The importance and movement of mud bacterial carbon within the symbiosis of the New Zealand sea anemone *Anthopleura aureoradiata*.

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Abstract

A. aureoradiata is New Zealand's only native cnidarian to form a phototrophic symbiosis with dinoflagellate microalgae. It is of particular interest as it can be found in estuarine mudflat habitats attached to cockles, where it spends a portion of the day submerged under the mud, either partially or completely. This scenario is very different to the situation in the tropics, where comparable symbioses (e.g. those with reef-building corals) live in brightly lit, clear waters. How *A. aureoradiata* maintains a stable symbiosis is therefore of considerable interest, with one potential mechanism involving the acquisition of carbon from the surrounding mud to counter the reduced availability of light and hence the reduced rate of photosynthesis.

In this thesis, I established the extent to which organic carbon in mud (especially bacteria) can be assimilated by *A. aureoradiata* and to what extent, if any, this carbon contributes to symbiosis nutrition and facilitates symbiosis stability under otherwise sub-optimal conditions. In the first instance, anemones were given access to ^{13}C glucose-labelled mud for 12 hours, in both the light and dark, and the extent of label incorporation (^{13}C enrichment) in both the host and symbiont was measured by mass spectrometry. Subsequently, *A. aureoradiata* was starved of planktonic food for six weeks in the presence of differing quantities of unlabelled mud ('no-mud', 'low-mud' and 'high-mud'), either with or without light, and a range of nutritional and biomass parameters measured. These included symbiont density, host protein content, and the accumulation of host lipid and symbiont starch stores.

Both the host anemone and its symbiotic algae showed signs of ^{13}C uptake from the mud. Host anemones maintained in the dark assimilated more ^{13}C label from the mud than did anemones incubated in the light, while the extent of label assimilation by the symbionts was unaffected by irradiance. Enhanced heterotrophic feeding in the dark is consistent with patterns reported for other symbiotic cnidarians, such as reef corals, where the host must counter the reduced availability of photosynthate from the symbiotic algae. However, the reason for the equal labelling of the symbionts in the light and dark is less clear. Nevertheless, factors such as reverse translocation in the dark (i.e. the transfer of organic carbon from host to symbiont), dark fixation of inorganic carbon, and a higher respiration rate of symbionts in the light than dark, could act either alone or in concert to produce the labelling pattern seen.

While the host and symbiont showed evidence of carbon uptake from the surrounding mud, mud quantity had no effect on either the host's or symbiont's storage products (% of starch in symbiont biomass, host protein content and lipid content), or on symbiont density. The lack of an effect of mud suggests that mud-derived bacteria comprise little of the host's natural diet. In contrast, increased light availability (independent of mud availability) did lead to elevated symbiont density and symbiont starch content, consistent with the phototrophic nature of this symbiosis. More surprising was that host protein content was highest in the dark, suggesting perhaps that the symbionts were less of an energetic drain on their host when starved in the dark due to their lower population density.

In summary, my thesis provides evidence that *A. aureoradiata* and its symbiotic algae can use organic carbon obtained from the surrounding mud for their nutrition, but that this carbon source is of only negligible importance. These results are consistent with previous findings for the uptake and role of mud-derived nitrogen in this system. Further work to establish how this symbiosis maintains its remarkable stability under apparently sub-optimal, low-light conditions is therefore needed.

Acknowledgements

I would like to thank my supervisor for all his help and patience throughout my thesis and to my lab members, especially Clint Oakley, who have helped me achieve the goals I set out to achieve in this thesis. I would also like to thank Sarah Bury and her team at NIWA for all their help and support with the processing of my samples. Also, I would like to thank Conor Neilson and all my friends who have helped support me and give me guidance through my time at Victoria University of Wellington and to all the staff at Victoria University who have helped me both during this thesis and throughout my academic career. Finally, I would like to thank my brother who has always been there for me through my thesis and throughout my life.

Chapter 1: General Introduction

1.1 Cnidarian-dinoflagellate symbiosis – an overview

1.1.1: Symbiosis

Symbiosis is the interaction of two or more organisms over a long timeframe. This ranges across a spectrum that includes interactions ranging from mutualism to parasitism (Fig. 1.1). Mutualism is the interaction of two organisms that results in mutual benefits for both partners. Conversely, parasitism is when one member in an interaction benefits and the other is harmed in some way. Along with this, symbiosis can change from one place along this continuum to another, such as from mutualism to a more parasitic interaction, depending on differing conditions (Davy et al., 2012).

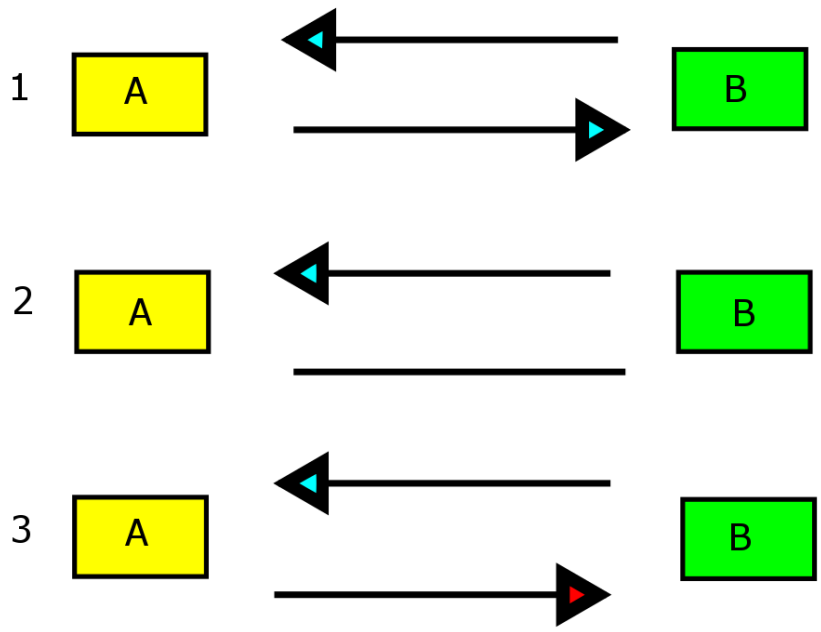

Figure 1.1: Diagrammatic representation of symbiosis interactions. Blue coloured arrows represent a positive gain by the organism to which the arrow points, while red represents a negative interaction. A and B represent two hypothetical organisms: 1) mutualism, where both organisms A and B gain from the interaction; 2) commensalism, where one organism gains and the other is unaffected; and 3) parasitism, where one organism gains at the expense of the other.

There are many forms of symbiosis depending on whether a partner benefits, is harmed, or is unaffected by this interaction (Davy et al., 2012). Furthermore, symbiosis can also be described as endo- or ectosymbiotic. Endosymbiosis is when one partner resides within the other, in which case the smaller of the pair is referred to as a symbiont, while the organism that houses the symbiont is called the host. Ectosymbiosis, by contrast, is when both partners reside outside each other and also interact with the wider environment. Endosymbionts can further be divided into extracellular endosymbionts, which reside between the cells of their host, or intracellular, where the symbiont resides within the cells of their host (Bradford & Schwab, 2012).

1.1.2: Cnidarians and zooxanthellae

Cnidarians belong to an animal phylum (Cnidaria) that include such organisms as hydra, hard and soft-bodied corals, hydrocorals, sea anemones and jellyfish (Davy et al., 2012) (Fig. 1.2). Symbiosis is widespread in this phylum, especially with endosymbiotic algae. Symbiotic cnidarians contain either symbiotic green or dinoflagellate algae (commonly known as either zoochlorellae or zooxanthellae, respectively) (Verde & McCloskey, 1996; Dimond et al., 2011). Both are photosynthetic, with zooxanthellae being the more common partner. Zooxanthellae are dinoflagellate protists and belong to the genus *Symbiodinium*. *Symbiodinium* includes 9 genetic clades (A-I), which are delineated by mostly genetic markers and occasionally morphological differences (Rowan, 1998; Muller-Parker & Davy, 2001; Weis et al., 2001; Baker, 2003; Barneah et al., 2004; Coffroth & Santos, 2005; Stat et al., 2006; Yamashita et al., 2011). Clade A is thought to be the most ancestral clade, diverging approximately 65 to 50 million years ago (MYA), with each clade subsequently diverging from each other until approximately 15 MYA (Pochon & Gates, 2010). Each clade has a large amount of within-clade diversity, with each clade having many different sub-clades or "types" (Coffroth & Santos, 2005).

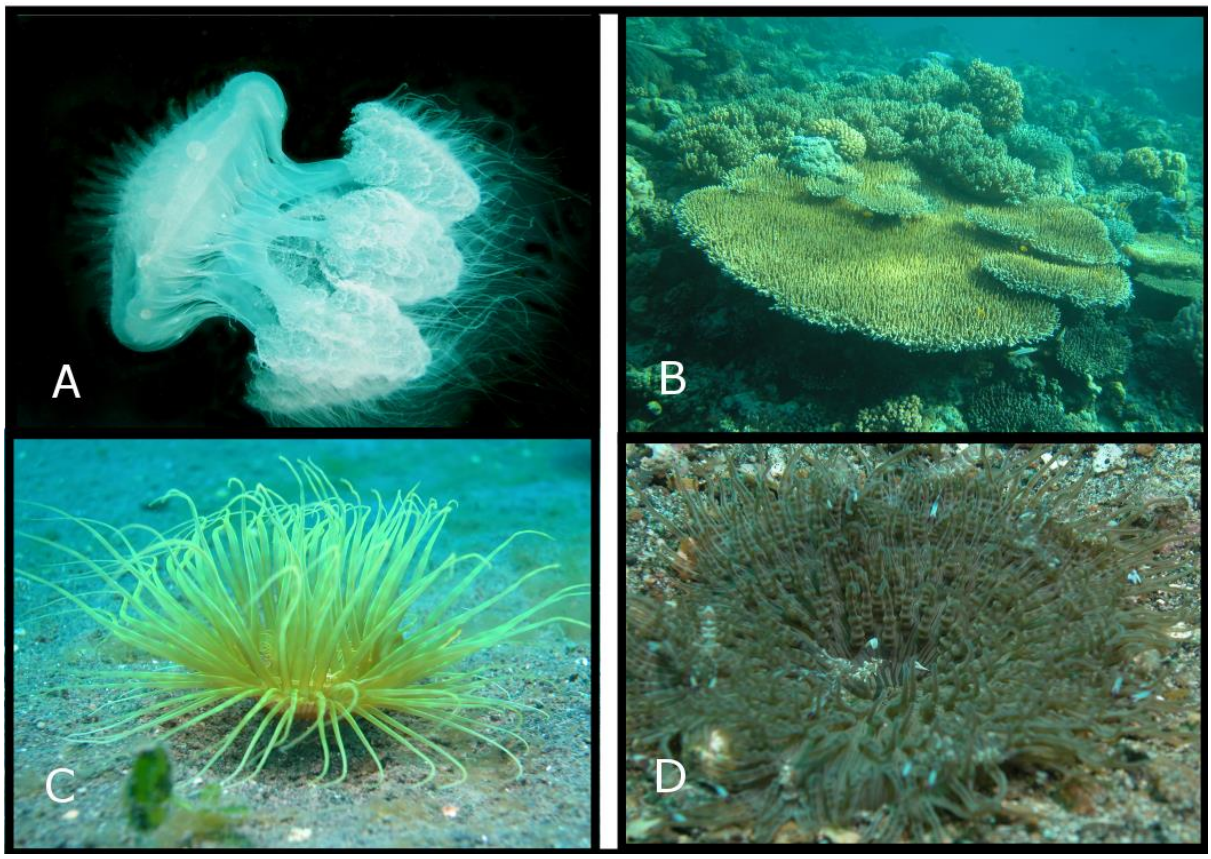

Figure 1.2: Various examples of animals belonging to the phylum Cnidaria. (A) Jellyfish; (B) hard corals; (C) black corals; and (D) sea anemones. Pictures curtesy of Teresa (Zubi) Zuberbühler (copy write).

Symbiodinium also form symbioses with many other marine organisms from different taxa, such as molluscs, foraminiferans, and sponges (Fay, 2010). This relationship can be mutualistic, parasitic or anywhere along this spectrum, though they are typically beneficial, and mutualism shifting into parasitism, at least in the long term, is thought to be rare (Sachs & Simms, 2006).

Dinoflagellate symbionts are acquired by the host *via* either "vertical" or "horizontal" transmission. In vertical transmission, symbionts are passed directly from parent to offspring in the gametes of the parent, with offspring having a similar symbiont composition to their parents. In contrast, horizontal transfer is when larval cnidarians take up symbionts from the free-living population of zooxanthellae and can occur before or after settlement onto the benthos (Farrant and Borowitzka, 1987; Weis et al., 2001; Davy & Turner, 2003; Barneah et

al., 2004; Banaszak et al., 2013). These two acquisition strategies have differing advantages and disadvantages. Vertical transfer is beneficial as it guarantees that young cnidarians will have symbionts to acquire energy from immediately. However, having a similar mixture of symbiont types as their parent means that they could be less adaptable to changing conditions (Weis et al., 2001). Cnidarians that utilise horizontal transfer, however, may host a wide range of *Symbiodinium* types across the host species, allowing some conspecifics to more easily adapt and persist in the face of changing abiotic conditions. The problem is that larvae must acquire compatible symbionts in a relatively short time to survive; this can be problematic as the symbionts must bypass the host's immune system (Dunn & Weis, 2009).

Another problem with having symbionts in the larval stage is the oxidative stress on larvae. However, larvae that undergo only horizontal transfer may have food stores that allow them to grow larger before having to acquire symbionts, reducing the level of stress they experience (Weis et al., 2001; Barneah et al., 2004; Banaszak et al., 2013).

Due to these two general forms of acquisition and the specificity mechanisms associated with symbiont retention, cnidarians can maintain the same symbiont types across generations and species ranges. For example, many scleractinian and octocorals species have the same type of symbionts no matter the depth where they are found or the symbiont types found in other coexisting coral species (Coffroth et al., 2001).

Once the cnidarian-zooxanthella symbiosis is established, symbionts are housed in the host's gastrodermal cells, within a membrane complex made up of several membranes of differing origins. Most of these membranes are algal in origin while the outermost one is derived from the host; this animal derived membrane is known as the perisymbiotic or perialgal membrane. All communication and exchange between the two symbiotic partners must occur across this membrane (Allemand et al., 1998; Wakefield & Kempf, 2001; Davy et al., 2012).

1.1.3: Ecological importance of the cnidarian-zooxanthella symbiosis

Cnidarians and their symbiotic dinoflagellates are large contributors to diverse ecosystems around the world. They aid in the recycling and input of energy into marine systems, both in the tropics or temperate locations (Davy et al., 2012).

It is in the tropics where this relationship has been most studied, given its pivotal role in enabling the survival and success of coral reefs in nutrient-poor waters; indeed, the environmental conditions encountered in the tropics versus temperate latitudes have led to cnidarian-algal symbiosis being much more common in the tropics (Muller-Parker & Davy, 2001). The input of autotrophically-derived energy, in an oligotrophic environment with low levels of dissolved nutrients and low prey abundance, helps maintain the host's energy budget, so allowing for host growth and reproduction. Moreover, the symbiosis facilitates the recycling and conservation of nitrogen in oligotrophic tropical seas and accelerates the rate of skeletogenesis in coral hosts (i.e. calcium carbonate deposition), so allowing the net accretion of the coral reef framework (reviewed by Davy et al., 2012). These nutritional interactions are described in detail below (Section 1.2).

By contrast, illumination levels are lower in temperate regions, especially at depth, but prey abundance is often much higher than in the tropics, allowing for the maintenance of temperate symbiotic cnidarians' energy budgets even in otherwise less-than-favourable environments (reviewed by Muller-Parker & Davy, 2001). Given this, temperate symbiotic cnidarians can still be responsible for considerable bioherms and provide habitat for many fish species (Elliott, 1992; Schiller, 1993; Ferrier-Pagès et al., 2015), even though far fewer species of temperate cnidarians have formed phototrophic symbioses than in the tropics. For example, of more than 70 species of cnidarians in British waters, just five contain symbiotic algae (Davy et al., 1996), while only one species is known to contain symbiotic algae in New Zealand waters (Morar et al., 2011).

Along with aiding host and symbiont nutrition, the cnidarian-dinoflagellate relationship likely reduces grazing pressure on the algae and helps to keep them in a light-rich environment in the

water column (Muscatine et al., 1977; Davy et al., 2012), though these aspects have not been well studied.

1.2: Nutritional Flux in Cnidarian-Zooxanthella Symbiosis

1.2.1: Carbon movement

Carbon flux between host and symbiont is a key feature of their symbiosis, and the mechanisms involved are quite different than those utilised by free-living microalgae which exchange carbon directly with the ocean (Allemand et al., 1998; Davy et al., 2012).

For microphytes and macrophytes, CO_2 is essential for the fixation of carbon through election transport for the production of energy. Like land plants, algae, including zooxanthellae, use the enzyme ribulose-1, 5-bisphosphate carboxylase oxygenase (RubisCO) to fix CO_2 into useful carbon products (Davy et al., 1996; Rowan et al., 1996; Leggat et al., 2002). In free-living algae, dissolved inorganic carbon (DIC) is usually actively transported across the alga's chloroplast envelope or into its cytoplasmic membrane directly, using a pump located at the interface between the cell and its external environment (Allemand et al., 1998). In contrast, symbiotic zooxanthellae are not directly exposed to seawater. Because of this, the host and symbiont have developed a sophisticated system of rapid transport of usable DIC to the symbionts, termed carbon concentrating mechanisms (CCMs) (Allemand et al., 1998; Davy et al., 2012).

Host respiratory CO_2 is a major DIC source for the symbionts, but not the only one. Benazet-Tambutté et al. (1996) found that, though symbionts of the sea anemone *Anemonia viridis* could utilise recycled CO_2 from host dark respiration, the amount supplied was not enough to support the rates of photosynthesis shown in this or other anemones. Therefore they concluded that CO_2 must be obtained from other forms of DIC taken up from the external seawater by the host, and made available to the symbiont *via* CCMs.

HCO_3^-, CO_3^{2-}, and bicarbonate are major sources of this external DIC. HCO_3^- is transported into the host and then transformed into CO_2. This uptake is facilitated by H^+-ATPase. H^+-ATPase is located in the cell membrane and secretes H^+ ions into the external medium, which causes HCO_3^- to be converted to carbonic acid and then dehydrated into CO_2 by a membrane enzyme, carbonic anhydrase (CA) (Tansik et al., 2015). This CO_2 can then passively diffuse into the cell where it is thought to be converted back to HCO_3^- so that it is trapped. The exact mechanism whereby HCO_3^- is transported from the host gastrodermal cells to symbiont cells is still unknown. CA aids in the interconversion of HCO_3^- and CO_2, and has been found in over 22 species of symbiotic cnidarians (Davy et al., 2012 see Fig 1.3). CAs are also found in other locations in the symbiosis. such as in the symbiotic dinoflagellates, and aid in the transport of carbon in the form of CO_2 to RubisCO, as part of symbiont CCMs (Tansik et al., 2015). Along with this, the efficiency of such enzymes differs depending on the type of dinoflagellate symbiont being examined. For example, different types of *Symbiodinium* may have CCMs that differ in their effectiveness at different temperatures (Oakley et al., 2014).

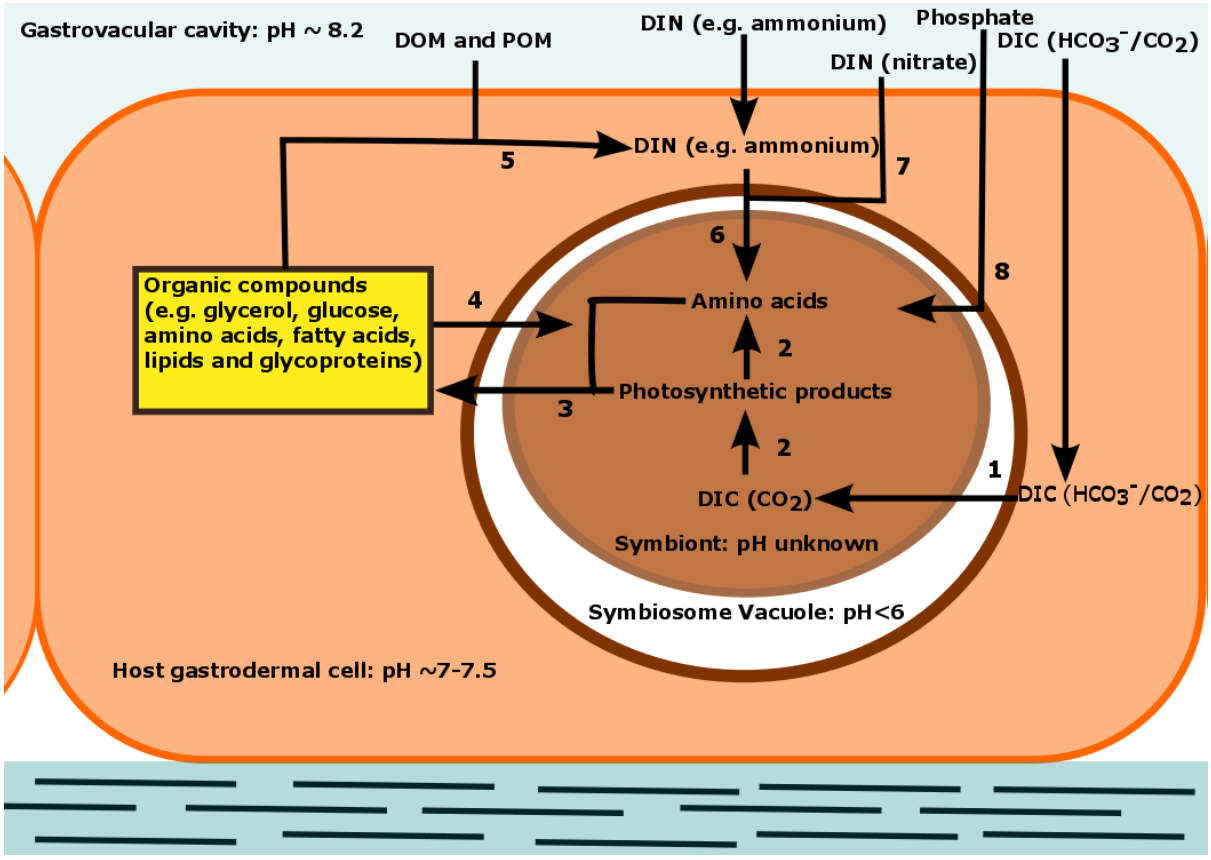

Figure 1.3: Diagrammatic representation of nutritional movement and interactions in the cnidarian-dinoflagellate symbiosis. 1) The uptake of dissolved inorganic carbon (DIC), either in the form of bicarbonate (HCO_3^-) from the

surrounding seawater, or as CO_2 from the seawater or host metabolism/calcification. Unlike CO_2 however, HCO_3^- must first be converted to CO_2 prior to photosynthesis by the dinoflagellate symbiont. 2) Photosynthesis. CO_2 is photosynthetically fixed through the Calvin-Benson cycle (i.e., the C3 pathway), with the dinoflagellate synthesising a range of organic compounds or photosynthate, including amino acids. 3) Translocation of photosynthate from the symbiont to the host. 4) Possible reverse translocation, where organic compounds are translocated from the host to the symbiont. Reverse translocated products could arise from host metabolism or be in the same forms as those originally translocated by the symbiont. 5) Host metabolism, where translocated compounds are used, alongside dissolved organic matter (DOM) and particulate organic matter (POM) taken up from seawater. 6) Ammonium assimilation, made possible by the catabolism of nitrogenous compounds in the host, ultimately leading to the generation of ammonium. Excretory and seawater ammonium can be assimilated by both the host cell (pathway not shown) and the symbiont, with translocated organic compounds providing carbon skeletons necessary for host assimilation. 7) Nitrate assimilation. Though nitrate is taken up from the seawater by the host, only the symbiont can convert it to ammonium for subsequent assimilation into amino acids. 8) Phosphate assimilation, where phosphate is likewise taken up from seawater and can be assimilated by the dinoflagellate symbiont. Figure redrawn from Davy et al. (2012).

One of the most studied and important steps in carbon movement between host and symbiont is the movement of products fixed by photosynthesis (photosynthate). The amount and type of products have been extensively studied for the past 60 years but have never been exhaustively quantified and qualified. Research shows that vastly differing quantities of photosynthate are translocated in different host-symbiont pairings, with as much as 90% of products produced being translocated to the host (Steen et al., 1984; Day, 1994; Davy et al., 1996).

The percent of fixed carbon that cnidarians obtain from their symbionts varies with prey abundance. When the host is feeding, it often receives a lower percent of the total carbon fixed by their symbionts, which reduces the burden placed on symbionts. For example, the temperate sea anemone *Anthopleura elegantissma* only obtains approximately 13% of the fixed carbon from its symbionts when well fed, while starved these anemones will obtain about 45% (Fitt et al., 1982). Light, temperature and the type of symbiont present also have a large effect on carbon and energy movement between symbiont and host. For instance, Muscatine et al. (1983) demonstrated that under well-lit conditions both temperate and tropical cnidarians can receive >90% of their carbon budget from their symbionts.

Starzak et al. (2014) showed that symbiont type could also cause a difference in the amount of carbon that a host cnidarian receives. Differences were found in a model tropical cnidarian

(*Aiptasia* sp.) with respect to photosynthate translocation depending on the associated type of *Symbiodinium*. For instance, the type B1 provided the most carbon to its host while types F5.1 and E, though they underwent the highest rates of photosynthesis, respired far more than type B1 and therefore provided less carbon to their host. This difference in photosynthate translocation between *Symbiodinium* clades may be more important for host survival depending on the life stage of the host. For example, Cantin et al. (2009) found that juveniles of the coral *Acropora millepora* showed differing rates of photosynthate incorporation depending on the clade of symbiont they hosed. Those housing clade C1 received more fixed carbon than those housing symbionts of clade D this was associated with an improved rate of electron transport in photosystem II in clade C1. The researchers suggested that this could lead to a competitive advantage in juvenile survival in their natural habitat.

The ability of symbionts to fix carbon from CO_2 can cause even hosts with low symbiont abundances to receive comparable levels of photosynthate as those with high symbiont abundances. Davy & Cook (2001) found that both fed and unfed anemones (*Aiptasia pallida*) translocated the same proportion of their photosynthate, a constant 16% of the total. It was suggested that this is due to lowered symbiont densities in starved anemones, which allowed for increased CO_2 availability *per* algal cell due to reduced CO_2 competition, and hence an increase in energy fixed *per* symbiont (Davy & Cook 2001). Evidence for this exists in other model species, such as the hydra *Chlorohydra viridissima*, which was shown to survive equally well with only 20% of its usual symbiont abundance (Muscatine & Lenhoff, 1965).

There are not only uncertainties about the amount of photosynthate being translocated but also the specific compounds that comprise this photosynthate. Traditionally, glycerol has been thought to be the major product transported (Davy et al., 1996; Davy et al., 2012). Glycerol is transported along with a suite of other photosynthetic products, such as glucose, glucose-6-phosphate, malate, amino acids (phenylalanine, alanine, tyrosine, isoleucine, leucine, histidine, lysine, and valine), lipids, and organic acids (Davy et al., 1996; Wang & Douglas, 1999; Whitehead & Douglas, 2003; Davy et al., 2012). However, there is debate surrounding the importance of glycerol owing to problems with *ex hospite* studies. For instance, Burriesci et al. (2012) suggested, through the labelling of ^{13}C bicarbonate in the intact symbiosis and isolated symbionts, when exposed to light, that glycerol was most probably produced as a response to the stress of being isolated from the holobiont, while in the intact symbiosis the majority of photosynthate translocated to the host was glucose, not glycerol.

The regulatory mechanisms for the translocation of photosynthate from the symbiont to the host are not fully understood. There are, however, two main theories as to how this is achieved. One is that cnidarians regulate the growth and cell division of zooxanthellae housed in their cells, for example through the restriction of nitrogen to the symbiont (Davy & Cook 2001a and b). Due to this, symbionts have more available photosynthate for release than they would if they grew and divided unimpeded (Rodriguez-Lanetty et al., 2006; Dunn et al., 2007). Evidence against this hypothesis exists, however, as studies on some symbiotic cnidarians have shown that symbiont cells translocate the same quantity of photosynthetic products regardless of zooxanthellar abundance and growth. One such species is the temperate coral *Cladocora caespitosa,* where the number of symbionts did not drastically alter the output of autotrophic energy produced by the zooxanthella population (Hoogenboom et al., 2010).

The second hypothesis is that the host produces a chemical or chemicals, called a host release factor or factors (HRF) that cause their symbionts to release and/or produce excess photosynthate, rather than directly controlling symbiont abundance (Davy et al., 2012). This HRF may vary between cnidarian species. For example, papers by Gates et al. (1995, 1999) showed that in the coral *Pocillopora damicornis* and the tropical sea anemone *Aiptasia pulchella,* free amino acids induced the release of photosynthate as well as increased CO_2 fixation by the symbionts. Not all symbiotic algae respond as readily to free amino acids, however (Withers et al., 1998), and doubts have been cast about their role as an HRF *in hospite*. Furthermore, it could be that the mechanisms used to control the release of photosynthate differ between symbiotic cnidarians (Lehnert et al., 2014); indeed, the response to HRF is not uniform in all symbiont types (Sutton & Hoegh-Guldberg, 1990).

Though there is still debate over symbiont to host translocation, even less is known about reverse translocation, where the host translocates organic compounds to the symbionts (Davy et al., 2012). Nevertheless, research suggests that it does occur. In particular, researchers have shown that nitrogen, sulphur and potentially carbon can be translocated from the host to the symbionts (Cook, 1972; Steen, 1986; Piniak et al., 2003). For example, Cook (1972) found that for the green hydra *Chlorohydra viridissima* that, when fed with ^{14}C-enriched food in the dark, that ^{14}C was incorporated into their symbionts where there was only negligible light and thus negligible rates of photosynthesis. This helps to further provide evidence for the existence of reverse translocation.

1.2.2: Nitrogen and phosphorus flux

Though photosynthetic products are essential in tropical environments to maintain coral reefs, the acquisition and conservation of nitrogen are also vital. The primary site for inorganic nitrogen assimilation, obtained from seawater, is the zooxanthellae (Piniak & Lipschultz, 2004). In comparison, organic nitrogen is obtained *via* passive filter feeding, prey acquisition in the form of plankton, and the acquisition of dissolved inorganic nitrogen (DIN) and dissolved organic nitrogen (DON) (Hoegh-Guldberg & Williamson, 1999; Piniak & Lipschultz, 2004). Most of the nitrogen from organic sources is kept in host tissues, though some are passed to the symbiont (Piniak & Lipschultz, 2004).

The cnidarian-dinoflagellate symbiosis allows for a degree of uptake, assimilation, and recycling of nitrogen that is not possible for either partner alone. Ammonium is often excreted and built up more in symbiotic cnidarians when symbiont abundance or photosynthetic activity are depressed, such as by darkness (Wang & Douglas, 1998; Miller and Yellowlees, 1989; Wang & Douglas, 1999). This if often thought to suggest that nitrogen is being recycled in the form of ammonia and transferred to the symbionts as needed, then transformed into amino acids that are released back to the host (Wang & Douglas, 1998; Wang & Douglas, 1999). Some research suggests, however, that host nitrogen is also conserved. In this nitrogen conservation process, it is proposed that photosynthate is used as an alternative respiratory substrate by the host, instead of amino acids, and also provides fixed carbon for use in amino acid synthesis (Wang & Douglas, 1998 see Fig. 1.4). Wang and Douglas (1998) presented evidence for this being the case, where they removed or suppressed symbiont abundance in symbiotic sea anemone *Aiptasia pulchella*. By adding exogenous carbon to the anemone, amino acid degradation and ammonium accumulation in host tissues decreased. Piniak and Lipschultz (2004) also provided evidence for nitrogen conservation through the effects of labelled prey on both starved and fed cnidarians. In this study, labelled nitrogen was consistent across both the host and symbionts, regardless of the host's nutritional regime. These results are consistent with the nitrogen conservation paradigm but not that of nitrogen recycling where labelled nitrogen should be recycled back into the host and out of the symbiont. In the nitrogen conservation hypothesis, nutritional regime in the light should have no effect on protein stores as the host catabolised fixed carbon from their symbionts in preference to amino acids from protein catabolism (Piniak & Lipschultz, 2004).

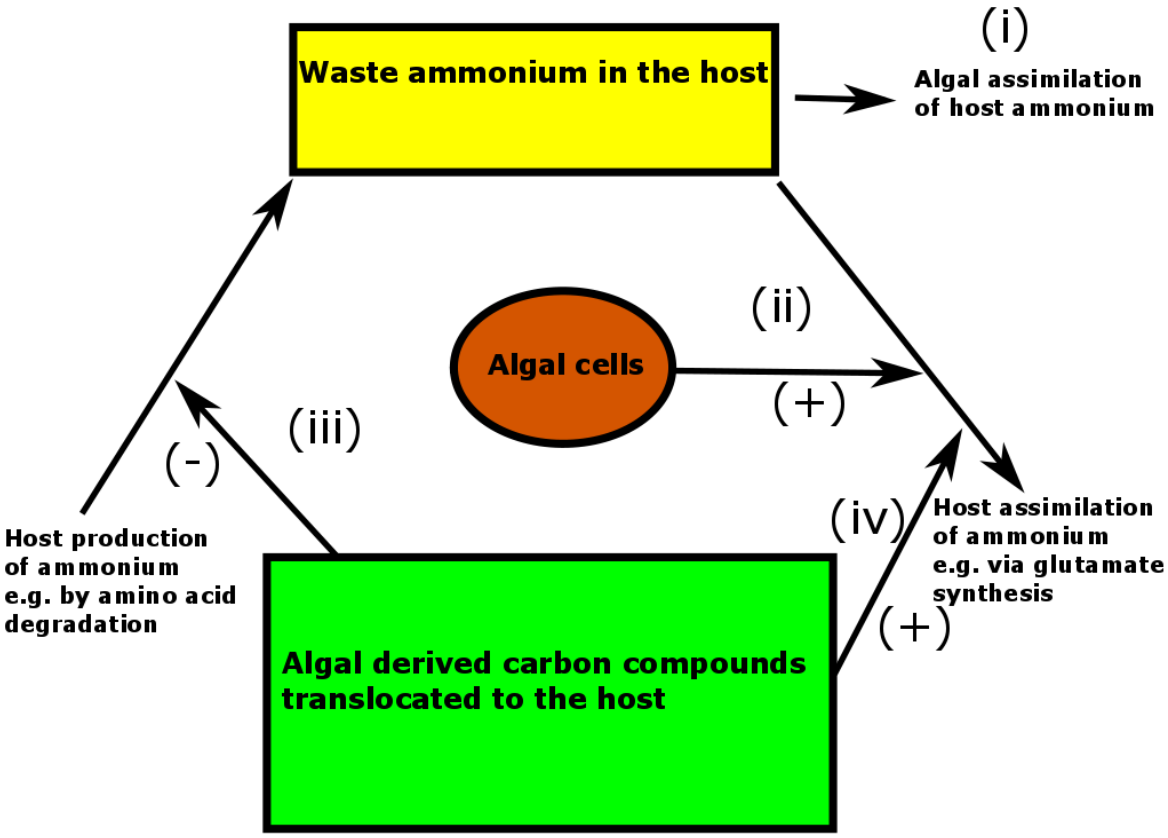

Figure 1.4: A diagrammatical representation of the proposed processes that influence the ammonium content of the host tissues and ammonium efflux from the host. (i) Symbiont assimilation of host-derived ammonium, as predicted by the nitrogen recycling hypothesis. (ii) An increase (+) in ammonium assimilation by the host resulting in nitrogen limitation of symbiont proliferation, as predicted by the nitrogen limitation hypothesis. (iii) A decrease (−) in ammonium production by amino acid deamination in the host through the preferential utilisation of photosynthate as respiratory substrates, as predicted by the nitrogen conservation hypothesis; and (iv) Promotion (+) of ammonium assimilation in the host by symbiont-derived photosynthate. Figure modified from Wang & Douglas (1998).

External nitrogen from the surrounding medium also plays a large part in allowing the symbiont and host to accumulate their required nitrogen. Nitrogen in the surrounding ocean comes most commonly in the forms of dissolved organic nitrogen (DON) such as urea, dissolved inorganic nitrogen (DIN) such as ammonium and nitrate, and finally particulate organic nitrogen (PON), often in the form of plankton (Davy et al., 2012).

Both partners can take up ammonium, but the symbionts are the chief site for ammonium assimilation (Davy et al., 2012). The host, without this association, would not be able to accumulate as much ammonium. Many corals require large quantities of carbon products from

their symbionts to form the structures needed for this assimilation of ammonium (Davy et al., 2012). Once ammonium is assimilated into the host, it is transformed into glutamate, an amino acid, and a precursor of many other amino acids, using the NADP-glutamate dehydrogenase pathway (Miller and Yellowlees, 1989; Roberts et al., 2001).

As with carbon product translocation, the nitrogen transport mechanisms in the symbiosis are not well understood. Recent work has, however, shown that various transport proteins on the symbiosome membrane are used to facilitate this transfer and many could theoretically help stimulate the transport of nitrogen from host to the symbiont (Peng et al., 2010).

Phosphorus is also very important and in short supply in tropical oligotrophic waters, though usually abundant in temperate waters. Phosphorus is incorporated into many vital compounds such as DNA, RNA and phospholipids in all cells. Phosphorus occurs in several forms: condensed phosphates, dissolved orthophosphates (DOPs) and phosphates bound to a range of other molecules. Free-living photoautotrophs, including members of the genus *Symbiodinium*, can usually only access DOPs and dissolved phosphates (Godinot et al., 2009). The symbiosis aids zooxanthellae greatly, as the host's feeding can help provide different forms of phosphorus, acquired from heterotrophy, that they otherwise would not have access to if they were free-living, at least in tropical environments (Godinot et al., 2009). It has been suggested, due to phosphate levels within hosts and symbionts that both members of this symbiosis have transporter proteins or anion pumps that help with this exchange of phosphate, and that zooxanthellae are large sinks for acquired phosphate (Godinot et al., 2009). Along with this, evidence exists for enzymatic activity (phosphatase P-1 and P-2) in zooxanthellae for the potential transport of phosphate from the host to the symbiont (Jackson et al., 1989).

1.3: Heterotrophic feeding – balancing the symbiosis energy budget

Host heterotrophy has a large effect on the maintenance, growth, and photosynthetic output of symbiont populations, and hence the success of the entire cnidarian-algal symbiosis. As stated above, this is especially due to the need for nitrogen obtained *via* heterotrophic feeding (Piniak & Lipschultz, 2004).

Indeed, over long periods of starvation, symbionts and hosts have been shown to stop growing altogether, even when exposed to light levels that allow for maximum photosynthetic rates (Fitt

& Cook, 2001). Conversely, increased heterotrophic feeding is important in suboptimal conditions for photosynthesis, such as low light and high turbidity, and after bleaching events where some anemones and corals, such as *Montipora capitata*, have been found to increase feeding rates sufficiently to restore energy reserves depleted by bleaching stress (Grottoli et al., 2006; Houlbrèque & Ferrier-Pagès, 2009). Other corals, however, such as *Porites compressa* and *Porites lobata*, do not increase their feeding rates to compensate after bleaching, which may cause them to be less resistant to bleaching and take longer to recover (Grottoli et al., 2006).

These studies show that tropical symbiotic cnidarians require heterotrophically-derived nutrients, and can alter their feeding behaviour to facilitate their survival in sub-optimal conditions. However, whether such compensatory behaviour could also play a role at temperate latitudes is unknown. In particular, research is needed to understand how temperate symbiotic cnidarians, compensate for turbid, low-light regimes, and whether the relative stability of these symbioses under such conditions is linked to heterotrophic food acquisition. Of particular note in this regard, temperate symbioses typically have greater access to planktonic food than do their tropical counterparts (Muller-Parker & Davy 2001), while there is evidence from tropical corals and temperate sea anemones that sedimentary particles can be consumed and supply nutrients to the host and its symbiotic algae (Mills et al., 2004; Morar et al., 2011). These temperate associations and their feeding behaviour are the focus of this thesis and are introduced in more detail below.

1.4: Temperate symbiotic cnidarians

1.4.1: Temperate symbiotic cnidarian distribution and diversity

Temperate symbiotic cnidarians exist from as high as 60°N in Alaska and Scotland to as low as 45°S in New Zealand (Muller-Parker & Davy, 2001). Though not as numerous as their tropical counterparts, there are still many species of symbiotic cnidarians in temperate environments. Evidence suggests that these cnidarians are able to survive in these temperate climates due to both their own and their symbionts' ability to adapt to the relatively low temperature and light regimes of such environments. This can, in part, be through the ability

of symbionts to recover from low temperatures (Thornhill et al., 2008), and the host's ability to supplement its own diet with heterotrophy when the light is limiting (Ferrier-Pagès et al., 2015).

In temperate locations, several clades of *Symbiodinium* are present. Many temperate cnidarians from the Atlantic coasts of the UK and France, and the Mediterranean appear to house 'temperate A' *Symbiodinium*, while at least one species from this region houses clade B (Visram et al., 2006). However, in the temperate region of Japan, symbiotic cnidarians predominantly house members of clade C, while clades D and F are also present (Lien et al., 2007; Lien et al., 2012). As in Europe, *Symbiodinium* clade A is prevalent in New Zealand, where it associates with the sea anemone *Anthopleura aureoradiata* from Stewart Island in the far south to Cape Reinga in the far north (Howe, 2012).

1.4.2: Characteristics of temperate environments

In comparison to the tropics, where irradiance is high and waters are nutrient poor, temperate regions are characterised by lower, more variable irradiances, high nutrient concentrations, and large amounts of plankton (Muscatine et al., 1977; Muller-Parker & Davy, 2001). The highest concentrations of nutrients and food occur in the spring and autumn, as water becomes more turbid and nutrients are stirred up into the photic zone (Andersson et al., 1994; Muller-Parker & Davy, 2001; Domingues et al., 2005).

In temperate areas, light does not penetrate as deep into the water column as in tropical regions, and although daily and annual photosynthetically active radiation (PAR) is comparable at the surface, it is more variable than in the tropics and decreases drastically with depth (Muller-Parker & Davy 2001). This has the potential to seriously limit the autotrophic potential of temperate cnidarian-dinoflagellate associations. For example, Davy et al. (1996) measured the irradiance at a temperate location (Lough Hyne, Ireland) under sunny and cloudy conditions at two different depths (1.5 and 9 m), and modelled the contribution of photosynthetic carbon from the symbionts to host respiration under these differing conditions in the sea anemones *Cereus pedunculatus*, *Anthopleura ballii* and *Anemonia viridis*, and the zoanthid *Isozoanthus sulcatus*. All species received the required energy from their symbionts to maintain a positive

carbon budget when exposed to sunny conditions in shallower waters, but their symbionts provided little under cloudy conditions and at greater depth. These authors suggested that increased heterotrophy could be utilised by these anthozoans to survive under suboptimal conditions. Similarly, the temperate anemone *Eunicella singularis* has a much lower rate of autotrophic carbon acquisition than tropical corals of the same genus, though the reduced acquisition of photosynthetic carbon from its symbiotic dinoflagellates is counterbalanced by utilising increased heterotrophy and reduced respiration, so maintaining a positive carbon balance (Ferrier-Pagès et al., 2015).

The ability to completely counter a decline in photosynthetic production at reduced irradiance is seasonally variable, however. In particular, during summer, when irradiance levels are high, symbiotic temperate cnidarians can maintain positive carbon budgets, but this pattern reverses in winter when irradiances decline despite greater prey abundance, most likely because colder temperatures also limit photosynthetic performance (Szmant-Froelich & Pilson, 1980; Engebretson and Muller-Parker, 1999; Dimond & Carrington, 2007; Ferrier-Pagès et al., 2011).

1.4.3: Temperate symbiosis stability

Temperate cnidarian-dinoflagellate symbioses are very stable to changes in temperature and irradiance when compared to tropical symbioses. Tropical symbiotic cnidarians show large shifts in their symbiont density across the seasons, with the highest density of symbionts for most species being found in the winter months. For example, five different corals - *Montastrea annularis, Montastrea faveolata, Acropora cervicornis, Montastrea franksi, Acropora palmata and Acropora cervicornis* all showed high densities of zooxanthellae in the winter months and lowest densities in the late summer-autumn months (Fitt et al., 2000).

Similar effects were found in a long-term study (years) of *Acropora formosa*. In this study algal densities in autumn and winter were three times those in spring and summer, due to increased temperature, nitrate levels and other unknown factors that change with season (Fagoonee et al., 1999).

In comparison, temperate symbiotic cnidarians are often found to be quite stable with season and variations in irradiance (Rodriguez-Lanetty et al., 2003; Muller-Parker & Davy, 2001;

Dimond et al., 2011). For example, in the American sea anemone *Anthopleura elegantissima*, even when the division frequency of symbionts was three to four times more in July (mid-summer) and November (late autumn) than other months, symbiont density was relatively constant (Dimond et al., 2011). Furthermore, temperate sea anemones such as the European *Anthopleura ballii* retain a stable symbiont population even when covered entirely by sediment, often for many weeks or months (Davy, pers. comm). This stability is not always the case, however. In particular, a study by Dimond & Carrington (2008) showed that the temperate coral *Astrangia poculata* showed large variations in symbiont density and mitotic index (MI) over seasons, housing more symbionts which divide at a much faster rate during the summer than the winter. The authors proposed that this is because of a change in the host's control mechanisms on their symbionts, which allow for more symbionts during the most advantageous, well-lit and warm months.

Symbionts in temperate environments can also be quite tolerant of fluctuations in temperature, and thermal extremes (especially cold extremes). For example, a study by Thornhill et al. (2008) measured the quantum yield of photosystem II (Fv/Fm) and the relative electron transport rate (ETR) of a temperate *Symbiodinium* type and compared it to several tropical types during and after cold shock, similar to conditions felt in temperate regions in late winter. Their results showed that, although Fv/Fm decreased to similar levels during the cold shock experiments in all types, only the temperate type demonstrated recovery once the temperature was increased to normal. The trend for temperate cnidarians to be thermally tolerant is further supported by Muller-Parker et al. (2007). In this study the temperate cnidarian *Anthopleura elegantissima* were tested to determine how thermally tolerant their symbionts were. It was found that the light-saturated photosynthetic rates were constant from 12 to 24°C and decline greatly after 26°C. With 26°C being 5-14°C higher than locations where this species is normally found during summer, highlighting the thermal stability of *A. elegantissima's* symbionts.

1.5: Anthopleura aureoradiata

Anthopleura aureoradiata is New Zealand's only native sea anemone that contains symbiotic alga. This species is most commonly found on mudflats, though it can also be found on rocky

shores. On the rocky shore, *A. aureoradiata* is often found clustered together in crevices amongst rocks and in rock pools in the intertidal zone (Morar et al., 2011).

On mud flats, however, most *A. aureoradiata* attach to the shell or shell debris of the cockle *Austrovenus stutchburyi* (Morar et al., 2011) (Fig. 1.5 A & B). These cockles live just below the surface in the aerobic layer of mud, allowing the anemones to remain oxygenated and to appear on the surface of the mud during low tide.

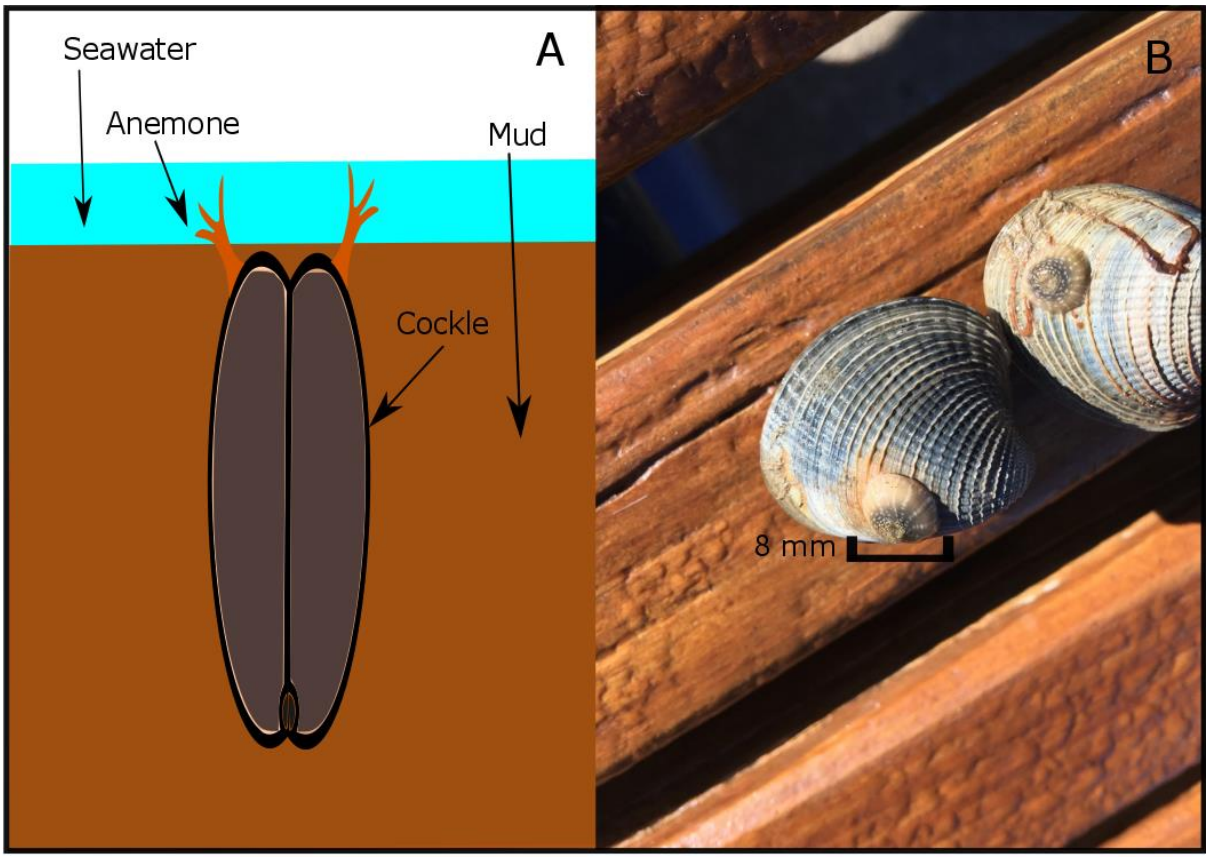

Figure 1.5: A: A diagrammatic representation of *Anthopleura aureoradiata* connected to a cockle at low tide in its native mudflat habitat, modified from Morar et al. 2011. B: *A. aureoradiata* on cockles collected from Pauatahanui Inlet, New Zealand. Photograph by Tess Keough.

Previous work on *A. aureoradiata* has focused on the flux and physiological importance of nitrogen in the symbiosis (Morar et al. 2011). This study showed that nitrogen taken up *via* heterotrophic feeding on mud bacteria is then incorporated into the host's tissues, before being translocated to its algal symbionts. This suggests that *A. aureoradiata* potentially utilises mud

bacteria to supplement its nitrogen budget, though contrary to this the nitrogen status of the symbiotic algae was not enhanced when the anemone was incubated in mud for up to a month *versus* in seawater alone. This does not preclude, however, carbon acquired heterotrophically from the surrounding mud from helping to sustain the symbiont population. This potential nutritional input could play an important role in symbiosis stability given the tendency for these anemones to be found buried in sediment and the low light/high turbidity environment in which they live. No work has been done on this aspect of the *A. aureoradiata-Symbiodinium* symbiosis.

1.6: Aims and Objectives

This study aimed to establish whether uptake of exogenous particulate organic carbon (POC) from the surrounding mud can support the symbiont population of the sea anemone *Anthopleura aureoradiata*. The specific objectives were:

1) To determine whether the sea anemone can take up organic carbon from sediment and whether this carbon is then made available to the host anemone's endosymbiotic dinoflagellates.

Hypothesis: Anemones in the dark will take up POC from sediment, with some of this carbon ultimately being incorporated by the symbionts.

2) To determine whether the uptake of exogenous organic carbon from mud helps to support the nutritional status of the symbiotic dinoflagellates, and hence the stability of the symbiosis, under sub-optimal, low-light conditions.

Hypotheses: (1) Anemones and their symbionts will exhibit fewer signs of starvation in the dark when provided with mud than when no mud is supplied, due to increased food availability; (2) Enhanced photosynthesis and autotrophy in the light will reduce the need for exogenous carbon sources, resulting in lowered incorporation of POC from the mud.

Chapter 2: Materials and Methods

2.1: Anemone collection and maintenance

Anemones were collected at low tide from the Pauatahanui Inlet mud flat, a branch of the Porirua Harbour estuary, near Wellington, New Zealand (Fig. 2.1 and 2.2). After collection, all anemones were housed in one glass bowl (1500 ml volume) with 1000 ml of 0.22-µm filtered seawater (FSW). Anemone were illuminated under a 12 h light/ 12 h dark cycle with 220 ± 10 µmol photons m^{-2} s^{-1} irradiance (Stareal 15 F54W/840) during the light phase, and acclimated for one week prior to experiments. Anemones were kept at a constant 19°C and fed once every 7-10 days with *Artemia* sp. nauplii prior to use in experiments.

I conducted two experiments, one a ^{13}C labelling/tracking experiment, the other a feeding/starvation experiment (named hereafter as Experiments 1 and 2, respectively). Anemones designated for Experiment 1 were starved for one week prior to their use, to increase the likelihood of a feeding response, while anemones for Experiment 2 were fed once every 7-10 days with *Artemia* sp. nauplii until the start of the experiment.

Figure 2.1: A map of the New Zealand study site, Pauatanui Inlet. The black rectangle indicated the location I collected samples from (41° 6’00’’S, 174° 52’16’’E). Map modified from the LINZ Data Service https://data.linz.govt.nz/set/4702-nz-aerial-imagery/ and licensed by LINZ for re-use under the Creative Commons Attribution 3.0 New Zealand licence.

Figure 2.2: Pauatahanui Inlet, Porirua, Wellington, New Zealand. Photograph by Tess Keough.

2.1.2: Preliminary studies

Before work on Experiment 1 was undertaken preliminary studies were performed to determine the correct molar concentration of ^{13}C-glucose, to optimise host and symbiont enrichment, if it indeed occurred. Preliminary tests were run over the course of ~ 4 months, with tests being comprised of anemones in 5 or 20 g of mud and 40 ml of FSW, in the light. In all of these preliminary tests, mud was collected and processed as detailed below in sections 2.1.3 and 2.2, and anemones collected from the same field site as both experiments.

Host tissue and symbionts were processed as outlined below in sections 2.1, and 2.3 while sediment labelling was also measured to ensure that enrichment of mud bacteria was taking place. This sediment was washed and acidified with 2 ml 95% HCL overnight to ensure that any inorganic carbonate present in the mud did not interfere with mass spectrometer readings.

In the first instance molar concentrations started at 1.5 mM and were increased through the optimisation tests (1.5, 2, to 6 mM), as each previous concentration did not give a high enough enrichment to be readably detectable above ambient ^{13}C levels in the host, even though some enrichment was taking place. In addition a range of different incubation times (6, 8, 12h), stirring intensities, mud quantity (20 g), and egestion times were trailed (12 h *vs* 18 h) to further optimise the protocol. The results of these various preliminary studies are provided in the Appendix.

At 6 mM ^{13}C-glucose, anemones were still not enriched to a level that was satisfactory, but further preliminary tests at higher concentrations were not possible as the mass spectrometer tolerance threshold for enrichment was near its limit for the enriched mud. Because of this, a decision was made to increase the incubation concentration three-fold, to 18 mM, with no confirmation of the extent of labelling in the sediment. This conclusion was used for the subsequent experiments.

Additionally, the homogeneity of ^{13}C-labelling in the mud was confirmed following labelling at 6 mM. After labelling 100 g of mud at 6 mM, six 1 g subsamples were randomly selected

and the ^{13}C content assessed by mass spectrometry as described in below in section 2.2.3. Based on the results of this test, the mixing protocol was considered as reliable (see Appendix).

2.1.3: Sediment collection

For Experiment 1, 400 ml of mud were collected from the top 1-10 cm of sediment at Pauatahanui Inlet. This was done to ensure that the mud used for both experiments would be comparable to mud in the anemone's natural habitat. The mud was sieved to a grain-size of 80 µm at the site to remove any large particulate material and then returned to the lab and left to settle, for approximately 30 min. The water was then decanted and the sediment rinsed with FSW two more times to remove any remaining estuarine water. Each time the mud was rinsed, it was allowed to settle for a further 30 min. It was assumed that during the washing process some bacterial biomass would be lost, but that sufficient bacteria would remain to form a food source for the anemones.

For Experiment 2, a further 1000 ml of mud were collected at a different time. This mud was washed to remove estuarine water as described above, after which it was freeze-dried (Virtus Freeze Dryer, VWR/Global) and stored at -80° C, to ensure there was no metabolic activity of bacteria.

2.2: Experiment 1: Tracking of POC uptake by A. aureoradiata *and its fate in the symbiosis*

2.2.1: ^{13}C labelling

Sediment was enriched with ^{13}C glucose to trace its fate and movement in the symbiosis. Once a day for three days 1.005 g of ^{13}C labelled glucose was added (Cambridge Isotopes, Andover, USA) to 300 ml FSW. This ensured that the 300 ml FSW remained at a concentration of approximately 18 mM every day throughout the enrichment procedure, allowing bacteria to maintain a high level of enrichment. The 400 ml of mud were stirred continuously in this ^{13}C-enriched FSW *via* a magnetic bar stirrer, to keep all mud in suspension and thus facilitate homogenous ^{13}C-labelling.

After three days of labelling the mud was rinsed three times with FSW, using the same procedure as when the mud was initially rinsed after collection from the field. This was done to remove any unincorporated label and any waste products, such as ammonium, that had accumulated in the mud. The mud was then immediately freeze-dried to halt any further processing of the label and stored at -80°C until its use in Experiment 1.

2.2.2: ^{13}C uptake from sediment by anemones

This experiment was designed to test whether POC carbon would be taken up by anemones and their symbionts from the bacteria in the surrounding mud, and what effect irradiance had on this uptake. Anemones (6-7 mm oral disk diameter) were placed individually into 80 ml containers kept in a room with a constant air temperature of 19°C. Each container contained 10 g of dried mud and 40 ml FSW.

The containers were then placed on an orbital shaker at a speed that was just sufficient to keep the mud in suspension while not stressing the anemones too much (as evidenced by the full extension of their tentacles). The containers were held at an irradiance of 220 ± 10 μmol photons m^{-2} s^{-1} on a 12-h light/ 12-h dark cycle, or in the dark continuously (Fig. 2.3). The anemones remained unfed other than *via* the mud for the duration of the experiment. These treatments both allowed the anemones access to the mud surface and, in the case of the light treatment, to the light for photosynthesis. The dark treatment, by comparison, was more representative of the situation when the anemones are buried or in very turbid water.

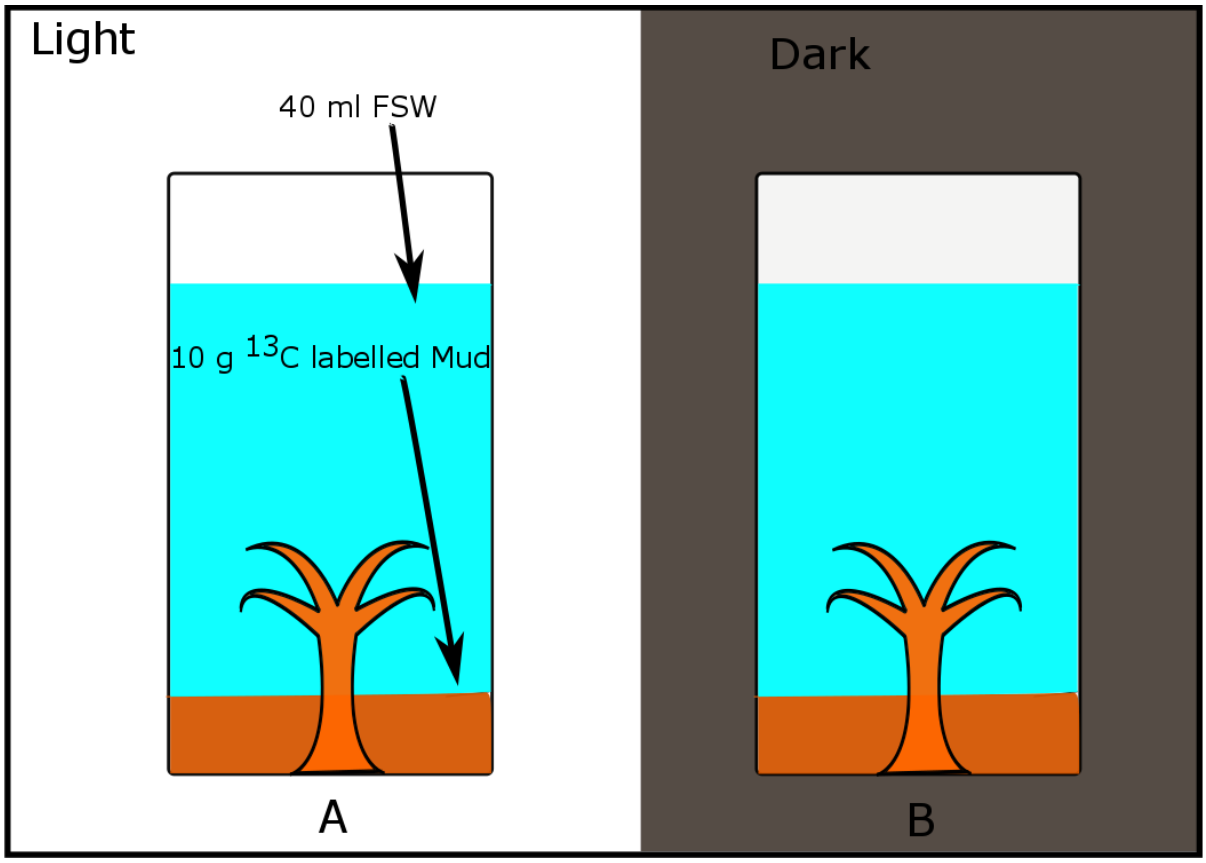

Figure 2.3: Diagrammatic representation of the two treatments in Experiment 1. Both contained 10 g of ^{13}C-labelled mud in 80-ml containers, and each contained 40 ml of 0.2-µm filtered seawater (FSW). (A) light treatment; (B) dark treatment. Diagram inspired by Morar et al. 2011.

Experiment 1 ran for 12 hours, during which time 2 ml of sediment suspension (stock: 5 g of sediment/ to 35 ml of FSW) were pipetted into each container each hour, to guarantee the availability of exogenous ^{13}C throughout. After 12 hours the anemones were removed from their containers, rinsed with FSW, placed in new containers filled with FSW and left to egest any undigested particles over the course of 12 hours, to ensure that all ^{13}C measured had been incorporated by the symbiosis.

Upon sampling, anemones were homogenised using a sonicator (Thermo-Fisher Scientific sonicator) in approximately 3 ml FSW and centrifuged at a speed of 400 × g at 5 °C for 5 min. The host fraction was then decanted into a separate tube and stored at −80 °C prior to further processing. The remaining symbiont pellet was resuspended with 1 ml of FSW and centrifuged the same as above. After this, FSW was removed from the pellet. This was repeated two more

times to remove any remaining host material. Symbiont pellets were then frozen at −80 °C. After this host and symbiont samples were freeze-dried, packed and sent to the National Institute of Water and Atmospheric Research (NIWA) for ^{13}C analysis as outlined below.

2.2.3: ^{13}C enrichment analysis

Once freeze dried samples were received by NIWA, they used subsamples of the powdered host and symbiont tissue samples to determine AT % (atom %. This means the amount of a specified atom (isotope) of a chemical element divided by the total amount of atoms of the element within the mixture, here 13C divided by the total carbon in the sample (Coplen 2011) values for each. Subsamples were processed through a Delta Plus continuous flow isotope ratio mass spectrometer (Thermo-Fisher Scientific, Bremen, Germany) linked to an NA1500 elemental analyser (Fisons, Italy) using an AS200 autosampler (Thermo-Fisher Scientific, Bremen, Germany).

These subsamples were combusted at 1020°C in a flow of oxygen and He carrier gas. Before being added to the mass spectrometer detector. N_2 and CO_2 gases were separated on a Porapak Q gas chromatograph column *via* an open split Conflo IV interface (Thermo-Finnigan, Bremen, Germany). To each subsample measured in the mass spectrometer, CO_2 was added as a reference gas standard. ISODAT (Thermo-Fisher Scientific) software was used to calculate $\delta^{13}C$ values against the CO_2 reference, relative to the National Bureau of Standards 19 – calcite (NBS19-calcite) standard (calibrated against Vienna Pee Dee Belemnite: VPDB), correcting for ^{17}O.

National Institute of Standards and Technology (NIST) standards USGS40 L-glutamic acid (C and N) and NIST8542 sucrose (C) (using 2 point normalisation) were used to normalise delta ^{13}C isotope values. A solid laboratory standard (DL-leucine (DL-2-amino-4-methylpentanoic acid, C6H13NO2, Lot 127H1084, Sigma, Australia) was used to calculate percent C values at the beginning of each run. NIST standards analysis was repeated on each run to produce data accurate to within 0.3 ‰ for $\delta^{13}C$ and a precision of >0.1 ‰ C. For % C content, data are accurate to within 0.4%, with a precision usually better than 0.2% for C (Leduc et al., 2015).

2.3: Experiment 2: Importance of POC from mud on symbiosis nutritional status and stability in A. aureoradiata

This experiment aimed to establish whether POC obtained from mud helps to maintain the nutritional status of the symbiotic algae and their population density in the host anemone, especially when darkness inhibits photosynthetic production. Furthermore, I tested whether increased light availability and hence photosynthesis led to a reduced uptake of POC from mud, as has been observed in reef corals (see Introduction).

2.3.1: Experimental setup

Anemones (6-8 mm oral disc diameter) were placed into unsealed 80 ml containers, containing 40 ml FSW, and maintained under the same light and thermal regime described for Experiment 1. To provide sufficient sample for the subsequent analyses without over-stocking each container with anemones, each replicate (n = 4 *per* treatment) consisted of 12 anemones divided equally across three containers (i.e. four anemones *per* container; see Fig. 2.2); these 12 anemones were then pooled for analysis.

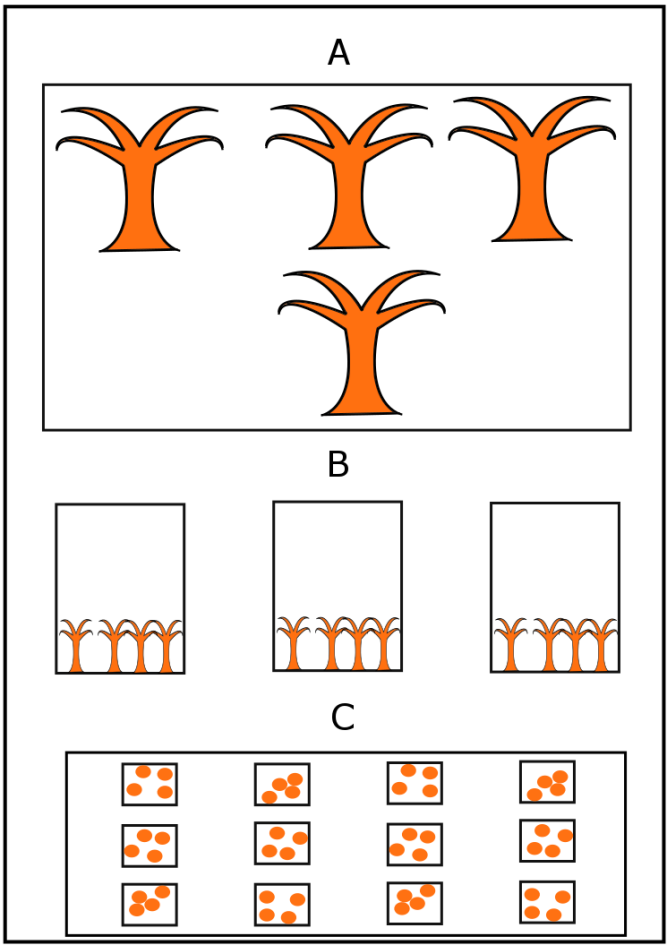

Fig. 2.4: Summary of replication design for Experiment 2. (A) shows the number of anemones *per* container (n = 4), (B) shows the number of containers *per* replicate (n = 3), and (C) shows the number of replicates *per* treatment (n = 4). Each replicate had 12 anemones across 3 containers and each treatment contained 4 replicates.

Unlabelled freeze-dried mud, prepared as described in Section 2.2.1, was then added to the containers to provide three different treatments: 'high mud' (20 g mud), low mud', (5 g mud), and 'no mud' (Fig. 2.5). Anemones remained in these treatments for 6 weeks and were unfed, other than *via* the availability of mud, for this period.

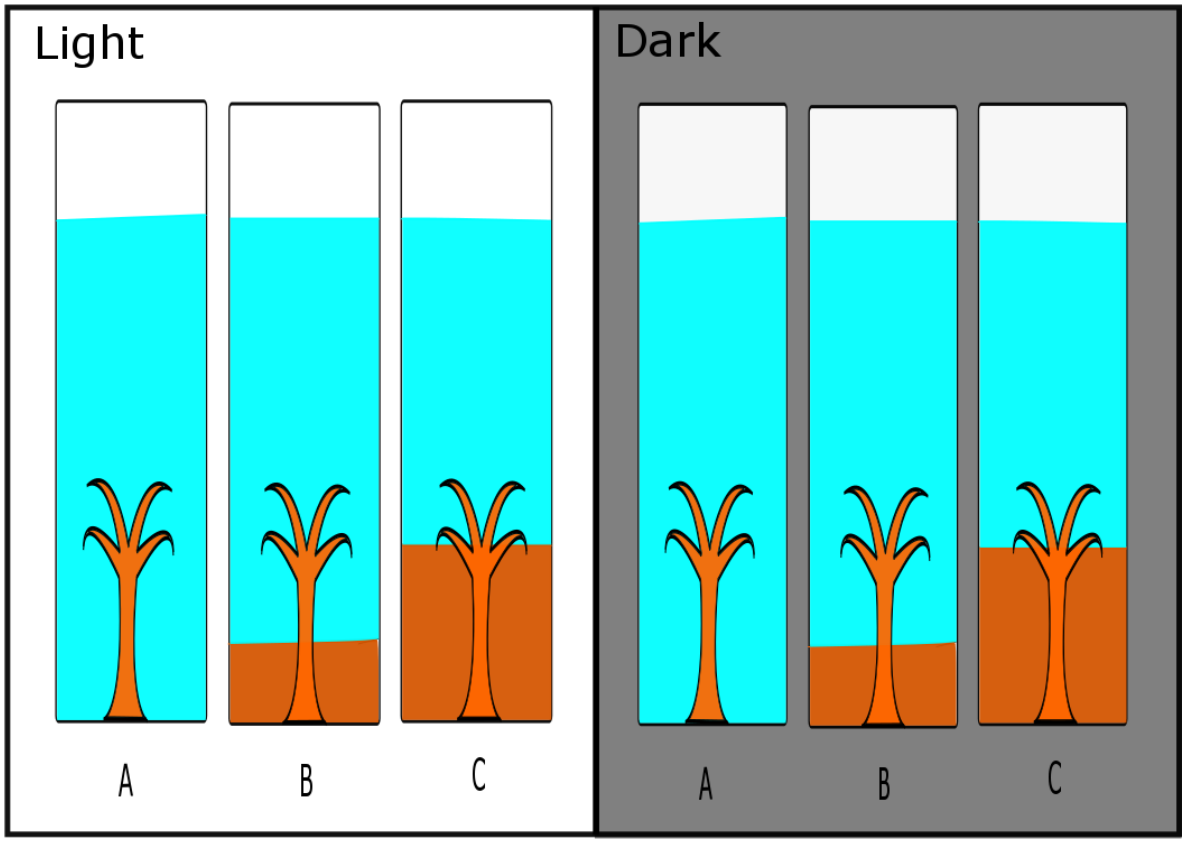

Figure 2.5: Diagrammatic representation of the different mud quantity treatment types in Experiment 2. A is no sediment, B is low sediment (5 g), and C is high sediment (20 g). Brown shading represents mud level and blue shading represents the 40 ml FSW added to each container. These treatments are either in the light or dark. Diagram inspired by Morar et al. 2011.

During the experiment, containers were cleaned of algal growth and FSW changed twice weekly, with the mud gently re-suspended each time. Furthermore, salinity was checked twice daily using a salinity meter and adjusted to approximately 30 ppt with distilled water if necessary, to prevent salinity stress. Container position was rotated each time FSW was changed (i.e. twice weekly) to allow for slight variability across the light field, while all handling of the dark anemones was conducted at very low light levels (< 1 μmol photons m^{-2} s^{-1}).

After the experiment, all anemones were removed from their containers, washed with FSW, and left to egest all undigested material for 12 h in new containers containing 40 ml FSW each. After this, anemones were pooled into their respective replicates (Figure 2.4) and homogenised using a sonicator. The host and symbiont fractions were separated and washed (symbiont only)

via centrifugation, as described in Section 2.3.3. A 50 µl aliquot of the host fraction was transferred into an Eppendorf tube for protein analysis, and the remainder retained for lipid analysis (see below); these samples were all stored at -80° C.

Symbiont pellets were re-suspended in 500 µl FSW and 200 µl of this suspension taken for symbiont cell counts; 20 µl of 100% formalin were added as a preservative to this cell count sample and the symbiont sample stored at 4° C. The remainder of the symbiont suspension was freeze-dried prior to analysing starch content (see below).

2.3.2: Lipid isolation and quantification

Tests for total lipid content were conducted to determine the effect that different light and mud levels had on anemones. The protocol was based on the standard approach of Bligh and Dryer (1959) and Kellogg and Patton (1983), and with modifications provided by the Munkacsi lab at Victoria University of Wellington.

Prior to lipid isolation, each empty Eppendorf tube was weighed separately, for future estimations of lipid dry weight in these tubes. Multiple tubes *per* replicate were used due to the large amount of anemones in each replicate in Experiment 2, with the anemone homogenate being evenly spread across these tubes.

To each tube, 216.5 µl of chloroform and 433.5 µl of methanol were added, and mixed thoroughly using a vortexer. After which, to each tube, 300 µl of chloroform and 200 µl KCL were added, and the tubes vortexed for 30 s after which they were chilled on ice for 1 min. This mixing was then repeated two more times.

After mixing, all tubes were centrifuged for 2 min at 13,000 rpm at 4°C to separate phases. The organic lower phase of each tube was transferred to a new tube, combing each replicate into one tube, and its volume measured. The volume of the non-organic upper phase of each tube were also measured, after replicates were combined, for subsequent normalisation of lipid

content. To ensure that no organic phase remained with the upper phase 400 µl of chloroform was added to the upper phase and the fractionalisation of the phases was repeated as above.

The combined organic fraction layer was vacuum dried (Eppendorf concentrator, 5301) and the tubes again weighed. These weights were subtracted from the weight of the empty tube to calculate the weight of the lipid in the aliquot. The below calculation was then performed, after which total lipid was standardised to each individual anemone and then to host protein content.

$$Total\ lipid\ (g) = \frac{Weight\ of\ lipid\ in\ aliquot\ \times Volume\ of\ chloroform\ layer}{Volume\ of\ aliquot}$$

2.3.3: Protein assays, cell counts, and starch analysis

The Bradford protein assay was conducted to determine the difference in protein content of host tissue across treatments, and to standardise cell counts and lipid content to host size. The Bradford assay was conducted as stated in the manufacturer's guide (Sigma- Aldrich, Saint Louis, USA). Cell counts were conducted using a haemocytometer (Improved Neubauer, deep 1/10 mm) at 125* magnification, with 10-16 cell counts *per* sample to ensure that variance was low.

For starch analysis, the symbiont fraction was dry weighed and analysed *via* the Amylase/Amyloglucosidase method, as stated in the manufacturer's guide with modifications (see below) (Starch Assay Kit, Amylase, Sigma-Aldrich, Saint Louis, USA).

Starch analysis

Sample preparation

Dry-weighed symbiont pellets were placed into individual tubes, and 5.0 ml of 80% ethanol were added to each and incubated for 5 min at 80-85°C. After this, tubes were mixed and another 5.0 ml of ethanol were added. The tubes were centrifuged for 10 min at 1,000 X g, after which the supernatant was discarded.

Once the ethanol was fully discarded, 10 ml of ethanol were added to each tube and the tubes were centrifuged again at the same speed and for the same timeframe. After this, the supernatant was again discarded. This procedure was conducted to remove any maltodextrins and glucose present in the symbiont cells before the remainder of this procedure breaks down starch, so that these products do not contribute to starch % calculations.

Starch digestion

After the sample preparation steps were completed, digestion of starch into detectable carbohydrates began. An 0.02 ml aliquot of 80% ethanol was added to each sample and to an empty tube labelled 'blank'. Each tube was then mixed thoroughly.

0.3 ml of water and 0.002 ml of α-amylase reagent were then added to each tube, and each tube was mixed and incubated in a boiling water bath (Julabo TW12) for 5 min, after which the tubes were removed and left to cool back to room temperature. The volume of each tube was then made up to 1 ml through the addition of filtered water.

0.1 ml from each tube was taken and placed in a new tube. To each of these new tubes, 0.1 ml of starch reagent was added and these tubes were placed again in the same water bath, now at a temperature of 60°C, for 15 min, mixing each tube every minute to allow for a thorough reaction to take place.

Glucose assay

The water bath was adjusted to 37°C. A standard blank (SB), standard (S), and a reagent blank (RB) were prepared. To SB, 0.1 ml of water was added; to S, 0.095 ml of deionised 0.22-um (FSW) was added along with 0.005 ml of glucose standard reagent; and to RB, 0.1 ml of liquid from the tube labelled 'blank' was added.

Once the water bath reached 37°C, tubes were placed into the water bath one at a time, one minute apart. Just before each tube was placed into the water bath, 0.2 ml of the glucose assay reagent was added to the tube. Each tube was kept in the water bath for exactly 29 min, after which each one was removed and had their reaction stopped by the addition of 0.4 ml 12 N sulphuric acid.

After this, 0.01 ml from each tube was added in triplicate to a 96-well plate (Cellstar cat-No. 655 180), and the plate was run through a plate reader (SCIMED plate reader) at 540 nm. The below calculation was then used to determine the percentage of starch present in the symbiont cells across treatments and replicates.

$$Starch\ \%\ in\ symbiont\ biomass = \frac{(\Delta_{ATest})(F)(V)(SF)(VGA)(MWF)(100)}{(Conversion\ Factor\ for\ \mu g\ to\ mg)(Sample\ Weight\ in\ mg)}$$

$\Delta A_{Standard} = A_{Standard} - A_{Standard\ blank}$

$\Delta A_{Test} = A_{Test} - A_{Reagent\ blank}$

VGA=

F = µg glucose in standard ÷ $A_{Standard}$ at 540 nm

V= Indicial sample volume (from starch preparation)

SF= Total assay volume from starch assay/sample volume from starch assay

SDF= Dilution factor from the end of the starch digestion

VGA= Initial Sample Volume from Glucose Assay

MWF= Molecular weight of starch monomer/Molecular weight of glucose= 162/180 = 0.9

2.4: Statistical analysis

All statistical analysis were conducted with R (version 3.3.1), with significant differences determined at the 5% level. Data was first checked to ensure that they were normality distributed (QQ-plots) and had equal variance (i.e. all Levene's test results were $p > 0.05$).

For Experiment 1, host tissue and symbiont enrichment across treatments were tested using one-way ANOVA, while each tested parameter in Experiment 2 (protein content, lipid content, percentage starch % in symbiont biomass, and zooxanthellar density) across treatments was compared using two-way ANOVA.

Furthermore, for Experiment 1, one-way ANOVA was used to compare AT % values across treatments for both host and symbiont. Due to the low variance found in unlabelled anemone tissues and zooxanthella cells, values for each were pooled across treatments and were then compared to the pooled enriched values for host and symbionts, respectively.

Lastly, for Experiment 2, two-way ANOVA was used to determine the effect of irradiance and mud quantity across treatments in terms of host protein content, host lipid content, starch % in symbiont biomass, and zooxanthellar density.

Chapter 3: Results

3.1.1: Preliminary data– evidence for incorporation of ^{13}C-glucose by mud microbes and optimisation of the labelling protocol

Preliminary tests showed that enrichment of mud increased gradually as the concentration of ^{13}C-glucose increased, but that host tissue and symbionts showed only a marginal increase in ^{13}C enrichment by comparison. As a result, the concentration of ^{13}C was tripled to 18 mM for Experiment 1. Label incorporation was not greatly affected by the modification to the egestion time, incubation time, or mud quantity (see Appendix, A1).

3.1.2: Experiment 1: Tracking of POC uptake by A. aureoradiata *and its fate in the symbiosis:*

When maintained in ^{13}C enriched mud, the host tissues of anemones were almost 2* more enriched than natural, unlabelled host tissues (one-way ANOVA: $F_{1, 22} = 51.83$, $p < 0.001$). Moreover, the host tissues of dark starved anemones were only 1.2* more enriched than those incubated in the light (one-way ANOVA: $F_{1, 18} = 16.269$, $p < 0.001$; Fig.3.1). In contrast symbiont cells were equally enriched regardless of irradiance level (one-way ANOVA: $F_{1, 18} = 1.211$, $p = 0.286$; Fig.3.1), but they were still greater than one fold more enriched than the natural, unlabelled symbionts (one-way ANOVA: $F_{1, 18} = 61.176$ $p < 0.001$).

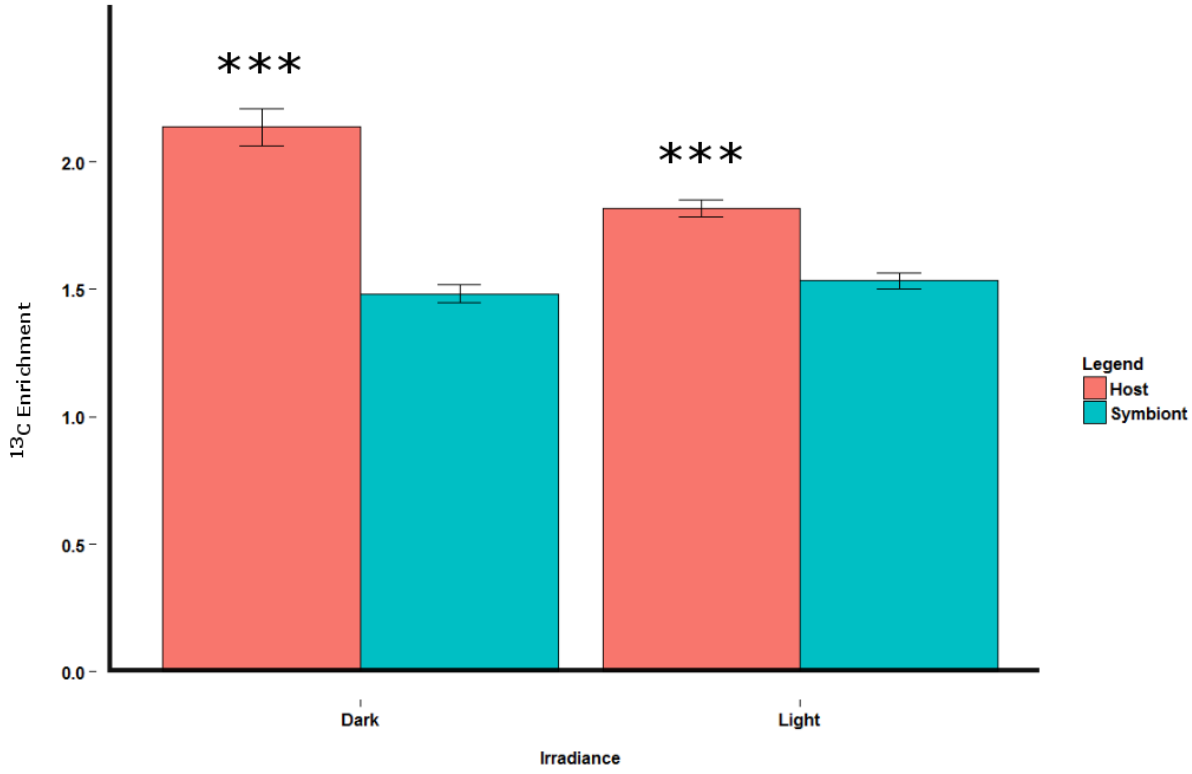

Figure 3.1: Uptake of ^{13}C-labelled particulate organic carbon from mud by the sea anemone *A. aureoradiata* and its symbionts, as shown by ^{13}C-enrichment of host tissue and symbionts (AT %). Values are means (values are means, n =10) ± SE. * indicates a significant effect with p <0.05, be that of irradiance, mud quantity or an interaction effect between them, ** indicates a significant effect or interaction of the above parameters with p <0.01, and *** indicates a significant effect or interaction of the above parameters with p <0.001.

3.2: Experiment 2: Importance of POC from mud on symbiosis nutritional status and stability in A. aureoradiata

3.2.1: Symbiont density

Symbiont density was much higher in the light than the dark, with anemones cultured in the light having a symbiont density that was >3.5 fold greater than that of anemones cultured in the dark (Two-way ANOVA: $F_{1,15} = 8.3984$, $p = 0.011$; Fig.3.2). Furthermore, while not statistically significant, anemones in the high mud x light had higher symbiont densities than anemones in any of the other treatments.

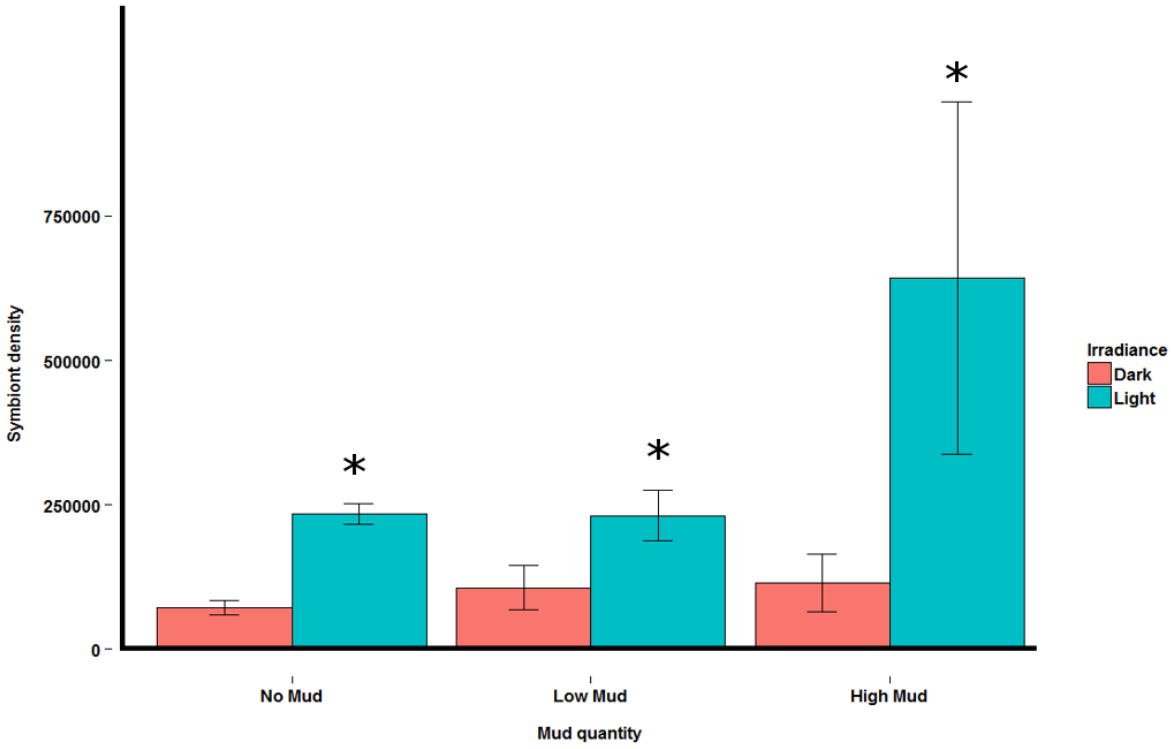

Figure 3.2: Density of zooxanthellae in *A. aureoradiata* (values are means, n = 4, ± SE), for the effects of mud quantity and irradiance, when incubated for 6 weeks with/without mud, in the light or the dark. * indicates a significant effect of mud or light on symbiont density or an interaction of these terms with $p < 0.05$, ** indicates a significant effect or interaction of the above terms with $p < 0.01$, and *** indicates a significant effect or interaction of the above terms with $p < 0.001$.

3.2.2: Host protein content

Host protein content was highest in the dark and was not affected by mud quantity across treatments (two-way ANOVA: $F_{1, 18} = 6.250$, $p < 0.05$; Fig. 3.3). The greatest disparity between light and dark protein content was in the high mud treatment, where host tissue contained ~1.2 fold more protein in the dark than the light.

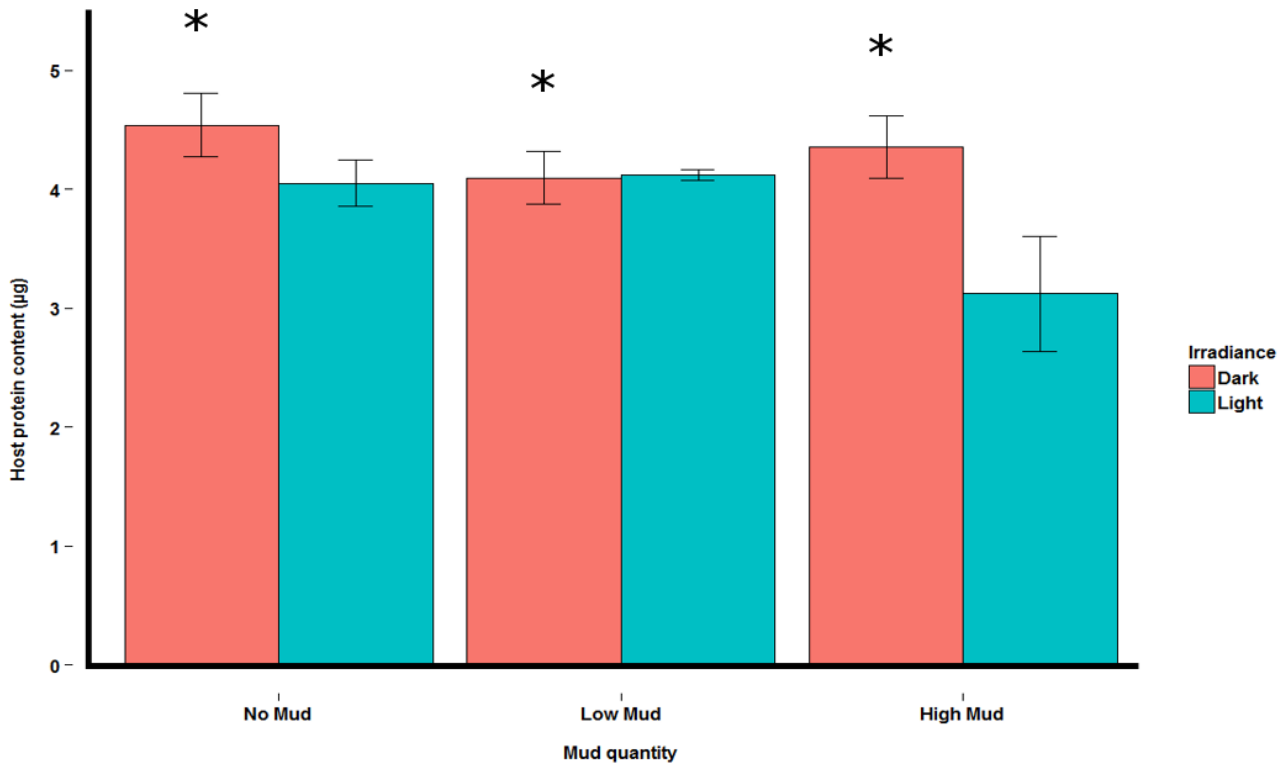

Figure 3.3: Protein content of *A. aureoradiata* cultured over 6 weeks with/without mud and in the light or the dark (values are means, n = 4, ± SE). * indicates a significant effect of mud quantity or light on host protein content or an interaction effect of the two with p <0.05, ** indicates a significant effect or interaction of the above parameters with p <0.01, and *** indicates a significant effect or interaction on the above parameters with p <0.001.

3.2.3: Host lipid and symbiont starch analysis

Lipid content of host tissue was unaffected by either irradiance or mud quantity (two-way ANOVA: $p > 0.1$; Fig. 3.4) though, like symbiont density, there did seem to be a non-significant effect of mud quantity in the high mud x light treatment, where anemones had the highest lipid content of any treatment. In comparison, % starch content of the symbionts was significantly affected by irradiance (two-way ANOVA: $F_{1, 16} = 5.933$, $p = 0.027$; Fig: 3.5). Percent starch values were >1.5 fold more in the light than the dark, irrespective of mud quantity treatment.

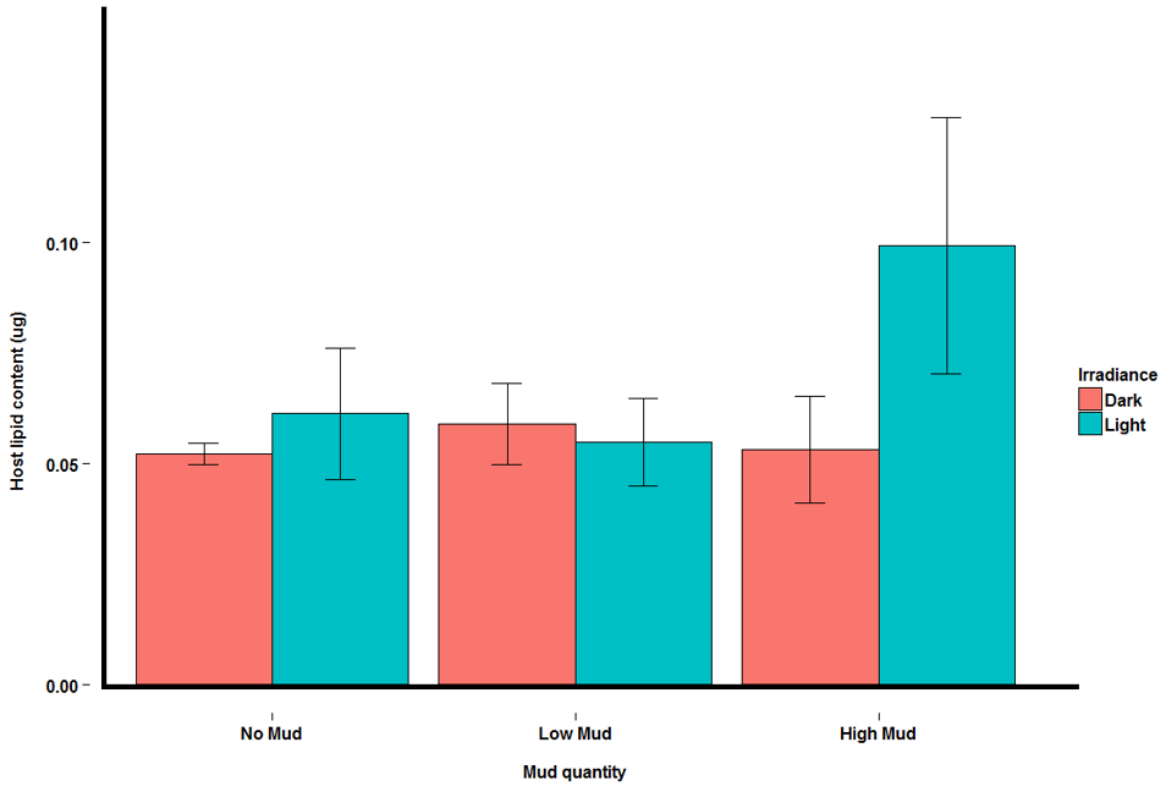

Figure 3.4: Lipid content of *A. aureoradiata* tissues when anemones were cultured for 6 weeks with/without mud and in the light or the dark (values are means, n = 4, ± SE). * indicates a significant effect of either light or mud quantity or an interaction of both with p <0.05, ** indicates a significant effect or interaction of the above parameters with p <0.01, and *** indicates a significant effect or interaction of the above parameters p <0.001.

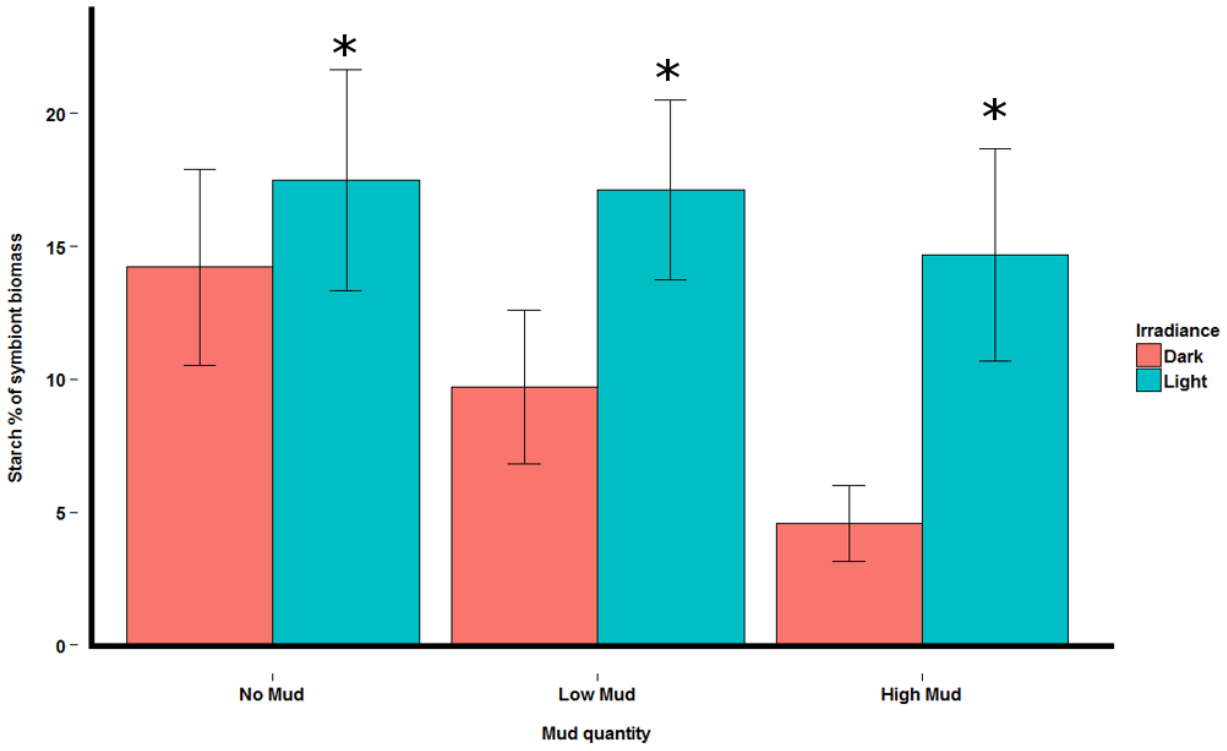

Figure 3.5: % starch of symbiont biomass in *A. aureoradiata* cultured over 6 weeks with/without mud and in the light or in the dark (values are means, n = 4, ±SE). * indicates a significant interaction with $p < 0.05$, ** indicates a significant interaction with $p < 0.01$, and *** indicates a significant interaction with $p < 0.001$.

Chapter 4: Discussion

4.1: Experiment 1: Tracking of POC uptake by Anthopleura aureoradiata *and its fate in the symbiosis*

4.1.1: Host Enrichment

This experiment aimed to determine if bacteria are heterotrophically taken up by *A. aureoradiata*, and where heterotrophically-derived carbon is moved and utilised within the symbiosis. It was expected that *A. aureoradiata*, both in the light and the dark, would incorporate carbon from mud bacteria, causing ^{13}C-enrichment of host tissues. However, it was expected that anemones maintained in the light would be less enriched due to increased translocation of photosynthate from their symbionts, and hence less need for feeding on exogenous carbon sources to support metabolism. ^{13}C enrichment was indeed greater in dark-treated anemones than their light-treated counterparts, supporting this hypothesis. Interestingly, however, symbiont enrichment in both light and dark was similar (~1.7 AT %), even though symbionts were clearly enriched above their background levels (~0.9 to 1 AT %); this pattern is discussed in more detail below.

Increased ^{13}C-enrichment in the dark in response to the reduced availability of photosynthate from symbionts has also been observed in other symbiotic cnidarians. For example, increased feeding on plankton has been reported during winter and spring months in temperate regions when light levels are sub-optimal (Ribes et al., 1999; Ferrier-Pagès et al., 2011), as well as during bleaching events in tropical corals (Houlbrèque & Ferrier-Pagès, 2009). This inverse relationship between symbiont photosynthesis and host feeding is not always apparent, however. For example, Hiebert & Bingham (2012) showed that symbiont presence had no effect on feeding in the temperate American anemone *Anthopleura elegantissima*.

Further research, identifying any qualitative differences between the carbon acquired from mud *versus* plankton *versus* photosynthesis, and tracking the fate of this carbon within the host and

overall symbiosis, will shed further light on the relative importance and use of these different carbon sources in *A. aureoradiata*.

4.1.2: Symbiont Enrichment

4.1.2.1: Prior Expectations on Symbiont Enrichment

It was expected that symbionts would be enriched with ^{13}C, especially in the light, due to photosynthetic incorporation. In the dark, carbon uptake from the host could still occur *via* dark fixation of $^{13}CO_2$ or 'reverse translocation' of organic carbon to the symbionts, though both of these processes are thought to be quantitatively minor in comparison to photosynthetic fixation and the translocation of photosynthetic products from symbiont to host (Falkowski and Raven, 2007; Davy et al., 2012). However, the data here show that, while the symbionts were enriched with ^{13}C relative to unlabelled background controls, there was no evidence of a light-driven effect (Fig: 4.1). Three possible explanations for the similar symbiont labelling in light and dark are presented here.

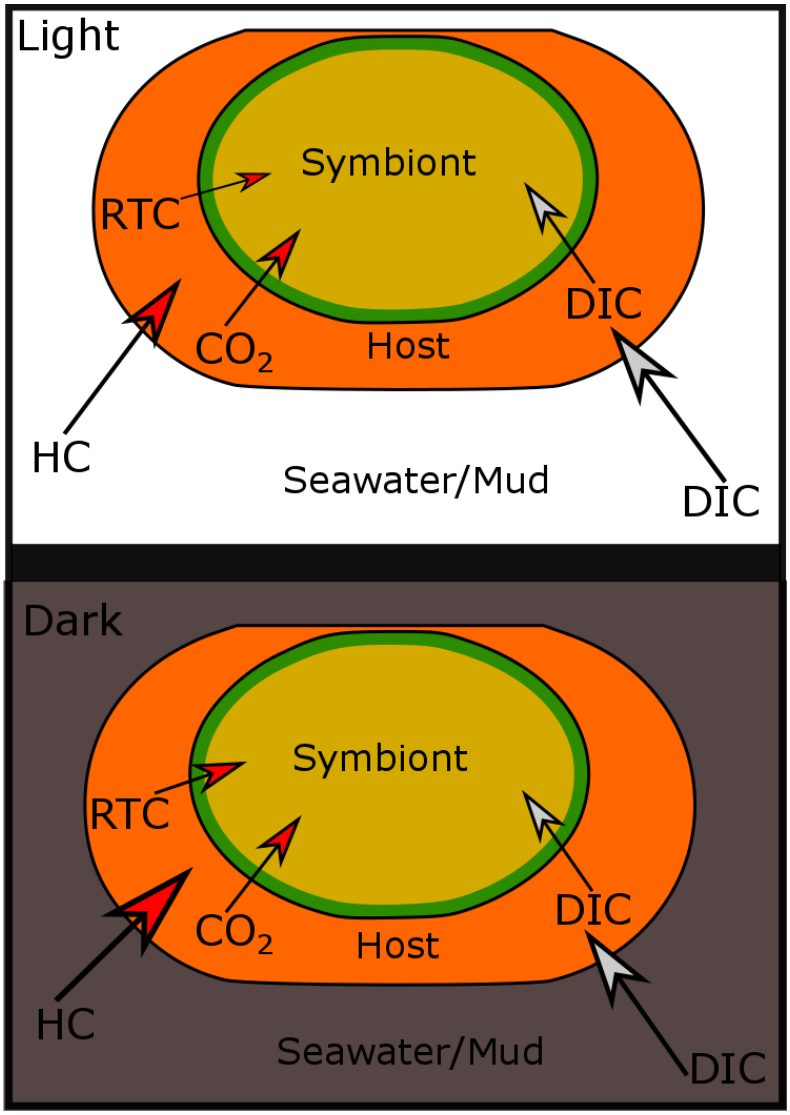

Figure 4.1: A diagrammatic representation of potential carbon flux from the ambient environment (seawater and/or Mud) to the host to the symbiont in Experiment 1. Heterotrophically-derived carbon (HC) is shown as red-tipped arrows, while exogenously-derived dissolved inorganic carbon (DIC) is shown as grey-tipped arrows. RTC = reverse translocated organic carbon from the host to the symbiont. Arrow size/weight indicates the relative proportion of products moved from the external seawater/ and or mud to the host and then to the symbiont.

4.1.2.2: Reverse translocation of organic carbon from host to symbiont

The symbionts may have taken up more organic carbon products from their host in the dark, so countering the reduced photosynthetic assimilation. Evidence for reverse translocation of products from host to symbiont is still understudied in comparison to the translocation of photosynthate from symbionts to their cnidarian hosts. What is known is that hosts provide nitrogen, phosphorus, and some CO_2 to their symbionts to allow photosynthesis to take place, and for symbiont maintenance and growth (Furla et al., 2005). For example, Piniak et al (2003)

showed that nitrogen moved from host to symbionts using ^{15}N-labelling. They found that in the coral *Oculina arbuscula*, the maximum proportion of the total heterotrophic nitrogen intake that was reverse translocated from host to symbiont was approximately 20%. Further evidence of reverse translocation of products from host to symbiont was found by Steen (1986), who fed ^{35}S-labelled prey to the sea anemone *Aiptasia pulchella* and estimated that approximately 2.8-6.4% of the symbionts' protein needs were supplied in this way. This author also provided indirect evidence of reverse translocation, with aposymbiotic anemones having a higher protein biomass and a lower rate of mortality than anemones that were colonised by symbionts but held in the dark. Lastly,^{14}C-enriched food was incorporated in the dark in symbionts (*Chlorella*) of the freshwater green hydra, consistent with the findings of the current study (Cook, 1972).

The potential uptake of organic carbon from the host by symbionts in the dark is perhaps consistent with a parasitic tendency in these algae, at least under sub-optimal conditions. Indeed, zooxanthellae are photosynthetic dinoflagellate protists and are closely related to many heterotrophic and parasitic dinoflagellate species (Lesser et al., 2013); the dinoflagellates as a whole are furthermore closely related to the apicomplexan parasites, which include *Plasmodium*, the protist that causes malaria (Davy et al., 2012). Evidence for parasitism by *Symbiodinium* has been suggested in many studies. For example, Starzak et al. (2014) showed that some *Symbiodinium* types elevate their host's respiratory rate to such an extent that it cannot be supported by the photosynthate provided by symbionts. Similarly, the giant clam *Strombus gigas*, which also houses *Symbiodinium*, showed signs of an energetic cost being imposed by its symbionts when the host reached adulthood. This included a possible draining of host nutrients heterotrophically by symbionts from their host (Banaszak et al., 2013). Such impacts on host resources may also have knock-on effects on host disease susceptibility and health, as shown in the Hawaiian coral *Acropora cytherea*, where colonies housing *Symbiodinium* clade A symbionts were less healthy and showed increased disease prevalence compared to those colonies harbouring *Symbiodinium* clade C Stat et al (2008). Indeed, it is interesting to note that *A. aureoradiata* also contains a type of *Symbiodinium* clade A (Howe, 2012). This clade is thought to have diverged from other *Symbiodinium* types early in their evolution and may have been the first to establish a symbiosis with cnidarians. Because of this, more parasitic, heterotrophic traits may still be partially present in at least some members of this clade (Pochon & Gates, 2010). This requires further examination in *A. aureoradiata*, however, as it is important to acknowledge that some members of clade A are in fact rather

beneficial to their hosts. For example, the sea anemone *Condylactis gigantea* were less likely to bleach during warmer or more variable temperature regimes when housing clade A than clade B, while translocating the same percentage of fixed carbon as clade B (Loram et al., 2007; Venn et al., 2008). Moreover, much greater information is needed about the mechanism and control of reverse translocation in the cnidarian-dinoflagellate symbiosis in general.

4.1.2.3: Host respiration and the uptake of inorganic carbon from the host

It is conceivable that *A. aureoradiata* could regulate the supply of CO_2 to its symbionts to facilitate the rate of dark fixation (Fig. 4.1). Dark carbon fixation occurs in all organisms, through β-carboxylation in the dark by enzymes such as phosphoenolpyruvate carboxykinase to produce intermediary products and allow for competing pathways to co-exist (Mortain-Bertrand et al., 1988; Falkowski & Raven 2007). Dark carbon fixation has been known to produce only small amounts of fixed carbon. For example, *Nitzschia turgiduloides* can produce 6.7, 8.3 and 12.7% of the carbon fixed *via* photosynthesis through dark fixation, while *Skeletonema costatum* in the dark produces nearly 12% of its photosynthetic rate (Mortain-Bertrand et al., 1988). Certainly, dissolved inorganic carbon (DIC) flux in the cnidarian-dinoflagellate symbiosis is an active process that utilises carbon concentrating mechanisms (CCMs) such as carbonic anhydrase and ion pumps to move CO_2 and bicarbonate to the algal cells, and thereby maintain a partial pressure of CO_2 for fixation (Leggat et al., 2002; Venn et al., 2008; Yellowlees et al., 2008; Davy et al., 2012).

The source of this DIC is two-fold. First, the metabolism of the organic carbon acquired heterotrophically by the host will produce respiratory CO_2. Second, carbon uptake can occur from the surrounding seawater, with the carbon being transported across several membranes to reach the algal cell (Fig: 4.1). The relative importance of these DIC sources is a little unclear, though Furla et al. (2000) proposed that it is the seawater, while Clayton & Lasker (1984) found that feeding (and hence likely enhanced host respiration) did not elevate the rate of symbiont photosynthesis. In contrast, Harland & Davies (1995) reported a strong link between the rate of host respiration and symbiont photosynthesis in the temperate sea anemone *Anemonia viridis*. If the majority of DIC taken up by the symbionts was originating from the surrounding seawater in my experiment, however, then it would mean that most of the carbon being incorporated into the symbionts was unlabelled. Moreover, whatever the source of the DIC,

dark carbon fixation is usually utilised to replenish intermediate products in energy pathways, or to allow two pathways that require the same or similar products to coexist, and usually only provides a small amount of fixed carbon products that only last for a short time (Falkowski & Raven, 2007). This further suggests that the amount of labelled, heterotrophically-derived carbon measured in the symbionts in the dark (and indeed the light) was small compared to the overall levels of fixation that likely occur in the light using DIC from other sources. Further work with labelled seawater sources will shed light on this matter, but in the case of being held in the dark, it could still be that some dark carbon fixation is better than none with respect to symbiont growth and survival.

4.1.2.4: Host respiration in the light versus *dark, and the availability of heterotrophically-derived carbon to the symbionts*

One final factor that could help to explain the equal levels of ^{13}C-enrichment of the symbionts in the light and dark is the differential rate of symbiont respiration under these two conditions. Typically, respiration of both host and symbiont is elevated in the light due to the increased availability of energy-rich photosynthate (i.e. respiratory substrate) and the production of O_2 in photosynthesis (Harland & Davies, 1995). If this also occurred in *A. aureoradiata* in the light, then the greater respiratory demands of the symbionts in particular could have led to a greater proportion of the available ^{13}C-labelled organic material being utilised. In doing so, this process may have caused loss of label from the symbionts as $^{13}CO_2$, and reduced the extent of ^{13}C-label found in the symbionts in the light to a level more comparable to that found in the dark.

The possible reasons for why the symbionts were equally labelled with ^{13}C in the dark and light are therefore complex, and could relate to enhanced uptake of organic carbon and/or enhanced assimilation of DIC by the symbionts in the dark, as well as a relatively greater loss of ^{13}C-labelled carbon through symbiont respiratory processes in the light. Given how little is currently known about the likes of reverse translocation, dark carbon fixation, and the links between photosynthesis and respiration in the cnidarian-dinoflagellate symbiosis, the relative importance of these various processes in generating the patterns seen in the current study cannot be determined at present, though this topic warrants further investigation.

4.2: Experiment 2: Importance of POC from mud on symbiosis nutritional status and stability in A. aureoradiata.

4.2.1: Experiment 2 hypotheses and outcomes

Experiment 2 was conducted to determine whether access to mud in the presence or absence of light affected the accumulation of storage products and/or symbiont density in *Anthopleura aureoradiata*. It was predicted that the host, when given ready access to mud in the light, would accumulate a higher concentration of lipid, and attain a higher protein content and symbiont density than in the other treatments, while the starch content of symbiont biomass would be highest in this high mud/light treatment.

In reality, there was significant, but slight, increase in the protein content of anemones in all dark treatments regardless of mud availability. Interestingly mud had no effect on zooxanthellar density. It was expected, prior to this study that higher mud quantity would correlate to increased host storage products and zooxanthellar density, as increased heterotrophy by the hosts would allow for more transported nitrogen to the symbionts. As nitrogen is required for the growth and reproduction of zooxanthellae, an increase in nitrogen would be expected to aid in symbiont proliferation. Alternatively, the expected increase in zooxanthellar density and starch content in the light compared to the dark was significant, with dark anemones having a lower zooxanthellar density and starch content than their illuminated counterparts. Though not significant, there was a trend towards greater mud availability being related to higher zooxanthellar densities. In comparison, host lipid content was relatively constant across treatments but lipid content did also show a non-significant trend towards being highest in the presence of mud plus light.

4.2.2: What is the effect of mud quantity on Anthopleura aureoradiata?

The effect, as stated of mud quantity on either the host or its symbionts appeared to be non-existent. There could be several reasons for this lack of an effect on host and symbiont storage products or symbiont density. Potentially, a reason that mud did not have an effect on host reserves such as protein and lipid content, could be that microbes in the mud were consumed

and exhausted relatively quickly in this experiment, after which the anemones would experience the same conditions as their 'no mud' counterparts in terms of exogenous nitrogen availability. If this is true, then the influx of nitrogen at the beginning of the experiment may have had little effect on long-term nitrogen stores.

Also if *A. aureoradiata* decreased its metabolism in response to starvation, then a reduced respiration rate could have led to an increased retention of storage products and a masking of the effect of 'no mud' in Experiment 2. Evidence exists for a response in metabolism in suboptimal conditions. For example, Muscatine et al. (1984) found that in the tropical coral *Stylophora pistillata*, when conditions are suboptimal such as at depth, this usually autotrophic species decreases its respiration rate which helps compensate for a decrease in translocated photosynthate from the symbionts. If *A. aureoradiata* reacted similarly to a lack of incoming energy, here bacterial mud, then the 'no mud' anemones would have experienced a decline in respiration rate, while those in mud would have had normal metabolic rates and most probably utilised incoming nitrogen and carbon from prey without accumulating storage products.

Another reason why the presence of mud did not significantly affect any metrics in this experiment could be that mud bacteria are only a minor part of the natural diet of *A. aureoradiata*. Indeed, there is typically a strong, positive link between food availability and host and symbiont biomass, but this was not seen here (Borell et al. 2008). A similar lack of an effect of mud bacteria on *A. aureoradiata* was shown by Morar et al. (2011). In this study, where the authors studied nitrogen sufficiency and flux in *A. aureoradiata*, mud appeared to have no significant effect on whether symbionts of *A. aureoradiata* were nitrogen sufficient or not. Indeed, nitrogen sufficiency of the symbionts was only affected when anemones were fed with shrimp. Given this, although mud bacteria are a source of organic carbon for both host and symbiont, as shown in Experiment 1, their importance to symbiosis nutrition may be limited.

Either of these reasons for why mud quantity had no significant effect in Experiment 2 could be correct. However, as there was a non-significant trend in zooxanthellar density of the highest symbiont densities being in the high mud and light treatment, there may have been an effect of

mud. Any such effect would have been masked by the low replicate number. With more replicates this could have been clarified, but for the purpose of this thesis, any such effect of mud will be assumed to be non-existent.

4.2.3: What was the effect of light on Anthopleura aureoradiata?

In comparison to the effect of mud availability on host and symbiont performance, light did affect several aspects of the symbiosis; namely protein content of the host, the starch composition of the symbionts, and zooxanthellar density. This was expected given that the symbionts are photosynthetic and that enhanced photosynthate release from the symbionts supports host growth, reproduction, and retention of pre-existing protein, i.e. nitrogen conservation (Fitt & Pardy, 1981; Dubinsky and Berman-Frank, 2001).

As stated the lack of an effect of mud quality or light presence on lipid stores in *A. aureoradiata* is unexpected. However, this results could be due to the naturally large quantity of storage products found in temperate symbiotic cnidarians. In Experiment 2, anemones and their symbionts may have had such large stores of lipid, utilised to survive in a relatively healthy state over the long-term, especially if dark cultured anemones decreased their metabolism due to lowered respiration in the suboptimal treatments of low mud and no light. Such a lack of change in long-term storage products and zooxanthellar density have been shown in other temperate symbiotic cnidarians. For example, Davy et al (2006) showed that the temperate cnidarian *Plesiastrea versipora* was slow to reach a state of nitrogen deficiency when compared to its tropical counterparts, with *P. versipora* not showing nitrogen deficiency in its symbionts until after four-weeks of starvation, compared to one-week in some tropical cnidarians. Though this is for symbionts, not the host, this could still be telling of a more general trend towards higher storage reserves in temperate cnidarians than tropical ones.

Along with temperate cnidarians taking a much longer time to become nitrogen deficient than their tropical counterparts, temperate symbiotic dinoflagellates are also thought to be naturally nitrogen sufficient. This is probably due to greater DIN and DON availability in temperate waters (Roberts et al., 2001; Davy et al., 2006). Because of this, temperate symbionts may have more nitrogen to draw upon internally than do the symbionts of tropical cnidarians. This could

have caused any effect of starvation or low mud supply on symbionts to be less apparent in the current study. Furthermore, this could help to explain why there was a trend towards anemones in the high mud treatment containing more storage products.

This lack of an effect of irradiance on the host lipid content is particularly unusual when compared with another closely related cnidarian species, *Anthopleura elegantissima*. Fitt & Pardy (1981) showed that aposymbiotic *A. elegantissima* immediately utilised lipid reserves when starved, while those anemones with symbionts used photosynthate translocated from their symbionts for up to a month before having to begin using their reserves for survival. Why lipid reserves were apparently unaffected in my study by the availability of light while they were affected by the presence/absence of phototrophic symbionts in this closely related species is unknown, but it could be that *A. aureoradiata* reduced its metabolic rate in the dark to conserve its lipid stores.

Higher host protein content in the dark than the light was unexpected. It was hypothesised prior to Experiment 2, that light cultured anemones would have the higher protein biomass. Indeed, the opposite trend would seem more intuitive given the positive links between symbiont photosynthesis, carbon translocation and host biomass (Muscatine & Falkowski, 1984; Falkowski & Raven, 2001). One possibility is that the symbionts are a greater drain on the nitrogenous resources (e.g. proteins) available in the host when in the light than in the dark, which they then use to support their own growth; this could be linked to the assimilation of ammonium (a by-product of protein catabolism) by the symbionts being a light-driven process (Fig. 4.2), as well as there being a lower density of symbionts in the dark. However, such a scenario is inconsistent with the nitrogen conservation hypothesis, which suggests that the provision of energy-rich respiratory substrates (i.e. photosynthate) by the symbionts to the host reduces the rate of protein and amino acid catabolism by the host (Wang & Douglas 1998).

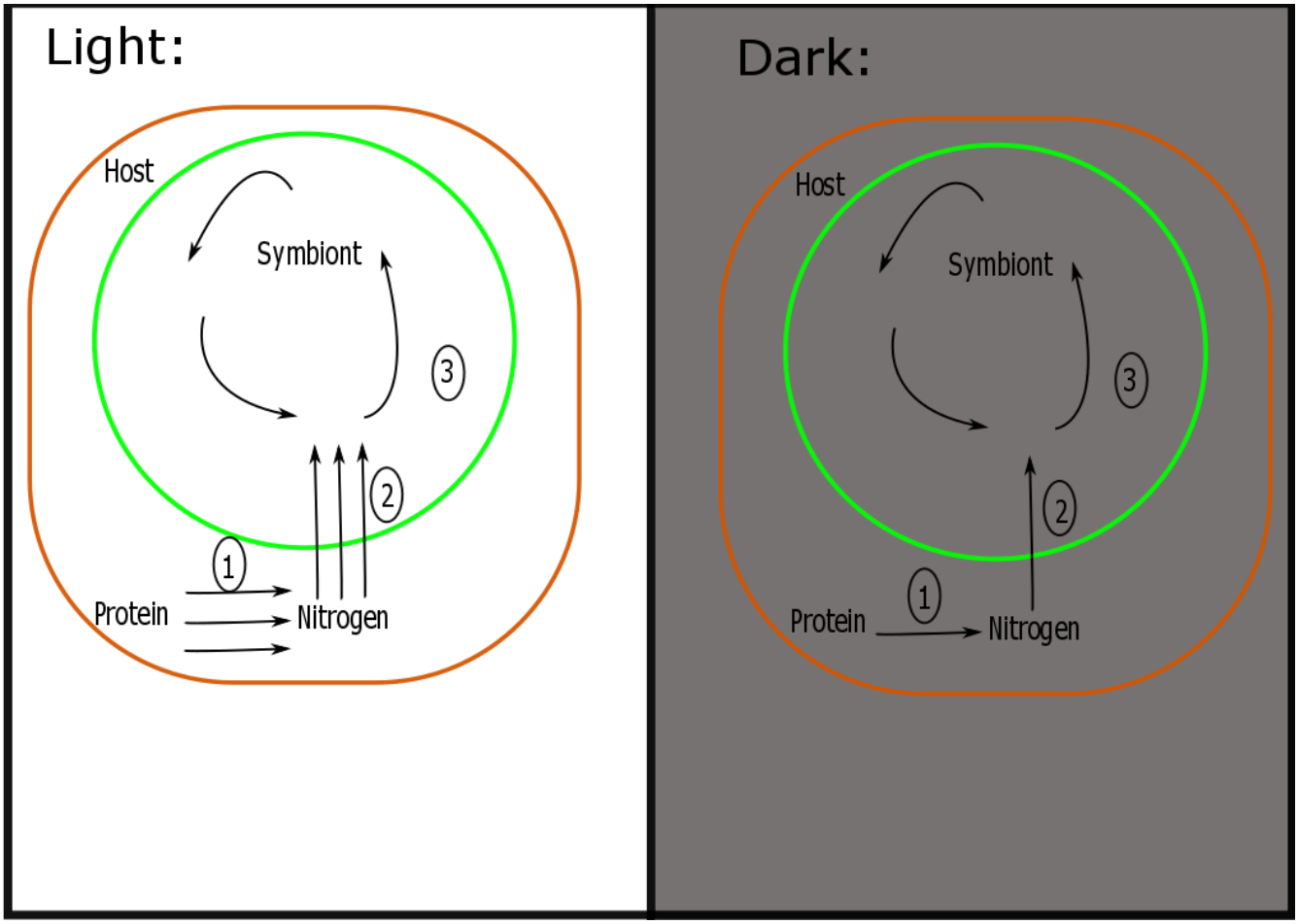

Figure 4.2: A diagrammatic representation of potential nitrogen flux from protein sources in the host to symbionts in *A. aureoradiata* in the light and dark. 1: Protein degradation into nitrogenous compounds such as amino acids and ammonium; 2: flux of these nitrogenous compounds to the symbiont, which is stronger in the light; and finally 3: use of the nitrogen within the symbiont for maintaining function and cell growth. The number of arrows represents the relative strength of the flux from host to the symbiont.

As dark cultured symbiont density was lower than those in the light it would be expected that symbionts must cause some type of an energetic burden to their hosts. Evidence for the energetic burden of zooxanthellae on their host has long been suggested to exist in suboptimal photosynthetic conditions. For example, Steen (1986) found that when symbiotic individuals of the tropical sea anemone *Aiptasia pulchella* was starved in the dark, the metabolic burden imposed by the symbionts causing a reduction in host protein content; aposymbiotic anemones, by comparison, did not show this loss of biomass. In response to this metabolic cost, hosts in the field may adjust their symbiont population. For instance, Muscatine et al. (1984) suggested that a decrease in zooxanthellar density with depth in the coral *S. pistillata* is due to an increasing energetic burden as the amount of photosynthate translocated from the symbionts

declines, while in the temperate coral *Astrangia poculata*, a decline in symbiont density and photosynthetic pigmentation, from summer to winter could ease the energetic burden when rates of photosynthesis are low (Dimond & Carrington, 2007, 2008).

Reduction in zooxanthellar density in response to starvation has been seen in other species. For example, Titlyanov et al (1996) studied several hermatypic coral species and found that, under natural conditions, corals degraded and removed zooxanthellae on a daily basis, as well as released healthy zooxanthellae. Degradation and expulsion were approximately equal to the rate of symbiont reproduction. Under starvation, however, it was found that these corals greatly increased their rates of degradation and release of symbionts. Another study by Titlyanov et al (2000) also showed a similar response, with symbiont density in hermatypic corals decreasing by 50% after 40 days of starvation. Along with this, photosynthetic rates decreased and symbiont degradation by hosts increased. Titlyanov et al. (1996) also showed that cell division almost stopped after long periods of starvation, most probably due to a lack of nutrients being transported from the host. This most likely occurred in *A. aureoradiata* as well, helping to explain the lowered symbiont density in the dark, irrespective of the availability of mud. If so, then *in hospite* degradation of symbionts could have also helped to maintain host protein biomass in the dark, as the symbionts would themselves have been a source of nitrogen.

Reduced symbiont density in dark anemones is, therefore, most probably due to a lack of light leading to less autotrophically fixed carbon, plus less nitrogen being translocated from the hosts. The anemones may have utilised one or more mechanisms to remove their symbionts and hence reduce the energetic burden. These include various cell death processes such as apoptosis, autophagy and/or cell necrosis (Dunn et al., 2002; Dunn et al., 2007; Dunn &Weis 2009; Fig. 4.3). Evidence also exists that at least some cnidarians may digest some of their symbionts targeted for removal (Titlyanov et al., 1996). Combined with their lower energy input, this would explain why anemones in the dark housed much lower levels of symbionts than those in the light. Whether these mechanisms are operating in *A. aureoradiata* and what their relative importance is, however, requires further investigation.

Symbiont starch reserves were significantly different between irradiance treatments, with symbionts accumulating the greatest amount of starch in the light. This is most probably due to any surplus fixed carbon from photosynthesis that was not used for cell growth and division, or translocated to the host, being stored as starch (Muller-Parker et al., 1996). It is also worth noting that nitrogen deficiency may cause starch storage to increase, as reduced symbiont growth and division increases carbon availability for storage. There is evidence for this in other free-living algae such as *Chlorella vulgaris* (Dragone et al., 2011) and *Scenedesmus obliquus* (Ho et al., 2012), as well as symbiotic dinoflagellates (Muller-Parker et al., 1996). Therefore, while the nitrogen status of the algae in *A. aureoradiata* was not measured here, if the mud was not acting as a major food source for the anemones, then nitrogen limitation in response to starvation or low food supply could also have contributed to symbiont starch accumulation both in the light and dark. Unfortunately starch content was not measured at the start of the experiment, however, so this potential effect cannot be confirmed from my data.

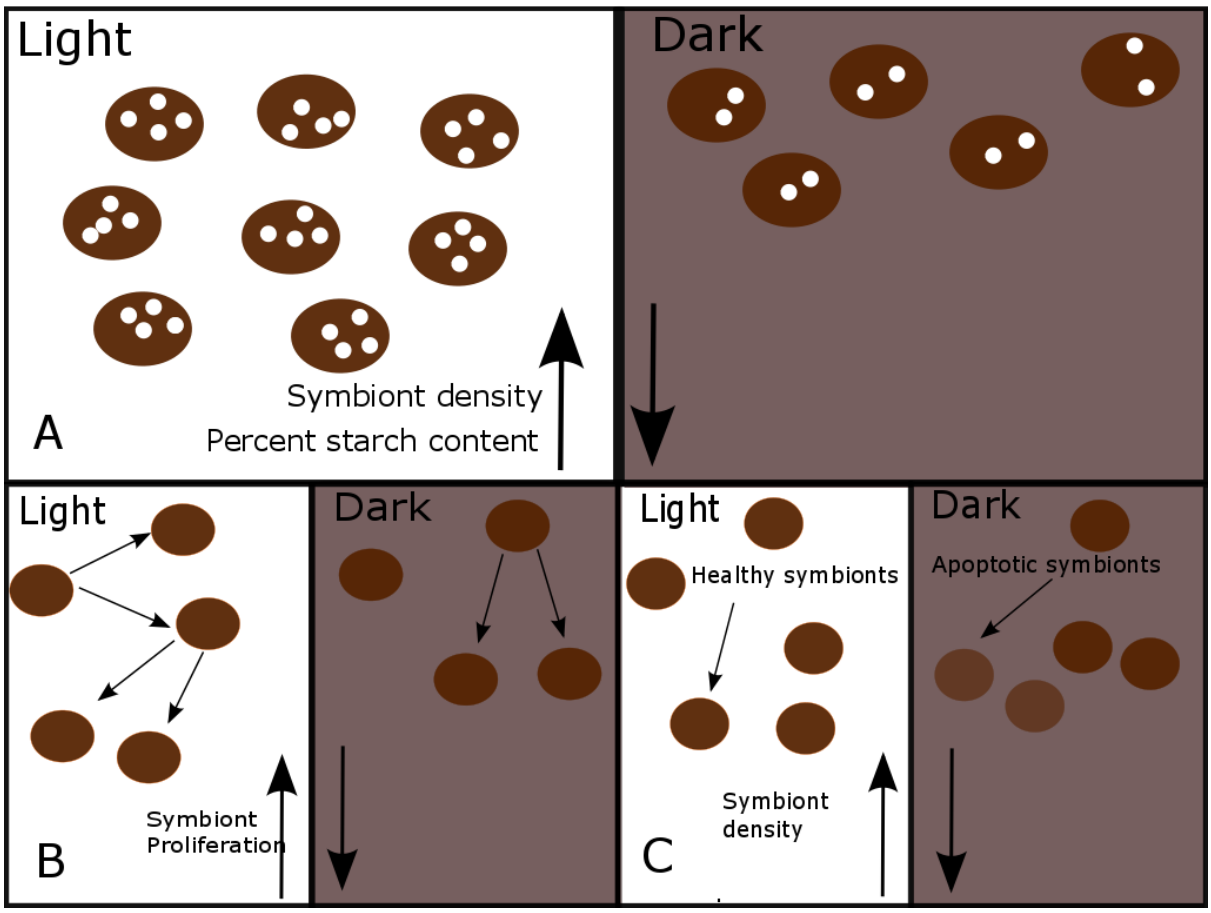

Figure 4.3: (A) A diagrammatic representation of zooxanthellar densities and percent symbiont starch content in the light and dark. Brown circles represent symbionts and white circles are stylised representations of starch bodies

within the symbiont cell. (B and C): two potential mechanisms for controlling zooxanthellar density in *A. aureoradiata*. (B) A model of increased or steady proliferation of symbionts in the light compared to a much lower level of symbiont proliferation in the dark. (C) Removal of symbionts *via* programmed cell death, autophagy and/or cell necrosis in the host to reduce symbiont abundance in the dark.

4.3: Conclusion

In summary, the purpose of this thesis was to determine what effect mud availability has on New Zealand's only native symbiotic anemone, *A. aureoradiata*, when starved under differing mud and irradiance regimes. In particular, I established whether organic carbon could be assimilated from the mud and where in the symbiosis (i.e. host and/or symbiont) this carbon ultimately resided. Consistent with the original hypotheses, I revealed that *A. aureoradiata* can assimilate organic carbon from mud, that this carbon is partially transferred to the symbionts, and that the host takes up more exogenous carbon in the dark than the light to counter the reduced availability of photosynthetically-fixed carbon. However, contrary to expectations, this carbon uptake is not of major value to the symbiosis with respect to its nutrition and stability. Further work is therefore needed to establish how this symbiosis maintains its remarkable stability under such apparently sub-optimal, low-light conditions.

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Appendices

Appendix 1: Preliminary tests for optimisation of ^{13}C-glucose labelling

Justification and methods

Prior to the experimental phase of this project, several preliminary tests were conducted to optimise the ^{13}C-labelling approach. In particular, incubation time, egestion time, mud quantity, and glucose label concentration were optimised (Table 1). These preliminary tests were conducted across a period of several months.

Table 1: Enrichment results on host tissue, symbiont, and mud bacteria, utilising ^{13}C-labelled glucose.

Experiment	mM addition	Incubation (hours)	Egestion (hours)	Sample type	AT% 13C/12C
1	1.5	6	18	Soil 5 g	2.68
				Host	1.17
				Symbiont	1.2
2	1.5	8	12	Soil 5 g	3.6
				Host	1.11
				Symbiont	1.14
3	1.5	12	12	Soil 5 g	3.07
				Host	1.11
				Symbiont	1.14
4	1.5	12	12	Soil 5 g	2.89
				Host	1.07
				Symbiont	1.10
5	2	12	12	Soil 5 g	2.78
				Host	1.12
				Symbiont	1.16
6	6	6	12	Soil 5 g	7.23
				Host	1.19

				Symbiont	1.25
				Soil 20 g	7.23
				Host	1.19
7	6	6	12	Symbiont	1.25
8	6	6	12	Mean mud value	3.86 +/- 0.68 SD

All preliminary tests were conducted as described in section 2.1, 2.2 and 2.3, unless otherwise stated. Mud was acidified with HCL, using NIWA protocol, prior to processing by NIWA. To determine the optimal label concentration and duration of label exposure, differing concentrations of ^{13}C-glucose were tested (1.5, 2, and 6 mM) on different dates, with exposure for 6, 8 or 12 hours. These times were based on the 12-hour timeframe used by Morar et al. (2012) for assessing ^{15}N flux through *A. aureoradiata*.

Egestion times tested were either 12 or 18 hours, with these tests again being run on separate dates.

Mud quantity for the ^{13}C labelling experiment was optimised by testing label incorporation when anemones were incubated in 5 or 20 g of mud.

Through all preliminary tests, shaker table settings and mixing procedures were modified and calibrated to ensure adequate mixing during experiments.

Results

Preliminary tests showed that enrichment of mud increased steadily as the concentration of ^{13}C-glucose increased, but that the host tissue and symbionts showed only a marginal increase in ^{13}C enrichment by comparison. As a result, the concentration of ^{13}C was tripled to 18 mM for Experiment 1. This was done to obtain adequate enrichment of host tissues, without the need to test ^{13}C labelling at higher concentrations than 6 mM given that this concentration was already close to the maximum amount tolerable by the mass spectrometer. Furthermore, homogeneity of labelling was confirmed by sub-sampling 6, 1 g mud samples, prior to mass spectrometric analysis.

Label incorporation was not affected by the modifications to the egestion time, incubation time, or mud quantity. Due to this, the 12-hour ingestion and 12-hour egestion timeframe, and a mud quantity of 10 g were used.

Appendix 2: Statistical tables

All statistical tables generated for this thesis across both experiments are detailed below. Df = degrees of freedom, Sum Sq = sum of squares values, and Mean Sq = the mean of squares. * indicates a significant value that is <0.05, ** indicates a value that is significant to <0.01, and *** indicates a value that is significant to <0.001.

Table 2: Host enrichment, light *VS* dark

Response of host enrichment to irradiance

	Df	Sum Sq	Mean Sq	F-value	*p*-value
Irradiance:	1	0.51200	0.51200	16.269	0.0007795***
Residuals:	18	0.56648	0.03147		

Table 3: Host enrichment, labelled *VS* unlabelled

Response of host enrichment to irradiance

	Df	Sum Sq	Mean Sq	F-value	*p*-value
Enrichment:	1	2.5433	2.54334	51.83	3.246e-07***
Residuals:	22	1.0796	0.04907		

Table 4: Symbiont enrichment, Light *VS* dark

Response of symbiont enrichment to irradiance

	Df	Sum Sq	Mean Sq	F-value	*p*-value
Irradiance:	1	0.013005	0.013005	1.2106	0.2857
Residuals:	18	0.193370	0.010743		

Table 5: Symbiont enrichment, labelled *VS* unlabelled

Response of symbiont enrichment to irradiance

	Df	Sum Sq	Mean Sq	F-value	*p*-value
Enrichment:	1	0.57408	0.57408	61.176	8.568e-08***
Residuals:	22	0.20645	0.00938		

Table 6: Host protein content

Response of host protein content to mud quantity, irradiance and their interaction effect

	Df	Sum Sq	Mean Sq	F-value	*p*-value
Mud quantity:	2	1.2781	0.63904	2.0733	0.1548
Irradiance:	1	1.9265	1.92646	6.2504	0.0223*
Mud quantity*Irradiance:	2	1.5804	0.79018	2.5637	0.1048
Residuals:	18	5.5479	0.30822		

Table 7: Zooxanthellar density

Response of zooxanthellae density to mud quantity, irradiance and their interaction effect

	Df	Sum Sq	Mean Sq	F-value	*p*-value
Mud quantity:	2	2.1164e+11	1.0582e+11	2.6177	0.10588
Irradiance:	1	3.3950e+11	3.3950e+11	8.3984	0.01104*
Mud quantity*Irradiance:	2	1.5867e+11	7.9337e+10	1.9626	0.17494
Residuals:	15	6.0637e+11	4.0425e+10		

Table 8: Host lipid content

Response of host lipid content to mud quantity, irradiance and their interaction effect

	Df	Sum Sq	Mean Sq	F-value	*p*-value
Mud quantity:	2	0.0020019	0.00100093	1.0740	0.3625
Irradiance:	1	0.0017508	0.00175079	1.8786	0.1873
Mud quantity*Irradiance:	2	0.0027158	0.00135791	1.4571	0.2591
Residuals:	18	0.0167750	0.00093194		

Table 9: Symbiont starch content

Response of symbiont starch content to mud quantity, irradiance and their interaction effect

	Df	Sum Sq	Mean Sq	F-value	*p*-value
Mud quantity:	2	98.03	49.014	1.1087	0.35406
Irradiance:	1	262.30	262.297	5.9331	0.02693*
Mud quantity*Irradiance:	2	40.77	20.385	0.4611	0.63871
Residuals:	16	707.35	44.209		