The control of carbon translocation in a sea anemone-dinoflagellate symbiosis from New Zealand

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Abstract

Anthopleura aureoradiata, a common sea anemone of New Zealand's intertidal mudflats and rocky shores, hosts symbiotic dinoflagellates of the genus *Symbiodinium*. This study investigated the control of photosynthetic carbon translocation in this symbiosis, and in particular the presence and operation of so-called 'host release factor' (HRF). Evidence for HRF exists in a number other algal-invertebrate symbioses, where tissue extracts of the host stimulate carbon release by isolated algal symbionts. However, its identity remains elusive and it has never been studied before in *A. aureoradiata*.

Translocation of photosynthetically-fixed carbon in the intact symbiosis and in the presence of host tissue extract was measured using a ^{14}C label. Zooxanthellae in the intact symbiosis released around 40% of their photosynthetically-fixed carbon to the anemone. Isolated zooxanthellae, however, translocated only 8%, even less than the amount of photosynthate liberated by zooxanthellae in FSW alone (11%). Photosynthetic rates per algal cell were similar in the intact symbiosis and both host homogenate and FSW incubations, meaning that the total amount of photosynthetically-fixed carbon released (in pg C/cell/h) by the zooxanthellae in these different situations reflected the %translocation values.

Given the failure of homologous zooxanthellae (i.e. those from *A. aureoradiata*) to respond to homogenized host tissue, it was tested whether zooxanthellae from other host species (i.e. cultured heterologous algae) responded. Heterologous zooxanthellae representing 5 clades (A-E) of *Symbiodinium* were incubated in host tissue homogenate and photosynthate release again measured with ^{14}C . The %translocation varied from 12-51% in *A. aureoradiata* homogenate and 17-67% in FSW, again suggesting a lack of an active HRF in the homogenized tissues of this sea anemone. Photosynthetic rates amongst the different heterologous algae also varied widely with, for instance, freshly isolated zooxanthellae from *A. aureoradiata* having 6-fold higher photosynthetic rates than cultured algae from the same clade (clade A).

The zooxanthellae of *A. aureoradiata* are known to be N-sufficient in the field, and studies with other species have demonstrated that N-deficient zooxanthellae release more photosynthate in response to HRF than do N-sufficient ones. Therefore,

induction of an HRF effect was attempted by starving sea anemones, and hence their zooxanthellae, prior to incubation of freshly isolated zooxanthellae in homogenized tissue. However, even after 8 weeks of starvation, the zooxanthellae showed no signs of N-deficiency (as indicated by the extent to which ammonium enhanced the rate of dark ^{14}C fixation), meaning that the relationship with HRF activity could not be examined. The ability of these temperate zooxanthellae to maintain their N-sufficiency, even after relatively long periods of food deprivation, may indicate a lower reliance on host feeding for nitrogen than is seen in tropical zooxanthellae, or a greater capacity to use internal stores of nitrogen.

The lack of photosynthate release by both homologous and heterologous zooxanthellae in host homogenate, as opposed to substantial carbon released in the intact symbiosis, suggests that control of carbon translocation in *A. aureoradiata* is not related to the activity of an HRF; alternatively, if an HRF is present, its activity is hindered when the symbiosis is disrupted. Further study is needed to determine what is responsible for the control of photosynthate translocation in the *A. aureoradiata*-*Symbiodinium* symbiosis.

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Introduction

1.1 - Symbiosis

Symbiosis was first defined in 1879 by Anton de Bary, who described it as the ‘living together of unlike organisms’ (Douglas and Smith 1987). Originally discovered in plants, symbioses have now been found to be an essential part of the biosphere. Examples of symbioses have been found in nearly every type of environment from the deep seas to volcanic cones and everywhere in between. The smaller partner in the symbiosis is usually referred to as the ‘symbiont’ and the larger organism as the ‘host’, but these distinctions are not always clear. The resulting partnership is known as the holobiont (Castro and Huber 2005). Most symbioses can be placed into four broad categories: commensalism, a relationship that is beneficial to one party without affecting the second party; ammensalism, when one party is unaffected by the presence of the other, but there are deleterious effects on the second party; parasitism, in which one party gains and the other party is negatively affected; and mutualism, a relationship in which both parties benefit (Douglas and Smith 1987). Symbiotic relationships can occur both intracellularly (within the host cells) or extracellularly (outside the host cells); they can also be defined as an ectosymbiosis (occurring outside the host) or an endosymbiosis (occurring within the host) (Douglas and Smith 1987). Symbioses are additionally defined as being either facultative symbioses, in which both partners can survive without the other, or obligate symbioses, in which one or both partners depend on the other for survival (Stachowicz 2001).

1.2 - Invertebrate-Algal Symbioses

Symbioses between invertebrates and unicellular algae have been recognized in the marine environment since the 19th century. Named ‘zooxanthellae’ by Brandt in 1881, some of these algae were defined as dinoflagellates after further work by Klebs (1884), Brandt (1885), Chatton (1923), and Hovasse (1924) (cited in Coffroth and Santos 2005). One of the most well-known invertebrate-dinoflagellate symbioses in the marine environment is that which occurs in coral reef ecosystems. Cnidarian corals form a symbiotic relationship with photosynthetic dinoflagellates of the genus *Symbiodinium*. This intracellular symbiosis is an obligate mutualism in which the dinoflagellates photosynthesize and translocate photosynthetically-fixed carbon, typically in the form of glycerol and other simple molecules, to the host at a rate capable of meeting its respiratory demands (Muscatine et al. 1984; Porter et al. 1984). *Symbiodinium* cells also promote the conservation and recycling of nitrogen within the symbiosis, making it available for incorporation by the dinoflagellates (Ambariyanto and Hoegh-Guldberg 1996).

Known as photosymbioses, such photosynthetic symbiotic relationships between algae and cnidarians are likely responsible for the survival and growth of coral reefs in the nutrient-poor waters of the tropics. Tropical regions are known for having high light levels, high temperatures, and low food and nutrient levels (Stoddart 1969). This lack of readily available food in the water column of the tropics would not sustain corals without the photosynthetic carbon provided by the zooxanthellae, which supports the idea that the basis of the symbiosis is nutritional, with the dinoflagellates playing a significant role in the nourishment and physiology of the host (Muscatine and Porter 1977). These tropical ecosystems are also often nitrogen-limited, so the dinoflagellates’ survival may depend on the nitrogen made available by the host (Muscatine and Porter 1977; Muller-Parker and D’Elia 1997). The symbiosis between *Symbiodinium* and invertebrates is not limited to coral species, as *Symbiodinium* spp. also form photosymbiotic relationships with other Cnidaria (e.g. sea anemones, jellyfish and zoanthids) and Mollusca (e.g. giant clams) (Trench 1993; Rowan 1998; Lobban et al. 2002).

Photosymbiotic relationships with zooxanthellae have been found in temperate waters as well, which usually have seasonally reduced irradiances and a greater

supply of inorganic nutrients and particulate food (Muller-Parker and Davy 2001). Temperate regions have inorganic nutrient concentrations an order of magnitude higher than those in tropical waters, which would seem to make the need for photosymbiotic relationships low (Muller-Parker and Davy 2001). However, these relationships have been found in many temperate species, including the anemones *Anthopleura ballii* (Davy et al. 1996) and *Anthopleura elegantissima* (Engebretson and Muller-Parker 1999), hard corals e.g. *Plesiastrea versipora* (Davy et al. 2006), and the soft corals such as *Capnella gaboensis* (Sutton and Hoegh-Guldberg 1990).

1.3 - *Symbiodinium*

Formally described in 1962 by Freudenthal, *Symbiodinium* is a genus of photosynthetic dinoflagellates with a worldwide distribution. Found in high abundance (possibly $>10^6$ algal symbionts per cm^2 in a healthy coral), understanding *Symbiodinium* is critical to understanding the evolution, present distribution, and future of coral reefs (Muscatine & Porter 1977; Hoegh-Guldberg 1999). Ranging in diameter from 5-10 μm , zooxanthellae are usually found in complex vacuoles within host gastrodermal cells of cnidarians (Trench 1979; Fitt and Trench 1983; Wakefield et al. 2000); in giant clams the zooxanthellae reside extracellularly in elaborate tubular systems (Norton et al. 1992). Algae within the host cells are surrounded by a host-derived symbiosome (Rands et al. 1993). The symbiosome has been defined as a membrane-bound compartment of the host cell, located in the cytoplasm of eukaryotic cells, which contains one or more symbionts and certain metabolic components (Roth et al. 1988) (Figure 1.1A, B). The outer membrane of the symbiosome is host derived and allows for control of nutrient availability to the algae (Miller and Yellowlees 1989). Zooxanthellae maintained in culture for multiple generations lack symbiosomes (Trautman et al. 2002).

Figures 1.1A & B: 1.1A) TEM picture of zooxanthellae in *Aiptasia pallida* tissue (M = symbiosome membrane P = pyrenoid; N = nucleus; S = starch; C = chloroplast; V = perialgal vacuole; H = host animal tissue Photo: Gisele Muller-Parker 1.1B) - Tentacle of *Anthopleura aureoradiata* with visible zooxanthellae areas Photo: Chris Gibbons.

To avoid digestion by host cells, zooxanthellae must be recognized as ‘self’ by the host, which is important when considering the acquisition of zooxanthellae and their incorporation into the cytoplasmic environment of the host cell (Trench 1979).

1.4 - Diversity

From their classification in 1962 until the late 1970s, all symbiotic dinoflagellates of the genus *Symbiodinium* were classified as one species, *Symbiodinium microadriaticum*. In 1980, Schoenberg and Trench published a series of papers that challenged the single species idea based on behavioral, infectivity, physiological and ultrastructural experimental evidence (Schoenberg and Trench 1980a-c). With advances in molecular genetic techniques, huge diversity has now been discovered within the *Symbiodinium* genus (Rowan and Powers 1991a,b; LaJeunesse 2001; Santos et al. 2001; reviewed by Baker 2003). Early studies of genetic diversity used DNA/DNA hybridization and allozymes, finding differences within *Symbiodinium* as large as those between algae in different classes (Blank and Huss 1989). Subsequent work was done using sequences derived from nuclear small subunit ribosomal DNA (18S-rDNA). This work also showed very high diversity, comparable to orders of free-living dinoflagellates (Rowan and Powers 1991a,b,

1992). This led to the implementation of a classification system that divides *Symbiodinium* into several clades i.e. *Symbiodinium* clades A, B, C (Rowan and Powers 1991) D (Carlos et al. 1999), E, F (LaJeunesse 2001), G (Pochon et al. 2001), and H (Pochon et al. 2004). The phylogeny of these clades has been described as in Figure 1.2:

Figure 1.2: The phylogenetic relationships between the major clades of *Symbiodinium*. The arrangement is a cladogram synthesized from Pochon et al. (2004). The positioning of *Symbiodinium* clades B, C, F and H varies depending on the method of tree generation and the molecules analyzed (sourced from Coffroth and Santos 2005).

These groupings have been supported by further work with chloroplast and mitochondrial genes (Santos et al. 2001; Takabayashi et al. 2004). This has shown considerable within-clade diversity, giving rise to the idea that each clade is comprised of multiple strains or “species” (Rowan 1998). This diversity has predominantly been explored using 18S, 5.8S and 28S rDNA (Baker and Rowan 1997; Darius et al. 1998; Carlos et al. 1999; Loh et al. 2001; Burnett 2002; Barneah et al. 2004; Karako-Lampert et al. 2004;) and, most extensively, the more variable ribosomal internal transcribed spacer (ITS) regions have been studied (LaJeunesse 2001; Santos et al. 2001; LaJeunesse 2002; Diekmann et al. 2003). For instance, LaJeunesse (2001) used sequence variation within the ITS regions to find as many as 76 sequence differences just within clade A.

Physiological differences between zooxanthellar ‘types’ may generate variability in host-symbiont pairings given the correlation of environmental parameters with symbiont distribution seen in many surveys (Rodriguez-Lanetty et al. 2001; LaJeunesse et al. 2004a). Mapping the physiology of zooxanthellae onto current knowledge of their diversity has not received the attention it deserves, but physiological variation clearly exists, and is responsible for some variation in host-

symbiont pairings. For example, the different ‘types’ of zooxanthellae differ in their responses to environmental changes and have different thermal tolerances (Iglesias-Prieto and Trench 1997; Kinzie et al. 2001; Perez et al. 2001; Bhagooli and Hidaka 2004). The differences in thermal tolerance have been documented for *Symbiodinium* of clade D, which dominates at sites with elevated temperatures and on reefs that have suffered temperature-induced bleaching in the past (Chen et al. 2003; Fabricius et al. 2004; Berkelmans and van Oppen 2006). Some studies even attributed distinctive phenotypes to various phylotypes of *Symbiodinium*; for example, C was defined as a ‘specialist’, E as ‘stress-tolerant’, and A as ‘invasive or opportunistic’ (Rowan 1998). However, not all studies have concluded that clade level identity correlates with physiological function (Kinzie et al. 2001; Savage et al. 2002; La Jeunesse et al. 2003; Tchernov et al. 2004). Tchernov (2004) found that algae from different clades responded similarly to elevated temperatures while algae from the same clade showed significantly different responses (Tchernov et al. 2004). The study by Savage et al. (2002) found significant variation in irradiance responses among isolates from within each of clade A, B and C from Bermuda, suggesting that variation within clades could contribute to the persistence of symbiosis in the face of climate change (Savage et al. 2002).

1.5 - Acquisition

Most known host species (~85%) acquire zooxanthellae from environmental pools (known as ‘open’ or ‘horizontal’ transmission); the remaining species acquire zooxanthellae parentally (known as ‘closed’ or ‘vertical’ transmission) (Schwarz et al. 2002). Although some species can survive without their symbiont or host in habitats with sufficient external nutrition, most cannot. Thus, the prevalence of horizontal transmission is surprising, as it leaves both organisms involved to live separately for a time. However, it may allow opportunity for a symbiosis to be established with a symbiont that may be better suited to the current local environmental conditions (Buddemeier and Fautin 1993; Van Oppen, 2004).

Symbiont specificity is determined at various life stages, depending on host taxa. With most cnidarians, zooxanthellae are acquired early in life by either vertical or horizontal transmission. In some taxa, such as Caribbean octocorals, host polyps

initially take up multiple clades of symbionts and establish a dominant clade as the polyp develops (Coffroth et al. 2001), while in many other taxa, host species are passed a ‘type’ of zooxanthella from the parent and do not change this symbiont type over time (Coffroth et al. 2001). Larvae of the coral *Fungia scutaria* acquire symbionts by horizontal transmission but, interestingly, take up algae of the same symbiont type as that found in the adult, even though they do not live in close proximity (Rodríguez-Lanetty et al. 2004). In some host species, it appears that the mode of transmission is correlated with the ‘type’ of symbiont. In the Red Sea, host species with horizontal transmission tend to associate with clade C symbionts, while scleractinians and others with vertical transmission tend to have clade A zooxanthellae (Barneah et al. 2004; Karako-Lampert et al. 2004); this trend has not been seen in other regions with clades B, C and D, despite horizontal transmission of symbionts. Levels of symbiont diversity within acroporid corals do not differ with the method of transmission, suggesting that the mode of transmission does not affect symbiont diversity within a host species (van Oppen 2004). This is contentious, with LaJeunesse (2004) suggesting that vertical transmission selects for host-symbiont specificity, leading to symbiont specialization and greater diversity of zooxanthellae on the reef (LaJeunesse et al. 2004a, b).

1.6 - Specificity

Specificity refers to the different ‘types’ of zooxanthellae that can associate with a particular species of host, or how many species of host a particular type of zooxanthella can associate with. A first step to studying specificity is to investigate the distribution of *Symbiodinium* diversity among the various host species and phyla (e.g. Cnidaria, Mollusca, Foraminifera) with which it forms associations. With exceptions, a survey of host-symbiont pairings shows that symbiont types are not randomly distributed among hosts, and that each species generally associates with the same type across its individuals (Schoenberg and Trench 1980a, b, c; Rowan and Powers 1991a, b). Early studies suggested that the same symbiont is found within a host throughout a geographical distribution, while symbionts differ between host species (Schoenberg and Trench 1980a, b, c). Genetic data have shown that in many cases, a particular type of *Symbiodinium* associates with only one or a few host

species, while in other cases, either the host or symbiont is more flexible and can associate with varied host species/symbiont types (reviewed in Baker 2003). For example, LaJeunesse (2002) documented 69 types of *Symbiodinium* on a single reef, but the majority of host species associated with a particular symbiont type (LaJeunesse 2002). It should be noted that though the majority of host species associate with a particular type of symbiont, but the symbiont found in a species is really just a subset of the types that can possibly be present in the host. Many studies have found that hosts can be induced to take up different types of zooxanthellae (Trench 1971a; Kinzie 1974; Kinzie and Chee 1979; Schoenberg and Trench 1980a, c; Colley and Trench 1983; Coffroth et al. 2001; Santos et al. 2001). The ability of the host to harbour multiple types of zooxanthellae suggests that specificity of host-symbiont relationships seen in the field may be partially dependent on the local environment (Rowan and Knowlton 1995; LaJeunesse and Trench 2000; Rodriguez-Lanetty et al. 2001).

Host-Symbiont Interactions

1.7 - Photophysiology

Zooxanthellae within host tissues fix inorganic carbon into organic carbon through photosynthesis. As suggested by Muscatine et al. (1984), carbon not used by the zooxanthellae for respiration or growth may be translocated to the host predominantly as glycerol, glucose, organic acids, amino acids, and possibly lipids, which may be used to create lipid stores in the host (Muscatine 1967; Trench 1971; Muscatine et al. 1972; Schmitz and Kremer 1977; Kellogg and Patton 1983; Battey and Patton 1987; Muscatine et al. 1994; Grant et al. 1997; Wang et al. 1997; Whitehead and Douglas 2003). These translocated photosynthetic products are then potentially available for respiration, growth and reproduction of the host, though some may be released from the symbiosis as mucus or dissolved organic matter (Muscatine et al. 1981; Edmunds and Davies 1986; Rinkevich 1989; McCloskey et al. 1994).

In 1967, Muscatine used ^{14}C labeling to measure release of photosynthetic products from *Symbiodinium* freshly isolated from the clam *Tridacna crocea* and the coral *Pocillopora damicornis* incubated in homogenized host tissue. It was found that

zooxanthellae release significant amounts of soluble photosynthate to the animal homogenate. This method, however did not quantify amounts of photosynthate released, nor did it assess the extent to which the needs of the host were satisfied by the released product. Later, Trench (1971a-c) ran a series of experiments using ^{14}C to measure release of photosynthetic products in the sea anemone *Anthopleura elegantissima*, both in the intact symbiosis, and in the presence of host homogenate. Throughout the three experiments, about 40-50% of the fixed ^{14}C was liberated both in the intact symbiosis and in host homogenate incubations (Trench 1971a-c).

In 1981, Muscatine et al. derived an equation to calculate the fractional contribution of translocated zooxanthellar carbon to the host's daily respiratory carbon requirements (CZAR):

$$\text{CZAR} = \frac{\text{Zooxanthellae fixed carbon} \times \% \text{ of C translocated to the animal}}{\text{Daily carbon respiration of the animal}}$$

This equation incorporates O_2 -flux measures of photosynthesis and respiration, which are converted into carbon equivalents, and in the first instance employed a ^{14}C tracer to measure the percent of photosynthetically-fixed carbon translocated to the host. Subsequently, however, Muscatine et al. (1984) estimated the percent of fixed carbon translocated using the so-called 'growth rate method', which assumes that all carbon not used for zooxanthellar growth (measured through microscopy techniques) is translocated to the host. Use of this method led to estimates of >90% translocation in several species, from both temperate and tropical regions (Muscatine et al. 1984; Steen and Muscatine 1984; Davy et al. 1996) (Table 1.1), though it is important to note that this method does not account for possible photosynthetic products stored by the zooxanthellae.

Table 1.1: Translocation percentages in temperate and tropical sea anemones determined with both ^{14}C -bicarbonate and the growth rate methods.

Species	Translocation % ^{14}C Method	Translocation % Growth Rate Method	Reference
<i>Anemonia viridis</i> Temperate	45.4	75.3-98.6 low-high light	Davy et al. 1996, 1997
<i>Anthopleura ballii</i> Temperate	33.8	46.8-96.5 low-high light	Davy et al. 1996, 1997
<i>Anthopleura elegantissima</i> Temperate	29	92.7	Fitt et al. 1982; Engelbreton and Muller-Parker 1999
<i>Cereus pedunculatus</i> Temperate	48.6	27.9-97.7 low-high light	Davy et al. 1996, 1997
<i>Aiptasia pulchella</i> Tropical	12-22.4	98.0-99	Muller-Parker 1985, unpubl. (sourced from Muller-Parker and Davy 2001)

When the CZAR is estimated for a whole day, it shows that in many cnidarian species with zooxanthellae, more than sufficient carbon is fixed and translocated by zooxanthellae to satisfy the host's carbon demand for respiration (McCloskey et al. 1994; Davy et al. 1996), except where light is limited by cloud cover, season or habitat (e.g. sediment or depth) (Davies 1991; Davy et al. 1996).

1.8 - Nitrogen Fluxes and Status

Zooxanthellar symbioses have been found mainly in tropical regions, where low levels of nutrients are often found. Zooxanthellae provide carbon products for the host, but host animals also require nitrogen, which is often the nutrient in shortest supply in the marine environment (Thomas 1970; Ryther and Dunstan 1971). One way to satisfy this requirement is by feeding on zooplankton in the water column, but this would supply only a fraction of the required nutrients in tropical seas. This potential lack of nutrients emphasizes the importance of nitrogenous excretions from host animals to zooxanthellar growth and survival (Cook and D'Elia, 1987; Rees, 1991). Nitrogen can be acquired from a variety of sources, 1- dissolved inorganic nitrogen in seawater; 2- dissolved organic nitrogen in seawater; 3- plankton and suspended particulate matter caught by the host. Dissolved inorganic nitrogen can be taken up from seawater as both ammonium (NH_4^+) (Kawaguti 1953; Muscatine and D'Elia 1978; Wilkerson and Trench 1986; Grover et al. 2002) and nitrate (NO_3^-)

(Franzisk 1973; Wilkerson and Trench 1986). Both host and zooxanthellae are capable of assimilating ammonium, though it is done by different pathways. In the animal, ammonium assimilation takes place via the NADP-glutamate dehydrogenase (NADP-GDH) pathway (Miller and Yellowlees 1989; Roberts et al. 2001). The zooxanthellae have been found to assimilate ammonium by the glutamine synthetase/glutamine 2-oxoglutarate amido transferase (GS/GOGAT) pathway. Both pathways convert the ammonium to the amino acid glutamate that can be used to synthesize other amino acids (Wilkerson and Muscatine 1984; Roberts et al. 2001). Nitrate is taken up by both the animal and the zooxanthellae, but only zooxanthellae are capable of converting it into ammonium for assimilation into amino acids (Miller and Yellowlees 1989).

Nitrogen converted into amino acids can then be incorporated into proteins and used for growth. Organic nitrogen can also be passed from zooxanthellae to the animal; for example, the amino acid alanine has been identified in photosynthetic products released by zooxanthellae in host homogenate incubations (Lewis and Smith 1971; Trench 1971; Sutton and Hoegh-Guldberg 1990). Zooxanthellae have been found to take up nitrogenous compounds from the animal, though quantities have not been established (Muscatine and D'Elia 1978).

Most host-symbiont pairings cannot take up sufficient nitrogen from the surrounding environment, and must rely either on nitrogen conservation or recycling to sustain enough nitrogen for growth and survival. Nitrogen conservation is best described as the conservation of nitrogen as the result of the preferential use of photosynthetic carbon received from algal cells for host respiration over amino acids (Wang and Douglas 1998). Nitrogen recycling is a complex procedure involving the transfer of the animal nitrogenous waste to the algal cells, and the translocation of these compounds back to the animal after assimilation of the nitrogen into useable compounds of nutritional value to the animal (Lewis and Smith 1971). There is not much evidence for recycling of nitrogen, beyond the translocation of small quantities of amino acids, though it is often alluded to (Muscatine and Porter 1977). Nitrogen conservation was originally proposed for a freshwater symbiosis (the hydra-*Chlorella* symbiosis) by Rees (1986), but it is applicable to marine cnidarian symbioses as well (Wang and Douglas 1998). Once assimilated, the nitrogen may be used by the zooxanthellae for growth, or they may release some of it back to the host as amino

acids (Trench, 1971a, b, c; Rahav et al. 1989; Sutton and Hoegh-Guldberg 1990; Markell and Trench 1993; McAuley and Cook 1994).

Nitrogen deficiency, as applied to algae, is defined as “algae that have been maintained in a growth-limiting concentration, or in the absence, of a given nutrient, but in the presence of a utilizable source of carbon, which is usually accumulated photosynthate” (Rees, 1991). Zooxanthellae appear to be nitrogen limited, as densities and growth rates increase when nitrogen is supplied in the form of particulate food or supplemental ammonium or nitrate (Hoegh-Guldberg and Smith 1989; Muscatine et al. 1989; Stimson and Kinzie 1991). Evidence for nitrogen deficiency in tropical symbioses was first evidenced by Rees, who used the general characteristics of nutrient-deficient unicellular algae to determine that the host might withhold nutrients from the zooxanthellae (Rees 1991). Since then, much stronger evidence for nutrient deficiency in zooxanthellae has been found. Cook et al. (1992) used ammonium enhancement of dark carbon fixation to measure nutritional status of zooxanthellae from Atlantic corals, and found evidence of nitrogen deficiency in the field; this method has been widely used to estimate nitrogen status of zooxanthellae (Cook et al. 1992, 1994; Davy and Cook 2001; Davy et al. 2006). Moreover, the fact that the temperate anemone *Anthopleura elegantissima* excretes ammonia into the surrounding seawater rather than retaining it all also suggests nitrogen sufficiency (Jensen and Muller-Parker 1994).

1.9 - Host Release Factor

In many marine invertebrates containing symbiotic zooxanthellae, there is substantial movement of photosynthetic products from the algae to the animal tissues (Muscatine & Hand 1958; Muscatine 1967; von Holt & von Holt 1968; Muscatine & Cernichiari 1969). Muscatine, in his 1967 study, found that zooxanthellae released significant amounts of photosynthate when incubated in host homogenate, when compared to incubation in seawater, giving rise to the idea of a ‘host release factor’ response (Muscatine, 1967). This idea was supported by later experiments by Trench (1971c), who suggested that the ‘host release factor’ response is not just an experimental artifact, but may well represent the result of an interaction between animal host and algal symbiont which occurs in the intact symbiosis (Trench, 1971c).

These findings have been supported by later works (Muscatine et al. 1972; Masuda et al. 1994; Engebretson and Muller-Parker 1999), all indicating the presence of some 'host release factor' (HRF) in host tissue homogenates. This HRF may be either a chemical signal present in the tissues of the host organisms that stimulates the release of photosynthetic products from the algae, or it might contain a substrate that can be directly metabolized by the zooxanthellae to produce the compounds that are then released (Biel et al. 2007). Both temperate and tropical species have shown effects of a host release factor, though there have been some species, eg. the soft coral *Capnella gaboensis* whose tissue illicit no increased translocation and even induce negative effects on isolated algae (Sutton and Hoegh-Guldberg 1990). The compound has not yet been conclusively defined, and its properties appear variable in different hosts. For example, the properties of the HRF have been described as heat labile or heat stable, enhancing carbon fixation or not, and behaving like a substance with a molecular mass <10 kDa or ~1 kDa (Gates, 1995). Gates proposed that the HRF in the Hawaiian reef coral *P. damicornis* is a set of free amino acids (FAAs), and found that incubation of algae in a mixture of mycosporine-like amino acids, as well as synthetic FAA mixtures induced the elevated release of photosynthate (Gates 1995; Gates 1999). Withers et al. (1998), incubated algae in a low molecular weight fraction of the animal homogenate and in an homogenate containing the free amino acids found in the low molecular weight fraction: aspartate, glutamate, serine, histidine, arginine, glycine, threonine, alanine, tyrosine, valine, phenylalanine, leucine, and lysine. The free-amino acids did induce the increased release of photosynthate, but not to the same extent as the original homogenate, raising questions about the validity of free amino acids as the main constituent of the HRF. Cook and Davy (2001) tested a synthetic host factor made up of glycine, alanine, and glutamic acid and found a significant response only with alanine, increasing with concentrations up to 50 mM; neither of the other amino acids had significant effects on translocation rates (Cook and Davy 2001), again raising doubts over the role of FAAs. Some possible ways that HRF stimulates the release of photosynthate from zooxanthellae include changing the permeability of the algal cell membrane to glycerol (Muscatine 1967; Trench 1971; Muscatine et al. 1972), stimulation of glycerol synthesis, and inhibition of further processing of glycerol into energy stores within algae (Hinde 1988) or inducing the release of glycerol from storage compounds (Muscatine 1967).

The diversion of carbon compounds away from storage within the zooxanthellae is consistent with the extent of HRF induced release increasing as zooxanthellae become increasingly nitrogen deficient when starved (Davy and Cook 2001b).

1.10 - *Anthopleura aureoradiata*

The sea anemone *Anthopleura aureoradiata* was first described in 1908 by Stuckey, who put it in the family *Bunodidae* and genus *Bunode* (Figure 1.3).

Figure 1.3: *Anthopleura aureoradiata* as seen through dissecting microscope.
Photo courtesy of Chris Gibbons

Originally found at Oriental Bay, Wellington (Stuckey 1908), it has since been found to range from Stewart Island in the south, through New Zealand to Cape Reinga in the far north. Ranging in size from about 5-12 mm wide, it is found on both rocky intertidal shores and mudflats, where it lives on the shells of the cockle *Austrovenus stutchburyi*; these cockles are usually found 5-10 cm deep in the mud at low tide. At high tides, the cockles migrate up to the surface of the mud such that the anemone can extend its tentacles above the surface. *A. aureoradiata* is symbiotic with zooxanthellae of clade A (Phillips 2006) throughout its range.

1.11 - Aims and Objectives

The aim of this study was to determine if host release factor is present in *Anthopleura aureoradiata*, and to ascertain if any response is specific for homologous zooxanthellae or can induce a response in heterologous symbionts.

Specifically, this aim was achieved by:

- 1- Determining if tissue from *Anthopleura aureoradiata* exhibits a host release factor response with freshly isolated homologous zooxanthellae, and comparing this host release factor response with translocation rates in the intact symbiosis;
- 2- Establishing if the host release factor response (if any) seen with homologous zooxanthellae, is also seen in heterologous zooxanthellae (cultured from other host species);
- 3- Establishing whether host release factor response, if any, is enhanced by nitrogen deficiency and hence reduced algal growth.

Photosynthetic carbon release in *Anthopleura aureoradiata*-host release factor activity vs. in hospite photosynthate release

2.1 - Assay for Host Release Factor Activity

Translocation of photosynthetic products from symbiotic zooxanthellae to host animal is known to be the basis of all invertebrate-dinoflagellate symbioses. Zooxanthellae translocate photosynthetically fixed carbon to their hosts for use in respiration, growth and reproduction (Muscatine et al. 1983, 1984; McCloskey et al. 1994). The products are translocated most commonly as glycerol, though glucose, organic acids, amino acids, and even lipids have been identified as translocated compounds (Trench 1971a-c; Muscatine et al. 1972; Kellogg and Patton 1983; Battey and Patton 1987; Muscatine et al. 1994; Grant et al. 1997; Wang et al. 1997; Whitehead and Douglas 2003). Muscatine (1967) discovered that the algae also release these photosynthetic products when isolated from the host, but only in the presence of host tissue. This indicates the presence of some ‘host release factor’ (HRF) in the host tissues that stimulates photosynthate release in the zooxanthellae. First observed in the giant clam *Tridacna crocea* and the coral *Pocillopora damicornis* by Muscatine (1967), it has since been observed in many invertebrate-algal symbioses, both temperate and tropical, including *Plesiastrea versipora*, *Zoanthus robustus*, *Aiptasia pallida*, *Montastraea annularis*, and *Anthopleura elegantissima*. There are some species which have been observed to have no HRF in the tissues, and at least one (*Capnella gaboensis*), in which the host homogenate actually causes the algal cells to lyse (Sutton and Hoegh-Guldberg 1990). The actual identity of the HRF has not yet been determined, though it has been suggested to be a mixture of free amino acids (FAAs) (Gates et al. 1995, 1999). However, some

authors argue against the role of FAAs, (Wang and Douglas 1997; Withers et al. 1998; Cook and Davy 2001).

Also of note, Grant et al. (2001) ran trials looking at HRF in the coral *Plesiastrea versipora* and discovered a photosynthesis inhibiting factor (PIF), which partially inhibits carbon fixation in freshly isolated symbiotic algae. PIF was isolated from the coral *P. versipora*, and has effects on symbiotic algae from several symbioses and on cultured algae (Grant et al. 2001).

This chapter aims to determine using radioisotopic (^{14}C) labelling if there is evidence for host release factor in the temperate sea anemone *Anthopleura aureoradiata*; this anemone has never been surveyed for HRF activity. Furthermore, this chapter aims to compare photosynthetic and translocation rates of isolated zooxanthellae with those in the intact symbiosis.

Materials and Methods

2.2 - Experimental Organisms

All specimens of *Anthopleura aureoradiata* were collected from Pauatahanui Inlet mudflats, located 30 km north of Wellington, New Zealand. Pauatahanui is an east-west running arm of the Porirua Harbor, with much of the shores having been filled in by sand and silt from the surrounding hills. Sampling sites were near the mouth of the inlet, on the mudflats (Figures 2.1A&B).

Figures 2.1 A&B: 2.1A) Satellite photo of Pauatahanui Inlet Image: Google Earth 2.1B) Photo of the mudflats at Pauatahanui Inlet. Photo courtesy of Milena Palka

Anemones were found on the shells of the cockle *Austrovenus stutchburyi* dug from between 5 and 10 cm below the mud surface at low tide. Anemones were then picked off the shells of the cockles and taken back to the lab where they were transferred into bowls containing 1 μ m-filtered seawater (FSW). Water was changed 24 hours later and anemones were moved into an incubator, where they were maintained until use in experiments.

Anemones were maintained at 17°C $\pm$ 1°C in a Contherm incubator on a 12 h light/ 12 h dark cycle. The irradiance was $\sim$ 250 μ mol photons/m²/s. Anemones were fed *Artemia* sp. nauplii once weekly for standard maintenance. Water was changed and debris removed 24 hours after each feeding. Feeding schedules were adjusted when required for experiments.

All anemones used to study translocation in the intact symbiosis and in host homogenate were collected and maintained as described previously, with weekly feedings. Anemones were used within 3 weeks of collection throughout trials.

2.3 - “Host Release Factor” Activity

To test for evidence of a host release factor effect in the temperate *A. aureoradiata* symbiosis, freshly collected anemones were employed. Host-tissue homogenate was prepared by blending 5-10 symbiotic anemones of the same nutritional history in a handheld Wheaton glass tissue grinder. The resulting homogenate was centrifuged at 12,000 g for 5 minutes to separate out the zooxanthellae. The animal-tissue supernatant was poured off and saved for protein content analysis. This analysis was done using the Shakir modification of the Lowry procedure (Shakir et al. 1994). The host homogenate was stored in the refrigerator (4°C) during preparation of the algae, or in the freezer (-17.9°C) if being saved for experiments on subsequent days. All animal homogenates were adjusted to the same protein concentration with FSW. Zooxanthellae were re-suspended in 5 mL FSW and then centrifuged again at 12,000 g for 5 minutes to further eliminate any residual animal tissue. After re-suspending again in 4 mL FSW, 6 hemacytometer counts were taken for each algal cell suspension and 300 μ L pipetted into each of 10x 1.6 mL microcentrifuge tubes. Samples (600 μ L) of animal homogenate were added to each of 4 tubes, 600 μ L FSW added to each of 4 control tubes, and 600 μ L of 20%

formalin (buffered with sodium borate) were added to 2 tubes for estimating background activity (i.e. for formalin-killed cells). All tubes were then labelled with 16 μL $\text{NaH}^{14}\text{CO}_3$ (containing 6 μCi activity). Samples (100 μL) were immediately taken from the formalin vials and were pipetted into 20 mL scintillation vials to which 5 mL OptiScint Hi-Safe II Scintillation Cocktail (Perkin-Elmer) were added and radioactivity read for background values. Remaining vials were incubated on a light bank (Figure 2.2) (irradiance of 250-300 $\mu\text{mol photons/m}^2/\text{s}$); this light level is sufficient to saturate photosynthesis (C. Gibbons, new unpublished data), for 30 minutes.

Figure 2.2: Light bank used for all ^{14}C incubations.

Tubes were vortexed every 10 minutes to ensure continued suspension of algae in the incubation medium. After incubation, tubes were vortexed and 300 μL samples were taken of the combined zooxanthellae and animal mixture, and pipetted into 20 mL glass scintillation vials. All tubes were then centrifuged at 12,000 g for 2 minutes to separate the animal and algal fractions. Three hundred μL of the supernatant (animal homogenate, FSW or formalin) was pipetted into 20 mL scintillation vials. All vials were then acidified with 300 μL of 1N HCl and allowed to dry to eliminate unincorporated ^{14}C . When dry, 300 μL dd H_2O were added,

followed by 5 mL OptiPhase Hi-Safe II scintillation fluid. Radioactivity counts were then taken with a Wallac 1409 liquid scintillation counter.

Fixed carbon, translocated carbon, and percent translocation were calculated using the host and algal fractions and dissolved organic carbon (DOC) as in equations 1, 2, and 3 below.

$$\text{Carbon fixed} = \text{Host+Algal fraction} + \text{DOC*} \quad (\text{Eq. 1})$$

$$\text{Translocated carbon} = \text{Supernatant 1 + 2 (i.e. total animal fraction)+ DOC*} \quad (\text{Eq. 2})$$

$$\% \text{ Translocation} = (\text{Translocated carbon/Fixed Carbon}) \times 100 \quad (\text{Eq. 3})$$

* - DOC only used in measurements in the intact symbiosis

Photosynthesis and carbon translocation rates, both calculated in grams C/cell/h, were determined using the mean specific activity (grams C/dpm), the number of zooxanthellae, and the incubation time. Mean specific activity was calculated using an assumed inorganic carbon content of 0.024g C per liter of seawater (Davy and Cook 2001a).

2.4 - Carbon Translocation in the Intact Symbiosis

To study carbon translocation in the intact symbiosis, three anemones were allowed to settle overnight in separate 20 mL glass scintillation vials with 5 mL 1 µm-filtered seawater (FSW). A fourth vial was filled with 5 mL of FSW alone and served to provide background values for the ambient medium. Before the incubations, the FSW was replaced with fresh FSW and 16 µL of $\text{NaH}^{14}\text{CO}_3$ (~6 µ Ci) were added to all 4 vials and the contents mixed. Two samples of 100 µL each were immediately taken from the background seawater vial and 5 mL of OptiPhase Hi-Safe II scintillation fluid added. The specific activity of the added ^{14}C was measured with these samples. Anemones were then incubated on the light bank as previously described at an irradiance of ~250 µmol photons /m²/s for 30 minutes. After the incubation, all work was carried out in low light (<1 µmol photons/m²/s). Two samples of 200 µL FSW were taken from each vial for measurement of dissolved organic carbon (DOC) release by the anemones. Anemones were then removed from the scintillation vials and rinsed in unlabelled FSW to eliminate excess $\text{NaH}^{14}\text{CO}_3$.

Each anemone was homogenized in 1.5 mL FSW in a Wheaton ground glass tissue grinder. Of this homogenate, 1.3 mL was transferred into a 1.6 mL microcentrifuge tube and the remainder saved for zooxanthella counts. Tubes were vortexed and 200 μ L (n=2) of homogenate (algae + animal) from each tube were pipetted into clean 20 mL glass scintillation vials. Tubes were then centrifuged at 12,000 g for 1 min to separate animal and zooxanthella fractions. Two samples per anemone (200 μ L) of the supernatant were taken from each tube and placed in clean 20 mL glass scintillation vials. The algal pellet was then re-suspended in 1 mL FSW and re-centrifuged. Two samples (200 μ L) were taken of this second supernatant and kept separate from the first in clean 20 mL glass scintillation vials. The 200 μ L set aside from the original homogenate was centrifuged at 12,000 g for 1 minute and the supernatant poured off. The algal pellet was re-suspended in 0.5 mL FSW and 6 cell counts were taken with a hemacytometer. All vials, except the added activity ones, were acidified with 200 μ L of 1N HCl to drive off unincorporated ^{14} C. Vials were then left to dry completely and 5 mL OptiScint Hi-Safe II scintillation fluid added. Radioactivity was determined with a liquid scintillation counter as before.

The photosynthetic rate, carbon release rate and percentage release were calculated as for the host homogenate incubations.

2.5 - Influence of HRF on Photosynthetic Health

In light of the lysing and photoinhibition of zooxanthellae when exposed to *Capnella gaboensis* tissue homogenate (Sutton and Hoegh-Guldberg 1990), and the lack of host release factor response seen in homogenate experiments, questions were raised about the possibility of the animal homogenate affecting the photosynthetic health of the zooxanthellae. Pulse Amplitude Modulated (PAM) fluorometry (Figure 2.3) was employed to measure photosynthetic health of the zooxanthellae in host homogenate. PAM fluorometry has been used in many previous studies (e.g. Beer et al. 1998; Jones et al. 1999; Ralph et al. 1999) to measure health of zooxanthellae in corals and other hosts, and is a useful tool to quickly check if low translocation rates are due to photoinhibition or mortality of the algae.

Figure 2.3: Heinz Walz Imaging PAM unit with well-plate in position for fluoremetry readings of zooxanthellae and host homogenate/FSW/Formalin incubations.

PAM fluorometry measures photosynthetic health by flashing a range of light intensities at a sample and measuring the fluorescence energy emitted by the sample. The measurement used to represent photosynthetic health is F_v/F_m , where F_m is the maximum fluorescence and F_v is the difference between the minimum (dark) fluorescence and the maximum fluorescence. To check for effects of the host tissue homogenate on the zooxanthellae, anemones were homogenized in a glass tissue grinder and the zooxanthellae extracted by centrifuging for 5 mins at 12000 g. The supernatant was poured off and the zooxanthellae resuspended in 5 mL FSW and centrifuged again to rinse off any residual animal tissue. The algae were then resuspended in 6 mL FSW and 6 hemocytometer counts taken. Animal tissue homogenate was prepared in advance as before and diluted down to 50% and 25% strength with FSW. Zooxanthellae (300 μ L) were then pipetted into each of 20 wells on a well plate, and 600 μ L host homogenate (100, 50, or 25% strength)/FSW/formalin was added as shown in Figure 2.4:

Figure 2.4: Well-plates as filled for PAM readings, all with 300 µL zooxanthellae (zoox.), and 600 µL of either FSW, host homogenate (HH), or formalin. Formalin wells are on a separate plate to avoid contamination of other samples by formalin fumes.

Imaging PAM fluorometer readings were taken every 30 minutes for 5 hours, measuring the photosynthetic health (F_v/F_m) of the zooxanthellae over time when in the presence of the animal homogenate. Zooxanthella concentration was 1.41 million cells per mL in the first trial and 1.67 million cells per mL in the second trial. All PAM fluorometry was carried out using a Heinz Walz Imaging PAM unit, and all samples dark incubated for ~2 min before F_v/F_m was measured.

2.6 – Statistical Analysis

Photosynthetic rate, translocation rate, and percent translocation were compared between isolated and in hospite zooxanthellae using one-way ANOVA and Tukey post hoc analysis. Significant differences were determined at the 5% level. The same statistical methods were used to compare photosynthetic health among treatments.

Results

2.7 - Carbon Translocation in the Intact Symbiosis and Isolated Zooxanthellae

Zooxanthellae in the intact symbiosis had an average photosynthetic rate of 1.175 ± 0.452 pg C/cell/h. Isolated zooxanthellae incubated in *Anthopleura aureoradiata* host homogenate had an average fixation rate of 1.48 ± 0.417 pg C/cell/h, and isolated zooxanthellae incubated in FSW had an average fixation rate of 2.08 ± 1.36 pg C/cell/h (Figure 2.5). None of the differences in fixation rates were significantly different (one-way ANOVA, $p > 0.05$). Note that all analyses of HRF/freshly isolated zooxanthellae include data from the freshly isolated zooxanthellae used in the cladal diversity/HRF experiments (see page 32).

Figure 2.5: Photosynthetic rates of zooxanthellae in the intact symbiosis (n=9), when incubated in host homogenate (n=6), and when incubated in FSW (n=6). All values are mean + 1 s.d

Percentage translocation values for the three treatments ranged from 40% +/- 11% in the intact symbiosis to 11% +/- 8.5% in the freshly isolated seawater controls and 8% +/- 5.5% in the freshly isolated homogenate incubations. Translocation values were significantly higher in the intact symbioses than with both trials of isolated zooxanthellae (Tukey *post hoc* one-way ANOVA, $p < 0.05$)(Figure 2.6).

Figure 2.6: Translocation percents for the three treatments: zooxanthellae in the intact symbiosis (n=9), isolated in host homogenate (n=6), and incubated in FSW (n=6). All values are mean + 1 s.d.

Translocation rates varied, with the rate in the intact symbiosis being 0.48 ± 0.23 pg C/cell/h, the rate in host homogenate 0.128 ± 0.11 pg C/cell/h, and the rate in FSW 0.243 ± 0.24 pg C/cell/h. However, the only significant difference was the higher rate in the intact symbiosis than when the freshly isolated zooxanthellae were incubated in host homogenate (Tukey *post hoc* one-way ANOVA, $p < 0.05$)(Figure 2.7).

Figure 2.7: Translocation rates of zooxanthellae in the intact symbiosis (n=9), incubated in host homogenate (n=6), and incubated in FSW (n=6). All values are mean + 1 s.d.

2.8 - Influence of HRF on Photosynthetic Health

Photosynthetic health of zooxanthellae freshly isolated from *A. aureoradiata* was measured with Imaging PAM (Figure 2.8). Health was measured as F_v/F_m and plotted against time. Trials in FSW were consistently lower than those in host tissue, but were only significantly different from the 25% dilution. Values dipped at 4 hours, but then recovered at 5 hours. Table 2.1 shows the significant differences among these data (Tukey *post hoc* one-way ANOVA, $p < 0.05$).

Figure 2.8: Photosynthetic health (F_v/F_m) of freshly isolated zooxanthellae incubated for 330 minutes in one of: FSW, 100% host homogenate, 50% dilute host homogenate, or 25% dilute host homogenate. No measurement was taken at 4:30. Values are means $\pm$ 1 s.d. All $n=8$.

Table 2.1: Significant differences (Tukey *post hoc* one-way ANOVA $p < 0.05$) between F_v/F_m values at timepoints (20-330 min) when freshly isolated zooxanthellae were incubated in filtered seawater (FSW) or 25%, 50%, or 100% host tissue homogenate.

Time point	Significantly different between:	
30 min	100%	25%
90 min	FSW	50%
	FSW	25%
	100%	25%
120 min	FSW	50%
	FSW	25%
	100%	25%
150 min	FSW	25%
180 min	FSW	50%
	FSW	25%
210 min	FSW	25%
	100%	25%
240 min	FSW	50%

Discussion

2.9 - Host Release Factor

Zooxanthellae in the intact symbiosis released 40% +/- 11% of their fixed carbon to the host. This figure is comparable to those found in previous studies; for example, the temperate anemones *Anemonia viridis* and *Anthopleura elegantissima* have been found to have values of 40-50% translocation (Trench 1971b; Stambler and Dubinsky 1987). Translocation percentages for other temperate species vary, with the soft coral *Capnella gaboensis* showing around 18%, and the temperate zoanthid *Zoanthus robustus* showing 11-35% (Sutton and Hoegh-Guldberg 1990). Values in tropical species tend to be similar; for example, algae in the tropical anemone *Condylactis gigantea* translocate 27% to the host, and the subtropical/tropical anemone *Aiptasia pallida* has shown translocation percentages from 40-82% (von Holt and von Holt 1968; Clark and Jensen 1982). With percent translocation values as found, there is great potential for autotrophy under well-lit conditions in the field, particularly on rocky shores. However, *Anthopleura aureoradiata* is often found under the surface of mud, so it seems unlikely that autotrophy is essential for this species.

Freshly isolated zooxanthellae from *Anthopleura aureoradiata* did not show any conclusive effects of an HRF when incubated in host homogenate as compared to FSW incubations, as they released only 8% when incubated in host homogenate and 11% when incubated in FSW. However, zooxanthellae in the intact symbiosis released ~40% of their fixed carbon to the host, so nutrient exchange is present in this symbiosis. Muscatine (1967) found that host tissue homogenates of the giant clam *Tridacna crocea* and scleractinian coral *Pocillopora damicornis* induced homologous algae to release nearly 40% of carbon fixed during host tissue homogenate incubations. In agreement with the results of Muscatine (1967), Withers et al. (1998) found in their trials with the coral *Plesiastrea versipora* that algae incubated in host homogenate consistently released more photosynthate than algae incubated in FSW, even when photosynthetic rates were lower in the host homogenate. Zooxanthellae from *P. versipora* released 13-41% of the total fixed carbon when incubated in host

homogenate, as compared to 1-2% when incubated in FSW (Withers et al., 1998). Similar results were found by Davy and Cook (2001) with the sub-tropical anemone *Aiptasia pallida* which released approximately 14% of photosynthate when incubated in host homogenate, and only 5% when incubated in FSW.

Translocation rates were highest in the intact symbiosis and lowest in the host homogenate incubation. This shows that the zooxanthellae do release photosynthate to the host, but that homogenate of the host's tissue does not have the effect on the zooxanthellae as found in previous studies. Sutton and Hoegh-Guldberg (1990) looked at 4 temperate invertebrates: the soft coral *Capnella gaboensis*, the zoanthid *Zoanthus robustus*, the hard coral *Plesiastrea versipora*, and the nudibranch mollusc *Pteraeolidia ianthina*, and found significant effects of HRF only in *P. versipora*, leading them to conclude that host factors are likely not a universal property of symbiotic associations with zooxanthellae.

These differences of translocation rates and percentages between the intact symbiosis and the isolated symbionts could be due to some part of the homogenizing process damaging the HRF compound or zooxanthellae, or changing the physiology of the zooxanthellae to not respond to the HRF compound. If the HRF compound is damaged by the homogenization process, it perhaps argues against the HRF being a free amino acid and in favor of a more complex and thus, larger compound. Zooxanthellae in the intact symbiosis typically live in membrane-bound vacuoles that isolate them from the cytoplasm of host cells (Trautman et al. 2002). Not much is known about the conditions within the vacuoles, but it is possible that the membrane controls the compounds that the zooxanthellar cells are exposed to, and controls the contact of the zooxanthellae with the HRF compound; homogenization likely disrupts this symbiosome membrane.

It has previously been found that tissue extracts which induce HRF responses also increase photosynthetic rates in isolated zooxanthellae (Cook and Davy, 2001a). Photosynthetic rates in zooxanthellae isolated from *A. aureoradiata* were around 1pg C/cell/h, which is comparable to photosynthetic rates found by Davy and Cook (2001), who found rates of around 1pg C/cell/h in zooxanthellae isolated from *Aiptasia pallida*. However, the data presented here show no increase in photosynthetic rate for *A. aureoradiata* when incubated in host tissue. They also show no significant decrease in photosynthetic rate in the presence of the host

homogenate, so it does not appear that the host homogenate causes the zooxanthellae cells to lyse, as with *C. gaboensis* (Sutton and Hoegh-Guldberg, 1990), or to be photosynthetically inhibited as with *Plesiastrea versipora* homogenate (Grant et al. 2001; Grant et al. 2004). This was supported by the PAM experiments, which examined the photosynthetic health of the zooxanthellae in the presence of host homogenate. It was found that the lowest values of Fv/Fm were for the zooxanthellae incubated in FSW, though these values were only significantly lower than the 25% and 50% dilute homogenate incubations, and only at a few time points. Values of all algae at the first reading were consistent with those of healthy photosynthetic algae from previous studies (Jones et al. 1998; Fitt et al. 2001; Hill et al. 2004; Ralph et al. 2005). The initial drop in the Fv/Fm ratio can possibly be attributed to light shock of the algae (Fitt et al. 2001). This, however, cannot explain the dip after 4 hours, which may be an experimental artifact, as the values are averages of the two trials. It is interesting to note that photosynthetic rates appeared highest in FSW incubations, but that photosynthetic health was highest in host homogenate, possibly because the host homogenate slows photosynthetic fixation, while not affecting the health or potential of the algae to perform photosynthesis. This could be interpreted as evidence for a photosynthetic inhibiting factor (PIF) in *A. aureoradiata* host tissues, though it was not as evident in the 100% dilution incubation as would be expected. Grant et al. (2001) looked at photoinhibition by a fraction of host homogenate, but did not examine if it affected photosynthetic potential, or just limited fixation. In *A. aureoradiata* there may be some form of PIF that affects carbon fixation rate without damaging photosynthetic capabilities.

In conclusion, zooxanthellae from *Anthopleura aureoradiata* release 40% of fixed carbon in the intact symbiosis, but show no evidence for a host release factor when isolated and incubated in host tissue homogenate. Translocation and photosynthetic rates were consistently lower in host homogenate incubations when compared to FSW controls. The lack of an HRF response in homologous zooxanthellae implies either the absence of such a compound in *A. aureoradiata* tissues, or that the isolated homologous algae lack the capacity to respond to it. The next chapter aims to determine if heterologous algae may be able to respond to the HRF of *A. aureoradiata*, or if it is not active in homogenized tissues of this anemone.

Heterologous Algae and HRF Response in *A. aureoradiata* Host Tissue Homogenate

3.1 - Heterologous zooxanthellae and host factor assays

Exceptional diversity within the genus *Symbiodinium* has been discovered through advances in molecular techniques as discussed in the introduction (LaJeunesse 2001; Santos et al. 2001). Physiological variation amongst the molecularly defined types of zooxanthellae clearly exists, and may be responsible for some variation in host-symbiont pairings. Even with this diversity, some hosts have been found to pair with multiple types of zooxanthellae across their range, and can also be induced to take up different types of zooxanthellae (Trench 1971a; Kinzie and Chee 1979; Coffroth et al. 2001; LaJeunesse 2002).

Davy and Cook (2001b) found similar responses to host homogenate incubations of homologous and heterologous zooxanthellae, and suggested this could be indicative of a similar release mechanism in a number of host species. Such similarities could be important if hosts acquire a new type of zooxanthellae, for example after bleaching events (Buddemeier and Fautin 1993). In this case, HRF would need to have an effect on heterologous symbionts to induce the release of photosynthetic products to the host. Muscatine (1967) found that the host homogenate from *Pocillopora damicornis* induced release of photosynthate from zooxanthellae isolated from *Tridacna crocea*. A similar trend was also found by Sutton and Hoegh-Guldberg (1990) who found that zooxanthellae isolated from *Plesiastrea versipora* and *Zoanthus robustus* both showed HRF effects when incubated in host tissue homogenate from *P. versipora*.

In Chapter 2 there was no HRF response of *Anthopleura aureoradiata* homogenate in freshly isolated zooxanthellae (FIZ). This could have been because the algae do not respond, and/or because an active HRF compound is not present in *A.*

aureoradiata tissues. This chapter aims to establish if host tissue homogenate from *A. aureoradiata* contains HRF that induces responses from heterologous zooxanthellae of clades A-E.

Materials and Methods

3.2 - Algal Clades from Culture

Cultures were acquired from Dr. Scott Santos (Auburn University, USA) and maintained in a Contherm incubator at 26°C on a 12 h light/ 12 h dark cycle (Figure 3.1).

Figure 3.1: Cultured zooxanthellae as maintained in the Contherm incubator

Cultures were subcultured every 3-6 weeks and antibiotics were added about every 6 months to eliminate unwanted bacteria. Cultures used for experiments were used within 2 months of subculturing. All cultures were kept in f/2 silica-free medium (Sigma) to eliminate growth of diatoms. The zooxanthellar types used for the HRF/zooxanthellae diversity experiments are shown in Table 3.1 (next page).

Table 3.1: Table of host species, locations, clades, and sources of each of the zooxanthellar types used in HRF/zooxanthellae diversity experiments. All cultures except for zooxanthellae from *Anthopleura aureoradiata*.

Clade	Abbreviation	Host	Organism	Location	Source
A	FLCass	<i>Cassiopeia xamachana</i>	Jellyfish	Long Key, FL	S.R. Santos
B	Pk13	<i>Plexaura kuna</i>	Soft Coral	Three Sisters, FL	M.A. Coffroth
B	Pd	<i>Pocillopora damicornis</i>	Hard Coral	Hawaii	M.A. Coffroth
C	Mp	<i>Mastigias paupa</i>	Jellyfish	Palau	R.A. Kinzie
C	Sin	<i>Sinularia</i> sp.	Soft Coral	Guam	
D	A001	<i>Acropora</i> sp.	Hard Coral	Okinawa	M. Hidaka
E	CCMP421	Free-Living		Wellington, NZ	Bigelow Marine Lab
A	FIZ	<i>Anthopleura aureoradiata</i>	Sea Anemone	Wellington, NZ	S. Phillips

3.3 - HRF Response in Heterologous vs. Homologous Zooxanthellae

HRF incubations were run as in chapter 2. Each trial consisted of 3 replicates of each of 2 clades of zooxanthellae and 3 replicates of freshly isolated zooxanthellae (FIZ) as a comparison. FIZ were prepared as in chapter 2. Zooxanthellae from cultures were prepared by swirling the culture and pipetting 10 mL into a clean 15 mL centrifuge tube and centrifuging at 12,000 g for 5 minutes. The culture-medium supernatant was poured off and the remaining algal pellet was re-suspended in 1.2 mL 1 μ M FSW to concentrate the algal suspension. Host homogenate for all experiments was prepared as in Chapter 2 and frozen for continued use in all trials. All experiments were completed in 14 days to limit effects of differences in growth cycles of the algae. Rates of photosynthesis and translocation were estimated as in Chapter 2.

3.4 – Statistical Analysis

Differences between host homogenate and FSW of the clades were analyzed using the students t-test. Photosynthetic and translocation rates, as well as percentage translocation were compared significance between clades using one-way ANOVA and Tukey post-hoc analyses. Significance was determined at the 5% level.

Results

3.5 - HRF Effect vs. Cladal Diversity

Heterologous zooxanthellae were incubated in *Anthopleura aureoradiata* host tissue homogenate to ascertain if there is evidence for HRF in *A. aureoradiata* tissue. Table 3.2 shows the clades of *Symbiodinium* that had photosynthetic or translocation rates that were significantly lower in host tissue homogenate incubation than the control FSW incubation. With regards to photosynthetic fixation rates (Figure 3.2A), all FSW incubations had higher fixation rates than host homogenate incubations, but were only statistically different as shown in Table 3.2.

Table 3.2: Summary of t-test comparisons between host homogenate incubations and FSW incubations with regards to photosynthetic rates and translocation rates of zooxanthellae from clades A-E. Significance was defined by t-test. (n=6 (clade A) n=3 (all other clades)). ** indicates a significant difference, p<0.05.

Clade	Photosynthetic rate	Translocation rate
A	↓ **	↓ **
B	↓ ns	↓ Ns
C	↓ **	↓ **
D	↓ **	↓ **
E	↓ **	↓ Ns
Freshly Isolated (A)	↓ ns	↓ **

Significantly different photosynthetic fixation rates in FSW incubations were 1.2-1.8 times higher than rates in host tissue incubations. When compared to one another, rates were different between clade A and the FIZ (also clade A), where fixation rates in the freshly isolated zooxanthellae were 6.5 times higher than those from cultured clade A. Rates were also different between the freshly isolated zooxanthellae and those from clade D, where the fixation rate of the FIZ was 3.7 times that of the clade D zooxanthellae (Figure 3.2).

Figure 3.2: Photosynthetic rates of the clades A-E from culture and freshly isolated zooxanthellae (FIZ) (clade A), when incubated in host homogenate of *Anthopleura aureoradiata* and FSW. All values are mean $\pm$ 1 s.d. (clade A n=6, clades B-E n=3).

Translocation rates (pg C/cell/hour) tended to be higher in the FSW incubations than in the host homogenates for all cultured algae, but these differences were only significant for clades A, C, D, and the freshly isolated algae (clade A) (t-test $p < 0.05$)(Figure 3.3). Translocation rates in FSW incubations were 1.7-4 times higher than rates in host homogenate incubations. Differences between the clades with respect to translocation rates were only evident amongst the FSW controls, and only between clades A and C (Tukey *post hoc* one-way ANOVA, $p < 0.05$). Clade C had translocation rates 3 times those in the cultured clade A.

Figure 3.3: Translocation rates of clades A-E from culture and freshly isolated zooxanthellae (FIZ) (clade A), when incubated in *A. aureoradiata* host homogenate and FSW. All values are mean +1 s.d. (clade A n=6, clades B-E n=3).

Percentage translocation showed the same trends as photosynthetic fixation and translocation rates, with percentages for FSW incubations 1.2-2.5 times higher than those from host homogenate incubations (Figure 3.4). Differences were only significant for the cultured clade A, and clade C (t-test, $p < 0.05$). Percentage translocation differences amongst the clades in host homogenate were only significant between the cultured clade A and all others except for clade E, with translocation percents more than 1.5 times greater than the rest. In FSW incubations, the cultured clade A again had translocation percents ranging from 1.3- nearly 4 times higher than all other clades. These differences were only significant, however, between the cultured clade A and clades B, C, and FIZ (Tukey *post hoc* one-way ANOVA, $p > 0.05$).

Figure 3.4: Percent translocation of zooxanthellae from 5 different clades (A-E) incubated for 30 minutes in *Anthopleura aureoradiata* homogenate or FSW. All values are mean + 1 s.d. (clade A n=6, clades B-E n=3).

Discussion

3.6 - Diversity

Anthopleura aureoradiata tissues were tested for evidence of HRF by incubating heterologous zooxanthellae in *A. aureoradiata* host tissue homogenate.

All of the clades measured (A-E) showed higher photosynthetic rates and translocation rates in FSW incubations, with host tissue homogenate incubations usually significantly lower. These decreased photosynthetic rates of heterologous zooxanthellae incubated in host homogenate support the idea that there is a compound present in the host homogenate that inhibits carbon fixation. Grant et al. (2001) found evidence of a photosynthetic inhibiting factor (PIF) present in host tissue homogenate of *Plesiastrea versipora*; this is the only species that has been studied for PIF. Later studies showed that PIF in *P. versipora* tissue affected homologous zooxanthellae and some heterologous zooxanthellae (Grant et al. 2006). Evidence for PIF in *A. aureoradiata* was found in every clade except for “B”, the only clade where the photosynthetic rates in the FSW incubations were not significantly higher than those in host homogenate.

No PAM fluorometry experiments were run on the heterologous zooxanthellae to see if, like the homologous zooxanthellae in Chapter 2, the photosynthetic health was still intact. It is interesting to note that the freshly isolated zooxanthellae, which are clade A, and the cultured clade A (from the jellyfish *C. xamachana*) had very different responses to the host homogenate. While both showed no increase in the rates of translocation or photosynthesis in host homogenate, the photosynthetic rates were very different: 0.267 pg C/cell/h for the cultured clade A vs. 1.71 pg C/cell/h for the freshly isolated clade A. Stat et al. (2008) looked at differences between clades A and C and determined that clade A is more closely related to free-living dinoflagellates, and closer to being parasitic than are other clades. This difference could be attributed to the inherent variation between a cultured symbiont and a freshly isolated one. Previous studies have shown physiological differences between cultured and freshly isolated zooxanthellae, perhaps resulting from the lack of the host-derived symbiosome membrane around cultured zooxanthellae (Trautman et al. 2002). These differences could also be attributed to the diversity within clades, though inter- and

intra-cladal differences in photosynthesis and photosynthate release have not been studied in other systems to date (Rowan 1998).

Many differences have been found between HRF for different host species in terms of specificity, thermal stability, effects on the amount of fixation, and the type of compounds released by algae in their presence. As evidenced by Muscatine (1967), some HRFs are not specific to hosts and can stimulate photosynthate release in algae isolated from different symbiotic associations. Some are specific, as demonstrated by Sutton and Hoegh-Guldberg (1990) when HRF from *Plesiastrea versipora* did not elicit any response from algae isolated from *Pteraeolidia ianthina*, though it had an effect on its own algae. Thermal stabilities of HRF are also variable: Gates et al. (1995) found that boiling the host homogenate of *Pocillopora damicornis* had no effect on the HRF activity, while boiling the host homogenate of *Anthopleura elegantissima* and *P. versipora* destroyed their HRF activity (Trench 1971). This suggests that HRF may not be the same compound across different host species. However, the success of other experiments to induce HRF responses in heterologous zooxanthellae indicate that there must be some similarities between host species and the composition of any HRF present.

Data from this chapter continue to support the suggestion that there is no HRF present in *Anthopleura aureoradiata*. There was no response measured with either homologous or heterologous zooxanthellae. However, preliminary evidence suggested that different types of zooxanthellae have differing photo-physiologies and may release differing amounts of photosynthate. This could have implications for the health of the host organism.

Carbon translocation and nutritional status

4.1 - Nutritional Status and Carbon Translocation

It has been suggested previously that carbon translocation from zooxanthellae to host may be inversely related to the nutritional status of the zooxanthellae. Nitrogen-limited zooxanthellae do not have the required nutrients to grow, and thus are thought to translocate carbon originally meant for growth to the host (Falkowski et al. 1993; Dubinsky and Jokiel 1994). Nutritional status of zooxanthellae is often measured using ammonium enhancement of dark carbon fixation (Cook et al. 1992, 1994; Davy and Cook 2001). Ammonium enhancement of dark carbon fixation to measure nutritional status of zooxanthellae is based on previous evidence that NH_4^+ stimulates dark fixation in cells with low nitrogen supply (Morris et al. 1971; Yentsch et al. 1977).

Previous experiments have shown no ‘host release factor’ effects on either heterologous or homologous zooxanthellae incubated in *Anthopleura aureoradiata* host tissue homogenate. It is thought that as zooxanthellae are starved of nitrogen, they do not have the necessary nutrients for growth, and thus would release surplus carbon to the host. Davy and Cook (2001a) found that as they starved the sea anemone *Aiptasia pallida* over 114 days there was no change in the percent translocation in the intact symbiosis. However, Davy and Cook (2001b) ran a similar starvation experiment on freshly isolated zooxanthellae in host homogenate incubations for the sea anemones *Aiptasia pallida* and the coral *Montastraea annularis* and found that starvation increased percent translocation, and decreased photosynthetic rates. It follows that potentially starving zooxanthellae from

Anthopleura aureoradiata could induce an HRF response in host homogenate incubations.

Nitrogen sufficiency in the field of temperate algal-invertebrate symbioses has not been widely studied, but the studies that exist show nitrogen sufficiency in temperate waters, and recent work by S. Morar (VUW; unpublished data) shows nitrogen sufficiency of the zooxanthellae in *A. aureoradiata* from Pauatahanui Inlet. If *A. aureoradiata* is nitrogen sufficient in the field and when well-fed in the lab, it could be inferred that the zooxanthellae have sufficient nutrients to grow, and may not have surplus carbon to release to the host. This chapter investigates the effects of starvation on nitrogen status of zooxanthellae isolated from *A. aureoradiata* and further, the possibilities of inducing a ‘host release factor’ response from zooxanthellae isolated from *A. aureoradiata* by limiting their nitrogen supply and hence growth.

Materials and Methods

4.2 - Experimental Organisms

Anthopleura aureoradiata specimens for nitrogen status experiments were collected from Pauahatanui Inlet on 11 September 2007, three weeks before the beginning of the experiment. All anemones were fed three times a week for the 3 weeks leading up to the experiment. Anemones used were kept in various size bowls containing 500 mL – 1 L nitrogen-free artificial seawater (N-free ASW), which was made following the procedure of Kester et al. (1967). This recipe was made up as in table 4.1. Anemones were kept under the same environmental regime as each other.

Table 4.1: Recipe used for nitrogen-free artificial seawater used throughout trials.

Salt	g/kg of distilled water
NaCl	23.9
Na ₂ SO ₄	4.01
KCl	0.677
NaHCO ₃	0.196
KBr	0.098
H ₂ BO ₃	0.026
NaF	0.003

4.3 - Nitrogen Status of Zooxanthellae

Dark carbon fixation rates were measured using $\text{NaH}^{14}\text{CO}_3$ incubations. The following method was adapted from the original design of Cook et al. (1992), and was repeated at 2 week intervals for 8 weeks to measure nutritional status of *A. aureoradiata* when kept in nitrogen-free artificial seawater and starved.

As outlined in figure 4.1, at each time point, 5 anemones (~5-8 mm oral disc diameter) were homogenized separately in a Wheaton ground glass tissue grinder in N-free ASW. This homogenate was then centrifuged at 12000 g for 5 minutes and the supernatant poured off. The algal pellet was re-suspended in 5 mL FSW and centrifuged again at 12000 g for 5 minutes to rinse any residual animal tissue from the zooxanthellae. The supernatant was then decanted off once more and the zooxanthellae were re-suspended in 1.6 mL N-free ASW. Six hemocytometer counts were then made of all 5 algal suspensions and zooxanthella concentrations were normalized to match the lowest recorded by dilution with N-free ASW. Plastic scintillation vials (6 mL) were wrapped in black electrical tape to prevent light exposure for dark fixation samples. For each of the anemones, 5 vials were filled with 0.5 mL of algal suspension and 0.5 mL of either N-free ASW (x2) or 40 μM NH_4Cl in N-free ASW (x2). All remaining zooxanthellae were combined, and 4 vials filled with 0.5 mL zooxanthellae and 0.5 mL of 20% formalin, saturated with sodium tetraborate; these acted as 'background' activity vials. Vials with black tape were then placed in the dark to pre-incubate for 45 minutes. Then in low light levels ($<1 \mu\text{mol photon/m}^2/\text{s}$) to minimize ^{14}C incorporation, 20 μL dilute $\text{NaH}^{14}\text{CO}_3$ stock (1 μCi) was added and the vials sealed. Vials were incubated in darkness for 4 hours. All dark incubations were at room temperature (17° C), with vials kept in a dark box. Incubations were ended when 0.5 mL of 1N HCl was added to drive off unincorporated ^{14}C . Samples were left to dry on a heating block at 37° C covered to ensure no light enhancement of carbon fixation.

During the pre-incubation of the dark fixation vials, incubations in saturating light were carried out. Twenty μL of dilute $\text{NaH}^{14}\text{CO}_3$ stock (1 μCi) was added to 5 vials to be incubated in the light. 100 μL was immediately sampled from one formalin vial for added activity readings. Vials were then incubated for 30 minutes on a light rack, which consisted of a clear plexiglass box above 4 fluorescent bulbs

and next to 4x40-W cool-white lamps, producing an irradiance of $\sim 250 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. This incubation was ended by the addition of 0.5 mL 1N HCl. Vials were then left on a heating block at 37° C until dry to eliminate unincorporated ^{14}C .

Figure 4.1: Flow chart of nitrogen status determination method

When all vials were dry, 0.5 mL ddH₂O was added, followed by 5 mL OptiPhase Hi-Safe II Scintillation Cocktail (Perkin-Elmer). Black tape was then removed from vials and all radioactivity was counted on a Wallac 1409 Liquid Scintillation Counter.

4.4 - 'HRF' Activity vs. Nitrogen Status

Measuring for a host release factor response was done following the procedure described in chapter 2, but with a few modifications. Instead of the zooxanthellae being isolated from several anemones homogenized together, each of 5 anemones was homogenized separately. Zooxanthellae were counted with 6 hemocytometer counts and all 5 algal suspension concentrations were normalized to the least concentrated extract. The animal tissue homogenate was prepared the day before the trials began and frozen until needed for weeks 0-2. A new batch of homogenate was prepared at week 4 and used thereafter. This new homogenate was adjusted to be the same protein concentration as the first batch, again using the Shakir (1994) modification of the Lowry procedure. Photosynthetic rates, translocation rates and percentages of photosynthate released were again calculated using the mean specific activity (g C per dpm) which was estimated from the added activity and an assumed inorganic carbon content of 0.024 g C per litre of seawater (Cook et al., 1992, Davy and Cook 2001a).

4.5 - Data Analysis

Nitrogen status was analyzed using the ammonium enhancement ratio (dark NH₄⁺ fixation rate/dark FSW fixation rate); calculated after all values were converted to grams carbon and zooxanthella counts were used to determine photosynthetic and dark fixation rates in grams carbon/cell/hour (as in Cook et al. 1992). Nitrogen status was also measured as the ratio $V_D: V_L$, where V_D = (dark NH₄⁺ fixation rate – dark FSW fixation rate) and V_L = light carbon fixation rate in FSW (Goldman and Dennett 1986).

Dark fixation rates in FSW and NH₄⁺ were compared at each time point with the Students t-test. All time points were compared using one-way ANOVA, followed by a pairwise Tukey test. In HRF experiments, the Students t-test was used to test for

differences between homogenate incubations and FSW incubations at each time point. Differences from FSW controls were tested with one-way ANOVA, again followed by pairwise Tukey comparisons.

Results

4.6 - Nutritional History and Zooxanthellar Nitrogen Sufficiency

Throughout the 8-week starvation period, the dark carbon fixation rates of zooxanthellae incubated in 40 μ M ammonium chloride were never significantly different than the controls incubated in filtered seawater (t-test, all p values > 0.05). (Figure 4.2)

Figure 4.2: Dark fixation rates of zooxanthellae incubated in FSW or 40 μ M NH_4^+ every 2 weeks throughout the 8-week starvation period. Values are mean + s.d. (n=5).

Throughout these trials, little evidence of nutritional deficiency of the zooxanthellae was seen in the ammonium enhancement ratios. No significant differences were detected throughout the 8 weeks of starvation, and the final value of 0.85 was no different from the value of the well-fed control group (0.87). (one-way ANOVA, $p > .05$).

Figure 4.3: Ammonium enhancement ratio of zooxanthellae isolated from *A. aureoradiata* and incubated in 40 μM NH_4^+Cl or FSW (dark NH_4^+ fixation rate/dark fixation in FSW rate) over 8-week starvation period. Values are mean + 1 s.d. ($n=5$).

Photosynthetic rates throughout the starvation period dropped from 11.8 pg C/cell/h to 3 pg C/cell/h over the first 2 weeks of starvation. The photosynthetic rate at week 0 was significantly higher than all other weeks, and the algae that were well fed throughout the 8 weeks had significantly higher photosynthetic rates than those starved for 2-6 weeks. They were, however, not different from starved zooxanthellae at week 8 (Tukey *post hoc* one-way ANOVA, $p < 0.05$) (Figure 4.4A).

Nitrogen status was also assessed by measuring $V_D:V_L$ (Figure 4.4B). This value was initially 0.007 and increased slightly before decreasing to -0.01 over the duration of the experiment. The only significant difference was for the starved algae at week 2 and week 8 (Tukey *post hoc* one-way ANOVA, $p < 0.05$).

A)

B)

Figures 4.4A&B: Figure 4.4A) Photosynthetic fixation rates of zooxanthellae in FSW isolated from *A. aureoradiata* incubated in the light every 2 weeks over the 8-week starvation period. Figure 4.4B) $V_d:V_l$ over 8 weeks of starvation for zooxanthellae isolated from *A. aureoradiata*. V_d = dark fixation rate for NH_4^+ incubation – dark fixation rate for FSW incubation; V_l = photosynthetic rate from FSW incubation in the light. All values are mean + 1 s.d. (n = 5)

4.7 - Translocation Rates and Starvation

Photosynthetic rates of zooxanthellae incubated in host homogenate were always lower than photosynthetic rates of those incubated in FSW throughout the 8-week starvation period, but this difference was only significant at weeks 2 and 8 (t-test $p < 0.05$). The photosynthetic rate did not change significantly from week 0 to week 8, with the only significant difference being at week 4 for both the control and the homogenate incubations (Tukey *post hoc* one-way ANOVA, $p < 0.05$) (Figure 4.5).

Figure 4.5: Photosynthetic rates of zooxanthellae isolated from *Anthopleura aureoradiata* incubated in host homogenate or in FSW controls over 8-week starvation period. All values are mean + 1 s.d. (n = 5)

Translocation rates of photosynthetic carbon from zooxanthellae were significantly lower in homogenate incubations than FSW incubations throughout the trial period (t-tests, all p values < 0.05). Week 4 had significantly higher translocation rates than any other week in both the control and host homogenate incubations, with a control rate nearly 5 times that of the next highest (Tukey *post hoc* one-way ANOVA, $p < 0.05$)(Figure 4.6A).

The percent of total fixed carbon released by the zooxanthellae (Figure 4.6B) appeared higher in FSW than host homogenate incubations in all weeks except for week 6, in which the two percents were nearly equal (t-test $p < 0.05$). The only differences that were significant were at week 2 and week 4. (Tukey *post hoc* one-way ANOVA, $p < 0.05$)

A)

B)

Figures 4.6A&B: 4.6A) Translocation rates of *A. aureoradiata* zooxanthellae incubated in either host homogenate or FSW and measured every 2 weeks over the 8-week starvation period. 4.6B) Translocation percent of zooxanthellae isolated from *A. aureoradiata* and incubated in either host homogenate or FSW. Percents measured every 2 weeks over the 8-week starvation period. All values are mean + 1 s.d. (n = 5).

Discussion

4.8 – Nitrogen Status

Typically the ammonium enhancement ratio and V_D/V_L rise throughout the starvation period as less nitrogen becomes available for zooxanthellar growth and creates more surplus carbon for potential release to the host. Furthermore, photosynthetic rates have been found to drop with starvation in some species. For example, Cook et al. (1992) found a 50% decline in the photosynthetic rate per cell when starved, and attributed it to decreasing chlorophyll *a* levels. Davy et al. (2006) also have attributed decreases in photosynthetic rates from nitrogen limitation to reduced levels of photosynthetic pigments in the algae (Davy et al. 2006). However, starvation experiments on the intact symbiosis by Davy and Cook (2001b) found that photosynthetic rates were sustained and even increased after prolonged starvation. This was attributed to increased CO₂ availability as zooxanthellar densities declined (Davy and Cook 2001b), though when isolated in FSW there is less likely to be CO₂ limitation, so this increase in photosynthetic rate would not be expected.

Zooxanthellae isolated from starved *Anthopleura aureoradiata* never appeared to be nitrogen deficient, even by the end of the 8-week starvation period. This may imply that it takes longer than 8 weeks of starvation to see evidence of a decrease in nitrogen sufficiency. It has been shown in previous studies of temperate species that starvation can take much longer than in tropical species (Davy et al 2006). One study using the coral *Plesiastrea versipora* only found evidence for nitrogen deficiency after 2 weeks of starvation (Davy et al. 2006), while another study starved the European sea anemone *Anemonia viridis* for 47 days in both dissolved inorganic nitrogen-enriched and low dissolved inorganic nitrogen conditions, and found continuing nitrogen sufficiency (Roberts et al. 2001). This suggests that zooxanthellae from temperate anemones might be less reliant on host feeding as a source of nitrogen, or that the host might have greater internal stores of nitrogen to supplement zooxanthellae when exogenous sources are less available (Cook et al. 1994; McAuley and Cook 1994). McAuley and Cook (1994) have also suggested that

zooxanthellae in some hosts might be more efficient at utilizing their own internal pools of nitrogen.

The lack of any response to nitrogen starvation in zooxanthellae from *Anthopleura aureoradiata* in these trials could also be due to some unknown external source of nitrogen, such as contamination of the nitrogen-free seawater, or even mortality and subsequent decomposition of other individuals in the bowl adding nitrogenous products to the water. During the first week of starvation, several of the anemones released brooded juveniles, some of which may have died during the 8 weeks and added ammonium to the water as they decomposed; in hindsight, removal of those juveniles may have resulted in a different response to starvation.

4.9 - Effects of Starvation on HRF Effects

In these trials, nitrogen deficiency was never achieved for the zooxanthellae, so conclusions cannot be drawn as to the influence of starvation on HRF response in the *Anthopleura aureoradiata*-zooxanthellae symbiosis. Previously, Davy and Cook (2001b) carried out a study looking at the effects of nitrogen limitation on translocation in starved zooxanthellae isolated from the anemone *A. pallida* and incubated in either FSW, *A. pallida* host tissue homogenate, or tissue homogenate from the coral *Montastraea annularis*. The results were markedly different from those found in the intact symbiosis. Translocation percentages in both host homogenate incubations increased from 14%-25% in *A. pallida* homogenate and 10%-30% in *M. annularis* homogenate over the 120-day starvation period. This was offset by a decrease in photosynthetic rate to about 72% of the original value over the starvation period. This decrease is such that the actual amount of photosynthate released was similar to or greater than the amount from well-fed zooxanthellae (Davy and Cook 2001b).

A second study of starvation on the percent translocation in intact *Aiptasia pallida* (a sea anemone) found no effects of starvation on translocation over a 114-day starvation period (Davy and Cook 2001a). It was also found that photosynthetic rates initially decreased, but then increased significantly after 14 days, which was attributed to increased CO₂ availability (due to lower densities of algae) that counteracted any

decline in chlorophyll levels. Translocation rates per cell followed the same trend as photosynthetic rates, resulting in the total amount of fixed carbon per mg host protein being unaffected by prolonged starvation (Davy and Cook 2001a).

Another previous study by McGuire and Szmant (1997) found that the coral *Porites astreoides* maintained in nitrogen-enhanced seawater (10 μ M) had decreased translocation rates when compared to controls in FSW. This could lend evidence to the theory that zooxanthellae release more carbon when they have more surplus from growth; in high-nitrogen conditions, zooxanthellae have more nitrogen available for growth and hence would utilize more carbon.

These studies suggest an ability to cope with sustained periods with low food availability, however the mechanisms involved seem very different in the intact symbiosis and in isolated zooxanthellae. This raises the issue that some responses of isolated zooxanthellae may be experimental artifacts, and further study is needed to determine the specific causes for these outcomes (Davy and Cook 2001b).

Discussion

5

This study found that in the intact *Anthopleura aureoradiata*-zooxanthellae symbiosis, zooxanthellae release 40% of their photosynthetically fixed carbon for use by the anemone. This concurs with results for translocation percentages found in other symbioses (Muscatine 1967; Davy et al. 1996; Engebretson and Muller-Parker 1999). Unlike other species, however, isolated zooxanthellae incubated in host homogenate showed no effects of any host release factor (HRF). Furthermore, incubations of heterologous zooxanthellae in *A. aureoradiata* tissue homogenate also showed no effects of HRF. The translocation of a substantial amount of fixed carbon in the intact association, but lack of any HRF response when the zooxanthellae are in isolation, raises two questions: 1) How might *Anthopleura aureoradiata* benefit from photosynthetic carbon in the field; and 2) How is carbon translocation regulated if there is no HRF operating in the anemone tissues? There was also evidence in one trial for PIF.

5.1 - How does the symbiotic relationship with zooxanthellae benefit *Anthopleura aureoradiata*?

Anemones living on mudflats are found beneath the surface of the mud at low tide. This means that about half of their time is spent with no sunlight, and limited access to particulate food. At high tide, the cockles that *A. aureoradiata* attaches to rise to the surface of the mud, allowing for feeding of anemones on planktonic food, and exposing the zooxanthellae to light. This exposure is limited, as water around mudflats is often turbid and sunlight in temperate zones is highly variable. This argues for mudflat anemones being predominantly heterotrophic.

A. aureoradiata living on rocky intertidal shores is exposed to much higher irradiance, more competition for space, and possibly fewer sources of exogenous carbon and nitrogen than when living on the mudflats. Rocky shores provide a greater potential for photosynthesis and thus may translocate more carbon to the anemones. This suggests that rocky shore anemones may be more autotrophic than mudflat anemones.

Even with the arguments for heterotrophy in *A. aureoradiata*, the carbon translocated may provide a competitive advantage for the anemones in all environments, especially those on the rocky shores. This is supported by several observations. This anemone is abundant and seems to be a dominant species in the environments in which it is found. In the mudflats the attachment to cockles allows the anemones to be above the surface of the mud at high tide and be exposed to sunlight, whereas on the rocky shores, anemones are found in positions with high exposure to sunlight, suggesting that they actively seek out sun exposure. Lastly, these anemones inherit zooxanthellae maternally through a closed system, so if there was no advantage to having the algae, it seems doubtful that they would go to such lengths to maintain the symbiosis. The benefit of translocated carbon may only be important to the anemones during periods of limited food availability, as even small amounts of translocated carbon can be stored and increase survival of anemones during periods of limited food supply.

Zooxanthellar carbon contributions may also only be important for rocky intertidal anemones, as with higher irradiance and more exposure to sunlight, there is greater potential for photosynthesis and more carbon translocated. Anemones living on mudflats may only have zooxanthellae because there is no pressure to get rid of them.

It is also possible that the zooxanthellae are actually parasitic, using the anemone for protection and nutrients, releasing carbon in the intact symbiosis to bypass defense mechanisms of the host. The zooxanthellae may also be an evolutionary artifact of tropical symbioses, or may have evolved to be of no consequence to the anemone while receiving protection in its tissues.

5.2 - How is carbon translocation regulated?

No evidence was found throughout the previous experiments for the presence of a host release factor compound. The homogenization process for the host tissues has been shown to damage animal cell structure, and thus may destroy the compound responsible for inducing photosynthate release in zooxanthellae (Muscatine et al. 1994). It has also been shown that zooxanthellae isolated from the host undergo physiological changes that may affect their ability to respond to signals (Goiran et al. 1997). There is also no evidence for a host release factor compound in any intact symbiosis (Davy and Cook 2001b). However, 40% of fixed carbon is translocated to the animal by zooxanthellae in the intact symbiosis, which indicates some form of control by the host. If there is no specific HRF compound, how is the release of carbon from zooxanthellae controlled?

If zooxanthellae in the field were deficient in nitrogen, expectations would be to see surplus carbon released by zooxanthellae without stimulation, but as recent studies have shown that zooxanthellae in *A. aureoradiata* are nitrogen sufficient in the field (S. Morar, unpublished), this is unlikely to play a role in translocation control in this species. It has previously been suggested that carbon translocation in the intact symbiosis is regulated by the host reducing the permeability of the symbiosome membrane (Muscatine 1983; Rands et al. 1993). This is possible, but there is no empirical evidence for it and further study would be needed. Another possibility is the presence of some other type of signaling molecule in the host tissues, perhaps a protein, or other complex molecule that has not yet been considered in the release of carbon in the intact symbiosis, but is destroyed by the homogenization process.

With advances in genomic technologies and understanding, the role genetics might play in this symbiosis has been questioned. Current technologies make it possible to examine changes in gene expression of thousands of genes with changing conditions, such as a change in symbiotic state, change in age or form, or with the onset of environmental stress. Once identified by genomic studies, target genes and their associated pathways may be subjected to further empirical studies at the cellular level to uncover their role in symbiosis (Weis et al. in press.). On this topic, recent work has been done investigating the hypothesis that there are genes present in either the host or the zooxanthellae that are “switched on” when the symbiosis is formed

that could possibly regulate translocation (Weis and Levine 1996, Weis and Reynolds 1998). DeBoer et al. (2007) found limited evidence for a change in protein profiles during the establishment of a symbiosis in the coral *Fungia scutaria*. Further studies have shown that protein profiles in established symbiotic and aposymbiotic anemones are different, and symbiotic anemones produce a unique set of proteins (Weis and Levine 1996; Rodriguez-Lanetty et al. 2006). Genetic techniques have led to the characterization of a “symbiosis protein” known as sym32 (Reynolds et al. 2000). This protein was discovered to be the most abundantly expressed symbiosis-enhanced protein in *Anthopleura elegantissima* and has been suggested to be involved in regulation of symbiosis (Reynolds et al. 2000). However, further work is needed to clarify the role of genetics in the control of translocation in symbiotic relationships.

In conclusion, *Anthopleura aureoradiata* shows no evidence of a host release factor present in homogenized tissues, but still receives photosynthetic carbon from symbionts in the intact symbiosis. This work suggests that not all organisms have an HRF that operates in vitro, but does not preclude the potential for its existence in the intact symbiosis. More work is needed to determine the controlling mechanism for translocation in cnidarian-dinoflagellate symbioses. With recent advances in genomic and molecular techniques to study gene expression, the host factor can be thoroughly studied to see if it is a protein expressed by a gene in symbiotic hosts only, or if it is an amino acid or other compound found in both symbiotic and aposymbiotic organisms. Only when the host factor in the intact symbiosis is defined can it be determined if host release factor in isolation experiments is an experimental artifact.

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Appendix

Re-infection of *A. aureoradiata* with heterologous and homologous zooxanthellae

To test whether any different responses to host homogenate by the cultured heterologous or freshly isolated homologous zooxanthellae were affected by the capacity to form a symbiosis with *Anthopleura aureoradiata*, aposymbiotic (anemones with no algal symbionts present) were established by heat-shocking symbiotic anemones by immersion in 32°C sea water for 6 hours, then placement in darkness for 6-12 months. During this period, anemones were fed *Artemia* sp. *nauplii* every 2 weeks and bowls were cleaned out in low light 24 hours after each feeding. Aposymbiotic status was established by fluorescent microscopy (Olympus Pro AX 70) and 6 hemocytometer counts done by homogenizing single anemones in a Wheaton ground glass tissue grinder with 2 mL FSW. Anemones were then fed once weekly with *Artemia* sp. *nauplii* and bowls were again cleaned out 24 hours after each feeding. This more regular feeding was to prevent too much shrinkage of the anemones. All feeding and cleaning was carried out under red light to prevent re-growth of residual zooxanthellae.

To establish a time frame for full re-infection of anemones, a preliminary re-infection study (Experiment 1) was conducted. Twelve aposymbiotic anemones were infected with 4 different clades of zooxanthellae: 3 of these clades were from cultures: Pd, Sin, A001 (see Table 3.1 p.32) for clade assignment and geographical information), with the fourth clade (clade A) being represented by the freshly isolated zooxanthellae (FIZ) from *A. aureoradiata*. FIZ were obtained by homogenizing 15 anemones in a Wheaton ground glass tissue grinder. The homogenate was centrifuged at 12,000 g for 5 minutes and the supernatant poured off. The algal pellet was resuspended and then spun again at 12,000 g for 5 minutes and the supernatant decanted. The algal pellet was then resuspended in 5 mL FSW and 8 hemocytometer counts conducted. Cultures were prepared by swirling the culture flask and pipetting 10 mL into a clean centrifuge tube. Zooxanthellae were then centrifuged at 12,000 g

and resuspended in 3 mL FSW to concentrate algal cells. 8 hemocytometer counts were taken of each culture and FSW added to normalize algal suspensions to 2.78 million cells/mL (the lowest cell concentration). All infections were carried out under a dissecting microscope. Anemones were allowed to settle each in an individual container overnight. Infection was done using a 1mL syringe with the sharp tip trimmed off the needle for better control (Figure A1.1). 1mL of concentrated zooxanthellae extract (2.78 million algal cells per mL) was injected into the mouth of the anemone. This was followed with an injection of *Artemia* sp. *nauplii* extract, prepared by homogenizing then freezing concentrated *Artemia* sp. *nauplii* to facilitate zooxanthellae uptake (Davy et al. 1997). Water was changed 24 hours after re-infection to remove any zooxanthellae and *Artemia* sp. not captured by the anemone. Zooxanthellar concentrations for this re-infection were standardized to 2.78 million cells per mL for all clades.

Figure A1.1 – Re-infection technique for infection of *A. aureoradiata* with homologous and heterologous zooxanthellae.

Infection status was checked after 2 weeks by homogenizing anemones in 5 mL FSW in a Wheaton ground glass tissue-grinder, centrifuging for 5 minutes at 12,000 g and resuspending in FSW, then centrifuging and resuspending in FSW a second time to ensure that little animal material remained. Cells were then counted using a Fuchs Rosenthal hemocytometer ($n = 8$). Infection status was re-checked after 4 weeks, still using 8 hemocytometer counts for each anemone. The trial was ended at this point, as no zooxanthellae were detected in re-infected anemones.

After preliminary attempts to re-infect *A. aureoradiata* with heterologous or homologous zooxanthellae were unsuccessful, concerns were raised about the accuracy of the placement of zooxanthellae into the mouth, and the possibility that *A. aureoradiata* would not take up heterologous zooxanthellae. The infection method was modified to use magnesium chloride (MgCl_2) as an anesthetizing agent prior to re-infection. MgCl_2 was used by Davy et al. (1997) with successful re-infection and no apparent negative or ongoing effects of the MgCl_2 on the anemones. In experiment 2, five mL MgCl_2 were added to each container 4 hours before the reinfection. Eight anemones were re-infected with freshly isolated zooxanthellae at a density of 3.13 million cells per mL using the same technique as before. Symbiotic status was checked 2 and 4 weeks after re-infection with 8 hemocytometer cell counts and anemone squashes under the fluorescent microscope.

Re-infection following continued exposure of aposymbiotic anemones to symbiotic anemones or to a suspension of zooxanthellae was tested in experiment 3. Three treatments were used: a control consisting of 5 aposymbiotic anemones, each in a separate dish; Five dishes, each containing 1 re-infected anemone and 1 symbiotic anemone; Five dishes, each containing 1 aposymbiotic anemone that was re-infected daily, as described previously. Symbiotic status was checked after 2 weeks using 8 hemocytometer counts of each anemone.

It is possible that there is incorporation of the algae on a short-term timescale, then subsequent expulsion at a later point in time. Experiment 4 was done to test for this short-term re-infection potential. Nine aposymbiotic anemones were re-infected as described previously, with the zooxanthellae at a concentration of 3.66 million cells per mL. Water was changed 1 hour after re-infection. Symbiotic status was checked at 0 hours, 12 hours, 24 hours, and 48 hours using hemocytometer counts. For each count, each anemone was homogenized separately in 2 mL FSW and centrifuged at 12,000 g for 5 minutes to isolate zooxanthellae. The algal pellet was resuspended in 1.5 mL FSW and counts taken from this. Anemones checked at 0 and 12 hours were cut in half and the gut was rinsed to eliminate unincorporated zooxanthellae before homogenization.

A fifth experiment was run to further investigate the possibility of short-term incorporation of the zooxanthellae. Aposymbiotic anemones (n=45) were allowed to settle, each in separate dishes, overnight. Freshly isolated zooxanthellae were prepared at a concentration of 2.98 million cells/mL as previously described. Anemones were re-infected as before using a 1 mL syringe, and the FSW was changed after 1 hour. Five control aposymbiotic anemones and 5 re-infected anemones were homogenized separately in 5 mL FSW in a Wheaton ground glass tissue grinder and counted with a hemocytometer at 0 hours. Counts of 5 re-infected anemones were then taken at 1, 2, 4, 6, 12, and 24 hours. Anemones at time points from 0 to 6 hours were cut in half and the gut rinsed before counts to wash away unincorporated algae.

Results

Re-Infection

None of the first three re-infection attempts showed evidence of incorporation after either 2 or 4 weeks. All attempts were checked with 8 hemocytometer counts per anemone, and none came up with a value greater than 2500 cells per mL when anemone was homogenized, centrifuged and re-suspended in 1 mL FSW, which was judged to indicate lack of incorporation by *A. aureoradiata*.

The continued exposure trial (experiment 3) also found no evidence for uptake of homologous zooxanthellae and was ended when all stock of photosynthetic anemones to provide freshly isolated zooxanthellae were used. This trial lasted 10 days and no incorporation was seen.

In experiment 4, 8 hemocytometer counts were taken 0 hours, 12 hours, 24 hours, and 48 hours after re-infection (Table A1.1).

Table A1.1: Average of 8 hemocytometer counts taken at timepoints after re-infection of aposymbiotic *A. aureoradiata* with homologous zooxanthellae

Timepoint	Count (cell/ mL)
0 h control	0
0 h	2000
12 h	35800
24 h	5000
48 h	5000

Experiment 5

Figure A1.2: Cell counts of homologous zooxanthellae in reinfected *Anthopleura aureoradiata* at 0, 1, 2, 3, 6, 12, and 24 hours after infection from experiment 5 as described above.

Short-term re-infection, as in experiment 5, showed decreasing incorporation for 4 hours and then an increase until 12 hours, when it seemed to level off (Figure A1.3).

Discussion

Aposymbiotic hosts from other species have previously been successfully re-infected with both homologous and heterologous zooxanthellae (Berner et al. 1993; Davy et al. 1997; Rodriguez-Lanetty et al. 2003). None of the attempted re-infections of aposymbiotic *Anthopleura aureoradiata* were successful as carried out above.

This is potentially due to short experiment lengths, incorrect counting, and assuming the infection had failed when there were in fact small numbers of zooxanthellae. Davy et al. (1997) found the sea anemone *Cereus pedunculatus* re-infected with heterologous and homologous zooxanthellae to have detectable numbers within 4 days, and to have levels similar to natural symbioses within 4 weeks. Rodriguez-Lanetty et al. (2003) judged re-infection of the anemone *Heteractis* sp. to be when the anemones showed color, and was found after up to 60 days. Trials in this section were often abandoned after 4 weeks if levels of zooxanthellae were low, raising the possibility that re-infection is possible in *A. aureoradiata*, it just needs more time and better designed experiments.

There is also the possibility that this anemone cannot be re-infected once it is rendered aposymbiotic. Rodriguez-Lanetty (2003) also found a few specimens that did not re-infect after 4 months of exposure. Perhaps aposymbiotic specimens of *A. aureoradiata* lose some cell-signalling molecule or fail to recognize zooxanthellae.

These studies above were often cut short and were side experiments meant as an introduction to a longer experiment. As re-infection did not appear successful after 4 weeks, the side experiments were abandoned completely and no further work was carried out to investigate if the lack of re-infection was based on poor experimental design or some change in the anemones themselves.