feeding trial. The commercial nursery diet would have lasted slightly longer; even with spore release events it was still reduced to insufficient biomass by week 16 (≈9 – 10 mm shell length). This was longer than the 14 weeks required too reduce the cover of *U. lens* to 11 % in Section 5.1.3 and the 9 weeks it took juvenile abalone in Daume & Ryan (2004), both from similar initial *U. lens* cover. In order to maintain commercially viable growth rates of juvenile greenlip abalone greater than 9 – 10 mm shell length throughout the later nursery phase, new seeded algal plates had to be introduced. Through the incorporation of new seeded algal plates both the mixed sporeling diet and the commercial nursery diet were able to provide sufficient algal biomass, resulting in improved growth rates and allowing the juvenile abalone to remain in the nursery system until they reached 14 – 15 mm shell length.

Juvenile abalone growth rates on the mixed sporeling, commercial nursery and artificial diets were significantly and positively correlated with the increase in mean seawater temperature during the 26-week trial. When the temperature was below 18^{o}C abalone growth rates were similar across all diets. As the temperature rose after week 14 the abalone growth rates became significantly faster on the three diets mentioned above. This pattern in growth rates was also evident with juvenile *H. laevigata* feeding on an *Ulva* sporeling/*U. lens*/*N. jeffreyi* diet when the temperature passed 16^{o}C (Daume *et al.* 2007). These results indicate that juvenile abalone growth rates may increase with rising temperatures if diets can provide sufficient algal biomass during the later nursery phase. Abalone growth rates were fastest at the end of the trial when the temperature reached 22.8^{o}C. Growth rates of *H. discus hannai* (>4.6 mm shell length) consuming *U. lens* and benthic diatoms have been shown to increase with temperature and exhibited superior growth rates at 22.5^{o}C (Uki *et al.* 1981). Juvenile *H. tuberculata* (3.22 mm shell length) produced faster growth rates when cultured at 22^{o}C compared to 15 and 18^{o}C, while *H. iris* (10 mm shell length) recorded maximum growth at 22^{o}C (Lopez *et al.* 1998,

Searle *et al.* 2006). Britz *et al.* (1997) determined that growth of *H. midae* (17.5 mm shell length) on an artificial diet significantly increased with temperature until 20°C after which a sharp decline occurred, indicating the possibility of temperature thresholds. However, a 50 % critical thermal maximum of 27.5°C and an optimum growth temperature of 18.3°C has been reported for *H. laevigata* (Gilroy & Edwards 1998). The optimal temperature occurred during week 14 of this feeding trial when juvenile abalone growth rates on the mixed sporeling, commercial nursery and artificial diets began to increase, indicating that juvenile *H. laevigata* benefit from being cultured at seawater temperatures greater than 18°C. Unfortunately culturing juvenile greenlip abalone during the later nursery phase at higher temperatures would be an unrealistic possibility for many commercial farms given their southern location and subsequent cool incoming seawater (<18°C). Also in Australia, greenlip abalone are generally spawned in the later part of the year and based on post settlement abalone growth rates and available nursery tank space, the juvenile abalone (>9 – 10 mm shell length) are not able to be maintained in the nursery system during the period of warmer seawater.

The irradiance received by the growing algae in the nursery system diets was significantly different to that received by the weaner system, however this did not translate into disparity in abalone growth rates between the two systems. The variation in abalone growth parameters between the WMT and the artificial diet in the weaner system were not due to irradiance, as there was no difference between the irradiance the two diets' tanks received. Irradiance did have an effect in the nursery system, however the effect was not between diets but rather between the replicate tanks within each diet. The variation from east to west across the nursery tank banks produced a significant increase in standardised growth and survival of juvenile abalone in the bank that received the greatest irradiance. This indicated that irradiance has an important effect on the algal biomass present and subsequently juvenile abalone growth. The effect of irradiance on diatom diets has been shown to be of particular importance in postlarval feeding and development (Daume *et al.* 2004, Gorrostieta-Hurtado & Searcy-Bernal 2004). Randomisation of nursery tanks across the irradiance gradient played an important role in removing irradiance as an environmental factor that altered the juvenile abalone response to a specific nursery algal diet.

The half-year, multi diet, semi-commercial feeding trial demonstrated that the nursery and weaner systems were equally effective in producing juvenile greenlip abalone from 5 to 15 mm shell length. The variation in all juvenile abalone growth and survival parameters through the later nursery phase were not affected by the system utilised to rear the animals, but rather by the type and biomass of diet they were able to consume. The faster juvenile abalone growth rates (shell length and weight) over the 26-week feeding trial produced on the mixed sporeling diet (*Hypnea*/*Ulva*), allowed it to be considered a suitable alternative to the current commercial nursery (*U. lens*/*N. jeffreyi*) and weaner (artificial) diets. Incorporating new seeded plates of the mixed macroalgal sporeling diet provided adequate algal biomass to sustain the intense grazing pressure of juvenile abalone and produced commercially viable growth rates, permitting the juvenile *H. laevigata* to remain in the nursery system throughout the entire later nursery phase.

CHAPTER 6
GENERAL DISCUSSION

The reliable supply of suitable algal species in sufficient biomass has been shown to be fundamentally important to the nutrition and development of juvenile *Haliotis laevigata* in the commercial nursery system. The use of inappropriate algal varieties or a reduction in algal biomass on the PVC plates can have significant impacts on the growth and survival of post settlement abalone. This in turn, has considerable implications for the later development of abalone and subsequently, the overall commercial production and profitability of abalone culture in land based facilities. Therefore, the central aim of this thesis was of particular importance given it was to identify, develop and evaluate alternative macroalgal diets for juvenile greenlip abalone, that supply sufficient algal biomass to maintain commercially viable abalone growth rates throughout the later nursery phase. In addressing the central aim, this research was the first in juvenile abalone culture to:

1) Identify carpospore liberation and vegetative propagation as macroalgal propagation methods for incorporating Rhodophyta species into juvenile abalone diets within the nursery system.

2) Utilise vegetative fragments of a red macroalga with the aid of a specifically developed artificial adhesion protocol for a juvenile abalone diet on a semi-commercial scale in the nursery system.

3) Incorporate high macroalgal biomass by seeding sporelings as a diet to address the intense grazing pressure of juvenile abalone and maintain growth rates above current diets on a semi-commercial scale in the nursery system.

By accomplishing these three outputs, juvenile greenlip abalone of 5 mm shell length were able to be cultured to 15 mm shell length at commercially appropriate growth rates, therefore improving the later nursery phase production of abalone aquaculture.

In assessing the ability of various red macroalgae to be incorporated into the juvenile abalone nursery system, three propagation methods of spore liberation, protoplast production and vegetative propagation were examined. All three propagation methods were able to produce *in vitro* cultures of a variety of red macroalgal species that would be suitable for juvenile abalone consumption (Section 3.3). Even though high-density macroalgal biomass could be theoretically attained to seed the nursery plates, it was not only the amount of biomass produced but also a range of factors that determined the propagation methods suitability to develop juvenile abalone diets. Biological factors including the source macroalgae biomass required, propagule/fragment characteristics and adhesion ability were taken into consideration, while the methodology, costs and ability to be utilised can have considerable bearing on whether a commercial abalone farm can incorporate the propagation methods into their standard operating procedures (Table 24).

Protoplast production was a prime example of being biologically capable of producing high propagule biomass to seed a juvenile abalone diet on the vertical PVC plates. However, its methodology and costs associated made it impractical for utilisation in an abalone nursery (Table 24). Given only 1 g blotted wet weight of *Gracilaria* sp. thalli produced 28.83 ± 8.69 $x10^5$ protoplasts (Section 3.3.2), it significantly reduced the amount of macroalgal biomass required too seed the nursery plates. This was the greatest number of propagules produced per gram alga out of three propagation methods and had the added benefit of being attained from any section of the thallus, from any phenotypic stage of the alga. The benefits associated with the source macroalgal biomass required are impressive, as it would remove the pressure on harvesting wild macroalgal populations, as well as reducing the costs associated with on site macroalgal culture. However, these benefits for abalone nurseries would be offset by the limited and time-consuming regeneration capacity of protoplasts (e.g. Yan & Wang 1993, Yeong *et al.* 2008), as well as the requirement for an artificial adhesion protocol to attach the protoplasts/callus formations to the plates. These two factors both add to protoplast productions already specialised and costly methodology, therefore, outweighing the biological benefits associated with propagule production and the reduced biomass of source macroalgae required. Thus making the development of a

juvenile abalone nursery diet through protoplast production an unfeasible proposition in a commercial abalone farm (Table 24).

Vegetative propagation through fragmentation can also utilise any part of the thalli from any phenotypic stage of macroalgae, making the harvest or culture of algal biomass relatively quick and easy (Table 24). This removes the problems associated with the triphasic life cycle of Rhodophyta and the collection of fertile material, hence its use as a propagation method in commercial macroalgal cultivation through the regenerative capacity of thalli fragments (e.g. Santelices & Varela 1995, Oliveira *et al.* 2000). Fragment cultures of *Hypnea* sp. and *Gracilaria* were established with low fragment mortality and adequate growth rates after the initial wound-healing period (Section 3.3.3). Given the limited attachment rate of the macroalgal fragments to a substratum, artificial adhesion protocols were developed to allow the *Hypnea* and *Gracilaria* fragments to be attached to the PVC plates utilised in the abalone nursery system. An active immobilisation protocol incorporating the sulphated galactan agar as a gelling agent and a spraying methodology, allowed an agar/macroalgal fragment mixture to be adhered onto the plates (Section 4.1). The need for artificial adhesion protocols dictated the characteristics of macroalgal fragments that could be incorporated; hence successful *in vitro* cultivation alone did not necessarily translate into a macroalgal species' suitability for use as a juvenile abalone diet.

Laurencia sp. actually proved the most appropriate alga for incorporation into the artificial adhesion protocols and was able to be examined as an alternative macroalgal diet in the later nursery phase. The *Laurencia*/agar diet was comparable to the current commercial nursery diet (*Ulvella lens*/*Navicula* cf. *jeffreyi*) for juvenile *H. laevigata* growth (8 – 11 mm shell length) and survival during a 12-week feeding trial (Section 4.2.3). Weight gain was the exception, with the abalone consuming the commercial nursery diet being significantly heavier at the end of the trial, causing implications for the total production biomass of an abalone farm when the animals reach marketable size. However, both algal diets examined were unable to accommodate the high grazing pressure of the juvenile *H. laevigata* once they had reached 10.5 mm shell

length. This was evident by the decline in growth rates and lack of algal biomass available towards the end of the feeding trial, consequently restricting the diets use in commercial abalone farms during the later nursery phase. Overall the *Laurencia*/agar diet produced slightly fewer and smaller abalone than the commercial nursery diet. However, it was the inability for the diet to remain adhered to the plates and therefore, the need for regular re-application that defined the *Laurencia*/agar diets ineffectiveness during the later nursery phase (Table 25).

Harnessing the natural recruitment of macroalgae through attachment and germination of spores can be an effective means of attaching a high biomass of red macroalgae to the PVC plates. This removes the need for artificial adhesion protocols, which in turn significantly reduces the labour and costs associated with creating an algal diet for juvenile abalone in the nursery system (Table 24). Even without the artificial adhesion protocols costs, cultivation of macroalgae through vegetative propagation can generally be more expensive than via the release of spores (Alveal *et al.* 1997). Spore liberation utilises simple equipment, while vegetative propagation methods currently used in commercial cultivation can be cumbersome, labour intensive, time consuming and inefficient (Halling *et al.* 2005, Mantri *et al.* 2009). The economic benefits of utilising spore liberation over vegetative propagation are clear, as seeding the PVC plates in the abalone nursery system with spores reduces the costs associated with methodology, labour, equipment, while also removing the need for time consuming and costly artificial adhesion protocols (Table 24).

The biological benefits of spore liberation are also important as a relatively small amount of algal biomass can produce large numbers of spores as seed. The mean carpospore production for *Hypnea* from all three induction treatments of temperature, dark and osmotic pressure was 67.23 ± 10.19 $x10^3$ carpospores.g^{-1} alga, while the temperature treatment and culture conditions of 25 ± 2^oC and 10 µmol photons.m^{-2}.s^{-1} liberated 140.21 ± 19.73 $x10^3$ carpospores from only 1 g of carposporophytic thalli (Section 3.3.1). These carpospore liberation rates allow the nursery plates to be seeded using far less macroalgal material than by vegetative propagation, which can consume

significant amounts of biomass. For example, 430 g of carposporophytic *Hypnea* was able to seed ninety, 60 x 26 cm PVC plates condensed in one nursery tank, to a sporeling density of up to 44.5 ± 5.5 sporelings.cm^{-2} present after 2 months of culturing (Section 5.2.2). The use of vegetative fragments from any phenotype of *Laurencia* thalli, required 1.8 kg to seed sixty, 60 x 26 cm PVC plates to a maximum density of 3.06 ± 0.09 fragments.cm^{-2} directly after application (Section 4.2.2). Given the sporelings were cultured for 2 months they were a similar length but slightly thinner than the fragments, however the difference in size does not counter act the substantial difference in density. The variation in biomass of source macroalgae required really becomes evident when an abalone nursery diet of macroalgal sporelings produced from carposporophytic *Hypnea* can last months being grazed by juvenile abalone (Section 5.2.3), while the *Laurencia*/agar diet had to be re-applied weekly. This re-application meant nearly 60 kg of *Laurencia* biomass was required for the 12-week *Laurencia* Fragment/Agar Diet Feeding Trial (Section 4.2), which scaled up to a nursery system under full production at a commercial abalone farm would be an unacceptable amount of macroalgal biomass to source.

The excessive macroalgal biomass required for cultivation using vegetative propagation has lead to the commercial macroalgae industry looking at seed production through spore release as a way of improving mass culture. Seeding by spore liberation has been shown to reduce the initial stock biomass required and have high production potential with uniform sporeling densities, indicating the possibility for use in commercial field cultivation (Alveal *et al.* 1997, Mantri *et al.* 2009). A direct comparison of spore and vegetative seeded rope cultivation of the red alga *Gracilaria chilensis* by Halling *et al.* (2005), indicated that during the first month the propagation methods had comparable alga biomass growth. However, after a biomass loss event from the spore-seeded ropes caused patchy low densities, the vegetative seeded ropes produced higher alga productivity. The biomass loss was attributed to the alga's morphology as it lacked a developed attachment disk and this combined with the open water location of the trial, resulted in the attachment strength being insufficient to maintain the macroalga on the ropes (Halling *et al.* 2005). Given juvenile abalone aquaculture occurs in tanks the algal biomass doesn't deal with excessive natural forces, allowing lower attachment strengths to be utilised. However, adhesion strength of the

algal biomass can't be minimal, as it's still required to have sufficient grazing resistance to accommodate the intense grazing pressure of juvenile abalone while not be rapidly depleted (Dyck *et al.* 2011). This further highlights the importance of appropriate macroalgal adhesion to the unique vertical PVC plate substrata in the abalone nursery system, as even with a propagation method incorporating natural attachment properties care still needs to be taken when selecting the most suitable macroalgal species.

Even though relatively little red macroalgal biomass was required to produce high amounts of propagules through the release of spores, the biomass harvested must be from specific phenotypic stages, namely the carposporophyte or tetrasporophyte stage. Given these phenotypes can have varying abundances in the wild population due to seasonality and sexual maturity (e.g. Kain 1982, Engel *et al.* 2001), it can be difficult to harvest sufficient appropriate phenotypes for spore liberation. This was evident in the collection of fertile *Plocamium mertensii* for the preliminary, small-scale spore production experiment (Section 3.3.1) and was taken into account when utilising spore liberation as a method of seeding the plates in the nursery system. This can be overcome to some degree by cultivation on site and utilising carposporophyte developed in culture (e.g. *G. chilensis*), even if the carpospore production capacity may be slightly reduced (Section 3.3.1). Harvesting/culturing of fertile phenotypes does produce limitations for the use of spore liberation in creating a diet for juvenile abalone. However, these limitations can be countered by numerous other benefits, including the propagule characteristics, adhesion ability, ease of methodology, and the reduced costs of production (Table 24). Even though all three macroalgal propagation methods (spore liberation, protoplast production and vegetative propagation) can produce *in vitro* cultures, given the plethora of factors associated, spore liberation was considered the most desirable propagation method for developing juvenile abalone diets in commercial abalone nurseries (Table 24).

Table 24: A summary comparison of the characteristics of the three algal propagation methods, spore liberation, protoplast production and vegetative propagation for their use in developing a juvenile *Haliotis laevigata* diet on the PVC settlement plates in the nursery system.

(✓✓ = very good, ✓ = good, x = bad, x x = very bad, na = not applicable)

Propagation Characteristic		**Spore**	**Protoplast**	**Vegetative**
Source Macroalgal Biomass Required	Amount	✓	✓✓	x
	Fertile	✓✓	✓✓	✓✓
	Non Fertile	x x	✓✓	✓✓
	Culture	✓	✓✓	✓✓
	Wild Harvest	✓	✓✓	✓✓
Propagule / Fragment Characteristics	Amount	✓✓	✓✓	✓
	Size	✓✓	✓✓	x
	Regeneration	✓✓	x x	✓
Adhesion Required	Natural	✓✓	x x	x x
	Artificial	na	✓	✓
Propagation Method	Simplicity	✓	x x	✓
	Equipment	✓✓	x x	✓
Associated Costs	Propagation	✓✓	x x	✓
	Adhesion	na	x	x
	Labour	✓	x x	x
	Utilisation	✓✓	x x	x
Abalone Diet	Algal Density	✓✓	x x	✓
	Farm Ability	✓✓	x x	x
Overall		✓✓	x	✓

By utilising the macroalgal natural adhesion methods of spore liberation, settlement and germination it allows algae from other Phylum to be developed into juvenile abalone diets. The Chlorophyte *Ulva* was able to produce a high density, macroalgal sporeling diet on the PVC plates within the nursery system as its reproductive and morphological characteristics facilitated a high spore liberation capacity (Hoxmark 1975, Hiraoka *et al.* 2003, Mantri *et al.* 2011). The *Ulva* spp. sporeling diet was comparable to the current commercial nursery diet for the survival and growth of juvenile abalone, producing a similar growth rate and specific growth rate over a 14-week feeding trial (Section 5.1.3). The growth of juvenile abalone in the range of 3.5 to 6.5 mm shell length was shown to be significantly greater when feeding on the *Ulva* sporeling diet. This implies the *Ulva* sporelings had a lower grazing resistance than *U. lens*, which can be difficult for juveniles less than 3 mm shell length to feed on due to its high attachment strength (Daume *et al.* 2000, Dyck *et al.* 2011). Importantly, the use of *Ulva* sporelings improved the transition from a diatom-based diet to a macroalgae-based diet juvenile abalone exhibit around 5 – 10 mm shell length. This transition between algal forms can be a problem in commercial nurseries and the use of *Ulva* sporelings could be included from settlement as a means of reducing the transitional impacts on juvenile abalone (<5 mm shell length) growth (Table 25).

The high biomass *Ulva* sporeling diet was not able to accommodate the growth requirements of larger juvenile abalone in the later nursery phase, even though it had the ability to improve early nursery phase production (Table 25). The commercial nursery diet produced significantly larger growth for juveniles in the range of 6.5 to 10 mm shell length and subsequently, bigger abalone at the end of the trial (Section 5.1.3). The inability of the *Ulva* sporeling diet to maintain growth comparable to the current nursery diet for larger juvenile abalone may have been due to its monospecies nature and biochemical composition. The commercial nursery diet was shown to have a biochemical composition closer to the optimum levels required for juvenile abalone growth, while the *Ulva* sporeling diet may of become limited in particular nutrients important to abalone growth.

The grazing pressure of the juvenile abalone (9 – 10 mm shell length) towards the end of the feeding trial became too great for both diets and biomass limitation resulted, consequently the growth of juvenile abalone suffered. Even utilising the high biomass and natural adhesion properties of *Ulva* sporelings, the diet was still unable to overcome the problem associated with biomass limitation in the later nursery phase. To tackle the reduction in biomass and nutrient limitation due to a monospecies diet, a mixed sporeling diet consisting of *Hypnea* and *Ulva* sporelings was developed. By utilising a red macroalga as part of a mixed sporeling diet, it reduced the number of plates in the nursery system to be seeded with *Hypnea* sporelings. This decreased the amount of fertile phenotype source biomass required, which was identified as the only limitation to utilising spore liberation as a propagation method for seeding a Rhodophyta species into a juvenile abalone diet within the nursery system (Table 24).

The mixed sporeling diet produced growth rates similar to the commercial nursery diet during the first 14 weeks of the Multi Diet and System Feeding Trial (Section 5.2.3). Given that the monospecies, *Ulva* sporeling diet could not maintain growth rates similar to the commercial nursery diet over 14 weeks, this indicated the inclusion of the red macroalgal (*Hypnea*) sporelings improved the nutrient profile of a sporeling diet and removed the nutrient limitation associated with larger juvenile abalone consuming single species diets. Therefore, commercial abalone farms would benefit from suppling a mixture of macroalgae rather than a single species, to produce a well-balanced juvenile abalone nursery diet.

All nursery system diets examined in the Multi Diet and System Feeding Trial (Section 5.2) – mixed sporeling, commercial nursery, *Phormidium* sp. – produced comparable growth rates for the first 14 – 16 weeks, indicating that a wide variety of algal species can be utilised by commercial abalone farms to grow juvenile *H. laevigata* to 9 mm shell length (Table 25). Algal biomass still became limiting, just as with the *Ulva* sporeling and *Laurencia*/agar diets, signifying 9 – 10 mm shell length can be a critical point in the production of juvenile abalone in the nursery system. To overcome the algal biomass limitation new seeded plates of the diets were introduced to the

nursery system, increasing algal biomass and by doing so demonstrated the ability of the mixed sporeling diet to improve juvenile abalone production in the later nursery phase. The mixed sporeling diet was able to produce the largest, heaviest and fastest growing juvenile abalone throughout the entire nursery phase from 5 to 15 mm shell length, therefore, accomplishing the central aim of this research. Even though the growth of juvenile abalone consuming the mixed sporeling diet were greater, they were not significantly different to the abalone on the current commercial diet. These results indicate the mixed sporeling diet with new seeded plates introduced, would be a viable alternative diet for commercial abalone farms to utilise while rearing juvenile abalone in the nursery system (Table 25).

Comparing different juvenile abalone management strategies by changing system design from the nursery to weaner system, did not improve production of juvenile abalone. The artificial diet utilised in the weaner system produced slightly smaller and lighter abalone with a slower growth rate compared to the mixed sporeling diet in the nursery system (Section 5.2.3). Both diets were considered similar for rearing juvenile abalone less than 9 mm shell length, which was unusual given growth rates and survival directly after weaning onto artificial diets are usual lower than on natural diets (Daume & Ryan 2004, Daume *et al.* 2007), as was the case in the *Laurencia* Fragment/Agar Diet Feeding Trial (Section 4.2). If no new algal biomass can be introduced to the nursery system, artificial diets in the weaner system are considered the most suitable strategy for rearing juvenile abalone over 9 mm shell length (Section 4.2.3). Given that algal biomass was maintained by incorporating new seeded plates in the Multi Diet and System Feeding Trial (Section 5.2), the mixed sporeling and commercial nursery diets both produced comparable growth to weaning juvenile abalone onto an artificial diet. In a cost benefit analysis of juvenile abalone production via the nursery system compared to early weaning onto artificial diets using the exact same systems as in this study, the running costs of the two management strategies were shown to be fairly comparable (Cook 2007). Overall, the mixed sporeling and commercial nursery diets with the inclusion of new algal biomass in the nursery system and the artificial diet in the weaner system are appropriate management strategies to rear juvenile abalone in the Australian abalone aquaculture industry (Table 25).

Table 25: A summary comparison of the five nursery system diets; current commercial nursery, mixed sporeling (*Hypnea*/*Ulva*), *Ulva* sporeling, *Laurencia* fragment/agar and *Phormidium*, and the two weaner system diets; Whole Marcoalgal Thallus (WMT) and an artificial diet, trialled for culturing juvenile *Haliotis laevigata* during the later nursery phase (5 to 15 mm shell length).

(✓✓ = very good, ✓ = good, x = bad, x x = very bad, na = not applicable)

Characteristic	**Growth**		**Survival**		**Algal Biomass**		**Consumption**		**Labour**		**Cost**		**Overall**	
Abalone size (mm)	**< 10**	**> 10**	**< 10**	**> 10**	**< 10**	**> 10**	**< 10**	**> 10**	**< 10**	**> 10**	**< 10**	**> 10**	**< 10**	**> 10**
Diet														
Commercial nursery	✓	✓	✓	✓	✓✓	✓	✓✓	✓	✓✓	✓	✓✓	✓	✓✓	✓
Mixed sporelings	✓✓	✓✓	✓	✓	✓	✓	✓✓	✓✓	✓	✓	✓✓	✓	✓✓	✓+0.5
Ulva sporeling	✓✓	x	✓	x	✓	x	✓✓	✓	✓	✓	✓✓	✓	✓✓	x
Laurencia / agar	✓	x	✓	x	x x	x x	x	x x	x x	x x	x x	x x	x	x x
Phormidium	✓	x x	✓	x	x x	x x	✓✓	x x	✓✓	x x	✓✓	✓	✓	x x
WMT	✓	x x	✓	x	✓	x x	✓	x x	x	x	✓	✓	✓	x x
Artificial	✓	✓✓	x	✓✓	na	na	na	na	✓	✓✓	✓	✓✓	✓	✓✓

In conclusion, propagation methods (spore liberation) that utilise natural adhesion can produce substantial macroalgal biomass, efficiently and cheaply on the PVC settlement plates utilised in the current abalone nursery system. Given the nutritional dietary transitions juvenile abalone experience due to the ontogenetic changes in their development, commercial abalone nurseries would be best served by incorporating a succession of algal forms and species that are simple to culture, provide high biomass and are of an appropriate grazing resistance. Daume (2006) proposed a timeline for abalone nutrition from settlement to 15 mm shell length in aquaculture. By examining macroalgal propagation methods this research was able to expand on the limited information available for juvenile *H. laevigata* nutrition (>3 mm shell length) in the later nursery phase and develop the timeline into a detailed feeding regime (Table 26). This regime can be considered an important expansion for the abalone aquaculture industry, as it would allow the most appropriate nutrition source to be available to juvenile abalone at all times during the nursery phase. Thus, removing any reduction in growth rates or increase in mortality due to monospecies diets, lack of algal biomass or weaning onto artificial diets at an inadequate size. The design of the Australian abalone nursery system would be able to accommodate such a feeding regime, as plates seeded with different algal species and biomass can be interspersed throughout tanks at different times and densities with relative easy. Through a staggered, multi algal species feeding regime (Table 26) the nursery phase would be able to produce uniform, large, high quality juvenile abalone biomass. This improved nursery management strategy would have the considerable flow on effect of increasing growth and abalone production throughout the entire commercial abalone farm.

Table 26: Timeline of juvenile *Haliotis laevigata* nutrition and the appropriate corresponding diet supplied (feeding regime) in the nursery system for optimal commercial, juvenile abalone production.

Abalone	**Size (mm)**	**Nutrition Source**	**Nursery System Diet**
Larvae	Settlement		*Ulvella lens*
Post Larvae	0 – 0.4	Yolk + DOM	
	0.4 – 0.7	Biofilm + Bacteria	
	0.7 – 1.5	Biofilm + Diatom	*Navicula* cf. *jeffreyi*
	1.5 – 3	Diatom	*N. jeffreyi* + *Ulva* sporeling
Transition	3 – 5	Diatom + Macroalgal sporeling	*N. jeffreyi*, *Ulva* sporeling + *U. lens*
Juveniles	5 – 10	Macroalgal sporeling	*Ulva* sporeling, *Hypnea* sporeling + *U. lens*
	10 – 15	Macroalgal sporeling + Macroalgal thalli	*Hypnea* sporeling + *U. lens* or Artificial diet (weaner system)
Adult	>15	Macroalgal thalli	Weaner System – Artificial diet and / or Macroalgal thalli

6.1 FUTURE STUDIES

The examination into macroalgal propagation methods for providing algal biomass for juvenile abalone nutrition, will assist Australian abalone aquaculture to improve commercial production in the later nursery phase and provide a solid basis for a range of research possibilities. This study not only contributes to the available knowledge on juvenile *H. laevigata* growth and survival in the nursery system, but also the significance of juvenile macroalgae in the dietary transition of juvenile abalone from a diatom-dominated to a macroalgal-dominated diet at approximately 5 – 10 mm shell length, while further advancing macroalgal cultivation methods for aquaculture.

The present study has shown that the algal propagation method of spore liberation was the most suitable methodology for developing macroalgal diets on the PVC settlement plates in the nursery system (Table 24). Given the requirements of specific phenotypes for spore liberation of Rhodophyta species and the variability of these in wild populations, on site macroalgal cultivation was identified as a possibility to improve the source algal biomass supply. Cultivation of macroalgal species already occurs around the world as the supply of natural stocks can't meet the current demand, particularly in the commercially important agarophyte algae such as *Gracilaria* (Zemke-White & Ohno 1999, Reddy *et al.* 2008b). A majority of this cultivation occurs using vegetative propagation on suspended rope systems in the ocean, shallow ponds or tanks (Santelices & Doty 1989, Oliveira *et al.* 2000). This cultivation method can be extremely useful in an integrated aquaculture system with fish farm cages, and has been shown to increase biomass production of the Rhodophyta species *G. chilensis* and *Macrocystis pyrifera*, while also increasing the algae's nutrient content through bioremediation (Troell *et al.* 1997, Buschmann *et al.* 2008).

The principles of integrated aquaculture systems are not limited to ocean culture and can be applied to a combination of commercial important species including fish, shrimp, abalone, sea urchin, other shellfish and algae in land based systems (Neori *et al.* 2004). Generally aquaculture facilities treat carnivore production waste with trophically lower organisms, which can then create revenue-generating cultivation of the secondary

organism (Neori 2008). This methodology can be applied to abalone aquaculture where the waste produced from can be utilised for macroalgal production, subsequently developing a low cost feed for abalone. These integrated abalone, macroalgae aquaculture systems are currently being explored and utilised in countries such as South Africa, where abalone culture utilises a high biomass of macroalgae as feed with the dominant specie of kelp *(Ecklonia maxima*) now reaching maximum sustainable limits (Troell *et al.* 2006). Given abalone growth improves with mixed rather than monospecies diets, other macroalgal species including *Gracilaria* and *Ulva* are being produced through integrated cultivation and examined as possible alternative algal diets for abalone in South African farms (Robertson-Andersson *et al.* 2008, Bolton *et al.* 2009). This developing body of research on integrated abalone and macroalgal cultivation can be transposed to Australian abalone production and would allow on-site culturing of the macroalgal phenotypic biomass required for spore liberation. This would reduce the reliance on wild harvest algae and allow the propagation method of spore liberation to be extremely effective in producing high biomass macroalgal sporeling diets on the nursery plates for juvenile abalone culture.

On farm cultivation of macroalgae allows a diversification of species to be cultured and subsequently utilised as mixed sporeling diets. Research into the spore production of the Rhodophyte *Palmaria palmata* for abalone hatcheries has been carried out (Le Gall *et al.* 2004), while sporelings of the brown algae *Eisenia bicyclis*, *Ecklonia cava* and *Undaria pinnatifida* have shown positive results for juvenile *Haliotis diversicolor* (<10 mm shell length) growth on a very small-scale (Onitsuka *et al.* 2010, 2011). The present study found that the mixed sporeling diet of *Hypnea* and *Ulva* was a viable alternative diet for raising juvenile *H. laevigata* in the nursery system (Table 25), which opens up future research into the myriad of algal combinations possible for juvenile abalone diets. A composite *Ulva* sporeling, *U. lens* and *N. jeffreyi* diet has already been developed based on research within this thesis and was shown to produce significantly larger juvenile *H. laevigata* than the current commercial nursery diet (Daume *et al.* 2007). These multi species diets, given the improvement in juvenile abalone growth appear to be the future for commercial abalone production.

Another area of important research to come out of this study would be to utilise these macroalgal sporeling diets not only in various combinations but also in conjunction with artificial diets, to further reduce the effects of the weaning process. A study incorporating the benthic diatom *Nitzschia longissima* and *U. lens* with artificial feeds in specifically designed tanks has already shown marked improvement in growth and survival of juvenile *Haliotis iris* during the weaning process (Dyck *et al.* 2010). By combining algae and artificial diets together it allows the juvenile abalone to seamlessly transition from one diet to the other without the stress of harvesting and weaning. Supplementation of artificial diets with wild adult macroalgal thalli has been shown to significantly improve growth for post weaning (>12 mm shell length) *Haliotis midae* (Dlaza *et al.* 2008), however, these juvenile abalone were cultured in baskets to incorporate the characteristics of both diets. The nursery and weaner/growout systems in Australian abalone farms are currently treated as separate entities and utilising a combination of algae and artificial diets will require significant research into an optimal combined system design. Before an entirely new system design would be utilised in Australian abalone farms, characteristics including tank design, water flow, irradiance and husbandry protocols would have to investigated and produce significant improvements on current culture practices.

The present study has developed a new macroalgal sporeling diet for juvenile abalone nutrition in the nursery system and demonstrated its' ability for commercial abalone production. This research provides a solid basis for future investigation into integrated culture of grazing tolerant macroalgal sporelings for juvenile abalone less than 3 mm shell length in the early nursery phase, as well as the combination of algal species and artificial diets to improve weaning of juvenile abalone greater than 15 mm shell length from the nursery system to the growout system in commercial abalone farms. The research presented in this thesis provides a significant step forward in commercial production of juvenile greenlip abalone (*H. laevigata*) in the current Australian abalone aquaculture systems.

REFERENCES

Ahuia, M.R. (1982) Isolation, culture, and fusion of protoplasts: problems and prospects. Silvae Genetica, 31: 66-77.

Ajisaka, T. & Chiang, Y.M. (1993) Recent status of *Gracilaria* cultivation in Taiwan. Hydrobiologia, 260/261: 335-338.

Alveal, K., Romo, H., Werlinger, C. & Oliveira, E.C. (1997) Mass cultivation of the agar-producing alga *Gracilaria chilensis* (Rhodophyta) from spores. Aquaculture, 148: 77-83.

Aoyagi, H., Sakamoto, Y., Asada, M. & Tanaka, H. (1998) Indole alkaloids production by *Catharanthus roseus* protoplasts with artificial cell walls containing of guluronic acid rich alginate gel. Journal of Fermentation and Bioengineering, 85: 306-311.

Aoyagi, H. & Tanaka, H. (1999) Development of simple methods for preparation of yeast and plant protoplasts immobilized in alginate gel beads. Biotechnology Techniques, 13: 253-258.

Araki, T., Hayakawa, M., Tamaru, Y., Yoshimatsu, K. & Morishita, T. (1994) Isolation and regeneration of haploid protoplasts from *Bangia atropurpurea* (Rhodophyta) with marine bacterial enzymes. Journal of Phycology, 30: 1040-1046.

Araki, T., Lu, Z. & Morishita, T. (1998) Optimization of parameters for isolation of protoplasts from *Gracilaria verrucosa* (Rhodophyta). Journal of Marine Biotechnology, 6: 193-197.

Avila, M., Piel, M.I., Caceres, J.H. & Alveal, K. (2011) Cultivation of the red alga *Chondracanthus chamissoi*: sexual reproduction and seedling production in culture under controlled conditions. Journal of Applied Phycology, 23: 529-536.

Azanza, R.V. & Ask, E.I. (2003) *Kappaphycus alvarezii* (Doty) Doty carposporeling growth and development in the laboratory. In: Chapman, A.R.O., Anderson, R.J., Vreeland, V.J. & Davison, I.R. (Eds.), Proceedings of the 17th International Seaweed Symposium, 28th January - 2nd February 2001, Cape Town, South Africa. Oxford University Press, New York, pp. 95-99.

Azanza-Corrales, R. & Dawes, C.J. (1989) Wound healing in cultured *Eucheuma alvarezii* var. *tambalang* Doty. Botanica Marina, 32: 229-234.

Bardos, D.C. (2005) Probabilistic Gompertz model of irreversible growth. Bulletin of Mathematical Biology, 67: 529-545.

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

Bautista-Teruel, M.N., Koshio, S.S. & Ishikawa, M. (2011) Diet development and evaluation for juvenile abalone, *Haliotis asinina* Linne: lipid and essential fatty acid levels. Aquaculture, 312: 172-179.

Ben-Amotz, A., Tornabene, T.G. & Thomas, W.H. (1985) Chemical profile of selected species of microalgae with emphasis on lipids. Journal of Phycology, 21: 72-81.

Björk, M., Ekman, P., Wallin, A. & Pedersén, M. (1990) Effects of growth rate and other factors on protoplast yield from four species of *Gracilaria* (Rhodophyta). Botanica Marina, 33: 433-439.

Björk, M., Gómez-Pinchetti, J.L., García-Reina, G. & Pedersén, M. (1992) Protoplast isolation from *Ulva rigida* (Chlorophyta). In: Björk, M. (Ed.), Protoplast isolation from marine macroalgae and applications to studies of inorganic carbon utilization. PhD Thesis, Uppsala University, Uppsala, Sweden, pp. 1-11.

Bligh, E.G. & Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37: 911-917.

Boarder, S.J. & Shpigel, M. (2001) Comparative performances of juvenile *Haliotis roei* fed on enriched *Ulva rigida* and various artificial diets. Journal of Shellfish Research, 20: 653-657.

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

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

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

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

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

Brown, M.R. (1991) The amino-acid and sugar composition of 16 species of microalgae used in mariculture. Journal of Experimental Marine Biology and Ecology, 145: 79-99.

Brown, M.R. & Jeffrey, S.W. (1995) The amino acid and gross composition of marine diatoms potentially useful for mariculture. Journal of Applied Phycology, 7: 521-527.

Brown, M.R., Jeffrey, S.W., Volkman, J.K. & Dunstan, G.A. (1997) Nutritional properties of microalgae for mariculture. Aquaculture, 151: 315-331.

Buschmann, A.H., Westermeier, R. & Retamales, C.A. (1995) Cultivation of *Gracilaria* on the sea-bottom in southern Chile: a review. Journal of Applied Phycology, 7: 291-301.

Buschmann, A.H., Correa, J.A. & Westermeier, R. (1999) Recent advances in the understanding of the biological basis for *Gigartina skottsbergii* (Rhodophyta) cultivation in Chile. Hydrobiologia, 398/399: 427-434.

Buschmann, A.H., Correa, J.A., Westermeier, R., Hernández-González, M.C. & Norambuena, R. (2001) Red algal farming in Chile: a review. Aquaculture, 194: 203-220.

Buschmann, A.H., Varela, D., Cifuentes, M., Hernández-González, M.C., Henríquez, L., Westermeier, R. & Correa, J.A. (2004) Experimental indoor cultivation of the carrageenophytic red alga *Gigartina skottsbergii*. Aquaculture, 241: 357-370.

Buschmann, A.H., Varela, D.A., Hernández-González, M.C. & Huovinen, P. (2008) Opportunities and challenges for the development of an integrated seaweed-based aquaculture activity in Chile: determining the physiological capabilities of *Macrocystis* and *Gracilaria* as biofilters. Journal of Applied Phycology, 20: 571-577.

Buttery, M.J. (2000) Culture studies of two toxic dinoflagellate species, *Alexandrium minutum* and *Gymnodinium catenatum*. PhD Thesis, Murdoch University, Perth, Western Australia. 237 pp.

Cabrita, M.T., Vale, C. & Rauter, A.P. (2010) Halogenated compounds from marine algae. Marine Drugs, 8: 2301-2317.

Carlson, P.S. (1973) The use of protoplasts for genetic research. Proceedings of the National Academy of Sciences of the USA, 70: 598-602.

Castell, L. (2003) Gastropod Molluscs. In: Lucas, J.S. & Southgate, P.C. (Eds.), Aquaculture: farming aquatic animals and plants (2nd Edition). Wiley-Blackwell Publishing, Chichester, pp. 567-582.

Chao, W.R., Huang, C.Y. & Sheen, S.S. (2010) Development of formulated diet for post-larval abalone, *Haliotis diversicolor supertexta*. Aquaculture, 307: 89-94.

Chaoyuan, W., Li, R., Lin, G., Wen, Z., Dong, L., Zhang, J., Huang, X., Wei, S. & Lan, G. (1993) Some aspects of the growth of *Gracilaria tenuistipitata* in pond culture. Hydrobiologia, 260/261: 339-343.

Chen, L.C.M. (1986) Cell development of *Porphyra miniata* (*Rhodophyceae*) under axenic culture. Botanica Marina, 29: 435-439.

Chen, Y.C. & Chiang, Y.M. (1994) Development of protoplasts from *Grateloupia sparsa* and *G. filicina* (Halymeniaceae, Rhodophyta). Botanica Marina, 37: 361-366.

Chen, Y.C. & Shih, H.C. (2000) Development of protoplasts of *Ulva fasciata* (Chlorophyta) for algal seed stock. Journal of Phycology, 36: 608-615.

Chen, Y.C. (2001) Immobilized microalga *Scenedesmus quadricauda* (Chlorophyta, Chlorococcales) for long-term storage and for application for water quality control in fish culture. Aquaculture, 195: 71-80.

Chen, Y.C. (2003) Immobilized *Isochrysis galbana* (Haptophyta) for long-term storage and applications for feed and water quality control in clam (*Meretrix lusoria*) cultures. Journal of Applied Phycology, 15: 439-444.

Cheney, D.P., Mar, E., Saga, N. & van der Meer, J. (1986) Protoplast isolation and cell division in the agar-producing seaweed *Gracilaria* (Rhodophyta). Journal of Phycology, 22: 238-243.

Chirapart, A. & Ohno, M. (1993) Growth in tank culture of species of *Gracilaria* from the southeast Asian waters. Botanica Marina, 36: 9-13.

Clayton, M.N. (1992) Propagules of marine macroalgae: structure and development. British Phycological Journal, 27: 219-232.

Collantes, G., Melo, C. & Candia, A. (2004) Micropropagation by explants of *Gracilaria chilensis* Bird, McLachlan and Oliveira. Journal of Applied Phycology, 16: 203-213.

Cook, P. (2007) The costs and benefits of early weaning and rotational farming for greenlip abalone. In: Daume, S. (Ed.), Improvement and evaluation of greenlip abalone hatchery and nursery production. Final Report to Fisheries Research and

Development Corporation on Project No. 2003/203. Fisheries Research Contract Report No. 16, Department of Fisheries, Western Australia, pp. 132-138.

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

Coon, D.A., Neushul, M. & Charters, A.C. (1972) The settling behavior of marine algal spores. In: Nisizawa, K. (Ed.), Proceedings of the 7th International Seaweed Symposium. University of Tokyo Press, Tokyo, pp. 237-242.

Coote, T.A., Hone, P.W., Van Barneveld, R.J. & Maguire, G.B. (2000) Optimal protein level in a semipurified diet for juvenile greenlip abalone *Haliotis laevigata*. Aquaculture Nutrition, 6: 213-220.

Corazani, D. & Illanes, J.E. (1998) Growth of juvenile abalone, *Haliotis discus hannai* Ino 1953 and *Haliotis rufescens* Swainson 1822, fed with different diets. Journal of Shellfish Research, 17: 663-666.

Correa, J.A., Beltrán, J., Buschmann, A.H. & Westermeier, R. (1999) Healing and regeneration responses in *Gigartina skottsbergii* (Rhodophyta, Gigartinales): optimization of vegetative propagation for cultivation. Journal of Applied Phycology, 11: 315-327.

Courtois de Vicose, G., Viera, M.P., Huchette, S. & Izquierdo, M.S. (2012) Improving nursery performances of *Haliotis tuberculata coccinea*: nutritional value of four species of benthic diatoms and green macroalgae germlings. Aquaculture, 334-337: 124-131.

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

Daume, S., Brand-Gardner, S. & Woelkerling, W.J. (1999a) Settlement of abalone larvae (*Haliotis laevigata* Donovan) in response to non-geniculate coralline red algae (Corallinales, Rhodophyta). Journal of Experimental Marine Biology and Ecology, 234: 125-143.

Daume, S., Brand-Gardner, S. & Woelkerling, W.J. (1999b) Preferential settlement of abalone larvae: diatom films vs. non-geniculate coralline red algae. Aquaculture, 174: 243-254.

Daume, S., Krsinich, A., Farrell, S. & Gervis, M. (2000) Settlement, early growth and survival of *Haliotis rubra* in response to different algal species. Journal of Applied Phycology, 12: 479-488.

Daume, S. & Ryan, S. (2002) Improving nursery production of the abalone *Haliotis laevigata* in a commercial abalone nursery in Western Australia. In: Fleming,

A.E. (Ed.), Proceedings of the 9th Annual Abalone Aquaculture Workshop, 29th - 31st July 2002, Queenscliff, Australia. Fisheries Research and Development Corporation, Abalone Aquaculture Subprogram, Canberra, Australia, pp. 123-133.

Daume, S. (2003) Early life history of abalone (*Haliotis rubra*, *H. laevigata*): settlement, survival and early growth. Final Report to Fisheries Research Development Corporation on Project No. 1998/306, Fisheries Research Contract Report No. 3, Department of Fisheries, Western Australia. 110 pp.

Daume, S., Long, B.M. & Crouch, P. (2003) Changes in amino acid content of an algal feed species (*Navicula* sp.) and their effect on growth and survival of juvenile abalone (*Haliotis rubra*). Journal of Applied Phycology, 15: 201-207.

Daume, S., Huchette, S., Ryan, S. & Day, R.W. (2004) Nursery culture of *Haliotis rubra*: the effect of cultured algae and larval density on settlement and juvenile production. Aquaculture, 236: 221-239.

Daume, S. & Ryan, S. (2004) Nursery culture of the abalone *Haliotis laevigata*: larval settlement and juvenile production using cultured algae or formulated feed. Journal of Shellfish Research, 23: 1019-1026.

Daume, S. (2006) The roles of bacteria and micro and marco algae in abalone aquaculture - a review. Journal of Shellfish Research, 25: 151-157.

Daume, S., Davidson, M., Ryan, S. & Parker, F. (2007) Comparisons of rearing systems based on algae or formulated feed for juvenile greenlip abalone (*Haliotis laevigata*). Journal of Shellfish Research, 26: 729-735.

Dawes, C.P. (1995) Suspended cultivation of *Gracilaria* in the sea. Journal of Applied Phycology, 7: 303-313.

Day, R.W. & Fleming, A.E. (1992) The determinants and measurement of abalone growth. In: Shepherd, S.A., Tegner, M.J. & Guzmán Del Próo, S.A. (Eds.), Abalone of the world: biology, fisheries and culture. Blackwell Scientific Publications, Oxford, pp. 141-168.

Day, R.W. & Cook, P. (1995) Bias towards brown algae in determining diet and food preferences: the South African abalone *Haliotis midae*. Marine and Freshwater Research, 46: 623-627.

Day, R.W., Gilmour, P. & Huchette, S. (2004) Effects of density and food supply on postlarval abalone: behaviour, growth and mortality. Journal of Shellfish Research, 23: 1009-1018.

Destombe, C., Valero, M., Vernet, P. & Couvet, D. (1989) What controls haploid-diploid ratio in the red alga, *Gracilaria verrucosa*? Journal of Evolutionary Biology, 2: 317-338.

Destombe, C., Godin, J., Lefebvre, C., Dehorter, O. & Vernet, P. (1992) Differences in dispersal abilities of haploid and diploid spores of *Gracilaria verrucosa* (Gracilariales, Rhodophyta). Botanica Marina, 35: 93-98.

Dixon, M.G. (1992) The effect of temperature and photoperiod on the digestive physiology of the South African abalone *Haliotis midae*. Masters Thesis, Rhodes University, South Africa. 85 pp.

Dixon, P.S. (1973) Biology of the Rhodophyta. Oliver & Boyd, Edinburgh, 285 pp.

Dlaza, T.S., Maneveldt, G.W. & Viljoen, C. (2008) Growth of post-weaning abalone *Haliotis midae* fed commercially available formulated feeds supplemented with fresh wild seaweed. African Journal of Marine Science, 30: 199-203.

Dorsey, T.E., McDonald, P. & Roels, O.A. (1978) Measurements of phytoplankton-protein content with the heated biuret-folin assay. Journal of Phycology, 14: 167-171.

Dunstan, G.A., Baillie, H.J., Barrett, S.M. & Volkman, J.K. (1996) Effect of diet on the lipid composition of wild and cultured abalone. Aquaculture, 140: 115-127.

Dunstan, G.A., Volkman, J.K. & Maguire, G.B. (2000) Optimisation of essential lipids in artificial feeds for Australian abalone. Final Report to Fisheries Research Development Corporation on Project No. 94/85, CSIRO Marine Research. 68 pp.

Dunstan, G.A., Brown, M.R., Volkman, J.K. & Maguire, G.B. (2002) Formulated feeds for newly settled juvenile abalone based on natural feeds (diatoms and crustose coralline algae). Final Report to Fisheries Research Development Corporation on Project No. 1996/386, CSIRO Marine Research. 78 pp.

Durazo-Beltrán, E., Viana, M.T., D'Abramo, L.R. & Toro-Vazquez, J.F. (2004) Effects of starvation and dietary lipid on the lipid and fatty acid composition of muscle tissue of juvenile green abalone (*Haliotis fulgens*). Aquaculture, 238: 329-341.

Dyck, M., Roberts, R. & Jeffs, A. (2010) Use of algal diets to aid early weaning in the abalone *Haliotis iris*. Journal of Shellfish Research, 29: 613-620.

Dyck, M., Roberts, R. & Jeffs, A. (2011) Assessing alternative grazing-tolerant algae for nursery culture of abalone, *Haliotis iris*. Aquaculture, 320: 62-68.

Engel, C., Åberg, P., Gaggiotti, O.E., Destombe, C. & Valero, M. (2001) Population dynamics and stage structure in a haploid-diploid red seaweed, *Gracilaria gracilis*. Journal of Ecology, 89: 436-450.

Erickson, K.L. (1983) Constituents of *Laurencia*. In: Scheuer, P.J. (Ed.), Marine natural products: chemical and biological perspectives, Vol 5. Academic Press, New York, pp. 131-257.

Faafeng, B.A., van Donk, E. & Källqvist, S.T. (1994) *In situ* measurement of algal growth potential in aquatic ecosystems by immobilized algae. Journal of Applied Phycology, 6: 301-308.

Fenical, W. (1975) Halogenation in the Rhodophyta a review. Journal of Phycology, 11: 245-259.

Fenical, W. (1976) Chemical variation in a new bromochamigrene derivative from the red seaweed *Laurencia pacifica*. Phytochemistry, 15: 511-512.

Fleming, A.E. (1992) Some aspects of the nutritional biology of the blacklip abalone *Haliotis rubra*. In: Hone, P.W. (Ed.), Proceedings of the Biotechnology Aquaculture Workshop: Abalone Artificial Diets, 15th July 1992, pp. 20-25.

Fleming, A.E. (1995a) Digestive efficiency of the Australian abalone *Haliotis rubra* in relation to growth and feed preference. Aquaculture, 134: 279-293.

Fleming, A.E. (1995b) Growth, intake, feed conversion efficiency and chemosensory preference of the Australian abalone, *Haliotis rubra*. Aquaculture, 132: 297-311.

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

Fletcher, R.L. & Callow, M.E. (1992) The settlement, attachment and establishment of marine algal spores. British Phycological Journal, 27: 303-329.

Foale, S. & Day, R. (1992) Recognizability of algae ingested by abalone. Australian Journal of Marine and Freshwater Research, 43: 1331-1338.

Friedlander, M. & Dawes, C.J. (1984) Studies on spore release and sporeling growth from carpospores of *Gracilaria foliifera* (Forsskål) Børgesen Var. *Angustissima* (Harvey) Talyor. I. Growth responses. Aquatic Botany, 19: 221-232.

Fujimoto, N., Sudo, R., Sugiura, N. & Inamori, Y. (1997) Nutrient-limited growth of *Microcystis aeruginosa* and *Phormidium tenue* and competition under various N : P supply ratios and temperatures. Limnology Oceanography, 42: 250-256.

Galun, E. (1981) Plant protoplasts as physiological tools. Annual Review of Plant Physiology, 32: 237-266.

Garcia-Esquivel, Z. & Felbeck, H. (2009) Comparative performance of juvenile red abalone, *Haliotis rufescens*, reared in laboratory with fresh kelp and balanced diets. Aquaculture Nutrition, 15: 209-217.

Garland, C.D., Cooke, S.L., Grant, J.F. & McMeekin, T.A. (1985) Ingestion of the bacteria on and the cuticle of crustose (non-articulated) coralline algae by post-larval and juvenile abalone (*Haliotis ruber* Leach) from Tasmanian waters. Journal of Experimental Marine Biology and Ecology, 91: 137-149.

Garza-Sánchez, F., Zertuche-González, J.A. & Chapman, D.J. (2000) Effect of temperature and irradiance on the release, attachment and survival of spores of *Gracilaria pacifica* Abbott (Rhodophyta). Botanica Marina, 43: 205-212.

Gilroy, A. & Edwards, S.J. (1998) Optimum temperature for growth of Australian abalone: preferred temperature and critical thermal maximum for blacklip abalone, *Haliotis rubra* (Leach), and greenlip abalone, *Haliotis laevigata* (Leach). Aquaculture Research, 29: 481-485.

Gómez-Montes, L., García-Esquivel, Z., D'Abramo, L.R., Shimada, A., Vásquez-Peláez, C. & Viana, M.T. (2003) Effect of dietary protein:energy ratio on intake, growth and metabolism of juvenile green abalone *Haliotis fulgens*. Aquaculture, 220: 769-780.

Gómez-Pinchetti, J.L., Fernández, E.C., Díez, P.M. & Reina, G.G. (1998) Nitrogen availability influences the biochemical composition and photosynthesis of tank-cultivated *Ulva rigida* (Chlorophyta). Journal of Applied Phycology, 10: 383-389.

Gordon, H.R. & Cook, P.A. (2004) World abalone fisheries and aquaculture update: supply and market dynamics. Journal of Shellfish Research, 23: 935-939.

Gordon, N., Shpigel, M., Harpaz, S., Lee, J.J. & Neori, A. (2004) The settlement of abalone (*Haliotis discus hannai* Ino) larvae on culture layers of different diatoms. Journal of Shellfish Research, 23: 561-568.

Gordon, N., Neori, A., Shpigel, M., Lee, J. & Harpaz, S. (2006) Effect of diatom diets on growth and survival of the abalone *Haliotis discus hannai* postlarvae. Aquaculture, 252: 225-233.

Gorrostieta-Hurtado, E. & Searcy-Bernal, R. (2004) Combined effects of light condition (constant illumination or darkness) and diatom density on postlarval survival and growth of the abalone *Haliotis rufescens*. Journal of Shellfish Research, 23: 1001-1008.

Guillard, R.R.L. & Ryther, J.H. (1962) Studies of marine planktonic diatoms: I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. Canadian Journal of Microbiology, 8: 229-239.

Guillemin, M.L., Faugeron, S., Destombe, C., Viard, F., Correa, J.A. & Valero, M. (2008) Genetic variation in wild and cultivated populations of the haploid-diploid red alga *Gracilaria chilensis*: how farming practices favor asexual reproduction and heterozygosity. Evolution, 62: 1500-1519.

Guimarães, M., Plastino, E.M. & Oliveira, E.C. (1999) Life history, reproduction and growth of *Gracilaria domingensis* (Gracilariales, Rhodophyta) from Brazil. Botanica Marina, 42: 481-486.

Gupta, V., Kumar, M., Kumari, P., Reddy, C.R.K. & Jha, B. (2011) Optimization of protoplast yields from the red algae *Gracilaria dura* (C. Agardh) J. Agardh and *G. verrucosa* (Huds.) Papenfuss. Journal of Applied Phycology, 23: 209-218.

Guzmán-Urióstegui, A. & Robledo, D. (1999) Factors affecting sporulation of *Gracilaria cornea* (Gracilariales, Rhodophyta) carposporophytes from Yucatan, Mexico. Hydrobiologia, 398/399: 285-290.

Haddon, M., Mundy, C. & Tarbath, D. (2008) Using an inverse-logistic model to describe growth increments of blacklip abalone (*Haliotis rubra*) in Tasmania. Fisheries Bulletin, 106: 58-71.

Hahn, K.O. (1989a) Survey of the commercially important abalone species in the world. In: Hahn, K.O. (Ed.), Handbook of culture of abalone and other marine gastropods. CRC Press, Boca Raton, Florida, pp. 3-11.

Hahn, K.O. (1989b) Nutrition and growth of abalone. In: Hahn, K.O. (Ed.), Handbook of culture of abalone and other marine gastropods. CRC Press, Boca Raton, Florida, pp. 135-156.

Halling, C., Aroca, G., Cifuentes, M., Buschmann, A.H. & Troell, M. (2005) Comparison of spore inoculated and vegetative propagated cultivation methods of *Gracilaria chilensis* in an intergrated seaweed and fish cage culture. Aquaculture International, 13: 409-422.

Han, T., Han, Y.S., Kim, K.Y., Kim, J.H., Shin, H.W., Kain, J.M., Callow, J.A. & Callow, M.E. (2003) Influences of light and UV-B on growth and sporulation of the green alga *Ulva pertusa* Kjellman. Journal of Experimental Marine Biology and Ecology, 290: 115-131.

Helidoniotis, F., Haddon, M., Tuck, G. & Tarbath, D. (2011) The relative suitability of the von Bertalanffy, Gompertz and inverse logistic models for describing growth in blacklip abalone populations (*Haliotis rubra*) in Tasmania, Australia. Fisheries Research, 112: 13-21.

Helidoniotis, F. & Haddon, M. (2012) Growth model selection for juvenile blacklip abalone (*Haliotis rubra*): assessing statistical and biological validity. Marine and Freshwater Research, 63: 23-33.

Hernández-González, M.C., Buschmann, A.H., Cifuentes, M., Correa, J.A. & Westermeier, R. (2007) Vegetative propagation of the carrageenophytic red alga *Gigartina skottsbergii* Setchell *et* Gardner: indoor and field experiments. Aquaculture, 262: 120-128.

Hertzberg, S. & Jensen, A. (1989) Studies of alginate-immobilized marine microalgae. Botanica Marina, 32: 267-273.

Hiraoka, M., Shimada, S., Ohno, M. & Serisawa, Y. (2003) Asexual life history by quadriflagellate swarmers of *Ulva spinulosa* (Ulvales, Ulvophyceae). Phycological Research, 51: 29-34.

Hommersand, M.H. & Fredericq, S. (1990) Sexual reproduction and cystocarp development. In: Cole, K.M. & Sheath, R.G. (Eds.), Biology of the red algae. Cambridge University Press, Cambridge, pp. 305-346.

Hone, P.W. (1992) Preliminary results of artificial diet trials. In: Hone, P.W. (Ed.), Proceedings of the Biotechnology Aquaculture Workshop: Abalone Artificial Diets, 15th July 1992, pp. 14-20.

Hoxmark, R.C. (1975) Experimental analysis of the life cycle of *Ulva mutabilis*. Botanica Marina, 18: 123-129.

Huggett, M.J., de Nys, R., Williamson, J.E., Heasman, M. & Steinberg, P.D. (2005) Settlement of larval blacklip abalone, *Haliotis rubra* in response to green and red macroalgae. Marine Biology, 147: 1155-1163.

Hurtado, A.Q. & Cheney, D.P. (2003) Propagule production of *Eucheuma denticulatum* (Burman) Collins *et* Harvey by tissue culture. Botanica Marina, 46: 338-341.

Hurtado, A.Q. & Biter, A.B. (2007) Plantlet regeneration of *Kappaphycus alvarezii* var. adik-adik by tissue culture. Journal of Applied Phycology, 19: 783-786.

Hurtado-Ponce, A.Q. (1990) Vertical rope cultivation of *Gracilaria* (Rhodophyta) using vegetative fragments. Botanica Marina, 33: 477-481.

Inoue, A., Kagaya, M. & Ojima, T. (2008) Preparation of protoplasts from *Laminaria japonica* using native and recombinant abalone alginate lyases. Journal of Applied Phycology, 20: 633-640.

Ismail, I., Jones, C.L.W., Britz, P.J. & Esterhuizen, A.J. (2009) Towards an abalone weaning diet: evaluation of agar-bound fishmeal and spirulina as dietary ingredients. African Journal of Marine Science, 31: 103-106.

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

Kain, J.M. (1982) The reproductive phenology of nine species of Rhodophyta in the subtidal region of the Isle of Man. British Phycological Journal, 17: 321-331.

Kain, J.M. (1986) Plant size and reproductive phenology of six species of Rhodophyta in subtidal Isle of Man. British Phycological Journal, 21: 129-138.

Kain, J.M. (1991) Cultivation of attached seaweeds. In: Guiry, M.D. & Blunden, G. (Eds.), Seaweed resources in Europe: uses and potential. John Wiley & Sons, Chichester, pp. 309-377.

Kain, J.M. & Destombe, C. (1995) A review of the life history, reproduction and phenology of *Gracilaria*. Journal of Applied Phycology, 7: 269-281.

Kaliaperumal, N., Chennubhotla, V.S.K., Kalimuthu, S. & Ramalingam, J.R. (1986) Growth phenology and spore shedding in *Gracilaria arcuata-var-arcuata* and *Gracilaria corticata-var-cylindrica* Rhodophyta. Indian Journal of Marine Sciences, 15: 107-110.

Kates, M. & Volcani, B.E. (1966) Lipid components of diatoms. Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism, 116: 264-278.

Kawamura, T., Saido, T., Takami, H. & Yamashita, Y. (1995) Dietary value of benthic diatoms for the growth of post-larval abalone *Haliotis discus hannai.* Journal of Experimental Marine Biology and Ecology, 194: 189-199.

Kawamura, T. & Takami, H. (1995) Analysis of feeding and growth rate of newly metamorphosed abalone *Haliotis discus hannai* fed on four species of benthic diatom. Fisheries Science, 61: 357-358.

Kawamura, T. (1996) The role of benthic diatoms in the early life stages of the Japanese abalone (*Haliotis discus hannai*). In: Watanabe, Y., Yamashita, Y. & Oozeki, Y. (Eds.), Proceedings of an International Workshop: Survival strategies in early life stages of marine resources, 11th - 14th October 1994, Yokohama, Japan. A.A. Balkema, Rotterdam, pp. 355-367.

Kawamura, T., Roberts, R.D. & Nicholson, C.M. (1998a) Factors affecting the food value of diatom strains for post-larval abalone *Haliotis iris.* Aquaculture, 160: 81-88.

Kawamura, T., Roberts, R.D. & Takami, H. (1998b) A review of the feeding and growth of postlarval abalone. Journal of Shellfish Research, 17: 615-625.

Kawamura, T., Takami, H., Roberts, R.D. & Yamashita, Y. (2001) Radula development in abalone *Haliotis discus hannai* from larva to adult in relation to feeding transitions. Fisheries Science, 67: 596-605.

Keesing, J.K. & Wells, F.E. (1989) Growth of the abalone *Haliotis roei* Gray. Australian Journal of Marine and Freshwater Research, 40: 199-204.

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

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

Kochert, G. (1978) Carbohydrate determination by the phenol-sulfuric acid method. In: Hellebust, J.A. & Craigie, J.S. (Eds.), Handbook of phycological methods: physiological and biochemical methods. Cambridge University Press, Cambridge, pp. 95-97.

Kong, C.S.L. & Ang, P.O. (2004) Seasonal occurrence and reproduction of *Hypnea charoides* (Rhodophyta) in Tung Ping Chau, N.T., Hong Kong SAR, China. Hydrobiologia, 512: 63-78.

Krsinich, A., Daume, S., Farrell, S., Gervis, M. & Thompson, P. (2000) Towards intensification of an abalone (*Haliotis rubra*) nursery operation via inoculation with a benthic diatom *Navicula* sp. and seeding with the macroalgae *Ulvella lens*. In: Fleming, A.E. (Ed.), Proceedings of the 7th Annual Abalone Aquaculture Workshop, 27th - 30th August 2000, Dunedin, New Zealand. Fisheries Research Development Corporation, Abalone Aquaculture Subprogram, Canberra, Australia, pp. 86-99.

Kuwano, K., Matsuka, S., Kono, S., Ninomiya, M., Onishi, J. & Saga, N. (1998) Growth and the content of laurinterol and debromolaurinterol in *Laurencia okamurae* (Ceramiales, Rhodophyta). Journal of Applied Phycology, 10: 9-14.

Lafontaine, N., Mussio, I. & Rusig, A.M. (2011) Production and regeneration of protoplasts from *Grateloupia turuturu* Yamada (Rhodophyta). Journal of Applied Phycology, 23: 17-24.

Landau, M. (1992) Introduction to aquaculture. John Wiley & Sons, New York, 440 pp.

Lawlor, H.J., Borowitzka, M.A. & McComb, J.A. (1991) A rapid and inexpensive method for surface sterilisation of *Ecklonia radiata* (Phaeophyta) for tissue culture. Botanica Marina, 34: 261-264.

Le Gall, L., Pien, S. & Rusig, A.M. (2004) Cultivation of *Palmaria palmata* (Palmariales, Rhodophyta) from isolated spores in semi-controlled conditions. Aquaculture, 229: 181-191.

Lebeau, T., Moan, R., Turpin, V. & Robert, J.M. (1998) Alginate-entrapped *Haslea ostrearia* as inoculum for the greening of oysters. Biotechnology Techniques, 12: 847-850.

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

Leighton, D. & Boolootian, R.A. (1963) Diet and growth in the black abalone, *Haliotis cracherodii.* Ecology, 44: 227-238.

Levinton, J.S. (1995) Marine biology: function, biodiversity, ecology. Oxford University Press, New York, 420 pp.

Lewin, J.C. & Guillard, R.R.L. (1963) Diatoms. Annual Review of Microbiology, 17: 373-414.

Liu, Q.Y., Chen, L.C.M. & Taylor, A.R.A. (1992) Ultrastructure of cell wall regeneration by isolated protoplasts of *Palmaria palmata* (Rhodophyta). Botanica Marina, 35: 21-33.

Liu, W.S., Tang, Y.L., Liu, X.W. & Fang, T.C. (1984) Studies on the preparation and on the properties of sea snail enzymes. Hydrobiologia, 116/117: 319-320.

Lopez, L.M., Tyler, P.A. & Viana, M.T. (1998) The effect of temperature and artificial diets on growth rates of juvenile *Haliotis tuberculata* (Linnaeus, 1758). Journal of Shellfish Research, 17: 657-662.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193: 265-275.

Lüning, K. & Pang, S. (2003) Mass cultivation of seaweeds: current aspects and approaches. Journal of Applied Phycology, 15: 115-119.

Maesako, N., Nakamura, S. & Yotsui, T. (1984) Food effect of brown and green algae of early developmental stage and blue green algae for the growth of the juvenile abalone, *Haliotis discus* Reeve (in Japanese with English abstract). Bulletin of Nagasaki Prefectural Institute of Fisheries, 10: 53-56.

Mai, K., Mercer, J.P. & Donlon, J. (1994) Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino II. Amino acid composition of abalone and six species of macroalgae with an assessment of their nutritional value. Aquaculture, 128: 115-130.

Mai, K., Mercer, J.P. & Donlon, J. (1995a) Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. IV. Optimum dietary protein level for growth. Aquaculture, 136: 165-180.

Mai, K., Mercer, J.P. & Donlon, J. (1995b) Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. III. Response of abalone to various levels of dietary lipid. Aquaculture, 134: 65-80.

Mai, K., Mercer, J.P. & Donlon, J. (1996) Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. V. The role of polyunsaturated fatty acids of macroalgae in abalone nutrition. Aquaculture, 139: 77-89.

Manahan, D.T. & Jaeckle, W.B. (1992) Implications of dissolved organic material in seawater for the energetics of abalone larvae *Haliotis rufescens*: a review. In: Shepherd, S.A., Tegner, M.J. & Guzmán Del Próo, S.A. (Eds.), Abalone of the world: biology, fisheries and culture. Blackwell Scientific Publications, Oxford, pp. 95-106.

Mantri, V.A., Thakur, M.C., Kumar, M., Reddy, C.R.K. & Jha, B. (2009) The carpospore culture of industrially important red alga *Gracilaria dura* (Gracilariales, Rhodophyta). Aquaculture, 297: 85-90.

Mantri, V.A., Reddy, C.R.K. & Jha, B. (2010) Reproductive processes in red algal genus *Gracilaria* and impact of climate change. In: Israel, A., Einav, R. & Seckbach, J. (Eds.), Seaweeds and their role in globally changing environments. Cellular origin, life in extreme habitats and astrobiology. Springer, Dordecht, London, pp. 319-338.

Mantri, V.A., Singh, R.P., Bijo, A.J., Kumari, P., Reddy, C.R.K. & Jha, B. (2011) Differential response of varying salinity and temperature on zoospore induction, regeneration and daily growth rate in *Ulva fasciata* (Chlorophyta, Ulvales). Journal of Applied Phycology, 23: 243-250.

Martín, L.A., Boraso de Zaixso, A.L. & Leonardi, P.I. (2011) Biomass variation and reproductive phenology of *Gracilaria gracilis* in a Patagonian natural bed (Chubut, Argentina). Journal of Applied Phycology, 23: 643-654.

Martínez-Ponce, D.R. & Searcy-Bernal, R. (1998) Grazing rates of red abalone (*Haliotis rufescens*) postlarvae feeding on the benthic diatom *Navicula incerta.* Journal of Shellfish Research, 17: 627-630.

Masuda, M., Abe, T., Sato, S., Suzuki, T. & Suzuki, M. (1997) Diversity of halogenated secondary metabolites in the red alga *Laurencia nipponica* (Rhodomelaceae, Ceramiales). Journal of Phycology, 33: 196-208.

McLachlan, J.L. (1991) General principles of on-shore cultivation of seaweeds: effects of light on production. Hydrobiologia, 221: 125-135.

McShane, P.E. (1992) Early life history of abalone: a review. In: Shepherd, S.A., Tegner, M.J. & Guzmán Del Próo, S.A. (Eds.), Abalone of the world: biology, fisheries and culture. Blackwell Scientific Publications, Oxford, pp. 120-138.

McShane, P.E., Gorfine, H.K. & Knuckey, I.A. (1994) Factors influencing food selection in the abalone *Haliotis rubra* (Mollusca: Gastropoda). Journal of Experimental Marine Biology and Ecology, 176: 27-37.

McShane, P.E. (1996) Recruitment processes in abalone (*Haliotis* spp.). In: Watanabe, Y., Yamashita, Y. & Oozeki, Y. (Eds.), Proceedings of an International Workshop: Survival strategies in early life stages of marine resources, 11th - 14th October 1994, Yokohama, Japan. A. A. Balkema, Rotterdam, pp. 315-324.

Mercer, J.P., Mai, K. & Donlon, J. (1993) Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* Linnaeus and *Haliotis discus hannai* Ino I. Effects of algal diets on growth and biochemical composition. Invertebrate Reproduction and Development, 23: 75-88.

Mercz, T.I. (1994) A study of high lipid yielding microalgae with potential for large-scale production of lipids and polyunsaturated fatty acids. PhD Thesis, Murdoch University, Perth, Western Australia. 278 pp.

Mgaya, Y.D. & Mercer, J.P. (1994) A review of the biology, ecology, fisheries and mariculture of the European abalone *Haliotis tuberculata* Linnaeus 1758 (Gastropoda: Haliotidae). Biology and Environment: Proceedings of the Royal Irish Academy, 94: 285-304.

Mollet, J.C., Verdus, M.C., Kling, R. & Morvan, H. (1995) Improved protoplast yield and cell wall regeneration in *Gracilaria verrucosa* (Huds.) Papenfuss (Gracilariales, Rhodophyta). Journal of Experimental Botany, 46: 239-247.

Montaño-Vargas, J., Viana, M.T., D'Abramo, L.R., Shimada, A. & Vásquez-Peláez, C. (2005) Growth and energy utilization of juvenile pink abalone *Haliotis corrugata* fed diets containing different levels of protein and two starch:lipid ratios. Journal of Shellfish Research, 24: 1179-1185.

Moreno-Garrido, I. (2008) Microalgae immobilization: current techniques and uses. Bioresource Technology, 99: 3949-3964.

Mshigeni, K.E. (1976) Effects of the environment on developmental rates of sporelings of two *Hypnea* species (Rhodophyta: Gigartinales). Marine Biology, 36: 99-103.

Naidoo, K., Maneveldt, G., Ruck, K. & Bolton, J.J. (2006) A comparison of various seaweed-based diets and formulated feed on growth rate of abalone in a land-based aquaculture system. Journal of Applied Phycology, 17: 211-217.

Najmudeen, T.M. & Victor, A.C.C. (2004) Seed production and juvenile rearing of the tropical abalone *Haliotis varia* Linnaeus 1758. Aquaculture, 234: 277-292.

Nash, C.E. (1991) Abalone culture. In: Nash, C.E. (Ed.), Production of aquatic animals: crustacean, molluscs, amphibians and reptiles. World Animal Science C4, Elsevier Science Publishers, New York, pp. 173-181.

Neori, A., Chopin, T., Troell, M., Buschmann, A.H., Kraemer, G.P., Halling, C., Shpigel, M. & Yarish, C. (2004) Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture, 231: 361-391.

Neori, A. (2008) Essential role of seaweed cultivation in integrated multi-trophic aquaculture farms for global expansion of mariculture: an analysis. Journal of Applied Phycology, 20: 567-570.

Nishihara, G.N., Terada, R. & Noro, T. (2004) Photosynthesis and growth rates of *Laurencia brongniartii* J. Agardh (Rhodophyta, Ceramiales) in preparation for cultivation. Journal of Applied Phycology, 16: 303-308.

Nordby, Ø. (1977) Optimal conditions for meiotic spore formation in *Ulva mutabilis* Føyn. Botanica Marina, 20: 19-28.

Oakes, F.R. & Ponte, R.D. (1996) The abalone market: opportunities for cultured abalone. Aquaculture, 140: 187-195.

Oliveira, E.C., Alveal, K. & Anderson, R.J. (2000) Mariculture of the agar-producing Gracilarioid red algae. Reviews in fisheries Science, 8: 345-377.

O'Mahoney, M., Mouzakitis, G., Doyle, J. & Burnell, G. (2011) A novel konjac glucomannan-xanthan gum binder for aquaculture feeds: the effect of binder configuration on formulated feed stability, feed palatability and growth performance of the Japanese abalone, *Haliotis discus hannai*. Aquaculture Nutrition, 17: 395-407.

Onitsuka, T., Kawamura, T., Ohashi, S., Horii, T. & Watanabe, Y. (2004) Morphological changes in the radula of abalone *Haliotis diversicolor aquatilis* from post-larva to adult. Journal of Shellfish Research, 23: 1079-1085.

Onitsuka, T., Kawamura, T., Iwanaga, S., Ohashi, S. & Horii, T. (2010) Dietary value of gametophytes and juvenile sporophytes of the brown macroalga *Eisenia bicyclis* for juvenile abalone *Haliotis diversicolor*. Fisheries Science, 76: 619-623.

Onitsuka, T., Kawamura, T., Ohashi, S., Iwanaga, S. & Horii, T. (2011) Growth and survival of juvenile abalone *Haliotis diversicolor* fed on brown macroalgal gametophytes, a potential alternative initial diet. Aquaculture, 313: 79-83.

Orduña-Rojas, J. & Robledo, D. (1999) Effects of irradiance and temperature on the release and growth of carpospores from *Gracilaria cornea* J. Agardh (Gracilariales, Rhodophyta). Botanica Marina, 42: 315-319.

Orduña-Rojas, J. & Robledo, D. (2002) Studies on the tropical agarophyte *Gracilaria cornea* J. Agardh (Rhodophyta, Gracilariales) from Yucatán, México. II. Biomass assessment and reproductive phenology. Botanica Marina, 45: 459-464.

Oza, R.M., Tewari, A., Rajyaguru, M.R. & Goswamy, S. (1994) Laboratory and field culture of marine red algae *Gracilaria verrucosa* (Gracilariaceae, Rhodophyta). Indian Journal of Marine Sciences, 23: 157-161.

Pang, S.J., Zhang, Z.H., Bao, Y. & Gao, S.Q. (2006) Settling abalone veliger larvae in a free-swimming microalgal culture. Aquaculture, 258: 327-336.

Parker, F., Davidson, M., Freeman, K., Hair, S. & Daume, S. (2007) Investigation of optimal temperature and light conditions for three benthic diatoms and their suitability to commercial scale nursery culture of abalone (*Haliotis laevigata*). Journal of Shellfish Research, 26: 751-761.

Peduzzi, P. & Herndl, G.J. (1991) Mucus trails in the rocky intertidal: a highly active microenvironment. Marine Ecology Progress Series, 75: 267-274.

Pintner, I.J. & Provasoli, L. (1958) Artificial cultivation of a red-pigmented marine blue-green alga, *Phormidium persicinum*. Journal of Genetic Microbiology, 18: 190-197.

Polifrone, M., De Masi, F. & Gargiulo, G.M. (2006) Alternative pathways in the life history of *Gracilaria gracilis* (Gracilariales, Rhodophyta) from north-eastern Sicily (Italy). Aquaculture, 261: 1003-1013.

Polne-Fuller, M. & Gibor, A. (1984) Developmental studies in *Porphyra*. I. Blade differentiation in *Porphyra perforata* as expressed by morphology, enzymatic digestion, and protoplast regeneration. Journal of Phycology, 20: 609-616.

Polne-Fuller, M. & Gibor, A. (1986) Calluses, cells, and protoplasts in studies towards genetic improvement of seaweeds. Aquaculture, 57: 117-123.

Poore, G.C.B. (1972a) Ecology of New Zealand abalones, *Haliotis* species (Mollusca: Gastropoda) 1. Feeding. New Zealand Journal of Marine and Freshwater Research, 6: 11-22.

Poore, G.C.B. (1972b) Ecology of New Zealand abalones, *Haliotis* species (Mollusca: Gastropoda) 2. Seasonal and diurnal movement. New Zealand Journal of Marine and Freshwater Research, 6: 246-258.

Provasoli, L. (1968) Media and prospects for the cultivation of marine algae. In: Watanabe, A. & Hattori, A. (Eds.), Culture and Collections of Algae, Proceedings of the US-Japan Conference, Hakone, September 1966. Japanese Society of Plant Physiology, pp. 63-75.

Qi, Z., Liu, H., Li, B., Mao, Y., Jiang, Z., Zhang, J. & Fang, J. (2010) Suitability of two seaweeds, *Gracilaria lemaneiformis* and *Sargassum pallidum*, as feed for the abalone *Haliotis discus hannai* Ino. Aquaculture, 300: 189-193.

Reddy, C.R.K., Migita, S. & Fujita, Y. (1989) Protoplast isolation and regeneration of three species of *Ulva* in axenic culture. Botanica Marina, 32: 483-490.

Reddy, C.R.K., Dipakkore, S., Kumar, G.R., Jha, B., Cheney, D.P. & Fujita, Y. (2006) An improved enzyme preparation for rapid mass production of protoplasts as seed stock for aquaculture of macrophytic marine green algae. Aquaculture, 260: 290-297.

Reddy, C.R.K., Gupta, M.K., Mantri, V.A. & Jha, B. (2008a) Seaweed protoplasts: status, biotechnological perspectives and needs. Journal of Applied Phycology, 20: 619-632.

Reddy, C.R.K., Jha, B., Fujita, Y. & Ohno, M. (2008b) Seaweed micropropagation techniques and their potentials: an overview. Journal of Applied Phycology, 20: 609-617.

Robaina, R.R., Garcia-Reina, G. & Luque, A. (1990) The effects of the physical characteristics of the culture medium on the development of red seaweeds in tissue culture. Hydrobiologia, 204/205: 137-142.

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

Roberts, R.D., Kawamura, T. & Takami, H. (1999b) Morphological changes in the radula of abalone (*Haliotis iris*) during post-larval development. Journal of Shellfish Research, 18: 637-644.

Roberts, R.D. (2001) A review of settlement cues for larval abalone (*Haliotis* spp.). Journal of Shellfish Research, 20: 571-586.

Roberts, R.D., Lapworth, C. & Barker, R.J. (2001) Effect of starvation on the growth and survival of post-larval abalone (*Haliotis iris*). Aquaculture, 200: 323-338.

Roberts, R.D., Kaspar, H.F. & Barker, R.J. (2004) Settlement of abalone (*Haliotis iris*) larvae in response to five species of coralline algae. Journal of Shellfish Research, 23: 975-987.

Roberts, R.D., Kawamura, T. & Handley, C.M. (2007) Factors affecting settlement of abalone (*Haliotis iris*) larvae on benthic diatom films. Journal of Shellfish Research, 26: 323-334.

Roberts, R.D., Barker, M.F. & Mladenov, P. (2010) Is settlement of *Haliotis iris* larvae on coralline algae triggered by the alga or its surface biofilm? Journal of Shellfish Research, 29: 671-678.

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

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

Romo, S. & Pérez-Martínez, C. (1997) The use of immobilization in alginate beads for long-term storage of *Pseudanabaena galeata* (Cyanobacteria) in the laboratory. Journal of Phycology, 33: 1073-1076.

Rusig, A.M. & Cosson, J. (2001) Plant regeneration from protoplasts of *Enteromorpha intestinalis* (Chlorophyta, Ulvophyceae) as seedstock for macroagal culture. Journal of Applied Phycology, 13: 103-108.

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

Santelices, B. & Doty, M.S. (1989) A review of *Gracilaria* farming. Aquaculture, 78: 95-133.

Santelices, B. & Varela, D. (1995) Regenerative capacity of *Gracilaria* fragments: effects of size, reproductive state and position along the axis. Journal of Applied Phycology, 7: 501-506.

Santelices, B. & Aedo, D. (1999) Evaluating substances that facilitate algal spore adhesion. Hydrobiologia, 398/399: 241-246.

Searle, T., Roberts, R.D. & Lokman, P.M. (2006) Effects of temperature on growth of juvenile blackfoot abalone, *Haliotis iris* Gmelin. Aquaculture Research, 37: 1441-1449.

Serviere-Zaragoza, E., Mazariegos-Villareal, A., Ponce-Díaz, G. & Montes Magallón, S. (2001) Growth of juvenile abalone, *Haliotis fulgens* Philippi, fed different diets. Journal of Shellfish Research, 20: 689-693.

Sharma, N.K., Tiwari, S.P., Tripathi, K. & Rai, A.K. (2011) Sustainability and cyanobacteria (blue-green algae): facts and challenges. Journal of Applied Phycology, 23: 1059-1081.

Shen, S., Wu, X., Yan, B. & He, L. (2010) Tissue culture of three species of *Laurencia* complex. Chinese Journal of Oceanology and Limnology, 28: 514-520.

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

Shepherd, S.A. & Hearn, W.S. (1983) Studies on southern Australian abalone (Genus *Haliotis*). IV Growth of *H. laevigata* and *H. ruber*. Australian Journal of Marine and Freshwater Research, 34: 461-475.

Shepherd, S.A. & Cannon, J. (1988) Studies on southern Australian abalone (genus *Haliotis*) X. Food and feeding of juveniles. Journal of the Malacological Society of Australia, 9: 21-26.

Shepherd, S.A., Clarke, S.M. & Dalgetty, A. (1992) Studies on southern Australian abalone (genus *Haliotis*) XIV. Growth of *H. laevigata* on Eyre Peninsula. Journal of the Malacological Society of Australia, 13: 99-113.

Shepherd, S.A. & Steinberg, P.D. (1992) Food preferences of three Australian abalone species with a review of the algal food of abalone. In: Shepherd, S.A., Tegner, M.J. & Guzmán Del Próo, S.A. (Eds.), Abalone of the world: biology, fisheries and culture. Blackwell Scientific Publishing, Oxford, pp. 169-181.

Shepherd, S.A. & Daume, S. (1996) Ecology and survival of juvenile abalone in a crustose coralline habitat in South Australia. In: Watanabe, Y., Yamashita, Y. & Oozeki, Y. (Eds.), Proceedings of an International Workshop: Survival strategies in early life stages of marine resources, 11th - 14th October 1994, Yokohama, Japan. A.A. Balkema, Rotterdam, pp. 297-313.

Shpigel, M., Ragg, N.L., Lupatsch, I. & Neori, A. (1999) Protein content determines the nutritional value of the seaweed *Ulva lactuca* L. for the abalone *Haliotis tuberculata* L. and *H. discus hannai* Ino. Journal of Shellfish Research, 18: 227-233.

Simpson, B.J.A. & Cook, P.A. (1998) Rotation diets: a method of improving growth of cultured abalone using natural algal diets. Journal of Shellfish Research, 17: 635-640.

Slattery, M. (1992) Larval settlement and juvenile survival in the red abalone (*Haliotis rufescens*): an examination of inductive cues and substrate selection. Aquaculture, 102: 143-153.

Smidsrød, O. & Skjåk-Braek, G. (1990) Alginate as immobilization matrix for cells. Trends in Biotechnology, 8: 71-78.

Steffensen, D.A. (1976) The effect of nutrient enrichment and temperature on the growth in culture of *Ulva lactuca* L. Aquatic Botany, 2: 337-351.

Steinberg, P.D. (1988) Effects of quantitative and qualitative variation in phenolic compounds on feeding in three species of marine invertebrate herbivores. Journal of Experimental Marine Biology and Ecology, 120: 221-237.

Steinberg, P.D. (1989) Biogeographical variation in brown algal polyphenolics and other secondary metabolites: comparison between temperate Australasia and North America. Oecologia, 78: 373-382.

Steinberg, P.D. & van Altena, I. (1992) Tolerance of marine invertebrate herbivores to brown algal phlorotannins in temperate Australasia. Ecological Monographs, 62: 189-222.

Stepto, N.K. & Cook, P.A. (1996) Feeding preferences of the juvenile South African abalone *Haliotis midae* (Linnaeus, 1758). Journal of Shellfish Research, 15: 653-657.

Stott, A., Takeuchi, T., Koike, Y., Yamakawa, H. & Imada, O. (2002) Using micro particle diets to replace diatoms for feeding postlarval abalone *Haliotis discus discus* (Reeve.). Fisheries Science, 68: 1088-1093.

Stott, A., Takeuchi, T., Koike, Y. & Imada, O. (2003a) Settling and raising postlarval abalone *Haliotis diversicolor supertexta* (Lischke) on microparticulate diets embedded in a layer of alginate. Aquaculture Research, 34: 561-567.

Stott, A.E., Takeuchi, T., Koike, Y. & Imada, O. (2003b) The effect of three different application methods for an artificial micro particulate diet on the survival and growth of post-larval abalone *Haliotis discus discus* (Reeve). Suisan Zoshoku, 51: 197-204.

Stott, A.E., Takeuchi, T. & Koike, Y. (2004a) Testing various substances that have been bound to plastic plates with agar to induce larval settlement and metamorphosis of abalone *Haliotis discus discus* (Reeve). Aquaculture, 231: 547-557.

Stott, A.E., Takeuchi, T. & Koike, Y. (2004b) An alternative culture system for the hatchery production of abalone without using livefood. Aquaculture, 236: 341-360.

Stott, A.E., Takeuchi, T. & Koike, Y. (2004c) Performance of a new artificial abalone hatchery culture system in terms of settlement of larvae and growth and survival of postlarval *Haliotis discus discus* (Reeve). Fisheries Science, 70: 1070-1081.

Stott, A.E., Takeuchi, T. & Koike, Y. (2004d) Growth and survival of post-larval abalone *Haliotis diversicolor supertexta* (Lischke) using an alternative culture method in the light and dark. Journal of Shellfish Research, 23: 957-961.

Strain, L.W.S. (2003) Algal sporelings for abalone aquaculture. Honours Thesis, Murdoch University, Perth, Western Australia. 110 pp.

Strain, L.W.S., Borowitzka, M.A. & Daume, S. (2005) Red algae fragments (*Laurencia* sp.) as an alternative feed for juvenile greenlip abalone (*Haliotis laevigata*). In: Fleming, A.E. (Ed.), Proceedings of the 12th Annual Abalone Aquaculture Workshop, 1st - 3rd August 2005, McLaren Vale, Australia. Fisheries Research and Development Corporation, Abalone Aquaculture Subprogram, Canberra, Australia, pp. 111-124.

Strain, L.W.S., Borowitzka, M.A. & Daume, S. (2006) Growth and survival of juvenile greenlip abalone (*Haliotis laevigata*) feeding on germlings of the macroalgae *Ulva* sp. Journal of Shellfish Research, 25: 239-247.

Strain, L.W.S., Isdepsky, A., Borowitzka, M.A. & Daume, S. (2007) Three algal propagation methods assessed to create a Rhodophyta diet for juvenile greenlip abalone (*Haliotis laevigata*) in the later nursery phase. Journal of Shellfish Research, 26: 737-744.

Stratmann, J., Paputsoglu, G. & Oertel, W. (1996) Differentiation of *Ulva mutabilis* (Chlorophyta) gametangia and gamete release are controlled by extracellular inhibitors. Journal of Phycology, 32: 1009-1021.

Stuart, M.D. & Brown, M.T. (1994) Growth and diet of cultivated black-footed abalone, *Haliotis iris* (Martyn). Aquaculture, 127: 329-337.

Sudatti, D.B., Fujii, M.T., Rodrigues, S.V., Turra, A. & Pereira, R.C. (2011) Effects of abiotic factors on growth and chemical defenses in cultivated clones of *Laurencia dendroidea* J. Agardh (Ceramiales, Rhodophyta). Marine Biology, 158: 1439-1446.

Takahashi, K. & Koganezawa, A. (1988) Mass culture of *Ulvella lens* as a feed for abalone, *Haliotis discus hannai*. NOAA Technical Report NMFS, 70: 29-36.

Takami, H., Kawamura, T. & Yamashita, Y. (1997a) Contribution of diatoms as food sources for post-larval abalone *Haliotis discus hannai* on crustose coralline alga. Molluscan Research, 18: 143-151.

Takami, H., Kawamura, T. & Yamashita, Y. (1997b) Survival and growth rates of post-larval abalone *Haliotis discus hannai* fed conspecific trail mucus and / or benthic diatom *Cocconeis scutellum* var. *parva.* Aquaculture, 152: 129-138.

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

Takami, H., Kawamura, T. & Yamashita, Y. (2000) Starvation tolerance of newly metamorphosed abalone *Haliotis discus hannai*. Fisheries Science, 66: 1180-1182.

Takami, H. & Kawamura, T. (2003) Dietary changes in the abalone, *Haliotis discus hannai,* and relationship with the development of the digestive organ. Japan Agricultural Research Quarterly, 37: 89-98.

Takami, H., Muraoka, D., Kawamura, T. & Yamashita, Y. (2003) When is the abalone *Haliotis discus hannai* Ino 1953 first able to use brown macroalgae? Journal of Shellfish Research, 22: 795-800.

Tanaka, H., Yamashita, Y., Aoyagi, H., Yamamoto, Y. & Fukunaga, Y. (1996) Efficient production of chitinase by *Wasabia japonica* protoplasts immobilized in double-layered gel fibers. Journal of Fermentation and Bioengineering, 81: 394-399.

Taylor, M.H. & Tsvetnenko, E. (2004) A growth assessment of juvenile abalone *Haliotis laevigata* fed enriched macroalgae *Ulva rigida*. Aquaculture International, 12: 467-480.

Thongrod, S., Tamtin, M., Chairat, C. & Boonyaratpalin, M. (2003) Lipid to carbohydrate ratio in donkey's ear abalone (*Haliotis asinina*, Linne) diets. Aquaculture, 225: 165-174.

Titlyanov, E.A. & Titlyanova, T.V. (2006) Production of plantlets of the red alga of *Gelidium* genus (Rhodophyta) from thallus fragments. Russian Journal of Marine Biology, 32: 284-288.

Troell, M., Halling, C., Nilsson, A., Buschmann, A.H., Kautsky, N. & Kautsky, L. (1997) Integrated marine cultivation of *Gracilaria chilensis* (Gracilariales, Rhodophyta) and salmon cages for reduced environmental impact and increased economic output. Aquaculture, 156: 45-61.

Troell, M., Robertson-Andersson, D., Anderson, R.J., Bolton, J.J., Maneveldt, G., Halling, C. & Probyn, T. (2006) Abalone farming in South Africa: an overview with perspectives on kelp resources, abalone feed, potential for on-farm seaweed production and socio-economic importance. Aquaculture, 257: 266-281.

Troynikov, V.S., Day, R.W. & Leorke, A.M. (1998) Estimation of seasonal growth parameters using a stochastic Gompertz model for tagging data. Journal of Shellfish Research, 17: 833-838.

Tung, C. & Alfaro, A.C. (2011) Effects of dietary protein source and amount on shell morphology of juvenile abalone *Haliotis iris*. Journal of Fisheries and Aquatic Science, 6: 107-118.

Tutschulte, T.C. & Connell, J.H. (1988) Feeding behavior and algal food of three species of abalones (*Haliotis*) in southern California. Marine Ecology Progress Series, 49: 57-64.

Uki, N., Grant, J.F. & Kikuchi, S. (1981) Juvenile growth of the abalone, *Haliotis discus hannai*, fed certain benthic micro algae related to temperature. Bulletin of Tohoku Regional Fisheries Research Laboratory, 43: 59-63.

Uki, N. & Watanabe, T. (1992) Review of the nutritional requirements of abalone (*Haliotis* spp.) and development of more efficient artificial diets. In: Shepherd, S.A., Tegner, M.J. & Guzmán Del Próo, S.A. (Eds.), Abalone of the world: biology, fisheries and culture. Blackwell Scientific Publishing, Oxford, pp. 504-517.

Umamaheswara Rao, M. (1976) Spore liberation in *Gracilaria corticata* J. Agardh growing at Mandapam. Journal of Experimental Marine Biology and Ecology, 21: 91-98.

Uppalapati, S.R. & Fujita, Y. (2002) A simple method for mass isolation of protoplasts from species of *Monostroma*, *Enteromorpha* and *Ulva* (Chlorophyta, Ulvales). Journal of Applied Phycology, 14: 165-168.

Vadas, R.L., Johnson, S. & Norton, T.A. (1992) Recruitment and mortality of early post-settlement stages of benthic algae. British Phycological Journal, 27: 331-351.

Van Alstyne, K.L., Ehlig, J.M. & Whitman, S.L. (1999) Feeding preferences for juvenile and adult algae depend on algal stage and herbivore species. Marine Ecology Progress Series, 180: 179-185.

Van Alstyne, K.L., Whitman, S.L. & Ehlig, J.M. (2001) Differences in herbivore preferences, phlorotannin production, and nutritional quality between juvenile and adult tissues from marine brown algae. Marine Biology, 139: 201-210.

Van Barneveld, R.J., Fleming, A.E., Vandepeer, M.E., Kruk, J.A. & Hone, P.W. (1998) Influences of dietary oil type and oil inclusion level in manufactured feeds on the digestibility of nutrients by juvenile greenlip abalone (*Haliotis laevigata*). Journal of Shellfish Research, 17: 649-655.

Vandepeer, M.E. & Van Barneveld, R.J. (2002) The effect of different dietary digestible protein: digestible energy ratios on growth of juvenile blacklip abalone (*Haliotis rubra)*. In: Fleming, A.E. (Ed.), Proceedings of the 9th Annual Abalone Aquaculture Workshop, 29th - 31st July 2002, Queenscliff, Australia. Fisheries Research and Development Corporation, Abalone Aquaculture Subprogram, Canberra, Australia, pp. 50-60.

Viana, M.T., López, L.M. & Salas, A. (1993) Diet development for juvenile abalone *Haliotis fulgens*. Evaluation of two artificial diets and macroalgae. Aquaculture, 117: 149-156.

Viana, M.T., Correa, G., Lazo, J.P., Frías-Díaz, R., Durazo-Beltrán, E. & Vasquez-Pelaez, C. (2007) Digestive physiology and metabolism of green abalone *Haliotis fulgens* from postlarvae to juvenile, fed three different diatoms. Aquaculture, 271: 449-460.

Viera, M.P., Gómez-Pinchetti, J.L., Courtois de Vicose, G., Bilbao, A., Suárez, S., Haroun, R.J. & Izquierdo, M.S. (2005) Suitability of three red macroalgae as a feed for the abalone *Haliotis tuberculata coccinea* Reeve. Aquaculture, 248: 75-82.

Viera, M.P., Courtois de Vicose, G., Gómez-Pinchetti, J.L., Bilbao, A., Fernandez-Palacios, H. & Izquierdo, M.S. (2011) Comparative performances of juvenile abalone (*Haliotis tuberculata coccinea* Reeve) fed enriched vs non-enriched macroalgae: effect on growth and body composition. Aquaculture, 319: 423-429.

Wells, F.E. & Keesing, J.K. (1989) Reproduction and feeding in the abalone *Haliotis roei* Gray. Australian Journal of Marine and Freshwater Research, 40: 187-197.

West, J.A. & McBride, D.L. (1999) Long-term and diurnal carpospore discharge patterns in the Ceramiaceae, Rhodomelaceae and Delesseriaceae (Rhodophyta). Hydrobiologia, 398/399: 101-113.

Westphalen, G. & Cheshire, A.C. (1995) Macro-algal digestion survival in two commercial species of abalone (*Haliotis laevigata* Donovan and *Haliotis rubra* Leach). Botanica Marina, 38: 53-59.

Xiuliang, L. & Meizhen, L. (1989) Experiment on releasing quantity and optimal temperature period for releasing of *Gracilaria asiatica* carpospores. Chinese Journal of Marine Drugs, 8: 42-46.

Yan, X.H. & Wang, S.J. (1993) Regeneration of whole plants from *Gracilaria asiatica* Chang et Xia protoplasts (Gracilariaceae, Rhodophyta). Hydrobiologia, 260/261: 429-436.

Yeong, H.Y., Khalid, N. & Phang, S.M. (2008) Protoplast isolation and regeneration from *Gracilaria changii* (Gracilariales, Rhodophyta). Journal of Applied Phycology, 20: 641-651.

Yunque, D.A.T., Tibubos, K.R., Hurtado, A.Q. & Critchley, A.T. (2011) Optimization of culture conditions for tissue culture production of young plantlets of carrageenophyte *Kappaphycus*. Journal of Applied Phycology, 23: 433-438.

Zemke-White, W.L. & Ohno, M. (1999) World seaweed utilisation: an end-of-century summary. Journal of Applied Phycology, 11: 369-376.