The influence of symbiont diversity on the functional biology of a model sea anemone

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Abstract

Cnidarian–dinoflagellate symbioses, particularly those between anthozoans and dinoflagellates of the genus *Symbiodinium* (commonly referred to as zooxanthellae) are widespread in the marine environment. They are responsible for the formation of coral reefs and are thus of great ecological importance. In recent years there has been an increase in the frequency and severity of episodes of coral bleaching resulting in degradation and mortality of coral reefs on a global scale. In order to gain a deeper understanding of how corals can adapt to changing environmental conditions, the effect that symbiont type has on the persistence and physiology of an association needs to be ascertained. The aim of this research was to determine how different symbiont types affect the nutritional biology and intracellular physiology of the symbiosis when in association with the sea anemone *Aiptasia pulchella*. The specific objectives of the study were to; (1) determine whether different symbiont types are equally as adept at supporting the energetic demands of the same host; (2) determine if internal pH (pHi) is a reflection of symbiont type and whether the optimal pH for photosynthesis coincides with the host cell pHi; and (3) test the influence of *Symbiodinium* type on host tissue glycerol and glucose pools.

In order to answer these questions, aposymbiotic (i.e. symbiont-free) sea anemones were infected with different *Symbiodinium* types and the relationship between symbiont type, photosynthetic performance and autotrophic potential was tested. A range of 'normal' and novel cnidarian–dinoflagellate symbioses was also used to measure host cell pHi and to determine the optimal pHi of isolated intact symbiosomes (i.e. the vacuoles that house the symbionts), as well as to compare the amounts of free glycerol and glucose (metabolites) present in the host tissues. Different host-symbiont combinations were found to have different photosynthetic and respiratory attributes. Earlier onset of full autotrophy (i.e. when all metabolic carbon demands of the symbiosis were met by photosynthesis) and higher CZAR values (i.e. the contribution of zooxanthellae to animal respiration) were demonstrated by symbioses hosting *Symbiodinium* B1 both from the original (homologous) and different (heterologous) host. The study showed that

Symbiodinium types differ in their pH optima and that the optimal pHi for photosynthesis does not always match the actual measured pHi. Symbiont type was also shown to have an effect on host tissue glycerol and glucose pools, with the associations harbouring the homologous *Symbiodinium* B1 attaining the highest concentrations of both metabolites.

Findings from this study suggest that corals may be able to maintain an association with a range of *Symbiodinium* types, and hence potentially switch as a consequence of bleaching. The new symbiont type may not be as nutritionally advantageous as the original type however, which could have implications for the growth and survivorship of the coral, unless it is able to supplement its carbon demands heterotrophically. The rapid proliferation of some of the heterologous *Symbiodinium* types (e.g. *Symbiodinium* E2) inside the host indicates that, after bleaching, there is potential for fast symbiont establishment. The reduced carbon contribution of these heterologous symbionts may not be a major concern should the coral be able to reinstate the more nutritionally advantageous symbiont as the dominant type during bleaching recovery. Finally, the rapid proliferation demonstrated by the heterologous *Symbiodinium* types and the associated metabolic cost to the host, could be an indication of the opportunistic nature of some of these types and may indicate a shift towards parasitism. It is imperative to extend this type of work to corals in the field to determine how these associations behave in nature. Also, in order to get a clearer picture of the diversity in symbiosis physiology, a wider range of *Symbiodinium* types needs to be investigated.

Contributions and Publications

This thesis is written as a series of manuscripts which are to be submitted in the near future. All laboratory work and writing were conducted by the Author.

Chapter 2

This chapter is formatted as a stand-alone manuscript and is going to be submitted for publication: Starzak, D. E., Quinnell, R. G., Nitschke, M. R., Davy, S. K. (in prep.). The influence of symbiont diversity on the photophysiological performance of novel cnidarian–dinoflagellate symbioses. All laboratory work and analyses were completed by the Author. M. R. Nitschke assisted with data collection for the *A. pulchella*-cultured homologous *Symbiodinium* association. R. G. Quinnell and S. K. Davy, advised on experimental set-up, analyses and writing.

Chapter 3

This chapter is formatted as a stand-alone manuscript and is going to be submitted for publication: Starzak, D. E., Gibbin, E., LaFlamme, A. C., Quinnell, R. G., Davy, S. K. (in prep.). The influence of symbiont type on host cell pH and physiology of a novel cnidarian–algal association. All laboratory work and analyses were completed by the Author. E. Gibbin assisted with the confocal microscopy for the *A. pulchella*–homologous *Symbiodinium* association. R. G. Quinnell, A. C. LaFlamme and S. K. Davy, advised on experimental set-up, analyses and writing.

Chapter 4

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Abbreviations

2-D:	two dimensional
ANOVA:	analysis of variance
APX:	ascorbate peroxidase
ATP:	adenosine triphosphate
CO2:	carbon dioxide
CZAR:	fractional contribution of translocated zooxanthellar carbon to the host's daily respiratory carbon requirements
DAPI:	6-diamidino-2-phenylindole dihydrochloride, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride
DIC:	dissolved inorganic carbon
DMSO:	dimethyl sulfoxide
FAAs:	free amino acids
FITC:	fluorescein isothiocyanate
FSW:	0.44 µm filtered seawater
HCl:	hydrochloric acid
HCO_3^-:	bicarbonate
HRF:	host release factor
ITS:	internal transcribed spacer
LSD:	least squares difference
MI:	mitotic index
NADPH:	nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH:	sodium hydroxide
NCBI:	National Center for Biotechnology Information
OH^-:	hydroxide
PAR:	photosynthetically-active radiation
P_{Net}:	net photosynthetic production
PCR:	polymerase chain reaction
P/DOC:	particulate and/or dissolved organic carbon
P_{Gross}:	total photosynthetic production
pHi:	internal pH inside the host cells
PIF:	photosynthesis-inhibiting factor

P:R ratio:	ratio of maximum gross photosynthesis (P) to symbiosis respiration (R) over 24 hours
P_{zNet}:	total net carbon fixed photosynthetically by zooxanthellae
R:	546/635 nm fluorescence intensity ratio
R_a:	host animal respiration
RNAi:	RNA interference
ROI:	region of interest
ROS:	reactive oxygen species
RuPB:	ribulose-bis-phosphate
Rz:	zooxanthellar respiration
SOD:	superoxide dismutase
T:	percentage of total net carbon (P_{zNet}) translocated to the animal
TCA:	trichloroacetic acid
TEM:	transmission electron microscope
UVR:	ultraviolet radiation

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Chapter 1: General introduction

1.1. Symbiosis

Symbiotic associations were first formally described by the German mycologist Heinrich Anton de Bary, who coined the term "symbiosis". He defined symbiosis to be a relationship that is "constant, intimate and between dissimilar species" (de Bary, 1879). Symbiotic relationships include those associations in which one organism lives on another (ectosymbiosis, such as mistletoe), or where one partner lives inside the other (endosymbiosis, such as bacteria in humans or symbiotic algae in corals). Three broad types of symbiotic relationships have been recognized; mutualism, parasitism and commensalism (Smith, 1993). Mutualism is defined as a biological interaction between two different species where both partners derive a fitness benefit such as increased survival. Parasitism is a relationship whereby one partner derives a benefit but the symbiosis comes at a cost to the other partner. Commensalism, on the other hand, occurs when one partner benefits from the association and the other does not but is not harmed by it. However, many symbiotic associations are complex or poorly understood and do not fit neatly into a single category (Douglas, 1994). Therefore, it is better to visualise symbiosis as a continuum that spans from parasitism to mutualism (Figure 1.1; Dimijian, 2000) with a given relationship being able to shift gradually or abruptly along the continuum. Symbiosis can be further defined as facultative, where the relationship is beneficial but not essential to the survival of either partner; or obligate, where the relationship is essential to the survival of at least one partner (Vega *et al*., 2006).

1.2. Cnidarian–dinoflagellate symbioses

Cnidarian – dinoflagellate symbioses are widespread in the marine environment and consist of associations between anthozoans (e.g. corals, sea anemones, zoanthids and gorgonians) and dinoflagellates (commonly referred to as zooxanthellae) of the genus *Symbiodinium* (Trench, 1987; Furla *et al*., 2005). These symbioses are responsible for the formation of coral reefs which, in turn, maintain a rich

biodiversity in shallow tropical waters. They are of great ecological importance as coral reefs are vital in sustaining the coastal communities in these regions (Peterson and Lubchenco, 1997; Linden *et al.,* 2002).

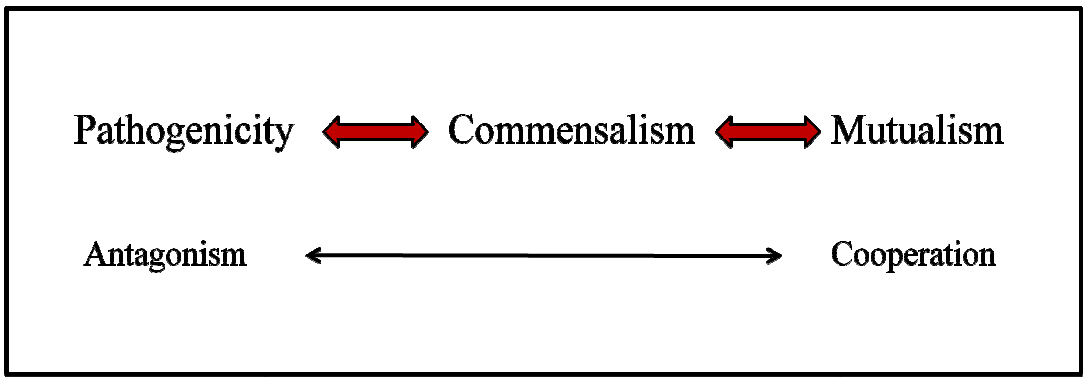

Figure 1.1. A schematic diagram representing the continuum that exists between antagonistic and cooperative symbiotic relationships. Antagonistic relationships occur between hosts and parasites (or pathogens), cooperative relationships between mutualists, and mixed interactions between commensals (adapted from Dimijian, 2000).

The presence of the dinoflagellate symbionts contributes substantially to the productivity, survival and success of their hosts (Muscatine and Porter, 1977). A true mutualistic relationship exists between the host and symbiont, in that the symbiont receives inorganic nutrients from the host and provides the host with translocated photosynthetic products (Muscatine, 1990). In Cnidaria, the *Symbiodinium* cells are generally intracellular, located in cells of the endodermis (Schoenberg and Trench, 1980a; Douglas, 2003), and a perisymbiont membrane of animal origin separates them from the host cytoplasm (Wakefield and Kempf, 2001).

Freudenthal (1962) formally described the taxonomy, life cycle and morphology of these dinoflagellates and erected the genus *Symbiodinium* to encompass the symbiotic dinoflagellates associated with a phylogenetically diverse range of invertebrate hosts. It was first considered that symbiotic dinoflagellates were members of a single pandemic species, *Symbiodinium microadriaticum* (Freudenthal, 1962; Taylor, 1971 and 1974). In the mid-1970s, however, evidence began to accumulate that these dinoflagellates in fact exhibit a high degree of genetic diversity

and, at present, eleven formally described species plus multiple clades and sub-clades have been identified in the genus *Symbiodinium* (Baker, 1999; Coffroth and Santos 2005; Pochon and Gates, 2010).

Molecular studies using small subunit ribosomal DNA (ssrDNA) sequences have yielded nine distinct clades (A-I) of *Symbiodinium* (reviewed in Baker, 2003 and Coffroth and Santos, 2005; Pochon and Gates, 2010). Five of these clades (A-D, F) are known to occur in corals (Rowan and Powers, 1991; Baker, 1999; Carlos *et al*., 1999; LaJeunesse, 2001; Toller, 2001a, b; Ulstrup and van Oppen, 2003). With the advent of more sophisticated molecular tools, the biodiversity of the *Symbiodinium* genus has become increasingly well documented. Analyses of the internal transcribed spacer (ITS) region have revealed within-clade variation amongst *Symbiodinium* isolates (Hunter *et al.,* 1997; LaJeunesse 2001, 2002; Santos *et al.* 2001; van Oppen *et al.* 2001; Diekmann *et al.* 2003; LaJeunesse *et al*., 2003, 2004a, b). Although numerous studies have assessed the biodiversity of both the coral host and symbiont partners individually (Vollmer and Palumbi, 2002; Coffroth and Santos, 2005), the diversity of host-symbiont combinations is still poorly understood (Baker, 2003; Goulet, 2006), and a better understanding of the mechanisms that confer recognition and specificity between the host and symbiont is necessary (Weis *et al*., 2008). Physiological differences (e.g. in thermal tolerance) have been detected between these *Symboidinium* ITS types (Iglesias-Prieto and Trench 1997; Kinzie *et al*., 2001; LaJeunesse *et al*., 2003). The presence of these differences has led to the suggestion that these symbiont types may affect their host's sensitivity to environmental conditions and/or that changes in environmental conditions may affect the distribution of symbiont types (van Oppen *et al*., 2005).

1.3. Symbiosis structure

There are numerous examples of symbioses in nature where one organism lives inside the cell cytoplasm of another i.e. endocytobiotic associations. These include: prokaryote-eukaryote associations (Jeon and Jeon, 1976; Lerouge *et al.,* 1990; Regensburg-Tuink and Hooykaas, 1993; Udvardi and Day, 1997; Ferrari *et al.,*1999),

and eukaryote-eukaryote associations such as protozoan parasite-vertebrate symbioses (Sibley and Krahenbuhl, 1989; Hall *et al.,* 1991; Russell *et al.,* 1992; Sam-Yellowe, 1992; Collins *et al.,* 1997; Forero *et al.,* 1999) and microalgal-invertebrate symbioses (reviewed by Lee and Anderson, 1991; Trench, 1993). In most symbiotic associations, membranes surround the microsymbiont and separate it from the host cell cytoplasm. The terms "symbiosome" and "symbiosome membrane" were introduced by Roth *et al*. (1988) to describe the intracellular symbiotic compartment in which the symbiont resides and its surrounding membrane. In cnidarians, the symbiosome has been defined as the host-derived outer membrane, *Symbiodinium* algal cell and the space between the two, including a series of multilayered membranes (Wakefield *et al*., 2000; Wakefield and Kempf, 2001). The barrier between the host and symbiont may thus be more accurately described as a "membrane complex", with the outer host-derived membrane acting as the "definitive symbiosome membrane" (Figure 1.2; Kazandjian *et al*., 2008). The origin and nature of the multilayered membranes immediately surrounding the cell wall of the alga have been debated, with several studies suggesting that they are derived from the host (Tripodi and Santisi, 1982; Colley and Trench, 1983; Palincsar *et al.,* 1988; Rands *et al.,* 1993), while others have proposed an algal origin (Kevin *et al.,* 1969; Taylor, 1971; Trench and Winsor, 1987; Wakefield *et al.,* 1998, 2000). Using immunocytochemical methods, Wakefield and Kempf (2001) confirmed that the membranes originate from the algal symbiont and are held in place by the presence of a single host-derived membrane.

1.4. Symbiont transmission, uptake and establishment

There are two modes of transmission of symbionts from host to host: (1) From parent to offspring (vertical transmission) and (2) the acquisition of symbionts from the environment (horizontal transmission) (Trench, 1979; Douglas, 1994). Whereas vertical transmission occurs within the confines of the host's sexual cycle, horizontal transmission occurs via phagocytosis resulting in the formation of a symbiont-containing host-derived vesicle (Smith and Douglas, 1987; Wakefield and Kempf, 2001). Free-living *Symbiodinium* cells are ingested probably via the host coelenteron.

The cells are captured by the tentacles and transported by ciliated grooves on the tentacles and/or taken into the coelenteron with incoming seawater (Fitt and Trench, 1983). Inside the coelenteron, the algal cells are phagocytosed by the host gastrodermal cells and invested with a host-derived membrane (Kinzie, 1974; Fitt, 1984). Molecular mechanisms are likely to be involved in host-symbiont recognition. The identity of both the host and the symbiont partner has been shown to determine the success of the symbiosis (Coffroth *et al*., 2001; Weis *et al*., 2001; Belda-Baillie *et al*., 2002; Baker, 2003; Rodriguez-Lanetty *et al*., 2004, 2006a). Past studies have shown that cnidarian hosts are specific towards a unique symbiont type and that surface recognition plays a role in host-symbiont discrimination (Jolley and Smith, 1980; Schoenberg and Trench, 1980b; Colley and Trench, 1983; Logan *et al*., 2010; Bay *et al*., 2011). There is also biochemical and molecular evidence for the presence of glycoproteins on the cell surface of symbiotic dinoflagellates (Markell *et al.,* 1992; Jimbo *et al.,* 2000; Bay *et al*., 2011; Logan *et al*., 2010), while some studies have identified and characterised lectins on the surface of host cells that may be involved in a lock-and-key type recognition process (Fenton-Navarro *et al.,* 2003; Koike *et al.,* 2004; Jimbo *et al.,* 2005; Wood-Charlson *et al*., 2006; Wood-Charlson and Weis, 2009; Kvennefors *et al*., 2010).

The onset of symbiosis in cnidarian/algal associations has been studied in a wide range of partnerships such as symbioses between: (i) the freshwater hydroid *Hydra viridis* and a chlorophyte (Muscatine *et al*., 1975, reviewed by Jolley and Smith 1980); (ii) the scyphozoan *Cassiopeia xamachana* and the dinoflagellate *Symbiodinium microadriaticum* (Colley and Trench 1983, 1985; Fitt and Trench 1983); and (iii) a variety of anthozoans and *Symbiodinium* spp. (e.g. Kinzie 1974; Schoenberg and Trench 1980b; Davy *et al*., 1997; Coffroth *et al*., 2001; Belda-Baillie *et al*., 2002; Rodriguez-Lanetty *et al*., 2003; Logan *et al*., 2010; Rodriguez-Lanetty *et al*., 2006a). From these studies it was established that a winnowing process occurs in cnidarian/algal symbioses (Jolley and Smith, 1980; McAuley and Smith, 1982; Dunn and Weis, 2009). This process includes interaction between putative molecular signals on the surfaces of symbiont and hosts cells (Meints and Pardy, 1980; Markell *et al*., 1992; Lin *et al*., 2000; Koike *et al*., 2004), discrimination by the host early in infection between its own symbionts (homologous symbionts) and those from other host species (heterologous symbionts) (Pool, 1979;

Jolley and Smith, 1980; Schoenberg and Trench, 1980b; Weis *et al*., 2001; Belda-Baillie *et al*., 2002; Rodriguez-Lanetty *et al*., 2004; Markell and Wood-Charlson, 2010), and a succession of different algal phylotypes later in host development (Rahat, 1991; Coffroth *et al*., 2001; Little *et al*., 2004; Gómez-Cabrera *et al*., 2008).

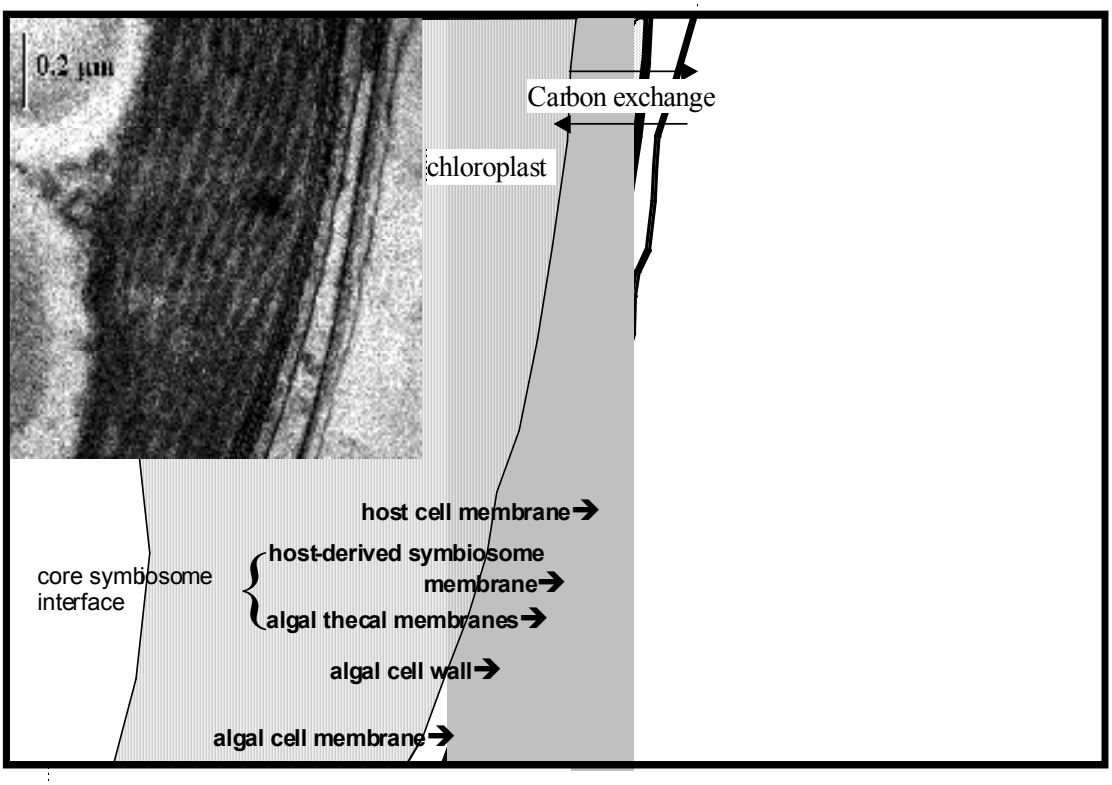

Figure 1.2. The symbiosome membrane complex – a transmission electron microscope (TEM) and graphic representation (obtained from Dr Rosanne Quinnell).

Little is known, however, about the cellular and molecular signals involved in maintaining the intracellular cnidarian – dinoflagellate symbiosis (Rodriguez-Lanetty *et al*., 2006b). How, for example, is the algal symbiont able to escape the host's immune response once inside the host cell, allowing for the successful persistence of a cnidarian-dinoflagellate symbiosis? It is possible that dinoflagellates may be utilizing or modifying the cnidarian's innate immune signalling pathways in order to avoid digestion of the phagosome in which they reside. Past studies of animal-algal symbioses have shown that, unlike phagosomes containing food particles, or dead or photosynthesis-impaired algae, the phagosomes containing live and functional algae do not show any signs of lysosomal fusion (Hohmann *et al*., 1982; Fitt and Trench,

1983). This evidence indicates that live and functional zooxanthellae possess the ability to interfere with the normal path of phagosomal maturation so as to survive inside cnidarian cells (Chen *et al*., 2003, 2004, 2005; Hong *et al*., 2009). Studies of prokaryote-eukaryote symbioses have shown that, after entering the host cell, certain bacteria such as *Mycobacterium tuberculosis* (Armstrong and Hart, 1975), *Chlamydia psittaci* (Friis, 1972) and *Legionella pneumophila* (Horwitz, 1983) are able to avoid digestion by preventing fusion of the phagosome (vacuole containing the symbiont) with the host's lysosomes. The protozoan *Toxoplasma gondii* (Jones *et al*., 1972) and the alga *Chlorella* sp. (Hohman *et al*., 1982) are also capable of avoiding digestion in this way. Kim *et al*., (1994) identified a 96 kDa protein and endosymbiont-derived lipopolysaccharides on the symbiosome membrane in a symbiosis between *Amoeba proteus* and a strain of gram-negative bacteria that were shown to be involved in the prevention of lysosome-symbiosome fusion.

Intracellular vesicular transport during endocytosis and exocytosis is thought to be mediated by Rab small GTP-binding proteins (peripheral membrane proteins). These proteins have been localized to distinct intracellular structures (Pfeffer, 2001; Zerial and McBride, 2001) and regulate fusion between various endocytic compartments by cycling between the GTP-bound, membrane-associated active state and the GDP-bound, cytosolic inactive state (Pfeffer, 2001; Zerial and McBride, 2001). Rab 5 and Rab 7 have been shown to act coordinately and sequentially to facilitate the traffic of internalized materials to lysosomes. Rab 5 regulates fusion between clathrin-coated vesicles and early endosomes and between early endosomes (Bucci *et al*., 1992; Barbieri *et al*., 1994; Mukhopadhyay *et al*., 1997), whereas Rab 7 controls the fusion between early and late endosomes (Feng *et al*., 1995; Mohrmann and van der Sluijs, 1999), and between late endosomes and lysosomes (Papini *et al*., 1997; Vitelli *et al*., 1997; Mohrmann and van der Sluijs, 1999). In past studies, ApRab7 and ApRab5, Rab7 and Rab5 homologues of the sea anemone *Aiptasia pulchella* were cloned and it was established that the intracellular persistence of the zooxanthella-containing phagosomes involves specific algal-mediated interference with the expression of ApRab5 and ApRab7, which results in the selective retention of the former on (Chen *et al*., 2003) and exclusion of the latter from the organelles (Chen *et al*., 2004). Later, Chen *et al*. (2005) found that the homologue of Rab11 (ApRab11), a key endocytic recycling regulator, was excluded from phagosomes in which healthy zooxanthellae

reside. More recently ApRab3 was identified as a new member of the Rab3 subfamily, associating with symbiosomes and accumulating on the maturing phagosomes in the *A. pulchella* digestive cells. It is has been shown to participate in the biosynthetic trafficking pathway, and symbiosome biogenesis has been demonstrated to involve interaction with ApRab3-positive vesicles (Hong *et al*., 2009). Thus, it appears that zooxanthellae must interfere with the normal recycling process required for efficient phagosome maturation, and thereby secure their intracellular persistence and consequently their endosymbiotic relationship with their cnidarian host (Chen *et al*., 2005).

The examination of either broad-scale patterns of RNA or protein expression in symbiotic cnidarians or the identification of specific genes that play a role in signal transmission between host and symbiont are still in their early stages (Rodriguez-Lanetty *et al*., 2006b; Bay *et al*., 2009, Voolstra *et al*., 2009, Ganot *et al*., 2011). A number of studies have, however, attempted to compare the gene expression patterns of symbiotic *versus* aposymbiotic individuals to determine which genes may play a role in governing the symbiotic association. Using two-dimensional proteomic analysis to compare symbiotic and naturally occurring aposymbiotic individuals, Weis and Levine (1996) revealed a number of proteins that were up- or down-regulated in the symbiotic state of the US Pacific coast temperate anemone *Anthopleura elegantissima*. One of the strongly expressed symbiotic proteins, sym32, was later identified as belonging to the Fasciclin I protein family which includes proteins that function in cell-cell interactions or cell adhesion in other organisms (Reynolds *et al*., 2000). Further immunocytochemistry and immunoblot studies by Schwarz and Weis (2003), using an anti-sym32 antibody, found a putative homologue in the symbionts. Their study revealed that sym32 is relocated from gastrodermal vesicles (where it was found in aposymbiotic anemones) to the symbiosome membrane when symbionts are phagocytosed by host cells. Thus the cnidarian host and its symbiont may utilise heterophilic Fasciclin I interactions as a means of inter-partner signalling (Schwarz and Weis, 2003; Rodriguez *et al*., 2006b). However, the studies by Barneah *et al*. (2006) and deBoer *et al*. (2007) revealed virtually no changes in the host proteome before and after infection with symbionts. This suggests that during the first days and weeks of symbiosis, translational and

post-translational changes specific to the symbiotic state may not be occurring in the host (Barneah *et al*., 2006).

The symbiosome membrane complex acts as a barrier between the host cell cytoplasm and the symbiont thus an understanding of the communicative processes between the algal symbiont and its cnidarian partner, which are no doubt crucial to the nutrition, stability and persistence of the symbiotic relationship, is necessary. It is essential to characterise the properties of the symbiosome membrane as the transport properties of this membrane govern what passes between the host and symbiont. Kazandjian *et al.* (2008) isolated the symbiosome membrane from the zoanthid, *Zoanthus robustus*, using a combination of mechanical disruption and sucrose gradient centrifugation. Using confocal microscopy and TEM they confirmed that the symbiosome interface surrounds the alga and also established that it is multi-layered, robust and displays a level of heterogeneity in terms of thickness. Since then Peng *et al*. (2010) undertook a proteomic analysis of the membrane and were able to determine some of the major membrane proteins involved in membrane formation, signalling, molecular transport between the symbiont and host, symbiont recognition, photosynthesis, and host and symbiont cell cycle regulation. More studies of this kind aimed at examining the membrane composition and membrane trafficking using fluorescent markers are necessary to gain an understanding of the symbiosome-membrane modification that occurs during symbiosis (Davy *et al*., 2012).

1.5. Host–symbiont biomass regulation

In order for an association to persist in the long-term, both host cell growth and symbiont population proliferation has to be well regulates (Davy *et al*., 2012). As the number of symbionts present inside the host increases so the host must accommodate them by growing additional cells or regulating their numbers (Muscatine *et al*., 1998). The potential mechanisms that have been suggested to regulate algal cell numbers in algal-invertebrate symbioses can be divided into two groups; pre-mitotic mechanisms and post-mitotic mechanisms. Pre-mitotic mechanisms that regulate symbiont numbers may include density-dependent negative feedback by limiting

nutrients or space (Jones and Yellowlees, 1997), host-induced release of photosynthate from the symbionts (Gates *et al*., 1995), and host manipulation of the symbiont cell cycle (Smith and Muscatine, 1999). In the green *Hydra* symbiosis has been shown to be regulated by restrictions on the cell cycle that limit progression through interphase and into the M phase (Douglas and Smith, 1984; McAuley, 1985a and b; McAuley and Muscatine, 1986; but see Pardy, 1981). Past studies have yielded four possible mechanisms for the control of the cell cycle in *Hydra*:

(1) pH stimulated release of photosynthate (maltose) to the host leading to a reduction in symbiont growth (Douglas and Smith, 1984; McAuley, 1985c) The internal pH inside the host cells (pHi) is known to play a role in the regulation of the cell growth cycle in some organisms and could provide a mechanism by which host cnidarians regulate cell division by their symbionts (Davy *et al.* 2012). *Chlorella* cells for example, have been shown to release maltose in response to acidifiacation (Cernichiari *et al*., 1969; Mews, 1980; Muscatine *et al*., 1967) and those *Chlorella* cells that release large amounts of maltose have been shown not to grow at low pH in vitro (Douglas and Smith, 1984). These finding suggests that small changes in the pH of the symbiosome space (space between the symbiont and host), could affect how much photosynthate is translocated from symbiont to host. This in turn could directly or indirectly influence the cell cycle. However, evidence for this is yet to be demonstrated *in hospite* (Davy *et al*., 2012).

(2) Host-regulated supply of a metabolite(s) (McAuley, 1982, 1985a, 1985b; McAuley and Muscatine, 1986). Studies by McAuley (1985a, b) and McAuley and Darrah (1990) suggested that restriction by the host to a pool or pools of metabolites derived from host digestion, may limit symbiont division.

(3) Regulation by the host of inorganic nutrient supply to the symbiotic algae (Muscatine and Pool, 1979; McAuley, 1982, 1985c; Blank and Muscatine, 1987). Studies have shown that by limiting the supply of certain inorganic nutrients to the symbiont, the host is able to restrict the growth of the

symbiont population. Rees (1986), for example, demonstrated that *Hydra* has the ability to assimilate and withhold ammonium from its symbiont.

(4) Algal production of a density-dependent inhibitor (Muscatine and Pool, 1979; McAuley, 1982). Experimental support for this mechanism is still lacking.

Premitotic mechanisms in cnidarian-dinoflagellate associations are thought to operate in a similar manner especially with respect to the restriction of the supply of inorganic nutrients to the symbiont and loss of photosynthetic carbon to the host (Davy *et al*., 2012), however the studies of these mechanisms in these symbioses is limited. In the *Aiptasia pulchella*, nutrient supply has been shown to influence the biomass, composition and physiology of the algal symbionts however, it did not limit symbiont growth (Smith and Muscatine, 1999). Instead it appears that the cellular growth of the host and the progression of the symbiont through the cell cycle is linked (Smith and Muscatine, 1999). A similar conclusion was reached by Fitt and Cook (2001) for the hydroid, *Myrionema amboinense*. Light has also been found to be important for the division of symbiotic dinoflagellates (Fitt, 2000). In a cultured *Symbiodinium* sp. belonging to clade B from the coral *Euphyllia glabrescens,* the light/dark period was demonstrated to encourage progression from the G1 phase to the S phase, and then to the G2 and M phases (Wang *et al*., 2008). Wang *et al*. (2008) found that a green fluorescent protein in the tissues of this coral dissipates blue light but, when added to a *Symbiodinium* culture, it inhibits progression through the G1 phase to S phase to G2/M phase but not cytokinesis. This suggests that the host may be able to control symbiont growth through the manipulation of the light regimen (Davy *et al*., 2012).

Post-mitotic mechanisms involve the removal of existing zooxanthellae by degradation and digestion of symbionts (Titlynanov *et al*., 1996) or the expulsion of intact symbionts (Hoegh-Guldberg *et al*., 1987). In *Hydra* there is good evidence that the expulsion of algal cells plays an important role in symbiont population control (Fishman *et al*., 2008). In cnidarian-dinoflagellate associations, expulsion (Reimer, 1971; Trench, 1974; Steele, 1975 and 1977; Hoegh-Guldberg *et al*., 1987; Hoegh-Guldberg and Smith, 1989; Muscatine *et al*., 1991; Stimson and Kinzie, 1991; Jones

and Yellowlees, 1997) also appears to be employed with there being less evidence for symbiont cell degradation. Smith and Muscatine (1999) proposed that expulsion is associated with host cell turnover (Gates *et al*., 1992; Jones and Yellowlees, 1997). Baghdasarian and Muscatine (2000) later concluded that expulsion is one of the primary regulators of the symbiont density in some cnidarian-dinoflagellate symbioses. Evidence for this was demonstrated in the anemone, *Anthopleura elegantissima*, in which the expulsion of algal cells can exceed the daily growth of the symbionts at irradiances of less than full sunlight (McCloskey *et al*., 1996). In the temperate Atlantic coral *Astrangia poculata*, relatively high rates of expulsion allow the coral to sometimes persist in a stable, near-nonsymbiotic state (Dimond and Carrington, 2008). The cellular mechanisms behind the expulsion mechanism however, are still largely unknown (Davy *et al*., 2012). Dead symbionts cells and cellular debris have been observed in the tissues of reef corals (Titlyanov *et al*., 1996, 2004 and 2006), other anthozoans (Trench, 1974; Steele and Goreau, 1977) as well as the hydroid *Myrionema amboinense* (Fitt and Cook, 1990; Fitt, 2000). Degraded symbionts and cellular debris have also been identified in pellets extruded by various tropical sea anemones (Steele, 1977; Steele and Goreau, 1977). However, the mechanisms of symbiont degradation have been examined only in the context of stress and bleaching and not as part of a homeostatic mechanism (Davy *et al*., 2012).

1.6. Carbon production and translocation

The carbon fixed by the zooxanthellae (probably by the C3 pathway) is used to support their respiration and growth (Streamer *et al*., 1993). A portion of this carbon is translocated to the host (Muscatine *et al*., 1983; Davies, 1984) in the form of glucose, glycerol (Muscatine, 1967; Trench, 1974; Schmitz and Kremer, 1977) and lipid (Kellogg and Patton, 1983; Patton *et al*., 1983; Muscatine *et al*., 1984; Peng *et al*., 2011). The translocated carbon that is made available to the host is used for host respiration, growth and reproduction or is released from the association (Crossland *et al*., 1980a, b; Muscatine *et al*., 1981; Edmunds and Davies, 1986; Rinkevich, 1989; McCloskey *et al*., 1994).

Muscatine and Hand (1958) were the first to demonstrate photosynthate translocation in the symbiotic, temperate sea anemone *Anthopleura elegantissima* by using autoradiography to track radiolabeled organic carbon in the anemone's tissues. An attempt to measure the release of photosynthetic products from *Symbiodinium* cells was later made by Muscatine (1967). He incubated freshly isolated *Symbiodinium* cells in homogenized host tissue and found that the zooxanthellae released significant amounts of soluble photosynthate to the host homogenate. In order to quantify the amount of photosynthate released by the zooxanthellae, Trench (1971a-c) ran a series of experiments using ^{14}C to measure the release of photosynthetic products in *Anthopleura elegantissima*. He concluded that approximately 40-50% of the fixed ^{14}C was made available to the host. Figure 1.3 illustrates an example of the daily budget for photosynthetically fixed carbon in a symbiotic cnidarian.

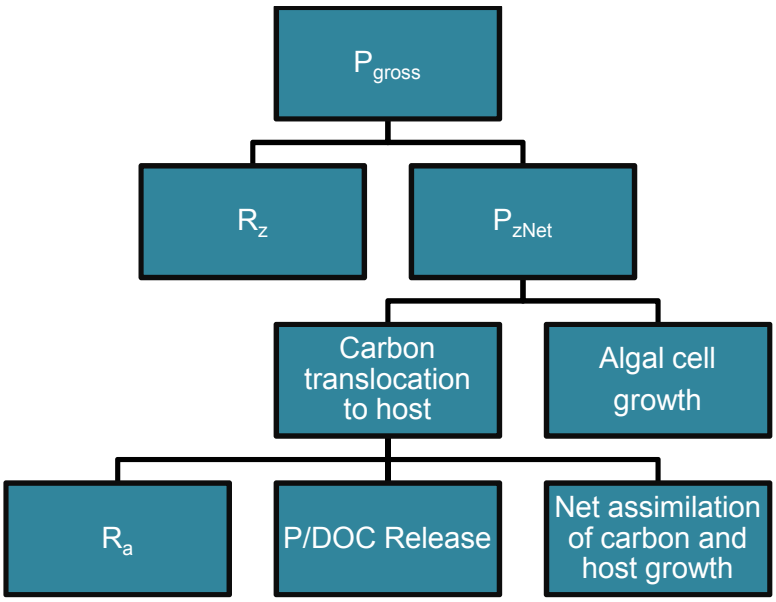

Figure 1.3. Representation of the daily budget for photosynthetically fixed carbon in a symbiotic cnidarian. P_{gross} is the total photosynthetic production, P_{zNet} is net photosynthetic production after allowing for zooxanthellar respiration (R_z), R_a is host animal respiration and P/DOC is particulate and/or dissolved organic carbon released by the host (reproduced from Muscatine *et al*., 1983).

The ratio of maximum gross photosynthesis (P) to symbiosis respiration (R) over 24 hours is a useful measure of the autotrophic potential of a symbiotic association

(Davies, 1977; Leletkin, 2000). A P:R value that exceeds 1 assumes that the symbiosis is autotrophic with respect to carbon and that there is a surplus of photosynthetically-fixed carbon available. A P:R value of less than 1, on the other hand, indicates a polytrophic symbiosis which can only be partially fulfilled photosynthetically and relies on an external carbon source (Muscatine *et al*., 1981).

Most cnidarian-dinoflagellate symbioses from tropical regions have been found to be autotrophic with respect to carbon (Muscatine *et al*., 1984). Temperate symbioses, on the other hand, generally cannot meet the demands of respiration through photosynthesis alone and as a result must acquire additional carbon from heterotrophic sources (Muller-Parker and Davy, 2001). The external carbon is sourced through predation on zooplankton, uptake of particulate and dissolved organic material from the environment (Lewis and Price, 1975), mucus feeding, and feeding on micro-organisms associated with detritus and mucus (Muscatine *et al*., 1984). However, the external uptake of carbon by a cnidarian host, as a means of compensating for insufficient carbon translocation by its symbionts, is most likely to be species dependent (Anthony and Fabricius, 2000)

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

$$CZAR = \frac{P_{zNet} * T}{R_a} \qquad \textbf{Equation 1}$$

Where P_{zNet} is the total net carbon fixed photosynthetically by the zooxanthellae, T is the percentage of P_{zNet} translocated to the animal, and R_a is the carbon respired daily by the animal. The major components used to estimate the CZAR are photosynthesis, respiration, algal reproduction (cytokinesis), and algal translocation (Verde and McCloskey, 1996a). This equation has been applied to a number of symbiotic associations (e.g. Muscatine *et al*., 1984; Edmunds and Davies, 1986 and 1989, Davy

et al., 1996; Verde and McCloskey, 2002), suggesting that up to 99% of carbon fixed in photosynthesis is translocated to the host (Davy *et al*., 1996).

1.7. Translocation products

There is still much uncertainty about the compounds that are translocated to the host (see review by Davy *et al*., 2012). Muscatine (1967) was the first to isolate zooxanthellae from corals and giant clams and identify both the intracellular and released products by two dimensional (2-D) radiochromatography after incubation of the algal cells in host tissue homogenate. The study revealed that glycerol (up to 90% of the total carbon released) was the major product released by these isolated zooxanthellae, with glucose and a ninhydrin-positive compounds also being released. Since then, many other studies have applied this same method to cnidarian-dinoflagellate associations and have also found glycerol to be the main compound released, with other metabolites such as glucose, the amino acid alanine, and organic acids such as fumarate, succinate, malate, citrate, and glycolate being released in smaller amounts (Von Holt and Von Holt, 1968; Trench 1971a, b and 1974; Sutton and Hoegh-Guldberg, 1990). Patton and Burris (1983) and Kellog and Patton (1983) also identified fat droplets being released from dinoflagellate cells isolated from the coral *Stylophora pistillata* and the sea anemone *Condylactis gigantean*, indicating that lipids may also be translocated.

However, since isolated zooxanthellae do not behave in the same manner as when they are inside the host (Roberts *et al*., 2001; Davy *et al*., 2012), many studies have also attempted to identify translocated compounds *in hospite*. This has proved to be very challenging, both due to the intracellular nature of zooxanthellae when in symbiosis, and the potentially rapid catabolism of translocated compounds in the host tissue. Nevertheless, a number of indirect approaches have been employed and have included, but are not limited to, studies that track the incorporation of ^{14}C-labelled compounds into the host tissues (Muscatine and Cernichiari, 1969; Trench, 1971c; Blanquet *et al*., 1979; Battey and Patton, 1984 and 1987), microscopic analyses of cnidarian tissues (Harland *et al*., 1991, Gautret *et al*., 1997; Wang and Douglas., 1999; Luo *et al*., 2009; Chen *et al*., 2012), and the proteomic and

ultrastructural analysis of "lipid bodies" isolated from symbiotic coral cells (Peng *et al*., 2011). More recently the entire host genome of the coral *Acropora digitifera* was analysed providing more information about the types of compounds translocated from symbiont to host (Shinzato *et al*., 2011). The outcome of this research again indicated that the major components of the translocated material are glycerol, amino acids and various lipids.

An alternative approach involved the addition of potential translocated compounds to the medium surrounding a ^{14}C-labelled symbiosis and the saturation of any uptake sites (Lewis and Smith, 1971). This stimulated the release of ^{14}C-labelled compounds into the surrounding medium and again supported the translocation of glycerol, glucose and alanine; lipids were not considered. Whitehead and Douglas (2003) used a similar approach to identify the compound translocated in the anemone *Anemonia viridis*, by comparing ^{14}C-labelled compounds in the host tissue between treatments treated with [^{14}C]bicarbonate under photosynthesizing conditions and when exogenous ^{14}C-labelled substrates were applied in the dark. They assumed that when the labelling patterns produced by both treatments were similar, then the exogenous compounds matched (or were similar to) the translocated compounds. This study identified glucose, succinate and fumarate as potential translocated metabolites but, contrary to previous studies, it found no evidence for glycerol translocation. Indeed, there is debate as to whether glycerol is translocated at all, with other studies (of the giant clam-*Symbiodinium* symbiosis) also suggesting that it does not form part of the translocate (Rees *et al*., 1993; Ishikura *et al*., 1999). More recently Burriesci *et al*. (2012) labled intact *Aiptasia* sp. in the light with ^{13}C-bicarbonate and, after homogenization and separation of animal and algal fractions, found labelled glucose in the animal fraction. However, they found no significant labelling of glycerol in the animal tissues thus supporting previous studies that indicated that glycerol does not form a major part of the translocate. Discrepancies exist between the compounds translocated in different symbioses as well between the compounds released by isolated *Symbiodinium* cells and those *in hospite* (Davy *et al*., 2012). Much more research is therefore necessary for us to conclusively state which compounds are translocated, and what qualitative and quantitative differences exist between different symbioses.

1.8. Translocation signals

Information about what stimulates and controls the translocation of photosynthetically fixed carbon from symbiont to host is still, to a large extent, lacking. A few hypotheses have, however, been proposed. One suggestion is that, by actively "blocking" symbiont mitosis or restricting the supply of nutrients to the symbiont, the host is able to limit the growth of the symbionts and in so doing 'free up' more carbon for translocation (Muscatine *et al*., 1983; Falkowski *et al*., 1984). A range of studies have also demonstrated that isolated zooxanthellae can be stimulated to release a substantial portion of their photosynthetically fixed carbon when incubated in host tissue homogenate (Trench, 1971c; Muscatine *et al*., 1972; Muscatine *et al*., 1984; Hinde, 1988; Sutton and Hoegh-Guldberg, 1990; Grant *et al*., 1997; Davy and Cook, 2001a), suggesting that the host produces some sort of signal that initiates this release. The signal molecule(s) that is thought to mediate this process has been termed the "host release factor" or HRF. The identity of the HRF is still unknown despite attempts to characterize it. Gates *et al*. (1995) proposed that it consists of a range of free amino acids (FAAs), though this idea has been disputed, with other studies suggesting that unknown small host compounds (<100 Da) act as HRFs (Withers *et al*., 1998; Cook and Davy, 2001). Continuing research by Grant and co-workers (Ritchie *et al*., 1993; Grant *et al*., 1997, 1998, 2001, 2006a, b) has identified two water-soluble signalling molecules of 1000 kDa or less, one that stimulates photosynthate release (HRF) and another that partially inhibits photosynthesis, termed the "photosynthesis-inhibiting factor" (PIF). They demonstrated that HRF from the coral, *Plesiastrea versipora*, stimulates translocation by either increasing glycerol synthesis or diverting it away from the symbiont's triglyceride stores (Grant *et al*., 1997). The limitation of HRF studies, however, is that they do not replicate the *in hospite* environment and thus the HRF factor may simply be an experimental artifact (Davy and Cook 2001a, b; see review by Davy *et al*., 2012).

1.9. Coral bleaching

Coral bleaching refers to the whitening of corals due to the mass expulsion of symbiotic algae and/or the loss of photosynthetic pigments within individual zooxanthellae (see Figure 1.4). Photosynthetic pigments are contained within the chloroplasts of the symbionts and thus corals that have lost their symbionts appear transparent with their white calcium carbonate skeletons visible through the tissue layers (Glynn, 1991: Baker, 2003; Lesser and Farrel, 2004). In recent years there has been an increase in the frequency and severity of episodes of coral bleaching resulting in degradation and mortality of coral reefs on a global scale (Baker, 2003; Lesser and Farrel, 2004; Obura, 2005; Hoegh-Guldberg *et al*., 2007; Hoegh-Guldberg and Bruno, 2010). A variety of stressors are thought to be responsible for coral bleaching, the most important being increased sea temperature, solar radiation, and a combination of both factors (Brown, 1997; Lesser and Farrell, 2004). Because symbioses with zooxanthellae are commonly found in habitats at 1-2 °C below the temperature which triggers breakdown of the symbiosis, they are extremely sensitive to increases in temperature (Douglas, 2003). A number of cellular mechanisms have been proposed for coral bleaching. These include: the *in situ* degradation of zooxanthellae; the loss of algae into the coelenteron and hence ambient seawater; and the release of intact endodermal cells containing intracellular zooxanthellae (Gates *et al*., 1992; Brown, 1997; Weis, 2008). It is likely that the pathway of zooxanthellar degradation may differ depending on the intensity and duration of stress and also the host species (Mise and Hidaka, 2003). Smith *et al*. (2005) suggested that hydrogen peroxide generated within zooxanthellae may have a signalling role in triggering the mechanisms that result in expulsion of the zooxanthellae from the coral.

Photosynthetically-active radiation (PAR) and/or ultraviolet radiation (UVR) may act in concert with elevated temperature to elicit a bleaching response (Brown, 1997; Warner *et al*., 1999; Lesser and Farrel, 2004). The role that UVR may play in increasing future levels of coral bleaching is one of the main areas of concern. The amount of UVR that submerged corals are exposed to is related to stratospheric ozone levels, changing weather patterns and the attenuation of UVR in the water column (Brown, 1997). Therefore, increases in ozone depletion, global warming and

anthropogenic influences that affect both particulate loads and dissolved organic carbon (DOC) in oceans, all contribute to coral bleaching (Brown, 1997). Lesser and Farrel (2004) showed that corals exposed to supra-optimal solar irradiance and elevated seawater temperatures exhibit greater damage to the photosynthetic apparatus. An increase in water temperature results in a decrease in the photosynthetic activity of the symbiosis, most probably associated with a decrease in the ability of *Symbiodinium* spp. to process captured light. This causes dysfunction in photosystem II. Damage to the chloroplast and photosynthetic apparatus can occur in at least three inter-related ways that act together to start the bleaching cascade (Weis, 2008): (1) Damage to the D1 protein can occur during elevated temperature in *Symbiodinium*, the D1 (Warner *et al*., 1999) and heat may also damage the protein's repair mechanism itself (Takahashi *et al*., 2004). D1 damage results in a backup in excitation energy and the dysfunction of photosystem II (Warner *et al*., 1999). (2) Both heat and light have been shown to negatively impact the dark reaction of photosynthesis such that carbon fixation declines (Jones *et al*., 1998). This is because the enzyme responsible for primary carboxylation, ribulose bisphospate carboxylase oxygenase (Rubisco), may be damaged (Lesser, 1996) resulting in reduced consumption of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) coming from the light reactions. Excitation energy can back-up due to this leading to the dysfunction of photosystem II (Jones *et al*., 1998; Venn *et al*., 2008). (3) The thylakoid membranes can also be damaged by elevated temperature and light (Tchernov *et al*., 2004) causing the energetic uncoupling of electron transport in both photosystems. This inhibits the production of ATP and NADPH. All three of these mechanisms can lead to the build up of electrons which is thought to ultimately lead to the generation of multiple reactive oxygen species (ROS) in the symbiont. The fluxes of ROS overwhelm host and algal antioxidant defense systems, resulting in damage to both the light (photosystem II) and dark reactions (carbon fixation) in photosynthesis (Lesser and Farrel, 2004; Weis, 2008). Under high temperature and light conditions, antioxidants such as superoxide dismutase (SOD) and ascorbate peroxidase (APX) that quench and convert superoxides into less harmful molecules are saturated, resulting in damage to cellular components (Hoegh-Guldberg and Smith 1989; Glynn and D'Croz 1990; Lesser *et al.,* 1990; Iglesias-Prieto *et al.,* 1992; Lesser, 2004; Fitt and Warner, 1995). As a result, ROS are able to further damage photosynthetic membranes, as described

above, in an escalating positive feedback loop (Lesser, 2006; Weis, 2008). It is also able to diffuse into the host tissue where the damage continues and ultimately leads to bleaching (Weis, 2008).

There is evidence that both the host and symbiont have developed protective mechanisms to tolerate stress. These mechanisms include an increased expression of heat shock proteins and antioxidant enzymes as well as the production of protective pigments (Coles and Brown, 2003; Douglas, 2003; Venn *et al*., 2008; Weis, 2008). Tolerance to stress appears to vary within a population or between species of either or both hosts and symbionts (Coles and Brown, 2003; Douglas, 2003; Venn *et al*., 2008). Single host species associated with different algal genotypes have been shown to vary in bleaching susceptibility (Rowan *et al*., 1997; Perez *et al*., 2001). *Symbiodinium* communities have also been shown to vary in their photobiological characteristics and different coral-*Symbiodinium* associations may display different photophysiological responses (Robinson and Warner, 2006; Hennige *et al*., 2009). Photoacclimation refers to the phenotypic response of the symbiont to changes in the light environment (Falkowski and LaRoche, 1991) and occurs as a result of the symbiont being able to alter the components of its photosynthetic apparatus (Falkowski and LaRoche, 1991; MacIntyre *et al*., 2002). Thus this finding has implications for bleaching as it suggests that different host-symbiont combinations will have varying photoacclimatory abilities. However, it remains to be seen whether the differential tolerance demonstrated by different host-symbiont associations, translates into differential survival and adaptation to climate change (Venn *et al*., 2008; Weis, 2008).

Figure 1.4. Photograph of a partially bleached *Acropora palifera* colony (obtained from Stefanie Pontasch).

The "adaptive bleaching hypothesis" proposed by Buddemeier and Fautin (1993) is based on the premise that bleaching is a deliberate strategy that allows corals to switch their symbionts as an adaptation to changing environmental conditions. It has generated much controversy in the field (Baker, 2001; Goulet, 2006; Hoegh-Guldberg *et al*., 2002) as support for this hypothesis in the wild is still lacking. Although some corals are capable of acquiring symbionts from the water column, this acquisition has been found to be temporary as these infections are not maintained as stable symbioses (Coffroth *et al*., 2010). And, despite evidence for post-bleaching shifts in the dominant member of a mixed symbiont population (symbiont "shuffling"; Baker, 2001; Baker *et al*., 2004; Berkelmans and van Oppen, 2006; Mieog *et al*., 2007; Jones *et al*., 2008), it is still unknown if the specific host–symbiont combinations found in nature can change with time at a rate fast enough to match the pace of the changing conditions associated with climate change (Weis, 2008). Figure 1.5 illustrates the proposed symbiont "switching" and "shuffling" mechanisms

1.10. The model system approach

The fields of molecular, cell and developmental biology have undergone great progress as a result of researchers focusing on a small number of intensively studied model organisms: the bacterium *Escherichia coli* and its viruses, the yeasts *Saccharomyces cervisiae* and *Schizosaccharomyces pombe*, the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the small mustard plant *Arabidopsis thaliana*, the mouse *Mus musculus* and a few lines of cultured mammalian cells (reviewed by Davis, 2004; Weis *et al*., 2008). This model system approach creates rapid progress in a given field by allowing for fast development of new methods and effective collaboration between laboratories (Weis *et al*., 2008). Recently, research on cnidarian-dinoflagellate symbioses has moved toward the use of a model system. The small tropical anemone *Aiptasia* sp. that is, like corals, symbiotic with dinoflagellates of the genus *Symbiodinium*, was selected by investigators as the model system on which to focus (Weis *et al*., 2008).

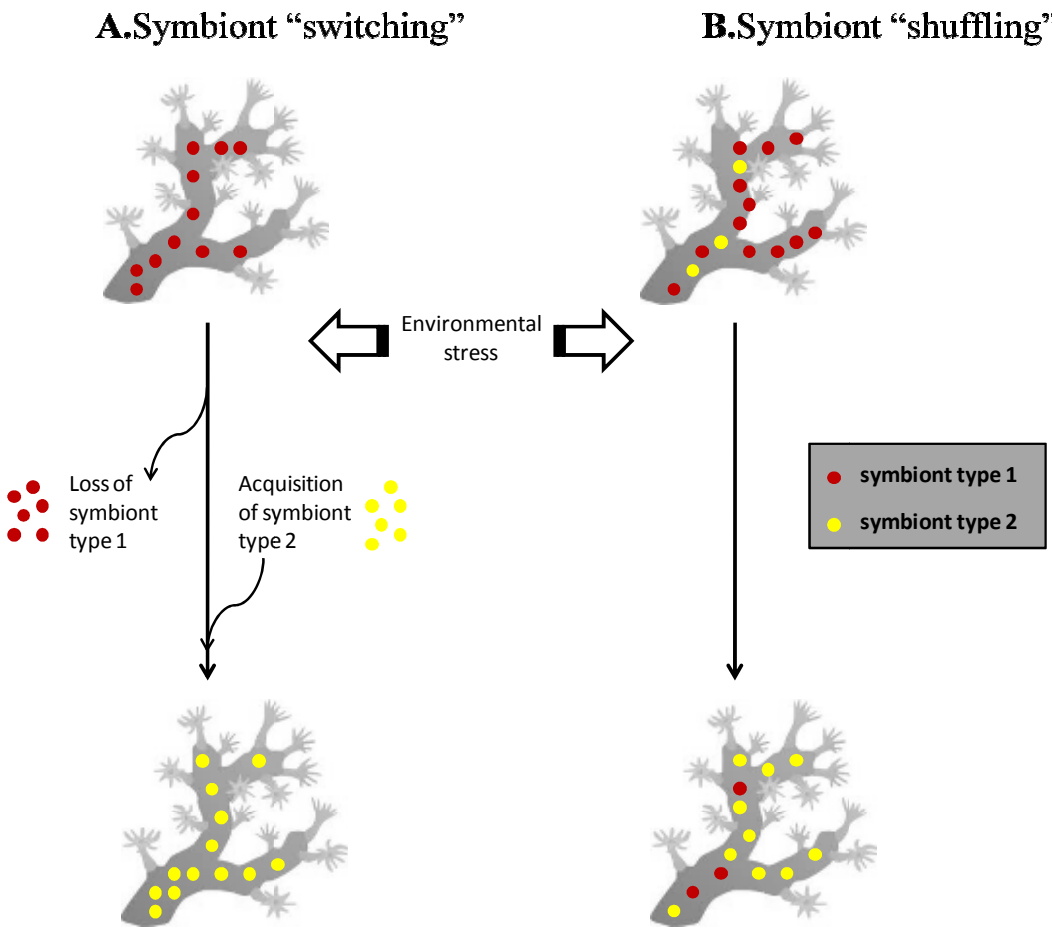

Figure 1.5. Schematic diagram illustrating symbiont (A) "switching" and (B) "shuffling", the two processes that can account for changes in the population of *Symbiodinium* in a cnidarian host (adapted from Stat *et al*., 2006).

The two well-studied *Aiptasia* species are *A. pallida* (found in the Atlantic) and *A. pulchella* (found in the Pacific). Individuals from both species undergo sexual and asexual reproduction, and have male and female forms. Sexual reproduction involves the release of gametes into the water column followed by fertilisation and the development of planula larvae. Asexual reproduction, on the other hand, occurs by basal laceration that necessitates the budding of small pieces of tissue from the anemone base (Belda-Baillie *et al*., 2002).

Aiptasia sp. is a common tropical aquarium pest due to its robust nature and ability to produce large clonal populations. These qualities also contribute to its appropriateness as a model organism. The main advantages of using *Aiptasia* sp. as a model are that, unlike corals, it can be quickly and easily grown in large numbers in

any laboratory making it available for experimentation (Hunter, 1984; Veron, 2000). Because they lack a skeleton, whole anemones can be homogenized or extracted, making *Aiptasia* sp. accessible for biochemical and molecular biological procedures (Kuo *et al*., 2004); its cells are also accessible to microscopy (Perez and Weis, 2006). There have also been some promising results from studies with *Aiptasia* sp. for RNA interference (RNAi) work, which involves suppressing specific genes of interest in order to identify the components necessary for a particular cellular pathway (Perez and Weis, 2006; Dunn *et al*., 2007). Most importantly, *Aiptasia* sp. can be fully cleared of its symbionts by temperature shock and dark-treatment (Belda-Baillie *et al*., 2002; Perez and Weis, 2006), maintained in the symbiont-free state for months or years (Kinzie *et al*., 2001), and re-infected at will with a variety of *Symbiodinium* types (Schoenberg and Trench, 1980b; Belda-Baillie *et al*., 2002) thus making it useful for studies aimed at investigating the cnidarian–dinoflagellate symbiosis (see Figure 1.6).

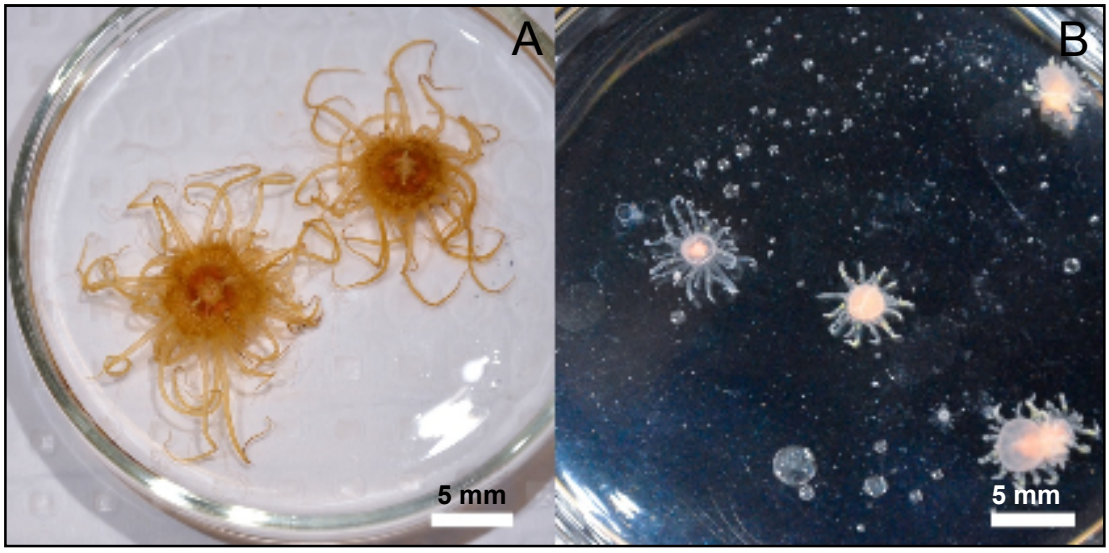

Figure 1.6. Photographs of the model anemone, *A. pulchella*, in the symbiotic (A) and aposymbiotic (B) state (photographs taken by Tom Hawkins).

1.11. Wider implications and aims

In order to gain a deeper understanding of how corals can adapt to changing environmental conditions, the effect that symbiont type has on the persistence and physiology of an association needs to be ascertained. This is particularly important when considering the impact of symbiont type on the recovery of an association from an environmental perturbation such as bleaching. Recovery of a symbiosis from such an event will depend on a number of factors: (1) the host's ability to either form a stable association with a novel symbiont type, or to allow a resident symbiont type that has previously been maintained at a low population level to proliferate; (2) the ability of that symbiont to provide enough photosynthetically fixed carbon to meet the host's respiratory demands; (3) the symbiont's ability to provide the required quality of translocate to the host; (4) the host's ability to control the symbiont population growth (and perhaps *vice versa*); and (5) the ability for both parties to communicate efficiently such that the above four points can be realised. The aim of this research was to determine how different symbiont types affect the nutritional potential and intracellular physiology of the symbiosis when in association with the sea anemone *Aiptasia pulchella* in order to establish what impact symbiont functional diversity has on the association.

Aim: To test whether *Symbiodinium* type influences the nutritional potential and intracellular physiology of the cnidarian host.

Objectives:

1. To determine whether different symbiont types are equally as adept at supporting the energetic demands of the same host. Chapter 2 tested the relationship between symbiont type, photosynthetic performance and autotrophic potential in a range of 'normal' and novel cnidarian-dinoflagellate symbioses.

2. To determine if pHi is a reflection of symbiont type and whether the optimal pH for photosynthesis coincides with the host cell pHi. These questions were

investigated in Chapter 3 by measuring the host cell pHi of associations with different symbiont types and assessing whether the optimal pH of different symbiont types falls within the range of host pHi values measured.

3. To test whether *Symbiodinium* type influences the glycerol and glucose pools of the host. Chapter 4 approached this objective by comparing the amounts of free glycerol and glucose (metabolites) in the host tissue of associations with different symbiont types, both prior to, and at different time points after photosynthesis began.

Chapter 2:

The influence of symbiont diversity on the photophysiological performance of novel cnidarian-dinoflagellate symbioses

2.1. Introduction

Cnidarian-dinoflagellate symbioses are widespread in the marine environment and are of great ecological importance (Furla *et al.,* 2005). The symbiosis between anthozoan cnidarians (e.g. corals, sea anemones) and dinoflagellates (commonly referred to as zooxanthellae) of the genus *Symbiodinium* is responsible for the formation of coral reefs which, in turn, maintain a rich biodiversity in shallow tropical waters and thus are vital for sustaining the coastal communities in these regions (Peterson and Lubchenco, 1997; Furla *et al.,* 2005).

The presence of dinoflagellate symbionts contributes substantially to the productivity, survival and success of their cnidarian hosts (Muscatine and Porter, 1977). A mutualistic relationship exists between the host and symbiont, in which the symbiont receives inorganic nutrients from the host and in return provides the host with translocated photosynthetic products (Muscatine, 1990). In most cnidarians, the symbiotic dinoflagellates are intracellular within the endodermis (Schoenberg and Trench, 1980a) and a perisymbiont membrane of animal origin separates them from the animal cytoplasm (Wakefield and Kempf, 2001). The genus *Symbiodinium*, to which these dinoflagellates belong, is characterised by a high degree of genetic diversity, with nine clades (A-I) and numerous sub-clades currently identified (Coffroth and Santos 2005; Stat *et al*., 2011).

The carbon fixed by the symbiotic dinoflagellates (probably by the C3 pathway) is used to support their own respiration and growth (Muscatine *et al*., 1981; Streamer *et al*., 1993). However, a portion of this carbon is translocated to the host (Muscatine *et al*., 1981; Davies, 1984), primarily in the form of glucose, glycerol and lipid

(Muscatine, 1967; Trench, 1974; Kellogg and Patton, 1983; Patton *et al*., 1983; Muscatine *et al*., 1984; Whitehead and Douglas, 2003; Peng *et al*., 2011). This translocated carbon is used for host respiration, growth and reproduction, or is released from the association (Muscatine *et al*., 1981; Edmunds and Davies, 1986; Rinkevich, 1989; McCloskey *et al*., 1994). It has been shown that up to 99% of carbon fixed in photosynthesis is translocated to the host (Steen and Muscatine, 1984; McCloskey *et al*., 1994; Day, 1994; Davy *et al*., 1996; see review by Muller-Parker and Davy, 2001) and that well in excess of 100% of the host's daily respiratory carbon demands can be met by autotrophy under well-lit conditions (Muscatine *et al*., 1981, 1983; Davies, 1991; Davy *et al*., 1996; Verde and McCloskey, 2002, 2007).

In recent years there has been an increase in the frequency and severity of episodes of coral bleaching (the whitening of corals due to the expulsion of their zooxanthellae and/or the loss of photosynthetic pigments) resulting in degradation and mortality of coral reefs on a global scale (Baker, 2003; Lesser and Farrell, 2004; Obura, 2005). However, our understanding of bleaching, and the potential for corals to withstand or recover from this phenomenon, has been hampered by our limited knowledge of the underlying mechanisms by which the cnidarian-dinoflagellate symbiosis is initiated and maintained (Weis *et al*., 2008; Weis and Allemand, 2009; Davy *et al*., 2012). For example, what is the potential for novel host-symbiont combinations to form and persist, and do they function optimally? Buddemeier and Fautin (1993) considered this issue and proposed that, after a bleaching event, corals have the capacity to acquire novel symbionts that are better suited to the new environmental regime than were the previous symbionts (i.e. symbiont 'switching'). Alternatively, lower density symbiont communities already inside the host may, in response to environmental change, increase in numbers and become the dominant component of the symbiont population (i.e. symbiont 'shuffling') (Baker, 2003; Fautin and Buddemeier, 2004). There is experimental evidence for such 'shuffling'in the field (Berkelmans and van Oppen, 2006; Jones *et al*., 2008), though symbiont 'switching' has so far only been reported conclusively in a laboratory situation (e.g. Schoenberg and Trench, 1980b; Davy *et al*., 1997; Lewis and Coffroth, 2004). In either case though, the potential for the symbiosis to survive and proliferate will be greatly influenced by the physiological performance of the symbiont type. Past

studies have provided evidence for this. For example, previous work on induced symbiosis of giant clams with zooxanthellae from various sources (different host species or fast-growing hosts) demonstrated differences in growth and survival rates in the laboratory (Fitt and Trench, 1981; Fitt, 1985), in outdoor culture tanks (Molea and Munro, 1994) and in the field (Belda-Baillie *et al*., 1999). Also, Stat *et al*., (2008) demonstrated that differences exist between *Symbiodinium* types belonging to clade A and clade C with respect to the amount of carbon that is fixed and released. However, to date the key question of whether different symbiont types are equally as adept at supporting the energetic demands of the same host species has not been quantified. In order to be able to predict whether novel host-symbiont combinations might arise and succeed in the future, this needs to be investigated.

The relationship between symbiont type, photosynthetic performance and autotrophic potential in a range of 'normal' and novel cnidarian-dinoflagellate symbioses was tested. The sea anemone *Aiptasia pulchella* was used as the experimental host; this anemone is a widely employed model laboratory system for coral research (Weis *et al*., 2008). Ultimately, the aim of the study was to gain insight into the influence of symbiont diversity on the nutritional physiology of the symbiosis and on the potential for that symbiosis to survive autotrophically.

2.2. Materials and methods

The sea anemone *Aiptasia pulchella* was used as the experimental host and infected with different *Symbiodinium* types (freshly-isolated and cultured *Symbiodinium* B1, heterologous *Symbiodinium* B1 as well as *Symbiodinium* A1.4, E2 and F5.1). After 12 weeks the maximum photosynthetic and dark respiratory O_2 fluxes were measured in order to calculate a daily carbon budget for the different symbioses tested.

2.2.1. Experimental organisms

The sea anemone *A. pulchella* (originally from the Indo-Pacific region) was cultured at 25 ^{o}C and an irradiance of 100 µmol photons m^{-2} s^{-1} (12 h light:12 h dark) for a

period of >1 year prior to use. The anemones were fed twice weekly with freshly-hatched *Artemia* sp. over this period. To render the anemones aposymbiotic (i.e. symbiont-free), 120 individuals (oral disc diameter ~10 mm) were placed in six 1-L light-proof containers (20 individuals in each) filled with 0.44 μm filtered seawater (FSW) and cold shocked at 4 ^{o}C for 24 hours. The cold-shock process was repeated at weekly intervals for five weeks and the feeding regime was retained between cold shocks, when care was taken to keep the anemones in low light (<1 μmol photons m^{-2} s^{-1}). The anemones were then maintained in the dark at 25 ^{o}C for approximately six months until the start of the experiment. The aposymbiotic state of the anemones was checked by exposure to light (12 h light:12 h dark) over a four-week period, with any change from white to brown colouration being indicative of a residual symbiont population. Fluorescence microscopy (Olympus Provis AX70 microscope using autofluorescence at 100× magnification) was used to further screen for the presence of symbionts in a sub-sample of 3 anemones per container (whole anemones were screened). Only anemones that showed no signs of symbiont infection were used for subsequent experiments.

Symbiotic dinoflagellates were freshly isolated from *A. pulchella* for further infection experiments. Three anemones were homogenised in 1 mL of FSW in a hand-held, glass tissue grinder for 5 min and the homogenate then centrifuged at ×400 g for 5 min to separate the animal and algal fractions. The supernatant was collected and the algal pellet was re-suspended in 100 μL of FSW to achieve a density of approximately 10^6 cells mL^{-1}.

Cultures of a range of heterologous *Symbiodinium* types (i.e. not originally isolated from the *A. pulchella* laboratory culture), as well as a culture of the homologous type that was isolated from the laboratory anemone culture, were grown at 25 ^{o}C and an irradiance of 100 μmol photons m^{-2} s^{-1} on a light regime of 12 h light: 12 h dark. The heterologous *Symbiodinium* types (refer to Table 2.1 for details) were selected such that a range of clades was represented. Furthermore, a heterologous B1 type was used to provide a comparison with the homologous B1 zooxanthellae. The zooxanthellae were sub-cultured from long-term laboratory stocks and grown in silica-free F⁄2 medium (Sigma-Aldrich, Auckland, New Zealand) for six weeks prior

to use in the infection study. All *Symbiodinium* types will be referenced by their cladal designation.

Table 2.1. Sub-clade, original host species and geographic origin of *Symbiodinium* cultures. Sub-clade genotyping was based on the ITS2 gene.

Culture I.D.	**Host**	**Geographical location**	**Sub-clade**
FlCass	*Cassiopea xamachana*	Long Key, Florida	A1.4
FlAp2 (heterologous)	*Aiptasia pallida*	Long Key, Florida	B1
Homologous	*Aiptasia pulchella*	Pacific (specific location unknown)	B1
CCMP421	Free-living (host, if any, unknown)	Wellington Harbour, NZ.	E2
Sin	*Sinularia* sp.	Guam	F5.1

Symbiodinium types (n = 3 samples per culture or isolate) were genetically identified prior to infection (see Appendix 3 for the ITS2 sequences). DNA was extracted from zooxanthellae as described by Logan *et al*. (2010). Polymerase chain reaction (PCR) was performed using the following *Symbiodinium*-specific ITS2 primers: forward primer (ITSD), 5'-GTG AAT TGC AGA ACT CCG TG-3'; reverse primer (ITS2rev2), 5'-CCT CCG CTT ACT TAT ATG CTT-3'. Reactions were performed in a total volume of 30 μL using the MyTaq^{TM} Mix with an amplification profile consisting of1 min 95 ^{o}C, 35 cycles of 1 min 95 ^{o}C, 15 s 55 ^{o}C, 10 s 72 ^{o}C, and a final hold temperature of 4 ^{o}C. Single strand conformation polymorphism (SSCP) was performed on all PCR products. PCR products were sequenced by Macrogen Inc. (Seoul, Korea). Sequences were aligned using the ClustalW plugin in Geneious Pro 4.8.5 and then edited. A blast search was then performed in the National Centre for Biotechnology Information (NCBI) database in order to identify the sequences (see Table A3.1).

2.2.2. Infection of aposymbiotic *A. pulchella* with different *Symbiodinium* types

Aposymbiotic specimens of *A. pulchella* (n = 60 per treatment) were infected with cultured or freshly-isolated *Symbiodinium* cells by pipetting a concentrated drop of dinoflagellate suspension (approximately 1 million cells/mL) onto the oral disc of each anemone, with a sharp-ended glass pipette. A drop of one-day old *Artemia* sp. culture was then pipetted onto the oral disc to induce a feeding response and encourage the uptake of the *Symbiodinium* cells, as described by Davy *et al*. (1997). Anemones were kept in FSW in 20 mL glass containers that received a water change 8 hours after infection. Five anemones, all infected with the same symbiont type, were allocated to each container (i.e. 12 containers per treatment). The physiological performance of the different symbioses was then assessed over a 12-week period as described below. The feeding regime during the 12-week period was maintained as per pre-infection as was the temeprature and light regime. After 12 weeks, zooxanthellae were isolated from three anemones per treatment and their genetic identity confirmed, using the isolation and molecular techniques described above. All treatments were found to harbour the same *Symbiodinium* type as the one introduced at the start of the experiment (see Appendix 3 for the ITS2 sequences).

2.2.3. Symbiont growth rate, density, size and biomass

At Weeks 1, 2, 4, 8 and 12, the growth rate of the zooxanthellae was measured. Individual anemones, each sampled from a different storage container at each time point, (n = 4 per treatment per time point) were homogenised in 1 mL of FSW in a hand-held, glass tissue grinder for 5 min and the homogenate then centrifuged at ×400 g for 5 min to separate the animal and algal fractions. The algal fraction was used to calculate the mitotic index. The percentage of algal cells (out of a total of 300 counted) appearing as doublets (mitotic index; MI) was measured by light microscopy at 400× magnification every 3 hours over a 24-h period and used to infer the algal growth rate (d^{-1}) (Muscatine *et al*., 1983).

At these same time-points, the algal fraction of the anemones used for the O_2 flux measurements (see below) was used to determine the algal cell density. A

haemocytometer and light microscope (100× magnification) were used to estimate the total number of algal cells per mL of anemone homogenate (n = 6 counts per anemone), and the total number of algae was normalised to host protein content, measured as described below. Average algal cell diameter (μm; n = 20 cells per anemone per treatment) was also measured by light microscopy (100× magnification), and the cell volume (μm^3) and carbon content (μg) were derived from this value (Strathmann, 1967; Muscatine *et al*., 1981).

2.2.4. Photosynthesis and respiration

Maximum photosynthetic and dark respiratory O_2 fluxes were measured for each treatment at Weeks 1, 2, 4, 8 and 12. Individual anemones were placed in a glass chamber of volume 10 mL, itself situated in a glass water bath set at 25 ^oC (four anemones, each sampled from a different storage container, were used per treatment at each time point to obtain four replicates). The chamber contained FSW that was stirred by a magnetic spin bar that rotated at the maximum speed at which the anemone did not appear stressed; the spin bar was overlaid by a perforated floor of nylon mesh on which the anemone sat. The chamber was sealed by a glass lid with rubber O-ring, into which an oxygen electrode (FIBOX 3 – fiber-optic oxygen meter, PreSens GmbH, Germany) and temperature probe were inserted. These were connected to the oxygen meter. The chamber was illuminated by a Thorn PAR (photosynthetically active radiation) 38 150 W sealed beam-reflector lamp and irradiance incident on the surface of the chamber was measured by a QSL-100 irradiance meter (Biospherical Instruments Inc.). The anemone was left to settle for an hour in the chamber prior to measurement so that it could attach to the grid and open its tentacles. The respiration rate (mL O_2 h^{-1}) was measured in darkness for about an hour, by which time a constant rate was observed. The oxygen tension was not allowed to fall below 50% saturation (Davies, 1984). The PAR lamp was then switched on and the rate of net photosynthesis by the intact association was measured at 400 μmol photons m^{-2} s^{-1} (light-saturation for photosynthesis), again until constant and for about an hour. A preliminary study was performed to check that there was no photoinihibation at 400 μmol photons m^{-2} s^{-1}.

The rate of gross photosynthesis was calculated by adding net photosynthesis to dark respiration, and converted into carbon equivalents by assuming photosynthetic and respiratory quotients of 1.1 and 0.9, respectively (Muscatine *et al*., 1981, 1983; Muscatine, 1990). Animal tissue and zooxanthellae were isolated by homogenisation and centrifugation as described before. The animal supernatant was collected and the algal pellet re-suspended in 1 mL FSW. Total animal protein was quantified by measuring absorbance of the supernatant at 280 nm using a ND – 1000 spectrophotometer (Nanodrop, Thermo Fisher Scientific Inc.); FSW was used for blank measurements. A preliminary study was performed to assess DNA contamination of the host tissue isolates. The ratio of absorbance at 260 nm vs 280 nm was measured for all samples at weeks 8 and 12. As the DNA content in the samples was found to be minimal (less than 5% in all cases), absorbance at 280nm was used to quantify protein content. The total gross photosynthetic and respiration rates were adjusted for the respiration chamber volume and normalised to animal protein content. The algal fraction was used to determine the algal cell density and volume as described previously.

2.2.5. Carbon budgets

The contribution of the zooxanthellae to animal respiration (CZAR) was estimated using the "growth rate method" of Muscatine *et al*. (1981), however a zooxanthellar C:N ratio of 6.1:1 was assumed in the current study (D'Elia *et al*., 1983). Algal cell protein was derived from carbon content by assuming a C:N ratio of 6.1:1 (D'Elia *et al*., 1983) and that algal cell protein is nitrogen x 6.25 (Muscatine *et al*., 1983). The "growth rate method" method assumes that all photosynthetic carbon not used for respiration or growth by the symbionts is translocated to the host and hence available to support its metabolism, and is calculated according to the equation: CZAR = $[(P_{zNet} \text{ x } T)/R_a] \times 100$, where P_{zNet} is net photosynthetic carbon fixation, T is the percentage of net photosynthetic production translocated to the host (calculated according to the equation: $T = (\mu_c - \mu \text{ x } 100)/ \mu_c$, where μ_c is the carbon specific growth rate and μ is the cell specific growth rate), and R_a is the carbon consumed in animal respiration. This method also assumes that R_z (zooxanthellar respiration) and R_a (animal respiration) are proportional to their relative biomasses. The CZAR and

its associated parameters were estimated as described in detail elsewhere (Muscatine *et al*., 1981, 1983; Davy *et al*., 1996), except that a daily light regime of 12 h saturating irradiance:12 h dark was assumed. The CZAR was calculated for all symbioses tested and at all time points.

2.2.6. Statistical analyses

Data were tested for normality using the Kolmogorov-Smirnoff test and for homogeneity of variance using the Bartlett test. Multiple comparisons of the various parameters (i.e. algal density, R_a, P_{gross}, P_{zNet}, MI, T and CZAR) between the different symbiotic associations and time points were made using one-way analysis of variance (ANOVA, $\alpha = 0.05$), followed by Least Squares Difference (LSD) *post hoc* tests for normal data using PASW (Predictive Analytics SoftWare, Version 18). Non-parametric permutation tests were conducted using the R statistics package, version 2.13.0 (http://www.r-project.org), for data that did not fit the normal distribution. All statistical comparisons provided in the results are one-way ANOVA with *post hoc* LSD testing unless otherwise stated. Due to high variability that was apparently masking trends for the other symbiont types, *Symbiodinium* F5.1 was removed from the analyses of algal density and total P_{gross} at Week 12.

2.3. Results

2.3.1. Symbiont density

Patterns of symbiosis establishment varied between the different *Symbiodinium* types, though all increased significantly between Weeks 1 and 4 (Figure 2.2 and Table 2.3; one-way ANOVA, $p < 0.05$ for all treatments). Densities of *Symbiodinium* A1.4 (Figure 2.2 C), E2 (Figure 2.2 D) and F5.1 (Figure 2.2 B), and both the freshly-isolated (Figure 2.2 E) and cultured homologous (Figure 2.2 F) zooxanthellae (*Symbiodinium* B1), increased by 67-95% between Weeks 1 and 4 (LSD, $p < 0.05$), before levelling off between Weeks 4 and 12 (LSD, $p > 0.05$). The heterologous *Symbiodinium* B1 (Figure 2.2 A) density increased by 91% between Weeks 1 and 4,

stabilised between Weeks 4 and 8 (LSD, $p > 0.05$) but then declined by 70% between Weeks 8 and 12 (LSD, $p < 0.05$).

At Week 12 and Week 1, significant differences were apparent between the densities of the various *Symbiodinium* types, though, in the case of Week 12, only once the highly variables values for type F5.1 (Figure 2.2 B) had been excluded from the analysis (Table 2.4; one-way ANOVA, $p < 0.005$; see Appendix 1, Table A1.1). At Week 1, *Symbiodinium* E2 (Figure 2.2 D) displayed a significantly higher density than all the other types (LSD, $p < 0.005$). At Week 12, *Symbiodinium* E2 (Figure 2.2 D) and A1.4 (Figure 2.2 C) displayed significantly higher densities than did the others (LSD, $p < 0.05$). These differences led to similar trends in the symbiosis biomass ratio (algal:total protein) ratio, which was again significantly different between various host-symbiont combinations (one-way ANOVA, d.f. = 19, $F = 4.6$, $p < 0.05$). Specifically, the biomass contribution of *Symbiodinium* E2 (Figure 2.1 D) was greater than that of all the B1 types (i.e. heterologous, and freshly-isolated and cultured homologous; Figure 2.2 A, E and F respectively), and the biomass contribution of *Symbiodinium* A1.4 (Figure 2.2 C) was greater than that of the cultured homologous and heterologous B1 types (Figure 2.2 E and F; LSD, $p < 0.05$ for all comparisons).

2.3.2. Symbiont mitotic index (MI)

All *Symbiodinium* types displayed a daily synchronicity in the timing of cell division (MI; see Appendix 1, Table A1.1). A peak in the MI was noted at 7 am (the start of the light period) for all types except the cultured homologous *Symbiodinium*, which displayed a peak at 10 am, and these patterns were consistent across the 12-week period (see Figure 2.1 for daily Week 1 MI graphs). However, the average daily MI decreased significantly with time for all *Symbiodinium* types during the 12-week experiment (Table 2.1; one-way ANOVA, $p < 0.05$ for all treatments), with the MI at Week 1 (2.3-4.4%) being significantly greater than at the subsequent time-points (0.009-1.9%) in all cases (LSD, $p < 0.05$).

There were differences between the average MI of the different *Symbiodinium* types at some of the time-points (Table 2.4; one-way ANOVA, $p < 0.05$). In particular, the average MI at Week 1 was higher for all of the B1 types (i.e., heterologous, and freshly-isolated and cultured homologous) than for the other types (LSD, $p < 0.05$), while at Week 12 the cultured homologous B1 had a lower MI than all of the other types (Table 2.2; LSD, $p < 0.005$).

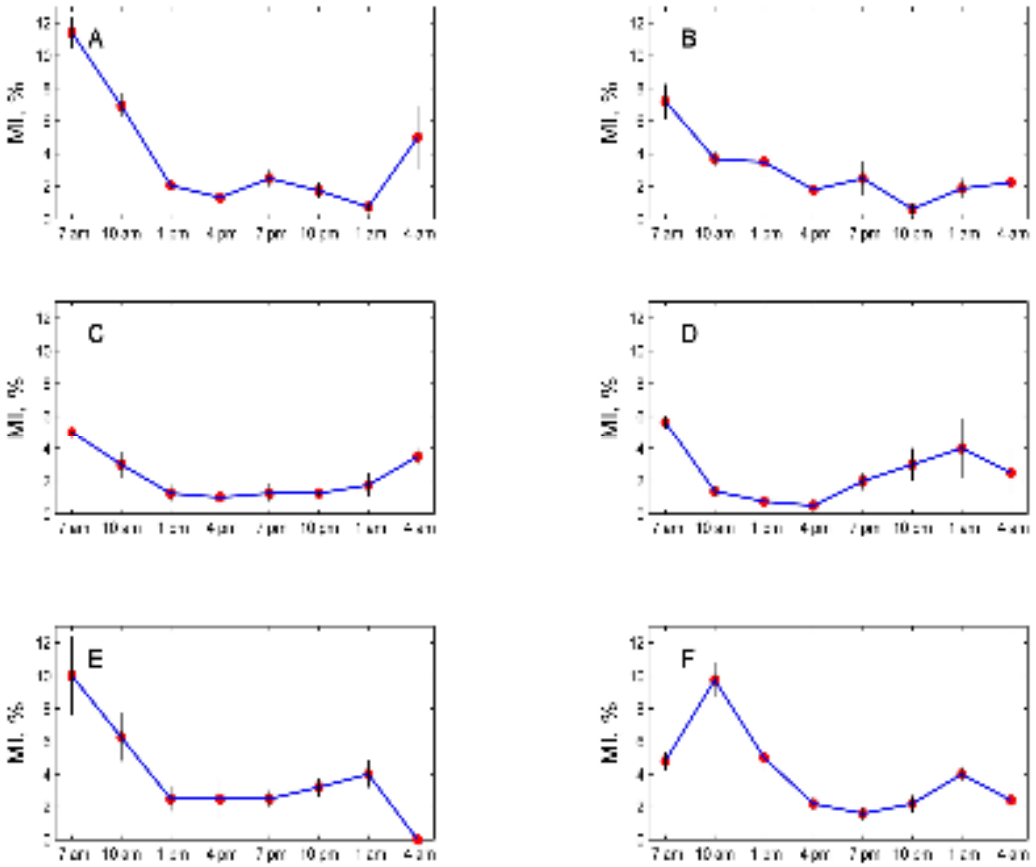

Figure 2.1. The Mitotic Index (MI) in *A. pulchella* calculated over a 24 hour period at Week 1 by a range of different cultured heterologous *Symbiodinium* types: (A) heterologous B1, (B) F5.1, (C) A1.4, and (D) E2. *A. pulchella* was also infected with homologous *Symbiodinium* type B1 that was either (E) freshly isolated or (F) cultured. Values are means ±SE; n = 5 per time point.

2.3.3. Photosynthesis, respiration and P:R

The algal biomass specific rate of gross photosynthesis (P_{gross}, μg C mm^{-3} day^{-1}) for the symbioses involving *Symbiodinium* A1.4, B1 (heterologous) and F5.1, as well as both the freshly isolated and cultured homologous *Symbiodinium* B1, decreased over the 12-week post-infection period (Figure 2.2 C, A, B, E and F respectively, Table 2.3; one-way ANOVA, $p < 0.05$). There was a significant decrease in P_{gross} between: Week 2 and Weeks 8-12 for *Symbiodinium* A1.4 (Figure 2.2 C); Week 1 and Weeks 4-12 for the heterologous *Symbiodinium* B1 (Figure 2.2 A); Week 2 and Weeks 4-12 for *Symbiodinium* F5.1 (Figure 2.2 B); Week 1 and Weeks 2-8 for the freshly isolated homologous B1 (Figure 2.2 E); and Week 1 and Weeks 2-12 for the cultured homologous B1 (Figure 2.2 F) (LSD, $p < 0.05$ for all comparisons). Of note, however, P_{gross} in *Symbiodinium* A1.4 (Figure 2.2 C) actually increased between Weeks 1 and 2 (LSD, $p < 0.05$) despite its subsequent decline, while P_{gross} in *Symbiodinium* E2 (Figure 2.2 D) remained unchanged throughout the experimental period (LSD, $p > 0.05$). Nevertheless, at Week 12, no significant difference in P_{gross} was observed between the *Symbiodinium* types (Table 2.4; one-way ANOVA, d.f. = 16, $F = 2.7$, $p > 0.05$ for all comparisons).

The total photosynthetic rate for the symbiosis (μg C mg^{-1} animal protein day^{-1}) did not change over the course of the 12 weeks for any of the symbiont types except for *Symbiodinium* A1.4 (Figure 2.3, Table 2.3; one-way ANOVA, $p < 0.005$), which displayed a significant increase between Weeks 1 and 2 (LSD, $p < 0.005$) followed by a significant decrease between Weeks 2 and 8 (LSD, $p < 0.05$). It appears that the lack of a significant increase in total P_{gross} for *Symbiodinium* B1, as well as *Symbiodinium* F5.1, may be a result of the effect of the lower initial algal density being countered by a high initial cell specific P_{gross} (Figure 2.2). At Week 12, the total photosynthetic rate in the different symbioses was dissimilar (Table 2.2, Table 2.4; one-way ANOVA, d.f. = 4; 12, $F = 4.2$, $p < 0.05$), with the symbiosis with *Symbiodinium* E2 (Figure 2.3 D) having a significantly higher photosynthetic rate than the other symbioses (LSD, $p < 0.05$); of note, while not included in this analysis due to its very high variability, the average P_{gross} of *Symbiodinium* F5.1 (Figure 2.3 B) was comparable to that of type E2.

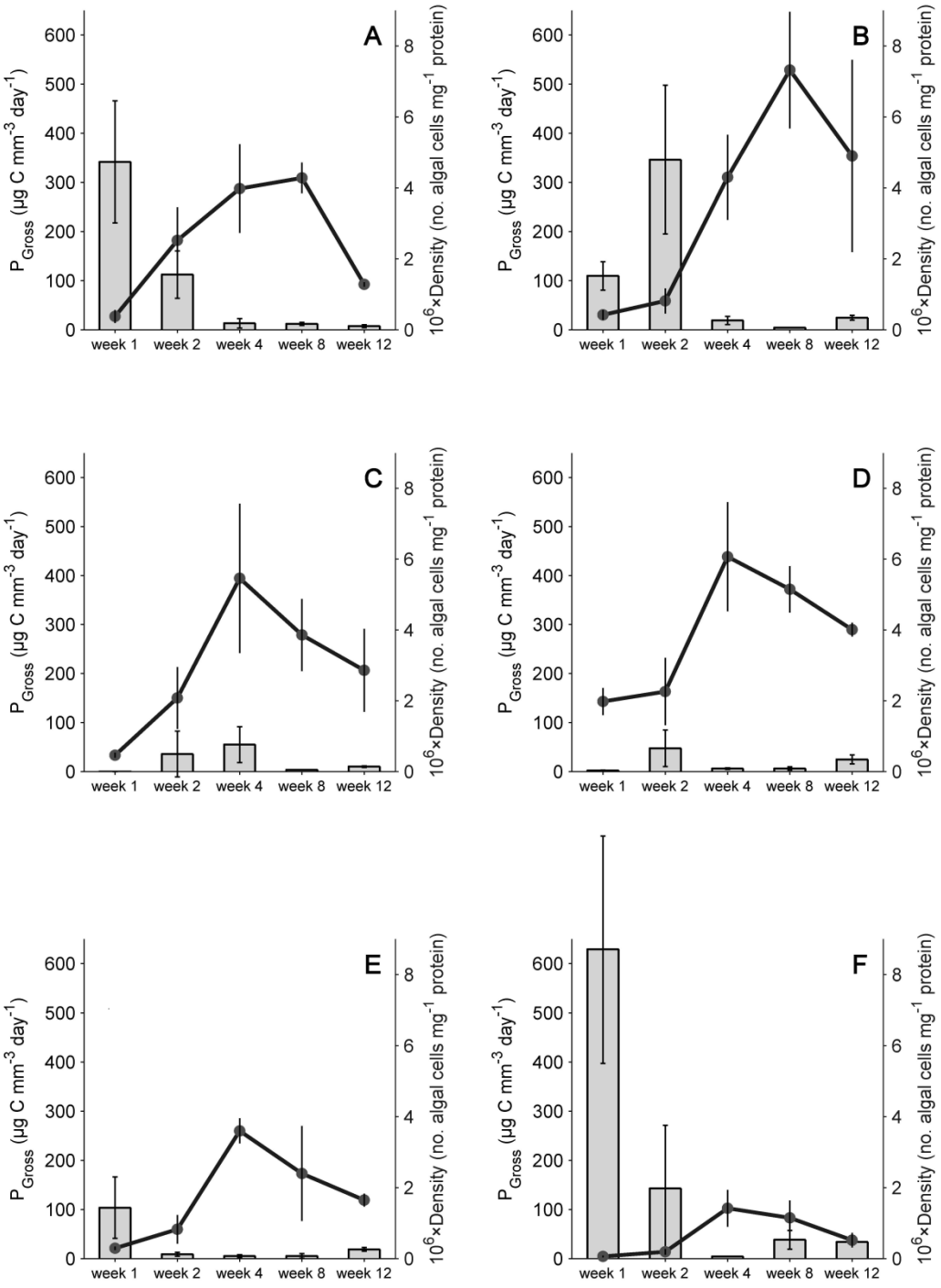

Figure 2.2. Symbiont density (solid line) and cell-specific gross photosynthesis (grey bars) in *A. pulchella* during infection by a range of different cultured heterologous *Symbiodinium* types: (A) B1, (B) F5.1, (C) A1.4, and (D) E2. *A. pulchella* was also infected with homologous *Symbiodinium* type B1 that was either (E) freshly isolated or (F) cultured. Values are means ±SE; n = 4-5 per time point.

Two general trends were observed with regards to symbiosis respiration (R_s) (Figure 2.3). First, in three cases (*Symbiodinium* F5.1, A1.4 and the freshly-isolated homologous B1) R_s changed with time since infection (Table 2.3; one-way ANOVA, $p < 0.05$), with an initial increase between Weeks 1 and 2 (A1.4, B1; Figure 2.3 C and A) or 4 (F5.1; Figure 2.3 B), and a subsequent decline to Week 12 (LSD, $p < 0.05$ and $p < 0.005$ for *Symbiodinium* A1.4 and B1, and F5.1, respectively). In the second group of remaining *Symbiodinium* types, R_s did not change with time (Table 2.3; one-way ANOVA, $p > 0.05$ for all types). At Week 12, significant differences in R_s were noted between the associations harbouring the different symbiont types (Table 2.4; one-way ANOVA, $p < 0.05$), with *Symbiodinium* E2 and F5.1 generating significantly higher R_s values than the other types (LSD, $p < 0.05$). Animal and zooxanthellar respiration (R_a, R_z) followed similar trends (see Appendix 1, Table A1.1 and Table 2.3 and 2.4), though of note R_z varied hugely at Week 12 (from 0.24 to 7.3 μg C mg^{-1} animal protein day^{-1}; one-way ANOVA, $p < 0.005$). This was largely a result of the significantly higher density of *Symbiodinium* E2 (see above) and the higher rate of R_s in this association, which led to a significantly higher R_z than for the other symbiont types (LSD, $p < 0.005$).

The ratio of total photosynthesis to total respiration (P:R, both μg C mg^{-1} animal protein day^{-1}; Figure 2.3) gives an indication of autotrophic potential. All the associations, except for those involving *Symbiodinium* E2 and F5.1 (which had much the highest rates of R_s) reached a P:R ratio of approximately 1 or higher by Week 12, and hence were autotrophic or near-autotrophic with respect to carbon. The association involving the heterologous *Symbiodinium* B1 attained a value of > 1 by Week 4, while the freshly-isolated and cultured homologous *Symbiodinium* B1 reached a P:R ratio of ~1 by Week 8.

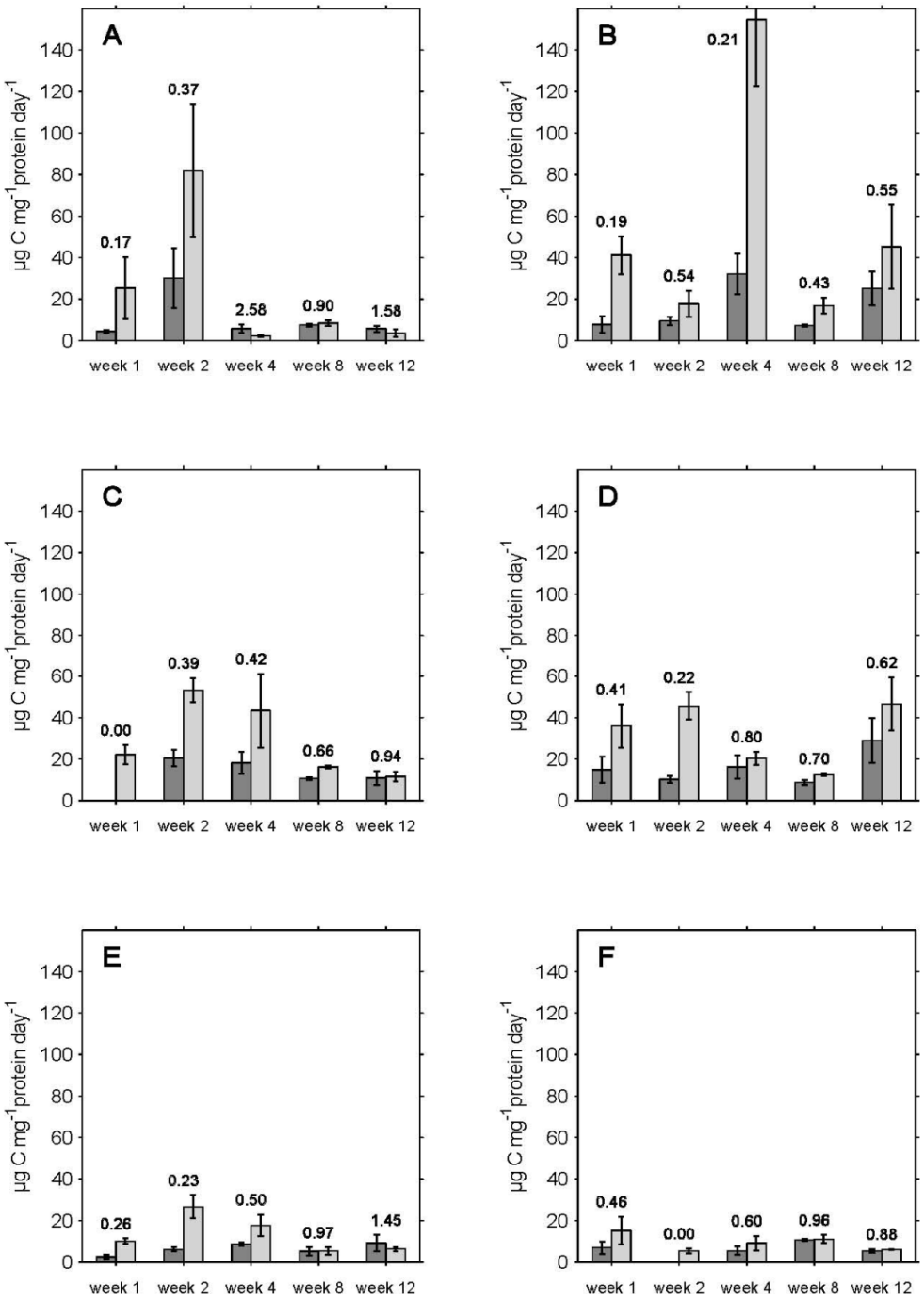

Figure 2.3. Gross photosynthesis (dark grey bars) and total symbiosis respiration (light grey bars) in an *A. pulchella – Symbiodinium* symbiosis during infection by a range of different cultured heterologous *Symbiodinium* types: (A) B1 (heterologous), (B) F5.1, (C) A1.4, and (D) E2. *A. pulchella* was also infected with homologous *Symbiodinium* type B1 that was either (E) freshly isolated or (F) cultured. The values above the bars are the P:R ratios (i.e. gross photosynthesis/total respiration). Values are means ±SE; n = 4-5 per time point.

2.3.4. Translocation

Symbioses with *Symbiodinium* E2 and the cultured homologous B1 displayed no significant change in the percentage of carbon available for translocation (T) over the course of reinfection (see Appendix 1, Table A1.1, Table 2.3; one-way ANOVA, $p > 0.05$). In the other symbioses, however, T declined significantly with time (see Appendix 1, Table A1.1; one-way ANOVA, $p < 0.05$ for all treatments), in part because of calculated increases in R_z (see above and Appendix 1, Table A1.1). *Symbiodinium* A1.4 and the freshly-isolated homologous *Symbiodinium* B1 showed a decrease of 3% and 30%, respectively between Weeks 1 and 4, while the heterologous *Symbiodinium* B1 and *Symbiodinium* F5.1 displayed a decrease of 9.3% and 5%, respectively, between Weeks 1 and 8 (LSD, $p < 0.05$ for all comparisons). At Week 12, the cultured homologous *Symbiodinium* B1 displayed a significantly higher translocation rate (99.9%) than did *Symbiodinium* B1 (heterologous), E2 and A1.4, and the freshly-isolated homologous B1 (96.4 – 98.8%; Table 2.4; one-way ANOVA with post hoc LSD, d.f = 19, $F = 3.7$, $p < 0.05$ for all comparisons); there were no other significant differences (Table 2.2).

2.3.5. CZAR

All the symbioses displayed a significant increase in the CZAR between Weeks 1 and 12 (ANOVA permutation test, $p < 0.05$ for all treatments; see Appendix 1, Table A1.1). The CZAR values obtained at Week 12 were: 176.8% for the freshly-isolated homologous *Symbiodinium* B1; 113.0% for the heterologous B1; 81.1% for the cultured homologous B1; and 74.9%, 72.4% and 52.3% for *Symbiodinium* A1.4, E2 and F5.1, respectively (Table 2.2). Despite this broad range of values, however, they were not significantly different (ANOVA permutation test, $p > 0.05$), most likely due to the high variability in the data for some of the associations.

Table 2.2. The CZAR in *A. pulchella* 12 weeks after infection with different *Symbiodinium* types. *Density* zooxanthellar cell density; *MI* mitotic index; μ cell-specific growth rate; P_{gross} gross photosynthetic rate; R_s symbiosis respiration per day; *P:R* ratio of total photosynthesis to total respiration; R_a derived animal respiration per day; R_z derived zooxanthellar respiration per day; P_{zNet} net photosynthesis per day by zooxanthellae; μ_c carbon-specific growth rate (fraction of the carbon standing stock added per day); *T* percentage of the net photosynthetic production translocated to host; *CZAR* potential contribution by zooxanthellae to animal's daily respiratory carbon requirements. The results represent an idealised day of 12 hours light exposure at saturating irradiance and 12 hours of darkness. Values are means ± SE (n = 4-5 for all parameters, except *algal cell diameter*, where n = 72 and *MI*, where n = 32).

***Symbiodinium* type**	**A1.4**	**B1**	**E2**	**F5.1**	**Homologous (B1) – freshly-isolated**	**Homologous (B1) – cultured**
Density (× 10^6 cells mg^{-1} protein)	2.9 ± 1.2	1.3 ± 0.1	4.1 ± 0.2	4.5 ± 2.9	1.6 ± 0.2	0.5 ± 0.2
Algal cell diameter (µm)	9.59 ± 0.25	9.20 ± 0.15	8.59 ± 0.30	7.72 ± 0.08	8.67 ± 0.07	8.29 ± 0.09
Derived algal carbon (pg C $cell^{-1}$)	73.14 ± 3.53	64.92 ± 2.83	54.69 ± 4.30	41.23 ± 1.06	55.89 ± 1.06	49.14 ± 2.71
MI (%)	1.1 ± 0.2	1.75 ± 0.6	1.4 ± 0.5	1.1 ± 0.2	1.6 ± 0.3	6.7×10^{-3} ± 1.3×10^{-3}
µ (d^{-1})	0.060	0.018	0.017	0.015	0.020	2×10^{-5}
P_{gross} (µg C mg^{-1} protein day^{-1})	10.88 ± 3.41	5.71± 1.46	29.02± 10.70	25.04 ± 8.10	9.22 ± 3.87	5.41 ± 0.71
R_s (µg C mg^{-1} protein day^{-1})	11.63 ± 2.40	3.61 ± 1.80	46.58 ± 12.79	45.13 ± 20.25	6.36 ± 1.00	6.13 ± 0.22
P:R	0.94	1.58	0.62	0.55	1.45	0.88
Algal:total protein ratio × 10^{-2}	15.93 ± 5.07	5.45 ± 0.88	17.24 ± 1.73	18.7 ± 5.10	6.56 ± 0.14	3.53 ± 0.97
R_a (µg C mg^{-1} protein day^{-1})	9.34 ± 1.02	3.43 ± 0.56	39.29 ± 9.70	44.56 ± 17.82	5.78 ± 0.74	5.91 ± 0.18
R_z (µg C mg^{-1} protein day^{-1})	2.29 ± 1.25	0.34 ± 0.20	7.30 ± 1.85	0.57 ± 0.39	0.58± 0.19	0.24 ± 0.08
Algal carbon standing stock: C' (µg C mg^{-1} protein)	21.33 ± 8.21	6.11 ± 1.04	22.15 ± 2.74	7.03 ± 1.44	7.48 ± 1.72	3.89 ± 1.12
P_{zNet} (µg C mg^{-1} protein day^{-1})	9.5 ± 3.5	5.1 ± 1.2	27.0 ± 10.2	19.6 ± 5.6	8.8 ± 3.7	5.4 ± 0.7
μ_c (d^{-1})	0.5 ± 0.09	0.83 ± 0.10	1.2 ± 0.40	1.63 ± 0.40	1.08 ± 0.20	1.71 ± 0.43
T (%)	96.4 ± 0.5	97.5 ± 0.6	97.4 ± 1.4	98.8 ± 0.5	98.0 ± 0.5	99.9 ± 0.001
CZAR (%)	74.9 ± 12.1	113.0 ± 10.9	72.4 ± 26.2	52.3 ± 8.3	176.8 ± 26.1	81.1 ± 7.1

Table 2.3. One-way ANOVA results for parameters; Density, MI (Mitotic Index), Volume-specific P_{Gross}, Total P_{Gross}, R_s (symbiosis respiration), R_z (zooxanthellar respiration) and T (translocation) indicating d.f., F values and p values for each *Symbiodinium* type.

Parameter	**Symbiont type**	**d.f.**	**F**	**p**
Density	Heterologous B1	4; 15	5.7	p = 0.005
	FI -Homologous B1	4; 11	3.4	p = 0.048
	C - Homologous B1	4; 15	7.1	p = 0.002
	A1.4	4; 14	3.4	p = 0.049
	E2	4; 14	3.3	p = 0.048
	F5.1	4; 13	5.6	p = 0.008
MI	Heterologous B1	4; 155	4.7	p = 0.0001
	FI -Homologous B1	4; 152	8.5	p = 0.0001
	C - Homologous B1	4; 195	64.1	p = 0.0001
	A1.4	4; 151	2.7	P = 0.030
	E2	4; 155	2.7	p = 0.031
	F5.1	4; 155	11.6	p = 0.0001
Volume specific P_{Gross}	Heterologous B1	4; 14	4.0	p = 0.023
	FI -Homologous B1	4; 10	3.0	p = 0.050
	C - Homologous B1	4; 10	5.9	p = 0.010
	A1.4	4; 12	3.1	p = 0.048
	E2	4; 10	0.6	p = 0.686
	F5.1	4; 10	3.0	p = 0.050
Total P_{Gross}	Heterologous B1	4; 13	2.0	p = 0.142
	FI -Homologous B1	4; 10	1.6	p = 0.248
	C - Homologous B1	4; 15	2.2	p = 0.117
	A1.4	4; 11	10.6	p = 0.001
	E2	4; 11	2.7	p = 0.083
	F5.1	4; 10	1.7	p = 0.225
R_s	Heterologous B1	4; 10	2.9	p = 0.078
	FI -Homologous B1	4; 9	5.3	p = 0.018
	C - Homologous B1	4; 12	1.8	p = 0.194
	A1.4	4; 11	4.2	p = 0.027
	E2	4;11	3.0	p = 0.064
	F5.1	4;9	13.1	p = 0.001
R_z	Heterologous B1	4; 10	4.5	p = 0.024
	FI -Homologous B1	4; 12	8.1	p = 0.072
	C - Homologous B1	4; 13	15.9	p = 0.097
	A1.4	4; 13	2.4	p = 0.0001
	E2	4; 10	3.0	p = 0.102
	F5.1	4; 12	2.5	p = 0.002
T	Heterologous B1	4; 15	4.6	p = 0.012
	FI -Homologous B1	4; 13	4.0	p = 0.030
	C - Homologous B1	4; 10	1.1	p = 0.390
	A1.4	4; 14	3.8	p = 0.042
	E2	4; 10	0.7	p = 0.629
	F5.1	4; 15	4.6	p = 0.018

Table 2.4. One-way ANOVA results for parameters; Density, MI (Mitotic Index), Volume-specific P_{Gross}, Total P_{Gross}, R_s (symbiosis respiration), R_z (zooxanthellar respiration) and T (translocation) indicating d.f., F values and p values for each time point.

Parameter	Week	d.f.	F	P
Density	1	5; 17	12.9	p = 0.0001
	2	5; 18	1.2	p = 0.333
	4	5; 17	2.0	p = 0.129
	8	5; 16	1.9	p = 0.143
	12	4; 12	6.9	p = 0.004
MI	1	5; 203	8.5	p = 0.024
	2	5; 194	0.2	p = 0.953
	4	5; 198	8.1	p = 0.0001
	8	5; 190	8.5	p = 0.0001
	12	5;178	12.2	p = 0.0001
Volume specific P_{Gross}	1	5; 13	4.2	p = 0.017
	2	5; 12	2.5	p = 0.094
	4	5; 12	1.5	p = 0.267
	8	5; 13	2.8	p = 0.063
	12	5; 11	2.7	p = 0.072
Total P_{Gross}	1	4; 12	5.9	p = 0.007
	2	4; 12	2.5	p = 0.098
	4	4; 10	2.4	p = 0.115
	8	4; 14	0.8	p = 0.543
	12	4; 12	4.2	p = 0.024
R_s	1	5; 15	1.8	p = 0.176
	2	5; 12	2.6	p = 0.085
	4	5; 11	16.9	p = 0.0001
	8	5; 14	5.1	p = 0.007
	12	5; 10	4.7	p = 0.018
R_z	1	5; 15	6.5	p = 0.002
	2	5; 13	2.0	p = 0.139
	4	5; 14	6.1	p = 0.003
	8	5; 14	2.6	p = 0.073
	12	5; 14	8.7	p = 0.001
T	1	5; 15	3.8	p = 0.021
	2	5; 15	0.4	p = 0.825
	4	5; 16	2.1	p = 0.120
	8	5; 16	1.8	p = 0.177
	12	5; 14	3.7	p = 0.023

2.4. Discussion

This is the first study to measure the influence of symbiont diversity and host-symbiont specificity on the autotrophic potential of novel cnidarian-dinoflagellate symbioses. It demonstrates that homologous (both cultured and freshly-isolated) *Symbiodinium* cells *A. pulchella* reach a stable state sooner than do heterologous

Symbiodinium types when infected into *A. pulchella*. Taking up homologous *Symbiodinium* cells also allows the host to achieve a fully autotrophic state at an earlier stage than when heterologous *Symbiodinium* types are taken up. Moreover, a marked trend was eveient towards the host anemones containing *Symbiodinium* B1 (both homologous and heterologous) benefiting most from their symbiotic partners. Of note, *A. pulchella* heterologous *Symbiodinium* B1was also successful, emphasising the specificity of *A. pulchella* for this symbiont type. The possible reasons for these differential patterns and their potential ecological significance will be considered here.

2.4.1. Infection dynamics

The finding of a peak in symbiont cell division just after dawn, as demonstrated by most of the *Symbiodinium* types, is supported by previous studies (Fitt and Trench, 1983; Fitt *et al*., 2000) and is a common characteristic of dinoflagellate growth (Chisholm, 1981). The delayed peak in the division at 10am of the cultured homologous symbionts has, to our knowledge, no parallels in the literature, but suggests an innate difference between the symbiont types. The peak in algal division at Week 1 and the following decrease in division over the 12-week period indicate that, while there is a rapid re-population of the anemones by the symbionts, their growth becomes constrained as the density of symbiont cells increases. This could arise for a number of reasons: (1) space limitation within the host cell (Muscatine and Pool, 1979; Jones and Yellowlees, 1997); (2) increasing competition for resources such as inorganic nitrogen (Cook and D'Elia, 1987) or carbon (Weis, 1993; Davy and Cook, 2001b); or (3) host control of symbiont cell growth and mitosis by an unknown mechanism (McAuley, 1981; Douglas and Smith, 1984; Bossert and Dunn, 1986).

Given that both the freshly-isolated and cultured homologous algae were originally isolated from *A. pulchella*, this might allow for more rapid integration with the host (e.g. via intracellular communication across the membrane complex at the host-symbiont interface; Wakefield and Kempf, 2001; Peng *et al*., 2010) and could explain their initially higher division rates during symbiosis establishment. The same

may be true for *Symbiodinium* B1 which was originally isolated from *A. pallida*, a close relative of the Indo-Pacific host species used here. Similarly, most infectivity studies have shown that homologous symbiont types are readily accepted by their hosts, and that heterologous types are either rejected or display lower rates of population growth if accepted (Kinzie, 1974; Kinzie and Chee, 1979; Schoenberg and Trench, 1980b; Trench, 1987, 1997; Davy *et al.,* 1997; Weis *et al*., 2001; Belda-Baillie *et al*., 2002; Rodriguez-Lanetty *et al*., 2003).

Despite the high initial division rates of the B1 types in this study, it was the heterologous *Symbiodinium* A1.4, E2 and F5.1 that attained the highest densities at Week 12. *Symbiodinium* E2 also displayed a higher density at Week 1 indicating that this type proliferated faster inside the host. This trend was different to that reported by Schoenberg and Trench (1980b), who found that homologous symbionts in *Aiptasia tagetes* (= *pallida*) reached and maintained higher densities than did heterologous symbionts. One interpretation of this trend is that these *Symbiodinium* types had overshot the 'preferred' steady state for this symbiosis. Given that the B1 symbiont types divided more rapidly but reached lower densities at Week 12 than the heterologous types, indicates that they must have been expelled and/or degraded more rapidly. Cnidarians regulate their symbiont populations by controlling algal division rates, or by selectively destroying or expelling unwanted symbionts (Gates *et al*., 1992; Falkowski *et al*., 1993; Baghdasarian and Muscatine, 2000; Dunn *et al*., 2002; Dunn and Weis, 2009; Davy *et al.*, 2012). It is conceivable, therefore, that the effectiveness of such control mechanisms was hindered in the symbioses with the heterologous types, though we know too little about the underlying cellular processes (Weis *et al*., 2008; Davy *et al*., 2012) to speculate further. Of note, the heterologous *Symbiodinium* B1 also attained a relatively high density, but this eventually declined to a level similar to that in the homologous symbioses. A similar overshoot and subsequent decline was observed in the temperate sea anemone *Cereus pedunculatus* when infected with its homologous symbionts (Davy *et al.*, 1997). Far more research is required to unravel the complexities of host-symbiont biomass regulation in homologous and heterologous symbioses.

2.4.2. Photosynthesis and respiration

The decrease in the cell specific rate of gross photosynthesis at high symbiont densities may be the result of limited resources such as carbon dioxide (CO_2) (Davy and Cook, 2001; Hoogenboom *et al*., 2010). Self-shading of the dinoflagellate cells may also occur at high densities (Jones and Yellowlees, 1997). In the case of *Symbiodinium* E2, however, gross photosynthesis remained constant despite increases in the cell density; the reason for this is unknown, but it may be that this *Symbiodinium* type, when in association with *A. pulchella*, requires more time to adjust to the *in hospite* environment and, as a result, the initial peak in photosynthesis was not observed.

Symbiodinium E2 and F5.1 had the highest cell densities and total rates of P_{gross} at Week 12 (though the values for type F5.1 were not significantly higher due to considerable variability). This relative photophysiological success of these two heterologous *Symbiodinium* types at first seems surprising, but it is important to note though that this higher total P_{gross} was countered by a relatively high R_s rate and hence a lower P:R ratio. Photosynthetic stimulation of respiration has been noted previously in the sea anemone *Anemonia viridis* (Harland and Davies, 1995). Furthermore, the disproportionately higher rate of respiration *vs* photosynthesis in these two heterologous symbioses may indicate that it is more energetically expensive for *A. pulchella* to maintain these *Symbiodinium* types than the others. Of particular note, *Symbiodinium* E2 was originally cultured from a single free-living cell collected from Wellington Harbour (New Zealand), which may explain its unusual behaviour when *in hospite* and why the symbiosis with this *Symbiodinium* type never reached a fully autotrophic state (i.e. a P:R of 1); there is increasing evidence that such free-living *Symbiodinium* types are common in the field (Takabayashi *et al*., 2012). In contrast, all of the other symbioses except for that with *Symbiodinium* F5.1 became fully autotrophic or near-autotrophic at some stage, though the point at which this occurred differed between types. This finding has implications for post-bleaching events, as those associations that reach a fully autotrophic state sooner will be more likely to recover.

2.4.3. Translocation

Once a steady state had been reached, the percentage of carbon translocated ranged from 90 to 99.9% for the associations tested. The 'growth rate method' of estimating translocation has also been applied to numerous other symbioses, with similar estimates being derived (Muscatine *et al*., 1984; Steen and Muscatine, 1984; McCloskey *et al*., 1994; Day, 1994; Davy *et al*., 1996; Muller-Parker and Davy, 2001). However, only the association involving the cultured homologous *Symbiodinium* B1 displayed a significantly higher translocation rate at Week 12 than was seen in the other associations. This might suggest that, when in a stable symbiosis with its homologous symbiont, *A. pulchella* receives more translocated carbon than when in association with other symbiont types. However, the same pattern was not observed with freshly-isolated homologous symbionts, which grew faster and hence had less carbon available for translocation; this highlights the potentially different physiologies of cultured and freshly-isolated algae (Trench, 1971b; Domotor and D'Elia, 1984), which in this case was also apparent in the final CZAR values calculated for the homologous *Symbiodinium* cells.

Previous studies have demonstrated that different types of symbiotic dinoflagellates release different amounts of photosynthate and that they differentially influence incorporation patterns of translocated carbon in the host's tissues (Loram *et al*., 2007; Stat *et al*., 2008; Cantin *et al*., 2009). For example, Loram *et al*., (2007) found that when the sea anemone *Condylactis gigantea* hosted *Symbiodinium* clade A, a greater proportion of photosynthetically-fixed carbon was incorporated into animal lipids and amino acid pools than when conspecific anemones hosted clade B. Similarly, Cantin *et al.* (2009) showed that when the coral *Acropora millepora* harboured either *Symbiodinium* type C1 or D, it received more translocate from type C1. However, none of these studies attempted to measure the extent to which the photosynthate met the carbon demands of the host, and hence whether the observed differences had implications for host survival and proliferation. Moreover, further work is needed to identify the forms in which organic compounds are translocated *in hospite* and how these differ between *Symbiodinium* types (see review by Davy *et al*., 2012).

2.4.4. CZAR and ecological implications

A number of past studies have examined the contribution of photosynthetically-fixed carbon to cnidarian host respiration (Muscatine *et al*., 1981; Davies, 1984; Muscatine *et al*., 1984; Steen and Muscatine, 1984; Edmunds and Davies, 1986 and 1989; Smith and Muscatine, 1986; Farrant *et al*., 1987a and b; Davies, 1991; Davy *et al*., 1996; Verde and McCloskey, 1996a and b; Tremblay *et al*., 2012), however this is the first study to compare this parameter between symbioses involving the same host species but different symbiont types. The range of CZAR values obtained in this study is comparable to previous reports. Studies using the "growth rate method" (Muscatine *et al*., 1981) in sea anemones, as well other cnidarians such as jellyfish and zoanthids, estimated the CZAR to generally be >90% and sometimes >100% under well-illuminated conditions (Steen and Muscatine, 1984; McCloskey *et al*., 1994; Day, 1994; Davy *et al*., 1996). However, given the number of unsubstantiated assumptions involved in this method (e.g. that host /symbiont respiration rates are proportional to their protein ratio), the CZAR values obtained may well be over-estimates (Muscatine *et al*., 1981). Indeed, in the coral *Stylophora pistillata*, the more direct approach of measuring translocation by ^{14}C-radiolabelling yielded a much lower CZAR value than that derived via the growth rate method (40% *vs.* 90%; Muscatine *et al*., 1984), though this approach too has its shortcomings(for detailed discussion see Davy *et al*., 1996, 2012; Muller-Parker and Davy, 2001). Nevertheless, as a comparative tool, the growth rate method provides a valuable means of examining the relative performance of different symbiont types.

The trend of an increased CZAR over the experimental period for all the associations, suggests that more carbon becomes available to the host once the symbiosis reaches a stable state. This is of course not surprising given the considerable increase in the density of symbionts during symbiosis establishment; it was not the result of a corresponding decrease in animal respiration as no significant decrease in respiration was noted for any of the associations. The difference in photosynthetic performance between symbiont types has been widely studied (Rowan, 2004; Goulet *et al*., 2005; Berkelmans and van Oppen, 2006; Cantin *et al*., 2009) and is very relevant when assessing how a particular host-symbiont combination will fare both during and after

a bleaching event. However, it is not simply the photophysiology of the symbiont that will determine the long-term success of an association but more importantly the amount of carbon that is made available to the host when in symbiosis with a specific symbiont type and the metabolic demands of the host (Muscatine *et al*., 1981; Cantin *et al*., 2009). In the current study, while there were no significant differences in the CZAR between the symbioses tested, there was a marked trend towards the host anemones containing *Symbiodinium* B1 (both homologous and heterologous) benefiting most from their symbiotic partners. Greater replication would likely have clarified this trend further, however the difficulty in generating large numbers of aposymbiotic anemones made obtaining more replicates problematic. Nevertheless, the high CZAR values obtained for the symbioses with *Symbiodinium* B1, along with significantly higher Week-12 P:R ratios of these symbioses, suggest that anemones containing *Symbiodinium* B1 would likely grow faster and exhibit greater survivorship than those containing the other symbiont types. Similarly, Little *et al*. (2004) demonstrated that juveniles of the corals *Acropora tenuis* and *Acropora millepora* grow 2-3 faster when in symbiosis with *Symbiodinium* clade C than with clade D.

This study indicates that hosts with different-symbiont types have different photosynthetic and respiratory attributes, and that these impact the autotrophic potential of the symbiosis. This is demonstrated by the earlier onset of full autotrophy and the higher final CZAR values in the symbioses hosting *Symbiodinium* B1 (both homologous and heterologous). Indeed, our data highlights the functional diversity present amongst associations hosting different symbiont types. Future work is needed to determine whether the innate photosynthetic capacity of the symbiont or the influence of host-symbiont interplay on photosynthetic performance is more important for the onset and persistence of novel cnidarian-dinoflagellate symbioses. Similarly important is elucidation of the symbiosome membrane complex, across which all nutritional interplay must occur, and how its function is influenced by different host-symbiont pairings. Furthermore, it will be important to extend this type of work to corals in the field. Indeed, it should be noted that although some corals are capable of acquiring symbionts from the water column, this acquisition is temporary as these infections are not maintained as stable symbioses (Coffroth *et al*., 2010).

However, there is evidence for post-bleaching shifts in the dominant member of a mixed symbiont population (Baker, 2001; Baker *et al*., 2004; Berkelmans and van Oppen, 2006; Mieog *et al*., 2007; Jones *et al*., 2008) thus the differential performance of different symbiont types may still have a profound impact on the growth and survival of the host.

Chapter 3

The influence of symbiont type on host cell pH and physiology of a novel cnidarian-algal association

3.1. Introduction

Cnidarian-dinoflagellate symbioses are the foundation of coral reefs, which in turn support a wide diversity of other organisms (Linden *et al.,* 2002; Furla *et al.,* 2005). The dinoflagellate symbionts (genus *Symbiodinium*) contribute substantially to the productivity, survival and success of their coral hosts (LaJeunesse, 2002) by providing photosynthetic carbon (Muscatine *et al*., 1981; Davies, 1984), promoting the recycling and conservation of essential nutrients (Wang and Douglas, 1998), and by enhancing rates of calcification (Moya *et al*., 2008). However, despite the immense ecological importance of this symbiosis, little is still known about the cell biology that underlies symbiosis establishment, maintenance and dysfunction (Weis *et al*., 2008; Yellowlees *et al.,* 2008; Weis and Allemand, 2009; Davy *et al.,* 2012).

Intracellular pH (pHi) influences various facets of cell metabolism. The functioning of membrane channels and enzymes, intracellular signalling and the availability of ions are all modulated by pHi, so it is imperative that changes in pHi are minimized and tightly regulated (Busa and Nucitelli, 1984; Busa, 1986; Venn *et al*., 2009). pHi is therefore likely to play an important role in the cnidarian-dinoflagellate symbiosis, especially with respect to inter-partner signalling and transport across the symbiosome membrane and vacuolar space that separate the symbiont from its host (Wakefield and Kempf, 2001). For example, pHi influences the speciation of dissolved inorganic carbon (DIC), whose transport into the dinoflagellate symbionts is required for photosynthesis (Allemand *et al*., 1998; Leggat *et al*., 1999), as well as the speciation of inorganic nitrogen that is essential for growth (Miller and Yellowlees, 1989). pHi is also known to play a role in the regulation of the cell growth cycle in some organisms and could provide a mechanism by which host

cnidarians regulate cell division by their symbionts (Davy *et al.,* 2012). Despite the obvious importance of pHi, however, we know very little about this parameter in the cnidarian-dinoflagellate symbiosis, largely due to methodological limitations that are only just being overcome. Crucially, however, Venn *et al*. (2009) recently used a pH-sensitive fluorescent probe and confocal microscopy to measure pHi in host cells of the symbiotic anemone *Anemonia viridis* and the coral *Stylophora pistillata*. They found that host pHi was about 7.13 ± 0.24 in the dark and 7.41 ± 0.22 in the light for *S. pistillata* and 7.01 ± 0.27 in the dark and 7.29 ± 0.15 in the light for *A. viridis*. This important finding highlights the link between pHi of the host cell and the photosynthetic activity of its symbionts, and raises questions about how the differential photosynthetic performance of various symbiont types might influence pHi of the host cell cytoplasm. Furthermore, it highlights the need to test the generality of Venn *et al*. (2009)'s findings and whether the optimal pH of different symbiont types falls within the range of host pHi values measured. This study addressed these issues in the sea anemone *Aiptasia pulchella*, a widely employed model system for coral research (Weis *et al*., 2008). Figure 3.1 demonstrates four possible scenarios that depict how the total photosynthetic and respiration rates of the symbiont may affect the pHi of the host cell thus indicating the influence that different symbiont types may have on the intracellular physiology of the host. The aim of the study was to determine if pHi was a reflection of symbiont type and whether the optimal pH for photosynthesis coincides with pHi. In order to answer these questions, aposymbiotic (i.e. symbiont-free) sea anemones were infected with a range of *Symbiodinium* types and: (1) intact symbiosomes (i.e. the vacuoles that house the symbionts) were isolated, exposed to a range of ambient pHs, and photosynthetic performance of the symbiotic dinoflagellates measured; and (2) the pHi of the host cell was measured.

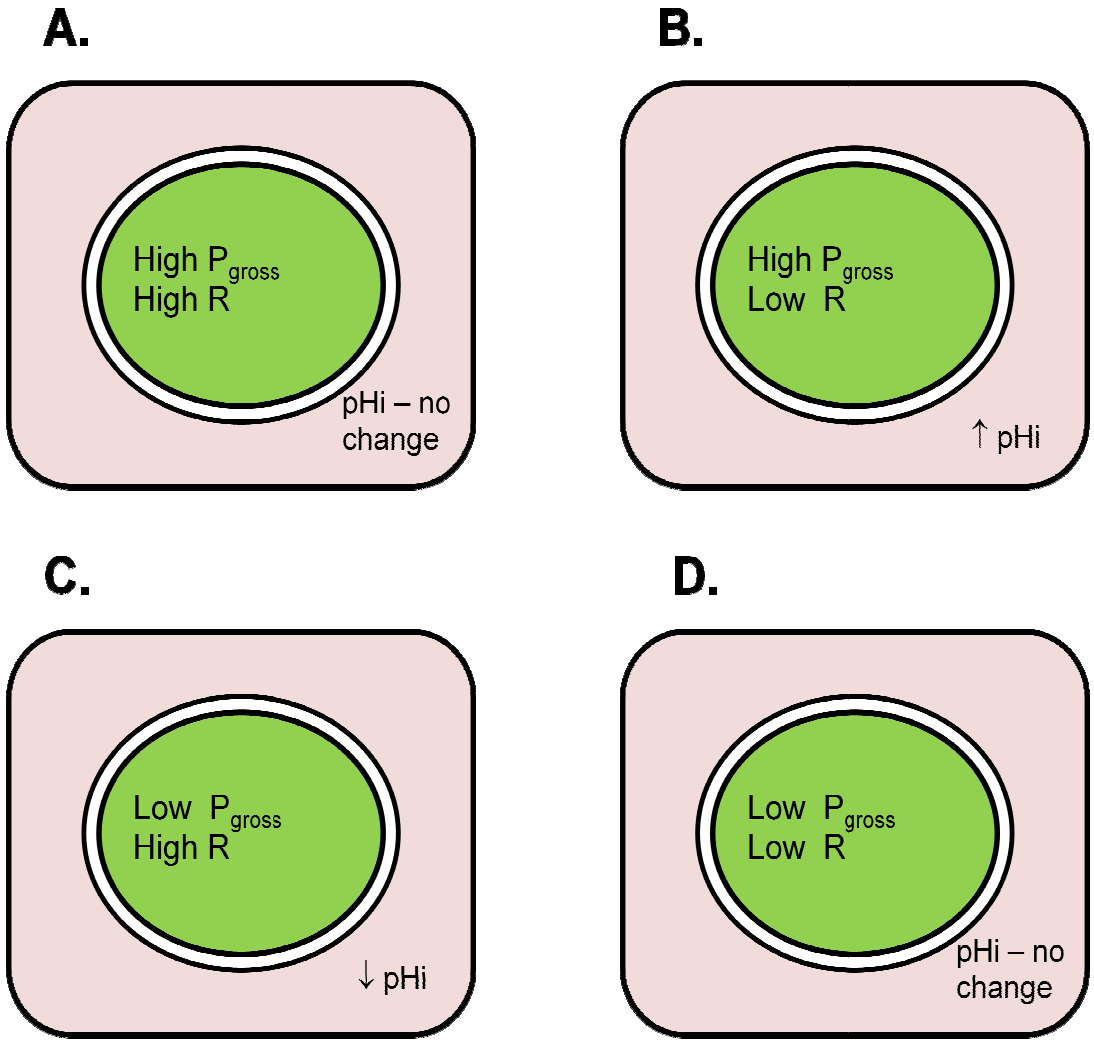

Figure 3.1. A conceptual diagram illustrating the potential relationship between photosynthetic and respiratory activity and internal host pH (pHi). Four scenarios are presented each representing a cnidarian host cell and its symbiont surrounded by the symbiosome membrane: A) High levels of photosynthesis and respiration balance each other out resulting in no change in the pHi. B) If levels of photosynthesis exceed respiration an increase in pHi is expected. C) If levels of respiration exceed photosynthesis a decrease in pHi is expected. D) Low levels of photosynthesis and respiration balance each other out resulting in no change in pHi.

3.2. Materials and methods

The sea anemone *Aiptasia pulchella* was used as the experimental host and infected with different *Symbiodinium* types (cultured *Symbiodinium* B1, heterologous *Symbiodinium* B1 as well as *Symbiodinium* E2). After 12 weeks intact symbiosomes were isolated from the infected anemones, exposed to a range of ambient pHs in ordre to simulate a change in host cell pHi, and the photosynthetic performance of the symbiotic dinoflagellates was measured. Host cells were also isolated from the anemones and the pHi of the host cell was measured using confocal microscopy.

3.2.1. Experimental organisms

The sea anemone, *A. pulchella* (originally from the Indo-Pacific region), was cultured at 25 ^{o}C and an irradiance of 100 µmol photons m^{-2} s^{-1} (12 h light:12 h dark) for a period of >1 year prior to use. The anemones were fed twice weekly with freshly hatched *Artemia* sp. To render the anemones aposymbiotic (i.e. symbiont-free), 120 individuals (oral disc diameter ~50 mm) were placed in six 1-L light-proof containers (20 individuals in each) filled with 0.44 µm filtered seawater (FSW) and cold shocked at 4 ^{o}C for 24 hours. The cold-shock process was repeated at weekly intervals for five weeks and the feeding regime was retained between cold-shocks, when care was taken to keep the anemones in low light (<1 µmol photons m^{-2} s^{-1}). The aposymbiotic anemones were maintained in the dark at 25 ^{o}C for approximately six months, when their aposymbiotic state was confirmed by exposure to a daily light cycle (12 h light:12 h dark) at an irradiance of 100 µmol photons m^{-2} s^{-1} for a four-week period; fluorescence microscopy (Olympus Provis AX70 microscope using autofluorescence) at 100× magnification was also used to screen for the presence of symbionts. Only anemones deemed to be completely free of their symbionts were used for subsequent experiments.

Two heterologous *Symbiodinium* cultures (i.e. from hosts other than the *A. pulchella* culture used here), as well as a culture of the homologous type (sub-clade B1), were grown at 25 ^{o}C and an irradiance of 100 µmol photons m^{-2} s^{-1}on a light regime of 12 h light: 12 h dark. The cultures were grown in silica-free f/2 medium at pH 8 (Sigma-

Aldrich, Auckland, New Zealand) over a six-week period prior to use in the infection study. The heterologous *Symbiodinium* sub-clades were *Symbiodinium* E2 (culture ID CCMP421), originally collected as a free-living alga from Wellington Harbour, New Zealand, and a heterologous *Symbiodinium* B1 (culture ID FlAp2), originally isolated from *A. pallida*, Long Key, Florida. The former of these types was selected because, in previous studies, its photosynthetic performance in symbiosis with the lab culture of *A. pulchella* had been particularly poor (see Chapter 2). The heterologous B1 allowed for a direct comparison between the same symbiont type but from different (though closely related) host species/geographic origins.

Symbiodinium types (n = 3 samples per culture or isolate) were genetically identified prior to infection (see Appendix 3 for the ITS2 sequences). DNA was extracted from zooxanthellae as described by Logan *et al*. (2010). PCR was performed using the following *Symbiodinium*-specific ITS2 primers: forward primer (ITSD), 5'-GTG AAT TGC AGA ACT CCG TG-3'; reverse primer (ITS2rev2), 5'-CCT CCG CTT ACT TAT ATG CTT-3'. Reactions were performed in a total volume of 30 µL using the MyTaq^{TM} Mix with an amplification profile consisting of 1 min 95 ^{o}C, 35 cycles of 1 min 95 ^{o}C, 15 s 55 ^{o}C, 10 s 72 ^{o}C, and a final hold temperature of 4 ^{o}C. Single strand conformation polymorphism (SSCP) was performed on all PCR products. PCR products were sequenced by Macrogen Inc. (Seoul, Korea). Sequences were aligned using the ClustalW plugin in Geneious Pro 4.8.5 and then edited. A blast search was then performed in the NCBI database in order to identify the sequences (see Table A3.1).

3.2.2. Infection of aposymbiotic *A. pulchella* with different *Symbiodinium* types

Aposymbiotic specimens of *A. pulchella* (n = 44 for each symbiont treatment for the respirometry experiments and n = 10 per treatment for the confocal fluorescence microscopy work) were infected with cultured *Symbiodinium* cells by pipetting a concentrated drop of the dinoflagellate suspension (approximately 1 million cells mL^{-1}) onto the oral disc of each anemone, with a sharp-ended glass pipette. A drop of one-day old *Artemia* sp. culture was then pipetted onto the oral disc to induce a feeding response and encourage the uptake of the *Symbiodinium* cells, as described

by Davy *et al*. (1997). Anemones were kept in FSW in 20 mL glass containers that received a water change 8 hours after infection and were maintained as before.

After 12 weeks, representative anemones (3 individuals per treatment) were selected from the pools of anemones reinfected with each of the different *Symbiodinium* types, to confirm symbiont identity. Individual anemones were homogenized in a hand-held, glass tissue grinder for approximately 5 min. The homogenate was then centrifuged at ×400 g for 5 min to separate the animal and algal fractions. DNA extraction and genetic typing were performed as described previously. All treatments were found to harbour the same *Symbiodinium* type as the one introduced at the start of the experiment (see Appendix 3 for the ITS2 sequences).

3.2.3. Symbiosome isolation and verification

Four anemones from each *Symbiodinium* type and pH treatment were homogenized in 1 mL of FSW in a tissue grinder and centrifuged at ×400 g. The supernatant was removed and the process repeated twice. The pellet was then passed through a step-wise sucrose gradient (30% - 80%) by centrifugation at ×3893 g for 1 hour (Figure 3.2). The 40% sucrose fraction (the symbiosome-enriched fraction) was collected fot further experiments. Three samples per symbiont treatment were also collected from each isolation and stained for 10 min with the FM1-43 lipophilic fluorescent probe (30 μM) as well as 10 μg mL^{-1} Hoechst 33342 for visualisation of the symbiosome membrane and nuclear material respectively; this enabled identification of the symbiosome membrane (Kazandjian *et al*., 2008). The presence of symbiosomes was confirmed with fluorescence microscopy (Olympus Provis AX70 microscope) at 100× magnification using a fluorescein isothiocyanate (FITC) filter to visualise FM 1-43 fluorescence and a 6-diamidino-2-phenylindole dihydrochloride, 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) filter to visualise Hoechst 33342 fluorescence. When examining the symbiosome-enriched fraction, all cells were noted to possess the surrounding symbiosome membrane. Dyes used for microscopy were purchased from Invitrogen (Auckland); other chemicals were purchased from Sigma-Aldrich (Auckland).

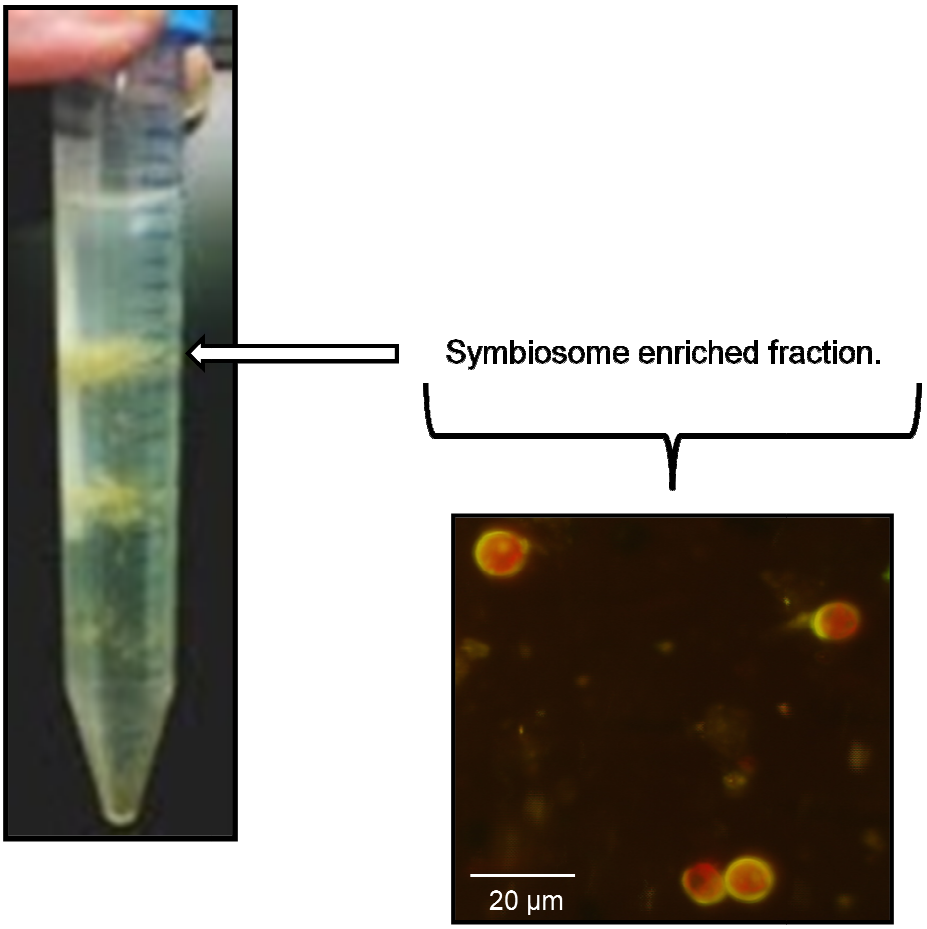

Figure 3.2. Photograph of the step-wise sucrose gradient depicting the 40% sucrose layer containing the symbiosome enriched fraction and the fluorescence microscope image of the symbiosome enriched fraction showing the symbiosome membrane (golden layer) after staining with FM1-43.

3.2.4. pH manipulation and determination of photosynthetic rates

The enriched symbiosome fractions isolated from individual anemones for each treatment were placed in a 10 mL glass chamber, itself situated in a glass water bath set at 25 ^{o}C (symbiosome fractions were isolated individually from 4 anemones per each pH and symbiont treatment). All the anemones used were for the isolations were of similar size. The density of the symbiosomes isolated from each anemone ranged from 1.5 × 10^{5} to 3.2 × 10^{6} cells mg^{-1} protein with differences in density arising from the variable infection success of the different symbiont types. The chamber contained FSW adjusted to a specific pH, ranging from 6 to 8.5 (in 0.25 pH

unit increments). The pH was adjusted using 2M HCl and 5M NaOH. A pilot study performed using anemones with a comparable symbiosome density showed that the pH remained stable over the course of the experiment. Unpublished data indicated that FSW buffered with PBS required a comparable volume of 2M HCl and 5M NaOH to adjust the pH to the desired increments, so buffering was determined to have no effect on the ion concentration and was deemed unnecessary. The cells were kept in suspension by gently stirring with a magnetic spin bar. The chamber was sealed by a glass lid with a rubber O-ring, into which an oxygen electrode (FIBOX 3 - fiber-optic oxygen meter, PreSens GmbH, Germany) and temperature probe were inserted; these were connected to the oxygen meter (see Figure 3.3 for experimental set-up). The chamber was illuminated by a Thorn38 PAR (photosynthetically active radiation) 150 W sealed beam-reflector lamp and irradiance incident on the surface of the chamber was measured by a QSL-100 irradiance meter (Biospherical Instruments Inc.). Maximum photosynthetic and dark respiratory O_2 fluxes were measured at each pH for symbiosomes isolated from all three associations. The respiration rate (mL O_2 h^{-1}) of each the different symbiosome suspensions was measured in darkness for about an hour or until a constant rate was observed. The oxygen tension was not allowed to fall below 50% saturation (Davies, 1984). The PAR lamp was then switched on and the rate of net photosynthesis by each symbiosome suspension was measured at 400 μmol photons m^{-2} s^{-1} (>light-saturation for photosynthesis), again for about an hour or until the rate was constant.

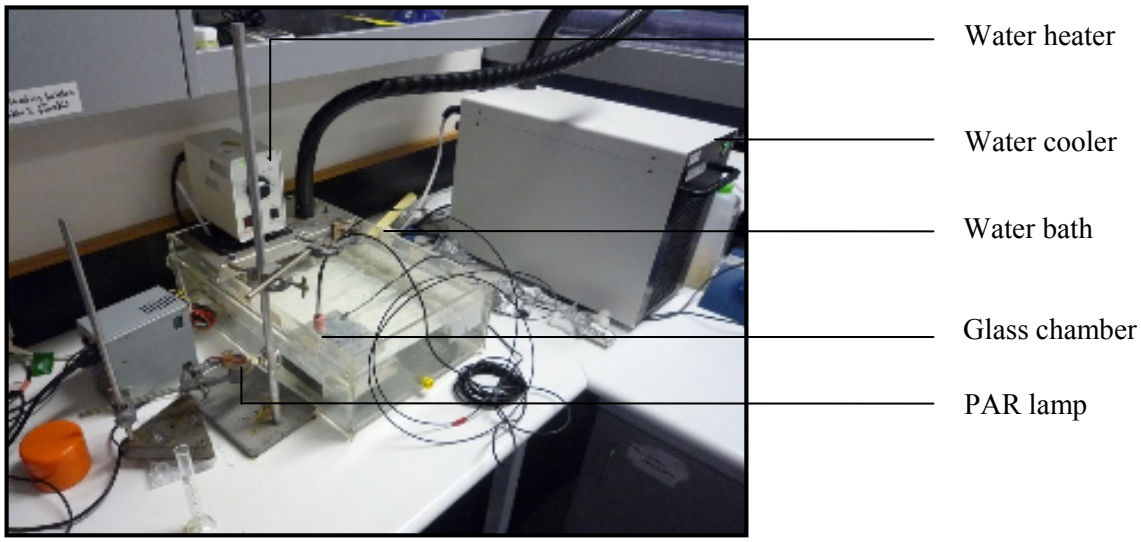

Figure 3.3. Photograph depicting the experimental O_2 flux set-up used to measure the photosynthetic and respiration rates of the isolated symbiosomes at a range of pH from pH 6 to pH 8.5 (photograph taken by Anne Wietheger).

The rate of gross photosynthesis was calculated by adding net photosynthesis to dark respiration, and converted into carbon equivalents by assuming photosynthetic and respiratory quotients of 1.1 and 0.9, respectively (Muscatine *et al*., 1981, 1983; Muscatine, 1990). The contents of the chamber were collected and centrifuged at ×400 g for 5 min to collect the symbiosomes. This fraction was then resuspended in 1 mL FSW. Algal cells were counted using light microscopy at 400× magnification. The gross photosynthetic and respiration rates were adjusted for the respiration chamber volume and normalised to the average algal cell volume.

3.2.5. *A. pulchella* cell isolation and staining

A. pulchella host cells were isolated based on a protocol adapted from Gates and Muscatine (1992). Anemones were first incubated in calcium-free seawater for 60 min. The tentacles from each anemone were then sliced off using a scalpel and cut into 1 to 2 mm sections. The sections were washed by transferring them from one glass slide to another, each with a drop of 3 U mL^{-1} lysozyme solution. This was repeated ten times to eliminate mucus. The chopped tentacles were then placed in a watch glass and the excess lysozyme solution pipetted off. A 300 µL 0.05% trypsin

solution (Type III, Sigma Chemical Co.; in Ca^{2+}-free FSW) was added to the tentacles. The watch glass was covered with parafilm to avoid evaporation and placed on an orbital shaker to allow the solution to agitate for 45 min. The contents were then transferred to a polylysine coated plastic dish and the isolated cells were left to settle for 15 min in the dark. The excess enzyme solution was then poured off.

Three different probe solutions were prepared as follows: 1) host nuclei and mitochondria – a mixture of 5 μg mL^{-1} Hoechst 33342 and 5 μM rhodamine; 2) host cell viability – a mixture of 10 μg mL^{-1} Hoechst 33342 and 10 μM fluorescein diacetate; 3) pHi determination – a 10 μM solution of the pH sensitive probe, carboxy SNARF-4f, 0.01% Pluronic F-127, and 0.1% dimethyl sulfoxide (DMSO). Cells were bathed in each solution in the dark at room temperature (approx. 22 °C) for 10 min with the exception of the Hoechst 33342 and fluorescein diacetate mix, which required 20 min. After dye loading, cells were washed with 200 μL Ca^{2+}free FSW. Cells were treated with 200 μL 1.5% carboxymethyl cellulose, so immobilizing them for confocal microscopy. All dyes used for microscopy were purchased from Invitrogen (Auckland); other chemicals were purchased from Sigma-Aldrich (Auckland).

3.2.6. Host cell viability and pHi

Isolated, stained host cells and their associated symbionts were visualized by confocal microscopy, conducted with an FV-1000 confocal microscope (Olympus, Auckland, New Zealand). All samples were imaged using 100× oil immersion lens, with excitation provided by a 405 nm laser for the Hoechst 33342 probe (emission at 460 nm ±10), 473 nm laser for fluorescein diacetate and rhodamine (emission at 520 nm ±10 and 530 nm ±10, respectively), and a 635 nm laser for chlorophyll autofluorescence. Cells loaded with SNARF-4f (emission at 546 and 635 nm) were excited at 473 nm, with a scan speed of 400 htz and the pinhole set at 1.51 units. Each cell was analyzed by capturing 10 frames in the x/y plane, throughout the depth (z plane) of the cell to produce a z-stack profile. Images were processed using Olympus FV10-ASW 1.7 software and analysed using ImageJ software (http://imagej.nih.gov/ij/). In order to empirically determine the viability of the

isolated cells, host cells were left to settle on the polylysine coated plates for up to 3 hours post-isolation, and were then stained with Hoechst and fluorescein at hourly intervals and visualized with confocal microscopy. Viable cells were identified every hour for 4 hours by green florescence of the host cell cytoplasm (as a result of the fluorescein probe) indicating an intact cell membrane. The percentage viability was determined from a count of 100 cells at 1, 2, 3 and 4 hours post host-cell isolation.

Measurements of pHi were performed by ratiometric analysis of SNARF-4f fluorescence (Venn *et al*., 2008). The ratio of fluorescence intensity of SNARF-4f at 546/635 nm was calibrated *in vivo* in the range pH 6 – 8.5, by washing host endodermal cells in calibration buffer adjusted to the desired pH by the addition of hydrochloric acid (HCl) or sodium hydroxide (NaOH). The ionophore, nigericin, was added at 30 μM to equilibrate pHi with the surrounding medium and eliminate pH gradients within the cell (Buckler and Vaughan-Jones, 1990). Calibration buffers contained the approximate cytosolic concentrations of major ions found in cnidarian cells (60 mM Na^+,200 mM K^+, 190 mM Cl^+, 25 mM Pipes (pH 6- 7.5) or Tricine (pH 8 – 8.5); Herrera *et al*., 1989; Goiran *et al*., 1997). Mannitol was added to adjust osmolarity to 1100 mosm L^{-1}. The calibration curve is provided in Appendix 2 (Figure A2.1).

The pH_i was then determined for dark-adapted host cells (after 20 min in the dark) as well as cells incubated for an hour under photosynthetically active radiation (PAR) that matched the conditions under which the anemones were cultured (100 μmol photons m^{-2} s^{-1}). The cells were stained with SNARF–4f and visualized using confocal microscopy (as described previously). Only endodermal cells that contained two algae were used as they were most suitable for visualizing the pHi of the host-cell cytoplasm and were far more numerous than cells with three algae. After inspection of each cell in the x, y and z planes, SNARF-4f fluorescence intensity at 546 and 635 nm was recorded within a region of interest (ROI) selected in an area of host cytoplasm that did not overlap with any algal cells; only the area that showed no autofluorescence was selected. R was calculated after subtracting background fluorescence recorded in a second ROI in the medium surrounding the

cell. The 546/635 nm fluorescence intensity ratio (R) was related to pH by the following equation:

$$pH = pK_A - \log [R - R_B/R_a - R \times F_{B(\lambda 2)}/F_{A(\lambda 2)}] \quad \textbf{[Equation 2]}$$

where (F) is fluorescence intensity measured at 635 nm (2) and the subscripts A and B represent the limiting values at the acidic and basic end-points of the titration, respectively (i.e., pH 6 and 8.5).

3.2.7. Statistical analyses

Data were tested for normality using the Kolmogorov-Smirnoff test and for homogeneity of variance using the Bartlett test. Multiple comparisons of R, P_{gross} and pHi between the different symbiotic associations and pH points were made using two-way analysis of variance ($\alpha = 0.05$), followed by LSD *post hoc* tests. A t-test was used to compare the pHi between the associations. PASW (Predictive Analytics SoftWare, Version 18) was used for all analyses.

3.3. Results

3.3.1. Determination of host cell pHi

Images of *Symbiodinium* cells within their host cells, stained with various fluorescent probes and visualized with confocal microscopy, are shown in Figure 3.4. Following 20 min of dark exposure the pHi of the host cells was 6.9 ± 0.04, 6.7 ± 0.14 and 6.6 ± 0.10 in the associations with the homologous B1, heterologous B1 and E2 symbionts respectively. After 1 hr of light exposure the pH increased to 7.0 ± 0.04, 6.9 ± 0.08 and 6.7 ± 0.08 respectively. Significant differences were noted between the dark- and light-adapted cells isolated from all the symbioses tested (T-test, $p < 0.05$ for all treatments). When comparing the pHi of the host cells isolated from the different associations, however, both after 20 min of darkness and after 1 hr of light

exposure, there were no significant differences between any of the symbioses (one-way ANOVA, d.f. = 2; 40, F =1.2-1.9, p> 0.05).

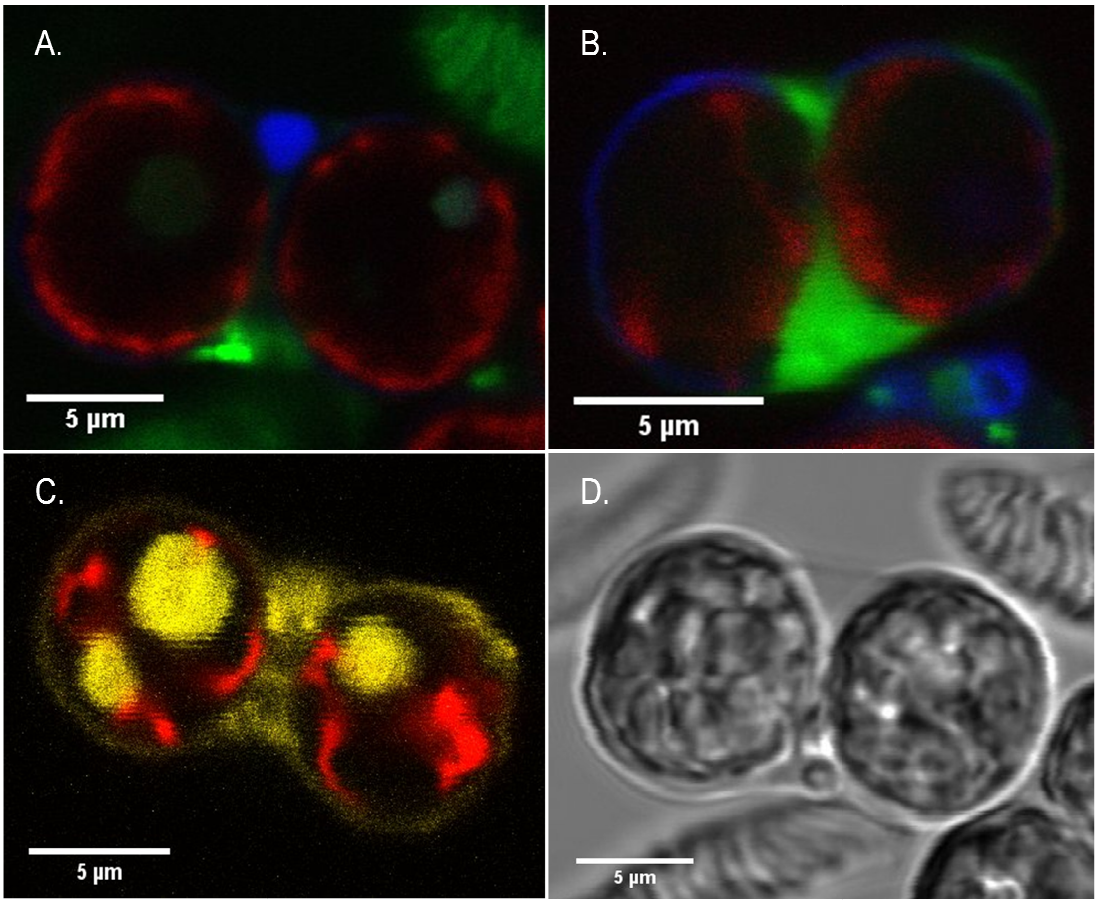

Figure 3.4. Cnidarian endodermal cells isolated from *A. pulchella* with two intracellular dinoflagellates. Confocal fluorescence microscope image of cells stained with (A) Hoechst 33342 and rhodamine 123, which stain nuclear material and mitochondria respectively. (B) Hoechst 33342 and fluorescein, which stain nuclear material and cytoplasm, respectively. (C) SNARF-4f for pHi determination. Colours represent the following: Red, autofluorescence of chlorophyll in intracellular algae; blue, nuclei stained with Hoechst 33342; green, mitochondria stained with rhodamine 123 (A) and cytoplasm stained with fluorescein (B); and yellow, cnidarian cytoplasm stained with SNARF-4f. (D) Light microscopy image.

3.3.2. Effect of pH on gross photosynthetic and respiration rates

Ambient pH had a significant effect on the rate of gross photosynthesis by the isolated symbiosomes. A positive interaction was observed between pH and symbiont type (two-way ANOVA, d.f. = 20, F = 1.8, $p < 0.05$). Significant differences in P_{gross} were observed both between different symbiont types and between different pH points (Figure 3.5A; two-way ANOVA, d.f. = 2, F = 5.6, $p < 0.005$ and two-way ANOVA, d.f. = 10, F = 5.9, $p < 0.005$ respectively). The symbiosomes isolated from the association infected with the homologous B1 type, displayed a significantly higher P_{gross} (0.0016 pg C $cm^{-3}h^{-1}$) at pH 7.5 than at any of the higher or lower pH values except for pH 8 (two-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons); P_{gross} at pH 8 was found to be significantly higher than the values at pH 7 and below (two-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons). The symbiosomes isolated from the association with heterologous B1 displayed significantly higher P_{gross} at pH 6.75, 7, 7.25 and 7.5 (0.0003 to 0.0008 pg C cm^{-3} h^{-1}) than the remaining pH values (two-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons) with the highest P_{gross} being achieved at pH 6.75 (two-way ANOVA with LSD post-hoc test, $p < 0.05$). The *Symbiodinium* E2 symbiosomes displayed the greatest P_{gross} at pH 8 (0.0012 pg C cm^{-3} h^{-1}), when it was significantly higher than the remaining pH values with the exception of pH 8.5 (two-way ANOVA with LSD post-hoc test, $p < 0.05$). At a pH below 7 the *Symbiodinium* E2 symbiosomes displayed significantly reduced P_{gross} (two-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons).

With respect to the respiration rate, no interaction between pH and symbiont type was observed (Figure 3.5B; two-way ANOVA, d.f. = 20, F = 1.2, $p > 0.05$). When looking at the association with the heterologous B1 only however, ambient pH was found to have a significant effect on the respiration of the isolated symbiosomes (Figure 3.5B; one-way ANOVA, d.f. = 10; 22, F = 5.1, $p < 0.005$). The respiration rate at pH 7.75, 8, 8.25 and 8.5 was significantly lower (0.0003 to 0.0004 pg C cm^{-3} h^{-1}) than at the lower pH values with the exception of pH 7.25 (one-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons). The pH at which the symbiosomes isolated from the different associations reached a total photosynthesis

to respiration ratio (P:R ratio) of 1 and above varied (Figure 3.6). The homologous B1 reached a P:R ratio of one and higher at pH 7.5, 7.75 and 8, while the heterologous B1 did not reach a P:R ratio of 1 but came close to it at pH 7-7.5. The symbiosomes with *Symbiodinium* E2 displayed a P:R ratio of 1 and greater from pH 8 upwards.

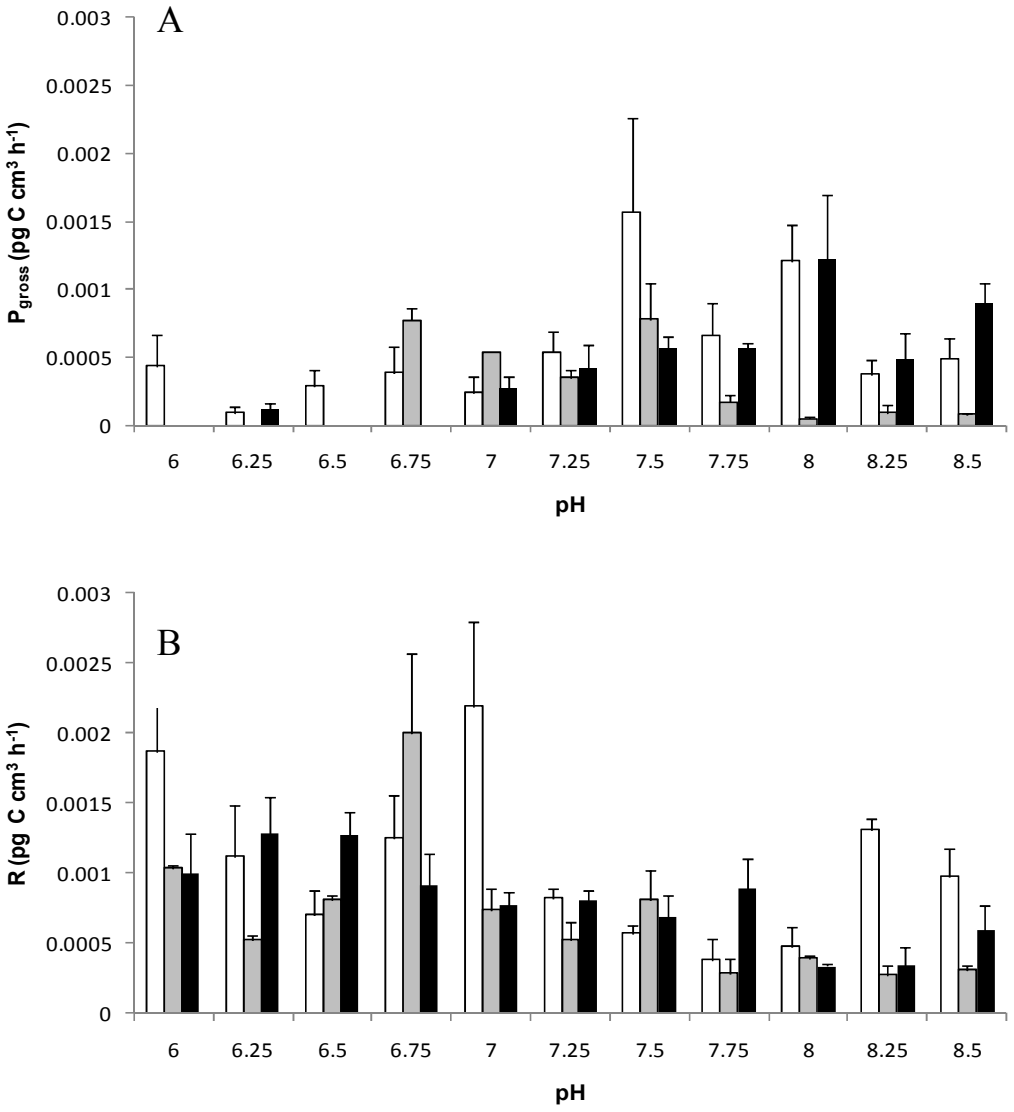

Figure 3.5. Relationship between gross photosynthetic rate (P_{gross}; A) and respiration rate (R; B) of symbiosomes isolated from *A. pulchella* when in association with three different *Symbiodinium* types, and the pH of the medium in which they were suspended. The white, grey and black bars represent the homologous B1, heterologous B1 and subclade E2 *Symbiodinium* respectively. Values are means ± SE, n = 4.

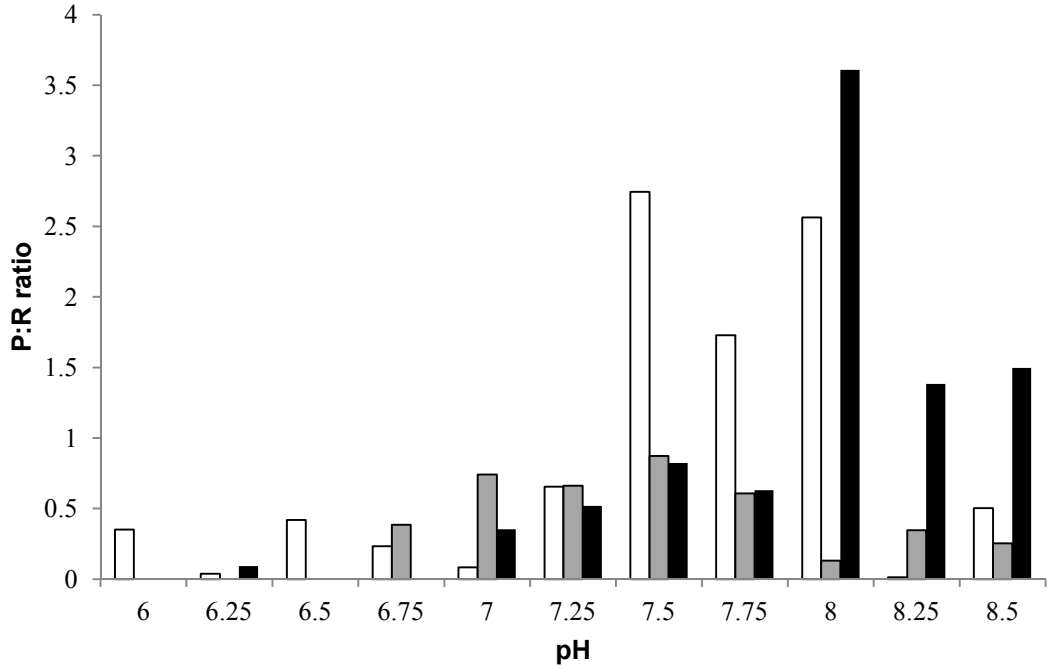

Figure 3.6. Relationship between the ratio of gross photosynthesis (P_{gross}) and respiration (R) of symbiosomes isolated from *A. pulchella* when in association with three different *Symbiodinium* types, and the pH of the medium in which they were suspended. The white, grey and black bars represent the homologous B1, heterologous B1 and subclade E2 *Symbiodinium* respectively (n = 4).

3.4. Discussion

This study reports the host-cell pHi in *A. pulchella*, an important model organism in symbiosis research (Weis *et al.,* 2008). More specifically, this study is the first to measure host pHi in the same host species but with different *Symbiodinium* types, and the pH optima of these different symbiont types. There have been few attempts to measure pHi in symbiotic cnidarians (Rands *et al*., 1993; Venn *et al.,* 2009; Venn *et al*., 2011), largely because the isolation of viable cnidarian cells is a complex process (Gates and Muscatine, 1992; Domart-Coulon *et al*., 2001; Helman *et al*., 2008) and because the imaging of these cells is equally challenging (Fang *et al*., 1997; Sawyer and Muscatine, 2001). This latter problem particularly arises due to the limited area of host cell cytoplasm available for visualization (Davy *et al.,* 2012) and the chlorophyll autofluorescence of the algal cells that can interfere with signals from

fluorescent probes. Rands *et al*. (1993) used an immunocytochemical approach to measure the pHi near the symbiotic dinoflagellates of the sea anemone *Anemonia viridis*, but while their method suggested an acidic intracellular environment (potentially as low as pH 5.7), it lacked sufficient resolution. However, the pioneering approach of Venn *et al*. (2009), which was used in the current study, has provided a platform to do this.

The pHi in *A. pulchella*, when in symbiosis with its homologous symbionts, was slightly lower than previously reported by Venn *et al.* (2009) for the symbiotic anemone, *A. viridis* and coral, *S. pistillata*, both in the light and dark. These authors found the pHi to range between 7.01 and 7.13 in the dark and 7.29 and 7.41 in the light. These values are all within the range reported for other marine invertebrates, with pHi being reported as anywhere between 6.7 for sea urchin eggs (Shen and Steinhard, 1978) to 7.6 for ascidian eggs (Russo *et al*., 1989). The reasons for the slight differences between these various symbiotic cnidarians is unknown, but it could result from the relative amounts of host-cell cytoplasm available for visualization and the ability to screen the region away from the symbiosome vacuole. That is, the relatively acidic region (pH < 6.0; Rands *et al.,* 1993; Venn *et al.,* 2009) immediately surrounding the symbiont cell, which coincides with the symbiosome vacuole, might have been incorporated into measurements here, so moderately lowering the pHi value measured. The increased host-cell pHi in *A. pulchella* in the light was, however, consistent with the findings of Venn *et al*. (2009). These authors proposed that this response is related to the dehydration of host cell bicarbonate (HCO_3^-) to CO_2 for photosynthesis by carbonic anhydrase. This process results in the production of hydroxide (OH^-) ions and, hence, the alkinization of the host cell cytoplasm (Allemand *et al*., 1998; Furla *et al*., 1998). While it is apparent that photosynthetic activity influences pHi, no significant differences were noted between the pHi of the host cells in response to hosting different symbiont types. Of note, however, the influence of *Symbiodinium* E2 does appear to lead to a trend of a lower pHi and it is highly likely that greater replication (always a problem given the difficulty of generating aposymbiotic cnidarians) would have led to a significant response.

The finding that photosynthetic production declined substantially below pH 6.75 for all symbiont types tested (although in the case of the homologous B1 type there was a drop off between pH 7.5 and 7.25 below which the P_{gross} was maintained at a low level) is consistent with previously reported trends for free-living algae. Thornton (2009), for example, who investigated photosynthetic production by the marine planktonic diatom *Chaetoceros muelleri*, found that photosynthesis declined with a corresponding decline in pH and that, at pH 6.8 and below, the growth rate of the diatom was negatively impacted. He suggested that this decline in photosynthesis during cellular acidosis may be due to less energy being available, which in turn can be linked to two mechanisms: 1) the effect of acidosis on mitochondrial function; and 2) the impact of acidosis on photosynthetic enzyme activity and thylakoid function. It is interesting to note that the algae in this study appear to be living at a pH below that predicted for seawater as a consequence of ocean acidification (pH 7.4; Caldeira and Wickett, 2003). However, symbiotic algae may be more adapted to a lower pH. The host cell cytoplasm, as reported in this and previous studies (Rands *et al*., 1993; Venn *et al*., 2009, 2011), is more acidic than the external seawater (pH ~7.0 *versus*~8.1), and hence symbiosomes typically function under such conditions without any significant impacts on the photosynthetic performance of the symbiotic alga within. However, whether the pH threshold changes (i.e. becomes less acidic) with time of exposure awaits further investigation. It would be valuable to look at the effect of pH on the holobiont and compare this between associations with different symbiont types, to identify potential inter and intra-cladal differences.

A range of responses to changes in pH was reported for the symbiosomes isolated from the different associations. In Chapter 2, the photosynthetic rates of *Symbiodinium* B1 (both homologous and heterologous) as well as *Symbiodinium* E2, when in association with *A. pulchella*, were shown to differ significantly and to range from 0.001 to 0.002 pg C cm^{-3} h^{-1}. A similar trend was noted in this study, in that the rate of P_{gross} reached by the different symbiont types at pH 6.75-7 (i.e. the pHi measured in the light), differed significantly. The heterologous B1 symbiosomes reached significantly higher rates of P_{gross} in this pH range than the symbiosomes containing the other symbiont types, and the peak value of 0.0008 pg C cm^3 h^{-1} was close to that noted in Chapter 2 (i.e. 0.001 pg C cm^{-3} h^{-1}). The association with

heterologous B1 was also the only association for which pHi in the light (i.e. pH 6.7) fell within the pH range (i.e. pH 6.75 to 7.5) at which the highest photosynthetic performance of the isolated symbiosomes was obtained. This suggests that the pHi enables the symbiont to perform optimally. For heterologous B1, the P:R ratios generally supported the trend observed for P_{gross} except that, at pH 6.75, a higher (though non-significant) R resulted in a lower P:R than at pH 7-7.5. This suggests that the association was nearing a state of being self-supporting with respect to carbon between pH 7 and 7.5, as a P:R ratio of close to 1 was attained (Muscatine *et al*., 1981).

In the case of the homologous B1 symbionts and *Symbiodinium* E2, the optimal ambient pH for photosynthesis did not coincide with the host-cell pHi in the light. In the case of *Symbiodinium* E2, this finding is consistent with its low cell-specific rate of photosynthesis when in symbiosis with *A. pulchella* (Chapter 2), though whether the low photosynthetic rate is caused by a less than optimal pHi or *vice versa* is unclear. It is possible that the high respiration rates observed in *A. pulchella* when in association with *Symbiodinium* E2 (Chapter 2) may lower the pHi. The improved performance of *Symbiodinium* E2 at a higher pH of 8, as evident from the rate of P_{gross} and the P:R, suggests that it would be able to function well in seawater given that the pH of seawater is approximately 8 (Ball and Stock, 1937). An increasing number of free-living *Symbiodinium* types are being identified and it has been suggested that the free-living state may be more common than originally suggested (Takabayashi *et al*., 2012). Therefore, given that type E2 was originally isolated from Wellington Harbour (New Zealand) and that its genotype does not match that of any known New Zealand dinoflagellates (J. Howe, Victoria University of Wellington, pers. comm.), these findings may be indicative of *Symbiodinium* E2's potential free-living origin.The disparity between the optimal pH of the homologous symbionts and the measured pHi is surprising but it could provide a mechanism by which symbiont productivity and hence growth are regulated *in hospite*. It is known that pre-mitotic controls influence symbiont growth, but what these mechanisms are, is unknown (see review by Davy *et al.,* 2012). Past studies have suggested that the cnidarian host possesses a photosynthesis inhibiting factor that is able to partially inhibit carbon fixation in freshly isolated symbiotic algae (Grant *et al*., 2006a). This has been

proposed to serve as a means of regulating the symbiont density (Grant *et al*., 2006a). The physiological nature and mechanism of this factor has however, not been established.

In conclusion, this study demonstrates that different *Symbiodinium* types have different pH optima with regards to photosynthetic performance and that the pHi of the host cells may vary depending on the *Symbiodinium* type present. The study also revealed that the optimal pHi for photosynthesis does not always match the actual measured pHi. This raises questions about the implications of this mismatch on the functioning of the symbiosis and if it could potentially provide a means of restricting symbiont growth. It should be recognised, however, that exposing isolated symbiosomes to an ambient medium of variable pH, is not a true reflection of the *in hospite* situation and thus the responses observed in this study may not be the result of pH change alone. This inability to replicate an *in hospite* environment is an ongoing problem in the field (Davy *et al*., 2012). Further work looking at the effect of changes in pHi on the photosynthetic rates of a variety of symbiont types (when in association with *A. pulchella*) is essential to more accurately assess the potential differences in physiology between them. Also, now that well established methods of measuring pHi in cnidarian cells are available (Venn *et al*., 2009), it would be interesting to apply them to a wider range of *A. pulchella–Symbiodinium* associations in order to more comprehensively determine the relationship between symbiont diversity and pHi.

Chapter 4:

The influence of symbiont type on the glycerol and glucose pools in *Aiptasia pulchella*

4.1. Introduction

Cnidarian–dinoflagellate symbioses consist of associations between anthozoan cnidarians (i.e. corals, sea anemones, zoanthids, gorgonians) and dinoflagellates (commonly referred to as zooxanthellae) of the genus *Symbiodinium* (Trench, 1987). A mutualistic relationship exists between the host and symbiont, in that the symbiont receives inorganic nutrients from the host and provides the host with translocated photosynthetic products (Muscatine, 1990). The dinoflagellate symbionts are located within a host-derived vacuole in the gastrodermal cells of the coral host and are separated from the host cytoplasm by a membrane of host origin (Roth *et al*., 1988) referred to as the symbiosome membrane. This membrane controls the transport of inorganic nutrients to the dinoflagellates (Rands *et al*., 1993) as well as the translocation of organic products to the host. Glycerol has been suggested as the major extracellular product of *Symbiodinium* (Muscatine, 1967; Trench, 1971 a, b, c; Grant *et al*., 1997, 1998; but also see Ishikura *et al*., 1999; Whitehead and Douglas, 2003; Burriesci *et al*., 2012), however a range of other mobile compounds have also been identified. These consist of sugars such as glucose and other hexoses (Muscatine *et al*., 1967; Bil *et al*., 1991; Ishikura *et al*., 1999; Whitehead and Douglas, 2003; Burriesci *et al*., 2012), amino acids such as alanine (Lewis and Smith, 1971; Wang and Douglas, 1999) and glycine (Von Holt, 1968), esters (Von Holt and Von Holt., 1968), alcohols and lipids (Trench, 1971c; Patton *et al*., 1977; Peng *et al*., 2011). Given the difficulty of identifying metabolites *in hospite*, most research to date has focused on the identification of compounds released from isolated dinoflagellates (see review by Davy *et al*., 2012), which do not provide a true reflection of their behaviour when in symbiosis (Sutton and Hoegh-Guldberg, 1990; Davy *et al*., 2012). Various approaches identify the translocated metabolites *in hospite* have been attempted. These include, but are not limited to, studies that track

the incorporation of ^{14}C-labelled compounds into the host tissues (Muscatine and Cernichiari, 1969; Blanquet *et al*., 1979; Trench, 1971b; Battey and Patton, 1984, 1987), microscopic analyses of cnidarian tissue (Harland *et al*., 1991, Gautret *et al*., 1997; Wang and Douglas., 1999; Luo *et al*., 2009; Chen *et al*., 2012), and the proteomic and ultrastructural analysis of "lipid bodies" isolated from symbiotic coral cells (Peng *et al*., 2011). The outcome of this research indicates that the major components of the translocated material are glycerol, amino acids and various lipids. However, there is much debate as to whether glycerol is in fact translocated, with some studies stating that there is no evidence for glycerol release (Rees *et al*., 1993; Ishikura *et al*., 1999; Whitehead and Douglas, 2003; Burriesci *et al*., 2012). More research is therefore necessary to confirm the nature of the compounds translocated from symbiont to host.

Much of the recent research on corals has focused on the interaction between the symbiotic dinoflagellates (genus *Symbiodinium*) and their coral host during periods of environmental disturbance or change (e.g. coral bleaching induced by elevated seawater temperature). Buddemeier and Fautin (1993) proposed that, following mass bleaching events, changes can occur in the symbiont community structure through recombination with symbionts acquired from the environment following ("switching"). However, although certain corals are capable of acquiring symbionts from the water column, this acquisition is temporary as these infections are not maintained as stable symbioses (Coffroth *et al*., 2010). There is evidence for post-bleaching shifts in the dominant member of a mixed symbiont population (Baker, 2001; Baker *et al*., 2004; Berkelmans and van Oppen, 2006; Mieog *et al*., 2007; Jones *et al*., 2008) and studies have shown that in some coral colonies, the symbiont composition may shift in favour of a *Symbiodinium* clade or sub-clade ('type') that is physiologically more suited to the new environmental conditions (Stat *et al*., 2009). For example, *Symbiodinium* clade D is thought to be more resistant to thermal stress than other clades and has been found to associate with coral colonies after bleaching episodes (Baker, 2001; Rowan, 2004; Abrego *et al*., 2008). However, this thermal resistance seems to come at a cost to the host, as studies have shown that *Symbiodinium* clade D may transfer significantly fewer metabolites to its host, leading to a reduction in host growth (Abrego *et al*., 2008; Cantin *et al*., 2009).

Different clades and sub-clades of symbiotic dinoflagellates have also been found to differentially influence incorporation patterns of translocated carbon in the host's tissues (Loram *et al*., 2007; Stat *et al*., 2008; Cantin *et al*., 2009). For example, Loram *et al*. (2007) found that when the sea anemone *Condylactis gigantea* hosted *Symbiodinium* clade A, a greater proportion of photosynthetically-fixed carbon was incorporated into animal lipids and amino acid pools than when conspecific anemones hosted clade B. These findings suggest that the interactions between specific host-symbiont combinations must play an important role in both the quality and quantity of translocate that is made available to the host. However, we know little about these qualitative and quantitative differences, and how they might influence the capacity of the host to acquire novel symbionts.

The aim of this study was to compare the quality of the photosynthetic translocate made available to the host when infected with different *Symbiodinium* types. Glucose and glycerol were selected as the metabolites to be tested due to previous studies identifying them as the major metabolites. Amounts of free glycerol and glucose present in the host's tissue both prior to and at different time points after photosynthesis began were analysed, and accumulation rates per symbiont cell calculated in an attempt to compare type-specific changes.

4.2. Materials and methods

The sea anemone *Aiptasia pulchella* was used as the experimental host and infected with different *Symbiodinium* types (cultured *Symbiodinium* B1, heterologous *Symbiodinium* B1 as well as *Symbiodinium* E2). After 12 weeks, the amounts of free glycerol and glucose present in the tissue of the infected anemones was measured.in the dark, and at different time points after light exposure.

4.2.1. Experimental organisms

The sea anemone, *Aiptasia pulchella*, (originally from the Indo-Pacific region) was cultured at 25 ^{o}C and an irradiance of 100 μmol photons m^{-2} s^{-1} (12 h light:12 h dark)

for a period of >1 year prior to use. The anemones were fed twice weekly with freshly hatched *Artemia* sp. over this period. To render the anemones aposymbiotic (i.e. symbiont-free), 120 individuals (oral disc diameter~10 mm) were placed in six 1 L lightproof containers (20 individuals in each) filled with 0.44 µm filtered seawater (FSW) and cold shocked at 4 ^{o}C for 24 hours. The cold-shock process was repeated at weekly intervals for five weeks and the feeding regime was retained between cold-shocks, when care was taken to keep the anemones in low light (<1 µmol photons m^{-2} s^{-1}). The anemones were then maintained in the dark at 25^{o}C for approximately six months until the start of the experiment. The aposymbiotic state of the anemones was checked by exposure to light (12 h light:12 h dark) over a four-week period, with any change from white to brown colouration being indicative of a residual symbiont population. Fluorescence microscopy (Olympus Provis AX70 microscope using autofluorescence at 100× magnification) was used to further screen for the presence of symbionts. A subsample of 3 anemones per container was tested. Only anemones that showed no signs of symbiont infection were used for subsequent experiments.

Cultures of *Symbiodinium* B1 (i.e. not originally isolated from the *Aiptasia* sp. laboratory culture but from the sea anemone, *Aiptasia pallida* collected in Long Key, Florida) and E1 (cultured from a single, free-living cell collected from Wellington harbour, New Zealand), as well as a culture of the homologous type (*Symbiodinium* B1) that was isolated from the laboratory anemone culture, were grown at 25 ^{o}C and an irradiance of100µmol photons m^{-2} s^{-1}on alight regime of 12 h light: 12 h dark. *Symbiodinium* types were chosen that had previously been shown to have very different photosynthetic performances when in association with *A. pulchella*, i.e. E1 performed poorly whilst both types of *Symbiodinium* B1 were relatively more successful (see Chapter 2). Furthermore, the heterologous B1 provided a comparison with the homologous B1. The algae were sub-cultured from long-term laboratory stocks and grown in silica-free F⁄2 medium (Sigma-Aldrich, Auckland, New Zealand) for six weeks prior to use in the infection study.

Symbiodinium types (n = 3 samples per culture) were genetically identified prior to infection (see Appendix 3 for the ITS2 sequences). DNA was extracted from

zooxanthellae as described by Logan *et al*., (2010) and PCR performed using the *Symbiodinium*-specific ITS2 primers: forward primer (ITSD), 5'-GTG AAT TGC AGA ACT CCG TG-3'; reverse primer (ITS2rev2), 5'-CCT CCG CTT ACT TAT ATG CTT-3'. Reactions were performed in a total volume of 30 μL using the MyTaq^{TM} Mix with an amplification profile consisting of 1 min 95 ^{o}C, 35 cycles of 1 min 95 ^{o}C, 15 s 55 ^{o}C, 10 s 72 ^{o}C, and a final hold temperature of 4^{o}C. Single strand conformation polymorphism (SSCP) was performed on all PCR products. PCR products were sequenced by Macrogen Inc. (Seoul, S. Korea). Sequences were aligned using the ClustalW plugin in Geneious Pro 4.8.5 and then edited. A blast search was then performed in the NCBI database in order to identify the sequences (see Table A3.1).

4.2.2. Infection of aposymbiotic *A. pulchella* with different *Symbiodinium* types

Aposymbiotic specimens of *A. pulchella* (n = 72 per treatment) were infected with cultured *Symbiodinium* cells by pipetting a concentrated drop of the dinoflagellate suspension (approximately 1 million cells/mL) onto the oral disc of each anemone, with a sharp-ended glass pipette. A drop of one-day old *Artemia* sp. culture was then pipetted onto the oral disc to induce a feeding response and encourage the uptake of the *Symbiodinium* cells, as described by Davy *et al*. (1997). Anemones were kept in FSW in 20 mL glass containers that received a water change 8 hours after infection. The infected anemones were then left for 12 weeks to form a stable association with their respective symbiont (see Chapter 2). The feeding regime during the 12-week period was maintained as per pre-infection. After 12 weeks, zooxanthellae were isolated from three anemones per treatment and their genetic identity confirmed, using the isolation and molecular techniques described above. All treatments were found to harbour the same *Symbiodinium* type as the one introduced at the start of the experiment (see Appendix 3 for the ITS2 sequences).

4.2.3. Levels of glycerol and glucose in host tissues

All the infected anemones were kept in darkness for 24 hours at a temperature of 25 ^{o}C. The anemones were last fed 1 week prior to the dark treatment. After the dark

adaptation period 36 anemones per treatment were exposed to light at 400 μmol photons m^{-2} s^{-1} (light-saturation for photosynthesis) and 6 anemones per treatment were used for the dark treatment (i.e. t = 0). Six anemones from each treatment were homogenized individually in 1 mL of FSW using a tissue grinder at each time point (i.e. 0, 1, 3, 6, 9, 12 and 24 hours after light exposure). A preliminary study was completed in order to demonstrate that 24 hours of continuous light did not negatively impact the photosynthetic rate of the symbionts. The homogenate was centrifuged at × 4500 g for 3 min. The supernatant was stored at -20 °C for quantification of glycerol and glucose as described below, as well as protein. Protein was quantified by measuring absorbance of the supernatant at 280 nm using a ND – 1000 spectrophotometer (Nanodrop, Thermo Fisher Scientific Inc.); FSW was used for blank measurements. The pellet was resuspended in 1 mL of FSW and centrifuged again at × 1000 g for 5 min. This was repeated three times. A haemocytometer and light microscope (100× magnification) were used to estimate the total number of algal cells per mL of anemone homogenate (n = 6 counts per anemone).

In a second, modified version of this experiment, anemones were treated with the respiratory inhibitor sodium cyanide (NaCN) in an attempt to minimise the influence of host metabolic activity on the accumulation of translocated carbon compounds. The experimental protocol was identical to that described above except that: (i) after the dark adaptation period, 18 anemones per treatment were placed in individual glass vials containing a 2.5×10^{-5} M NaCN solution (in FSW and adjusted to pH 8.2); and (2) 6 anemones per treatment were processed at 0, 30 and 60 min after light exposure.

4.2.4. Carbon fluxes in isolated *Symbiodinium*

To prepare a host tissue homogenate, five anemones of the same nutritional history were homogenized together in 3 mL of FSW using a tissue grinder. The homogenate was then centrifuged at ×1000g for 3 min, and the supernatant retained and stored at -20 °C. This homogenate was used for all subsequent incubations.

To obtain isolated *Symbiodinium* cells, 12 re-infected anemones per treatment were individually homogenized in 1 mL FSW using a tissue grinder. The homogenate was centrifuged at ×4500g for 3 min. The pellet was resuspended in 1 mL FSW and centrifuged again at ×1000g for 5 min. This washing process was repeated three times. The algal pellet was then diluted to 10^6 cells mL^{-1} in FSW. An aliquot (300 µL) of the algal suspension from each anemone was then placed in a 1.5-mL microcentrifuge tube together with 300 µL of the previously prepared host homogenate. The tubes were placed in darkness for 24 hours at a temperature of 25 ^oC, after which they were exposed to light for an hour (400 µmol photons m^{-2} s^{-1}; light-saturation for photosynthesis). At 0, 30 and 60 min post light exposure, 4 microcentrifuge tubes per treatment were individually vortexed and centrifuged at × 1000g for 3 min in order to separate the algal and animal fractions. The supernatant was stored at -20 ^oC for glycerol and glucose quantification as described below. The pellet was resuspended in 1 mL FSW and algal cell counts were performed as described earlier.

4.2.5. Glucose and glycerol assays

All samples were analysed using the Abcam Free Glucose and Glycerol Assay kits (Sapphire Bioscience, NZ, Ltd.) in order to measure the free glycerol and glucose concentrations. Samples and standards were prepared according to the protocol provided. The assays involve the enzymatic oxidation of glycerol/glucose to generate a product which reacts with a dye to generate colour (λ = 570 nm) in the case of glucose and fluorescence (Ex/Em = 535/587 nm) in the case of glycerol. A microtiter plate reader (EnSpire 2300 Multilabel Reader; PerkinElmer; Waltham, MA, USA) was used for these measurements.

In order to calculate the amount of glycerol or glucose present in each sample, the value derived from the blank glucose and glycerol standards was subtracted from all the sample readings to correct for background fluorescence. The standard curve was plotted as fluorescence *vs.* concentration of glycerol/glucose (nmol). The sample fluorescence/absorbance readings were applied to the standard curve in order to calculate the concentration of glycerol/glucose in each sample. For the intact

symbiosis experiments (both in the presence and absence of NaCN) the sample concentrations were normalised to the total host protein present in each sample (i.e nmol mg^{-1} protein or pmol mg^{-1} protein) in order to track the change in concentration in the host tissue. The values were also normalised to the algal cell density present in the association from which the sample was obtained (i.e. nmol/10^6 algal cells) so to to directly compare the contribution of the different symbiont types to the host tissue metabolite pools. The glycerol/glucose concentration at t = 0 was than subtracted from each subsequent time point in order to determine the increase in the metabolite concentration from the baseline level. Glycerol and glucose concentrations released by isolated *Symbiodinium* were normalised to algal cell density only (i.e. nmol/10^6 algal cells) and the change from the baseline level was calculated as described above.

4.2.6. Statistical analyses

Data were tested for normality using the Kolmogorov-Smirnoff test and for homogeneity of variance using the Bartlett test. Comparisons of glycerol and glucose host tissue concentration (normalised to host protein and to algal cell density) between the different symbiotic associations and time points (in the presence and absence of NaCN) were made using one-way analysis of variance (ANOVA, α = 0.05), followed by LSD *post hoc* tests for normal data using PASW (Predictive Analytics SoftWare, Version 18). Analyses of the glycerol and glucose concentrations released by isolated *Symbiodinium* were performed in the same manner.

4.3. Results

4.3.1. Glycerol and glucose levels in the host tissues

The average algal densities measured across all time points differed between the associations tested (one-way ANOVA, d.f. = 2; 15, F = 508.7, $p < 0.005$ for all treatments). The association with *Symbiodinium* E2 had a significantly higher algal density (4.00 x 10^6 cells mg^{-1} protein) than the associations with the other two types

(1.25 x 10^6 cells mg^{-1} protein and 0.52 x 10^6 cells mg^{-1} protein for the associations with heterologous *Symbiodinium* B1 and homologous *Symbiodinium* B1, respectively; one-way ANOVA, LSD post-hoc test, $p < 0.005$ for both treatments). Also, the association with heterologous *Symbiodinium* B1 had a significantly higher algal density than the association with homologous *Symbiodinium* B1 (one-way ANOVA, LSD post-hoc test, $p < 0.005$ for both treatments).

There was a significant increase in the host tissue glycerol concentration (nmol mg^{-1} protein) over time when the anemones with homologous and heterologous *Symbiodinium* B1 were incubated in the light (Figure 4.1A; one-way ANOVA, d.f. = 6; 15-21, F = 2.5-5.6, $p < 0.05$ for all treatments). The concentration increased from to 0.014 to 0.039 nmol mg^{-1} protein and 0.024 to 0.068 nmol mg^{-1}protein for associations with homologous B1 and the heterologous B1 respectively. The association with *Symbiodinium* E2 displayed no change in host tissue glycerol concentration over time (one-way ANOVA, d.f. = 6; 24, F = 0.6, $p > 0.05$). Differences in glycerol concentration between the associations were noted at 0 and 1 hour (one-way ANOVA, d.f. = 2; 8-9, F = 9.2-41.0, $p < 0.05$) with the heterologous *Symbiodinium* B1 and *Symbiodinium* E2 associations displaying significantly higher levels of glycerol (0.02 and 0.04 nmol mg^{-1} protein at 0 hours and 0.01 and 0.03 nmol mg^{-1} protein at 1 hour for associations with heterologous *Symbiodinium* B1 and *Symbiodinium* E2, respectively) than the association with homologous *Symbiodinium* B1 (0.01 and 0.009 nmol mg^{-1} protein for 0 and 1 hour, respectively; one-way ANOVA, LSD post-hoc test, $p < 0.05$ for both treatments). The concentration in the different associations did not differ after 24 hours in the light (one-way ANOVA, d.f. = 2; 5, F = 1.5, $p > 0.05$).

The trends in host tissue glycerol concentration seen when normalising to cell density were not the same as those observed when normalising to host protein as the algal cell densities present in the different associations varied. The cell-specific glycerol concentration (nmol/10^6 algal cells, measured as change from the baseline concentration at 0 hours) changed with time in all cases (one-way ANOVA, d.f. = 5; 12-16, F = 3.5-24.5, $p < 0.05$ for all comparisons), but the patterns of accumulation were again different between the different *Symbiodinium* types (Figure 4.1B). In the

case of homologous *Symbiodinium* B1, the cell-specific glycerol concentration was significantly higher at 1, 3, 6 and 9 hours, where it ranged from 25.10 to 40.53 nmol/10^6 algal cells, than at 12 and 24 hours (10.80 and 6.51 nmol/10^6 algal cells, respectively; one-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons). With heterologous *Symbiodinium* B1 type, on the other hand, the cell-specific glycerol concentration was significantly higher at 12 hours (20.65 nmol/10^6 algal cells) than at all the other time points where it ranged from 3.90 to 9.50 nmol/10^6 algal cells (one-way ANOVA with LSD post-hoc test, $p < 0.005$ for all comparisons); it was also higher at 24 hours (9.50 nmol/10^6 algal cells) than at 1, 3 and 6 hours where it ranged from 3.90 to 5.15 nmol/10^6 algal cells (one-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons). The association with *Symbiodinium* E2 had the highest cell-specific glycerol concentration at 9 hours when compared to the other time points (11.42 nmol/10^6 algal cells; one-way ANOVA with LSD post-hoc test, $p < 0.005$), while the concentrations at 6 and 12 hours (2.90 and 5.27 nmol/10^6 algal cells, respectively) were also higher than for the remaining time points (except 9 hours) where it ranged from 0.48 to 1.22nmol/10^6 algal cells (one-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons). These different accumulation patterns led to differences between the various *Symbiodinium* types at some of the time points (one-way ANOVA, d.f. = 2; 4-9, F = 8.0-16.7, $p < 0.05$ for all comparisons). In particular, the symbiosis with the homologous *Symbiodinium* B1 type contained more glycerol per symbiont cell (25.09-40.53 nmol/10^6 algal cells *versus* 0.48 to 5.15 nmol/10^6 algal cells) at 1, 3, 6 and 9 hours than the symbioses with the other two types (one-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons). At 12 hours, on the other hand, the glycerol concentration in the symbiosis with the heterologous *Symbiodinium* B1 was significantly higher than for the symbioses with the other two symbiont types (20.65 nmol/10^6 algal cells *versus* 10.80 and 5.27 nmol/10^6 algal cells for homologous *Symbiodinium* B1 type and *Symbiodinium* E2, respectively) (one-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons). At 24 hours no significant differences between the symbioses were displayed (one-way ANOVA, d.f. = 2; 8, F = 4.2, $p < 0.05$ for all comparisons).

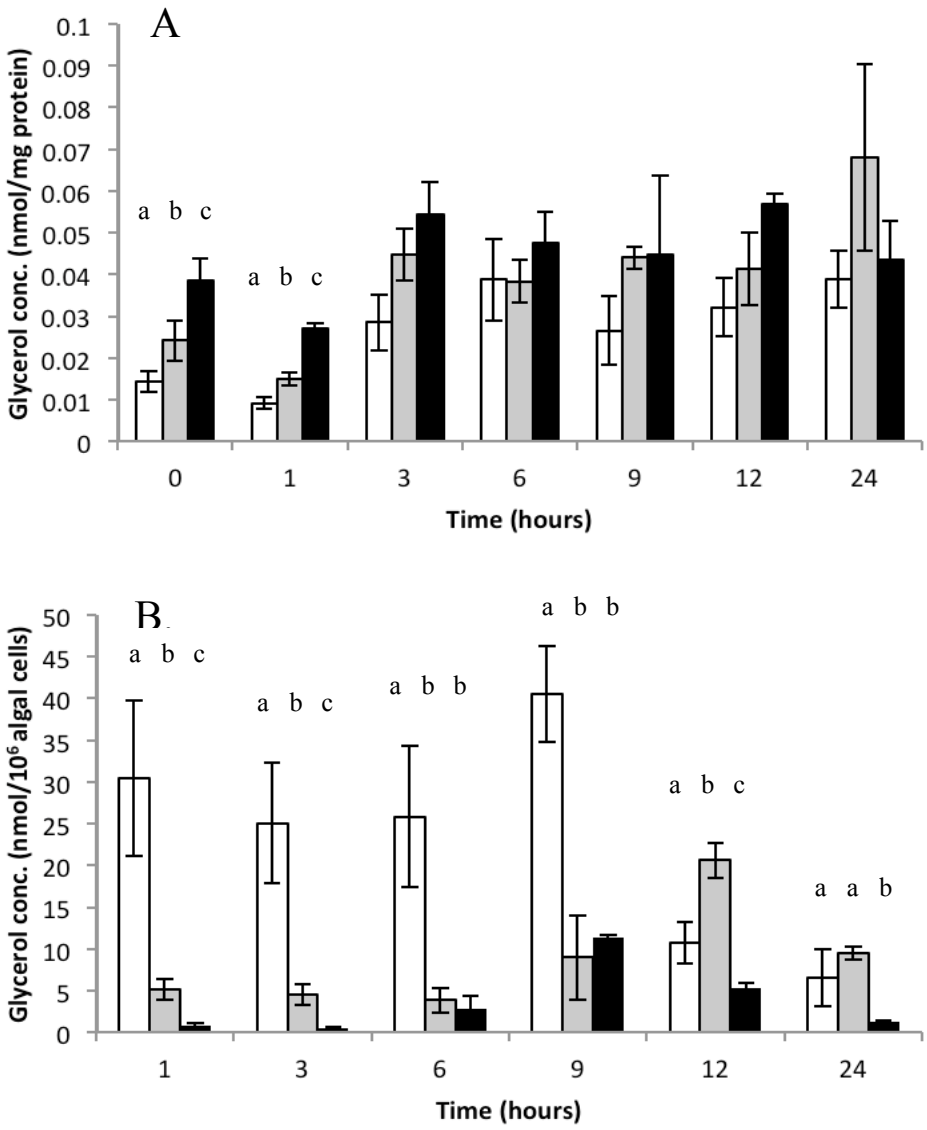

Figure 4.1. Concentration of glycerol in tissues of *A. pulchella* when infected with different *Symbiodinium* types *versus* time in the light. (A) Standardised to host protein content. (B) Standardised to algal cell number, after subtracting the baseline glycerol concentration at 0 h. White bars, homologous *Symbiodinium* B1; grey bars, heterologous *Symbiodinium* B1; black bars, *Symbiodinium* E2. At each time point, associations assigned different letters are significantly different from each other ($p < 0.05$). Values are means ±SE; n = 5 or 6 per time point. The baseline glycerol concentration was 27.81 ± 5.65, 6.96 ± 1.05 and 2.86 ± 0.12 nmol/10^6 algal cells for the homologous *Symbiodinium* B1, heterologous *Symbiodinium* B1 and *Symbiodinium* E2 respectively.

All three associations displayed a significant increase in the concentration of glucose in the host tissues over time in the light (Figure 4.2A; one-way ANOVA, d.f. = 6; 8-

24, F = 5.5-12.5, $p < 0.005$ for all treatments) increasing from 0.05to 0.6 pmol mg^{-1} protein for the association with the homologous *Symbiodinium*B1, 0.08 to 0.9 pmol mg^{-1} protein for the association with the heterologous *Symbiodinium* B1, and 0.17 to 0.63 pmol mg^{-1} protein for the association with *Symbiodinium* E2 (Figure 4.2A). For the association with the homologous B1, at 12 and 24 hours, the amount of glucose present (0.80 and 0.60 pmol mg^{-1} protein, respectively) was significantly higher than at the preceding time points where it ranged from 0.01 to 0.11pmol mg^{-1} protein (one-way ANOVA, LSD post-hoc test, $p < 0.05$ for all comparisons). The association with the heterologous B1 had significantly higher concentrations of glucose at 9, 12 and 24 hours (0.72, 0.68 and 0.89 pmol mg^{-1} protein, respectively) than at the earlier time points where it ranged from 0.03 to 0.18 pmol mg^{-1} protein (one-way ANOVA, LSD post-hoc test, $p < 0.005$ for all comparisons). The amount of glucose present in the host tissues of the *Symbiodinium* E2 symbiosis after 24 hours of light exposure (0.63pmol mg^{-1} protein) was significantly higher than at all of the preceding time points where it ranged from 0.05 to 0.38 pmol mg^{-1} protein (one-way ANOVA, LSD post-hoc test, $p < 0.05$ for all comparisons), and at 12 hours (0.35 pmol mg^{-1} protein) it was significantly higher than at 0, 1 and 6 hours (0.17, 0.05 and 0.20 respectively; one-way ANOVA, LSD post-hoc test, $p < 0.05$ for both comparisons). This increased accumulation of glucose with time in all three symbioses meant that there were few significant differences between them. Indeed, the only significant difference was at 9 hours, when the symbiosis with the heterologous *Symbiodinium* B1 had a greater concentration of glucose in its host tissues than did the other two associations (0.72 pmol mg^{-1} protein *vs.* 0.1 and 0.14 pmol mg^{-1} protein for the associations with homologous B1 and E2, respectively) (one-way ANOVA, d.f. = 2; 9, F = 32.1, $p < 0.005$ for both comparisons).

The trends in host tissue glucose concentration (as noted previously with glycerol) seen when normalising to cell density were not the same as that observed when normalising to host protein due to variable algal cell densities present in the different associations. When normalized to symbiont density (nmol/10^6 algal cells), there was a tendency towards an accumulation of glucose as time progressed for all the symbioses (Figure 4.2B; one-way ANOVA, d.f. = 5; 12-14, F = 3.0-11.2, $p < 0.005$). With homologous *Symbiodinium* B1, the cell-specific glucose concentration was

significantly greater at 12 hours (1.63 nmol/10^6 algal cells) than at any of the other time points where it ranged from -0.03 to 0.45 nmol/10^6 algal cells (one-way ANOVA with LSD post-hoc test, $p < 0.005$ for all comparisons), and the cell-specific glucose concentration in the association with heterologous *Symbiodinium* B1 was significantly higher at 9, 12 and 24 hours (0.49, 0.27 and 0.32 nmol/10^6 algal cells respectively) than at the previous time points where it ranged from 0.003 to 0.05 nmol/10^6 algal cells (one-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons). Similarly, with *Symbiodinium* E2 the cell-specific glucose concentration was significantly greater at 24 hours (0.06 nmol/10^6 algal cells) than at the earlier time points where it ranged from 0.003 to 0.02 nmol/10^6 algal cells (one-way ANOVA with LSD post-hoc test, $p < 0.005$ for all comparisons). Despite these similar trends, however, the rates of glucose accumulation differed between the different symbioses, with there being significant differences at 1, 9 and 12 hours (one-way ANOVA, d.f. = 2; 4-8, $F = 5.1\text{-}8.1$, $p < 0.05$ for all comparisons). At 1 hour, the cell-specific concentration of glucose was significantly greater in the presence of the heterologous *Symbiodinium* B1 than with the other two symbionts (0.04 nmol/10^6 algal cells *vs*. -0.03 and 0.01 nmol/10^6 algal cells for the association with homologous *Symbiodinium* B1 and *Symbiodinium* E2, respectively; one-way ANOVA with LSD post-hoc test, $p < 0.05$ for both comparisons). At 9 and 12 hours, the associations with both the homologous and heterologous *Symbiodinium* B1 showed significantly higher concentrations of glucose per symbiont cell (0.26 and 0.49 nmol/10^6 algal cells at 9 hours, and 1.63 and 0.27 nmol/10^6 algal cells at 12 hours, respectively) than did the association with *Symbiodinium* E2 (0.02 and 0.06 nmol/10^6 algal cells at 9 and 12 hours, respectively) one-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons). Of note, the average cell-specific glucose concentration was about 7-fold higher in the presence of the homologous *Symbiodinium* B1 at 12 hours than for the heterologous B1.

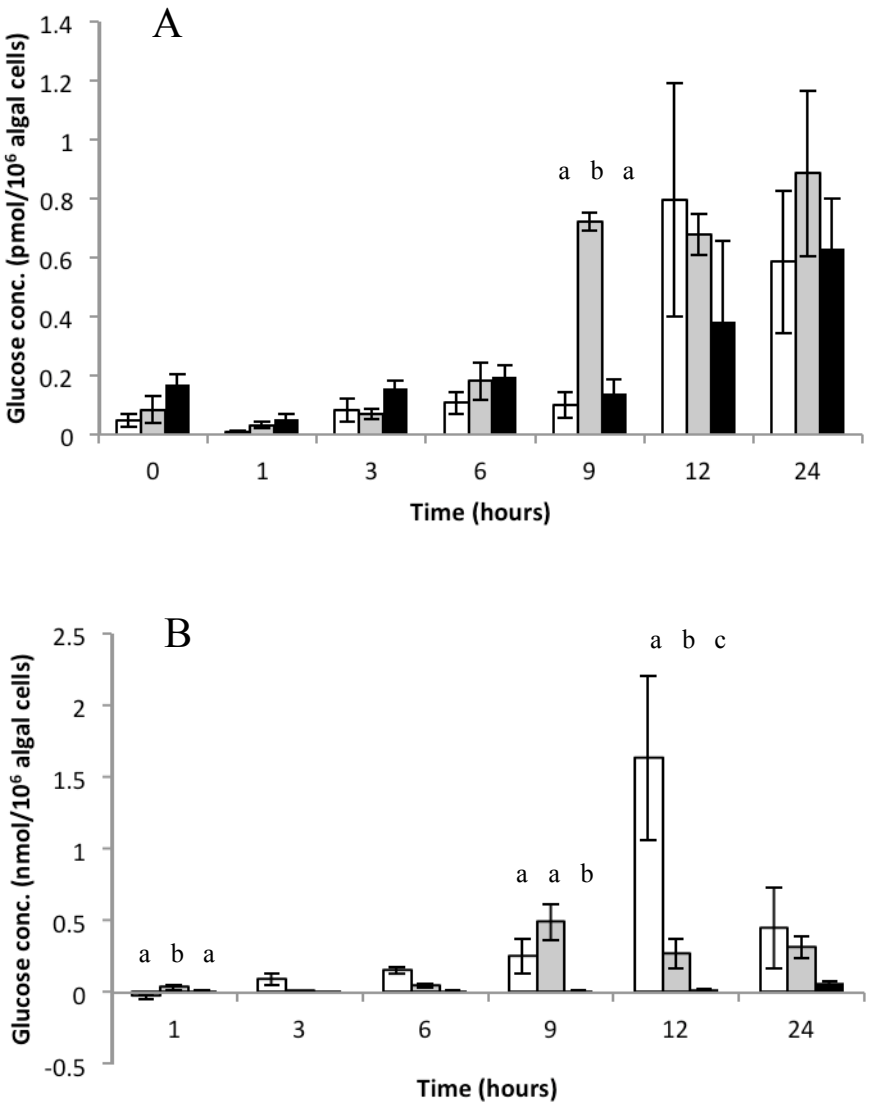

Figure 4.2. Concentration of glucose in tissues of *A. pulchella* when infected with different *Symbiodinium* types *versus* time in the light. (A) Standardised to host protein content. (B) Standardised to algal cell number, after subtracting the baseline glucose concentration at 0 h. White bars, homologous *Symbiodinium* B1; grey bars, heterologous *Symbiodinium* B1; black bars, *Symbiodinium* E2. At each time point, associations assigned different letters are significantly different from each other ($p < 0.05$). Values are means ±SE; n = 5 or 6 per time point. The baseline glucose concentration was 0.10 ± 0.04, 0.02 ± 0.003 and 0.006 ± 0.0008 nmol/10^6 algal cells for the homologous *Symbiodinium* B1, heterologous *Symbiodinium* B1 and *Symbiodinium* E2 respectively.

4.3.2. Glycerol and glucose levels in the host tissue in the presence of NaCN

When incubated with NaCN, the different associations displayed similar trends to each other with respect to the accumulation of glycerol (Figure 4.3A). In all the associations, the concentration of glycerol (nmol/mg protein) in the host tissues increased over the first 30 min of the the incubation (ranging from 0.006 to 0.012 nmol mg^{-1} at 0 min and 0.023 to 0.030 nmol mg^{-1} protein at 30 min), though in the case of the association with the homologous *Symbiodinium* B1 this increase only approached significance (one-way ANOVA, d.f. = 2; 6-9, F = 6.2-19.1, $p < 0.05$, except for homologous B1 where $p = 0.06$). Glycerol concentration then declined between 30 and 60 min in all cases (ranging from 0.023 to 0.030 nmol mg^{-1} protein at 30 min at 30min and 0.011 to 0.020 nmol mg^{-1}protein at 60 min; one-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons). No significant differences, however, were detected between the different associations at the various time points (one-way ANOVA, d.f. = 2; 7-10, F = 0.1-4.3, $p > 0.05$ for all comparisons).

In the case of the cell-specific glycerol concentration (nmol/10^6 algal cells, measured as change from the baseline concentration at 0 min), only the association with homologous *Symbiodinium* B1 changed significantly between 30 and 60 min of incubation, when it dropped from 58.2 to 0.4 nmol/10^6 algal cells (Figure 4.3B; one-way ANOVA, d.f. = 1; 5, F = 11.7, $p < 0.05$). Significant differences between the types were seen at both 30 and 60 min (one-way ANOVA, d.f. = 2; 7-9, F = 8.8-20.1, $p < 0.005$ for all comparisons). At 30 min, the associations with homologous and heterologous *Symbiodinium* B1 displayed a greater increase in host tissue glycerol concentration per cell (58.21 nmol/10^6 algal cells and 15.05 nmol/10^6 algal cells, respectively) than did the *Symbiodinium* E2 association (2.31 nmol/10^6 algal cells), with homologous B1 symbiosis exhibiting the greatest increase (one-way ANOVA with LSD post-hoc test, $p < 0.05$ for all comparisons). At 60 min, the association with the heterologous *Symbiodinium* B1 showed a significantly greater increase in glycerol concentration per cell (3.71 nmol/10^6 algal cells) than the other two associations (0.39 and -0.32 nmol/10^6 algal cells for the association with homologous

Symbiodinium B1 and *Symbiodinium* E2, respectively; one-way ANOVA with LSD post-hoc test, $p < 0.005$ for all comparisons).

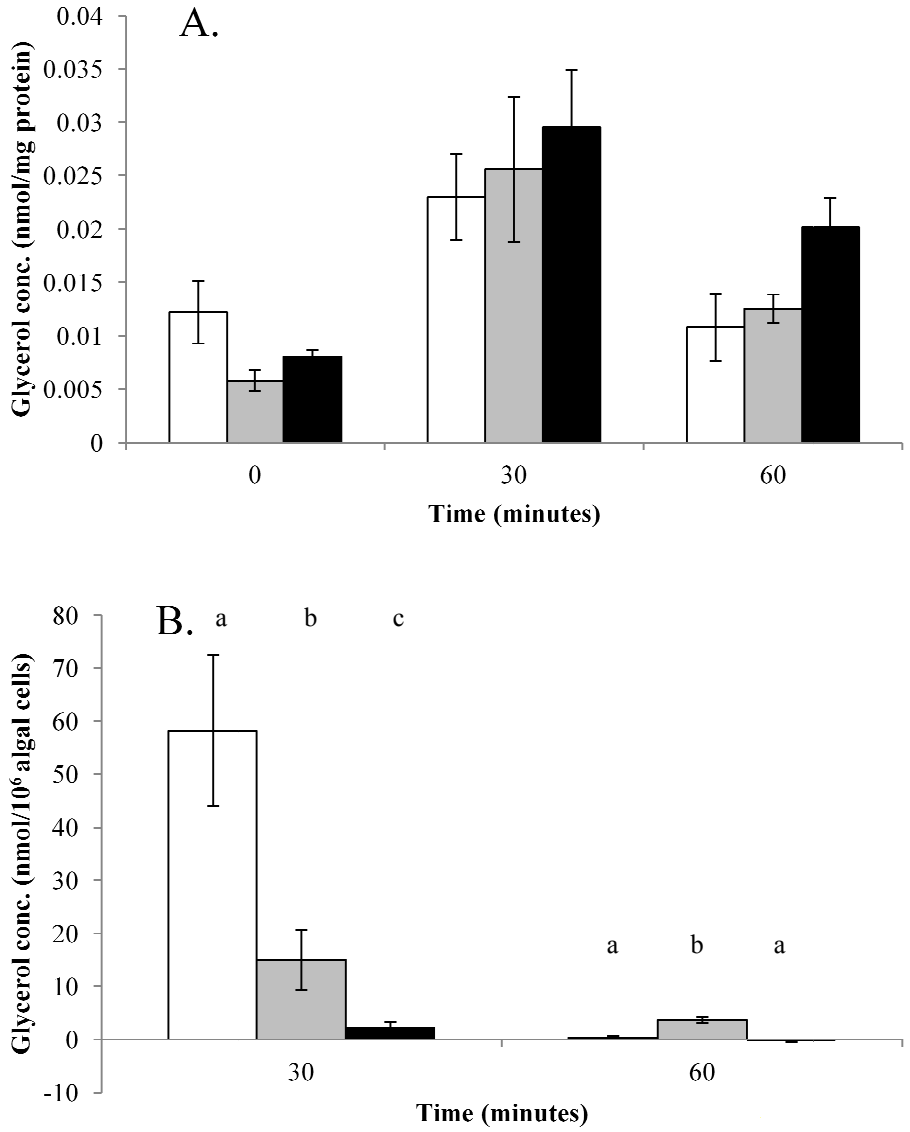

Figure 4.3. Concentration of glycerol in *A. pulchella* tissues when incubated for an hour in the light in the presence of the respiratory inhibitor NaCN. (A) Standardised to host protein content. (B) Standardised to algal cell number, after subtracting the baseline glycerol concentration at 0 min. White bars, homologous *Symbiodinium* B1; grey bars, heterologous *Symbiodinium* B1; black bars, *Symbiodinium* E2. At each time point, associations assigned different letters are significantly different from each other ($p < 0.05$). Values are means ±SE; n = 5 or 6 per time point.

In the case of glucose in the host tissues, all three associations demonstrated an increase in concentration per unit protein between 0 and 30 min, from 0.038-0.051 pmol/mg protein to 0.124-0.132 pmol/mg protein (Figure 4.4A; one-way ANOVA with LSD post-hoc test, d.f. = 2; 7-9, F = 7.1-24.8, $p < 0.05$ for all comparisons). Only the association with heterologous *Symbiodinium* B1, however, showed a further increase between 30 and 60 min, reaching a peak of 0.187 pmol/mg protein (one-way ANOVA with LSD post-hoc test, $p < 0.05$). This trend meant that at 60 min the association with heterologous B1 had a significantly higher concentration of glucose than the other two associations (one-way ANOVA with LSD post-hoc test, d.f. = 2; 8, F = 5.1, $p < 0.05$); no other significant differences were apparent between the three symbiont types (one-way ANOVA, d.f. = 2; 8-9, F = 0.1-0.7, $p < 0.05$).

With respect to the cell-specific glucose concentration (nmol/10^6 algal cells), only the association with the homologous *Symbiodinium* B1 changed between 30 and 60 min, with a drop from 0.20 to 0.16 nmol/10^6 algal cells (Figure 4.4B; one-way ANOVA with LSD post-hoc test, d.f. = 1; 5, F = 83.8, $p < 0.005$). However, significant differences were apparent between the associations at both 30 and 60 min (one-way ANOVA, d.f. = 2; 8-9, F = 19.9-3274.5, $p < 0.005$ for all treatments), with the homologous and heterologous *Symbiodinium* B1 associations having significantly greater cell-specific glucose concentrations (0.20 and 0.07 nmol/10^6 algal cells at 30 min, and 0.16 and 0.11 nmol/10^6 algal cells at 60 min for the associations with homologous and heterologous B1, respectively) than the *Symbiodinium* E2 association at both time points (0.02 and 0.02 nmol/10^6 algal cells for 30 and 60min, respectively; one-way ANOVA with LSD post-hoc test, $p < 0.005$ for all comparisons). Furthermore, the homologous B1 association had a greater cell-specific concentration of glucose than the heterologous B1 association at both time points (one-way ANOVA with LSD post-hoc test, $p < 0.05$ for both comparisons).

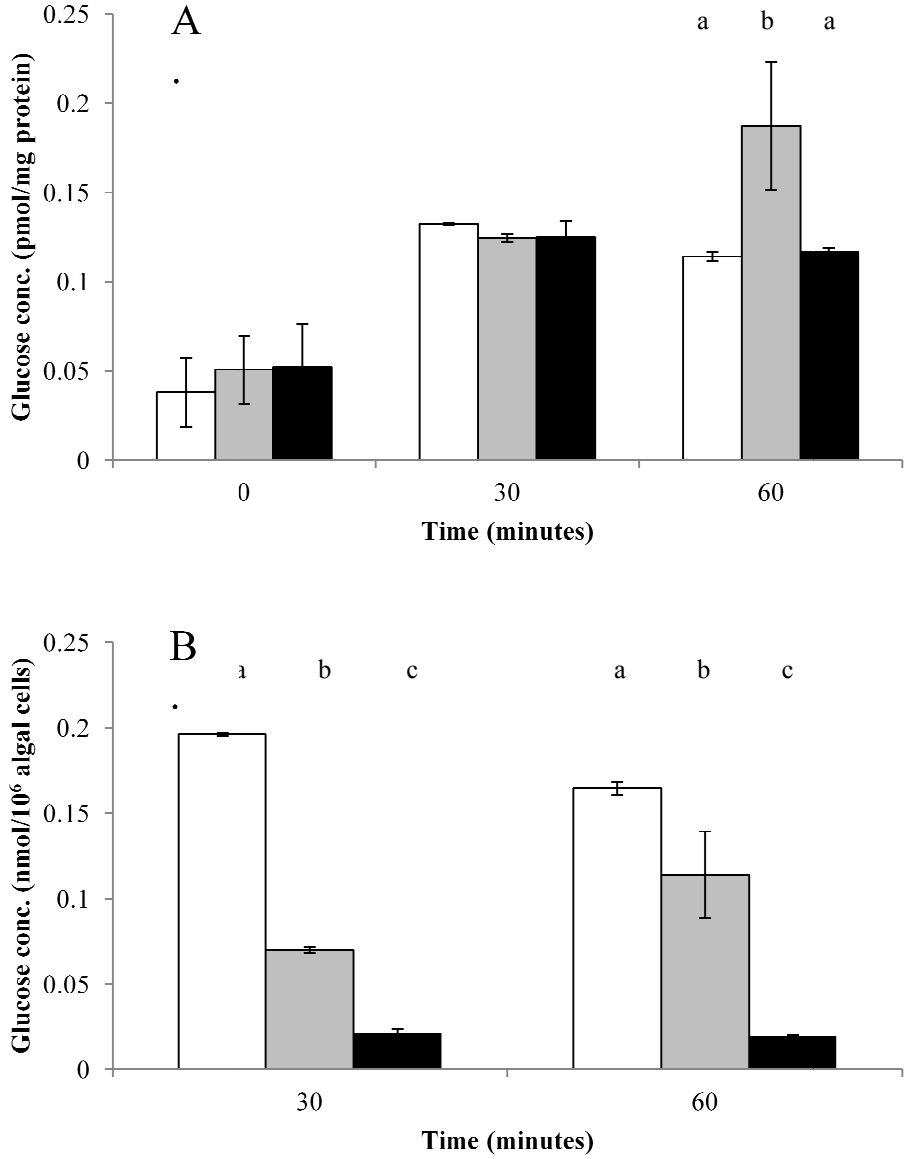

Figure 4.4. Concentration of glucose in *A. pulchella* tissues when incubated for an hour in the light in the presence of the respiratory inhibitor NaCN. (A) Standardised to host protein content. (B) Standardised to algal cell number, after subtracting the baseline glucose concentration at 0 min. White bars, homologous *Symbiodinium* B1; grey bars, heterologous *Symbiodinium* B1; black bars, *Symbiodinium* E2. At each time point, associations assigned different letters are significantly different from each other ($p < 0.05$). Values are means ±SE; n = 5 or 6 per time point.

4.3.3. Carbon fluxes in isolated *Symbiodinium*

When a host homogenate was used to stimulate metabolite release by the different *Symbiodinium* types, they all exhibited similar trends for both glycerol and glucose (Figure 4.5). Glycerol concentration increased from 14.4 nmol/10^6 algal cells at 0 min to 104.4, 64.5 and 72.0 nmol/10^6 algal cells at 30 min for the homologous *Symbiodinium* B1, heterologous *Symbiodinium* B1 and *Symbiodinium* E2, respectively (one-way ANOVA with LSD post-hoc test, d.f. = 2; 6, F = 4.9-8.5, $p < 0.05$ for all comparisons) and then remained unchanged between 30 and 60 min (one-way ANOVA with LSD post-hoc test, $p > 0.05$). Glucose concentration followed a similar pattern, with an increase from 0.29 nmol/10^6 algal cells at 0 min to 0.54, 0.41, 0.40 nmol/10^6 algal cells for the homologous *Symbiodinium* B1, heterologous *Symbiodinium* B1 and *Symbiodinium* E2, respectively (one-way ANOVA with LSD post-hoc test, d.f. = 2; 6, F = 10.4-31.8, $p < 0.05$ for all comparisons) and no change between 30 and 60 min (one-way ANOVA with LSD post-hoc test, $p > 0.05$).

There were differences in the amount of glycerol and glucose released by the different *Symbiodinium* types at 30 and 60 min (one-way ANOVA, d.f. = 2; 6, F = 5.3-51.1, $p < 0.05$ for all comparisons). By 30 min, the homologous *Symbiodinium* B1 had released significantly more glycerol and glucose than had *Symbiodinium* E2 (one-way ANOVA with LSD post-hoc test, $p > 0.05$), while by 60 min the homologous *Symbiodinium* B1 had released significantly more glycerol than had *Symbiodinium* E2 (one-way ANOVA with LSD post-hoc test, $p > 0.05$), and significantly more glucose than both *Symbiodinium* E2 and the heterologous *Symbiodinium* B1 (one-way ANOVA with LSD post-hoc test, $p > 0.005$ for both comparisons).

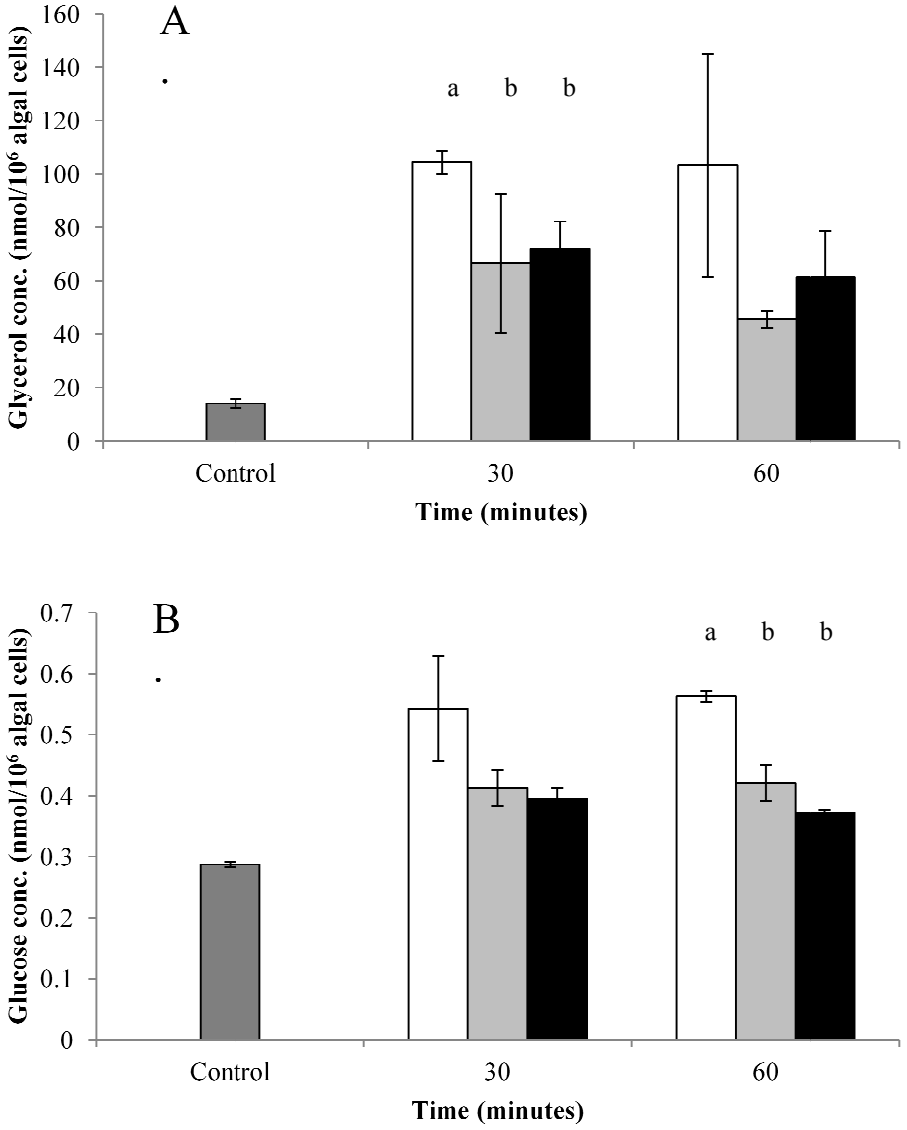

Figure 4.5. Concentration of (A) glycerol and (B) glucose in a host tissue homogenate of *A. pulchella* when incubated with different *Symbiodinium* types in the light. White bars, homologous *Symbiodinium* B1; grey bars, heterologous *Symbiodinium* B1; black bars, *Symbiodinium* E2. The dark grey bar represents the glycerol/glucose concentration in the host homogenate incubating medium at 0 min (control). At each time point, associations assigned different letters are significantly different from each other ($p < 0.05$). Values are means ±SE; n = 3 or 4 per time point.

4.4. Discussion

Overall, this study demonstrated that the presence of both homologous and heterologous symbiont types results in an increase in host tissue glycerol and glucose in the *A. pulchella–Symbiodinium* association although the accumulation patterns in the different associations of these two compounds varied. A significant increase in both metabolites was also observed when freshly-isolated *Symbiodinium* types were incubated in host tissue homogenate with homologous *Symbiodinium* B1 out-performing the other two types with regards to both glycerol and glucose release. Cell-specific metabolite concentrations revealed significant differences between the symbiont types with regards to their contribution to the glycerol and glucose pools, with the homologous *Symbiodinium* B1 contributing significantly more than the other two types and *Symbiodinium* E2 contributing the least. Given that the overall glycerol and glucose pools in the *Symbiodinium* B1 association (as demonstrated by the metabolite concentrations normalized against host protein content) were as high or higher than those in the symbiosis with *Symbiodinium* E2, it seems that a higher symbiont density (as in the case of type E2) can mask a relatively low contribution to the host metabolite pool per symbiont cell and *vice versa*.

4.4.1. Glycerol and glucose levels in the host tissue

A number of studies have investigated carbon dioxide fixation in *Symbiodinium* (Schmitz and Kremer, 1977; Trench, 1979; Streamer *et al*., 1993), though the identity of the compounds released and their quantities are still open to question (Davy *et al.* 2012). Of particular relevance to the current study, there is evidence for glycerol translocation *in vitro*, when freshly isolated *Symbiodinium* cells are incubated in a host tissue extract or "host release factor". (HRF; Grant *et al*., 1997, 1998), as well as *in hospite* in the sea anemone *Condylactis gigantea* (Battey and Patton, 1987). However, Ishikura *et al*. (1999) could not identify glycerol *in hospite* in the giant clam-*Symbiodinium* symbiosis, while Whitehead and Douglas (2003) could not detect it in the translocated material of the temperate sea anemone *Anemonia viridis*. A masters study by Hrdlicka (2000) that looked at metabolite transfer in the *Aiptasia pallida-Symbiodinium* association supported these findings. More recently, Burriesci

et al. (2012) also did not find any evidence of glycerol transfer in symbioses with *A. pulchella* thus further suggesting that glycerol does not form a major part of the translocate. Like previous studies however, they did find that isolated *Symbiodinium* cells released glycerol and suggest that the explanation for this may be that production of glycerol by these algae may be part of a stress response. The evidence for glucose translocation is stronger however: for example both Ishikura (1999) and Whitehead and Douglas (2003) identified it in their studies with intact symbioses. Burriesci *et al*. (2012) supported these findings when they labled intact *Aiptasia* sp. in the light with ^{13}C-bicarbonate and, after homogenization and separation of animal and algal fractions, found labelled glucose in the animal fraction. The current study is the first of its kind to not only measure both of these metabolites (i.e. glucose and glycerol) in the host tissues of a cnidarian-dinoflagellate symbiosis, but to also measure the effect of symbiont type on the internal pools of these metabolites. The study therefore adds to the evidence for both glycerol and glucose release *in hospite*, though the indirect nature of this evidence must still be acknowledged. Moreover, in all cases here the concentration of host tissue glucose was lower than that of glycerol, indicating that glycerol is likely to be the more significant metabolite present in the host tissue; this is consistent with the suggestion of Trench (1971a, b), based on radio-chromatographic studies in the temperate sea anemone *Anthopleura elegantissima*.

This study demonstrates that photosynthesis in the *A. pulchella–Symbiodinium* association results in an increased concentration of free glycerol and glucose in the host tissues, whatever symbiont type is present (except for the association with *Symbiodinium* E2 which did not demonstrate an increase in glycerol when normalized against host protein). However, the host-tissue concentrations of these two metabolites in the different symbioses were not always the same; for example, the symbiosis with *Symbiodinium* E2 contained higher levels of glycerol at 0 hours and in the early stages of the light incubation. Other studies have shown that hosts with certain *Symbiodinium* types grow faster than they do with others, which again suggests that they potentially receive a greater quantity of some or all metabolites from specific types (Fitt, 1985; Little *et al*., 2004). Little *et al*. (2004), for example, demonstrated that juveniles of the corals *Acropora tenuis* and *A. millepora* grow 2-3

faster when in symbiosis with *Symbiodinium* clade C than with clade D. However, in the current study the greater overall contribution of the symbiont population was not necessarily a reflection of the cell-specific contribution; the reasons and implications for this are discussed below.

While there was good evidence for both glycerol and glucose accumulation in the current study, the accumulation patterns of these two compounds differed. In particular, glycerol levels increased at first, but declined towards the end of the incubation period, while glucose levels only increased after several hours. The eventual decrease in glycerol may relate to its use for host metabolism. In particular, as suggested by Battey and Patton (1984), glycerol may be translocated to the host for short-term use as a respiratory substrate; these authors suggested that lipid droplets are more likely to be used during periods of reproduction and starvation. According to Battey and Patton (1987), host catabolism of glycerol is fairly rapid, with the turnover time of the glycerol pool in the sea anemone *Condylactis gigantea* estimated as 4 hours. The influence of host metabolism on the glycerol pool is supported by the elevated concentrations of glycerol in the presence of NaCN, though it should be noted that cyanide would also have influenced respiration by the symbionts and can induce excessive photosynthate release to the host (Davy and Cook, 2001a). Cyanide inhibits respiration by binding to the terminal cytochrome in the electron transport system, which then blocks the donation of electrons to oxygen (Wishnick and Lane, 1969), however it can also inhibit photosynthesis through the binding to the ribulose-bis-phosphate (RuBP) carboxylase complex and the blocking of the incorporation of carbon dioxide (Wishnick and Lane, 1969). This latter effect could have contributed to the decrease in glycerol concentration between 30 and 60 min seen in all three symbioses, especially if the existing glycerol was lost through leakage to the surrounding seawater; this is possible given the ease with which glycerol diffuses through membranes.

The increase in host glucose concentration after 9 hours or more of light incubation was markedly different to the pattern seen with glycerol. The reason for this pattern is unclear, but possibilities include more rapid respiration of glucose by the host in the earlier stages of incubation or reduced glucose synthesis and/or translocation with

time; alternatively, the glucose increase may have resulted from the breakdown of other translocation products. Given that the anemones incubated with NaCN accumulated more glucose by 30 and 60 min than the non-inhibited anemones did by 60 min, it seems that host respiration played a part in masking the true trend in glucose translocation (though the non-specific nature of NaCN and its potential impacts on carbon release should again be recognized).

4.4.2. Carbon fluxes in isolated *Symbiodinium*

The significant increase in the release of both glycerol and glucose in the presence of host tissue homogenate by all of the *Symbiodinium* types is consistent with the enhancement of photosynthate release seen in past studies (Muscatine 1967; Trench, 1971c; Muscatine *et al*., 1972; Muscatine *et al*., 1984; Hinde, 1988; Sutton and Hoegh-Guldberg, 1990; Gates *et al*., 1995; Grant *et al*., 1997, 1998; Withers *et al*., 1998; Ishikura *et al*., 1999; Davy and Cook, 2001a; Burriesci *et al*., 2012).This stimulation is thought to be due to the activity of a compound or combination of compounds present in the host tissues, referred to as a "host release factor" (HRF; Trench, 1971c; Muscatine *et al*., 1972; Hinde, 1988). The nature of this HRF is still uncertain (Davy *et al*, 2012) although studies by Grant *et al*. (1997; 1998, 2006b) suggest that it may be a water-soluble signalling molecule of 1000 kDa or less. The amount of glycerol released by the homologous *Symbiodinium* B1 in host tissue homogenate was 104.4 nmol/10^6 algal cells after 30-60 min. This release rate is of a similar magnitude, though slightly higher than, that reported for *Symbiodinium* cells isolated from the scleractinian coral *Plesiastrea versipora* which released 14-46 nmol glycerol/10^6 algal cells when incubated in a host-tissue extract of this same species for 4 hours in the light (Grant *et al*., 1997). Burriesci *et al*. (2012) found no detectable glucose release by freshly-isolated *Symbiodinium* and no comparable data from other studies are available for glucose.

The results with the isolated algae demonstrate that the homologous *Symbiodinium* B1 outperformed the other two types with regards to glycerol and glucose accumulation/release. It should be noted, however, that since HRF studies are conducted *in vitro*, with isolated dinoflagellates incubated in homogenised host

tissue, they are not representative of theenvironment in the intact symbiosis and it is possible that HRF is an experimental artifact (Davy *et al*., 2012). For example, translocation patterns in response to host (*Aiptasia pallida*) starvation when the symbionts were either exposed *in vitro* to HRF or retained *in hospite* were found to differ (Davy and Cook 2001a, b).

4.4.3. The role of metabolite transfer in host-symbiont recognition and specificity

While the protein-specific concentrations of glycerol and glucose provide insight into the nutritional potential of the different symbioses, the cell-specific response is far more informative with respect to the possible roles of metabolite exchange in host-symbiont recognition and specificity. Indeed, the two measures were shown to be quite different, reflecting the fact that a high symbiont density can mask a relatively low contribution to the host metabolite pool per symbiont cell and *vice versa*. For example, when in symbiosis with *Symbiodinium* E2, the individual symbionts contributed relatively little yet the overall glycerol and glucose pools were as high or higher than those in the symbiosis with the homologous *Symbiodinium* B1 because of the higher density of symbionts in the *Symbiodinium* E2 association. As described in Chapter 2, this high density of *Symbiodinium* E2 may indicate an overshooting of the 'steady state' due to the loss of control by the host of the symbiont population. Little is known of the mechanisms that underlie host-symbiont recognition, integration and the regulation of host-symbiont biomass, however it may well be that metabolite signals play a role. If so, then quantitative and qualitative aspects of the translocated photosynthate could signal to the host that it has acquired a compatible symbiont type, as they do in the green *Hydra* symbiosis where maltose release is required for successful symbiosis establishment (McAuley, 1982; McAuley and Smith, 1982). However, the disparity in the current study between the concentrations of glycerol and glucose in the presence of the different symbiont types and their infection success does not support this; though we cannot exclude roles for other mobile compounds.

One possibility is that *Symbiodinium* E2 was maintained at a higher density to meet the nutritional demands of the host, though we currently know too little about the regulation of symbiont biomass by the host (and *vice versa*) to know what the basis for this would be (Weis *et al*., 2008; Davy *et al*., 2012). It is of note, though, that *Symbiodinium* E2 was originally cultured from a single free-living cell collected from Wellington Harbour (New Zealand), which may explain its unusual behaviour when *in hospite*. There is increasing evidence that such free-living *Symbiodinium* types are common in the field (Takabayashi *et al*., 2012) and these results would suggest that they may perform differently when *in hospite* than symbiotic *Symbiodinium*. It is also important to note that, while *Symbiodinium* E2 overcame its limited cell-specific potential to enhance the metabolite pools of the host anemone by reaching a higher density, there may be energetic costs to the host of maintaining this higher density. Indeed, when the contribution of the symbiotic algal population to host respiration is calculated, the homologous and heterologous *Symbiodinium* B1 appear to be more effective than *Symbiodinium* E2 at supporting the host's metabolic needs due to differences in the respiratory rate (Chapter 2).

From this study it is clear that symbiont type has an effect on host tissue glycerol and glucose pools, however it remains to be seen whether this is a result of the increased translocation of these metabolites to the host, or simply the catabolism of other translocated products. Further work looking into metabolite translocation using more sophisticated approaches such as metabolite profiling (see review by Davy *et al*., 2012) in associations with different symbiont types is needed, as is further research on the potential roles of mobile compounds in host-symbiont recognition.

Chapter 5: General discussion

This thesis examined the relationship between the physiological diversity of different symbiont types and host-symbiont specificity in the model system *Aiptasia pulchella*. Each chapter focused on a different physiological aspect: Chapter 2, the ability of different symbiont types to meet the host's carbon demands; Chapter 3, how symbiont type effects host cell pHi and, in turn, how pHi effects the photosynthetic rate of different symbiont types when *in hospite*; and Chapter 4, how symbiont type impacts the quality and quantity of metabolites translocated from symbiont to host. A wide range of symbiont types was profiled initially (Chapter 2), namely *Symbiodinium* A1.4, both homologous and heterologous *Symbiodinium* B1, *Symbiodinium* E2 and *Symbiodinium* F5.1, before the more focused study of three types (homologous and heterologous *Symbiodinium* B1 and *Symbiodinium* E2) in Chapters 3 and 4.

The main findings were that: (1) Different symbiont types exhibit different proliferation rates when inside the host; (2) Symbiont type impacts the photosynthetic and respiratory attributes of the symbiosis which, in turn, affect the nutritional potential of the symbiosis; (3) Different *Symbiodinium* types have different pH optima, which do not necessarily match the pHi of the host cell and could lead to differential photosynthetic performances when inside the same host species; and (4) Symbiont type has an effect on the release of glycerol and glucose to the host, and the accumulation of these energy-rich compounds in the host's tissues. Here, the interrelationships between these findings will be discussed, as will their implications for our understanding of host-symbiont specificity and the potential for the establishment and persistence of novel associations in the field.

5.1. Mutualism vs parasitism

5.1.1. Shift along the symbiosis continuum

Although symbiotic relationships can be divided into mutualistic, commensal and parasitic interactions, many symbioses do not fit neatly into a single category (Douglas, 1994). Thus it is more accurate to describe symbiosis as a continuum that spans from parasitism to mutualism (Dimijian, 2000) with a given relationship being able to shift along the continuum from one end to the other. Past research suggests that algae can be costly to hosts, potentially acting as parasites. Douglas and Smith, (1983) demonstrated that when hydras that host *Chlorella* algae were grown in the dark, uninfected hosts were found to outgrow infected hosts. Cnidarian–dinoflagellate symbioses have been defined as being mutualistic interactions with the algae benefiting their hosts (Trench, 1993). Numerous cnidarian hosts, however, acquire symbiotic algae infectiously at each sexual generation (Fadlallah, 1983) and it is believed that the horizontal transmission of symbionts promotes the evolution of parasitism (Fine, 1975; Ewald, 1983; Bull, 1994). Muller-Parker and Davy (2001) described that, in some environments, algal symbionts grow and proliferate within hosts during winter despite there being minimal potential for photosynthesis. The evidence generated from this thesis indicates that the cnidarian–dinoflagellate symbiosis may not function strictly as a mutualistic interaction and may, under certain situations, become parasitic. The heterologous symbiont, *Symbiodinium* E2, displayed the highest rate of proliferation inside the host, overshooting the 'preferred' steady state for the symbiosis. This suggests an inability of re-infected *A. pulchella* to effectively regulate these algal populations. This opportunistic behaviour has been previously noted in clade A *Symbiodinium* present in corals recovering from bleaching events (Toller *et al*., 2001b; LaJeunesse, 2005). Also, in the scyphozoan, *Cassiopea xamachana*, acquisition of symbionts from the external environment, resulted in a rapid proliferation of these symbionts inside the host as well as a corresponding reduction in host growth rate (Sachs and Wilcox, 2006). The data from this thesis suggest a similar outcome when *Symbiodinium* E2 infects A. *pulchella*. A clear trend throughout the study was the marked difference in the behaviour of *Symbiodinium* B1 and E2 when in association with *A. pulchella.* This

was demonstrated by the earlier onset of a fully autotrophic state (with respect to carbon) and the higher CZAR values achieved in the symbioses hosting both the homologous and heterologous *Symbiodinium* B1 than type E2. *Symbiodinium* E2 also demonstrated a lower cell-specific release of glycerol and glucose, and associations with this type displayed lower levels of both metabolites in the host tissue metabolite pools. *Symbiodinium* types have been previously shown to differ with respect to the amount of carbon they release. Carbon fixation studies by Stat *et al*. (2008), for example, indicated that clade A *Symbiodinium* may not provide as much carbon to the host as clade C *Symbiodinium* and thus may not meet the host's nutritional requirements. This reduced contribution of carbon by the symbiont to the host may lead to a decline in fitness of the coral host and, in turn, the carbon retained by the symbiont may increase algal fitness and allow for rapid algal proliferation (as evidenced in the *A. pulchella–Symbiodinium* E2 association in this study). Stat *et al*. (2008) demonstrated that *Acropora cytherea* from Hawaii with clade A *Symbiodinium* exhibited suboptimal health states and an increased incidence of disease when compared to corals sampled on the same reef that harboured clade C *Symbiodinium*. Thus the acquisition of clade A *Symbiodinium* by *A. cytherea* may lead to a reduction in coral health or, alternatively, these opportunistic symbiont types may proliferate in health-compromised corals (Stat *et al*., 2008). Similarly, in the wild, it is possible that due to its ability to rapidly proliferate in *A. pulchella*, *Symbiodinium* E2 may be capable of out-competing other symbiont types. However, as suggested by this PhD study, the interaction of *A. pulchella* with *Symbiodinium* E2 may not be mutually beneficial and a shift to parasitism is indicated. A shift from mutualism to parasitism has been previously demonstrated by Sachs and Wilcox (2006), in the symbiosis between the scyphozoan *C. xamachana* and *Symbiodinium*. They showed that horizontally transmitted algae proliferated faster within hosts and had higher dispersal rates from hosts than vertically transmitted algae. However, the horizontally transmitted algae reduced host reproduction and growth. Steen (1986) demonstrated the parasitic nature of some zooxanthellae when he exposed *A. pulchella*, previously kept in the dark, to cultured zooxanthellae under photosynthesizing conditions. He observed that, after the establishment of a stable symbiosis, the symbiotic animals had a protein biomass significantly lower than did control aposymbiotic animals, suggesting that the reinfection of the symbiotic animals by zooxanthellae caused a decrease in animal biomass. He also showed that

aposymbiotic anemones starved in darkness suffered a lower mortality rate than symbiotic anemones under the same conditions, suggesting that zooxanthellae may impose a fatal metabolic burden on their hosts. Thus under certain circumstances, normally beneficial symbionts may become harmful, and thus a symbiont's status of being either a mutualist or a parasite is by no means stable.

5.1.2. Free-living *Symbiodinium*

The phylogenetic positioning of both clades A and E has been found to be either basal or sister to the other *Symbiodinium* clades (Carlos *et al*., 1999; Baillie *et al*., 2000; LaJeunesse, 2001; Pochon *et al*., 2004; Takabayashi *et al*., 2004; Chen *et al*., 2005; Stat *et al*., 2008). This positioning suggests that these clades were, either the first groups to initiate a symbiosis with invertebrates, or that they evolved from the ancestral dinoflagellate that first formed a symbiosis with invertebrates and are divergent lineages on a different evolutionary path to other *Symbiodinium* clades (Stat *et al*., 2008). Alternatively, it is possible that a free-living state is ancestral within the genus and a symbiotic lifestyle evolved independently in the two sister lineages. It certainly appears that the more derived *Symbiodinium* clades such as B and C are better able to form a mutualistic interaction with their hosts (e.g. the nutritional advantage of *A. pulchella* hosting *Symbiodinium* B1 demonstrated in this study). Clade E *Symbiodinium* is extremely rare and has only been identified in the temperate anemone *Anthopleura elegantissima* (LaJeunesse, 2001). This rarity indicates that Clade E either does not form symbioses readily, is becoming extinct, or it is an opportunistic dinoflagellate that is more abundant as a free-living entity (Stat *et al*., 2008). The *Symbiodinium* E2 used in this PhD study was originally isolated from Wellington Harbour (NZ) and its genotype does not match that of any known symbiotic dinoflagellates from New Zealand which belong to *Symbiodinium* Clade A (J. Howe, Victoria University of Wellington, pers. comm.). Therefore, it is suspected that it may be free-living in nature. Since the pH of seawater is approximately 8.1, the superior performance of *Symbiodinium* E2 at a pH of 8 (as evidenced in this study) provides further proof that it may be suited to a free-living environment. As more information is coming to light about the higher-than-expected incidence of free-living *Symbiodinium* (Takabayashi *et al*., 2012), so it is becoming more likely that the symbiotic state may not be the preferred state for all *Symbiodinium* types and

that, in fact, a large proportion of the genus may not be physiologically suited to symbiosis but rather to a free-living lifestyle

5.2. Will corals adapt to climate change?

5.2.1. Symbiont type and the infection process

This PhD study provided some insight into the effect that the acquisition of different symbiont types has on the infection process as well as the short and long-term maintenance of a stable association. Given that all the symbiont types (i.e. *Symbiodinium* A1.4, E2, F5.1 and both the homologous and heterologous B1) tested were taken up by *A. pulchella*, it is clear that this host can form an association with a wide range of symbiont types. Moreover, there was an apparent lack of discrimination by the host during the early stages of infection, at least with these symbiont types. Discrimination by the host between homologous and heterologous symbionts has in past studies been found to occur early in infection (Jolley and Smith, 1980; Schoenberg and Trench, 1980b; Weis *et al*., 2001; Belda-Baillie *et al*., 2002; Rodriguez-Lanetty *et al*., 2004; Markell and Wood-Charlson, 2010), however it has also been noted later in host development (Coffroth *et al*., 2001; Little *et al*., 2004; Gómez-Cabrera *et al*., 2008). The basis of this specificity is poorly understood, however inter-partner signalling has been shown to play an important role both in symbiont uptake (Coffroth *et al*., 2001; Belda-Baillie *et al*., 2002; Rodriguez-Lanetty *et al*., 2003; Logan *et al*., 2010) and symbiosis establishment (Chen *et al*., 2003; Chen *et al*., 2004; Chen *et al*., 2005), and some form of cellular signalling could also explain why the homologous B1 provided the host with significantly more glycerol and glucose than the heterologous B1 type and *Symbiodinium* E2, and ultimately contributed more to the host's metabolic carbon demands. It has been suggested that host release factor (HRF), which is present in homogenised host tissue, acts as a signal molecule and mediates the release of photosynthetically-fixed carbon by the symbiont (Trench, 1971c; Muscatine *et al*., 1972; Muscatine *et al*., 1984; Hinde, 1988; Sutton and Hoegh-Guldberg, 1990; Grant *et al*., 1997; Davy and Cook, 2001a). Given that homologous *Symbiodinium* B1 released proportionally more glucose and

glycerol in response to a host tissue homogenate than did heterologous *Symbiodinium* B1 and *Symbiodinium* E2, it would appear that this signal is symbiont-specific. HRF studies are not representative of the environment in the intact symbiosis however, and the HRF may be an experimental artifact (Davy *et al*., 2012). Also, at this stage the information about symbiosis-specific signal molecules is limited. As more becomes known about the host genome, so the identification of candidate signal molecules will progress (Davy *et al*., 2012).

The symbionts acquired also need to be compatible with the host cell intracellular environment. The intracellular environment of the host may also play a part in determining host-symbiont specificity; for example, pHi may influence symbiont function, as shown in this thesis. Host cell pHi has been shown to influence various facets of cell metabolism including the functioning of membrane channels and enzymes, as well as intracellular signalling (Busa and Nucitelli, 1984; Busa, 1986; Venn *et al*., 2009), so changes in pHi could influence the communication pathways between symbiont and host. Data from this study indicate that all the symbionts tested were functional at the intracellular pH in *A. pulchella*. However, for both homologous *Symbiodinium* B1 and *Symbiodinium* E2, the optimal ambient pH for photosynthesis did not coincide with the host-cell pHi measured in *A. pulchella* harbouring those types. This indicates that the situation is far more complex. Many other factors that are important for promoting a functional symbiosis need to be considered such as the functioning of membrane channels and enzymes and intracellular signalling. (Davy *et al*., 2012).

The ability of a symbiont to proliferate inside the host and increase its population size is critical for the formation of a stable association. However, the regulation of the symbiont population is equally important. If the symbiont density is too low then the symbiont population might be diluted and hence be less effective at supporting the host's metabolic needs (Neckelmann and Muscatine, 1983; Taylor *et al*., 1989), however excessive replication of the symbiont could turn out to be costly to the host and result in higher virulence (Sachs and Wilcox, 2006; Stat *et al*., 2008). This thesis demonstrated how critical it is for the host to be able to regulate its symbiont population. *Symbiodinium* A1.4, F5.1 and E2 were able to attain higher densities inside the host than *Symbiodinium* B1, however this rapid proliferation came at a cost

as demonstrated by the higher metabolic rates seen in the anemones infected with *Symbiodinium* E2 and F.51, as well as the less favourable P:R ratios and CZAR values of associations with types A1.4, F5.1 and E2. Cnidarians regulate their symbiont populations by controlling algal division rates, or by selectively destroying or expelling unwanted symbionts (Gates *et al*., 1992; Falkowski *et al*., 1993; Baghdasarian and Muscatine, 2000; Dunn *et al*., 2002; Dunn and Weis, 2009; Davy *et al.*, 2012). It appears that *A. pulchella* is unable to effectively regulate these heterologous symbiont populations, possibly due to the inhibition of some of the above mentioned mechanisms. How this may occur is uncertain though as currently we do not know enough about the underlying cellular process involved (Davy *et al*., 2012).

Metabolic exchange is central to the ecological success of the coral-dinoflagellate symbiosis (Muscatine, 1990) as, given that most coral reefs are found in nutrient poor waters (Dubinsky, 1990), the symbiont needs to be able to provide the host with enough carbon to meet its host's respiratory demands in order for the symbiosis to persist (Muscatine, 1990). The overall findings from this thesis suggest that it is *Symbiodinium* B1 or, more specifically, homologous *Symbiodinium* B1, that provides the greatest nutritional advantage to *A. pulchella* both in terms of meeting the host's carbon demands, and providing the highest concentration of two key metabolites, glucose and glycerol. This may be facilitated through effective communication pathways between the homologous symbiont and its host, which may not function optimally when a heterologous symbiont is present. Again, more information is necessary about the underlying cellular process involved in order to speculate further (Davy *et al*., 2012).

5.2.2. The implications on the recovery of corals from bleaching events

It has been proposed that corals are able to switch their symbionts as an adaptation to changing environmental conditions (the "adaptive bleaching hypothesis"; Buddemeier and Fautin, 1993). Thus, following bleaching events, the formation of novel symbioses could assist in coral reef recovery. As shown in this thesis, *A. pulchella* is able to form an association with a range of symbiont types, with some of the heterologous types even exhibiting greater proliferation rates inside the host than

the homologous types. This indicates that, should coral associations behave in a similar manner in the field, then they may also have the capacity to form novel symbioses. However, as mentioned previously, the ability to form an association is not the entire picture. In order for a functional association to persist, the symbiont needs to be able to meet the host's nutritional needs (Muscatine, 1990). The findings from this thesis indicate that there are metabolic costs associated with hosting heterologous symbionts and that, should a novel association form, the new symbiont type may not necessarily be as nutritionally advantageous as the homologous type, possibly leading to reduced growth and success of the association. Differences in the growth and survivorship of symbiotic hosts with different symbiont types have been noted in other studies (Fitt, 1985; Little *et al*., 2004). Thus, it is likely that, should a novel symbiosis arise after a bleaching event, it may function less successfully than the original association (at least under non-stressful conditions). Clearly then, whether corals are able to form novel associations in the field is not the only concern with respect to their capacity to recover from bleaching events; the type of symbiont they associate with, is equally important. However, the nutritional contribution of the symbiont may not be of as much significance if the coral is able to rely on heterotrophy to supplement its carbon demands. A recent study by Houlbrèque and Ferrier-Pagès (2008) suggested that heterotrophy is significant to the energy budget of corals and contributes up to 70% of daily carbon requirements when feeding on all possible food sources is taken into account. Moreover, corals have been shown to upregulate heterotrophic feeding when photosynthesis is suppressed (Hoogenboom *et al*., 2010), either due to decreased water clarity (Anthony and Fabricius, 2000) or when symbionts are lost from coral tissue (Grottoli *et al*., 2006). Therefore, a reduced carbon contribution of the resident symbiont population may not be of great importance should the coral be able to compensate for this through heterotrophy. This compensatory mode is particularly important in temperate symbioses as temperate corals are thought to be more heterotrophic than tropical corals (FitzGerald and Szmant, 1988; Tremblay *et al*., 2011).

Support for the "adaptive bleaching hypothesis" is lacking though, and despite some corals being capable of acquiring symbionts from the water column, this acquisition has been found to be temporary as these infections are not maintained as stable symbioses (Coffroth *et al*., 2010). It is more likely that a shift in the dominant

member of a mixed symbiont population will occur as a result of bleaching rather than the formation of a novel association (Baker, 2001; Baker *et al*., 2004; Berkelmans and van Oppen, 2006; Mieog *et al*., 2007; Jones *et al*., 2008). The findings from this thesis apply to the "symbiont shuffling" hypothesis too however, as it still involves a change in the dominant symbiont type. Thus a shift to a less nutritionally advantageous symbiont type may negatively impact the host. For example, *Symbiodinium* clade D is found in some corals that have survived episodes of severe bleaching and is thus considered to be a thermally-tolerant type (Baker *et al*., 2004; Rowan, 2004; Stat and Gates, 2011). Little *et al*. (2004), however, demonstrated that juveniles of the corals *Acropora tenuis* and *Acropora millepora* grow 2-3 faster when in symbiosis with *Symbiodinium* clade C than with clade D indicating that, despite the advantage of hosting type D2 during bleaching events, prolonged association with this type may be disadvantageous for the growth and persistence of the symbiosis. In support of this, Thornhill *et al*. (2005) demonstrated shifts in the dominant symbiont type present in certain colonies of *Montastrea annularis* and *Montastrea franksi* from the Florida Keys as a result of recovery from the stresses of the 1997–1998 El Niño Southern Oscillation (ENSO) event. Over several years, these authors found that stress-tolerant *Symbiodinium* clade D was progressively replaced in these colonies by other symbiont types. This suggests that certain corals may "shift" their algal population to a more stress resistant *Symbiodinium* type during periods of coral bleaching but then reinstate the more nutritionally advantageous symbiont as the dominant type during bleaching recovery. This indicates that the type of symbiont that the coral "shifts" to during the bleaching period may not be of much significance as, even if it does not meet the coral's carbon demands, its contribution may be enough to allow the coral to survive during this period before the more nutritionally advantageous symbiont is reinstated. Such a scenario is even more applicable given that corals appear to be able to supplement their carbon requirements through heterotrophy (Grottoli *et al*., 2006; Hoogenboom *et al*., 2010; Tremblay *et al*., 2011; Wijgerde *et al*., 2011).

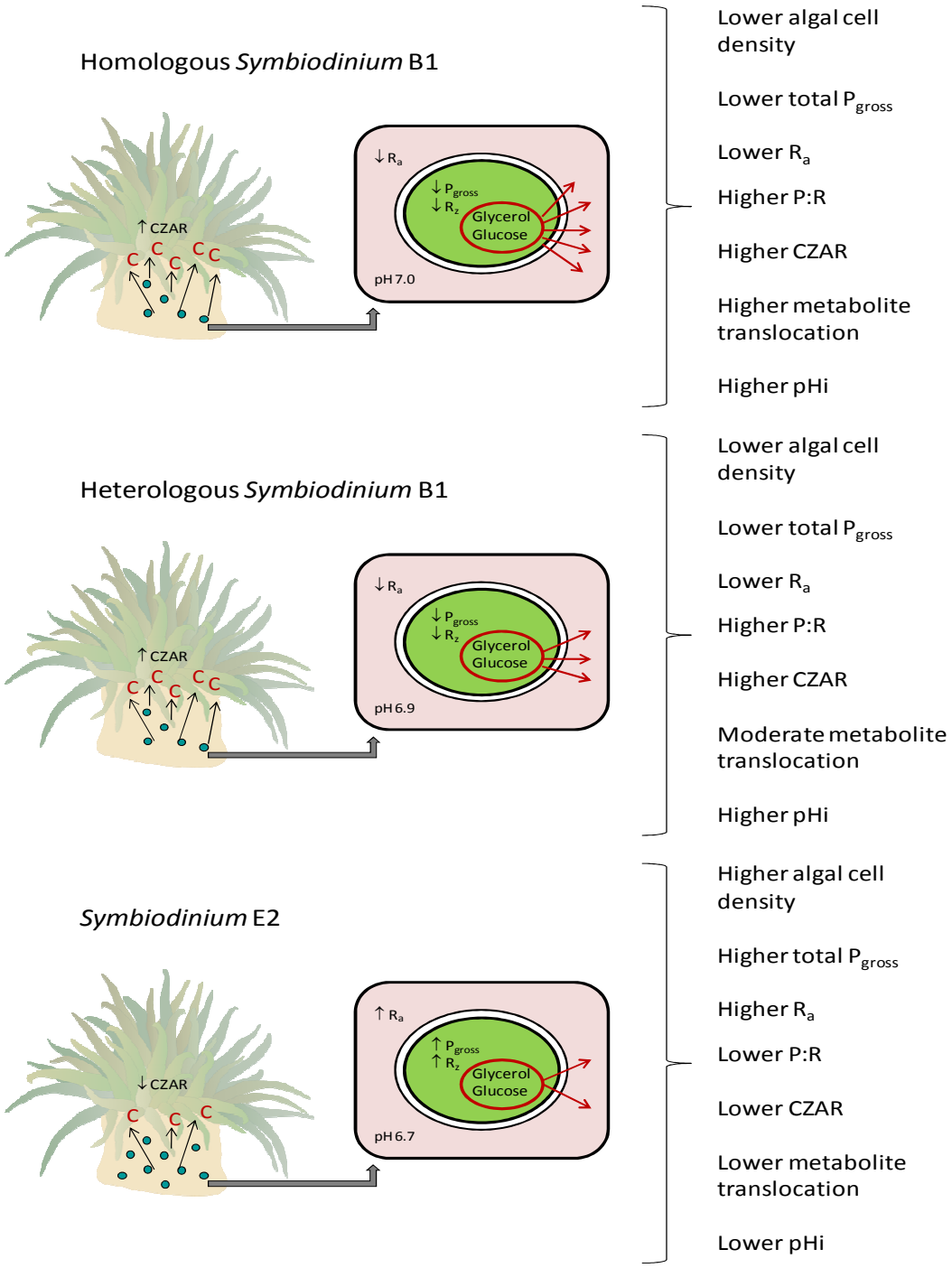

Figure 5.1. Schematic diagram illustrating the summary of the physiological differences and/or similarities between the *A. pulchella–Symbiodinium* associations investigated in this study. The physiological parameters compared include: algal cell density (represented by the green circles inside the anemone diagram), total P_{gross} (gross photosynthetic rate), R_a (host respiration), P:R (ratio of P_{gross} to R_a), R_z (zooxanthellar respiration), CZAR (contribution of translocated zooxanthellar carbon to the host's daily respiratory carbon requirements), metabolite translocation and host cell pHi. The terms "lower" and "higher" are relative terms based on statistical analyses between the three symbioses.

5.3. Future work

While the findings from this work indicate that the physiology of a symbiosis is symbiont type dependent, these studies only incorporated a small number of different *Symbiodinium* types. In order to get a clearer picture of the diversity in symbiosis physiology, a wider range of types needs to be investigated. Of equal importance, the above studies were all laboratory based and do not give a true representation of the behaviour of cnidarian–dinoflagellate symbioses in the field. Also, the studies were performed using the anemone, *A. pulchella,* and despite its applicability as a model system for corals, the contribution of zooxanthellar carbon to the host's respiratory requirements has been shown to vary, both between cnidarian hosts and with environmental parameters (McCloskey and Muscatine, 1984; Davy *et al*., 1996; Muller-Parker and Davy, 2001). It is therefore imperative to extend this type of work to corals in the field, to determine how these associations behave in nature.

Because the symbiosome membrane complex acts as a barrier between the host cell cytoplasm and the symbiont, an understanding of the communicative processes between the algal symbiont and its cnidarian partner, which are no doubt crucial to the nutrition, stability and persistence of the symbiotic relationship, is necessary. Despite recent attempts to identify some of the symbiosome membrane proteins involved in membrane formation, host-symbiont signalling, molecular transport and recognition, photosynthesis, and host and symbiont cell cycle regulation (Peng *et al*., 2010), more information is still necessary to comprehensively understand the interaction between the host and symbiont. In order to be able to elucidate how symbiont type affects the communication pathways between the symbiont and host (i.e. how, for example, a heterologous symbiont may negatively impact these pathways), insight into the influence of different host-symbiont pairings on membrane function as well as the host cell intracellular environment (e.g. the host cell pHi) is needed. Expanding our knowledge of symbiosome membrane composition will in turn provide much needed information about inter-partner signalling, and once the membrane proteins involved are identified, their function in associations with different symbiont types can be studied. Moreover, the use of modern chromatographic and mass spectrometric techniques, as well as comparative

metabolic profiling to characterize the metabolites transferred between partners during different phases of the symbiosis and with different host–symbiont combinations, will provide great insight both into host nutrition and inter-partner signalling (Davy *et al*., 2012). Finally, since a number of free-living *Symbiodinium* types have now been identified (Takabayashi *et al*., 2012), it would be interesting to determine if some of these types can be taken up by *A. pulchella* and are able to form a symbiosis and secondly, determine how they behave when *in hospite* with respect to the quantity and quality of carbon production, symbiosome membrane function and host cell pHi.

5.4. Conclusion

Evidence that corals are able to host multiple symbiont clades has increased in recent years. By surveying 26 coral species previously thought to be restricted to hosting a single *Symbiodinium* clade ('symbiotic specialists'), Silverstein *et al*. (2012) showed that the ability to associate with multiple symbiont clades is common in scleractinian corals. This finding provides further indication that reef corals may be able to adapt or acclimatize to environmental change via shifts in the dominant resident symbiont population (symbiont "shuffling"). Findings from the current study suggest that corals may be able to maintain an association with a range of *Symbiodinium* types and so, should a symbiont population shift occur within a coral as a result of bleaching, the new, dominant symbiont type will be able to successfully populate the host. However, the new symbiont type may not be as nutritionally advantageous as the original type which could have implications for the growth and survivorship of the coral, unless it is able to supplement its carbon demands heterotrophically. Given however, that corals have been shown to upregulate heterotrophic feeding when photosynthesis is suppressed (Grottoli *et al*., 2006; Hoogenboom *et al*., 2010; Tremblay *et al*., 2011) and to reinstate the more nutritionally advantageous symbiont as the dominant type during bleaching recovery, the reduced carbon contribution of the "interim" symbiont type may not be a major concern. Furthermore, it is likely that the metabolic cost associated with hosting the "interim" symbiont type as the dominant type may be compensated for by its rapid proliferation ability (as

demonstrated by some of the heterologous symbiont types tested in this study). The reason being is that, should the “interim” symbiont be more stress tolerant, its ability to rapidly populate the host would clearly be of benefit to the host during periods of bleaching. Finally, the rapid proliferation demonstrated by heterologous types and the associated metabolic cost to the host, could be an indication of the opportunistic nature of these types and may indicate an inability to form mutualistic associations; this is a fascinating area for future research.

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Appendix 1

The influence of symbiont diversity on the photophysiological performance of novel cnidarian–dinoflagellate symbioses; complete carbon budget over entire 12 week period after *A. pulchella* infection with different *Symbiodinium* types

Table A1.1. The CZAR in *A. pulchella* at Week 1 to Week 12 after infection with different *Symbiodinium* types. *Density* zooxanthellar cell density; *MI* mitotic index; μ cell-specific growth rate; P_{gross} gross photosynthetic rate; R_s symbiosis respiration per day; *P:R* ratio of total photosynthesis to total respiration; R_a derived animal respiration per day; R_z derived zooxanthellar respiration per day; P_{zNet} net photosynthesis per day by zooxanthellae; μ_c carbon-specific growth rate (fraction of the carbon standing stock added per day); *T* percentage of the net photosynthetic production translocated to host; *CZAR* potential contribution by zooxanthellae to animal's daily respiratory carbon requirements. The results represent an idealised day of 12 hours light exposure at saturating irradiance and 12 hours of darkness. Values are means ± SE (n = 4-5 for all parameters, except *algal cell diameter*, where n = 72 and *MI*, where n = 32).

***Symbiodinium* type**	**A1.4**					**Heterologous B1**					**E2**					**F5.1**					**Homologous (B1) - freshly isolated**					**Homologous (B1) - cultured**				
Week	**1**	**2**	**4**	**8**	**12**	**1**	**2**	**4**	**8**	**12**	**1**	**2**	**4**	**8**	**12**	**1**	**2**	**4**	**8**	**12**	**1**	**2**	**4**	**8**	**12**	**1**	**2**	**4**	**8**	**12**
Density (x 10^6 cells mg^{-1} protein)	0.5 ± 0.07	2.1 ± 0.9	5.5 ± 2.1	3.9 ± 1.0	2.9 ± 1.2	0.4 ± 0.2	2.5 ± 0.9	4.0 ± 1.2	4.3 ± 0.4	1.3 ± 0.06	2.0 ± 0.4	2.3 ± 1.0	6.1 ± 1.5	5.2 ± 0.7	4.1 ± 0.2	0.4 ± 0.1	0.8 ± 0.4	4.3 ± 1.2	7.3 ± 1.7	4.5 ± 2.9	0.3 ± 0.05	0.8 ± 0.4	3.6 ± 0.4	2.4 ± 1.3	1.6 ± 0.2	0.06 ± 0.02	0.2 ± 0.08	1.4 ± 0.5	1.2 ± 0.5	0.5 ± 0.2
Algal cell diameter (µm)	5.56 ± 0.32	5.88 ± 0.66	9.95 ± 0.55	10.22± 0.15	9.59 ± 0.25	5.83 ± 0.17	6.19 ± 0.34	10.63 ± 0.28	9.61 ± 0.33	9.20 ± 0.15	6.88 ± 0.78	6.92 ± 0.53	9.03 ± 0.34	9.01 ± 0.25	8.59 ± 0.30	4.97 ± 0.23	5.23 ± 0.15	8.49 ± 1.11	7.79 ± 0.28	7.72 ± 0.08	6.51 ± 0.19	8.95 ± 0.50	8.92 ± 0.42	9.74 ± 0.15	8.67 ± 0.07	8.68 ± 0.35	7.57 ± 0.12	9.32 ± 0.17	8.64 ± 0.36	8.29 ± 0.09
Derived algal carbon (pg C $cell^{-1}$)	19.72 ± 3.21	24.78 ± 8.67	81.85 ± 12.00	88.47 ± 2.67	73.14 ± 3.53	21.79 ± 2.10	28.18 ± 3.95	97.98 ± 5.85	73.12 ± 6.96	64.92 ± 2.83	64.45 ± 11.24	68.03 ± 27.79	64.94 ± 5.07	64.07 ± 4.78	54.69 ± 4.30	13.63 ± 1.80	16.01 ± 1.94	77.99 ± 19.67	46.11 ± 5.09	41.23 ± 1.06	22.11 ± 6.78	63.06 ± 9.34	61.38 ± 7.53	78.62 ± 3.91	55.89 ± 1.06	46.21 ± 13.64	39.22 ± 1.24	67.21 ± 3.30	55.81 ± 5.89	49.14 ± 1.35
MI (%)	2.3 ± 0.5	1.3 ± 0.7	1.2 ± 0.5	1.4 ± 0.5	1.1 ± 0.2	4.0 ± 1.3	1.9 ± 0.2	0.9 ± 0.2	1.3 ± 0.2	1.75 ± 0.6	2.5 ± 0.6	1.2 ± 0.3	1.7 ± 0.6	1.6 ± 0.5	1.4 ± 0.5	2.3 ± 0.5	1.3 ± 0.7	1.2 ± 0.5	1.4 ± 0.5	1.1 ± 0.2	4.4 ± 1.1	1.7 ± 0.5	1.6 ± 0.4	1.5 ± 0.6	1.6 ± 0.3	4.0 ± 0.9	1.9 ± 0.5	9.9×10^{-3} ± 2.0×10^{-3}	4.5×10^{-3} ± 6.1×10^{-4}	6.7×10^{-3} ± 1.3×10^{-3}
µ (d^{-1})	0.026	0.018	0.017	0.019	0.016	0.035	0.023	0.014	0.018	0.018	0.027	0.023	0.022	0.021	0.019	0.030	0.023	0.021	0.015	0.016	0.037	0.022	0.021	0.020	0.021	0.035	0.023	2×10^{-3}	1×10^{-4}	2×10^{-5}
P_{Gross} (µg C mg^{-1} protein day^{-1})	0	20.53 ± 3.88	18.27 ± 5.40	10.68 ± 0.73	10.88 ± 3.41	4.39 ± 0.67	30.14 ± 14.41	5.79 ± 1.99	7.51 ± 0.72	5.71 ± 1.46	14.92 ± 6.27	10.23 ± 1.60	16.25 ± 5.56	8.75 ± 1.14	29.02 ± 10.70	7.72 ± 4.09	9.48 ± 2.01	32.07 ± 9.68	7.25 ± 0.58	25.04 ± 8.10	2.62 ± 0.89	6.12 ± 1.04	8.79 ± 0.77	5.22 ± 1.96	9.22 ± 3.87	7.01 ± 2.99	0.01 ± 0.006	5.48 ± 2.02	10.63 ± 0.52	5.41 ± 0.71
R_s (µg C mg^{-1} protein day^{-1})	22.23± 4.52	53.18 ± 5.89	43.38± 17.71	16.28 ± 0.66	11.63 ± 2.40	25.30 ± 14.96	81.96 ± 32.05	2.25 ± 0.35	8.36 ± 1.31	3.61 ± 1.80	35.96 ± 10.41	46.62 ± 6.66	20.34 ± 3.21	12.53 ± 0.57	46.58 ± 12.79	41.16 ± 9.11	17.62 ± 6.38	154.84 ± 32.01	16.83 ± 3.80	45.13 ± 20.25	10.12 ± 1.32	26.68 ± 5.56	17.68 ± 5.28	5.40 ± 1.80	6.36 ± 1.00	15.22 ± 6.71	5.39 ± 1.18	9.19 ± 3.46	11.13 ± 1.99	6.13 ± 0.22
P:R	0	0.39	0.42	0.66	0.94	0.17	0.37	2.58	0.9	1.58	0.19	0.54	0.21	0.43	0.55	0.41	0.22	0.80	0.70	0.62	0.26	0.23	0.50	0.97	1.45	0.46	0.002	0.60	0.96	0.88
Algal:total protein ratio × 10^{-2}	0.89 ± 0.25	2.11 ± 0.68	14.37 ± 5.08	41.74 ± 2.33	15.93 ± 5.07	0.42 ± 0.11	6.00 ± 2.04	15.66 ± 4.35	23.21 ± 0.71	5.45 ± 0.88	8.05 ± 0.45	5.24 ± 1.75	30.86 ± 3.01	43.30 ± 6.77	17.24 ± 1.73	0.54 ± 0.18	1.40 ± 0.64	18.88 ± 3.17	27.33 ± 1.97	18.7 ± 5.1	1.48 ± 0.70	11.32 ± 2.38	46.65 ± 4.57	39.38 ± 18.84	6.56 ± 0.14	0.19 ± 0.06	0.72 ± 0.36	13.20 ± 3.09	8.55 ± 2.86	3.53 ± 0.97
R_a (µg C mg^{-1} protein day^{-1})	22.05 ± 4.50	52.21 ± 5.17	31.63± 13.36	11.14± 1.34	9.34 ± 1.02	25.23 ± 12.96	80.73 ± 32.56	1.77 ± 0.29	6.17 ± 0.84	3.43 ± 0.56	33.50 ± 9.87	43.66 ± 3.77	11.39 ± 2.21	8.28 ± 0.89	39.29 ± 9.70	40.19 ± 9.13	17.42 ± 5.39	117.27 ± 14.85	11.87 ± 2.82	44.56 ± 17.82	10.05 ± 1.14	25.85 ± 4.56	10.88 ± 4.52	4.50 ± 1.64	5.78 ± 0.74	15.21 ± 5.81	5.35 ± 1.32	7.25 ± 2.61	9.98 ± 1.76	5.91 ± 0.18
R_z (µg C mg^{-1} protein day^{-1})	0.18 ± 0.05	0.71 ± 0.26	13.30± 2.64	5.14 ± 1.42	2.29 ± 1.25	0.04 ± 0.008	1.63 ± 0.64	0.35 ± 0.10	2.20 ± 0.57	0.34 ± 0.20	2.46 ± 0.96	1.96 ± 1.13	4.05 ± 0.58	12.39 ± 6.14	7.30 ± 1.85	0.18 ± 0.07	0.29 ± 0.14	37.56 ± 14.14	4.96 ± 0.58	0.57 ± 0.39	0.07 ± 0.04	0.83 ± 0.40	6.79 ± 3.57	0.89 ± 0.43	0.58 ± 0.19	0.01 ± 0.007	0.05 ± 0.03	1.95 ± 0.11	1.14 ± 0.61	0.24 ± 0.08
Algal carbon standing stock: C' (µg C mg^{-1} protein)	0.95 ± 0.27	2.29 ± 0.75	32.65 ± 15.13	33.39 ± 7.77	21.33 ± 8.21	0.43 ± 0.12	6.85 ± 2.45	43.83 ± 18.80	30.60 ± 1.84	6.11 ± 1.04	12.24 ± 3.02	5.94 ± 2.07	57.16 ± 10.72	33.66 ± 4.85	22.15 ± 2.74	0.57 ± 0.19	1.51 ± 0.70	34.91 ± 12.59	39.99 ± 3.98	7.03 ± 1.44	0.74 ± 0.34	4.75 ± 1.90	39.45 ± 17.67	46.66 ± 19.41	7.48 ± 1.72	0.20 ± 0.07	0.47 ± 0.22	19.68 ± 3.77	10.22 ± 3.72	3.89 ± 1.12
P_{zNet} (µg C mg^{-1} protein day^{-1})	0	34.62 ± 13.58	31.32 ± 14.65	8.94 ± 0.68	9.50 ± 3.12	3.87 ± 0.95	9.93 ± 1.66	5.29 ± 1.49	3.12 ± 0.73	5.07 ± 1.25	7.50 ± 4.38	8.18 ± 2.49	15.09 ± 5.53	13.62 ± 5.95	26.96 ± 10.15	1.50 ± 0.91	9.43 ± 2.33	8.21 ± 2.04	6.72 ± 0.68	19.60 ± 5.95	2.60 ± 0.89	3.93 ± 1.69	8.11 ± 1.84	4.94 ± 2.03	8.82 ± 3.70	6.84 ± 2.12	2.16 ± 0.89	5.48 ± 2.02	8.78 ± 1.90	5.41 ± 0.71
μ_c (d^{-1})	0	18.34 ± 4.82	0.88 ± 0.50	0.30 ± 0.05	0.49 ± 0.09	17.72 ± 7.02	3.61 ± 1.05	0.16 ± 0.06	0.27 ± 0.12	0.83 ± 0.14	0.10 ± 0.05	0.02. ± 0.01	0.23 ± 0.04	0.50 ± 0.17	1.20 ± 0.41	48.09 ± 22.62	24.87 ± 9.23	0.79 ± 0.15	0.17 ± 0.02	1.63 ± 0.43	5.35 ± 2.69	0.83 ± 0.07	0.36 ± 0.13	0.32 ± 0.01	1.08 ± 0.19	28.86 ± 9.76	0.59 ± 0.05	0.35 ± 0.06	0.82 ± 0.30	1.71 ± 0.43
T (%)	0	99.33 ± 0.54	96.27± 2.26	92.17 ± 1.13	96.43 ± 0.55	99.54 ± 0.25	98.91 ± 0.55	90.18 ± 4.15	84.29 ± 5.52	97.50 ± 0.59	86.74 ± 6.54	88.85 ± 23.54	89.73 ± 1.92	83.20 ± 10.43	97.35 ± 1.41	96.41 ± 3.25	99.75 ± 0.17	98.32 ± 1.58	91.19 ± 1.50	98.74 ± 0.55	98.78 ± 0.44	97.33 ± 0.23	85.83 ± 9.98	70.00 ± 14.82	97.93 ± 0.37	99.85 ± 0.07	95.98 ± 3.76	99.89 ± 0.04	99.98 ± 0.01	99.99 ± 0.001
CZAR (%)	0	29.66± 8.33	39.21± 8.01	51.05 ± 3.14	74.92 ± 12.06	36.48 ± 5.46	32.13 ± 8.47	150.93 ± 60.76	62.68 ± 29.27	112.98 ± 10.85	13.83 ± 8.40	8.58 ± 3.04	63.07 ± 19.18	50.47 ± 12.84	72.44 ± 26.17	10.69 ± 5.46	52.57 ± 35.40	20.22 ± 5.02	40.58 ± 10.44	52.29 ± 8.34	24.15 ± 7.21	43.74 ± 3.42	49.97 ± 26.34	100.95 ± 0.57	176.78 ± 26.11	46.39 ± 1.45	22.82 ± 11.51	46.22 ± 8.20	91.66 ± 35.80	81.10 ± 7.05

Appendix 2.

Measuring the host cell pHi in *A. pulchella–Symbiodinium* associations with different symbiont types

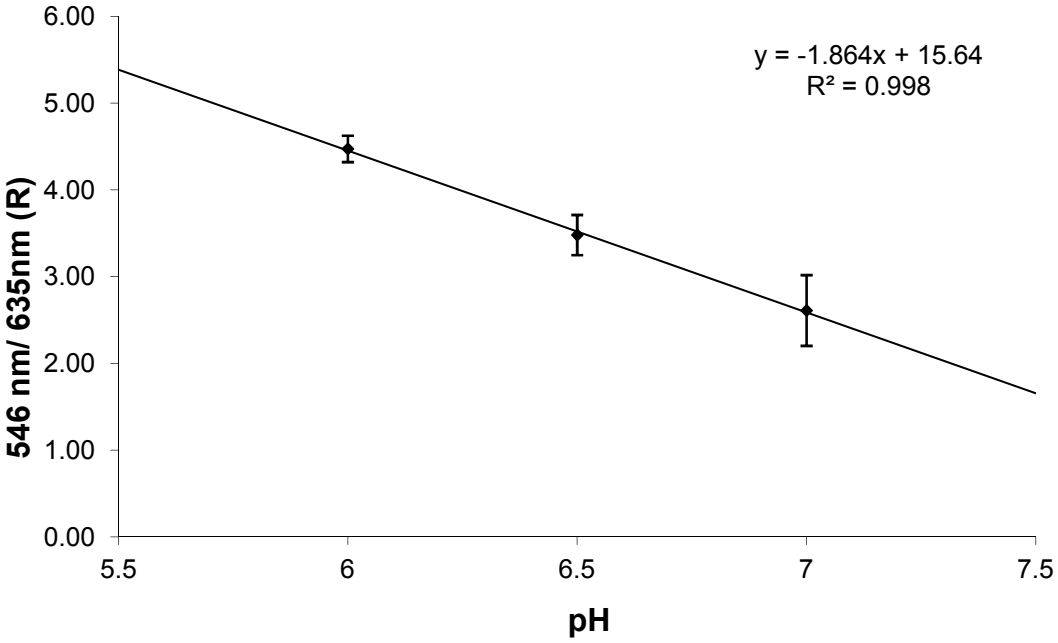

Figure A2.1. Calibration curve of the internal cellular pH (pHi) of *Aiptasia pulchella* cells with the SNARF-4f signal.

Appendix 3.

ITS2 sequences of *Symbiodinium* types used in this study

The *Symbiodinium* types (n = 3 samples per culture or isolate) used in the experiments in Chapter 2, 3 and 4 were genetically identified prior to infection of *A. pulchella*. After 12 weeks, zooxanthellae were isolated from three anemones per treatment and their genetic identity confirmed, using the isolation and molecular techniques described in the methods section of each chapter. All treatments were found to harbour the same *Symbiodinium* type as the one introduced at the start of the experiment. Both the freshly-isolated and cultured homolgous *Symbiodinium* B1, and the heterologous *Symbiodinium* B1 had identical ITS2 sequences. Table A3.1 shows the NCBI accession numbers of the ITS2 sequences that were used to identify the *Symbiodinium* types used in this study.

Sequences of the 5.8s ribosomal RNA gene (partial sequence), internal transcribed spacer ITS2 (complete sequence) and 28s ribosomal RNA gene (partial sequence) for *Symbiodinium* sub-clades (A) A1.4, (B) B1, (C) E2 and (D) F5.1.

(A)

```
  1         10         20         30         40         50
  |         |          |          |          |          |
  TCTGCTTCAGTGCTTCTACTTTCATTTTCTGCTGCTCTTGTCATCAGGAG
  CAGTGTTGCTGCATGCTTCTGCAAGTGGCACTGGCATGCTAAATATCAAG
  TTTTGCTTGCTGTTGTGACTGATCAACATCTCATGTCGTTTCAGTTGGCG
  AAACAAAAGCTCATGTGTGTTCTTAACACTTCCTAGCATGAAGTCAGACA
  A
```

(B)

```
  1         10         20         30         40         50
  |         |          |          |          |          |
  GCTGCTTCAGTGCTTAGCATTATCTACCTGTGCTTGCAAGCAGCATGTA
  TGTCTGCATTGCTGCTTCGCTTTCCAACAAGTCATCGATCGCTTTTGTGT
  TCGTAAATGGCTTGTTTGCTGCCTGGCCCATGCGCCAAGCTTGAGCGTAC
  TGTTGTTCCAAGCTTTGCTTGCATCGTGCAGCTCAAGCGCGCAGCTGTCG
  GGATGCTGATGCATGCCCTTAGCATGAAGTCAGACAAGAGAACCCGCTGA
  ATTTAAGCATATAA
```

(C)

```
1         10         20         30         40         50
|         |          |          |          |          |
TCTGCTTCAGTGCTTTTCATATCTTCGCAGTGCGGGCTTCCTGGAGAAGC
CTTGAGCCTCTTTGCGCGCTGCTGCATCAGAATTTGCAGCGGCGCGCTGA
ACACAAACCGGGAGGTAAGCTGGACTGATTTGTCGCGCATCACTGGGCAC
GTGTGTCCGTTTTGGCCCAATCATGCCAGCCTGCCAAGCAATTGGTGCTC
AAATACCAATCTTAGCATGAAGTCAGACAAGCAAACCCGCTGAATTTAAG
CATATAA
```

(D)

```
1         10         20         30         40         50
|         |          |          |          |          |
TCTGCTTCAGTGCTTAGCTTGCCCAATCTTGCGGATAGACTTTGTTTCTG
TCTTGCGCCCCTGTGAGCCATTGAGCGTCTAGTCAATGGCTTATTGAATG
ATTCGGTCTTGCAAAAGCTTTGCGCGCTTCTATTCAAGATTCCACCTTGG
AGTGGTATTGCTTGAGTGACGCTGCTCATGCTTGCAACTGCTGGGATGCT
AACGCATGCCTCTAGCATGAAGTCAGACAAGCGAACCCGCTGAATTTAAG
CATATAA
```

Table A3.1. NCBI accession numbers of the ITS2 sequences that were used to identify the *Symbiodinium* types used in this study.

Sequence	***Symbiodinium* type**	**NCBI accession number**
A	*Symbiodinium* A1.4	HE578977
B	*Symbiodinium* B1	EU074875
C	*Symbiodinium* E2	JN558086
D	*Symbiodinium* F5.1	AM748615

Appendix 4.

Symbiosome membrane isolation and proteomics

A4.1. Introduction

The symbiotic lifestyle involves mutual ecological, physiological, structural, and molecular adaptations between the partners, and mechanisms that allow for the recognition of the symbiont by the host (or *vice versa*) as well as for the establishment and persistence of the symbiosis. However, not much is known about these mechanisms (Furla *et al.,* 2005). Because the symbiosome membrane complex acts as a barrier between the host cell cytoplasm and the symbiont (Wakefield and Kempf, 2001), an understanding of the communicative processes between the algal symbiont and its cnidarian partner, which are no doubt crucial to the nutrition, stability and persistence of the symbiotic relationship, is necessary. It is essential to characterise the properties of the symbiosome membrane as the transport properties of this membrane govern what passes between the host and symbiont. Thus there is a level of specificity as to which symbiont type can successfully colonise the host resulting in an "effective" symbiosis. Kazandjian *et al,* (2008) isolated the symbiosome membrane from the zoanthid, *Zoanthus robustus*, using a combination of mechanical disruption and sucrose gradient centrifugation. Using confocal microscopy and transmission electron microscopy (TEM) they confirmed that the symbiosome interface surrounds the alga and also established that it is multilayered, robust and displays a level of heterogeneity in terms of thickness. More studies aimed at examining the membrane composition and membrane trafficking using fluorescent markers are necessary to gain an understanding of the symbiosome-membrane modification that occurs during symbiosis. Questions that need to be addressed include: How does transport across the membrane occur? Does the symbiosome interface have transporter/protein pores, dicarboxylate transporters and glucose transporters to facilitate this transport, and is the transport active or passive? Can the transporters be up- and down-regulated and is there transcription-level

regulation of the transport proteins? Furthermore, what happens to the symbiosome membrane when the symbiotic alga within divides? Does this membrane divide simultaneously with the alga? How is the division of the symbiosome membrane coordinated, so that overgrowth of the symbiosome by dividing algal symbionts is prevented? For how long can a symbiosome membrane lineage persist? Is the level of coordination maintained with all types of algal symbiont? The focus of this study was to address some of these key questions. Unfortunately, despite progressing to the point of successfully isolating the symbiosome membrane complex from the *A. pulchella–Symbiodinium* association and isolating the membrane proteins, due to funding and time constraints, the membrane proteomic work could not be completed.

The aim of the study was to characterise the symbiosome membrane complex and the impacts of symbiosis establishment on its functional properties.

Specifically, the objectives and hypotheses were:

1. To isolate the symbiosome membrane complex from *A. pulchella* using a modified version of the method of Kazandjian (2008).
2. To identify the key nutrient transporters present in the symbiosome membrane.
    a. Key nutrient transporters will be upregulated during the infection process.
3. To compare the key nutrient transporters present in the symbiosome membrane amongst symbioses incorporating different symbionts.
    a. The upregulation of nutrient transporters will vary amongst the different symbioses.
    b. Symbioses incorporating homologous symbionts will show greater upregulation of key nutrient transporters.

A4.2. Materials and methods

A4.2.1. Symbiosome membrane isolation

Four *A. pulchella* anemones were homogenized in 1 ml of 0.44-µm FSW in a tissue grinder and centrifuged at ×400 g. The supernatant was removed and the process repeated twice. The animal tissue was then mechanically disrupted to release symbiosomes by passing the extract vigorously seven times through a 23-gauge needle (0.33 mm inner diameter) into a Falcon tube. Symbiosomes were separated from gastrodermal cell debris, cnidoblasts and other animal cells by sucrose gradient density centrifugation. The deaggregated and broken gastrodermal homogenate was loaded onto a step-wise sucrose gradient (30% - 80%). The gradient was centrifuged at ×3893 g for 1 hour (Fig.) with slow acceleration/deceleration rates, at 12–15^{o}C. Gently disrupted material resolved into three bands of symbiosomes on the sucrose gradients: top, above 40% sucrose, middle, above 50% sucrose, and bottom, above 60% sucrose. Vigorously disrupted material resolved into a single large band above 40% sucrose. All symbiosome bands were were stainedwith FM 1-43 and assessed for purity using fluorescence and bright-field microscopy as described below. The fractions containing symbiosomes were then pooled diluted in PBS-50%FSW, pelleted (at ×3893 g for 30 min) and resuspended in PBS-50%FSW. The resuspended symbiosomes were vigorously disrupted, by strong manual pressure on, and seven repeat passes through, the 23 gauge needle described above, loaded on to a second sucrose gradient (30% - 80%). and centrifuged as described previously. The symbiosome membrane fraction was recovered as a golden layer of vesicles suspended in the load zone. The golden-layer material derived from both first and second sucrose gradients were stained with FM 1-43 and examined using fluorescence microscopy as described below.

A4.2.2. Symbiosome and symbiosome membrane visualisation

Fractions containing symbiosomes and the symbiosome membrane were fluorescence-labelled with FM 1-43 (Molecular Probes, U. S. A.) (λ_{ex} 479 nm, λ_{em} 598 nm). FM 1-43 is a vitally staining fluorochrome in the styryl family, used for

detecting vesicle trafficking in eukaryotic cells (Bolte *et al*., 2004). FM 1-43 was selected for labelling the symbiosome membrane because it identifies internalised orendocytosed membranes derived from the plasma membrane (Bolte *et al*., 2004). Stock solutions of FM 1-43 were made up in Milli-Q water at a concentration of 1 mM, and were kept in the dark at -20 ^{o}C. The 40% sucrose fraction (the symbiosome-enriched fraction) was collected and stained for 10 min with 30 μM FM 1-43 and10 μg ml^{-1} Hoechst 33342 for visualisation of the symbiosome membrane and nuclear material respectively. FM 1-43 and Hoechst 3342 fluorescence was observed with fluorescence microscopy (Olympus Provis AX70 microscope) at 100× magnification using a FITC filter to visualize FM 1-43 fluorescence and a DAPI filter to visualise Hoechst 33342 fluorescence.

A4.2.3. Digestion of isolated membranes

The symbiosome membranes fractions were pooled together. A 500 μL aliquot of the pooled fractions was incubated with 500 μL of 2% Triton X-100 (prepared in FSW), 40 μL of protease inhibitor cocktail (Roche Diagnostics, Manheim, Germany, Cat. No. 11697498001) and 5 μL of saturated NaCl solution (Sigma-Aldrich, Auckland). The solution was then mixed in a rotating mixer (rotating 90° right and left) for 30 min at room temperature. The mixture was centrifuged at ×50 g for 5 min in order to separate the Triton X-100-soluble fraction (i.e., the supernatant) from the insoluble fraction (i.e., the pellet). The Triton X-100-soluble fraction was collected for subsequent trichloroacetic acid (TCA) protein precipitation. The Triton X-100-insoluble pellets containing the membrane fractions were resuspended in 1 ml RIPA buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO4, 1 mM NaF) and 40 μL protease inhibitor cocktail. The solution was homogenized in liquid nitrogen and the homogenate centrifuged for 5 min at ×50g to obtain the supernatant in preparation for TCA protein precipitation. All dyes used for microscopy were purchased from Invitrogen (Auckland); other chemicals were purchased from Sigma-Aldrich (Auckland) unless otherwise stated.

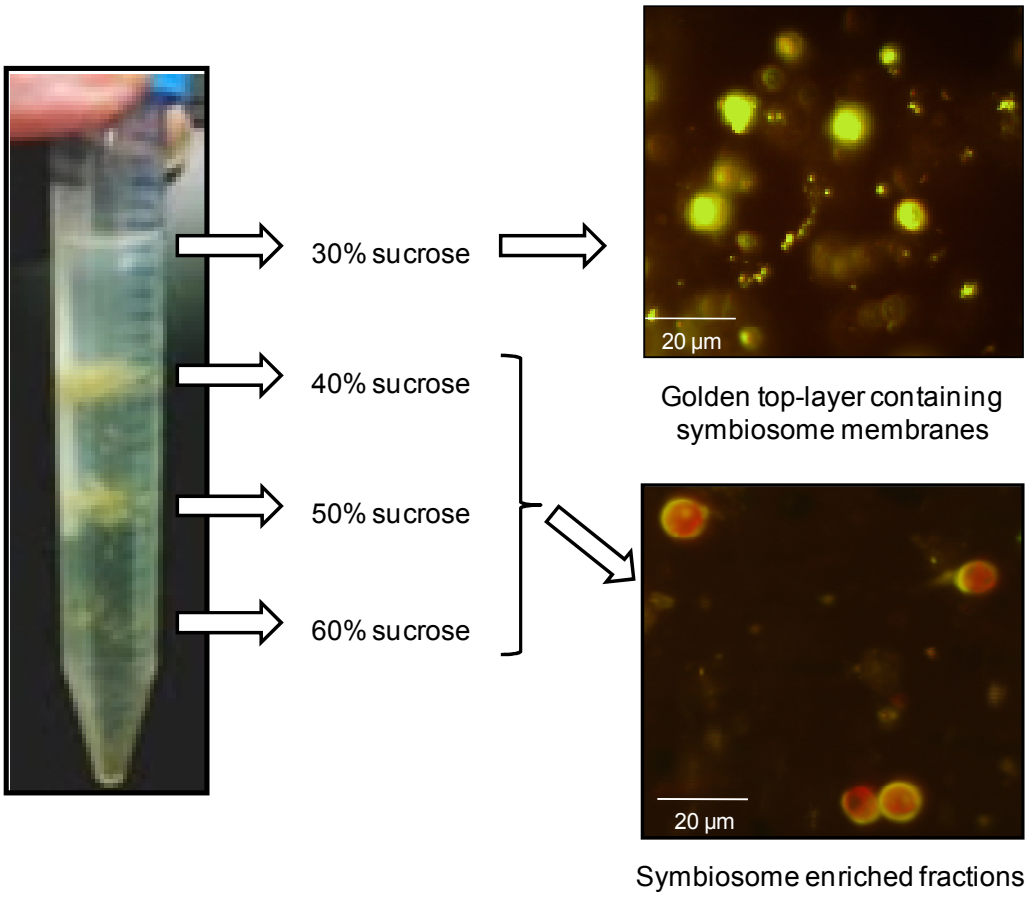

Figure A4.1. Photograph of the step-wise sucrose gradient used to isolate the symbiosome membrane. The various bands obtained include the golden layer enriched with symbiosome membranes above 30% sucrose, and the symbiosome enriched layers above 40%, 50% and 60% sucrose. The top fluorescence microscope image depicts FM 1-43 stained symbiosome membrane complex fragments. The bottom fluorescence image depicts FM 1-43 stained symbiosomes (the symbiosome membrane stains yellow).