**The Genetic and Physiological
Characteristics of the
Symbiodinium spp. in the endemic
anemone
*Anthopleura aureoradiata***

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Abstract

Photosynthetic dinoflagellates of the genus *Symbiodinium* form symbiotic relationships with many marine hosts, including cnidarian corals and sea anemones. This partnership is extremely successful in tropical waters leading to a great diversity of coral species and *Symbiodinium* types. Environmental condition in the tropics are stable, changes to which can lead to destabilization of the symbiotic interactions between the host and symbiont, which in turn can lead to total breakdown of the partnership and expulsion of the symbiont. Temperate symbiotic cnidarian species, especially sea anemones, are less common but locally abundant. Environmental conditions are highly variable with extreme differences in light and temperature. Adaptation to these conditions has led to the success of resilient partnerships, but also to less diversity of *Symbiodinium* types.

This study looked at the relationship between the endemic New Zealand anemone, *Anthopleura aureoradiata*, and its symbiotic relationship with the *Symbiodinium* cells it harbours. The aim was to determine why and how this symbiotic relationship is so resilient to the temperate conditions by 1) determining the molecular identity of the *Symbiodinium* spp. within the anemone, throughout its latitudinal range and through the seasons, and whether any seasonal changes differed between two habitats, the rocky shore and mudflats; 2) comparing the identity of the *Symbiodinium* spp. in New Zealand with those from four species of anemones from Europe (*Cereus pedunculatus*, *Anthopleura ballii* and *Anemonia viridis* from the south-west of England and *Aiptasia mutabilis* from Brittany (France)) to establish any differences or similarities between the northern and southern hemispheres; 3) determining whether resilience to environmental conditions is attributed to the *Symbiodinium* photoprotective mechanisms.

A. aureoradiata were collected in early autumn in five sites from the top (Parengarenga Harbour) to the bottom (Stewart Island) of New Zealand for the latitudinal study. Seasonal anemones were collected from a rocky shore in Wellington Harbour (Point Halswell, Kau Bay) and a mudflat at Pauatahanui Inlet. *Symbiodinium* types were identified to subcladal level using ITS2 sequencing. A low diversity of types was found, with all anemones harbouring algal cells identified as being similar, or identical to, *Symbiodinium* sp. Mediterranean clade A (Med clade A) and *Symbiodinium* sp. Amed

(Amed). 96.55% of the anemones from the latitudinal study, all the winter anemones, 87.50% of the summer anemones and almost 78% of the autumn anemones harboured *Symbiodinium* cells most similar or identical to Med clade A. All *Symbiodinium* sequences from the European anemones also were identified as being similar or identical to Med clade A or Amed, suggesting that the *Symbiodinium* in *A. aureoradiata* are likely not endemic. It is not known whether anemones harbour both types simultaneously and whether a change in dominant symbiont type occurs with seasons within anemones by “shuffling”.

The photophysiology of the *Symbiodinium* cells isolated from the anemones was studied using an Imaging-PAM fluorometer whilst being maintained in six light and temperature treatments. The photosynthetic rate of PSII, energy quenching by NPQ, and photosystem recovery were measured to determine whether the *Symbiodinium* cells had a strong capacity for photoprotection and were able to down-regulate quickly to reduce photodamage to the chloroplast. The main outcome of this study is that the *Symbiodinium* cells within *A. aureoradiata* are very effective in protecting themselves against photo-damage by activating an efficient NPQ system. Down-regulation of the quantum efficiency of PSII under high light conditions appeared to cease altogether. Whether this was a true measurement of down-regulation to stop photodamage, or whether these clade A types use an alternative electron transport that bypasses PSII, and can therefore not be measured with the I-PAM fluorometer technique used, needs to be addressed in future studies.

Declaration

STATEMENT OF ORIGINALITY

The following work presented in this thesis is, to the best of my knowledge, original. It is a representation of my own work throughout my Masters of Science course, except as acknowledged throughout the text. All figures presented represent original work unless otherwise stated. The contained material has not been submitted for any degree at this or any other institution.

Candidate's Signature

Date

Supervisor's Signature

Date

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Abbreviations

SSUrRNA	small subunit ribosomal RNA
RFLP	restriction fragment length polymorphism
ITS1 and ITS2	non-coding internal transcribed spacer regions 1 and 2
DNA	deoxyribonucleic acid
rDNA	ribosomal DNA
PCR	polymerase chain reaction
qPCR	quantitative real-time PCR
DGGE	denaturing gradient gel electrophoresis
SSCP	single-strand conformation polymorphism
MYA	million years ago
MAAs	mycosporine-like amino acids
BLAST	Basic Local Alignment Search Tool
Med clade A	Symbiodinium Mediterranean clade A
Amed	Symbiodinium Amed
FSW	filtered seawater
PSI and PSII	photosystems 1 and 2
ATP	adenosine triphosphate
NADPH	nicotinamide adenine dinucleotide phosphate
ROS	reactive oxygen species
SOD	superoxide dismutase
ABH	adaptive bleaching hypothesis

PAR	photosynthetically active radiation
I-PAM	imaging-pulse amplitude modulated fluorometer
F_o	minimal fluorescence yield during dark adaptation
F_m	dark-adapted maximum fluorescence yield
F_t	instantaneous fluorescence yield
F_m'	light-adapted maximum fluorescence yield
F_vF_m	photosynthetic efficiency or dark-adapted maximum quantum yield
$\Delta F/F_m'$	effective quantum yield
Φ_{PS}	also effective quantum yield
qN	the coefficient on non-photochemical quenching
RLC	rapid light curve
ETR	electron transport rate
rETR	relative electron transport rate
$rETR_{max}$	maximum observed relative electron transport rate
NPQ	nonphotochemical quenching
NPQ_{max}	highest nonphotochemical quenching value on the RLC
qE	energy-dependent quenching
qT	quenching due to state-transition
qI	photoinhibitory quenching
ΔpH	pH gradient
E_k	saturation radiance

Chapter 1 – Introduction

The Cnidarian-Dinoflagellate Symbiosis

1.1 Symbiosis

1.1.1 Definition and types of symbiosis

The term “symbiotism” was originally used by Albert Bernhard Frank in 1877 to describe the relationship between fungi and algae in lichens (Frank 1877, Trappe 2005). He used the term to describe a mutually beneficial relationship between participating partners. The concept of symbiosis was expanded in 1879 by de Bary to include close relationships that can be detrimental to a partner; this therefore included parasitism (de Bary 1879, Trappe 2005). Symbiosis now generally describes an association formed between individuals (symbionts) of different species that “persists throughout a life time or for the major part of it” (Douglas 2010). Partnerships can be between closely related, confamiliar species (Baeza 2007) or distantly related taxa, such as dinoflagellates and cnidarians (Baeza 2007). These include the scleractinian (stony) corals and sea anemones.

Biological interactions between organisms are often described as being of an antagonistic nature; the most common being competition, predation or parasitism. Over time many organisms respond to these hostile biotic stresses and stressful abiotic situations, such as nutrient deficiency, by forming alliances (Douglas 2010). These alliances often result in enhanced fitness for the individuals and increased population size leading to ecological success for the species involved (Connell and Gillanders 2007). Often symbiosis also leads to the symbionts increasing their key biochemical functions, such as recycling nutrients in nutrient poor habitats or using energy from breaking down H_2S to convert CO_2 into organic carbon around deep-sea vents, thereby allowing occupation of inhospitable environments and increasing the diversity and distribution of the species involved (Gast et al. 2003).

There are three categories to describe symbiotic relationships. ‘Mutualism’ describes a relationship where both partners benefit from the association equally and where the alliance is reciprocal. The value of the benefits received from the partner are greater than the value of what has been given away (Douglas 2010). ‘Commensalism’ is where the relationship is beneficial to one partner but neither advantageous nor detrimental to the other. ‘Parasitism’ occurs where one partner benefits with a loss of function or health to the other. The association’s impact on an organism varies with age, species, environmental conditions and the physical well-being of the partners (Douglas 2008). Therefore, in reality, the partnership can move along a gradient from mutualism through commensalism and onto parasitism as the situation changes, in which case the bond may be broken completely as the partnership breaks down.

Symbiosis can further be described as being ‘obligate’, in which at least one partner is dependent on the other for survival, a situation which has developed through co-evolution. It can also be described as being ‘facultative’, where the individuals can survive and reproduce without their partners but are better off together (Baeza 2007). Usually the relative sizes of the partners are unequal where the host is larger than the smaller symbiont. The symbiosis can also be described by the symbiont’s position; ectosymbiotic where the symbiont lives outside the host’s body, such as the clownfish and a sea anemone, or endosymbiotic, where it is found within the host’s body.

Figure 1.1: Forms of endosymbiosis as described by the position of the symbionts within the host

The symbiont within the host can be extracellular, where it can be found within a body cavity or between cells in the host's tissue, or intracellular within membrane-bound vacuoles in the host's cells (Smith and Douglas 1987) (Figure 1.1). Intracellular symbiosis is therefore the sharing of the same cytoplasmic environment by two different genomes from separate evolutionary origins (Trench 1979).

1.1.2 Cnidarian-dinoflagellate symbiosis

The exact origins of the cnidarian-dinoflagellate symbiosis are unknown but the partnership has been hugely successful. Cnidarians include the corals, sea anemones and jellyfish (Figure 1.2).

Figure 1.2: Cnidarians include corals, jellyfish and sea anemones. A) Coral reef at Heron Island, Australia; B) Jellyfish, C) clownfish and anemone and D) coral reef, all taken from reefs around Pulau Perhentians, Malaysia.

There is a hypothesis that the modern coral-dinoflagellate symbiosis began in the Late Triassic period, about 235 MYA, from information collected by skeletal stable isotopes and organic matrix analyses of fossilized corals (Stanley and Swart 1995, Muscatine et al. 2005, Stanley 2006). These corals probably originated from soft, non-reef-building anemone-like ancestors (Stanley and Fautin 2001). This photo-endosymbiotic relationship was important in shaping the co-evolution of the host and symbiont, leading to ecological success, especially for scleractinian corals in nutrient-poor waters (Stanley 2006). The sharing of nutrients between the partners provided a competitive metabolic advantage (Smith et al. 2005) leading to enhanced growth and laying down of calcified skeleton. This has led to the domination of tropical reefs by stony corals (Stanley 2006).

1.1.2.1 The symbiont and host

The photosynthetic unicellular dinoflagellate algae that form symbiotic relationships with cnidarians are mostly of the genus *Symbiodinium* (Baker 2001), though at least one species belongs to the genus *Gymnodinium* (Karako-Lambert et al. 2004). These microscopic algae are commonly known as ‘zooxanthellae’ due to the golden-brown colour that they give to their coral hosts (Brown et al. 2002, Perez 2007) (Figure 1.3 E). They can have two forms, a coccoid vegetative cyst of 5-15 µm in diameter and a motile zoospore (Stat et al. 2006). The coccoid *Symbiodinium* cells contain a large nucleus, in which the chromosomes are permanently condensed, a pyrenoid body and disk-shaped chloroplasts containing the photopigments chlorophylls *a* and *c2*, and xanthophylls unique to dinoflagellates, peridinin, dinoxanthin and diadinoxanthin (Jeffrey and Haxo 1968, Hackett et al. 2004). This haploid vegetative cyst (Santos and Coffroth 2003) is the form that the *Symbiodinium* cell takes when in an endosymbiotic relationship.

The cnidarian host has a very simple body plan composed of two tissue layers, an outer epidermis and an inner endodermis or gastrodermis. These are separated by an acellular mesogloea (Perez 2007, Weis et al. 2008). The body form is a tubular shape or polyp with a single digestive tract continuous with the hollow feeding tentacles.

Figure 1.3: The study organism *Anthopleura aureoradiata*, with its associated *Symbiodinium* spp. A) *A. aureoradiata* under a microscope; B) close-up of tentacles illustrating the brown colouration of the host but originating from the *Symbiodinium* cells; C) and D) a tentacle squash illustrating the dense assembly of *Symbiodinium* cells located within the endodermal tissue; E) *Symbiodinium* cells escaping the squashed tentacle, showing their coccoid shape and golden-brown colouration.

The *Symbiodinium* cells are localized within the endodermal cells (Grant et al. 2003, Yellowlees et al. 2008) in vacuoles surrounded by a host-derived membrane (Rands et al. 1993). Between this membrane and the algal symbiont there may be other membranes that are symbiont-derived (Wakefield and Kempf 2001). These membranous layers are composed of sloughed plasma membranes, thecal vesicles and plates. Altogether they are known as the symbiosome membrane complex (Kazandjian et al. 2008) and the entire vacuolar structure is called the symbiosome (Rands et al. 1993, Wakefield and Kempf 2001) (Figure 1.4). All communication between host and symbiont has to cross these membranes.

Figure 1.4: The arrangement of algal cellular structures, algal plasma membrane and cell wall in the cnidarian-*Symbiodinium* symbiosis, all surrounded by the host-derived membrane (Yellowlees et al. 2008).

1.2 Cellular basis of cnidarian-dinoflagellate symbiosis

1.2.1 Establishment of the symbiosis

Cnidarian hosts acquire their endosymbiont cells either vertically (i.e. passed on from their parents) or horizontally from the surrounding environment (Davy and Turner 2003). An estimated 85% of tropical corals acquire their symbionts horizontally during the host planula larva or juvenile polyp stages (Schwarz et al. 2002, Dunn and Weis 2009) whereas in temperate waters, maternal transmission is thought more likely to

occur due to the scarceness of *Symbiodinium* cells in the water column (Muller-Parker and Davy 2001). Horizontal transmission is dependent on there being a plentiful source of free-living *Symbiodinium* cells (Baker 2003) which have been released by other symbiotic invertebrates or from faeces of corallivorous predators (Muller-Parker 1984). Although there is the potential for the host to associate with many different species of *Symbiodinium*, there appears to be a high degree of specificity between host species and symbiont, and often with only one species of *Symbiodinium* per host (Dunn and Weis 2009). This suggests that ecological processes may have involvement in determining the extent of specificity or that there has to be a process of recognition between the cells of the symbiont and the host. This progressive process of identification and selection has been termed ‘winnowing’ (Dunn and Weis 2009, Voolstra et al. 2009) and probably involves the recognition of different glycoproteins, lectins and receptors on the host and symbiont membranes (Lin et al. 2000, Wood-Charleston et al. 2006, Kvennefors et al. 2008, Kerrigan and Brown 2009, Logan et al. 2010).

Once uptake of the genetically foreign symbiotic organism has occurred, there has to follow some form of resistance or avoidance of the host cell’s defence (Fitt and Trench 1983). Unwanted *Symbiodinium* cells within host cellular vacuoles are either recognised and broken down or the whole host cell is destroyed by apoptosis (Dunn and Weis 2009). Vacuoles containing required symbionts appear to avoid lysosomal fusion and may do so by membrane enzymes (Rab proteins) of the host and symbiont interacting (Chen et al. 2003) or by control in the host cell of vesicular trafficking (Dunn and Weis 2009). This prevents phagosomal maturation and stops the processes leading to vacuole degradation (Vieira et al. 2002). Host cells with appropriate *Symbiodinium* cells stop caspase activity, which controls the innate immune cellular pathways, thereby evading apoptosis (Dunn and Weis 2009).

1.2.2 Maintenance of the symbiosis

The success of this symbiotic relationship, particularly in the tropics, is based upon its enhanced nutritional potential. Here the host potentially receives more than 100% of its energy requirements from organic carbon fixed by symbiont photosynthesis (Steen and Muscatine 1984, Falkowski et al. 1993, McCloskey and Muscatine 1994, Wang and Douglas 1998). It is thought that the symbionts recycle respiratory CO₂ and other host

metabolic waste (e.g. inorganic nitrogen and phosphorus) and translocate up to 99% of their photosynthate (e.g. glycerol glucose, amino acids and fatty acids) to the host (Muscatine 1967, Muscatine et al. 1989, Davy et al. 1996, Hoegh-Guldberg and Jones 1999, Muscatine et al. 2005, Perez 2007). The host uses this for respiration, growth, calcification, mucus secretion, reproduction and regeneration (Smith and Bernays 1991, Hoegh-Guldberg 2004, Furla et al. 2005, Obura 2009). One or both partners may express regulatory mechanisms that control the amount of photosynthate translocated, used and shed by the host, and that manage biomass regulations between the two (Obura 2009), but these mechanisms are poorly understood (Davy et al. 2012).

Most Cnidaria live in nutrient-poor tropical waters and have developed adaptations for assimilating and conserving nitrogen. Ammonium resulting from digestion and metabolism of ingested food by the host is not excreted but re-assimilated by the symbiont and recycled as amino acids back to the host (Furla et al. 2005). This is the basis of the nitrogen recycling hypothesis where the sharing of nitrogen benefits both partners in symbiosis (Wang and Douglas 1998). In addition, a nitrogen conservation hypothesis proposes that photosynthetically-fixed carbon will be used preferentially in animal respiration instead of amino acids. The conserved nitrogenous compounds in the animal tissue can then be used instead for animal growth (Smith and Bernays 1991). The conservation of nitrogenous compound in the animal tissue would promote the persistence of the symbiosis in low-nitrogen environments (Wang and Douglas 1998).

1.2.3 Breakdown of the symbiotic partnership

The symbionts are present in extremely high densities within the host animal, and may fluctuate over time in response to external environmental factors such as temperature change, salinity, increased sedimentation, pollution, nutrient levels, infection by pathogens and changes in light quality (increased visible light or UV radiation) (Brown 1997, Gates and Edmunds 1999, Hoegh-Guldberg and Jones 1999, Douglas 2003, Mayfield and Gates 2007). Many of these factors have a synergistic effect on the interactive relationship between the symbiotic partners. Changes to these environmental factors can stress the symbiotic balance to the point where the partnership breaks down, leading to loss of the symbionts from the host cells. In corals this is called coral bleaching as they lose their dinoflagellate pigmentation and colour. The bleaching

process affects conspecific coral colonies and various species of corals differently, which may be due to the difference of *Symbiodinium* types within (Baker 2001, Ulstrup and Van Oppen 2003). If conditions are extreme enough or continue long enough to prohibit new partnerships to be formed, bleaching can lead to death of the coral polyps as their endosymbionts are essential for their wellbeing (Hoegh-Guldberg 1999, Weis 2008). There are five mechanisms suggested that explain the loss the symbiont (Edmunds and Gates 2003, Weis 2008) (Figure 1.5).

Figure 1.5: Normal host cells (H) are anchored to the acellular mesogloea (M). Symbionts (S) are contained within host vacuoles or symbiosomes. The five mechanisms of cellular breakdown are; 1) Insitu degradation via programmed cell death and autophagy of the symbiont by the host cell (Weis 2008, Dunn and Weis 2009); 2) Exocytosis or expulsion of the symbiont (Fang et al. 1997); 3) Host cell detachment from the mesogloea (Brown et al. 1995, Baghdasarian and Muscatine 2000, Sawyer and Muscatine 2001); 4) Host cell apoptosis, a form of programmed cell death (Lesser and Farrell 2004, Strychar et al. 2004, Dunn et al. 2007); and 5) Necrosis which is an uncontrolled host cell death from external stress factors, which can be reversed, if the stress is decreased (Dunn et al. 2004). Figure taken from Weis (2008).

1.3 Genetic diversity of *Symbiodinium*

1.3.1 Molecular identification within the genus *Symbiodinium*

Early classification of the members of the genus *Symbiodinium* was based on morphology. Study of the coccoid and non-motile symbiotic cells found them to be so similar that they were described as one, pandemic species, *Symbiodinium microadriaticum* Freudenthal (Freudenthal 1962, Taylor 1974). However, since then, molecular, physiological and ultra-microscopical analyses have determined that there is a diverse range of *Symbiodinium* types. Genetic studies in particular have shown that there is a tremendous diversity in this genus. Using the small subunit ribosomal RNA (SSUrRNA) (18S), Rowan and Powers (1991) were the first to divide the genus into 3 large groups or clades, each containing about half a dozen types based on restriction fragment length polymorphism (RFLP) and sequencing. Further work using the large subunit region (28S) (Rowan 1998), chloroplast large subunit (23S) (Santos et al. 2002) and mitochondrial protein-coding gene (cytochrome oxidase subunit 1 (*cox1*)) (Takabayashi et al. 2004) led to more cladal divisions. Studies using the non-coding internal transcribed spacer regions (ITS), consisting of ITS1, 5.8S and ITS2, led to a vast expansion of recognised types within the genus (Hunter et al. 1997, Baillie et al. 2000, LaJeunesse 2001) (Figure 1.6).

Figure 1.6: Schematic of nuclear and organelle genomes targeted for molecular differentiation of the genus *Symbiodinium* (Sampayo 2009).

The ITS regions are part of the eukaryotic nuclear rDNA gene and lie between the 18S small ribosomal subunit and the 28S large ribosomal subunit. A feature of this sequence is that there is a high level of divergence between species, but it is well conserved within a species, and therefore makes a suitable marker for low level phylogenetic analyses (Schultz et al. 2006, Stat et al. 2006).

After PCR amplification of the particular region of interest, rapid resolution of distinct *Symbiodinium* types can be made on denaturing gradient gel electrophoresis (DGGE) or single-strand conformation polymorphism (SSCP) non-denaturing gels. ITS1 and ITS2 amplification have been widely used to differentiate between types within a clade. The term ‘type’ is used to name genetically similar members of the genus *Symbiodinium*. These have unique genetic sequences at any particular locus of a gene or DNA sequence (Coffroth and Santos 2005). SSCP distinguishes between different types by relying on the folding of single-stranded DNA into specifically shaped “secondary structures” where nucleotide bases will match with only one of the other base types (adenine with thymine and cytosine with guanine), leaving some of the DNA strand free from a partner (Figure 1.7). The secondary structures migrate through the gel according to their shape and size. DNA strands of different sequences will generally not assume the same shape and so have distinct gel mobilities and final position (Sunnucks et al. 2000).

Figure 1.7: Proposed ITS2 secondary structure for *Symbiodinium* type A1 (Hunter et al. 2007)

Nine clades have now been recognised (Clades A-I) (Pochon and Gates 2010) with over 400 ITS2 types identified from sequencing and gel profiles (Silverstein et al. 2012). Clades A-H are found in scleractinian corals, A-D more commonly so (Baker 2003, LaJeunesse et al. 2010), clade E in anemones and some corals (LaJeunesse and Trench 2000), and clades C, D and F to I in Foraminifera (Pochon and Gates 2010). Radiation within the genus *Symbiodinium*, from the first clade, clade A, occurred about 50 MYA at the beginning of the Eocene period, probably relating to the divergence and radiation of the scleractinian corals at this time (Pochon et al. 2006) (Figure 1.8). Ancient ancestors of clade A may have been present 65 MYA (Tchernov et al. 2004) as symbionts within Palaeocene corals which managed to pass through the time-period of the K-T boundary (Pochon et al. 2006).

Figure 1.8: The phylogenetic relationships between the major clades of *Symbiodinium*. The cladal tree is a consensus from a maximum likelihood phylogram based on molecular analysis by Pochon et al (2006 and 2010).

Several studies have suggested that there are functional differences between the different clades and that prediction of habitat and geographical distribution, and tolerance to different irradiance and temperature regimes can be made from the genetic type (Rowan 1998, Toller et al. 2001). Clade A have been described as “invasive/opportunistic/parasitic”, B as “sunspecialists”, C as “shade specialists” and E as “stress tolerant” (Rowan 1998, Toller et al. 2001, Savage et al. 2002b). Differences in benefits for the host have been found where one clade has been reported to supply different proportions of metabolites and MAAs in photosynthate than another (Loram et al. 2007, Reynolds et al. 2008). However, cladal differences cannot always be used as a prediction of functionality, as molecular types within a clade have been found to present varying levels of thermal (LaJeunesse et al. 2003) and irradiance tolerance (Savage et al. 2002b).

1.3.2 Biogeographic and ecological variability in diversity

The increasing knowledge of molecular diversity within the genus *Symbiodinium* has revealed not only the extent of specificity between hosts but also the wide-scale distribution and patterns made by both global and small-scale ecological differences, such as depth, light and temperature (Sampayo et al. 2007, McDonald et al. 2008, Silverstein et al. 2012). Members of clade C dominates in the tropics with a transition to clades A, B and D with increasing latitude (Toller et al. 2001, Van Oppen et al. 2001, Savage et al. 2002a). Also, clade C types are present in most coral hosts in the Pacific whereas clade B dominates in the Caribbean with some clade A (LaJeunesse et al. 2003).

A single host species may have a change of clade with increasing latitude. This change could be attributed to changes in temperature, irradiance or turbidity (McDonald et al. 2008). For example the coral *Plesiastrea versipora* hosts clade C at low latitudes and B at the high-latitude margins of its distribution (Rodriguez-Lanetty et al. 2001). Similarly, from 6° N in Thailand to 35° N in Japan the symbionts in *Oulastrea crispata* change from being both clade C and D to just being clade C (Lien et al. 2007). Differences also occur at a sub-cladal level. In southeast Africa a shift with latitude was observed between six *Symbiodinium* types within clade C in the coral *Stylophora pistillata* (McDonald et al. 2008).

In the temperate anemones *Anthopleura elegantissima* and *Anthopleura xanthogrammica*, not only is there a change in *Symbiodinium* type with latitude but also a change in the ratio between *Symbiodinium* symbionts and a green symbiotic alga ('zoochlorella') (LaJeunesse and Trench 2000, Secord and Augustine 2000). Zoochlorellae are restricted to northern latitudes and coexist with *Symbiodinium muscatinei* (clade B), whereas further south the anemones host just *Symbiodinium californium* (clade E). Midway there are mixed populations of both *Symbiodinium* species (LaJeunesse and Trench 2000). Laboratory experimental evidence, field observations and transplant experiments have shown that the changes are influenced by light and temperature. Zoochlorellae grow and photosynthesise more at lower temperatures and irradiances, and zooxanthellae at higher temperatures and irradiances (Secord and Augustine 2000, Verde and McCloskey 2007). Therefore a change in composition was also found with shore height, with zoochlorellae more abundant on the lower shore and zooxanthellae on the higher shore (Bates 2000).

Shore height and depth differences in clades also occur on tropical reefs. Clades A and B were found in *Montastraea annularis* in the Caribbean in shallow, well-illuminated sites, but in deeper more dimly-illuminated sites the coral hosted clade C (Baker 2003). Depth, light and temperature influenced a change of clades in the anemone *Condylatis gigantea* in Bermuda, with clade A inshore in variable temperatures and high light, and clade B offshore, with mixed populations in between (Venn et al. 2008b). These authors found that population composition also changed due to season with clade A more prevalent in the summer and B in the winter.

There are also examples of *Symbiodinium* types that demonstrate a widespread global distribution, with some *Symbiodinium* isolates from the Caribbean being identical to some from the Red Sea or Western Pacific (LaJeunesse 2001). A single 'Temperate A' ribosomal haplotype is widely distributed in anemones, gorgonians, scleractinian, hydrozoans and hexacorals in the Mediterranean and north-eastern Atlantic (Bythell et al. 1997, Savage et al. 2002a, Visram et al. 2006, Focioli et al. 2011). Widely distributed *Symbiodinium* types tend to be host-generalists, with the ability to be in symbiosis not only with different cnidarian species but also with species from other

phyla such as molluscs. These are also, phylogenetically, more ancestral than the *Symbiodinium* types that have specialised hosts (LaJeunesse et al. 2004).

1.4 Photophysiology of *Symbiodinium*

Photosynthesis by the phototrophic endosymbionts brings in nutritional benefit for the host but can also be a potential source of stress. The most significant is when a warming of sea surface temperatures acts synergistically with high solar radiation to cause photoinhibition (long-term reduction in photosynthetic capacity) and damage to the chloroplast and photosystem apparatus (Weis 2008, Vidal-Dupiol et al. 2009). In optimum conditions *Symbiodinium* cells can undergo high rates of photosynthesis, but there has to be a balance between the rates of collecting energy from light and the use of the energy made (Venn et al. 2008a). Maximum photosynthesis and production of organic carbon molecules rely upon an efficient photosynthetic apparatus.

Photosynthesis in the *Symbiodinium* cell takes place in two stages: The energy transduction reaction (light stage) and the carbon fixation reaction (dark stage or Calvin-Benson Cycle). Two photosystems (PS) are involved in the light reaction, photosystem I (PSI) and photosystem II (PSII). Each PS constitutes an assembly of about 250-400 pigments and consists of closely-linked components, an antenna complex that gathers light and passes it onto a reaction centre. The reaction centre is made up of proteins and pigment molecules, chlorophylls *a* and *c*₂ and peridinin, that convert light energy into chemical energy (Iglesias-Prieto 2005).

PSII, within all plants and algae, is primarily located in the grana thylakoid membranes and PSI is almost entirely in the stroma thylakoids and outer portion of the grana. Therefore the two centres are spatially separated, and electrons passing from PSII to PSI require carriers such as plastoquinone and plastocyanin to pass them on via the electron transport chain. Energized electrons from PSII synthesise adenosine triphosphate (ATP) as they pass down the chain and are replaced by electrons from water molecules being split. A product of this split is O₂ gas. Light also excites PSI and the electrons from there are stored in nicotinamide adenine dinucleotide phosphate (NADPH). The stored electrons are replaced from PSII. ATP and NADPH are passed into the stroma to provide energy for the fixation of CO₂ by the enzyme ribulose biphosphate carboxylase

oxygenase (Rubisco) (Raven et al. 2005). *Symbiodinium* chloroplasts also have a Form II Rubisco, which is not efficient at discriminating between O_2 and CO_2 , and therefore needs a high concentration of CO_2 to be maintained to ensure continuous carbon fixation in the Calvin-Benson cycle (Wooldridge 2009).

Environmental stress affects the photophysiology of the *Symbiodinium* cell and leads to photoinhibition by dysfunction of the photosynthetic apparatus in three inter-related mechanisms in the chloroplast (Venn et al. 2008a, Nesa and Hidaka 2009) (Figures 1.9). These are damage to the D1 protein in PSII, disruption to the electron transport chain (energetic uncoupling) in the thylakoid membrane, and impairment of the carbon fixation cycle. All three sites of disruption potentially lead to synthesis of reactive oxygen species (ROS).

Figure 1.9: Representation of photosynthesis under ambient conditions and excessive temperature and light. When functioning optimally, PSI and PSII on the thylakoid membrane produce oxygen which diffuses to the host. Most reactive oxygen species (ROS) produced are converted back to oxygen. There are three proposed impacts of elevated temperature and light on the photosystem of *Symbiodinium* cells (shown with yellow flashes): 1) Dysfunction of PSII and degradation of the D1 protein; 2) energy uncoupling in the thylakoid membranes; 3) impairment of the Calvin-Benson Cycle leading to overproduction of ROS. Figure adapted from (Wicks 2009), which was adapted from Weis (2008) and Venn et al. (2008).

D1 are rapid-turnover proteins that bind the components of the reaction centres and electron transport chain together, and are part of the water-splitting complex in PSII (Miyao et al. 1995). They are a vulnerable component of PSII and have the highest rate of turnover of all the chloroplast proteins. The D1 proteins are degraded by high light but are constantly being repaired and replaced. Under normal conditions the capacity for repair equals the rate of damage but, under stress, the rate of damage exceeds that of repair (Smith et al. 2005, Takahashi et al. 2009). High temperatures may damage the repair mechanism itself (Takahashi et al. 2004), though recent studies have found that this is probably not the case (Hill et al. 2011). These authors found that the repair mechanism did increase the repair rate, but not enough to keep up with damage. Degradation occurs by there being more energy in the photosystems than can be used in the Calvin-Benson Cycle. The excess energy comes from a reduction in electron transport combined with continued absorption of light (Lesser 2006). The electrons contribute to the production of ROS and these powerful oxidants increase damage to the photosystem components (Franklin et al. 2005, Venn et al. 2008a). If these are produced in the plastiquinone binding sites as suggested, they would specifically degrade the D1 protein (Miyao et al. 1995).

It has also been suggested that heat and light initially damage the Calvin-Benson Cycle so that carbon fixation decreases. This causes a downstream impact damaging PSII (Jones et al. 1998). These authors suggested that the site of damage may be the enzyme Rubisco, which would lead to reduced carboxylation resulting in reduced consumption of ATP and NADPH from the light reaction. This would then lead to increased free electrons, which reduce oxygen, producing more ROS. However, Lesser and Farrell (2004) suggested that both photochemistry and carbon fixation are affected simultaneously in a feedback loop increasing the damage to PSII. The ROS formed also go on to inhibit the function of Rubisco, adding to the loop (Lesser and Farrell 2004).

Heat and high light may also damage the thylakoid membranes directly (Weis 2008). This leads to uncoupling of the electron transport chain and allows leakage of protons (Tchernov et al. 2004). As a result there is a cessation of ATP and NADPH synthesis but generation of electrons continues by light exciting PSI and PSII, with water molecules still being split. The O₂ generated by PSII can then react with the

photochemically-generated electrons in PSI to form more ROS in the Mehler reaction (Arora et al. 2002, Venn et al. 2008a). The ROS then oxidize the thylakoid membrane lipids (Tchernov et al. 2004). Differences in genetically predetermined lipid biochemistry between *Symbiodinium* types may be one of the reasons for variations in stress susceptibility (Franklin et al. 2005, Diaz-Almeyda et al. 2011). Sensitive types have lower concentrations of saturated polyunsaturated fatty acids which are more predisposed to attack from ROS (Berkelmans and van Oppen 2006). A higher concentration of the fatty acids allows the thylakoid membrane to remain more fluid, allowing for migration of energy-dissipating pigments.

The Mehler reaction may act as a protective mechanism providing an electron sink to help prevent the PSII electron acceptors from becoming fully reduced at high light intensities (Smith et al. 2005, Venn et al. 2008a). The Mehler reaction reduces O₂ to produce superoxide radicals. These are converted rapidly within the chloroplast into hydrogen peroxide (H₂O₂) by superoxide dismutase (SOD): H₂O₂ is then detoxified into water by ascorbate peroxidase (Downs et al. 2002, Smith et al. 2005). Under most conditions the rate of detoxification should exceed the rate of ROS generation but when under stress, the enzymes become overworked and cannot detoxify the ROS, which accumulate (Franklin et al. 2005). H₂O₂ then can readily diffuse across membranes disrupting other cell reactions (Tchernov et al. 2004, Lesser 2006) and can react with ferrous iron (Fe²⁺) to form the most reactive ROS, a hydroxyl radical (·OH) (Weis et al. 2008). ·OH attacks all biological molecules as it diffuses across the cell, oxidising membrane lipids and denaturing proteins and nucleic acids (Lesser 2006, Csaszar Jr et al. 2009).

As well as the ROS leaking from the damaged symbiont, there are also ROS produced from the host's mitochondria, damaged too from excessive heat and light. The host also has an antioxidant response which can become overwhelmed (Richier et al. 2006). The combined ROS cause photoinhibition and cellular damage to both host and symbiont, and lead to cell apoptosis and necrosis (Lesser and Farrell 2004). H₂O₂ is also known to act as a signal for digestion, apoptosis and exocytosis of the *Symbiodinium* cell by activating a mitogen-activated protein kinase (MAPK) or caspase-signalling cascade (Smith et al. 2005, Perez 2007, Tchernov et al. 2011). Tchernov et al. (2011) found that

the addition of caspase inhibitors prevented bleaching and death even in sensitive corals. ROS could also act as a signal to stimulate the host to produce nitric oxide (NO) during heat stress (Perez and Weis 2006). These authors proposed that the symbionts usually modulate the host's immune system and so act as a controlled infection, 'hiding' themselves from the host's immune system. By signalling their presence with the production of ROS when damaged, which triggers NO production, the host initiates an immune response to eliminate them, as it would a foreign pathogen (Perez and Weis 2006, Weis et al. 2008).

As radiance from the sun can be highly variable, the *Symbiodinium* cell must have efficient protective mechanisms to be able to photoacclimate, so optimizing productivity from the light available. The symbiont is able to dissipate excess light energy in PSII via the xanthophyll cycle in nonphotochemical quenching (NPQ) (Brown et al. 1999, Obura 2009). Protection by the NPQ mechanism occurs within seconds but can last for hours. NPQ can be divided into three component mechanisms: energy-dependent quenching (qE); quenching due to state-transition (qT); and photoinhibitory quenching (qI).

qE is a feedback control mechanism induced when the absorbed light intensity is greater than what can be used by electron transport in the thylakoid membrane. Too much light absorption leads to accumulation of protons in the thylakoid lumen. This results in thermal dissipation of absorbed energy via the pigment antenna of PSII. This is in turn dependent on the energisation of the thylakoid membrane or transthylakoid pH gradient (ΔpH) (Horton and Hague 1988). The gradient can relax very quickly (seconds to minutes) (Muller et al. 2001). During induction of qE, the build-up of ΔpH activates the synthesis of diatoxanthin (Goss et al. 2006), a xanthophyll pigment specific to dinoflagellates (Jeffrey and Haxo 1968). The synthesis of diatoxanthin decreases the rate that photons are passed onto the PSII RCs, passing on the energy as heat (Smith et al. 2005)

qT quenching, or state transition, is the ability to vary the distribution of excitation energy between the two photosystems, so maximizing the overall efficiency of photosynthesis (Hill et al. 2005). Irradiance is thought to induce phosphorylation of the chlorophyll-protein complexes so that part of the complex moves across from PSII to

PSI (Gorbunov et al. 2001). This component of NPQ has been shown to relax over a period of minutes upon darkening (Horton and Hague 1988, Hill et al. 2005). When ROS oxidises the thylakoid lipids, the membrane becomes less fluid affecting the ability for the chlorophyll-protein complexes to rearrange and dissipate energy as heat. This is why different compositions of saturated and unsaturated lipids in the different *Symbiodinium* types affect their sensitivity to thermal and light stresses (Tchernov et al. 2004).

qI, or photoinhibitory quenching, remains after dissipation of the ΔpH (qE) and qT, and normally becomes most active when light levels are in excess of that needed for photosynthesis (Baker et al. 2008). qI relaxes very slowly, over tens of minutes to hours (Hill et al. 2005). It is thought that this process is involved with inactivation of PSII, damage to PSII and quenching in the antenna pigments, as the carotenoids prevent activation of singlet oxygen in the chloroplast (Hill et al. 2005, Szabo et al. 2005)

Both partners in the symbiosis also use other protective mechanisms to tolerate stress. Both have pigments that may help in protection; algae have carotenoids and xanthins (Brown 1997), and the host has phytochromes, which lower the internal light environment, and chromoproteins that absorb photons before they reach the symbionts chloroplasts (Dove et al. 2008). Both partners can produce antioxidants to minimize the effects of oxidative damage on cellular components such as SOD and catalases (Lesser and Farrell 2004, Lesser 2006). There are several classes of SOD, some occurring only in the host and others only in the symbiont (Furla et al. 2005). Studies have found higher SOD activity in symbiotic anemones compared to aposymbiotic ones due to O₂ produced from photosynthesis by the symbiont (Richier et al. 2005).

The host has mycosporine-like amino acids (MAAs) which act as a sunscreen against ultraviolet radiation, some of which may originate from the symbiont, translocated with the photosynthate (Shick and Dunlap 2002, Furla et al. 2005). Shick and Dunlap (2002) found that free-living species of dinoflagellates produce a wide variety of MAAs compared to those in symbiosis and hypothesised that this is due to the host taking on some of the protective role. The host can also rapidly synthesise heat-shock proteins, a type of molecular chaperone, in response to increased temperature (Fitt et al. 2001a, Csaszar Jr et al. 2009). Studies have found that freshly isolated *Symbiodinium* cells have

a greater stress response than cultures of the same type, presumably because the host's protection has reduced the need for the algae to develop long-term photoacclimatory mechanisms (Abrego et al. 2008, Dove et al. 2008, Hennige et al. 2008).

1.5 Tolerance and acclimation to environmental stress by change of symbiont

Bleaching events are increasing in frequency on a global scale (Hoegh-Guldberg et al. 2007, Hoegh-Guldberg and Bruno 2010), but during a bleaching event there is never total elimination of all corals from an entire reef suggesting that there is resistance to environmental stress in some partnerships (Mayfield and Gates 2007). One reason for tolerance may depend on the type of *Symbiodinium* being harboured at the time. Changes between specific host and symbiont combinations, and loss or uptake of symbionts have been proposed as mechanisms leading to tolerance and acclimation. For an adult coral to survive bleaching, it must either retain some of the population it already hosts or acquire symbionts from the environment (Coffroth et al. 2010). The 'Adaptive Bleaching Hypothesis' (ABH) postulates that a change in environmental conditions will cause the loss of symbionts sensitive to present conditions and repopulation within the host of symbionts more tolerant to the conditions (Buddemeier and Fautin 1993, Berkelmans and van Oppen 2006). Tolerant symbionts can be changed or 'switched' with those from the outside environment or can be "shuffled" by increasing the relative abundance of 'cryptic' ones already present in the host that are better suited to the new environmental conditions (Baker 2001) (Figure 1.10).

Studies have found that there has been possible switching from the environment in anemones (Kinzie et al. 2001) and soft corals (Lewis and Coffroth 2004) after bleaching. It was thought that switching was the most likely mechanism for a change in the symbiont population as, using conventional molecular methods, only 25% of corals had been found to associate with multiple types (Silverstein et al. 2012).

Figure 1.10: Diagrammatic representation of how host polyps react to changes in the environment, in this case an increase in temperature, by using ‘switching’ and ‘shuffling’ of symbionts from either the pool of *Symbiodinium* types from the surrounding water, or from within the host itself, to acclimate to the new conditions. In column 1, before temperature increases, polyps A and B host *Symbiodinium* blue, and polyp C hosts a larger population of *Symbiodinium* blue and a ‘cryptic’ population of *Symbiodinium* red. As the temperature increases (column 2), polyps A and B lose their symbionts in the process of bleaching, whereas polyp C remains healthy but the ratio between *Symbiodiniums* blue and red changes as “shuffling” occurs. As the environment remains at the new temperature, column 3, polyp A dies due to loss of its obligate symbiont, polyp B recovers but has now ‘switched’ symbionts from the environment to *Symbiodinium* red, and polyp C remains healthy but has ‘shuffled’ the population within it to having a larger population of *Symbiodinium* red and a ‘cryptic’ population of *Symbiodinium* blue.

However, the advent of more sensitive, high-resolution, real-time PCR techniques, which can pick up very low abundances of symbionts in the host, has changed this point of view. A recent study found that at least one sample of all species tested (39 corals) hosted at least two clades of *Symbiodinium* and that 68% of all sampled colonies hosted at least two clades (Silverstein et al. 2012). This suggests that shuffling occurs more often than had been presumed, and also suggests that total bleaching, or loss of symbionts, does not have to occur for a differently structured population of symbionts in the host. However, when conditions return to normal for the area, many of the newly dominant types become competitively displaced by the original dominant symbionts (Lawler 2009, LaJeunesse et al. 2010).

The concept of an “ecospecies”, or a single host that can function differently due to the *Symbiodinium* type it associates with, has evident advantages for living in a changing environment (Buddemeier and Fautin 1993, Berkelmans and van Oppen 2006). In temperate regions, where conditions do change cyclically, flexibility in the *Symbiodinium* partnership would explain how cnidarian hosts might be able to have populations with such wide latitudinal distribution, and live in habitats where temperature and irradiance can be so varied.

1.6 Temperate vs tropical

Symbiotic relations between marine invertebrates and endosymbiotic dinoflagellates are abundant in oligotrophic tropical seas, where phototrophy, and conservation and recycling of essential nutrients are crucial for the survival of the host. The success of cnidarian-dinoflagellate symbiosis is such that the reefs dominate the shallow waters of the tropics with over 700 species of symbiotic corals found (Yellowlees et al. 2008). Coral reefs are found at lower latitudes, generally between 25°N and 25°S where mean winter sea surface temperatures (SST) do not fall below 18 °C (Savage et al. 2002a), and light penetration and aragonite saturation levels are sufficient (McDonald et al. 2008). In contrast, symbiotic relationships between invertebrates and symbiotic dinoflagellates are relatively uncommon in temperate seas (Muller-Parker and Davy 2001). Environmental conditions become more variable at higher latitudes, with seasonal changes in light, temperature and nutrient levels. However, even though fewer

cnidarian species form symbioses in temperate regions, symbiotic anemones are locally abundant and ecologically important (Muller-Parker and Davy 2001).

Inheritance of endosymbionts in temperate regions is commonly through vertical or closed systems passed on by the mother, possibly due to the rarity of potential donors of *Symbiodinium* cells and of viable cells in the water column (Davy and Turner 2003). In the tropics, *Symbiodinium* cells are more readily available either because symbiotic invertebrates regularly release them into the water or because viable cells are present in the faeces of corallivores (Muller-Parker 1984). Horizontal or open systems are therefore more common in the tropics. The density of endosymbiont cells is far less in temperate associations than in the tropics, where there may be several million per square centimetre of host tissue (LaJeunesse 2002). However, as mentioned previously, the partnership in tropical cnidarians is unstabilised by small fluctuations in environmental parameters whereas temperate symbioses are typified by stable populations, even when subjected to extreme conditions.

Temperatures at higher latitudes are generally lower than in the tropics but the differences in minimum and maximum temperature are pronounced between different seasons and even on a daily scale. Intertidal anemones in rockpools can be exposed to a change in temperature of 10 °C during a summer's day (Dingman 1998). The stability of temperate symbioses in such fluctuating thermal conditions suggests that temperate partnerships are more tolerant of temperature change than are tropical ones, which have been shown to bleach with only minor changes in temperature and live close to their thermal limits (Brown 1997, Fitt et al. 2001a, Dunn et al. 2004).

For symbiotic cnidarians, light is an important environmental parameter as it determines the amount of photosynthetic carbon fixed by the symbionts. Levels of photosynthetically active radiation (PAR) are similar between the tropics and temperate regions during the summer, but during winter levels, at high latitudes, drop significantly in temperate regions (Muller-Parker and Davy 2001). Due to lower densities of *Symbiodinium* cells in temperate hosts and reduced light, the host receives lower quantities of organic carbon from its symbionts in the winter (Verde and McCloskey 2007) and may rely more on heterotrophic sources of organic carbon from nutrients in the water (Davy et al. 1996). Tropical cnidarians, on the other hand, rely heavily on

their dinoflagellate symbionts, and would perish without the organic carbon and recycled nitrogen that they obtain from them (Smith and Douglas 1987, Wang and Douglas 1998, Furla et al. 2005).

Tropical waters are clear due to the lack of phytoplankton, and therefore zooplankton. However, tropical cnidarians may receive more than 100% of their energy requirements from their symbionts under well-lit conditions (Davies 1991, Falkowski et al. 1993). Temperate waters in contrast, are often turbid and nutrient rich. In temperate regions there are seasonal fluctuations in zooplankton, a food source for anemones, and consequently temperate anemones have a peak in both nutrient supplies, photosynthate from symbionts and zooplankton during summer (Muller-Parker and Davy 2001). Temperate anemones are able to rely solely on heterotrophic feeding (Davy et al. 1997b).

There have been few studies on temperate *Symbiodinium* diversity compared to those in the tropics, but they indicate it to be relatively low within hosts or among host species (LaJeunesse and Trench 2000, Savage et al. 2002a, Chen and Yang 2005, Focioli et al. 2011). For example there are only five anthozoans out of eighty around the United Kingdom that contain *Symbiodinium* algae (Davy et al. 1996). Symbiotic anemones can be found throughout temperate regions from 60°N in Alaska to 46°S in New Zealand (Buddemeier and Fautin 1996, Phillips 2006). This study will be on a small intertidal anemone, *Anthopleura aureoradiata* and the molecular identity and photophysiology of the *Symbiodinium* within.

1.7 The study organism –*Anthopleura aureoradiata*

Anthopleura aureoradiata is a small, brown coloured, symbiotic anemone, endemic to New Zealand. It was first described in 1909 from specimens found in Oriental Bay, Wellington and originally named *Bunodes aureoradiata* (Stuckey 1909). Stuckey gave a very full description of the anemone and noted the abundance of symbiotic dinoflagellates that could be seen in the endoderm. It is approximately 5-12 mm in diameter and is taller than it is wide, with 48 tentacles arranged in 4 circular rows. *A. aureoradiata* reproduces asexually and broods the young within its body cavity before they are expelled. The young are already in symbiosis with *Symbiodinium* cells when

released (J. Howe, per.obs), suggesting that they have inherited the algae from their mother.

The anemone can be found abundantly on rocky shores or on mudflats from the far north at Cape Reinga to the far south on Stewart Island (Phillips 2006). Amongst tidal pools it is more commonly found in cracks and crevices above the water rather than within pools (J. Howe, pers. obs). On the mudflats it is commonly found attached to the shell of the cockle *Austrovenus stutchburyi*, which are often buried in the sediment at low tide. When the cockles migrate up through the substrate, the anemones open up their tentacles along the surface. This enables the anemone to feed heterotrophically but also allows its symbionts to photosynthesise (Figure 1.11).

Figure 1.11: *Anthopleura aureoradiata*, which are approximately 5-12 mm in diameter. A) inside a crack on the rocks at Point Halswell, Wellington; B) extending its tentacles on the surface of the mudflat at Pauatahanui; C) attached to the cockle *Austrovenus stutchburyi*; and D) in a pool at Point Halswell.

1.8 Aims and objectives

A. aureoradiata was chosen as a model species as it has a symbiotic partnership with *Symbiodinium* cells. Unlike corals which bleach, or lose their symbionts with small changes in their environmental conditions, this anemone appears to be resilient to the extreme environmental conditions encountered in NZ. Of particular note, it survives the very cold winters in the far south and hot summer conditions in the far north without any evidence of bleaching. My research aim was to determine why and how this symbiotic relationship is so resilient.

The specific objectives were:

Objective 1 – To determine the molecular identity of the *Symbiodinium* spp. within the anemone, throughout its latitudinal range, and through the seasons, and whether seasonal changes differed in two habitats, the rocky shore and mudflats.

Hypothesis – That *Symbiodinium* diversity would be low but that there would be variance with latitude and season.

Objective 2 – To compare the molecular identity of the *Symbiodinium* spp. in New Zealand with those from four species of anemones from Europe (*Cereus pedunculatus*, *Anthopleura ballii* and *Anemonia viridis* from the south-west of England, and *Aiptasia mutabilis* from Brittany)

Hypothesis – The *Symbiodinium* spp. in New Zealand would be endemic, due to the isolation of these islands from other landmasses

Objective 3 – To determine whether resilience to the extreme differences in temperature and light in New Zealand is attributed to the *Symbiodinium* photoprotective mechanisms.

Hypothesis – The *Symbiodinium* photoprotective mechanisms would effectively and efficiently protect the photosystem and photosynthetic yield would therefore be low.

The two data chapters are structured as independent manuscripts. Consequently there is some overlap between these chapters and a degree of repetition, especially in their introductions.

Chapter 2

The biogeography of *Symbiodinium* spp. within the New Zealand endemic anemone, *Anthopleura aureoradiata*

2.1 Introduction

Photosynthetic dinoflagellates of the genus *Symbiodinium* form symbiotic relationships with many marine hosts, including species of the phyla Porifera, Mollusca, Foraminifera, Radiolaria and Cnidaria (Smith and Bernays 1991, Hackett et al. 2004). Symbiotic cnidarians include corals, anemones and jellyfish. The ecological success of the reef-building corals within the oligotrophic waters of the tropics is attributed to the nutritional interactions between host and symbiont, that support the coral's respiration, growth and reproduction (Trench 1971, Muscatine and Kaplan 1994). Cnidarian/dinoflagellate symbiosis becomes more uncommon with increasing latitude. At temperate latitudes, where days are seasonally shortened, irradiance is reduced from cloudy skies, sea temperatures fluctuate and are generally cooler, and waters are more turbid and nutrient rich, nutritional dependence on the symbiont is not essential for host survival, (Davy et al. 1996, Davy et al. 1997a, Muller-Parker and Davy 2001). Nevertheless, temperate symbiotic cnidarians (especially sea anemones) are locally abundant and ecologically important (Savage et al. 2002a) and are known to be distributed from 60 °N in Alaska to 47 °S in New Zealand (Buddemeier and Fautin 1996, Phillips 2006; J. Howe. pers. obs)

2.1.1 Diversity within the genus *Symbiodinium*

Members of the genus *Symbiodinium* are morphologically similar but are genetically diverse. To date there have been 9 molecular clades identified by analysing nuclear and chloroplast ribosomal DNA; these are clade A through to I (Pochon and Gates 2010). Within these clades, over 400 distinct internal-transcribed spacer-2 (ITS2) types have been detected (LaJeunesse 2002, LaJeunesse et al. 2004, Silverstein et al. 2012), though whether types are separate species is not clear (Stat et al. 2011). Restriction fragment

length polymorphism (RFLP) of PCR-amplified small subunit ribosomal RNA (SSU rRNA) was the first technique used to identify the differences between *Symbiodinium* spp. and place them in a cladal tree (Rowan and Powers 1991). These authors named and placed clade A at the base of the tree and found members of this clade to be as closely related to free-living dinoflagellates as to symbiotic *Symbiodinium*. Confusingly, the order of divergence of the clades over time cannot be assumed by the nomenclature given, as clade E probably originated directly after A, followed by G, then D, then I, B, F, and H and finally C (Pochon and Gates 2010).

2.1.1.1 Molecular techniques used to identify diversity in the genus *Symbiodinium*

The discovery of the magnitude of *Symbiodinium* diversity, distribution and behaviour has come about with the use of ever more subtle molecular techniques. These include restriction fragment length polymorphisms (RFLP) (eg Rowan and Powers 1991, Rowan and Knowlton 1995), direct sequencing (eg Loh et al. 2001), sequencing of bacterially cloned ribosomal DNA (rDNA) (eg Apprill and Gates 2007), quantitative real-time PCR (qPCR) (eg Mieog et al. 2007) and size-screening with denaturing gradient gel electrophoresis (DGGE) (eg LaJeunesse et al. 2004) and single-stranded conformation polymorphisms (SSCP) (eg Ulstrup and Van Oppen 2003). Sequence comparison of rapidly evolving genes, such as the large subunit of rDNA, the hypervariable regions of the chloroplast 23S rDNA, the microsatellite flanking regions and internal transcribed spacer regions of the rDNA (ITS1 and ITS2), make the distinction of subcladal types possible (LaJeunesse 2001, Loh et al. 2001, Rodriguez-Lanetty et al. 2001, Van Oppen et al. 2001, Santos and Coffroth 2003, Chen et al. 2005, Hunter et al. 2007, Sampayo et al. 2007).

The ITS2 region is part of the eukaryotic nuclear rDNA operon and lies between the 5.8S and the 28S rRNAs (Schultz et al. 2006, Hunter et al. 2007). The organization of the operon is similar among all eukaryotes but the ITS2 region is highly diverse between species. This makes it a very useful marker for low level phylogenetic analysis (Hunter et al. 1997). However, while the sequences of this region are variable amongst species, the secondary structures formed, when single strands fold back on themselves, are well conserved across eukaryotes and can also be used to identify species.

2.1.1.2 The secondary structure as an identification tool

The secondary structure has a similar four-fingered or four-helix shape across such divergent species as vertebrates and yeast, and green algae and higher plants (reviewed by Schultz et al. 2006) with the third helix being the longest. As the structural shape is a general feature across eukaryotes, it can be used as a way to differentiate the slower evolutionary time over which some clades diverged compared to the quicker divergence of one species from another (Schultz et al. 2006).

The secondary structure of *Symbiodinium* ITS2 takes one of two configurations. The more ancestral *Symbiodinium* clades and free-living dinoflagellates have the common ‘four-fingered’ configuration whereas those clades that diverged in more recent times have a sub-helix (III a), making it a ‘five-fingered’ structure (Gottschling and Plotner 2004) (Figure 2.1).

Figure 2.1: Representation of a *Symbiodinium* secondary structure with five helices. Figure taken from Hunter et al. (2007).

Analyses by Hunter et al. (2007) have found that clades A, E, G and D have four helices and clades B, C, F and H have five (Figure 2.2). Pochon et al. (2010) later identified another clade and named it clade I, placing it one branch above clade D and as a sister to the group including clades F, B, H and C. They also found that the secondary structure of the ITS2 from clade I folded into a five-helix configuration.

Figure 2.2: The two variations of secondary structures of *Symbiodinium* ITS2 and its relationship with the cladal phylogeny. The phylogenetic consensus cladogram comes from various studies (see Coffroth and Santos 2005). Figure comes from Hunter et al. (2007).

The study by Hunter et al. (2007) also found that sequences of base pairs (bp) at the bases of helix I and helix II show variability between *Symbiodinium* types but are conserved within a type, giving further support of the usage of secondary structures as an identification tool.

2.1.2 Distribution of the genus *Symbiodinium*

The diversity and distribution of *Symbiodinium* clades and types varies with geographical and latitudinal location, habitat type and depth. There is evidence of scleractinian corals from the Eastern Pacific hosting mainly clade C whereas in the Caribbean they host mainly clade B with some A and C (LaJeunesse et al. 2003). To the west of the Pacific, C is still the dominant clade with A, B and D in less than 15% of corals (LaJeunesse et al. 2003). On the reefs off Eilat (Red Sea), only A and C have been found (Karako-Lambert et al. 2004) and in the NE Atlantic and Mediterranean Sea, most *Symbiodinium* are of clade A (Savage et al. 2002a, Visram et al. 2006). Different phylotypes are thought to have distinctive physiological traits in response to different light and temperature conditions (Baker 2001). Consequently, as temperature and irradiance are the environmental factors that vary most with latitude, they are believed to have an influence on *Symbiodinium* distribution. For instance, clades that are present at high latitudes are probably better adapted physiologically to function with

the seasonally changing conditions (Rodriguez-Lanetty et al. 2001, McDonald et al. 2008).

Due to the apparent partitioning of cladal distribution within a habitat, studies have been carried out that suggest that genetically distinct *Symbiodinium* possess unique environmentally adaptive capabilities (Rowan 2004). Clade A algae are thought to be shallow-water specialists (LaJeunesse 2002) as they may have a competitive edge over other clades by producing mycosporine-like amino acids, thereby giving protection to their hosts and their own cells from UV light (Banaszak et al. 2000). They have also been characterized as being ‘invasive’ and ‘opportunistic’ (Rowan 1998, Toller et al. 2001, Stat et al. 2008b). Clade B algae have also been described as ‘sun specialists’, while clade C algae have been proposed as ‘shade specialists’, and E and D as ‘stress tolerant’ (Rowan 1998, Toller et al. 2001, Savage et al. 2002b). However, other studies have found that associating one clade with certain traits is too restrictive as there is variation among types within a clade (Iglesias-Prieto and Trench 1994, Tchernov et al. 2004, Hennige et al. 2008). Moreover, particular host-symbiont combinations also influence the position of the holobiont in the environment (Sampayo et al. 2007, Wicks et al. 2010b).

The ‘ecospecies’ concept proposes that a host species will function differently depending on the symbionts it associates with, and thereby can adapt to changing conditions (Buddemeier et al. 2004). Some cnidarian species are thought to react to changes in the environment by changing the type of symbionts within. The changes would function as an adaptive mechanism, as suggested by the ‘Adaptive Bleaching Hypothesis’ (Buddemeier and Fautin 1993), where changing environmental conditions trigger the loss of intolerant types from the host which are then ‘switched’ for more tolerant symbionts from the water column (Baker 2001, Berkelmans and van Oppen 2006). Full bleaching, or loss of symbionts, might not have to occur for a change of the *Symbiodinium* type present (Chen et al. 2005, Venn et al. 2008b) and new symbionts need not come from the external environment. Instead, a ‘shuffling’ of the *Symbiodinium* community within the host can occur as the relative abundance of one type over another ensues as competitive dominance changes (Baker 2001). Shuffling is thought to occur more often than once thought. The majority of coral colonies on the

Great Barrier Reef have been found to harbour multiple symbiont types, one dominant with a background level of other types (Berkelmans and van Oppen 2006, Mieog et al. 2007). Shuffling has been shown to occur with season (Chen et al. 2005), with latitude (LaJeunesse and Trench 2000) and habitat, and after natural bleaching events (Thornhill et al. 2006).

Some host species with long latitudinal distributions have been found to change symbionts from one type to another, often with both types present along a midway point. *Anthopleura elegantissima*, from the Washington and Oregon coastlines, hosts clade B (*Symbiodinium muscatinei*) which then becomes replaced by clade E (*Symbiodinium californium*) in Southern California (LaJeunesse and Trench 2000). Latitudinal patterns also occur in the corals *Plesiastrea versipora* and *Acropora millepora* along the eastern Australian coast (Rodriguez-Lanetty et al. 2001, Berkelmans and van Oppen 2006) and in *Oulastrea crispata* in its distribution from Thailand to Japan (Lien et al. 2007). Differences in habitat as the depth changes on the same reef have also been correlated to changes in symbionts. The Caribbean coral *Monastrea annularis* (sensu lato) has a change in *Symbiodinium* clade which is depth related (Rowan and Knowlton 1995) as does the Bermudan anemone *Condylactis gigantea* (Venn et al. 2008b). This anemone was also found to undergo a change in symbiont clade with the seasons (Venn et al. 2008b). More extraordinary are the anemones *Anthopleura elegantissima* and *Anthopleura xanthogrammica*, as these anemones may not only contain the two *Symbiodinium* endosymbionts mentioned above, but also another algal taxon, a symbiotic chlorophyte or ‘zoochlorella’. Light and, to a greater extent, temperature, affect the ratio of the three symbionts within *A. elegantissima*. Higher latitudes, lower shore positions and cooler seasons favour *S. muscatinei* and zoochloellae whereas lower latitude, higher shore positions and warmer seasons favour *S. californium* (Bates 2000, LaJeunesse and Trench 2000, Secord and Augustine 2000, Muller-Parker et al. 2007, Verde and McCloskey 2007).

There have been comparatively fewer studies of temperate than tropical *Symbiodinium* diversity. Most of these temperate studies have found a relatively low symbiont diversity, whether within a host species or among host species (LaJeunesse and Trench 2000, Savage et al. 2002a, Chen et al. 2005, Visram et al. 2006). Some suggest that

there is a trend for endemism in high latitude *Symbiodinium* types (Savage et al. 2002a, Wicks et al. 2010b) and host specificity due to the strong selection pressure imposed on the symbionts by seasonally variable environmental conditions (Wicks et al. 2010b, Foccoli et al. 2011).

2.1.3 The temperate anemone, *Anthopleura aureoradiata*

Anthopleura aureoradiata is a small, brown-coloured endemic anemone from New Zealand (Figure 2.3). It largely reproduces asexually and broods its young within its body cavity. The young are released harbouring *Symbiodinium* cells (J. Howe. pers. obs), suggesting that they inherit their dinoflagellates vertically from their mother. The anemones are found throughout NZ ranging from 34°S to 47°S (Phillips 2006) and can be found on the rocky shore and on mudflats.

Figure 2.3: *Anthopleura aureoradiata* as seen from (A) above and from (B) the side displaying its characteristic brown tentacles and thick column.

2.1.4 Aims and Objectives

The purpose of this study was to determine the molecular identity of the *Symbiodinium* cells within *A. aureoradiata* by sequencing and identifying the ITS2 region of the algae, using PCR and SSCP techniques. The objectives were:

1. To determine if there is a change in symbiont identity and/or population composition with latitudinal gradient within anemones throughout the length of NZ.
2. To determine if there is a change in symbiont identity and/or population composition through the seasons within anemones. A preliminary study was made to determine whether the variability is also correlated to habitat type (mudflat *versus* rocky shore).
3. To compare the genetic identities of the *Symbiodinium* cells in NZ anemones to four species of anemones from Europe (*Cereus pedunculatus*, *Anthopleura ballii* and *Anemonia viridis* from the south-west of England and *Aiptasia mutabilis* from Brittany, (France) to identify differences or similarities between *Symbiodinium* originating from a similar latitudinal distance away from the Equator. This comparison was based on the hypothesis that temperate *Symbiodinium* diversity is low and possibly closely related, due to the difficulties of living in extreme conditions.

The predictions for the New Zealand study were that the *Symbiodinium* diversity would be low, but that there may be genetic variation with latitude and season due to adaptation to the differences in climate with time and distance. The prediction was also that there would be endemism in the *Symbiodinium* type as a result of NZ being isolated from other landmasses.

2.2 Materials and Methods

2.2.1 Study Area and Sampling

The anemones collected for the latitudinal study came from five sites throughout New Zealand during the early southern autumn in March 2011 (Table 2.1). These were, from south to north, Stewart Island, Dunedin, Pauatahanui Inlet near Wellington, Raglan and Parengarenga Harbour (Figure 2.4). All sites were mudflats at the mouth of estuaries and the anemones collected were attached to *Austrovenus stutchburyi* cockles, which were shallow-buried in the sandy sediment.

Figure 2.4: Collection sites around New Zealand for the latitudinal molecular study. (Photograph of Raglan by Emma Gibbin).

The anemones for the temporal and habitat study were collected either from the mudflats at Pauatahanui or from the rocky shore at Point Halswell, Kau Bay, within Wellington Harbour (Figure 2.5). The anemones from Kau Bay were collected from the midlittoral zone, between 1-5 m above the low tide mark. The tidal range in Wellington is small, being 2 m or less, with 2 tides per day (Land Information New Zealand (LINZ)). Four anemones were collected in a 10 m² area before moving along a transect parallel to the shore for 10 m, to reduce the chances of collecting clonal anemones, before another four anemones were collected in the same sized area. These sites were visited once in the middle of each season (mid-April, mid-July and mid-January) for the year beginning in the autumn 2011 and finishing in the summer 2012 (Table 2.1).

Figure 2.5: Collection sites and habitats around Wellington. **A)** map of NZ; **B)** Close-up of the southern end of the North Island; **C)** and **D)** Collection site at Pauatahanui Inlet; **E)** *Anthopleura aureradiata* on the cockle *Austrovenus stutchburyi*, within a seagrass bed; **F)** Kau Bay in Wellington Harbour; **G)** The lighthouse on Point Halswell in Kau Bay; **H)** The rocky shore to the west of the lighthouse where the rocky shore collection was made. *Anthopleura aureoradiata* within **I)** shallow tidal pool or **J)** a rocky crevice. **B)**, **C)** and **H)** are from Google Earth.

Table 2.1 – Collection data for all anemones analysed in this study giving species, site collected, coordinates for the site and the date collected.

Anemone Collected	Collection Site	Coordinates	Date Collected
<u>For Latitudinal Study</u>			
<i>Anthopleura aureoradiata</i>	Stewart Island	46° 54' 346'' S, 168° 00' 589'' E	4 th March 2011
	Dunedin	45° 46' 60'' S, 170° 41' 60'' E	6 th March 2011
	Pauatahanui Inlet, Wellington	41° 6' 4.85'' S, 174° 52' 17'' E	10 th March 2011
	Raglan	37° 47' 52'' S, 174° 42' 36'' E	22 nd March 2011
	Parengarenga Harbour	34° 32' 5'' S, 172° 56' 43'' E	24 th March 2011
<u>For Temporal Study</u>			<u>Autumn</u>
<i>Anthopleura aureoradiata</i>	Pauatahanui Inlet, Wellington	41° 6' 4.85'' S, 174° 52' 17'' E	14 th April 2011
	Kau Bay, Wellington	41° 17' 2.42'' S, 174° 49' 33.89'' E	15 th April 2011
			<u>Winter</u>
	Pauatahanui Inlet, Wellington		13 th July 2011
	Kau Bay, Wellington		14 th July 2011
			<u>Summer</u>
	Pauatahanui Inlet, Wellington		18 th January 2012
	Kau Bay, Wellington		17 th January 2012
<u>European Collection</u>			
<i>Anthopleura ballii</i>	Private aquarium (originated, Scilly Isles, England)	49° 93' 63'' N, 6° 3' 23.0'' W	December 2010
<i>Anemonia viridis</i>	West Hoe, Plymouth, England	50° 21' 49'' N, 4° 8' 42.15'' W	14 th December 2010
	Firestone Bay, Plymouth	50° 21' 33.59'' N, 4° 9.57' 64'' W	2 nd December 2010
<i>Cereus pedunculatus</i>	West Hoe, Plymouth	50° 21' 49'' N, 4° 8' 42.15'' W	17 th December 2010
<i>Aiptasia mutabilis</i>	Roscoff, Brittany, France	48° 43' 38.46'' N, 3° 59' 17.68'' W	5 th May 2011

2.2.1.1 Climatic conditions in New Zealand

The mean seasonal temperatures in NZ range from 19 °C in the summer to 6.5 °C in the winter, though the lowest and the highest temperatures range from -4 °C in the south to 34 °C in the north. The mean daily minimum and maximum temperatures also has a wide range with latitude (Table 2.2). The UV radiation from the sun is intense with more sunlight reaching the surface around New Zealand in the spring and summer than autumn and winter. (More detailed climate data for New Zealand, compiled from daily local measurements from NIWA's National Climate Database (Cliflo Retrieved 6 June 2012), are provided in Appendix 1).

Table 2.2: Mean daily minimum and maximum temperature of the 5 years prior to the latitudinal collection of anemones throughout New Zealand. Three weather stations nearest to collection sites were chosen. Tiwai Point is the furthest south in New Zealand, Paraparaumu Aerodrome is in the middle and Cape Reinga is the furthest north. Data collected from the National Climate Database from NIWA (Cliflo Retrieved 6 June 2012)

Weather station site	Season	Mean daily minimum temperature	Mean daily maximum temperature
Tiwai Point, Southland	Winter	3.31 °C	9.82 °C
	Summer	10.54 °C	17.12 °C
Paraparaumu Aerodrome	Winter	5.45 °C	12.84 °C
	Summer	13.14 °C	20.70 °C
Cape Reinga, Northland	Winter	10.83 °C	15.06 °C
	Summer	16.06 °C	21.83 °C

2.2.1.2 European anemone collection

The anemones *Cereus pedunculatus* and *Anemonia viridis* from England were collected by SCUBA from the south-western coastline around Plymouth. The English anemone, *Anthopleura ballii*, had been held in an aquarium and was provided by Dr Keith Hiscock (Marine Biological Association of the UK). *Aiptasia mutabilis* was collected by SCUBA from the northern shore off Roscoff, Brittany and was provided by Station Biologique de Roscoff. (see Table 2.1 for collection details)

Each *A. aureoradiata* collected in New Zealand was placed immediately into a 15-ml tube containing approximately 14 ml of 99% DNA grade ethanol. This ethanol was changed the following day and the tubes then stored at -20 °C. Anemones from Europe were stored in 99% DNA grade ethanol for shipment to New Zealand. On arrival, the ethanol was changed and the tubes stored at -20 °C.

2.2.2 DNA extraction and *Symbiodinium* spp. identification

Symbiodinium cells were isolated by homogenizing a small piece of tissue from each anemone. The tissue was ground in individual 1.5-ml Eppendorf collection tubes using a tissue grinder attached to a Proxxon Micropower Driver in 200 µl extraction buffer (1 µM filtered seawater (FSW), 5 mM EDTA and 0.025% Triton X-100). The slurry was centrifuged at 3000 x g for 5 minutes and the supernatant discarded. The remaining pellet was re-suspended in 500 µl extraction buffer (FSW and 5 mM EDTA), centrifuged (3000 x g for 5 minutes) and the supernatant discarded again. This final stage was repeated another 3 times. An aliquot (200 µl) of guanidinium buffer (25 ml guanidinium isothiocyanate 4 M, 0.1514 g Tris 50 mM, 0.09306 g EDTA 10mM; heated to 65 °C; 0.5152 g Sarkosyl 70mM, 0.25 µl β-mercaptoethanol) was then pipetted into the tube and the contents vortexed and stored at -4°C for a week for DNA extraction (Stat et al. 2009). The tubes were then centrifuged, “short spin”, to collect all the liquid at the bottom of the tube and heated to 72 °C for 10 minutes, during which time they were vortexed every 2-3 minutes, before being centrifuged again (16000 x g for 5 minutes). Supernatant (100 µl) was transferred to new tubes and 100 µl isopropanol added to each. These tubes were stored overnight at -20 °C. The next day the tubes were centrifuged (16000 x g for 15 minutes), the supernatant removed, and the

tubes and DNA extract then “washed” in 100 µl 70% ethanol and centrifuged once more (16000 x g for 10 minutes). Supernatant was again removed and the tubes and extracted DNA dried under a laminar-flow hood for an hour to remove all traces of ethanol. Tris 0.1 M pH8 (50 µl) was added to each tube. These tubes were placed onto ice and vortexed every 15 minutes for an hour. The DNA samples were stored at -20 °C, ready for genotyping.

Table 2.3: Identity and codes given for anemones that were processed and sequenced. The last column has the number of *Symbiodinium* sequences that were good enough to be used to assemble phylogenetic trees.

Anemone name/place or habitat collected	Coding used to identify samples analysed	Number processed	Number used for tree construction
<u>European Study:</u>			
<i>Anthopleura ballii</i>	Ab	3	2
<i>Anemonia viridis</i>	Av	4	4
<i>Cereus pedunculatus</i>	C	5	5
<i>Aiptasia mutabilis</i>	AM	10	10
<u>Latitudinal Study: <i>Anthopleura aureoradiata</i></u>			
Stewart Island	S	6	6
Dunedin	D	5	5
Pauatahanui, Wellington	W	5	3
Raglan	R	5	2
Parengarenga Harbour	PH	6	4
<u>Temporal Study: <i>Anthopleura aureoradiata</i></u>			
Kau Bay, Autumn	KA	22	13
Pauatahanui, Autumn	PA	5	1
Kau Bay, Winter	KW	11	9
Pauatahauni, Winter	PW	3	0
Kau Bay, Summer	KU	6	4
Pauatahanui, Summer	PU	2	1

Not all anemones collected were used for molecular identification; rather, enough were used to generate as rigorous a dataset as possible within the timeframe available. The numbers processed and those that provided good enough sequences to use for phylogenetic tree constructions are given in Table 2.3.

Symbiodinium types were characterised using the ITS2 region of the nuclear ribosomal DNA with 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' (Pochon et al. 2007). PCR reactions were performed using MyTaqTM Mix (Bioline, Australia) in a total volume of 30 µl (1 µl DNA template, 13 µl dd H₂O, 15 µl MyTaqTM Mix and 0.5 µl of each primer 10 mM.) The amplification profile was adapted from the MyTaqTM Mix protocol and consisted of 3 minutes 95 °C (initial denaturation), 29 cycles of 15 seconds 95 °C (denaturation), 15 seconds 60 °C (annealing) and 15 seconds 72 °C (extension) with a final holding temperature of 4 °C. The PCR was run on a VeritiTM Thermal Cycler (PN4375786) machine.

The ITS2 amplified PCR products were run for 2 hours 20 minutes at 100 volts on a SSCP system with a vertically run polyacrylamide gel (1.5 ml polyacrylamide (40% acrylamide, 0.8% bisacrylamide), 1.2 ml 5x TBE buffer, 7.3 ml ddH₂O, 37.5 µl Temed and 100 µl APS solution (0.01g of crystal in 100 µl ddH₂O) per gel). The samples were first denatured by heating 8 µl of PCR-amplified DNA with 8 µl of formamide loading dye per tube (25 mM EDTA pH 8 with 50 mg/ml of blue dye, diluted with 100% formamide 4:1 ratio (Sambrock et al. 1989)) for 5 minutes at 95 °C, followed by snap-cooling for 10 minutes on ice. This allowed the two separated DNA strands to fold back onto themselves into their secondary structure. Along with the denatured PCR product, a double-stranded DNA sample was also run as a reference standard to help with band interpretation as not all the DNA in the denatured product would have split and folded.

To identify distinct *Symbiodinium* types, prominent bands were excised with a pipette tip under UV light. The tips were placed in 1.5-ml tubes with 20 µl of ddH₂O and swirled overnight on ice using a table-top rotary-shaker to dissolve the DNA. These samples were reamplified using the same amplification profile as before using a total volume of 30 µl (5 µl of dissolved DNA template, 9.5 µl ddH₂O, 15 µl MyTaqTM Mix

and 0.25 µl of each primer 10 mM). The PCR product was re-run on another SSCP gel to check band positions in relation to the position of the excised band from the previous gel.

Results of a SSCP gel rely on the size and shape of the secondary-structure fold of a denatured single-stranded DNA (Sunnucks et al. 2000, Sampayo et al. 2009). If there is only one type of *Symbiodinium* in the sample, then there should be two bands in the gel from the two halves of the DNA strands. Different size and shape folded structures will terminate at different distances along the gel. Differences of bases along a sequence, even just one base, can change the configuration of the fold and therefore affect the migration dynamics of the DNA strand. If there was number of types within a sample, even of the smallest proportion, it could result in the multiple bands. Therefore, four banded lanes would mark a sample that contained two *Symbiodinium* types or type variations and eight banded lanes, four types or type variations. The standard procedure for identifying types using a SSCP gel is that once a two band patterned sample has been sequenced and identified, all future samples that have the same band pattern, and have the same terminal position, can be inferred to be the same type. Early on in this study it was noted that samples sent for sequencing that had the same two-banded SSCP gel pattern were being identified as being similar or identical to two different *Symbiodinium* types. Therefore all samples were sent for sequencing and identity was not inferred by comparing SSCP gel band patterns.

Successful PCR amplifications were prepared for sequencing by cleaning 16-25 µl of the product with 1 µl of ExoSAP-ITTM (Exonuclease I and Shrimp Alkaline Phosphatase in buffer – USB Corporation, Australia). This was incubated for 30 minutes at 37 °C in the PCR machine and then at 80 °C for a further 15 minutes. The samples were sequenced in both directions at Macrogen Inc., Korea.

Chromatograms were checked and aligned and overhangs removed using the software Geneious Pro 5.5.7 (Drummond et al. 2011). Retrieved sequences were identified using the Basic Local Alignment Search Tool (BLAST) for nucleotide sequences (blastn) of the National Center for Biotechnology Information (NCBI) BLAST database. A search was also carried out in the ITS2 sequence database (GeoSymbio) from the Gates Laboratory at the Hawaii Institute of Marine Biology (HIMB) (Franklin et al. 2012).

Additional ITS2 sequences for the analyses of phylogenetic relatedness of the *Symbiodinium* types in this study were retrieved from GeoSymbio and NCBI. These included the free-living dinoflagellate *Gymnodinium simplex* (GenBank accession no: JN558104.1) and *Symbiodinium* clade A members (A1, A1.1, A1.2, A2, A3, A4, A5, A6, A7, A8, A9, A10, A11, A12, A13, A15, A16; GenBank accession no: AF333505, AF333504, AB207210, AF333506, DQ838546, AF333509, AF333508, AY686646, AY239360, AY258468, FJ224014, EU792882, FJ224013, EU792883, AF333504, EU792884 and EU792888).

2.2.3 Data analysis

2.2.3.1 Phylogenetic tree construction

Phylogenetic analyses were performed in Geneious Pro 5.6.2 (Drummond et al. 2011) using the plugins, PhyML v 2.0.12 (Vincent Lefort and Biomatters Ltd) (Guindon and Gascuel 2003) and MrBayes v 2.0.3 (Marc Suchard and Biomatters Ltd) (Huelsenbeck and Ronquist 2001). The Maximum Likelihood (ML) method tree topology was determined using a bootstrap analysis (1000 replicates) to infer tree topology robustness with PhyML. A GTR substitution model (discrete gamma), with four substitution rate categories and program values for all other parameters were chosen. The initial tree was obtained via BioNJ (Gascuel 1997). The GTR model specifications were: Likelihood: Loglk = -1275.61322, proportion of invariable sites: 0.000, relative rate parameters: A ↔ C: 1.47498, A ↔ G: 2.82482, A ↔ T: 1.54658, C ↔ G: 2.16469, C ↔ T: 3.27153, G ↔ T: 1.00000.

A bayesian tree reconstruction with posterior probabilities was inferred using MrBayes v 2.0.3 (Marc Suchard and Biomatters Ltd) (Huelsenbeck and Ronquist 2001) using the same datasets. This tree was rooted using *Gymnodinium simplex* as the outgroup. Four Markov chains were run for 2,000,000 generations with trees sampled every 400 generations, with 100,000 initial trees discarded as “burn-in”.

2.2.3.2 Secondary structure

ITS2 secondary structures were analysed after being folded by default on Geneious. This application did not use the templates set by Hunter et al. (2007). This meant that

neither the four-helix nor the five-helix configurations were made. However, the Geneious fold made the equivalent of Helix I and Helix II so that the sequences of base pairs at the root of the helices could be compared to the secondary structures from the 2007 study.

The 3' end of the base on Helix I of all ITS2 sequences within members of *Symbiodinium* clade A from Hunter et al.'s (2007) study have the base sequence variability of GAGCAG (Types A1, A2 A3 and A4) , GGGCAG (*Symbiodinium* sp. Amed), and GGGTGG ('Cryptic A') (see Appendix A2.4 for diagrammatic representations of secondary structures of clade A *Symbiodinium*).

The 3' end of the base on Helix II of all ITS2 sequences within members of *Symbiodinium* clade A from Hunter et al.'s (2007) study have the base sequence variability of ACTGGCATGCTA (Types A1, A2, A3 and A4), ACTGGCAGCGT (*Symbiodinium* sp. Amed), ATTGGCATGCTC ('Cryptic A').

The next six bases then read AATATC (Type A1), AGATTT (*Symbiodinium* sp. Amed), AGTACC (A3), AGTATC (Type A4), AGTATT ('Cryptic A') and AATATC (Type A2).

2.3 Results

2.3.1 PCR and SSCP Gels

Positive PCR bands were approximately in-line with the position of 350 base pairs as shown by the Hyperladder II. This is what was expected for *Symbiodinium* ITS2 amplifications. When the amplified products were run on SSCP gels, a range of band patterns were found (Figure 2.6). Winter and summer samples from the temporal/habitat study returned more than two bands. When more than two bands were present, and were excised and reamplified successfully, the resulting SSCP band patterns were the same (Figure 2.6 F)

Figure 2.6: Photographs of stained SSCP gels showing the patterns returned from the amplified PCR products. Gels A) and B) have 2 bands; D) has 4 bands and C) and E) have 8 bands. Gel F) shows the band pattern on reamplifying the excised bands from earlier gels.

Migration of the single strand DNA relies on the temperature of the buffer surrounding the gel, and this relies on the temperature of the room the SSCP equipment is situated (Nataraj et al. 1999, Sunnucks et al. 2000). Each SSCP gel-run in this study usually had the same band patterns in all lanes run i.e. all the lanes had 2, 4 or 8 bands. If band patterns were due to samples' sequence variation, due to some anemones hosting more than one type, there should have been a variation of band patterns in the lanes for each gel-run. Therefore, thermal instability in the lab could have also led to some inconsistencies in the SSCP gel results.

2.3.2 *Symbiodinium* identity for all anemones in this study

2.3.2.1 Sequencing of all samples

A total of 98 samples were sequenced (Table 2.3). The sequence BLAST searches identified all the study samples as being nearest to, or identical to, the ITS2 region in ‘*Symbiodinium* sp. Mediterranean clade A’ (Meron et al. 2012) (hereafter termed ‘Med clade A’) or ‘*Symbiodinium* sp. Amed’ (Hunter et al. 2007) (hereafter termed ‘Amed’) (Table 2.4) (see Appendix A2.2 and A2.3 for sequences). In other words, *A. aureoradiata* hosts two *Symbiodinium* types that are identical to or very closely related to those in anemones collected in England and in Brittany (France), and to Med clade A or Amed. Sixty-nine sequences were more than 95 % pairwise identical to either of these types (Table 2.5), and were “tidy” enough to be used to construct the phylogenetic trees. The ‘messiness’ of the other sequences may have been the result of the host anemone containing both *Symbiodinium* types simultaneously.

Table 2.4: Details of *Symbiodinium* types suggested by NCBI Database and GeoSymbio BLAST searches as being most similar or identical to all samples identified in this study

<i>Symbiodinium</i> species	Genbank accession no.	Journal citation	Location of species source	Host species
Mediterranean clade A	JN242191.1	(Meron et al. 2012)	Ischia Island, Italy	<i>Balanophyllia europaea</i>
Amed	DQ865210.1	(Hunter et al. 2007)	Eastern Mediterranean	<i>Anemonia</i> sp.

All specimens of *C. pedunculatus* and 75% of *A. viridis* from south-west England hosted *Symbiodinium* cells identical to Amed whereas all *A. ballii* and 90% of *A. mutabilis* from Brittany contained symbionts that were similar or identical to Med clade A (Table 2.5) 96.55% of the *A. aureoradiata* in the latitudinal study contained symbionts that were most similar to Med clade A; just one of these anemones from the far north of NZ contained symbionts that were more similar to Amed (Table 2.5). In the temporal study (Table 2.5), all winter-collected anemones from all the habitats hosted *Symbiodinium* cells that most closely matched to Med clade A. However, in summer

and autumn there were some anemones that hosted symbionts that were more closely matched to Amed, (12.5% and 22.22% of anemones respectively). The preliminary comparison of the two habitat types suggests that the presence of Amed with the seasonal change occurred at both sites (Table 2.6).

Table 2.5: Numbers of samples sequenced from the study identified by Geneious BLAST search as being most similar to, or identical to either Med clade A or Amed. The number of samples with more than 95% pairwise identical bases to these two types are also given. Only these sequences were used in the final molecular comparison of *Symbiodinium* types to make the phylogenetic trees. The sequences less than 95% identical may have been ‘messier’ due to the sample anemone hosting both *Symbiodinium* types simultaneously.

Total no. sequenced	Number identified as <i>Symbiodinium</i> sp. Mediterranean clade A			Number identified as <i>Symbiodinium</i> sp. Amed		
Total number sequenced:	Total identified as:	> 95% pairwise identical to:	100% identical to:	Total identified as:	> 95% pairwise identical to:	100% identical to:
European study						
<i>Anthopleura ballii</i>						
3	3	2	0	0	0	0
<i>Cereus pedunculatus</i>						
5	0	0	0	5	5	5
<i>Anemonia viridis</i>						
4	0	0	0	4	4	3
<i>Aiptasia mutabilis</i>						
10	9	9	3	1	1	1
Latitudinal study						
27	26	20	4	1	0	0
Habitat study in Autumn						
27	21	11	0	6	3	0
Habitat study in Winter						
14	14	9	0	0	0	0
Habitat study in Summer						
8	7	5	1	1	0	0

Table 2.6: Number of samples sequenced from each habitat in each season and the number of sequences identified as being clearly more similar, or identical, to either *Symbiodinium* sp. Med clade A or *Symbiodinium* sp. Amed.

Season, <i>Symbiodinium</i> type and no. collected	Number of <i>Symbiodinium</i> type collected from each habitat	
	Rocky shore	Mudflat
Autumn – 27 total	22	5
Med clade A	17	4
Amed	5	1
Winter – 14 total	11	3
Med clade A	11	3
Amed	0	0
Summer – 8 total	6	2
Med clade A	5	2
Amed	1	0

2.3.2.2 Secondary structure

The Geneious default secondary structure fold had the equivalent of Helix I and Helix II from the template configuration used by Hunter et al. (2007) and confirmed that the study samples were either identical to or very closely related to Med clade A or Amed (Figure 2.7)

Figure 2.7: ITS2 sequence of D5 (sample from Dunedin) folded with the Geneious default folding function. Helices I and II are in the top and top left of the configuration.

The 3' end of the base on Helix I of all the samples sequenced from the European and the New Zealand anemones in this study had the GGGCAG sequence which is the same as in the original fold of *Symbiodinium* sp. AMed by Hunter et al. (2007) (See Figure 2.8 A).

Figure 2.8: Secondary structures of ITS2 sequences for **A)** rRNA of *Symbiodinium* sp. Amed, which is why bases are labelled as “U” for uracil and not “T” for thymine; **B)** rDNA of C5 (*C. pedunculatus*); and **C)** rDNA of D5 (*A. aureoradiata* from Dunedin). Helix I in all three images has the same base sequences (as seen in the red ovals on the left of the three folds). Helix II, in Amed and C5, has the sequence ACTGGCATGC**G**T whereas Helix II in D5, which was more closely related to Med clade A, has the sequence ACTGGCATGC**T**T (as seen in the red ovals on the right of all three folds). All three then continue from the base of Helix II with the sequence AGATTT (as seen in the blue oval).

The 3' end of the base on Helix II of all the samples in this study that were identified as being most similar to *Symbiodinium* sp. AMed had the sequence ACTGGCATGC**G**T (Figure 2.8 A), the same as the original fold of *Symbiodinium* sp. AMed by Hunter et al. (2007). This is illustrated by the secondary structure fold of C5 (Figure 2.8 B). However, all the samples sequenced in this study that were identified as being most similar to Med clade A had the sequence ACTGGCATGC**T**T. This is illustrated by the secondary structure fold of D5 (Figure 2.8C). Then, in the original *Symbiodinium* sp. AMed fold (Hunter et al. 2007), and in the folds from all the samples in this study, the bases sequence read towards the 3' end with the sequence AGATTT (Figure 2.8), which is different to all other clade A secondary structure folds (see Appendix A2.3).

2.3.2.3 Phylogenetic analyses

The genetic relatedness of the *Symbiodinium* types identified in this study to other members of clade A, *Gymnodinium simplex*, *Symbiodinium* sp. AMed and *Symbiodinium* Mediterranean clade A was carried out using the Geneious programme. Before a tree was constructed, all the sequences being analysed were aligned and ends trimmed so that the sequences were mostly the length of the ITS2 sequence of Med clade A (260 bases). The resulting phylogenetic trees constructed under Maximum Likelihood (ML) (Figure 2.9) and Bayesian criteria (Figure 2.10) produced similar topologies.

Figure 2.9: Maximum-likelihood tree of sampled *Symbiodinium* types and reference types inferred from ITS2 amplification, using *Gymnodinium simplex* as the outgroup. Taxa codes for the reference types were obtained from GenBank. Other labels indicate sample codes (see Table 2.3). Numbers correspond to bootstrap values (> 40.0) for 1000 replicates obtained with ML analysis (except at the node showing division of the Amed group from the Med clade A group (bootstrap value 10.3)). (— samples identified with Med Clade A; — samples identified with Amed and; — highlights Med Clade A and Amed sequences from Genbank).

Figure 2.10: Bayesian tree of sampled *Symbiodinium* types and reference types inferred from ITS2 amplification.. This tree was rooted using *Gymnodinium simplex* as the outgroup. Taxa codes for the reference types were obtained from GenBank. Other labels indicate sample codes (see Table 2.3). Numbers correspond to posterior probabilities (> 0.50) inferred using MrBayes. (— samples identified with Med Clade A; — samples identified with Amed and; highlights Med Clade A and Amed sequences from Genbank).

Both trees placed *Symbiodinium* type A2 as a sister group to the Med clade A and Amed groups (bootstrap value: 59.0, posterior probability 0.68). The ML tree placed the *Symbiodinium* Amed group as a distinct group within the *Symbiodinium* Med clade A group (bootstrap value: 96.6) (Figure 2.9). The bootstrap value in this case means that in 96.6% of the bootstrap replications (1000 replicates in this analysis), the Amed group was placed as a group within the Med clade A group. The Bayesian tree places the Amed group as a distinct group within the Med clade A group too, with a posterior probability of 0.91 (Figure 2.10). The posterior probability value in this case means that the probability of a divergence between the Amed group and the Med clade A group is 91% certain, assuming that the model is correct, in all trees sampled. The two trees have very similar confidence that the two groups have diverged from each other, and that both groups have diverged from the other members of clade A (bootstrap value 58.1 and posterior probability 0.68)

2.4 Discussion

All the anemones in this study, both from Europe and New Zealand, hosted *Symbiodinium* with ITS2 sequences very similar to, or identical to, either *Symbiodinium* sp. Amed or *Symbiodinium* sp. Mediterranean clade A. This also means that in this study all the *Symbiodinium* types within anemones from New Zealand and Europe were similar or identical to each other, suggesting that the *Symbiodinium* in *A. aureoradiata* are likely not endemic. Nearly all the *A. aureoradiata* from the latitudinal collection and all the anemones from the winter collection hosted *Symbiodinium* similar to, or identical, to Med clade A. However, an eighth of the summer collection, and nearly a quarter of the autumn collection, hosted *Symbiodinium* more similar to Amed. The preliminary comparison of the two habitats where seasonal collections were made, suggests that the presence of Amed, with seasonal change, occurs at both sites.

2.4.1 Reliability of SSCP gels as a method of molecular identification

Multiple SSCP bands in a sample could have been due to the conditions the gels were run in as mentioned in the results section, but also have been due to intragenomic variation (van Oppen et al. 2005). The number of base differences does not always correlate to distances between bands on a SSCP gel. These authors found that a small

difference of bases in a sequence could finish up with a greater distance between bands on a gel compared to samples with a greater variation in bases, such as there would be between two clades. The eukaryotic nuclear rDNA region is repeated many times in a genome (Coleman 2003), and the ITS regions can be more variable than the 18S, 28S and 5.8S regions either side of them (Thornhill et al. 2007). The rapid rate of evolution in the ITS regions make them suitable for distinguishing between closely related taxa, but cannot always be relied upon (Coleman 2003) as the regions evolve relatively rapidly compared to the rest of the genome and this, combined with high levels of rDNA repetition, can lead to intragenomic and intraspecific variation (Stat et al. 2009). Remarkably, this variation can also be found within one *Symbiodinium* cell and can cause such ‘messy’ sequences after PCR amplification that they are unalignable, and can be so different that they could be identified as originating from different clades (van Oppen et al. 2005). Thornhill et al. (2007) found differing levels of intragenomic sequence variation when they cloned PCR products of amplified ITS2 regions from *Symbiodinium* types in clades A and E, the most ancestral of clades,. Some of these variants had base substitutions that resulted in different formations of the secondary structure, which would then travel and terminate at a different position on the SSCP gel. Thornhill et al. postulated that these were pseudogenes as secondary structure folding is an important part of the rDNA molecule and aides in the transcription into messenger RNA.

Multiple bands caused by intraspecific variation may come about by the sexual recombination of individual *Symbiodinium* cells within the same type. This theory was formulated by the discovery of variability in the genetic data of cultured isolates (Baillie et al. 2000, LaJeunesse 2001, Santos and Coffroth 2003). It was assumed that there would not be any variability in these cultures as they were made up of asexually reproduced cloned cells. However, Baillie et al. (2000) found more diversity within cultures of the northern hemisphere *Symbiodinium* A1 than has been seen in the sexually reproducing *Gymnodinium catenatum*. Sexual reproduction is probably more likely to occur in the free-living phase of *Symbiodinium* in the wild as meiosis has never been observed *in hospite* (LaJeunesse 2001, van Oppen and Gates 2006). Therefore, if sexual reproduction does occur in the *Symbiodinium* from *A. aureoradiata*, gametes should exist in the water column, as should free-living cells. Evidence for this has not

been verified. *A. aureoradiata* is known to pass on symbionts to the next generation vertically from mother to larvae, but the larvae or adult could perhaps take up fresh symbionts from the environment. Sexual recombination would give rise to variation within a population, either within an individual host or within populations of cnidarian hosts in the environment (LaJeunesse 2001). This variation could most likely generate populations with the capacity to adapt to fluctuating temperature and irradiance, as is experienced in NZ. Evidence of this has been found by Howells et al. (2012), who discovered differences in sensitivity and tolerance to heat between populations belonging to the same *Symbiodinium* type, within the same host, the coral *Acropora tenuis*, when measured along a latitudinal gradient on the Great Barrier Reef.

More samples from NZ would need to be analysed to gauge whether there is a pattern of base substitution in the sequences from *A. aureoradiata* with different latitude, season and habitat, or whether the differences are just random substitutions. The possible intraspecific variation of the *Symbiodinium* from the sampled anemones, as seen by the differences in the sequences, may be driven by adaptation in populations to local conditions, genetic isolation from other *Symbiodinium* types and short asexual generation times of the *Symbiodinium* within the host cells (Howells et al. 2012). This ecological specialization to variable environmental conditions would not just include the external habitat with its various temperature and irradiance levels, but also could depend on the location of the symbiont within the host (Finney et al. 2010). The host tissue could provide different levels of light attenuation to the symbiont but would probably not provide differences in temperature within *A. aureoradiata*, as the anemone is so small.

2.4.2 Change of symbiont with latitude or season

Analysis of the sequences was more difficult in the NZ anemones than the ones from Europe due to the numerous band patterns on the SSCP gels and the chromatograms on Geneious being “messy”, i.e. the peaks often had more than one base signature per position. The difficulty in reading the chromatogram could originate from an anemone hosting both Amed and Med clade A. Though both types are more similar each other than to other types of clade A, there are still 14 base pairs along the ITS2 sequence that do not match up. There were too few samples in this study to make a statistical

statement or predict a trend as to the distribution and change in ratio of *Symbiodinium* type within the anemones. However, there could be a possibility that there is a change from Med clade A to Amed with the warmer, sunnier seasons and to the warmer areas of NZ. In the autumn, *Symbiodinium* similar to Amed was present in approximately 25% of the sampled anemones, both from the rocky shore and the mudflat. In summer *Symbiodinium* ITS2 regions were only sequenced in eight anemones in total but one of these was identified as being similar to Amed. Could this be a case of symbiont “shuffling” in the anemone or “switching” from the environment for the holobiont to acclimate to warmer or colder conditions?

Shuffling is the most likely mechanism for a change in symbiont type. Only two studies have found that cnidarian hosts (anemone and an octocoral) recovered from bleaching by switching to new types from the environment (Kinzie et al. 2001, Lewis and Coffroth 2004) and in both papers the authors acknowledge that the presence and recovery of residual symbionts was a possibility. Switching is also unlikely in temperate waters due to the scarcity of *Symbiodinium* cells in the water column, and is particularly unlikely in *A. aureoradiata* as it maternally transmits *Symbiodinium* to the next generation (Muller-Parker and Davy 2001). A host harbouring multiple *Symbiodinium* types is not as rare as once thought (Silverstein et al. 2012). *A. aureoradiata* could harbour a small population of Amed during the winter, which becomes the more dominant type in warmer conditions that are perhaps more favourable to its well-being.

2.4.3 Similarities in *Symbiodinium* types between Europe and New Zealand

In their study of *Anemonia viridis*, Bythell et al. (1997) investigated the genetic variation in symbionts between intertidal and subtidal anemones in southwest England. Using PCR-RFLP of the small subunit rRNA (SSU rRNA) gene they found that the *Symbiodinium* cells at both depths/shore heights were not genetically different enough to be classified as different types and identified them as being a type within clade A. Savage et al. (2002a) likewise investigated the diversity of high latitude *Symbiodinium* types in Bermuda, the Mediterranean and NE Atlantic. They used the same PCR-RFLP techniques as Bythell et al. (1997) on the SSU rDNA, LSU rDNA and ITS1 sequences, and found that every sample of sea anemone they analysed from the NE Atlantic and the Mediterranean contained an undescribed group within clade A (Savage et al. 2002a).

They named the group “Temperate A” and postulated that it could be endemic to the region. Visram et al. (2006) subsequently characterised the molecular diversity of the *Symbiodinium* in ten species of scleractinian coral, sea anemone and scyphomedusan from the Mediterranean. They also used PCR-RFLP methods on SSU and LSU rRNA genes and found that only one of the species analysed, the sea anemone *Bunodeopsis strumosa*, harboured clade B whereas all the other species harboured Temperate A. They agreed with the previous study that the phylotype is endemic to the area. Forcioli et al. (2011) studied whether there was molecular diversity of Temperate A in a gorgonian, *Eunicella singularis*, at different depths and found that there was not.

It is probable that the group Temperate A includes ITS2 types Med clade A and Amed. This assumption is made because molecular identification of *Symbiodinium* cells from the same host species using either PCR-RFLP or ITS2 amplification from the studies just mentioned, as well as from Hunter et al. (2007), Meron et al. (2012) and now my study identified them all as either being Temperate A, when using PCR-RFLP, or ITS2 types Med clade A and Amed (Table 2.7). This is because analysing the ITS2 region provides better phylogenetic resolution at or below a species level, whereas the SSU and LSU rDNA sequences do not give such subtle phylogenetic differentiation (LaJeunesse 2001) (Figure 2.11). If the *Symbiodinium* types in *A. aureoradiata* in this study are the same as the ones in NE Atlantic and the Mediterranean, it would contradict Savage et al.’s (2002a) and Visram et al.’s (2006) hypothesis that Temperate A is endemic to the NE Atlantic and the Mediterranean but, rather, it has a distribution within both the southern and northern hemispheres. To confirm this, another molecular assessment of the same samples collected in my study could be carried out using the PCR-RFLP method on the SSU rDNA, LSU rDNA and ITS1 sequences.

Table 2.7: Comparison of *Symbiodinium* identification from different molecular sequence analyses in host anemones that come from the NE Atlantic and the Mediterranean. Data are from both the current and published studies.

			Molecular identification of <i>Symbiodinium</i> cells		
Authors	Host species	Molecule sequence analysed	Temperate A	Med clade A	Amed
Bythell et al. (1997)	<i>Anemonia viridis</i>	SSU rRNA	✓		
Savage et al. (2002)		SSU, LSU and ITS1 rDNA	✓		
Visram et al. (2006)		LSU rRNA	✓		
This study		ITS2			✓
Savage et al. (2002)	<i>Cereus pedunculatus</i>	SSU, LSU and ITS1 rDNA	✓		
Visram et al. (2006)		LSU rRNA	✓		
This study		ITS2			✓
Visram et al. (2006)	<i>Balanophyllia europaea</i>	LSU rRNA	✓		
Meron et al. (2012)		ITS2		✓

Figure 2.11: Diagrammatic representation of the different levels of phylogenetic resolution identified by the molecules analysed in *Symbiodinium* cells.

If Temperate A does indeed include both Med clade A and Amed, it demonstrates that these two similar types have a wide range of tolerance of similar climatic and light conditions found in northern and southern temperate regions. In the northern studies there was no significant genetic variation in the symbionts of *A. viridis* between 1-10 m depth (Bythell et al. 1997) or in the Mediterranean sea whip between 10-50 m (Focioli et al. 2011) or over a latitudinal range of approximately 15 °. The *Symbiodinium* cells in the anemones in the current study were collected over a 12 ° range and from quite varied habitats. Temperate *Symbiodinium* have been found in past studies to be less sensitive to variations in light and temperature than tropical types (Bythell et al. 1997). This may be because they are able to physiologically tolerate large variations in environmental parameters or possibly because the host is able to sustain them more in non-favourable conditions than can tropical hosts (Muller-Parker and Davy 2001).

2.4.4 *Symbiodinium* diversity within temperate anemones

There appears to be a low level of *Symbiodinium* diversity and high host specificity within temperate anemones. Those that are present appear to withstand the wide range of environmental conditions faced at different latitudes (Sanders and Palumbi 2011) and in different habitats (Bythell et al. 1997, Focioli et al. 2011). Being able to maintain a stable symbiotic partnership in highly-variable conditions may have been selected for, thereby providing evolutionary success to just a few *Symbiodinium* types (Rodriguez-Lanetty et al. 2003) in high latitude anemones. This successful partnership is then maintained by the maternal transmission of symbionts to the next generation by the

anemone (Muller-Parker and Davy 2001). This appears to be unlike the relationship between high-latitude corals and their symbionts. For example, a high diversity of *Symbiodinium* types and reduced host/symbiont specificity was found around the sub-tropical Lord Howe Island. On these reefs, changing symbiont types is thought to occur as an acclimatory mechanism to cope with changeable and stressful conditions (Wicks 2009).

Other temperate *Symbiodinium* have also been found to have a large biogeographical range. Two sea-anemone species, *Anthopleura japonica* and *Anthopleura kurogane* from Korea, harboured *Symbiodinium* in clade A for a latitudinal range of 4 ° along the east coast of Korea to Cheju Island, and did so regardless of season (Rodriguez-Lanetty et al. 2000). This was also true for *Symbiodinium muscatinei* in *A. elegantissima* (Sanders and Palumbi 2011). Several authors postulate that some *Symbiodinium* types have a global distribution; for example with the same type being found in the Caribbean and the Red Sea (LaJeunesse 2001). Some hypothesise that global distribution may be related to temperature climes present at certain latitudes (LaJeunesse and Trench 2000, Gou et al. 2003). For example, Gou et al. (2003) identified a free-living dinoflagellate from China as a *Symbiodinium* sp. that was so similar in molecular identity to *S. californium* from California (USA) and a free-living strain (*Gymnodinium varians*) from Wellington (NZ) that they could possibly be all one species. If this is the case, and if the *Symbiodinium* in *A. aureoradiata* are the same as from the NE Atlantic and Mediterranean, the intriguing questions would be how these dinoflagellates are spread from the northern hemisphere to the southern (or *vice versa*), crossing tropical waters which would probably be too warm for them to tolerate. Do they have host species that act as vectors across the Equator? Or are the clades so ancient that they spread across their global distribution millions of years ago before mass divergence of the genus *Symbiodinium* into all the other clades? (This is discussed further in Chapter 4). To answer some of these questions more analysis needs to be carried out on much larger sample sizes, both in NZ and in Europe, between the different habitats, during the different seasons and across a range of latitudes. To complement the molecular study, a comparison of symbiont morphology and physiology could be made. And to differentiate if there is ‘shuffling’ of Amed or Med clade A within individual anemones, more sensitive molecular methods, such as cloning single *Symbiodinium* cells from a

host sample, sequence analysis of microsatellite flanking regions or running real-time quantitative PCR, need to be undertaken.

Chapter 3

Photophysiology of isolated *Symbiodinium* cells from the anemone, *Anthopleura aureoradiata*

3.1 Introduction

The success of the cnidarian-*Symbiodinium* symbiosis relies on the flux of nutrients between the host and symbiont. Organic matter produced by photosynthesis within the *Symbiodinium* cell supports the host's metabolism, growth and reproduction, and, in return, inorganic waste excreted by the host sustains the symbiont and the photosynthetic process (Muscatine and Kaplan 1994, Dubinsky and Berman-Frank 2001, Rodriguez-Lanetty et al. 2006). This partnership is especially successful in the tropics and has led to the dominance of reef-building corals, but it is also found across temperate and subtropical regions (Muller-Parker and Davy 2001, Savage et al. 2002a).

The genus *Symbiodinium* is highly diverse, consisting of nine clades (A-I) (Pochon and Gates 2010) with hundreds of ITS types identified (LaJeunesse 2001, Silverstein et al. 2012). The different ITS types are not equally susceptible to environmental stress (Iglesias-Prieto and Trench 1994, Tchernov et al. 2004, Hennige et al. 2008). Early hypotheses were that each clade may have a particular ecological characteristic, such as being tolerant or sensitive to certain thermal or photic conditions (Toller et al. 2001, LaJeunesse 2002, Savage et al. 2002c, Rowan 2004, Coffroth and Santos 2005). Instead, however, there seems to be vast photophysiological diversity within clades (Tchernov et al. 2004, Robinson and Warner 2006, Hennige et al. 2008, Suggett 2008, Fitt et al. 2009, Diaz-Almeyda et al. 2011, Buxton et al. 2012) and even within types (Howells et al. 2012).

3.1.2 Light and the photosynthetic apparatus

Light provides the essential source of energy required for photosynthesis. However, the complex photosynthetic apparatus that converts light energy into chemical energy is vulnerable to damage when the quantity of light photons supplied is more than can be utilized for CO₂ assimilation (Kraus and Weis 1991, Muller et al. 2001, Hill et al. 2005, Szabo et al. 2005, Warner and Berry-Lowe 2006). Photoinhibition and inactivation of photosystem II (PSII) comes about by several processes that may include degradation of the D1 protein at a rate that exceeds its rate of repair (Smith et al. 2005, Takahashi et al. 2009, Hill et al. 2011), damage to the repair system itself (Takahashi et al. 2004), inhibition of the synthesis of new intrinsic light-harvesting antennae (chlorophyll *a*-chlorophyll *c*₂-peridinin protein complexes (acpPC) and carotenoids) (Burton 1990, Takahashi et al. 2004, Venn et al. 2006), damage to the thylakoid membranes causing uncoupling of electron transport due to membrane-lipid composition changes (Tchernov et al. 2004), and impairment to the enzymes of the Calvin-Benson Cycle (Jones et al. 1998, Hoegh-Guldberg and Jones 1999, Jones 2004). The D1 protein is most susceptible to damage as it is the site of the electron transfer chain in PSII (Kern and Guskov 2011). Regardless of the processes involved, the excess energy in the photosystem leads to the production of reactive oxygen species (ROS) which may lead to further disruption and impairment of photophysiological processes if not detoxified (Venn et al. 2008a, Wicks et al. 2010b). High light alone can cause up to 40% reduction in the photosynthetic process (photoinhibition) in shallow reef corals (Gorbunov et al. 2001) but often light reacts synergistically with temperature, either high or low, to exacerbate photosystem damage (Jones et al. 1998, Saxby et al. 2003, Smith et al. 2005).

3.1.3 Photoprotection

All photosynthetic organisms need to balance their rate of CO₂ assimilation in changing light conditions with the rate of damage and repair to the photosystems (Hill et al. 2011). Prevention from long term damage (chronic photoinhibition) to the photosystems can be accomplished through photoacclimation, a process of phenotypic modification of the light-harvesting apparatus in response to light (Hennige et al. 2008). Modifications can be through changes to the components of the light-harvesting antennae and to the size and number of the reaction-centre pools (Iglesias-Prieto and Trench 1994, Reynolds

et al. 2008, Hennige et al. 2009). Recovery from chronic photoinhibition is slow and may take many days or longer (Gorbunov et al. 2001). Conversely, relatively rapid recovery from damage to, and then renewal of the D1 protein is termed “dynamic photoinhibition” and can take just minutes to a few hours (Gorbunov et al. 2001). Furthermore, short-term decline in photosynthetic efficiency during inclement conditions may occur not from damage, but by the photosystem “down-regulating” or protecting itself, by diverting photon energy away from the photosynthetic apparatus (Fitt et al. 2001b). Instead of the energy being used for photosynthetic reactions (photochemical quenching, qP), it is dissipated away from the photosystems as heat by non-photochemical quenching (NPQ) (Horton and Hague 1988). There are three major processes of NPQ: energy-dependent quenching (qE), quenching from state transitions by phosphorylation of light harvesting complexes (LHC) in PSII (qT), and photoinhibitory quenching (qI).

Quenching by qE is a feedback control mechanism generated when more light photons are absorbed than can be used by photosynthesis (Hill et al. 2005). Excessive photon absorption leads to increased protons in the thylakoid lumen which in turn build up the transthylakoid pH gradient (ΔpH). This acidification of the thylakoid lumen activates the conversion of diatoxanthin (Dtx) from the xanthophyll pigment diadinoxanthin (Ddx) (Goss et al. 2006, Schubert et al. 2006), which dissipates excess energy as heat (Brown et al. 1999, Hill et al. 2011). The ΔpH can relax within seconds to minutes (Horton and Hague 1988) and can be so effective that it thermally dissipates energy 4-5 times faster than can be used for photosynthesis (Gorbunov et al. 2001).

State transition (qT) is where irradiance induces phosphorylation of the LHC in PSII, so that there is a rearrangement of the acpPC, as some components migrate to PSI. This redirects some of the excitation energy away from PSII, thereby protecting it (Gorbunov et al. 2001, Hill et al. 2005, Smith et al. 2005). This component of NPQ is able to relax over minutes once the irradiance has been removed (Horton and Hague 1988, Hill et al. 2005).

The third component of NPQ, photoinhibitory quenching (qI), relaxes very slowly once irradiance is removed, taking tens of minutes to hours, and remaining after ΔpH has dissipated and qT has finished (Hill et al. 2005). This quenching leads to reversible

photo-damage of the photosynthetic apparatus (Kraus and Weis 1991, Muller et al. 2001, Hill et al. 2005, Warner and Berry-Lowe 2006). Decreased photosynthetic activity at this point is mainly due to long-term down-regulation of PSII by damage to the D1 protein (Krause 1988, Muller et al. 2001).

Once there is damage to the photosystems from deleterious conditions, dysfunction to the symbiotic relationship sets in, which can lead to expulsion of the *Symbiodinium* cells from the host (Fitt et al. 2001a, Smith et al. 2005, Hill et al. 2009). This ‘bleaching’ phenomenon has become a major source of coral degradation and mortality in tropical seas, where changes of as little as 1-2 °C above or below average temperatures for an extended period, combined with high irradiance, can trigger the chain of cellular events that leads to symbiosis dysfunction (Hoegh-Guldberg 1999, Saxby et al. 2003).

3.1.4 Temperate resilience

In temperate regions where temperature, light intensity and duration of daylight are extremely variable in comparison to the tropics, the cnidarian-*Symbiodinium* relationship appears to be more resilient to environmental fluctuations. Some temperate anemones that host the same *Symbiodinium* type, function effectively across a wide latitudinal range and throughout the seasons (Bythell et al. 1997, Rodriguez-Lanetty et al. 2000, Sanders and Palumbi 2011). For example, the New Zealand anemone *Anthopleura aureoradiata* has not been seen to bleach in the wild (S. Davy. personal communication). This anemone is found throughout NZ, ranging from 34°S to 47°S, on rocky shores and mudflats, where it is usually found attached to cockles. The mean seasonal air temperatures in NZ range from 19 °C in the summer to 6.5 °C in the winter, though the lowest and the highest temperatures range from -4 °C in the south to 34 °C in the north (see Appendix 1). Solar irradiance and UV radiation levels can also be extremely high, especially for intertidal organisms. Of particular note, *A. aureoradiata* harbours just two *Symbiodinium* types across its broad latitudinal range, with just one type being apparent in nearly 97% of anemones (Chapter 2). This interesting observation raises the question of how these symbionts withstand such a broad environmental range without compromising their photosynthetic function and hence the success of the symbiosis. Furthermore, this apparent resistance to bleaching provides a

valuable parallel to more susceptible tropical corals and may provide important insight into which processes are significant in conferring environmental tolerance. This chapter addresses this question.

3.1.5 Aim of the study

This study aimed to establish whether the dinoflagellate symbiont, under high light at both low and high temperatures, has a strong capacity for photoprotection, and to test the hypothesis that the photosystem can down-regulate quickly and effectively to decrease the possibility of photodamage, resulting in a decrease in photosynthetic yield. This was tested by measuring chlorophyll *a* fluorescence with a Maxi Imaging-Pulse Amplitude Modulated (I-PAM) (Walz, Effeltrich, Germany) fluorometer; this equipment allowed for non-destructive and rapid measurement of the same samples over time (Hill et al. 2005, Ralph et al. 2010). The photosynthetic rate of PSII, energy quenching by NPQ, and photosystem recovery were measured.

3.2 Materials and Methods

3.2.1 Sampling

Anemones (*Anthopleura aureoradiata*, n=120) were collected on five occasions through the Austral summer of 2012 (end of January, and through February) from Pauatahanui Inlet, Wellington (41°06' 005''S, 174° 52' 017''E). The temperature and solar radiation profiles for Pauatahanui were collated for the five years before collection to determine the temperature range in the anemone's habitat and thereby determine the conditions to use in the experiment (Figure 3.1).

The anemones were scraped off their host cockles and kept in plastic containers (40 cm x 15 cm x 15 cm) in seawater. In the laboratory, the anemones were maintained in 1- μ m filtered seawater (FSW) in a temperature controlled room at $16^{\circ} \pm 1^{\circ}\text{C}$ on a 12 hour light/ 12 hour dark cycle (irradiance of $100 \pm 10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 2-3 weeks to acclimatize to the control experimental conditions. The anemones were fed five times per week with *Artemia* sp. nauplii and the water changed soon after feeding.

Figure 3.1: Monthly temperature and solar irradiance (mean $\pm$ SE) near Pauatahanui Inlet. Data were collected from the National Climate Database run by the National Institute of Water and Atmospheric Research Ltd (NIWA). Data came from a weather station in Paraparaumu (25 km from Pauatahanui). The daily highest and lowest temperatures, and the global diffuse and direct solar radiation data for one year prior and five years prior to sampling were used.

3.2.2 Experimental set-up

Three tanks were maintained at either 30 ± 0.1 °C (warm), 16 ± 1 °C (control) or 8 ± 0.2 °C (cold). Water was heated and maintained in the warm tank by a HAAKE C10 water heater, and the control and cold tanks were cooled and maintained by a HAAKE EK20 immersion cooler. There were two light levels per tank: high light (400 ± 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and low light (100 ± 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). These irradiances were

those incident on the surface of the experimental tubes, as measured using a LI-COR LI-250A light meter. The experiment therefore had six treatments with two factors, light (high and low) and temperature (8 (cold), 16 (moderate) and 30 °C (warm)). The treatments will be referred to in figures as: Low light, 8 °C (L8); low light, 16 °C (L16); low light, 30 °C (L30); high light, 8 °C (H8); high light, 16 °C (H16); and high light, 30 °C (H30).

3.2.3 Experimental procedure

The original intention of the study was to measure the photophysiology of the symbionts within the host as a true measure of how the holobiont responds to stress. This proved too difficult due to the frequent and unpredictable movement of the anemones (see Appendix 3). Therefore, to study the photophysiology of the symbionts, the *Symbiodinium* cells were isolated from the host.

A total of 105 anemones were blended simultaneously with FSW in a Breville BFP50 Whizz™ Chopper and Shake blender for 30 seconds. This tissue slurry was then homogenised further with a hand-held glass tissue grinder. The homogenate was made up to 200 ml with FSW and centrifuged (500 x g, 5 minutes), before the supernatant was decanted, and the algal pellet re-suspended in FSW (Figure 3.2); this procedure was repeated a further five times to wash the algal cells of host cell debris.

Figure 3.2: Algal pellet after centrifuging and algal suspension once re-suspended in FSW

The algae were then re-suspended in 128 ml of FSW and decanted into a 150 ml flask. The flask was returned to the temperature-controlled room under the low light regime for 3 hours. Preliminary studies indicated that the algal photosynthetic efficiency of PSII dropped for the first two hours after isolation before recovering, and that the cells remained viable for at least 12 hours after isolation (Appendix 4).

After this time, 0.5 ml of algal suspension was removed for cell counts, to measure the cell density at the onset of the experiment (see later). A sub-sample (20 ml) of the suspension was also pipetted into six 50-ml Falcon tubes. Three Falcon tubes containing the algae destined for the low-light treatments (i.e. L8, L16, L30) were attached to wire brackets by elastic bands, with one Falcon tube placed into each of the three tanks. The three Falcon tubes containing algae destined for the high-light treatments (i.e. H8, H16, H30) were placed into position one hour later. This difference in timing was necessary as I-PAM fluorometer measurements for each light treatment, including preparation time, took 50 min. The flask with the remaining suspension was dark adapted for 20 min. A 1-ml aliquot of algal suspension was then pipetted from the flask into each of 5 x 3-ml darkened cylindrical vials. These vials were then placed into a black, wooden rack and placed into position on the I-PAM stage for measurement.

An hour and 40 min after being placed in the tanks, the Falcon tubes were wrapped in aluminium-foil and returned to their respective tanks to dark adapt for 20 min, thereby maintaining their treatment temperatures. Aliquots of algal suspension were then pipetted into vials as described above. Once all 15 vials per light treatment (i.e. L8, L16 and L30, or H8, H16 and H30) were in place, the rack was positioned on the I-PAM stage (Figure 3.3). This protocol was carried out two more times for each treatment, so that I-PAM measurements were made 2, 4 and 6 h after the *Symbiodinium* cell suspension was placed in its respective treatment tank. At the end of the replicate run, 0.5 ml from each Falcon tube was collected and immediately frozen at -80°C for future cell counts. The mean of the five I-PAM data measurements for each replicate cell suspension/treatment were averaged for each time point. Each treatment was repeated four times over a period of four weeks.

Figure 3.3: Set-up of the I-PAM and the 15 vials measured for each light treatment at each time point. A) Raised red-perspex hood of illumination unit; B) stage on the mounting stand; C) Black rack; D) Darkened 3-ml vials.

3.2.4 Cell counts

Symbiont densities at the beginning and end of the experiment were estimated by using a haemocytometer (Improved Neubauer). Each sample was counted 24 times and averaged.

3.2.5 Photophysiological measurements

Measurements of chlorophyll fluorescence were carried out with a Maxi I-PAM (Figure 3.4) (settings: measuring intensity = 3, frequency = 1, gain = 4, damping = 3 NIR = 8, saturation pulse intensity = 10).

Figure 3.4: Imaging-PAM set-up: A) LED-array illumination unit; B) control unit; C) computer with ImagingWin-software

The first I-PAM measurement after dark adaptation was a fast dark-light induction curve (settings: delay before actinic light activated = 10 seconds, clock-time between saturation pulses = 60 seconds, duration of curve = 615 seconds, actinic light setting = 6 for low light ($111 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and 12 for high light ($396 \mu\text{mol photons m}^{-2} \text{s}^{-1}$)). The irradiance of actinic light depended on whether the samples came from the low-light treatments or the high-light treatments. Information collected gave the minimal fluorescence yield before the first saturation pulse (F_o), the dark-adapted maximum fluorescence yield at the peak of the first pulse (F_m), the instantaneous fluorescence yield throughout the induction curve (F_t), and the light-adapted maximum fluorescence yield at the peak of all subsequent pulses (F_m').

Using the data collected, the following parameters were calculated according to the equations of Genty et al. (1989) and Schreiber et al. (1994):

$$\text{Photosynthetic efficiency} = \text{maximum quantum yield} = (F_m - F_o)/F_m = F_v/F_m \text{ [Eq. 1]}$$

$$\text{Effective PSII quantum yield } (\Phi_{PS}) = (F_m' - F)/F_m' = \Delta F/F_m' \text{ [Eq. 2]}$$

$$\text{Coefficient of NPQ } (q_N) = (F_m - F_m')/(F_m - F_o) \text{ [Eq. 3]}$$

Immediately after the dark-light induction curve, a rapid-light curve (RLC) was recorded. A RLC is best run without samples being dark-adapted as it should not be dominated by induction effects (Heinz Walz GmbH 2006); the best time to run a RLC is after a dark-light induction curve, so retaining the original F_v/F_m . Measurements of F and F_m' were obtained through the application of a series of saturation pulses under increasing and then decreasing actinic irradiance (0, 1, 21, 56, 111, 186, 281, 336, 396, 461, 531, $701 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and back down again). Each step of actinic irradiance was maintained for 60 seconds. Measurements during the decreasing irradiance steps indicated levels of recovery from exposure. From the data recorded, the following parameters were calculated:

- (1) The electron transport rate (ETR). In terrestrial plants, this has been determined as $\Phi_{PSII} \times 0.84 \times 0.5 \times \text{PAR}$, where $\Phi_{PSII} = (F_m' - F)/F_m'$ and 0.84 and 0.5 are

absorption coefficients. As these absorption coefficients are unknown for the isolated *Symbiodinium* cells, a relative ETR (rETR) was calculated from each saturation pulse, where $rETR = (F_m' - F) / F_m'$ [Eq 4] (Ralph et al. 2002).

(2) Non-photochemical quenching (NPQ), where $NPQ = (F_m - F_m') / F_m'$ [Eq. 5].

NPQ_{max} was determined as the highest NPQ value on the RLC (Hill et al. 2004).

To compare the descriptive parameters α (a measure of the efficiency of light utilization, i.e. light limited photosynthetic rate), E_k (saturation radiance, i.e. the irradiance at the onset of saturation) and $rETR_{max}$ (the maximum observed relative electron transport rate), the RLCs were fitted by the function of Platt et al. (1980) using Sigmaplot 11.0 (Platt et al. 1980, Ralph et al. 2002, Hill et al. 2004) such that:

$$P = P_s [1 - \exp(-\alpha E_d / P_s)] \exp(-\beta E_d / P_s) \text{ [Eq. 6]}$$

where E_d is the downwelling irradiance, P_s is a scaling parameter defined as the maximum potential ETR if there is no photoinhibition, α is the initial slope of the light curve before the onset of saturation, and β is the slope of the light curve after the onset of photoinhibition or down-regulation (Figure 3.5). If there is no photoinhibition (i.e. $\beta = 0$), then Equation 6 is simplified to:

$$P = P_m [1 - \exp(-\alpha E_d / P_m)] \text{ [Eq. 7]}$$

The parameters $rETR_{max}$ and E_k were estimated using the equations:

$$rETR_{max} = P_s [\alpha / (\alpha + \beta)] / [\beta / (\alpha + \beta)]^{\beta / \alpha} \text{ [Eq. 8]}$$

$$E_k = rETR_{max} / \alpha \text{ [Eq. 9]}$$

Figure 3.5: A typical photosynthesis *versus* irradiance (P-I) curve. In the dark, there is net consumption of oxygen (C_0) as a result of respiration (R). Photosynthetic rate increases linearly (α) with irradiance until the saturation intensity (E_k) is reached and the rate plateaus at its maximum (P_{max}). Past this optimum irradiance, a decline in photosynthetic rate often occurs (β). Figure taken from Falkowski and Raven (2007).

3.2.6 Statistical analysis

For the final analysis, there were four replicates per treatment. However, if an outlier was found which could not be removed by transformation, this value was replaced with the mean of the other three replicates, as recommended by Quinn and Keough (2002). This occurred with about 5.5% of the data. Though not ideal, outliers could not just be removed because a repeated measure analysis cannot be performed if any values are missing.

Statistical analysis was carried out using PASW (version 18.00). Variation of fluorescence measurements was tested using a repeated measures one-way analysis of variance model (rmANOVA, $\alpha = 0.05$) with time of measurement as the within-subject factor, and light and temperature as the between-subject factors. Where assumptions of normality and equal variance were not satisfied, transformation of data was carried out using an arcsin or a reflect and square root method. The Mauchley Sphericity Test was used in all analyses to test for sphericity of data. As all data met assumptions of sphericity, *post hoc* tests were carried out using Tukey HSD ($\alpha = 0.05$).

3.3 Results

3.3.1 Algal cell density

There was a decrease of 22-37% in cell density from the start to the end of the experiment in all treatments, except in the high-light, warm treatment (Tukey *post hoc*, two-way ANOVA, $0.003 \leq p \leq 0.044$). At the end of the experiment there were no differences between the cell densities in any of the treatments (Tukey *post hoc*, two-way ANOVA, $0.625 \leq p \leq 1.000$: Figure 3.6) (see Appendix 5 for statistical data).

Figure 3.6: Density of cells at the beginning and the end of the experiment (0-6 h) (mean $\pm$ SE, $n = 4$). Notations mark treatments that are significantly different from the start measurements, where * $p < 0.05$ and ** $p < 0.01$.

3.3.2 Fluorescence parameter measurements

3.3.2.1 Maximum quantum yield (F_v/F_m)

Changes in maximum quantum yield (F_v/F_m) were evident after 2 h, with cold temperature at both light levels having the greatest affect (rmANOVA, $p < 0.001$); F_v/F_m decreased by 62 % and 73% in the cold under low and high light (rmANOVA, $p < 0.001$ in both treatments: Figure 3.7) (see Appendix 6 for all statistical data for this section). F_v/F_m continued to decline in response to cold treatment between 2-4 h, before stabilising in the low-light treatment and declining further in the high-light treatment between 4-6 h (Tukey *post hoc*, rmANOVA, $p = 0.001$). High light also had a negative impact in the first 2 h, causing a decrease in F_v/F_m in the other two temperature treatments (Tukey *post hoc*, rmANOVA, $p < 0.001$ for H16 and $p < 0.05$ for H30). F_v/F_m in the control, low-light, moderate treatment (L16), and the low-light, warm

treatment did not change over the 6 h. At the end of the 6-h experiment, there was a difference between the control and the two cold treatments (Tukey *post hoc*, two-way ANOVA, $p < 0.001$), and the high-light, moderate treatment (Tukey *post hoc*, two-way ANOVA, $p = 0.009$).

Figure 3.7: Maximum quantum yield (F_v/F_m) from the start of a dark-light induction curve following 20 min of dark adaption (mean $\pm$ SE, $n = 4$). Measurements were taken at the start of the experiment and 2, 4 and 6 h after. Initial measurements came from the same algal suspension before the suspension was divided between the six treatments with two factors (temperature and light). Notations indicate F_v/F_m that were different to the initial measurement (Tukey *post hoc*, rmANOVA, * $p < 0.05$ and ** $p < 0.001$), or difference in F_v/F_m between treatments and the control (L16) at 6 hours (Tukey *post hoc*, two-way ANOVA, • $p < 0.05$ and •• $p < 0.001$).

3.3.2.1 Effective PSII quantum yield ($\Delta F/F_m'$)

The series of dark-light induction curves (Figure 3.8) clearly demonstrates more reduction, by the end of the experiment, of effective PSII quantum yield ($\Delta F/F_m'$) in response to high light at all temperatures compared with low light at the same temperature (Tukey *post hoc*, two-way ANOVA, $p \leq 0.005$). $\Delta F/F_m'$ was present at all time-points in all of the low-light treatments. It decreased in the first 2 h in the control treatment (L16) (rmANOVA, $p = 0.009$) but recovered and remained the same as the start measurement by 4 and 6 h (Tukey *post hoc*, two-way ANOVA, $0.059 \leq p \leq 0.562$).

In comparison, $\Delta F/F_m'$ was consistently higher in the low-light, warm treatment than in the control (Tukey *post hoc*, two-way ANOVA, $p < 0.001$) and increased over time (rmANOVA, $p < 0.001$). $\Delta F/F_m'$ in the low-light, cold treatment declined below its initial value by 2 h, but then remained constant (Tukey *post hoc*, two-way ANOVA, $p \geq 0.952$) $\Delta F/F_m'$ was adversely affected by high light at all three temperatures (rmANOVA, $p < 0.05$). After 2 h of experimental treatment, and only after one minute of actinic light in the dark-induction curve, $\Delta F/F_m'$ had dropped to zero under high light at 16 and 30 °C, though in the cold, high-light treatment this parameter remained just above zero. After 4 h under high light exposure, $\Delta F/F_m'$ was zero at all temperatures. A small recovery was measured at 6 h, with $\Delta F/F_m'$ rising just above zero in the high-light, warm treatment.

3.3.2.2 Coefficient of non-photochemical quenching (qN)

qN in all treatments, except the low-light, warm treatment, increased rapidly and then stabilized one minute after exposure to actinic light, at all time-points (Figure 3.8). qN decreased markedly by 2 h in the low-light, warm treatment (Tukey *post hoc*, two-way ANOVA, $p = 0.016$), and was less than qN in the control treatment (L16) (Tukey *post hoc*, two-way ANOVA, $p < 0.001$). Over the next 4 h, in the low-light, warm treatment, qN continued to drop, mirroring the increase in $\Delta F/F_m'$ (rmANOVA, $p < 0.001$), and was less than in all other treatments by the end of the experiment (Tukey *post hoc*, two-way ANOVA, $p < 0.001$). qN in the last measurement of the dark-induction curve, under low light in the cold and moderate treatments, was less than under high light at the corresponding temperatures at all time-points (Tukey *post hoc*, two-way ANOVA, $p < 0.001$).

3.3.2.3 F_o , F_m and F_t

The series of graphs for the dark induction curves (Figure 3.8) show that the difference between F_o and F_m fluorescence remained relatively constant throughout the experiment for all of the moderate and warm treatments (mean difference between F_o and F_m , 0.1951). In the two cold treatments, particularly under high light, both F_o and F_m dropped over time, as did the difference between the two (mean difference between F_o and F_m , 0.0147). The values in the F_t curves, which monitor the response of chlorophyll.

Low Light

Start

High Light

Figure 3.8: Fluorescence parameters through dark-induction measurements with the I-PAM taken at the start and after 2, 4 and 6 h (mean, $n=4$). For clarity, SE lines have been omitted but SE values can be found in Table A6.4.1 in Appendix 6). Treatments in the low light (first three rows) were measured separately from the treatments in high light (last three rows). Parameters measured were F_m (dark-adapted maximum fluorescence yield), F_0 (minimum fluorescence yield) and F_v/F_m (maximum quantum yield) at the start of the induction curve, followed by $\Delta F/F_m$ (effective PSII quantum yield), qN (non-photochemical quenching) and F_t (instantaneous yield) throughout the induction curve with saturation pulses every minute for 10 minutes. Saturation pulses for low light were set at $111 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, and for high light, $396 \mu\text{mol photons m}^{-2} \text{s}^{-1}$.

fluorescence, were generally higher in the warmer treatments, and higher under low light.

3.3.2.4 Maximum observed relative electron transport rate ($rETR_{max}$)

$rETR_{max}$ was negatively affected by temperature and irradiance over the 6-h treatment (rmANOVA, $p = 0.01$). By 2 h, $rETR_{max}$ in all treatments had decreased, except in the low-light, warm treatment, and more so under low temperature and high light (Tukey *post hoc*, rmANOVA, $0.001 \geq p \leq 0.032$) (Figure 3.9). $rETR_{max}$ then increased between 2-6 h in both warm treatments, but more so in low light than high light (Tukey *post hoc*, rmANOVA, $p < 0.001$ in L30 and $p = 0.001$ in H30). This parameter remained relatively unchanged between 2-6 h in the other four treatments. At the end of the experiment, $rETR_{max}$ was greater than the control (L16) in both warm treatments (Tukey *post hoc*, two-way ANOVA, $p < 0.001$), and less than the control in both cold treatments and the high-light, moderate treatment (Tukey *post hoc*, two-way ANOVA, $p < 0.001$).

3.3.2.5 Saturation irradiance (E_k)

Changes to the saturation irradiance (E_k) were affected by low light in the first 2 h (rmANOVA, $p = 0.002$) (Figure 3.9) and by temperature in the first 4 h of the experiment (rmANOVA, $p < 0.05$), with a decrease in E_k in the cold treatment and an increase in the warm treatments. In the first 2 h, only E_k in algae from the cold, low-light treatment decreased (Tukey *post hoc*, rmANOVA, $p = 0.005$); it then remained unchanged over the next 4 h (Tukey *post hoc*, rmANOVA, $p = 0.99$). Between 2-4 h, high light affected E_k with a decrease in the cold treatment (Tukey *post hoc*, rmANOVA, $p = 0.024$) and an increase in the warm treatment (Tukey *post hoc*, rmANOVA, $p = 0.033$). E_k in the control treatment (L16) did not change over time ($0.078 \geq p \leq 1.00$). At the end of the experiment, only the E_k values in the warm treatments were different (greater) than in the control (L16) (Tukey *post hoc*, two-way ANOVA, $p < 0.001$).

Figure 3.9: Parameters of the rapid light curves (RLC). A) $rETR_{max}$; B) E_k and; C) NPQ_{max} in all six treatments after 0, 2, 4 and 6 h (means $\pm$ SE, $n=4$). All parameters were analysed with rmANOVA. Notations indicate differences from the initial value (Tukey *post hoc*, rmANOVA, * $p < 0.05$ and ‡ $p < 0.001$) or where there was a difference in measurements between treatments and the control (L16) after 6 h (Tukey *post hoc*, two-way ANOVA • $p < 0.05$ and •• $p < 0.001$). Note the difference in scales on the y-axis.

Relative Electron Transport Rate

0 Hours

Figure 3.10: Rapid light curve parameters measured were relative electron transport rates (rETR), effective quantum yield ($\Delta F/F_m'$) and non-photochemical quenching (NPQ) for all treatments at 0, 2, 4 and 6 h in the experiment (Mean, $n=4$). The six treatments were low-light 8 °C (L8); low-light, 16 °C (L16); low-light 30 °C (L30); high-light, 8 °C; high-light, 16 °C (H16) and; high-light, 30 °C (H30). Each irradiance level was maintained for sixty seconds.

3.3.2.6 Maximum non-photochemical quenching (NPQ_{max})

Changes in NPQ_{max} were affected in the first 2 h by temperature (rmANOVA, $p < 0.001$), with low temperature having the greatest effect under both light irradiances, where NPQ_{max} decreased from the initial measurement by approximately 67% in both cold treatments (Tukey *post hoc*, rmANOVA, $p < 0.001$) (Figure 3.9). NPQ_{max} in the cold, high-light treatment continued to decrease over the next 4 h (Tukey *post hoc*, rmANOVA, $p = 0.005$) whereas it stabilised in the cold, low-light treatment (Tukey *post hoc*, rmANOVA, $p = 0.115$). There was a decrease in NPQ_{max} in both 16 °C treatments by 4 h, but by 6 h only NPQ_{max} in the high-light, moderate treatment was less than the initial measurement (Tukey *post hoc*, rmANOVA, $p = 0.04$). At the end of the experiment, NPQ_{max} was less than the control (L16) in both cold treatments (Tukey *post hoc*, two-way ANOVA, $p < 0.001$) whereas all the other treatments were the same.

3.3.2.7 Rapid light curve parameters: rETR, $\Delta F/F_m'$ and NPQ

Measurements were also made to compare the trends between relative electron transport (rETR), effective quantum yield ($\Delta F/F_m'$) and non-photochemical quenching (NPQ) parameter profiles throughout the RLC as actinic light increased and then decreased (Figure 3.10). The NPQ measurements at 2, 4 and 6 h showed that four treatments (low and high light at 16 and 30 °C) had similar profiles to the profile at time 0. The NPQ profiles in the cold treatments were considerably lower at 2 h than at 0 h, with a decrease of the maximum measurement by 68%. This profile then remained the same at the next two time-points. In the 16 and 30 °C treatments, at all four time-points, NPQ increased with irradiance, but then relaxed rapidly as the irradiance decreased. $\Delta F/F_m'$, on the other hand, decreased to zero as irradiance increased and recovered once irradiance had dropped below 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the 8 and 16 °C treatments. In the warm treatments, recovery occurred at increasingly higher irradiances at 4 and 6 h. rETR increased over time in both of the warm treatments, but more so under low light. Over the four time-points in the experiment, there was also a decrease in the length of time when rETR ceased at higher irradiances in the

warm treatments. In the other four treatments there was an initial drop in the first peak of the rETR profiles, after which they remained constant.

3.4 Discussion

The main finding of this study is that *Symbiodinium* cells within *A. aureoradiata* are very effective in protecting themselves against photo-damage by activating an efficient NPQ system and down-regulating the quantum efficiency of PSII under different irradiances and at different temperatures. Temperate anemones are known to depend less on the photosynthetic abilities of their symbionts than those in tropical hosts (Muller-Parker and Davy 2001). As these symbionts had negligible photosynthetic activity measured, this would appear to be the case in *A. aureoradiata* too, though there may have been a different process occurring that was not measurable in the methods used.

3.4.1 Down-regulation

3.4.1.1 Maximum quantum yield (F_v/F_m)

Down-regulation of photosystem II to protect against any damage by light photons can be determined by comparing measurements of maximum quantum yield, or photosynthetic efficiency (F_v/F_m) (Krause 1988, Brown et al. 1999, Muller et al. 2001, Fisher et al. 2011). F_v/F_m was affected more by cold temperature than by warm conditions in this study and was considerably lower, especially under high light, than F_v/F_m in the other two temperatures at the 2-hour time-point. Lower quantum yield could indicate that the *Symbiodinium* cells were either under stress or that the photosystems had down-regulated. Calculations for the decreased F_v/F_m were made using the minimal fluorescence yield (F_o) measurements, which were much lower than those from the start. This drop in F_o is an indication of energy quenching by qE (Muller et al. 2001) or an uncoupling of energy transfer by a phase shift occurring in the thylakoid membrane (Diaz-Almeyda et al. 2011), both photoprotective mechanisms. If there had been a rise in F_o measurements instead, as maximum fluorescence yield (F_m) and quantum yield dropped, then this would have indicated damage occurring to the PSII reaction centres (Krause 1988, Muller et al. 2001,

Schreiber and Klughammer 2008). Therefore, down-regulation seems likely to have occurred in the photosystems in the algae from the cold treatments. Down-regulation was also noted by decreased F_v/F_m and F_o in the high-light, warm treatment after 2 hours, though this was not apparent by the end of the experiment as F_v/F_m had returned to initial levels.

Though maximum quantum yield dropped considerably between 0-2 hours in the 8 °C treatments and gradually continued to drop over time, there was only a small drop in yield in the other two temperature treatments by 2 hours. The yield in the 16 °C treatments then remained steady over the next 4 hours, while this same parameter increased steadily in the 30 °C treatments to such an extent that F_v/F_m in the low-light, 30 °C treatment was higher than the value measured at the start (0.4685 compared to 0.4516). This suggests that, though the cell counts had dropped by the end of the experiment, the remaining *Symbiodinium* cells were not physiologically compromised (Jones et al. 2000).

3.4.1.2 Non-photochemical quenching (NPQ) and the thylakoid membrane

Most protection by non-photochemical quenching (NPQ) occurred under high-light conditions at all temperatures tested. qN was rapidly induced and then stabilized as, simultaneously, effective PSII quantum yield decreased. The quick response by NPQ could prevent photoinhibition, while the drop in effective PSII quantum yield suggests the down-regulation of the photosystems. Down-regulation, and not damage, is also suggested by the rapid light curves recovering for both the cold treatments, as the measurements at the end of the RLCs indicated rapid increase in electron transport rate (ETR) and effective PSII quantum yield ($\Delta F/F_m'$), as the irradiance was lowered, suggesting that these *Symbiodinium* cells have adapted to rapidly changing conditions in their habitat. In most of the treatments, qN and $\Delta F/F_m'$ mirrored each other and almost added up to 1, indicating that the use of light photons in photochemical quenching and non-photochemical quenching was well balanced, and protected the photosystem. The only treatment in which photoinhibitory damage was suspected, rather than down-regulation, was the high-light, 30 °C treatment from 4 hours onwards. At 4 and 6 hours, maximum quantum yield remained as high as at

the start but $\Delta F/F_m'$ dropped to zero once the actinic light was on. At the same time, qN was neither induced nor stabilized as rapidly as the other parameters. This suggests that there may have been free electrons in the system which could have led to the formation of destructive reactive oxygen species.

Resilience to temperature and light-stress by increased photoprotection in these *Symbiodinium* cells could be due to the composition of the thylakoid membranes in the chloroplasts. Thermally-damaged membranes cause uncoupling of energy transfer, but do not affect the water-splitting capabilities of the chloroplast, leading to the presence of excessive oxygen (Tchernov et al. 2004). These authors analysed the lipid composition of various *Symbiodinium* types and found that sensitivity to thermal damage was determined by the saturation of the lipids within the membrane, with thermally-tolerant types having significantly lower concentrations of polyunsaturated fatty acids. Similarly, in two *Symbiodinium* clade A types tested, rigidity of the membranes due to cold could be reduced by increasing the levels of polyunsaturated fatty acids (Diaz-Almeyda et al. 2011). These authors found that the melting curve of thylakoid membranes is type-specific, even within *Symbiodinium* clades. Fluidity of the membrane allows dissociation of the pigments within the antenna complex from PSII to PSI facilitating dissipation of energy as heat, which is more evident in *Symbiodinium* in clade A (Reynolds et al. 2008). Another reason that thylakoid lipid content could affect resilience is that lipids within the membrane help to remove oxygen from PSII (Kern and Guskov 2011). Clumping together of the lipids allows rapid diffusion of oxygen across the membrane and away from the reaction centre region, so reducing oxidative stress.

Measurement of instantaneous fluorescence yield (F_t) allows an assessment of the integrity and thermostability of the thylakoid membrane by monitoring the reaction of chlorophyll fluorescence to actinic light (Hill et al. 2009). Thylakoid integrity is inferred as chlorophyll pigments are embedded within the membrane. This parameter was highest in the warmer temperatures at both light levels. Temperature may have affected the fluidity of the bi-lipid layers, which may then have affected the plasticity and resilience to the higher temperature by facilitating the repositioning of chlorophyll and carotenoid pigments (Tchernov et al.

2004). Indeed, this mechanism could have contributed to the increase in effective PSII quantum yield in the low-light, 30 °C treatment, and the eventual recovery in the high-light, 30 °C treatment. On the other hand, the decrease in q_N in both 30 °C treatments could have been caused by the change in the thylakoid membrane affecting the generation of the trans-thylakoid ΔpH , and thereby diminishing the activation of the xanthophyll cycle in the qE component of NPQ. This would have decreased the loss of photon energy by heat dissipation. The cold, however, could have affected the thylakoid membranes by reducing the fluidity of the bi-lipid layers, thereby leading to the uncoupling of the electron transport chain, as can be verified by the rETR profiles. Unlike Hill et al. (2009), who found that membrane sensitivity was temperature dependent but light independent, in this experiment F_t was continuously higher in low light than in high light at all temperatures, and effective PSII quantum yield increased gradually over time for all low-light treatments. This may be due to adaptation by the *Symbiodinium* cells to low-light environments as *A. aureoradiata* are found in cracks on the rocky shore and buried on the mudflat, and also adaptation to the environment within the thick tissue of the anemone.

The induction of NPQ in the high temperature and high light treatments in this study was expected, but the measurement of q_N indicated that there was almost as much NPQ being induced in both light irradiances in the cold temperature treatments too. Only a few studies have been carried out on the stressful effects of cold temperature on *Symbiodinium* photosystems (e.g. Thornhill et al. 2008, Kemp et al. 2011). Colder than normal conditions have been shown to cause bleaching in corals (Saxby et al. 2003) and photoinhibition in *Symbiodinium* cells (Saxby et al. 2003, Wicks et al. 2010a). Cnidarian-*Symbiodinium* partnerships at temperate latitudes must have adapted over time, and are able to acclimate to seasonal and daily changes, to survive in colder conditions. Higher plants in the cold are thought to keep Q_A , the primary quinone electron acceptor, more oxidized so that excessive light photons are unable to move into the photosystems (Huner et al. 1998, Bravo et al. 2007), and plants from the Antarctic and Andes have developed fast action NPQ to dissipate light energy away from the photosystems (Bravo et al. 2007). Likewise, dinoflagellates in polar regions protect their photosystems by having a higher ratio of

polyunsaturated fatty acids in their thylakoid membranes to maintain membrane fluidity in the cold (reviewed in Morgan-Kiss et al. 2006). This would aid energy dissipation by state transitioning of the chloroplast pigments. Thornhill et al. (2008) hypothesised that loss of thylakoid membrane fluidity decreased photosynthetic efficiency in cold-stressed *Symbiodinium* in their study. Conversely, it is also thought that *Symbiodinium* cells become more heat tolerant with higher lipid-saturation levels to maintain thylakoid membrane integrity at higher temperatures (Tchernov et al. 2004). For temperate *Symbiodinium* cells to survive, in the cold of winter and the heat of summer, changes in the ratio of lipid types in the membrane throughout the year may be necessary. However, this study with low and high temperature treatments was only carried out over 6 hours. Therefore, synthesis of new thylakoid lipids would have had to be completed in a very short time span and the possibility for this happening in *Symbiodinium* is unlikely (Tchernov et al. 2004). If synthesis of lipids is unlikely, then the *Symbiodinium* cells in my study would have had an increase of the qE component of the NPQ mechanism in both the colder and warmer treatments and not the qT, state transitioning component. Additional study on the ratio of the different NPQ components is needed to verify this.

3.4.2 Photosynthetic efficiency

High light decreased photochemical efficiency compared to low light at every temperature tested, with photosynthesis eventually ceasing in this study. Similarly, in previous studies, high irradiance also caused a decline in effective PSII quantum yield when corals were exposed to both high and low temperatures (Saxby et al. 2003, Wicks et al. 2010a). Photosynthesis may still be occurring in the *Symbiodinium* cells tested here, but via a different method within the chloroplast. The *Symbiodinium* cells in this study were identified as types from clade A, and clade A *Symbiodinium* types have been found to be able to upregulate PSII-independent cyclic electron transport (CET) that transfers energy around PSII to PSI, maintaining ATP synthesis and therefore conversion of carbon from its inorganic to organic form, even when PSII is inactivated (Reynolds et al. 2008). These authors found that most members of clade A demonstrated alternative methods of photosynthetic electron transport whereas the other *Symbiodinium* clades did not. This

alternative CET is an important pathway for amplifying NPQ in plants and may have a similar function in *Symbiodinium* dinoflagellates (Munekage et al. 2004, Warner and Berry-Lowe 2006). Clade A is the most ancestral of the *Symbiodinium* genera (LaJeunesse 2001) and has retained the alternative CET and the ability to dissociate pigments in the antennae complex, whereas the other clades have diverged from these physiological traits. Retaining these traits may explain why clade A is found in hosts that primarily inhabit shallow, high-light habitats (Finney et al. 2010). Further discussion on alternative CET is carried out in Chapter 4.

It was also interesting to note that the irradiance where the photosystems reached saturation point (E_k) was so low in the rapid light curves. E_k measurements indicate the irradiance at which the energy from absorbed light photons is transferred to NPQ for dissipation (Hill et al. 2004). The highest E_k in the rapid light curve of the present study of $61.05 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ was well below the irradiance in the high-light treatments of $400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, and less than the low-light treatment ($100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). E_k was also much lower than the radiance experienced on the mud flats from where the anemones were collected (summer maximum, $1614 \mu\text{mol photons m}^{-2} \text{s}^{-1}$; winter maximum, $795 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Gibbons 2008)). This could be due to the *Symbiodinium* cells within the anemones already being physiologically acclimatised to the low light conditions whilst being in the laboratory ($100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) or when covered by mud in the field, or it could be due to the *Symbiodinium* cells being acclimatised to the shading caused by their endosymbiotic habitat (Bhagooli and Hidaka 2003)

It could also be that the *Symbiodinium* cells functioned very differently than they would have done, had they been in their normal environment within the host. The host tissue would have protected them by reflecting and reducing light (Banaszak et al. 2000). Also the host cell cytoplasm would have provided the *Symbiodinium* cells with different nutrient and ionic concentrations than seawater (Seibt and Schlichter 2001), which could have induced a degree of stress. For example the photochemical efficiency of *Symbiodinium* cells isolated from four different hosts species decreased significantly and recovered more slowly than when the same *Symbiodinium* types were being tested within their hosts (Bhagooli and

Hidaka 2003). Similar to the results in my study, the 2003 study found that even exposure to low light ($70 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) caused a significant drop in photosynthetic efficiency at both control and higher temperatures. As the irradiance was increased, yield almost dropped to zero, which was similar to the zero quantum yield measured in the current study under higher irradiance ($400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). The present study tested photoprotection under a sudden change of light intensity and temperature, leading to short-term stress effects. On a daily basis, irradiance levels could change as rapidly as they did in the experiment but, in reality, temperature would change gradually through the day and through the seasons. Future experiments following the same PAM procedures from this study, but working with the holobiont would be valuable to determine if residing in the anemone affects the photosynthetic outcomes significantly.

There have not been many studies of light and temperature stress in high-latitude types of *Symbiodinium*. Wicks et al. (2010) carried out studies on the high latitude coral *Pocillopora damicornis* at Lord Howe Island. They placed nubbins of corals into six different temperature and light combinations and conducted measurements every three hours for three days using a Diving-PAM. They measured effective quantum yields ($\Delta F/F_m'$) and fast induction curves (FIC), and found that the *Symbiodinium* cells in the corals were more tolerant to a drop in temperature (-3°C) from the control compared to an equal rise in temperature above the control, though there was a drop in $\Delta F/F_m'$ at both temperatures, and more so in the high-light treatments. NPQ was also greater in high-light than low-light treatments. Wicks and co-workers concluded from this that the *Symbiodinium* cells were specialized to function in the cold as they originate from relatively high-latitude waters. The induction curves presented in this study also showed more deleterious effects under high light compared to low light at the same temperature. The main difference between the two studies was that, at the higher temperature, but in low light, the *Symbiodinium* cells in my study appeared to increase in photosynthetic health; NPQ decreased and $\Delta F/F_m'$ increased. Could this be because the anemones in New Zealand have had to also specialize to being in warm habitats such as rockpools or shallow pools on mudflats, whereas the shallowest the corals in Wicks et al.'s study were found was in 2-3 m depth in the sea?

This study has resolved some of the objectives set out at the start. The *Symbiodinium* cells do have strong photoprotective abilities, and can down-regulate the quantum efficiency of PSII quickly and effectively but do this at high and low temperatures. However, to learn how they have photoacclimated will need further studies. These could include molecular analysis to establish if genes are present and upregulated for chloroplast pigment and thylakoid lipid synthesis. The thylakoid membrane can be examined by high-performance liquid chromatography (HPLC) to measure the ratio of different pigment and lipid types present. Also measurement of the three components of NPQ will enable an assessment of which provided the most photoprotection.

Chapter 4

General Discussion

This study examined the molecular identity of the *Symbiodinium* types within the local symbiotic anemone, *Anthopleura aureoradiata*, and how biogeographical isolation, latitudinal distribution and local habitat could affect symbiont diversity. A comparison was also made with the *Symbiodinium* types found within four species of anemones from Europe to identify differences, or similarities, between types from similar latitudes. As predicted, diversity was low but, contrary to expectations, the isolation of New Zealand does not seem to have led to endemism of these symbionts, as there was a marked similarity between the *Symbiodinium* types from New Zealand, southern England, Brittany (France) and the Mediterranean. The *Symbiodinium* types from all the anemones in the study were identified as being similar or identical to ITS2 types *Symbiodinium* Amed, (“Amed”) and Mediterranean clade A (“Med clade A”). These two types were both originally sourced and identified from the Mediterranean Sea (Hunter et al. 2007, Meron et al. 2012)

The effects of different temperature and light regimes on the photophysiology of the *A. aureoradiata*-*Symbiodinium* symbiosis were also examined, to better understand how this association remains functional under a wide range of environmental conditions. As predicted, the photoprotective capacity of the non-photochemical quenching mechanism was quickly and effectively utilized by the algal cells but, surprisingly, there was little or no photosynthesis measured, except under low light at 16 and 30 °C. These *Symbiodinium* cells effectively protect their photosystems by converting photon energy into heat and not into chemical energy.

Two main questions arose from the outcomes of the study and will be discussed further in this section.

1. How did Med clade A and Amed *Symbiodinium* get to both hemispheres, and how long ago did the populations separate?
2. What is it about clade A photophysiology that makes it so suitable for temperate regions?

4.1 Population distribution – How and when?

4.1.1 Ancient Origins

There is evidence to suggest that the mutualistic relationship between anemone-like coral ancestors and *Symbiodinium*-like dinoflagellates began in the late Triassic period, about 235 million years ago (MYA) (Stanley and Fautin 2001, Muscatine et al. 2005, Stanley 2006). Estimations from molecular clock deductions suggest that *Symbiodinium* clade A originated from the Paleocene time, 65-50 MYA, and went through diversification within the clade and divergence into the other clades throughout the next 35-50 million years (Tchernov et al. 2004, Pochon et al. 2006). Throughout this time the position of the world's landmasses was very different to today (Figure 4.1).

There was a continuous band of sea around the northern hemisphere and open water between what was to become North and South America. Ocean currents flowed differently than they do today, and the temperature of the oceans was less differentiated between the furthest latitudes, as the establishment of cold water domains at the poles did not occur until 16 MYA ($\pm$ 8MYA) (Darling et al. 2000). Closure of the Mediterranean Sea to the east occurred about 20 MYA (Hrbek and Meyer 2003) and the final closure of the Isthmus of Panama occurred approximately 3.5 MYA (Coates et al. 1992), stopping direct links between the Atlantic Ocean and the Pacific and Indian Oceans.

Figure 4.1: Paleogeographic map of 65 MYA showing the open water linkage across the northern hemisphere, and a link between what will become the Atlantic and Pacific Oceans where the Isthmus of Panama will form. (Culver 2003).

There was a continuous band of sea around the northern hemisphere and open water between what was to become North and South America. Ocean currents flowed differently than they do today and the temperature of the oceans was less differentiated between the furthest latitudes, as the establishment of cold water domains at the poles did not occur until 16 MYA (± 8 MYA) (Darling et al. 2000). Closure of the Mediterranean Sea to the east occurred about 20 MYA (Hrbek and Meyer 2003) and the final closure of the Isthmus of Panama occurred approximately 3.5 MYA (Coates et al. 1992), stopping direct links between the Atlantic Ocean and the Pacific and Indian Oceans.

Establishment of the distribution for Med clade A and Amed *Symbiodinium* in New Zealand, England and what was to be the Mediterranean Sea, could have occurred before the waterways closed 3.5 MYA. The length of time from then to now and the apparent isolation of these populations from each other would suggest that genetic divergence would have occurred, as mutations over time would be expected in each population. However, studies of molecular data indicate that divergence can be delayed for millions of years

(Gould and Eldridge 1977, Soltis et al. 2002, LaJeunesse 2005) and separated populations can remain undifferentiated due to limited sexual reproduction (LaJeunesse 2005). For instance, ribosomal sequences in the ancestral “types” C1 and C3 in the Atlantic-Caribbean and Indo-Pacific regions have remained unchanged since at least the time of the Isthmus of Panama closure (LaJeunesse 2005), which may also be the case with northern and southern hemisphere populations of Med clade A and Amed *Symbiodinium*.

4.1.2 Vectors of connectivity

Bipolar distributions of the same species can be found in many organisms and many planktonic groups (Darling et al. 2000), and whole microplankton communities in the north and south are essentially alike, even though they have been separated since the Miocene (20MYA) by the tropical zone (Taylor et al. 2008). Northern and southern hemisphere dinoflagellate communities are virtually identical within similar latitudinal and climatic zones (Taylor et al. 2008). Connectivity between the hemispheres may have occurred in relatively recent times and may still be occurring today. However, for genetic exchange to occur between the high southern and northern latitudes, warm equatorial seas have to be crossed. Darling et al. (2000) suggested that connection between subpolar populations of planktonic foraminiferans is made possible by individuals moving with the cold boundary currents up the continents and crossing the tropics by submergence below the deep, cold thermocline. Antarctic and Arctic population of the ciliate *Euplotes nobilii* are also thought to migrate this way (Giuseppe et al. 2011). However, this does appear to be an unlikely route for photosynthesising dinoflagellates. Nonetheless, a study in 2003 found two strains of a species of a photosynthesising dinoflagellate, *Polarella glacialis*, in the Antarctic Ross Sea and Canadian Arctic waters (Montresor et al. 2003). These authors hypothesised that the dinoflagellate is able to cross tropical waters in the encysted resting stage of its lifecycle. Also of note, a new paper presents evidence of isolated symbiotic *Symbiodinium* cells feeding heterotrophically on bacteria and micro-algae (Jeong et al. 2012), and so not relying on the sun or a host as a food source. This ability would facilitate the migration of these dinoflagellates.

To cross great distances, *Symbiodinium* cells would have to be able to live independently in seawater and travel either in ocean currents or the ballast water of ships and boats. Many *Symbiodinium* types must have free-living stages as, in the majority of cases, host cnidarians acquire their symbionts via horizontal transmission. The presence of free-living *Symbiodinium* cells is more common than once thought (Stat et al. 2008a, Pochon et al. 2010), especially in sediment (Gou et al. 2003, Hirose et al. 2008, Takabayashi et al. 2012). Gou et al. (2003) found *Gymnodinium* dinoflagellates in water samples from Jiaozhou Bay, China that they identified as being possibly the same species as *G. varians* from Wellington Harbour and *S. californium* from Santa Barbara, USA. Whether these dinoflagellates naturally have such a wide distribution or were spread via ballast water is unknown. Nevertheless, the extent to which a *Symbiodinium* cell can remain healthy and survive in the sea would differ from one species or type to another (LaJeunesse et al. 2009). In the current case, it is unlikely that Med clade A and Amed would be able to last long enough in the water to be able to travel such distances as they have evolved to function as endosymbionts (LaJeunesse et al. 2009). Identification of free-living and symbiotic *Symbiodinium* types in the same habitat has shown little overlap between the two communities which leads to the proposal that *Symbiodinium* types may be restricted to having either long-term free-living or endosymbiotic modes of living (Hirose et al. 2008, Pochon et al. 2010, Takabayashi et al. 2012). Moreover the endosymbionts identified in this study are known to be maternally transmitted from an adult anemone to the young, in all but one of the anemone species sampled (*A. mutabilis* has not been studied yet) (Bythell et al. 1997, Davy et al. 1997a, Davy and Turner 2003), and so a long free-living stage would not be envisaged. However, the lack of genetic differences encountered from the samples analysed from the north to the south of NZ suggests sharing of genes, in which case, some free-living Med clade A and Amed *Symbiodinium* would be required with some horizontal transmission occurring in the *A. aureoradiata* population, (Focioli et al. 2011).

Conversely connectivity between the northern and southern latitudes may occur by these *Symbiodinium* types migrating as endosymbionts within pelagic hosts, whether naturally with currents or within the ballast waters or on hulls of boats, and being passed on from

these hosts into native animals. One of the organisms that hosts *Symbiodinium* clade Temperate-A is *Cotylorhiza tuberculata*, the Mediterranean fried egg jellyfish (Visram et al. 2006; Figure 4.2 A). Closely related species of jellyfish can be found in the Atlantic and Pacific Oceans with, for example the symbiotic jellyfish *Phyllorhiza punctata* (Figure 4.2 B) inhabiting New Zealand waters (Gordon 2009). It is conceivable that Med clade A and Amed may have colonized two distant regions by passing through a network of jellyfish hosts, though there is no evidence for this hypothesis.

Another consideration is that these clade A *Symbiodinium* types appear to be host-generalists in the Mediterranean and around the English coast. No other cnidarian-*Symbiodinium* partnerships have been identified in New Zealand and it would be interesting to discover if the jellyfish *Phyllorhiza punctata* does host Med clade A and Amed, or if there are any other host animals along the New Zealand coastline which also harbour *Symbiodinium* cells of these types.

Figure 4.2: A) *Cotylorhiza tuberculata*, the Mediterranean fried egg jellyfish (image from <http://fc09.deviantart.net/fs71/f/2010/009/r/0Cotylorhiza-tuberculata-6-149764996>) and; B) *Phyllorhiza punctata*, the Australian spotted jellyfish (image by K Kalani Patterson from www.gambassa.com/public/project/2066/PoojaPatelNotableSpecies.html)

An alternative idea is that the widespread distribution of Med clade A and Amed occurred together with the anemone host. *Anthopleura ballii* from England sampled in this study

hosted Med clade A, and *Symbiodinium* cells identified in *Anthopleura japonica* and *Anthopleura kurogane* from Korea hosted members of *Symbiodinium* clade A too (Rodriguez-Lanetty et al. 2000). This partnership between *Anthopleura* anemones and clade A *Symbiodinium* may be an ancient combination that took place before the landmasses parted and shifted into the geographical positions of today, whilst maternal transmission in these anemones has maintained the genetic similarities between the northern and southern distribution of these *Symbiodinium* types.

4.2 What is special about clade A as a temperate symbiont?

The species *Anthopleura aureoradiata* was found to host *Symbiodinium* Med clade A in the majority of cases with very few individuals hosting *Symbiodinium* Amed throughout the whole of its biogeographical range (97% Med clade A *versus* 3% Amed). Other studies have found that *Symbiodinium* type changes within host species with latitude (e.g. Lien et al. 2007), season (e.g. Venn et al. 2008b) and shore height (e.g. Baker 2003). However, the relationship that *A. aureoradiata* has with its symbionts appears to be stable even though the climatic conditions in New Zealand are so varied and changeable, and the habitats where this symbiosis is found are relatively diverse. Tropical cnidarian-*Symbiodinium* partnerships can break down with only a small change in temperature, sometimes as little as 1-2 °C above the norm (Hoegh-Guldberg 1999, Hughes et al. 2003), whereas temperate partnerships are more hardy as they have had to acclimate to the wide range of seasonal conditions (Verde and McCloskey 2007).

4.2.1 Partnership persistence with minimal nutritional benefits

Though *A. aureoradiata* hosts these two *Symbiodinium* types throughout the year, in this study it appeared that the algae were not photosynthetically productive in most of the conditions tested in the laboratory. Photosynthesis increased in warmer conditions but only when the light levels were low. High light conditions appeared to inhibit photosynthesis altogether. With such low rates of photosynthesis, the *Symbiodinium* cells in *A. aureoradiata* may only be contributing adequate carbon for the anemone's respiratory

requirements during certain times of the day or year. Other studies looked at translocation of carbon by temperate algae to their animal hosts (Davy et al. 1996, Verde and McCloskey 2007, Bergschneider and Muller-Parker 2008, Gibbons 2008) and found that, in most conditions, translocation was such that heterotrophic feeding would be necessary to meet animal respiratory requirements. This was particularly true in “winter” conditions, when for example Gibbons (2008) found that there was potentially no contribution from the algae to the animal host in *A. aureoradiata*. This current study did not look at the contribution of carbon to the host, but concluded that photosynthesis in all treatments was so minimal that there would be very little energy gain for the host under high irradiance. However, Gibbons’ (2008) work on *A. aureoradiata* measured oxygen production from *Symbiodinium* cells’ photosynthetic responses in increasing quantities up to 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, which then remained steady as irradiance was increased to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. If oxygen electrodes can detect oxygen production by *Symbiodinium* cells in *A. aureoradiata*, suggesting effective photosynthesis, why did the PAM fluorometry method in the current study not do so?

4.2.2 Alternative methods of making chemical energy

Reynolds et al. (2008) used a novel method of measuring photosynthesis with a PAM fluorometer, by measuring chlorophyll *a* fluorescence in response to serial irradiation pulses (SIP). They found that members of clade A *Symbiodinium* were capable of alternative photosynthetic electron-transported pathways, such as PSII-independent cyclic electron transport (CET) and chlororespiration, where oxygen serves as an alternative photosynthetic electron transport acceptor. What this means is that both CET and chlororespiration can continue ATP synthesis when PSII becomes inactivated, and therefore conversion of inorganic carbon to organic carbon continues too. They also found that PSII in clade A become inactivated quickly in high light (exposure for 100 minutes at 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) when up to half of the peridinin-chlorophyll *a*-protein (PCP) antenna complexes disassociated from PSII in a form of NPQ state transitioning. Another benefit is that chlororespiration may also decrease the probability of reactive oxygen species (ROS) formation, and the damage that can ensue, by using oxygen as an electron

carrier. They found that all of these qualities have been lost in the other *Symbiodinium* clades.

What this could mean in relation to *Symbiodinium* types Med clade A and Amed is that they may still be supplying carbon to the animal in high light, but in a process that is not detectable by the PAM fluorometry methods used here. The points at which I measured zero for $\Delta F/F_m'$ under high light ($400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) are about when Reynolds and co-workers suggested that PSII shuts down, due to efficient NPQ, and CET and chlororespiration pathways are used instead to bypass PSII and produce ATP. On the other hand, type A2 cells were found by the authors to not have CET and chlororespiration responses. In my study, the molecular analysis of ITS2 sequences from all samples tested placed Med clade A and Amed in a sister group to clade A2 in both Maximum Likelihood and Bayesian constructed phylogenetic trees. Type A2 and these sequences together are placed as a sister group to all the other clade A types. LaJeunesse (2001) also placed A2 as a sister group to the other clade A types. Therefore, the *Symbiodinium* types in the current study may share the same photophysiological traits as clade A2. The serial irradiation pulses method with the PAM fluorometer would need to be used on *Symbiodinium* from *A. aureoradiata* to confirm whether alternative photosynthetic electron-transport pathways are being used.

Clade A types have also been considered to be less beneficial to their hosts than other types (Stat et al. 2008b). Stat et al. (2008) worked with freshly-isolated clades A and C *Symbiodinium* and found that both fixed similar amounts of carbon in filtered seawater but once incubated in a synthetic 'host factor', clade C fixed and released four times as much carbon as clade A, which did not have any change in the amount of carbon fixed. This suggested that a host has no influence on photosynthate release from clade A *Symbiodinium*, and the fact that clade A was found in more diseased corals than was clade C, persuaded the authors to conclude that clade A algae are nutritionally less beneficial to their host than are other *Symbiodinium* clades. These authors theorized that clade A *Symbiodinium* cells have not fully evolved into mutualistic endosymbionts from their free-living ancestors, whereas more derived clades may have attained a more mutualistic state

over time. However, *A. aureoradiata*'s symbionts are passed onto the next generation vertically from the mother which, according to some theories, suggests that the symbionts are more likely to be mutualistic, with a substantial degree of host-symbiont integration (Sachs and Wilcox 2006). Moreover, carbon budgets constructed by Gibbons (2008) for this sea anemone suggest that, under well-lit conditions at least, the host may derive a substantial portion (sometimes > 100%) of its metabolic needs from its symbionts. For much of the year though, this same author demonstrated that *A. aureoradiata* receives much less photosynthetic carbon than it needs due to light limitation.

4.2.4 Persistence through vertical transmission

If there is only a moderate nutritional benefit to hosting these temperate *Symbiodinium* types, why has the partnership persisted across generations? It was thought that clade A *Symbiodinium* cells were found in cnidarian hosts predominantly located in shallow water habitats as they produced UV-absorbing mycosporine-like amino acids (MAAs), thereby protecting their host, whereas other clades did not offer this protection. (Banaszak et al. 2000, Reynolds et al. 2008, Finney et al. 2010). However, the study by Banaszak et al (2000) was done with *Symbiodinium* cultures; in a more recent study, it was found that most *Symbiodinium* clades *in hospite* produce MAAs and translocate these to their host (Banaszak et al. 2006). So hosting clade A *Symbiodinium* in shallow NZ waters would not necessarily give more protection from solar radiation than a *Symbiodinium* type from another clade. It may simply be the case that *A. aureoradiata* hosts Med clade A and Amed because there are no other types in New Zealand waters to associate with and associating with them for the few times that they are nutritionally beneficial is more cost effective than not associating with them at all. The times of year that the holobiont receives energy surplus to its requirements may give it a competitive edge over other organisms within its habitat and may go towards the production of anemone gametes (Muller-Parker and Davy 2001). As the symbionts are vertically transmitted, this would also mean increasing the population and distribution of the *Symbiodinium* algae. *Symbiodinium* cells may be maternally transferred due to the scarcity of *Symbiodinium* cells in temperate seas and/or a shortage of potential hosts (e.g. *A. aureoradiata* is the only known host of *Symbiodinium* in

New Zealand). Moreover, the stability of the partnership combined with its mode of symbiont acquisition may be the underlying reason why the holobiont has persisted and been an ecological success around New Zealand.

4.3 What can be done to further understand *Symbiodinium* types in clade A

Measuring the amount and ratio of different lipid types and pigments in the thylakoid membrane of the *Symbiodinium* chloroplast would help clarify the mechanisms of NPQ that aid the apparent resilience of the algae to thermal stress. Gas chromatography and high-performance liquid chromatography (HPLC) can be used to identify the lipid types within the membrane (Plante et al. 2011) and whether the polyunsaturated lipid content increases as latitude increases. Using the same samples, HPLC can be used to study the pigment profile of the membranes (Venn et al. 2006, Wakahama et al. 2012). The study of two *Symbiodinium* types in clade A by Kraemer et al. (2012) found that there was a reduction in light-harvesting pigments and an increase in the xanthophyll pool size when under thermal stress. Reducing the light-harvesting pigments would decrease the amount of photons in the photosystems, which in turn would decrease photochemical efficiency of PSII. This could have been the reason for the low to zero measurements in this study for the effective quantum yield ($\Delta F/F_m'$) of samples during dark-light induction, especially those in the highlight treatments, and also for the zero $\Delta F/F_m'$ for all treatments once the irradiance reached $400 \pm 50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the rapid light curves. Hennige et al. (2009) also found that *Symbiodinium* cells exposed to high light had a decrease in their photosynthetically active pigments, peridinin and chlorophylls *c* and *a*. Other papers also found an increase in the xanthophyll pool (diadinoxanthin and daitoxanthin) within the thylakoid membrane, which would allow for faster heat dissipation and dispersal of too much light energy in the photosystem (e.g. Venn et al. 2006, Warner and Berry-Lowe 2006, Ulstrup et al. 2008, Hennige et al. 2009, Kraemer et al. 2012).

Resilience studies would also have to include the anemone, whose behaviour and physiology could protect the symbionts. From personal observations of the anemone, it was difficult to measure fluorescence using the PAM fluorometer once the anemone had

withdrawn its tentacles, presumably due to the animal tissue being thick enough to reduce the amount of light reaching the algae. On mudflats, the anemones positioned on the surface were seen to be open in the early morning light but would close nearer noon. This was also seen in rockpools, though anemones on the rocky shore were more plentiful within cracks in the rocks. The anemone could also protect its symbionts by storing UV- absorbing mycosporine-like amino acids (MAAs) (Shick and Dunlap 2002, Lesser and Farrell 2004) and heat-shock proteins (Fitt et al. 2009) as well as animal tissue attenuating and reflecting light away from the algal cells (Banaszak et al. 2000).

4.4 Concluding Remarks

Anthopleura aureoradiata from New Zealand hosts two ITS2 types of *Symbiodinium* from clade A, which are similar to or identical to those in the anemones sampled from the South of England and from Brittany (France). All these types most closely match ITS2 sequences of *Symbiodinium* Amed and *Symbiodinium* Mediterranean clade A (Hunter et al. 2007, Meron et al. 2012), with most harbouring Med clade A. There was no clear latitudinal, seasonal or habitat-related pattern of symbiont distribution, or any pattern with respect to whether an anemone hosted just one symbiont type or both simultaneously. Further molecular analyses using more sensitive methods, such as quantitative PCR would be able to clarify this, especially now that the *Symbiodinium* types have been identified and unique primers can be designed. Likewise, running PCR-RFLP on some samples would conclusively show whether the ITS2 sequences from the *Symbiodinium* types here could also be classified as ‘phylotype Temperate A’, and so be included in the same group as the northern hemisphere *Symbiodinium* in cnidarians studied by Bythell et al. (1997), Savage et al. (1997), Visram et al. (2006) and Forcioli et al. (2011).

Like other temperate symbiotic anemones, *A. aureoradiata* relies less on its symbionts’ photosynthetic resources than tropical symbiotic cnidarians do (Muller-Parker and Davy 2001), though the benefits gained may give the host a competitive advantage in its habitat (Muller-Parker and Davy 2001). Resilience to temperate conditions by the partnership between *A. aureoradiata* and its symbionts, Med clade A and Amed, is probably due to the

physiological benefits the symbionts, and other clade A *Symbiodinium*, have to photoprotect themselves against harsh and changeable conditions. Episodes of bleaching in tropical regions are occurring more frequently, with climate changes affecting temperature and weather patterns (Hoegh-Guldberg and Bruno 2010). However, climate change is happening on a global scale and affects temperate regions too. Understanding the photophysiology of cnidarian-*Symbiodinium* symbiosis in temperate regions, and how it responds to environmental change, will help understand whether these organisms have as gloomy an outlook as their tropical counterparts, or whether the resilience built by adaptation to variable temperate conditions has made them more likely to survive and continue into the future. Temperate regions may become a refuge for the 235 million year-old cnidarian-*Symbiodinium* symbiosis if warming continues at a rate too fast for tropical partnerships to acclimate, adapt and survive.

Figure 4.3: Early morning collection of anemones on the mudflats of Pauatahanui Inlet.

Appendices

APPENDIX 1 – Climatic conditions in New Zealand for 5 years prior to molecular study

A1.1 Temperature and radiance for New Zealand

Figure A1.1.1: Mean ($\pm$ SE) daily temperature and radiance through the seasons for 5 years prior to the molecular latitudinal study (autumn 2007-summer 2012) for the 5 collection sites. Data for the 5 collection sites came from NIWA's National Climate Database (Cliflo Retrieved 6 June 2012). Tiwai Point station, Bluff for Stewart Island; Musselburch Ews for Dunedin; Paraparaumu Aero Aws for Pauatahanui; Port Taharoa (temperature) and Hamiton Ruakura 2 Ews (radiation) for Raglan and; Cape Reinga Aws for Parengarenga Harbour.

A1.2 Temperature and radiance at the seasonal and habitat sites

Figure A1.2.1: Mean ($\pm$ SE) daily temperature and radiance through the seasons for 5 years prior to the molecular seasonal and habitat study (autumn 2007–summer 2012) for the 2 collection sites. Data for the collection sites came from NIWA’s National Climate Database (Cliflo Retrieved 6 June 2012). Paraparaumu Aero Aws for Pauatahanui and; Wellington Kelburn for Point Halswell.

A1.3 Weather stations used to collect climate data

Table A1.3.1: Collection sites and their geographical coordinates for the molecular and photophysiology studies with the weather stations used to collect climatic data from NIWA's National Climate Database (Cliflo Retrieved 6 June 2012)

Collection Site	Coordinates	Weather Station	Database Station Agent Number
Stewart Island	46° 54' 346'' S, 168° 00' 589'' E	Tiwai Point Ews	5823
Dunedin	45° 46' 60'' S, 170° 41' 60'' E	Mussleburgh Ews	15752
Pauatahanui	41° 6' 4.85'' S, 174° 52' 17'' E	Paraparaumu Aero Aws	8567
Point Halswell, Kau Bay	41° 17' 2.42'' S, 174° 49' 33.89'' E	Wellington Kelburn	25354
Raglan	37° 47' 52'' S, 174° 42' 36'' E	Port Taharoa (Temp) Hamilton Ruakura (Rad)	2136 12616
Parengarenga Harbour	34° 32' 5'' S, 172° 56' 43'' E	Cape Reinga Aws (Temp) Kaitaia Ews (Rad)	1002 17067

APPENDIX 2 – Molecular sequences of *Symbiodinium* types in this study

A2.1 Coding used to identify samples in this study

Table A2.1.1: Coding used in this study to identify the species of anemone the *Symbiodinium* cells came from, the place and habitat they originated from and the season they were collected.

Sample code	Anemone species	Origin or place, habitat and season collected
Ab	<i>Anthopleura ballii</i>	Collector's aquarium in Plymouth, England
Av	<i>Anemonia viridis</i>	Plymouth, England
AM	<i>Aiptasia mutabilis</i>	Roscoff in Brittany, France
C	<i>Cereus pedunculatus</i>	Plymouth, England
D	<i>Anthopleura aureoradiata</i>	Dunedin, New Zealand
KA	<i>Anthopleura aureoradiata</i>	Kau Bay, autumn, midlittoral zone
KU	<i>Anthopleura aureoradiata</i>	Kau Bay, summer, midlittoral zone
KW	<i>Anthopleura aureoradiata</i>	Kau Bay, winter, midlittoral zone
PA	<i>Anthopleura aureoradiata</i>	Pauatahanui Inlet, autumn
PH	<i>Anthopleura aureoradiata</i>	Parengarenga Harbour, New Zealand
PU	<i>Anthopleura aureoradiata</i>	Pauatahanui Inlet, summer
PW	<i>Anthopleura aureoradiata</i>	Pauatahanui Inlet, winter
R	<i>Anthopleura aureoradiata</i>	Raglan, New Zealand
S	<i>Anthopleura aureoradiata</i>	Stewart Island, New Zealand
W	<i>Anthopleura aureoradiata</i>	Wellington, New Zealand

A2.2 Sequences of sample used for phylogenetic analysis

Table A2.2.1: DNA sequence alignment used for phylogenetic analysis obtained from Geneious v 5.6.2 (Drummond et al. 2011). First column is the identification code for each sample analysed.

	1	10	20	30	40	50	60
Ab1	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTTTGGT						
Ab2	AAGC----GTCTCT--AAAGCGCATTGAGCTCTCGGGGATTCTTGAGAGCATGTCTGCT						
Av1	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
Av2	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
Av3	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
Av11	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
AM1	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
AM2	AACCAATGGCCTCTTGAACGTGCATTGCGCTCT--GGGATATGCCTGAGAGCATGTCTGCT						
AM3	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
AM4	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
AM5	AACCAATGGCCTCTTGAACGTGCATTGCGCTCT--GGGATATGCCTGAGAGCATGTCTGCT						
AM6	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
AM7	AACCAATGGCCTCTTG--ACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
AM9	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
AM10	AACCAATGGCCTCTTG--ACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
C1	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
C3	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
C4	-----CCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
C5	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
C6	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
D1	AACCAATGGCCTYTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
D3	AACCAATGGCCTYTTGAACGTGCATTGCGCTYTTGGGATATGCCTGAGAGCATGTCTGCT						
D5	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
D9	AACCAATGGCCTNNTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTNTGCT						
D10	--CCAATGGCCTNNTGAACGTGCATTGCGCTNNTGGGATATGCCTGAGAGCATGTNTGCT						
KA21	AACCAATGGCCTTTTGAACGTGCATTGCGCTTTTGGGATANGCCTGAGAGCANGTNNNT						
KA25	AACCAATGGCCTNNTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KA37	AACCAATGGCCTNNTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KA22	AACCAATGGCCTNNTGAACGTGCANTGCGCTNNTGGGATATGCCTGAGAGCATGTCTGCT						
KA34	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						

	1	10	20	30	40	50	60
KA38	AACCAATGGCCTNNTTGAACGTGCATTGNGCTNNTTGGGATATGCCTGAGAGCATGTCTGCT						
KA11	A-CCAATGGCCTTTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KA15	A-CCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KA27	A-CCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KA31	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KA39	A-CCAATGGCCTNNTTGAACGTGCATTGNGCTNNTTGGGATATGCCTGAGAGCATGTCTGCT						
KA28	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KA40	AACCAATGGCCTNNTTGAACGTGCATTGNGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KU2	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
K38	AACCAATGGCCTTTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KU7	A-CCAATGGCCTCTTGAACGTGCATTGCGCTTTTGGGATATGCCTGAGAGCATGTCTGCT						
KU15	---CAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KW9	AACCAATGGCCTTTTGAACGTGCATTGCGCTTTTGGGATATGCCTGAGAGCATGTCTGCT						
KW13	AACCAATGGCCTNNTTGAACGTGCATTGTGCTNNTTGGGATATGCCTGAGAGCATGTCTGCT						
KW17A	AACCAATGGCCTYTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KW10	A-----ACGTGCATTGCGCTCTTGGGAT-NGCCTGAGAGCATGT-NGCN						
KW14	AACCAATGGCCTCTTGAACGTGCATTGNGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KW11	AACCAATGGCCTTTTGAACGTGCATTGCGCTTTTGGGATATGCCTGAGAGCATGTCTGCT						
KW15	AACCAATGGCCTNNTTGAACGTGCANTGNGCTNNTTGGGATANGCCTGAGAGCATGTCTGCT						
KW39A	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KW39B	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KW39C	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KW39D	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
KW39E	AACCAATGGCCTTTTGAACGTGCATTGCGCTTTTGGGATATGCCTGAGAGCAAGTCCACT						
KW39F	AACCAAT-GCCTTTTGAACGTGCATTGCGCT-TTGGGATATGCCTGAGAGCAAGTCCACT						
KW12	AACCAATGGCCTTTTGAACGTGCATTGCGCTCTTGGGATANGCCTGAGAGCANGTGAGCT						
PA10	AACCAATGGCCTTTTGAACGTGCANTGGGCTTTTGGGATTTGGCTGAGAGCATGTCTGCT						
PH1	AACCAATGGCCTYTTGAACGTGCATTGSGCTYTTGGGATATGCCTGAGAGCATGTCTGCT						
PH7	AACCAATGGCCTTTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
PH9	AACCAATGGCCTNNTTGAACGTGCATTGNGCTTTTGGGATATGCCTGAGAGCANGTNTGCT						
PU 10	AACCAAAGGCCTTTTGAATGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
R5	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
R7	AACCAATGGCCTTTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
S1	AACCAATGGCCTYTTGAACGTGCATTGCGCTYTTGGGATATGCCTGAGAGCATGTCTGCT						
S3	AACCAAWGGCCTCTTGAACGTGCATTGCGCTYTTGGGATATGCCTGAGAGCATGTCTGCT						
S5	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
S7	AACCAATGGCCTCTTGAACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						
S8	AACCAATGGCCTCTTG-ACGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCT						

	1	10	20	30	40	50	60
S9	A	A	C	C	A	A	T
S10	A	A	C	C	A	A	T
W1	A	A	C	C	A	A	T
W5	A	A	C	C	A	A	T
W6	A	A	C	C	A	A	T

	61	70	80	90	100	110
Ab1	T	C	A	G	T	G
Ab2	T	C	A	G	T	G
Av1	T	C	A	G	T	G
Av2	T	C	A	G	T	G
Av3	T	C	A	G	T	G
Av11	T	C	A	G	T	G
AM1	T	C	A	G	T	G
AM2	T	C	A	G	T	G
AM3	T	C	A	G	T	G
AM4	T	C	A	G	T	G
AM5	T	C	A	G	T	G
AM6	T	C	A	G	T	G
AM7	T	C	A	G	T	G
AM9	T	C	A	G	T	G
AM10	T	C	A	G	T	G
C1	T	C	A	G	T	G
C3	T	C	A	G	T	G
C4	T	C	A	G	T	G
C5	T	C	A	G	T	G
C6	T	C	A	G	T	G
D1	T	C	A	G	T	G
D3	T	C	A	G	T	G
D5	T	C	A	G	T	G
D9	T	C	A	G	T	G
D10	T	C	A	G	T	G
DQ865210 - Amed	T	C	A	G	T	G
JN242191 -Med Clade A	T	C	A	G	T	G
KA 21	T	C	A	G	T	G

	61	70	80	90	100	110
KA 25	TCAGTGCTTCTTATATCAATTTTTTTGNTGNTGNTCTTTTT	-----	CAAAGGG			
KA 37	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KA 22	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KA 34	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KA 38	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KA 11	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KA 15	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KA 27	TCAGTGCTTCTTATATCCACTTGTT-GCTGCTGCTCTTTYK	-----	TTAAAGGG			
KA 31	TCAGTGCTTCTTATATCCACTTGTT-GCTGCTGCTCTTNT	-----	TYAAAGGG			
KA 39	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KA 28	TCAGTGCTTCTTATATCCACTTGTT-GCTGCTGNTCTTT	--CG---	TNAAAGGG			
KA 40	TCAGTGCTTCTTATATCAATTTTTTTGNTGNTGNTCTTTTT	-----	CAAAGGG			
KU 2	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KU 38	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KU 39	TCAGTGCTTCTTATATCAATTTTTTTGYKGCTGYTCTTTTT	-----	CAAAGGG			
KU 15	TCAGTGCTTCTTATATCAATTTTTTTG-TGCTGCTCTTTTT	-----	CAAAGGG			
KW 9	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KW 13	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KW 17A	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTYTTTTT	-----	CAAAGGG			
KW 10	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KW 14	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KW 11	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KW 15	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KW 39A	TCAGTGCTTCTTATATCMATTTTTTTGYTGCKGCTCTTTTT	-----	CAAAGGG			
KW 39B	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KW 39C	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KW 39D	TCAGTGCTTCTTATATCMATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KW 39E	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KW 39F	TCAGTGCTTCTTRTWKCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
KW 12	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
PA10	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	AAGGG			
PH1	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
PH7	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
PH9	TCAGTGCTTCTTATATCAATTTTTTTGTTGTTGNTCTTTTT	-----	CAAAGGG			
PU 10	TCAGTGCTTCTTATATCAATTTTTTTGYKGYKGCTCTTTTT	-----	CAAAGGG			
R5	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			
R7	TCAGTGCTTCTTATATCAATTTTTTTGTTGYTGCTCTTTTT	-----	CAAAGGG			
S1	TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT	-----	CAAAGGG			

S3 TCAGTGCTTCTTATATCAATTTTTTTGCTGYBGCTCTTTTT-----CAAAGGG
S5 TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT-----CAAAGGG
S7 TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT-----CAAAGGG
S8 TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT-----CAAAGGG
S9 TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT-----CAAAGGG
S10 TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT-----CAAAGGG
W1 TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGCTCTTTTT-----CAAAGGG
W5 TCAGTGCTTCTTATATCAATTTTTTKGYKGYTGCTCTTTTT-----YAAAGGG
W6 TCAGTGCTTCTTATATCAATTTTTTTGCTGCTGTTCTTTTT-----CAAAGGG

	115	125	135	145	155	163
Ab1	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGC---GTAGA					
Ab2	CAGCGTGGGKGCATGCTACTGCTTGGCAGCACTGGCATGC---GTAGA					
Av1	CAGCGTGGGTGCATGCTACTGCTTGCAGCACTGGCATGC---GTAGA					
Av2	CAGCGTGGGTGCATGCTACTGCTTGCAGCACTGGCATGC---GTAGA					
Av3	CAGCGTGGGTGCATGCTAC-GCTT-GCAGCACTGGCATGC---GTAGA					
Av11	CAGCGTGGGTGCATGCTACTGCTTGCAGCACTGGCATGC---GTAGA					
AM1	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
AM2	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
AM3	CAGCGTGGGTGCATGCTACTGCTTGCAGCACTGGCATGC---GTAGA					
AM4	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
AM5	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
AM6	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
AM7	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
AM9	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
AM10	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
C1	CAGCGTGGGTGCATGCTACTGCTTGCAGCACTGGCATGC---GTAGA					
C3	CAGCGTGGGTGCATGCTACTGCTTGCAGCACTGGCATGC---GTAGA					
C4	CAGCGTGGGTGCATGCTACTGCTTGCAGCACTGGCATGC---GTAGA					
C5	CAGCGTGGGTGCATGCTACTGCTTGCAGCACTGGCATGC---GTAGA					
C6	CAGCGTGGGTGCATGCTACTGCTTGCAGCACTGGCATGC---GTAGA					
D1	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
D3	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
D5	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
D9	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
D10	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
DQ865210 - Amed	CAGCGTGGGTGCATGCTACTGCTTGCAGCACTGGCATGC---GTAGA					
JN242191 -Med Clade A	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA					
KA 21	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TANA					

	115	125	135	145	155	163
KA 25	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KA 37	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KA 22	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KA 34	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KA 38	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KA 11	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KA 15	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KA 27	CAGCGTGGGTGCATGCTACTGCTTKGCAGCACTGGCATGC---	GTAGA				
KA 31	CAGCGTGGGTGCATGCTACTGCTTKGCAGCACTGGCATGC---	GTAGA				
KA 39	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KA 28	CAGCGTGGGTGCATGCTACTGCTTKGCAGCACTGGCATGC---	GTAGA				
KA 40	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KU 2	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KU 38	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KU 7	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KU 15	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 9	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 13	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 17A	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 10	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 14	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 11	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 15	CAGCGNGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 39A	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 39B	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 39C	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 39D	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 39E	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 39F	CAGCGTGGGTGCATGCTMCTGCTTGGCAGCACTGGCATGCT---	TAGA				
KW 12	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
PA10	CANCGNGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TANA				
PH1	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
PH7	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
PH9	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
PU 10	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
R5	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				
R7	CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---	TAGA				

S1 CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA
 S3 CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA
 S5 CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA
 S7 CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA
 S8 CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA
 S9 CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA
 S10 CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA
 W1 CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---YAGA
 W5 CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA
 W6 CAGCGTGGGTGCATGCTACTGCTTGGCAGCACTGGCATGCT---TAGA

	164	174	184	194	204	214
Ab1	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTKWATCAAAT-CTTGTC					
Ab2	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTCTATCAAAT-CTTGTC					
Av1	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
Av2	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
Av3	TTTGC-GCTTGCTCA-C-GGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
Av11	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
AM1	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
AM2	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
AM3	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
AM4	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
AM5	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
AM6	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
AM7	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
AM9	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
AM10	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
C1	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
C3	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
C4	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
C5	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
C6	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
D1	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
D3	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-MTTGTC					
D5	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
D9	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-NTTGTC					
D10	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					

	164	174	184	194	204	214
DQ865210 - Amed	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
JN242191 -Med Clade A	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KA 21	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-NTTGTC					
KA 25	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAATT-TTGTC					
KA 37	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-NTTGTC					
KA 22	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-NTTGTC					
KA 34	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KA 38	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KA 11	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KA 15	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-MTTGTC					
KA 27	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
KA 31	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
KA 39	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-NTTGTC					
KA 28	TTTGCTGCTTGCTCA-CTGGTTC-GA-TTGATCACTTCATCAAAT-CTTGTC					
KA 40	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KU 2	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KU 38	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KU 7	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KU 15	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KW 9	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-MTTGTC					
KW 13	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KW 17A	TTTGCTGCTTGCTCANCTGGTT-TGA-TTGATCACTTTWTCAAAT-MTTGTC					
KW 10	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KW 14	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-NTTGTC					
KW 11	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KW 15	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KW 39A	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KW 39B	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KW 39C	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KW 39D	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KW 39E	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KW 39F	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
KW 12	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
PA10	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					
PH1	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAATA-TTGTC					
PH7	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAATA-TTGTC					
PH9	TTTGCTGCTTGCTCA-CTGGTT-NGA-TTGATCACTTTATCAAAT-CTTGTC					
PU 10	TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC					

R5 TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCA-TTTATCAAAT-CTTGTC
 R7 TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC
 S1 TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC
 S3 TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC
 S5 TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC
 S7 TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC
 S8 TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC
 S9 TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC
 S10 TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-CTTGTC
 W1 TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAAT-MTTGTC
 W5 TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAATA-TTGTC
 W6 TTTGCTGCTTGCTCA-CTGGTT-TGA-TTGATCACTTTATCAAATCTTGTC

	217	227	237	247	257	267	276
Ab1	AGTATTGCA-AGTCTTGCGTAT-GTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
Ab2	AGTATTGCA-AGTCTTGCGTAT-GTGTTGT-GACACATCCTAGAAAAGAA--CACACACA						
Av1	AGTGTTGCA-AGTCTTGCGTAT-GTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
Av2	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
Av3	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
Av11	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
AM1	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
AM2	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
AM3	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
AM4	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
AM5	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
AM6	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
AM7	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
AM9	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
AM10	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
C1	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
C3	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
C4	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
C5	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
C6	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
D1	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						

	217	227	237	247	257	267	276
D3	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTASCATGAAGTCARACA-A						
D5	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
D9	AGTATTGCA-AGTCTTGNNGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCANACA-A						
D10	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTANCATGAAGTCA-ACA-A						
DQ86521-Amed	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
JN242191-Med Clade A	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
KA21	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACNTCCTANCNTGAANTCANACA-A						
KA25	AGTNTTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
KA37	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAANTCANACA-A						
KA22	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTANCATGAANTCANACA-A						
KA34	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTANCATGAANTCANACA-A						
KA38	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTANCATGAANTCANACA-A						
KA11	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGTCA-A						
KA15	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCAGACA-A						
KA27	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
KA31	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCANACA-A						
KA39	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTANCNTGAAGTCANACA-A						
KA28	AGTGTTGCA-AGTCTTGCGTA-TGTGTT-TTGACACATCCTAGCATGAAGTCAGACA-A						
KA40	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GACACATCCTAGCATGAAGTCANACA-A						
KU2	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
KU38	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
KU7	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
KU15	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
KW9	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAAACA-A						
KW13	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTANCATGAANTCANACA-A						
KW17A	AGTATTGCA-AGTCTTGTGTWK-GTGTTGT-GAC-ACWTCCTASCATGAAGWMARAMA-A						
KW10	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTANCATGAAGTCANACA-A						
KW14	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAANTCANACA-A						
KW11	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
KW15	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTANCNTGAANTCANACA-A						
KW39A	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
KW39B	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
KW39C	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
KW39D	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
KW39E	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAAACA-A						
KW39F	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAAACA-A						
KW12	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-NCNTCCNANCNTGAANTCANACA-A						

	217	227	237	247	257	267	276
PA10	AGTATTGCA-AGTCTTGTGT--TGTGTTGT-GAC-ACATCCTAGCATGAANNCANA-A-A						
PH1	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTARCATGAAGTCAGACA-A						
PH7	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAAACA-A						
PH9	AGTATTGCA-AGTCTTNGTA-TGTGTTNT-GAC-ACATCCTANCATGAAGTCANACA-A						
PU10	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGAC--A						
R5	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
R7	AGTATTGCA-AGTCTTGTGTA-TGTGTT-TTGAC-ACATCCTAGCATGAAGTCAGACA-A						
S1	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAKTCARACA-A						
S3	AGTATTGCA-AGTCTTGKGT-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCARACA-A						
S5	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
S7	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
S8	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						
S9	AGTATTGCA-AGTCTTNGTA-TGTGTTGT-GAC-NCATCCTAGCATGAAGTCANACA-A						
S10	AGTATTGCA-AGTCTTNGTA-TGTGTTGT-GAC-NCATCCTAGCATGAAGTCAGACA-A						
W1	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCWARCWYGAASTCASACA-A						
W5	AGTATTGCA-AGTCTTGTGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGAC--A						
W6	AGTATTGCA-AGTCTTGGTA-TGTGTTGT-GAC-ACATCCTAGCATGAAGTCAGACA-A						

A2.3 Sequences of Reference *Symbiodinium* Types

Table A2.3.2: DNA sequence alignment of reference *Symbiodinium* types used for phylogenetic analysis obtained from Geneious v 5.6.2 (Drummond et al. 2011). Sequences retrieved from the National Centre for Biotechnology Information (NCBI) BLAST database and from the ITS2 sequence database (GeoSymbio) from the Gates Laboratory at HIMB (Franklin et al. 2012)

		1	10	20	30	40	50	60
AF333504	- A1.1	A	A	A	A	A	A	A
AB207210	- A1.2	A	A	A	A	A	A	A
DQ838546	- A3	A	A	A	A	A	A	A
AF333509	- A4	A	A	A	A	A	A	A
AF333508	- A5	A	A	A	A	A	A	A
AY686646	- A6	A	A	A	A	A	A	A
AY239360	- A7	A	A	A	A	A	A	A
AY258468	- A8	A	A	A	A	A	A	A
FJ224014.1	- A9	A	A	A	A	A	A	A
EU792882	- A10	A	A	A	A	A	A	A
FJ224013.1	- A11	A	A	A	A	A	A	A
EU792883	- A12	A	A	A	A	A	A	A
AF333504	- A13	A	A	A	A	A	A	A
EU792884	- A15	A	A	A	A	A	A	A
EU792888	- A16	A	A	A	A	A	A	A
AF333505	- A1	A	A	A	A	A	A	A
JN558104.1-Gym simplex		A	A	A	A	A	A	A
AF333506	- A2	A	A	A	A	A	A	A
DQ865210	- Amed	A	A	A	A	A	A	A
JN242191-MedClade A		A	A	A	A	A	A	A
		61	70	80	90	100	110	120
AF333504	- A1.1	C	A	T	T	C	T	T
AB207210	- A1.2	T	C	A	G	T	C	T
DQ838546	- A3	T	C	A	G	T	C	T
AF333509	- A4	T	C	A	G	T	C	T
AF333508	- A5	T	C	A	G	T	C	T
AY686646	- A6	T	C	A	G	T	C	T
AY239360	- A7	T	C	A	G	T	C	T
AY258468	- A8	T	C	A	G	T	C	T
FJ224014.1	- A9	T	C	A	G	T	C	T
EU792882	- A10	T	C	A	G	T	C	T

	61	70	80	90	100	110	120
FJ224013.1 - A11	TCAGTGCTTCT--ACTA-TC---	TTTTCTGC-----	TGCTCTT----	GTTGTCA--GG-			
EU792883 - A12	TCAGTGCTTCT--ACT-TTCA--	TTTTCTGC-----	TGCTCTT----	GCTATCA--GG-			
AF333504 - A13	TCAGTGCTTCT--ACT-TTCA--	TCTTCTGC-----	TGCTCTT----	GTTATCA--GG-			
EU792884 - A15	TCAGTGCTTCT--ACT-TTC---	TTCTCTG-G-----	TGCTCTT----	GTTATCA--TG-			
EU792888 - A16	TCAGTGCTTCT--ACT-TTC---	TTTTCTGC-----	TGCTCTT----	GTTATCA--TG-			
AF333505 - A1	TCAGTGCTTCT--ACT-TTCA----	TTTT-C-TGC--	TGCTCT---	T-GTTATCA----	G		
JN558104.1-Gym simplex	TCAGTGCTTGTGGAATCCTCA-----	TGCATGCGTTGCTCT---	CCG-----				
AF333506 - A2	TCAGTGCTTCTA-ACTC-TCTATGTGT--	CC-TGC--	TGCTCTC-TCCCTT-TGA--	G--			
DQ865210 - Amed	TCAGTGCTTCTT-A-TA-TCCACTTGTT-GC-TGC--	TGCTCTT--	TCGTT---AAAGGG				
JN242191-Med Clade A	TCAGTGCTTCTT-A-TA-TCAATTTTTTGC-TGC--	TGCTCTTTTT-----	CAAAGGG				

	121	130	140	150	160	170	180
AF333504 - A1.1	-AGCAGTGTGCTGCATG---CTTCTGCAAGTG---	GCACTGGCAT-GCTAAATATCAAG					
AB207210 - A1.2	-AGCAGTGTGCTGCATG---CTTCTGCAAGTG---	GCACTGGCAT-GCTGAATATCAAG					
DQ838546 - A3	-AGCAGTGTGCTGCATG---CTTCTGCAATTG---	GCACTGGCAT-GCTAAGTACCAAG					
AF333509 - A4	GAGCAGTGTGCTGCATG---CTTCTGCAAAAG---	GCACTGGCAT-GCTAAGTATCAAG					
AF333508 - A5	-AGCAGTGTGCTGCATG---CTTCTGCAATGG---	GCACTGGCAT-GCTAAGTACCAAG					
AY686646 - A6	-AGCAGTGTGCTGCATG---CTTCTGCAATTG---	GCACTGGCAT-GCTAAGTACCAAG					
AY239360 - A7	-AGCAGTGTGCTGCATG---CTTCTGCAATTG---	GCACTGGCAT-GCTAAATACCAAG					
AY258468 - A8	-AGCAGTGTGCTGCATG---CTTCTGCAATTG---	GCACTGGCAT-GCTAAGTATCAAG					
FJ224014.1 - A9	-AGCAGTGTGCTGCGTG---CTTCTGCAAGTG---	GCACTGGCAT-GCTAAATATCAAG					
EU792882 - A10	-AGCAGTGTGCTGCAAG---CTTCTGCAAGTG---	GCACTGGCAT-GCTAAATATC--G					
FJ224013.1 - A11	-AGCAGTGTGCTGCATG---CTTCTGCAATTG---	GCACTGGCAT-GCTAAGTACCAAG					
EU792883 - A12	-AGCAGTGTGCTGCATG---CTTCTGCAAGCG---	GCACTGGCAT-GCTAAATATCAAG					
AF333504 - A13	-AGCAGTGTGCTGCATG---CTTCTGCAAGTG---	GCACTGGCAT-GCTAAATATCAAG					
EU792884 - A15	-AGCAGTGTGCTGCATG---CTTCTGCAATTG---	GCACTGGCAT-GCTAAGTACCAAG					
EU792888 - A16	-AGCAGTGTGCTGCATG---CTTCTGCAATTG---	GCACTGGCAT-GCTAGGTACCAAG					
AF333505 - A1	GAGCAGTGTGCTGCATG---CTTCTGCAAGTGGC---	ACTGGCAT-GCTAAATATCAAG					
JN558104.1-Gym simplex	-AGC-----GATGCATGTGTCTT-TG----	TG-----TGCCACAGCTTAGCAT----					
AF333506 - A2	-AGCAGTGTGCTGCATG---CTACTGCGCT--	GCTGCACTGGCAT-GCTCAATATCAAG					
DQ865210 - Amed	CAGC-GTG--GGTGCATG---CTACTGC--	TTTGACGCACTGGCAT-GC---GTA--GA-					
JN242191 -Med Clade A	CAGC-GTG--GGTGCATG---CTACTGC--	TTGGCAGCACTGGCAT-GCT---TA--GA-					

	181	190	200	210	220	230	240
AF333504 - A1.1	TTT--TGCTT-G-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
AB207210 - A1.2	TTT--TGCTT-G-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
DQ838546 - A3	TTT-C-GCTT-G-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
AF333509 - A4	TTT--TGCTT-G-CT-----	GTTCTGACT-GAT-----	CAACAT-CTCATGTC-				
AF333508 - A5	TTT--TGCTT-G-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
AY686646 - A6	TTT-C-GCT-CG-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
AY239360 - A7	TTT--TGCTT-G-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
AY258468 - A8	TTT--TGCTT-G-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
FJ224014.1 - A9	TTT--TGCTT-G-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
EU792882 - A10	TTT--TGCTT-G-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
FJ224013.1 - A11	TTT-C-GCTT-G-CT-----	GTTGTGAC-AGAT-----	CAACAT-CTCATGTC-				
EU792883 - A12	TTT--TGCTT-G-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
AF333504 - A13	TTT--TGCTT-G-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
EU792884 - A15	TTT--TGCTT-G-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
EU792888 - A16	TTT--TGCTT-G-CT-----	GTTGTGACT-GAT-----	CAACAT-CTCATGTC-				
AF333505 - A1	TTT--TGCTT--GCT-----	GTTGTGAC-TGATC-A-----	AC-AT-CTCATGTC-				
JN558104.1-Gym simplex	-----TGCTTCGGCTC-----	TGTG----	GAGCGAG---	TGGCAC-	ATT	CAGGTG	CTCT
AF333506 - A2	TTT-----T--GCCCA-CTGGTT-TGACTTGATC-ATC---	ATC-----T--TGTC-					
DQ865210 - Amed	TTTGCTGCTT--GCTCA-CTGGTTC-GA-TTGATC-A-CTTCATCAA-AT-CT--	TGTC-					
JN242191-Med Clade A	TTTGCTGCTT--GCTCA-CTGGTT-TGA-TTGATC-A-CTTTATCAA-AT-CT--	TGTC-					

	241	250	260	270	280	290	300
AF333504 - A1.1	-GT-TT-CAG----	TTG-GCGAAACA--	AAAGCTCATGTGTGTTCT--	TAACA-CTTCCT			
AB207210 - A1.2	-GT-TT-CAG----	TTG-GCGAAACA--	AAAGCTCATGTGTGTTCT--	TAACA-CTTCCT			
DQ838546 - A3	-GT-TT-CAG----	TTG-GCGAAACA--	AAGGCT--TGTGTGTTTTC---	CAACA-CTTCCT			
AF333509 - A4	-GT-TT-CAG----	TTG-GCGAAACA--	AAAGCTGAAGTGTGTTCT--	TAACA-CTTCCT			
AF333508 - A5	-GT-TT-CAG----	TTG-GCGAAACA--	AAGGCT--TGTGTGTTTTC---	CAACA-CTTCCT			
AY686646 - A6	-GT-TT-CAG----	TTG-GCGAAACA--	AAGGCT--TGTGTGTTTTC---	CAACA-CTTCCT			
AY239360 - A7	-GT-TT-CAG----	TTG-GCGAAACA--	AAGGCT--TGTGTGTTTTC---	CAACA-CTTCCT			
AY258468 - A8	-GT-TT-CAG----	TTG-GCGAAACA--	AAGGCT--TGTGTGTTTTC---	CAACA-CTTCCT			
FJ224014.1 - A9	-GT-TT-CAG----	TTG-GCGAAACA--	AAAGCTCATGTGTGTTCT--	TAACA-CTTCCT			
EU792882 - A10	-GT-TT-CAG----	TTG-GCGAAACA--	AAAGCTCATGTGTGTTCT--	TAACA-CTTCCT			
FJ224013.1 - A11	-GT-TT-CAG----	TTGA-TGAAAC---	AAGGCT--TGCGTGTTTTC---	CAACA-CTTCCT			
EU792883 - A12	-GT-TT-CAG----	TTG-GCGAAACA--	AAAGCTCATGTGTGTTCT--	TAACA-CTTCCT			
AF333504 - A13	-GT-TT-CAG----	TTG-GCGAAACA--	AAAGCTCATGTGTGTTCT--	TAACA-CTTCCT			
EU792884 - A15	-GT-TT-CAG----	TT-AGCGAAACA--	AAGGCT--TGTGTGTTTTC---	CAACA-CTTCCT			
EU792888 - A16	-GT-TT-CAG----	TTG-GCGAAACA--	AAGGCT--TGTGTGTTTTC---	CAACA-CTTCCT			
AF333505 - A1	-G--TTTCAG----	TTG-GCGAAACA--	AAAGCTCATGTGTGTTCTT--	AAC-ACTTCCT			
JN558104.1-Gym simplex	AG-ATTGC-----	CTTG-GCA-----	TCT----	ACA	ACTT---		
AF333506 - A2	----TTGCAGA---	TTGGGTAAATAGGAAAT-----	AGTGTT--	TGCAAC-	ACTTCCT		

DQ865210 - Amed AGTGTGCA-AGTCTTGCCTA---T-----GTGTT-TTG--AC-ACATCCT
 JN242191-Med Clade A AGTATTGCA-AGTCTTGTGTA---T-----GTGTTGT-G--AC-ACATCCT

	301	310	318
AF333504 - A1.1	AGCATGAAGTCAGACA-A		
AB207210 - A1.2	AGCATGAAGTCAGACA-A		
DQ838546 - A3	AGCATGAAGTCAGACA-A		
AF333509 - A4	AGCATGAAGTCAGACA-A		
AF333508 - A5	AGCATGAAGTCAGACA-A		
AY686646 - A6	AGCATGAAGTCAGACA-A		
AY239360 - A7	AGCATGAAGTCAGATA-A		
AY258468 - A8	AGCATGAAGTCAGATA-A		
FJ224014.1 - A9	AGCATGAAGTCAGACA-A		
EU792882 - A10	AGCATGAAGTCAGACA-A		
FJ224013.1 - A11	AGCATGAAGTCAGACA-A		
EU792883 - A12	AGCATGAAGTCAGACA-A		
AF333504 - A13	AGCATGAAGTCAGACA-A		
EU792884 - A15	AGCATGAAGTCAGATA-A		
EU792888 - A16	AGCATGAAGTCAGATA-A		
AF333505 - A1	AGCATGAAGTCAGACA-A		
JN558104.1 - Gym simplex	-G--TGAA-TCAG-CA-A		
AF333506 - A2	AGCATGAAGTCAGACA-A		
DQ865210 - Amed	AGCATGAAGTCAGACA-A		
JN242191 -Med Clade A	AGCATGAAGTCAGACA-A		

A2.4 ITS2 secondary structures for *Symbiodinium* Clade A

Figure A2.4.1: ITS2 secondary structures for *Symbiodinium* clade A as proposed by Hunter et al (2007)

A) ITS2 type A1; **B)** ITS2 type “Amed”; **C)** ITS2 type A3; **D)** ITS2 type A4 (*S. linucheae*); **E)** ITS2 type “Cryptic A”; **F)** ITS2 type A2 (*S. pilosum*).

APPENDIX 3- Preliminary trials on *Anthopleura aureoradiata*

A3.1 Introduction

The original proposal for my Masters' research was to carry out a set of experiments based on the study by Hill et al (2005). The ratios of the three components of NPQ in coral were measured after being placed in different treatments, with light and temperature as factors. The study found that the main component of NPQ was the energy dependent quenching (qE) which dissipates excess energy in non-bleaching stress conditions, either high light or high temperature. However, once bleaching conditions were enforced on the coral (high light and high temperature combined) total NPQ increased and the ratio of the different NPQ components changed. State transition quenching (qT) increased significantly compared to the other two components so that it became an equal contributor for energy dissipation as qE. In all of the treatments, photoinhibitory quenching (qI) did not change in proportion to the whole.

The method used by Hill et al was to measure fluorescence emissions using a PAM fluorometer and analyse the relaxation kinetics of the chloroplasts in the *Symbiodinium* cells by adding DCMU (3-(3,4-dichlorophenyl)-1, 1-dimethylurea) after the cells had been dark adapted and once a series of saturating pulses had been applied. By using the data from the curve, the three components could be calculated. DCMU inhibits the formation of the ΔpH across the thylakoid membrane by taking the place of the electron donor side on Q_B (Jones and Kerswell 2003, Hill et al. 2005). Once DCMU binds the electron, it stops the onwards movement of the electron through the electron transport chain (Jones 2004). Therefore, once added, the relaxation of the ΔpH can be measured and visualized on a graph and calculations made.

Figure A3.1.1: Chlorophyll fluorescence induction kinetics using DCMU to differentiate between the three components of NPQ. The kinetic curve depicts a control sample as produced in Hill et al's (2005) paper. SP – saturating pulse, AL – actinic light.

From the diagram, total NPQ is measured as the distance between the first two saturation pulses ($(F_m - F_m')/F_m'$). qE occurs between the top of the second pulse and the plateauing of the fluorescence measurement once DCMU is added ($qE = (F_E - F_m')/F_m'$). qT is measured from the plateauing at the end of qE , when the actinic light is turned off, and the plateauing of the saturation pulses, which are applied every 30 seconds ($qT = (F_T - F_E)/F_m'$). qI is measured by the remaining difference between F_T and F_m ($(F_m - F_T)/F_m'$).

A3.2 Trial Experiments

The first trial was to measure the concentration of DCMU needed to relax ΔpH in the *Symbiodinium* cells in *A. aureoradiata*. I placed 1 anemone within each 3 ml tube of filtered seawater (FSW) and placed 18 tubes within a holder in the PAM fluorometer. I added 6 different concentrations of DCMU to the tubes during the fluorescence run so that

there were 3 tubes with the same concentration. The first problem encountered was that the anemones were very active during the PAM fluorometer run. They contracted their tentacles when put in the dark for the dark adaption stage and extended and withdrew their tentacles under the different light settings. This made any comparison between treatments impossible.

The next trial was to try to “relax” the anemones in an open state so that they could not move during the PAM fluorometer run. I did this by adding MgCl to the FSW. Firstly the anemones were given time to attach themselves to the bottom of the tubes within 100 µl of FSW under a light source. After 15 minutes another 900 µl of FSW was slowly added. After another 15 minutes another 1000 µl of FSW was added. Once the anemones were all open, MgCl dissolved in FSW was added.

Different concentrations of MgCl were trialled (0.05 M, 0.1 M and 0.2 M). The two more concentrated solutions were too strong as the anemones curled up or became so flaccid that they no longer were able to retain attachment to the tubes. I also found that the addition of the 0.05 M MgCl solution caused some anemones to retract their tentacles due to the movement of water around them. Therefore, instead of adding MgCl solution to each 3 ml tube, I attached the anemones as before but then placed the tubes within a bath of FSW under a light source. The bath water was maintained at 20 °C. After 2 hours, when the anemones were open, MgCl solution was added to the bath so that the final concentration within the bath was 0.05 M.

I then ran trials to measure whether the addition of MgCl affected the photophysiology of the *Symbiodinium* cells in the anemone. I did this by running rapid light curves (RLC) to measure effective PSII quantum yield (Y(II)), quantum yield of regulated energy dissipation (Y(NPQ)) and quantum yield of non-regulated energy dissipation (Y(NO)) in anemones bathed in 0.05 M MgCl or just in FSW. The anemones were again attached in 3 ml tubes within a holder in the PAM. There were 4 replicate tests (n=6). Anemones were placed in their treatment under $100 (\pm 10) \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 7 hours before the RLC.

Figure A3.2.1: Mean ($n=6 \pm \text{SE}$) of Y(II), (YNPQ) and Y(NO) during four RLCs to test whether 0.05 M MgCl dissolved in FSW would affect the photophysiology of the *Symbiodinium cells* from within *A. aureoradiata* anemones. Asterisks mark significant differences between the means at specific irradiance levels (* $p < 0.05$, ANOVA, Tukey *post hoc*).

The results showed that under the majority of irradiance levels there was no significant difference in means between anemones in FSW and those in 0.05 M MgCl ($p=0.05$) (Figure A3.2.1). I concluded that using MgCl to relax anemones to stop them moving through the measurements of fluorescence induction kinetics was a viable option as the photophysiology of PSII of the anemones in FSW and MgCl reacted in a similar manner, and would not affect the induction kinetics for the DCMU tests.

I then re-ran tests to measure which concentration of DCMU could be used for the experiment. However, one of the tests was run with anemones in an unheated bath as the heater was on loan to another experiment. Even though the room temperature was not cold and would have been no more than 5°C below the previous tests, the anemones were all tightly closed. I decided that working with the whole anemone was proving to be too difficult and too varied. Controlling their behaviour, whilst not relaxing them too much or affecting their photophysiology was not possible. Therefore, further DCMU trials were run on *Symbiodinium* cells isolated from the anemones.

Four replicate tests were run with 25 anemones used in each. The anemones were broken up in a small liquidiser and the tissue then ground with a manual glass grinder to release the *Symbiodinium* cells from the animal cells. The cell suspension was spun five times in a centrifuge to separate the algal from the animal cells. The final supernatant was made up to 16 ml with FSW and then pipetted into 1 ml tubes. There were 4 treatments, a control with just FSW and 3 final concentrations of DCMU (10 μM , 20 μM and 30 μM). Therefore there were 4 tubes in each treatment. The actinic light setting for the run was set at 396 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. As the addition of DCMU was manually carried out at a non-specific time, when the kinetic curve had settled, a graph could not be drawn with the mean outcome for the 4 treatments. The objective was to have a quick and steep curve after adding DCMU but not enough to cause loss of saturation pulses after its addition. After running the four replicates and visually looking at the effects of the different concentrations of DCMU, I decided to use 10 μM concentration in the experiment planned (Figure A3.2.3)

Figure A3.2.3: Chlorophyll fluorescence induction kinetics of the DCMU concentration trials on isolated *Symbiodinium* cells. Each line represents the mean ($n=4$) of a treatment in the replicate.

A 25 ml DCMU solution was made up into a 1 mM stock by firstly dissolving 0.0058 g of DCMU in 2 ml of acetone and topped up to 25 ml with FSW. Therefore the next trial run was to measure whether the acetone had any effect on the photophysiology of the *Symbiodinium* cells. To do this, another RLC trial was run with 3 replicates (n=3). 14

anemones were ground and the isolated *Symbiodinium* cells were suspended in 8 ml of FSW from which 990 μ l was then pipetted into each of the tubes for the PAM run. 10 μ l of FSW was pipetted into 3 tubes (Control) and 10 μ l of diluted acetone to the other 3 tubes. The diluted acetone came from stock of 2 ml acetone with 23 ml FSW. The tubes were then dark adapted for 20 minutes before the RLC was run. There were no significant differences in mean at any of the irradiance levels in any of the parameters measured. Therefore no significant effect of the acetone on the photophysiology of the *Symbiodinium* cells can be assumed. (Figure A3.2.4)

Figure A3.2.4: Acetone dilution tests (Mean, n=3 $\pm$ SE) of Y(II), (YNPQ) and Y(NO) during four RLCs to test whether acetone diluted in FSW would affect the photophysiology of the *Symbiodinium* cells within *A. aureoradiata* anemones. There were no significant differences in means at any irradiance levels (p=0.05).

However, after all these trials there was still one problem that made running an experiment based on Hill et al's (2005) study unworkable. To be able to make any of the calculations measuring the three components of NPQ, the first saturation pulse spike had to be greater than any other fluorescence measurements made in the kinetic curve. In the majority of the trials this did not occur (Figure A3.2.5).

Figure A3.2.5: A chlorophyll fluorescence induction kinetic curve with 10 μ M DCMU added to a suspension of isolated *Symbiodinium* cells in FSW. This graph illustrates how the peak of the first saturation pulse, F_m , is lower than F_e and F_t , thereby making any calculations to measure the components of NPQ unworkable. SP – saturating pulse, AL – actinic light

A shorter saturation pulse than expected after dark adaptation suggests that the reaction centres in PSII are not all open. In other words all the electron in the primary quinone electron acceptor (Q_A) have not moved on through PSII and onto PS I (Wykoff et al. 1998). Therefore, on exposing the PSII reaction centres to a saturation pulse, the difference in F_m

and F_0 is smaller and the saturation pulse looks shorter than if the centres had all been open. To 'empty' PSII a far-red light (730 nm) can be used, instead of keeping the samples in the dark prior to the first saturation pulse, so as to stimulate PSI and to increase the flow of electrons out of PSII (Wykoff et al. 1998)

A final trial was run exposing isolated *Symbiodinium* cells to far-red light to measure if there was an increase in the saturation pulse peak at the beginning of a test. Five tests were run (n=3). *Symbiodinium* cells were isolated, re-suspended and then pipetted into 6 tubes for the PAM. Three were placed in the dark (control) and 3 were placed under an illuminated far-red filter for the same amount of time. In each test the time under the light was increased, starting at 10 seconds and ending at 60 seconds. There was no difference between the dark adapted or the far-red stimulated suspension.

Six months of trials to run the experiment originally planned were not successful. Therefore I changed the objective in my photophysiology experiment. Instead of measuring the ratio of the three components of NPQ I would measure the total energy dissipated by NPQ compared to energy used for carbon fixation, and measure how well PSII down-regulated to decrease damage to the chloroplast by light. The experiment is described in Chapter 3.

APPENDIX 4 – Time trials to determine how long isolated *Symbiodinium* cells remained photosynthetically healthy

The aim of these trials was to establish how long *Symbiodinium* cells isolated from *A. aureoradiata* would remain photosynthetically healthy and also the best time to start the photophysiology experiment. There were three trials.

Twenty-five anemones were crushed and the *Symbiodinium* cells resuspended, after isolation, in 30 ml of FSW. They were kept under $100 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ irradiance at 18°C . After settling for an hour, 2 ml of suspension was removed and divided into 2 tubes, and dark adapted for 20 mins before measuring F_v/F_m . A further 5 measurements were spread over the next 25 hours (Figure A4.1).

Figure A4.1: Time trials to measure the photosynthetic health of freshly isolated *Symbiodinium* cells (mean, $n = 3$). The first graph shows measurements over the first 26 hours after isolation and the second shows measurements over the first 11 hours.

The results indicated an immediate drop in photosynthetic health over the first three hours before a levelling out and maintenance of health to around 6 hours before F_v/F_m gradually dropped away again. Based on these trials I let the isolated *Symbiodinium* cells rest for 3 hours in the main experiment before taking the first PAM measurements and separating the algal suspension between the six treatment tanks. I also kept the suspension in the treatments for only a further 6 hours to reduce bias in the final measurements of the experiment. It should be noted, however, that the change in F_v/F_m over time is relatively small compared to the first measurement.

APPENDIX 5 – Cell Counts

Table A5.1: Estimated density of cells in the algal suspension used in the photophysiology experiment. Counts were made using a haemocytometer (Improved Neubauer) at the beginning and at the end of the experiment. Six replicates of each sample were counted 4 times and the final mean count expressed per ml of cell suspension.

No of <i>Symbiodinium</i> cells/ml of algal suspension								
Treatment		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Mean	SE
Beginning	16 °C Low	2371667	2910000	2565833	2573333	2692778	2622722	88357
End of Experiment – 6 hours later	8 °C Low	1862500	2165833	1906667	1310000	2342500	1917500	175284
	8 °C High	1607500	2245833	1742500	1897500	1802500	1859167	107503
	16 °C Low	1484167	1585833	1990000	1664167	1621667	1669167	85553
	16 °C High	1815833	2234167	2013333	1640000	1502500	1841167	130364
	30 °C Low	1787500	2510000	2277500	2010000	1606667	2038333	162746
	30 °C High	1890833	2155000	2517500	1889167	1755000	2041500	135545

A5.1 Statistical results for cell density

Table A5.1.1: PASW output for mean cell density ($n = 24 \pm \text{SE}$) using ANOVA where normality and variance had been assumed. Significance is assumed when $p < 0.05$.

Tests of Between-Subjects Effects

Dependent Variable: Cell Count

Source	Type III Sum of Squares	df	Mean Square	F	p value.
Corrected Model	2.075E12	6	3.458E11	4.770	0.003
Intercept	1.047E14	1	1.047E14	1444.296	< 0.001
Treatment	2.075E12	6	3.458E11	4.770	0.003
Error	1.522E12	21	7.248E10		
Total	1.083E14	28			
Corrected Total	3.597E12	27			

R Squared = .577 (Adjusted R Squared = .456)

Table A5.1.2: Post hoc tests using Tukey HSD, comparing the difference in mean ($n = 24 \pm \text{SE}$) of the cell density in each treatment ($n = 6$) between the start and finish for each treatment and between each of the treatments at the end of the experiment.

	Start	L8	L16	L30	H8	H16	H30
Start		0.021	0.003	0.044	0.007	0.006	0.117
L8	0.021		0.973	1.000	0.999	0.996	0.979
L16	0.003	0.973		0.882	1.000	1.000	0.625
L30	0.044	1.000	0.882		0.979	0.963	0.999
H8	0.007	0.999	1.000	0.979		1.000	0.837
H16	0.006	0.996	1.000	0.963	1.000		0.786
H30	0.117	0.979	0.625	0.999	0.837	0.786	

APPENDIX 6– Statistical Data for the Results in Chapter 3

The following data originates from statistical analyses using PASW (version 18.00). Variation of fluorescence measurements were tested using a repeat measure one-way analysis of variance (rmANOVA, $\alpha = 0.05$) with time of measurements as the within-subjects factor and light and temperature as the between-subjects factors. Where assumptions of normality and equal variance were not satisfied, transformation of data were carried out using an arcsin or a reflect and square root method. The Mauchley Sphericity Test was used in all analyses to test for sphericity of data. As all data met assumptions of sphericity, post hoc tests were carried out using Tukey HSD.

A6.1: Statistical Results for F_v/F_m

Table A6.1.1: PASW output for mean ($n=4 \pm SE$) arcsin transformed F_v/F_m using rmANOVA where normality, variance and sphericity had been assumed. Significance is assumed when $p < 0.05$.

Tests of Within-Subjects Effects

Measure: Arcsin F_v/F_m

Source	Type III Sum of Squares	df	Mean Square	F	p value
Time	0.389	3	0.130	228.831	< 0.001
Time * Light	0.016	3	0.005	9.418	< 0.001
Time * Temp	0.433	6	0.072	127.476	< 0.001
Time * Light * Temp	0.004	6	0.001	1.108	0.370
Error(Time)	0.031	54	0.001		

Table A6.1.2: PASW output for mean ($n=4 \pm \text{SE}$) arcsin transformed F_v/F_m tests of within-subjects contrasts to find the time-points between which there had been significant effects of factor interactions with time. Significance is assumed when $p < 0.05$.

Tests of Within-Subjects Contrasts

Measure: Arcsin F_v/F_m

Source	Time	Type III Sum of Squares	df	Mean Square	F	p value
Time	Start vs. 2 h	0.501	1	0.501	552.013	< 0.001
	2h vs. 4h	0.001	1	0.001	1.579	0.225
	4h vs. 6h	0.002	1	0.002	1.096	0.309
Time * Light	Start vs. 2 h	0.018	1	0.018	19.474	< 0.001
	2h vs. 4h	0.002	1	0.002	2.596	0.125
	4h vs. 6h	0.007	1	0.007	4.648	0.045
Time * Temp	Start vs. 2 h	0.366	2	0.183	201.725	< 0.001
	2h vs. 4h	0.027	2	0.014	15.702	< 0.001
	4h vs. 6h	0.005	2	0.002	1.611	0.227
Time * Light * Temp	Start vs. 2 h	0.001	2	0.000	0.471	0.632
	2h vs. 4h	0.001	2	0.001	0.582	0.569
	4h vs. 6h	0.002	2	0.001	0.624	0.547
Error(Time)	Start vs. 2 h	0.016	18	0.001		
	2h vs. 4h	0.016	18	0.001		
	4h vs. 6h	0.027	18	0.001		

Table A6.1.3: PASW output for mean ($n=4 \pm \text{SE}$) arcsin transformed F_v/F_m tests of between-subjects to find out if the two factors and their interaction had a significant effect on the experiment outcome. Significance is assumed when $p < 0.05$.

Tests of Between-Subjects Effects					
Measure: MEASURE_1 Arcsin F_v/F_m					
Transformed Variable: Average					
Source	Type III Sum of Squares	df	Mean Square	F	p value
Intercept	12.342	1	12.342	20631.052	< 0.001
Light	0.038	1	0.038	62.743	< 0.001
Temp	1.213	2	0.607	1014.060	< 0.001
Light * Temp	0.005	2	0.002	3.865	0.040
Error	0.011	18	0.001		

Table A6.1.4: Post hoc tests using Tukey HSD, as assumptions of sphericity were assumed, comparing the difference in mean ($n=4 \pm \text{SE}$) of arcsin F_v/F_m in each treatment between the time-points in a rmANOVA analysis. Significance is assumed when $p < 0.05$.

Low-light, 8 °C		Start	2 hours	4 hours	6 hours
	Start		< 0.001	< 0.001	< 0.001
	2 hours	< 0.001		0.065	0.281
	4 hours	< 0.001	0.065		0.795
Low-light, 16 °C		Start	2 hours	4 hours	6 hours
	Start		0.103	0.053	0.513
	2 hours	0.103		0.978	0.682
	4 hours	0.053	0.978		0.456
Low-light, 30 °C		Start	2 hours	4 hours	6 hours
	Start		0.205	0.571	0.342
	2 hours	0.205		0.856	0.011
	4 hours	0.571	0.856		0.042

Table 6.1.4 continued: Post hoc tests using Tukey HSD of arcsin Fv/Fm in each treatment between the time-points in a rmANOVA analysis. Significance is assumed when $p < 0.05$.

High-light, 8 °C		Start	2 hours	4 hours	6 hours
	Start		< 0.001	< 0.001	< 0.001
	2 hours	< 0.001		0.057	0.001
	4 hours	< 0.001	0.057		0.140
High-light, 16 °C		Start	2 hours	4 hours	6 hours
	Start		< 0.001	< 0.001	< 0.001
	2 hours	< 0.001		0.969	0.909
	4 hours	< 0.001	0.969		0.996
High-light, 30 °C		Start	2 hours	4 hours	6 hours
	Start		0.035	0.767	0.951
	2 hours	0.035		0.177	0.086
	4 hours	0.767	0.177		0.969

Table A6.1.5: Post hoc tests using Tukey HSD, as assumptions of sphericity were assumed, comparing the difference in mean ($n=4 \pm \text{SE}$) of arcsin F_v/F_m at 6 hours with an ANOVA analysis. Significance is assumed when $p < 0.05$.

	L8	L16	L30	H8	H16	H30
L8		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
L16	< 0.001		0.307	< 0.001	0.009	0.999
L30	< 0.001	0.307		< 0.001	< 0.001	0.492
H8	< 0.001	< 0.001	< 0.001		< 0.001	< 0.001
H16	< 0.001	0.009	< .001	< 0.001		0.004
H30	< 0.001	0.999	0.492	< 0.001	0.004	

A6.2 Statistical results for $\Delta F_v/F_m$ ' from the dark-induction curve

Table A6.2.1: Post hoc tests using Tukey HSD, as assumptions of sphericity were assumed, comparing the difference in mean ($n=4 \pm SE$) of the final measurement of $\Delta F_v/F_m$ ' at 6 hours with an ANOVA analysis. Significance is assumed when $p < 0.05$.

	L8	L16	L30	H8	H16	H30
L8		< 0.001	< 0.001	0.005	0.005	0.156
L16	< 0.001		< 0.001	< 0.001	< 0.001	< 0.001
L30	< 0.001	< 0.001		< 0.001	< 0.001	< 0.001
H8	0.005	< 0.001	< 0.001		1.000	0.535
H16	0.005	< 0.001	< .001	1.000		0.535
H30	0.156	< 0.001	< 0.001	0.535	0.535	

Table A6.2.2: Mean and standard error of the last $\Delta F_v/F_m$ ' of each treatment at each time-point

Low-light 8 °C		Mean	St Error	High-light 8 °C		Mean	St Error
	Start	0.1859	0.0109		Start	0.1859	0.0109
	2 hours	0.0615	0.0056		2 hours	0.0755	0.0755
	4 hours	0.0541	0.0064		4 hours	0	0
	6 hours	0.0650	0.0144		6 hours	0	0
Low-light 16 °C		Mean	St Error	High-light 16 °C		Mean	St Error
	Start	0.1859	0.0109		Start	0.1859	0.0109
	2 hours	0.1063	0.0056		2 hours	0	0
	4 hours	0.1120	0.0271		4 hours	0	0
	6 hours	0.1516	0.0208		6 hours	0	0
Low-light 30 °C		Mean	St Error	High-light 30 °C		Mean	St Error
	Start	0.1859	0.0109		Start	0.1859	0.0109
	2 hours	0.2082	0.0127		2 hours	0	0
	4 hours	0.2562	0.01		4 hours	0.01	0.0081
	6 hours	0.2956	0.0031		6 hours	0.026	0.006

A6.3 Statistical results for the coefficient of non-photochemical quenching (qN)

Table A6.3.1: Post hoc tests using Tukey HSD, as assumptions of sphericity were assumed, comparing the difference in mean ($n=4 \pm \text{SE}$) of the final measurement of qN in each treatment between the time-points in a rmANOVA analysis. Significance is assumed when $p < 0.05$.

Low-light, 8 °C		Start	2 hours	4 hours	6 hours
	Start		0.273	0.894	0.775
	2 hours	0.273		0.632	0.059
	4 hours	0.894	0.632		0.384
Low-light, 16 °C		Start	2 hours	4 hours	6 hours
	Start		0.006	0.05	0.895
	2 hours	0.006		0.606	0.019
	4 hours	0.05	0.606		0.161
Low-light, 30 °C		Start	2 hours	4 hours	6 hours
	Start		0.016	< 0.001	< 0.001
	2 hours	0.016		0.062	< 0.001
	4 hours	< 0.001	0.062		0.015
High-light, 8 °C		Start	2 hours	4 hours	6 hours
	Start		< 0.001	< 0.001	< 0.001
	2 hours	< 0.001		0.842	0.196
	4 hours	< 0.001	0.842		0.572
High-light, 16 °C		Start	2 hours	4 hours	6 hours
	Start		< 0.001	< 0.001	< 0.001
	2 hours	< 0.001		0.998	0.918
	4 hours	< 0.001	0.998		0.967
High-light, 30 °C		Start	2 hours	4 hours	6 hours
	Start		< 0.001	< 0.001	< 0.001
	2 hours	< 0.001		0.367	0.039
	4 hours	< 0.001	0.367		0.510

Table A6.3.2: Post hoc tests using Tukey HSD, as assumptions of sphericity were assumed, comparing the difference in mean ($n=4 \pm \text{SE}$) of the final measurement of qN at 6 hours with an ANOVA analysis. Significance is assumed when $p < 0.05$.

	L8	L16	L30	H8	H16	H30
L8		0.787	< 0.001	0.005	0.005	0.002
L16	0.787		< 0.001	< 0.001	< 0.001	< 0.035
L30	< 0.001	< 0.001		< 0.001	< 0.001	< 0.001
H8	< 0.001	< 0.001	< 0.001		0.973	0.122
H16	< 0.001	< 0.001	< .001	0.973		0.399
H30	0.002	0.035	< 0.001	0.122	0.399	

A6.4: Mean and SE of F_o , F_m and F_t

Table 6.4.1: Mean ($n=4$) and standard error of dark-adapted F_o , and F_m and the last measurement of F_t from each treatment at each time-point during a dark-light induction curve.

Treatment	Time (Hours)	F_o		F_m		F_t	
		Mean	SE	Mean	SE	Mean	SE
Low-light 8 °C	0	0.289	0.0016	0.5276	0.009	0.1856	0.0016
	2	0.1781	0.0073	0.2253	0.01	0.1094	0.0053
	4	0.1564	0.0071	0.1774	0.0083	0.1120	0.0035
	6	0.15	0.0073	0.1739	0.0085	0.1140	0.0044
Low-light 16 °C	0	0.289	0.0016	0.5276	0.009	0.1856	0.0016
	2	0.2541	0.0049	0.4407	0.0109	0.1258	0.0029
	4	0.241	0.0068	0.4142	0.0102	0.1462	0.0154
	6	0.2389	0.0061	0.4232	0.0057	0.1486	0.0075

Table 6.4.1 (cont'd): Mean (n=4) and standard error of dark-adapted F_o , and F_m and the last measurement of F_t from each treatment at each time-point during a dark-light induction curve.

Treatment	Time (Hours)	F_o		F_m		F_t	
		Mean	SE	Mean	SE	Mean	SE
Low-light 30 °C	0	0.289	0.0016	0.5276	0.009	0.1856	0.0016
	2	0.3104	0.0045	0.5447	0.0104	0.2427	0.0132
	4	0.3085	0.0072	0.554	0.0083	0.2972	0.0134
	6	0.31	0.016	0.5828	0.0248	0.3133	0.0118
High-light 8 °C	0	0.289	0.0016	0.5276	0.009	0.1856	0.0016
	2	0.1639	0.0032	0.2113	0.0267	0.0826	0.0017
	4	0.1431	0.0031	0.155	0.0047	0.07732	0.0019
	6	0.1323	0.00866	0.1376	0.0095	0.0757	0.0048
High-light 16 °C	0	0.289	0.0016	0.5276	0.009	0.1856	0.0016
	2	0.2481	0.0013	0.3865	0.0037	0.0861	0.0033
	4	0.2455	0.0055	0.387	0.0131	0.0875	0.0027
	6	0.2428	0.0062	0.3841	0.0082	0.0903	0.0058
High-light 30 °C	0	0.289	0.0016	0.5276	0.009	0.1856	0.0016
	2	0.2905	0.0047	0.4766	0.015	0.1073	0.0028
	4	0.303	0.007	0.5221	0.0162	0.1314	0.0119
	6	0.281	0.0092	0.5035	0.0196	0.0137	0.0112

A6.5: Statistical results for rETR_{max}

Table A6.5.1: PASW output for mean ($n=4 \pm \text{SE}$) reflect and square root transformed rETR_{max} using rmANOVA where normality, variance and sphericity had been assumed. Significance is assumed when $p < 0.05$.

Tests of Within-Subjects Effects

Measure: MEASURE_1

Source	Type III Sum of Squares	df	Mean Square	F	p value
Time	16.867	3	5.622	143.277	< 0.001
Time * Light	3.743	3	1.248	31.796	< 0.001
Time * Temp	28.126	6	4.688	119.457	< 0.001
Time * Light * Temp	0.737	6	0.123	3.132	0.010
Error(Time)	2.119	54	0.039		

Table A6.5.2: PASW output for mean ($n=4 \pm \text{SE}$) reflect and square root transformed rETR_{max} tests of within-subjects contrasts to find the time-points between which there had been significant effects of factor interactions with time. Significance is assumed when $p < 0.05$.

Tests of Within-Subjects Contrasts

Measure: MEASURE_1

Source	Time	Type III Sum of Squares	df	Mean Square	F	p value
Time	Start vs. 2 h	28.454	1	28.454	288.367	< 0.001
	2h vs. 4h	1.998	1	1.998	25.243	< 0.001
	4h vs. 6h	5.256	1	5.256	58.786	< 0.001
Time * Light	Start vs. 2 h	3.426	1	3.426	34.718	< 0.001
	2h vs. 4h	0.140	1	0.140	1.774	0.199
	4h vs. 6h	1.426	1	1.426	15.946	0.001
Time * Temp	Start vs. 2 h	11.236	2	5.618	56.934	< 0.001
	2h vs. 4h	1.637	2	0.818	10.340	0.001
	4h vs. 6h	7.775	2	3.888	43.477	< 0.001
Time * Light * Temp	Start vs. 2 h	0.054	2	0.027	0.274	0.764
	2h vs. 4h	0.021	2	0.011	0.133	0.876
	4h vs. 6h	1.061	2	0.531	5.935	0.010
Error(Time)	Start vs. 2 h	1.776	18	0.099		
	2h vs. 4h	1.424	18	0.079		
	4h vs. 6h	1.609	18	0.089		

Table A6.5.3: Post hoc tests using Tukey HSD, as assumptions of sphericity were assumed, comparing the difference in mean ($n=4 \pm \text{SE}$) of square root transformed rETR_{max} in each treatment between the time-points in a rmANOVA analysis

Low-light, 8 °C		Start	2 hours	4 hours	6 hours
	Start		< 0.001	< 0.001	< 0.001
	2 hours	< 0.001		0.997	0.999
	4 hours	< 0.001	0.997		1.000
Low-light, 16 °C		Start	2 hours	4 hours	6 hours
	Start		< 0.001	0.001	0.058
	2 hours	< 0.001		0.737	0.013
	4 hours	0.001	0.737		0.075
Low-light, 30 °C		Start	2 hours	4 hours	6 hours
	Start		0.605	0.004	< 0.001
	2 hours	0.605		0.038	< 0.001
	4 hours	0.004	0.038		< 0.001
High-light, 8 °C		Start	2 hours	4 hours	6 hours
	Start		< 0.001	< 0.001	< 0.001
	2 hours	< 0.001		0.891	0.358
	4 hours	< 0.001	0.891		0.749
High-light, 16 °C		Start	2 hours	4 hours	6 hours
	Start		< 0.001	< 0.001	< 0.001
	2 hours	< 0.001		0.073	0.511
	4 hours	< 0.001	0.073		0.566
High-light, 30 °C		Start	2 hours	4 hours	6 hours
	Start		0.032	0.632	0.001
	2 hours	0.032		0.004	< 0.001
	4 hours	0.632	0.004		0.01

Table A6.5.4: Post hoc tests using Tukey HSD, as assumptions of sphericity were assumed, comparing the difference in mean ($n=4 \pm \text{SE}$) of square root transformed rETR_{max} of all treatments at 6 h using an ANOVA analysis. Significance is assumed when $p < 0.05$.

	L8	L16	L30	H8	H16	H30
L8		< 0.001	< 0.001	0.001	0.731	< 0.001
L16	< 0.001		< 0.001	< 0.001	< 0.001	< 0.001
L30	< 0.001	< 0.001		< 0.001	< 0.001	0.001
H8	0.001	0.014	< 0.001		< 0.001	< 0.001
H16	0.731	< 0.001	< .001	0.014		< 0.001
H30	< 0.001	< 0.001	0.001	< 0.001	< 0.001	

A6.6: Statistical Results for E_k

Table A6.6.1: PASW output for mean ($n=4 \pm \text{SE}$) E_k using rmANOVA where normality, variance and sphericity had been assumed. Significance is assumed when $p < 0.05$.

Tests of Within-Subjects Effects

Measure: MEASURE_1 E_k

Source E_k	Type III Sum of Squares	df	Mean Square	F	P value
Time	640.907	3	213.636	12.589	< 0.001
Time * Light	347.451	3	115.817	6.825	0.001
Time * Temp	1987.484	6	331.247	19.520	< 0.001
Time * Light * Temp	123.116	6	20.519	1.209	0.316
Error(Time)	916.356	54	16.970		

Table A6.6.2: PASW output for mean ($n = 4 \pm SE$) E_k tests of between-subjects to find out if the two factors and their interaction had a significant effect on the experiment outcome. Significance is assumed when $p < 0.05$.

Tests of Between-Subjects Effects

Measure:MEASURE_1

Transformed Variable:Average

Source Ek	Type III Sum of Squares	df	Mean Square	F	p value
Intercept	34520.291	1	34520.291	3717.684	< 0.001
Light	235.706	1	235.706	25.384	< 0.001
Temp	856.989	2	428.495	46.147	< 0.001
Light * Temp	2.506	2	1.253	0.135	0.875
Error	167.138	18	9.285		

Table A6.6.3: PASW output for mean ($n=4 \pm SE$) E_k tests of within-subjects contrasts to find the time-points between which there had been significant effects of factor interactions with time. Significance is assumed when $p < 0.05$.

Tests of Within-Subjects Contrasts

Measure:MEASURE_1

Source Ek	Time	Type III Sum of Squares	df	Mean Square	F	p value
Time	Start vs. 2 h	254.150	1	254.150	7.561	0.013
	2h vs. 4h	306.023	1	306.023	6.776	0.018
	4h vs. 6h	332.640	1	332.640	10.000	0.005
Time * Light	Start vs. 2 h	429.260	1	429.260	12.770	0.002
	2h vs. 4h	13.651	1	13.651	0.302	0.589
	4h vs. 6h	66.169	1	66.169	1.989	0.175
Time * Temp	Start vs. 2 h	305.541	2	152.770	4.545	0.025
	2h vs. 4h	846.645	2	423.323	9.373	0.002
	4h vs. 6h	190.563	2	95.282	2.864	0.083
Time * Light * Temp	Start vs. 2 h	5.536	2	2.768	0.082	0.921
	2h vs. 4h	103.951	2	51.976	1.151	0.339
	4h vs. 6h	208.905	2	104.453	3.140	0.068
Error(Time)	Start vs. 2 h	605.043	18	33.613		
	2h vs. 4h	812.929	18	45.163		
	4h vs. 6h	598.781	18	33.266		

Table A6.6.4: Post hoc tests using Tukey HSD, as assumptions of sphericity were assumed, comparing the difference in mean ($n=4 \pm \text{SE}$) of E_k in each treatment between the time-points in a rmANOVA analysis.

Low-light, 8 °C		Start	2 hours	4 hours	6 hours
	Start		0.005	0.001	0.008
	2 hours	0.005		0.901	0.99
	4 hours	0.001	0.901		0.759
Low-light, 16 °C		Start	2 hours	4 hours	6 hours
	Start		0.078	0.088	0.699
	2 hours	0.078		1.00	0.408
	4 hours	0.088	1.000		0.444
Low-light, 30 °C		Start	2 hours	4 hours	6 hours
	Start		0.962	0.19	0.005
	2 hours	0.962		0.087	0.206
	4 hours	0.19	0.087		0.206
High-light, 8 °C		Start	2 hours	4 hours	6 hours
	Start		0.954	0.01	0.739
	2 hours	0.954		0.024	0.955
	4 hours	0.01	0.024		0.059
High-light, 16 °C		Start	2 hours	4 hours	6 hours
	Start		0.976	0.6	0.999
	2 hours	0.976		0.377	0.993
	4 hours	0.6	0.377		0.52
High-light, 30 °C		Start	2 hours	4 hours	6 hours
	Start		0.521	0.003	< 0.001
	2 hours	0.521		0.033	0.002
	4 hours	0.003	0.033		0.326

Table A6.6.5: Post hoc tests using Tukey HSD, as assumptions of sphericity were assumed, comparing the difference in mean ($n=4 \pm SE$) of E_k of all treatments at 6 h using an ANOVA analysis. Significance is assumed when $p < 0.05$.

	L8	L16	L30	H8	H16	H30
L8		0.704	< 0.001	0.282	0.163	< 0.001
L16	0.704		< 0.001	0.97	0.873	< 0.001
L30	< 0.001	< 0.001		0.001	0.002	0.180
H8	0.282	0.97	0.001		0.999	< 0.001
H16	0.163	0.873	0.002	0.999		< 0.001
H30	< 0.001	< 0.001	0.18	< 0.001	< 0.001	

A6.7: Statistical Results for NPQ_{max}

Table A6.7.1: PASW output for mean ($n=4 \pm SE$) NPQ_{max} using rmANOVA where normality, variance and sphericity had been assumed. Significance is assumed when $p < 0.05$.

Tests of Within-Subjects Effects

Measure:MEASURE_1

Source	Type III Sum of Squares	df	Mean Square	F	p value
Time	30.074	3	10.025	143.846	< 0.001
Time * Light	0.292	3	0.097	1.398	0.253
Time * Temp	33.228	6	5.538	79.467	< 0.001
Time * Light * Temp	0.486	6	0.081	1.161	0.341
Error(Time)	3.763	54	0.070		

Table A6.7.2: PASW output for mean ($n=4 \pm SE$) NPQ_{max} tests of between-subjects to find out if the two factors and their interaction had a significant effect on the experiment outcome. Significance is assumed when $p < 0.05$.

Tests of Between-Subjects Effects

Measure:MEASURE_1

Transformed Variable:Average

Source	Type III Sum of Squares	df	Mean Square	F	p value
Intercept	285.342	1	285.342	8020.169	< 0.001
Light	0.036	1	0.036	1.007	0.329
Temp	24.386	2	12.193	342.710	< 0.001
Light * Temp	0.234	2	0.117	3.288	0.061
Error	0.640	18	0.036		

Table A6.7.3: PASW output for mean ($n=4 \pm SE$) NPQ_{max} tests of within-subjects contrasts to find the time-points between which there had been significant effects of factor interactions with time. Significance is assumed when $p < 0.05$.

Tests of Within-Subjects Contrasts

Measure:MEASURE_1

Source	Time	Type III Sum of Squares	df	Mean Square	F	Sig.
Time	Start vs. 2 h	33.020	1	33.020	271.505	< 0.001
	2h vs. 4h	0.325	1	0.325	1.826	0.193
	4h vs. 6h	0.205	1	0.205	1.808	0.195
Time * Light	Start vs. 2 h	0.385	1	0.385	3.168	0.092
	2h vs. 4h	0.168	1	0.168	.942	0.345
	4h vs. 6h	0.082	1	0.082	.724	0.406
Time * Temp	Start vs. 2 h	37.202	2	18.601	152.945	< 0.001
	2h vs. 4h	0.926	2	0.463	2.606	0.101
	4h vs. 6h	0.654	2	0.327	2.890	0.082
Time * Light * Temp	Start vs. 2 h	0.958	2	0.479	3.940	0.038
	2h vs. 4h	0.278	2	0.139	.782	0.472
	4h vs. 6h	0.002	2	0.001	.008	0.992
Error(Time)	Start vs. 2 h	2.189	18	0.122		
	2h vs. 4h	3.200	18	0.178		
	4h vs. 6h	2.036	18	0.113		

Table A6.7.4: Post hoc tests using Tukey HSD, as assumptions of sphericity were assumed, comparing the difference in mean ($n=4 \pm \text{SE}$) of NPQ_{max} in each treatment between the time-points in a rmANOVA analysis. Significance is assumed when $p < 0.05$.

Low-light, 8 °C		Start	2 hours	4 hours	6 hours
	Start		< 0.001	< 0.001	< 0.001
	2 hours	< 0.001		0.207	0.115
	4 hours	< 0.001	0.207		0.982
Low-light, 16 °C		Start	2 hours	4 hours	6 hours
	Start		0.238	0.045	0.225
	2 hours	0.238		0.743	1.000
	4 hours	0.045	0.743		0.764
Low-light, 30 °C		Start	2 hours	4 hours	6 hours
	Start		0.212	0.883	0.465
	2 hours	0.212		0.547	0.935
	4 hours	0.883	0.547		0.865
High-light, 8 °C		Start	2 hours	4 hours	6 hours
	Start		< 0.001	< 0.001	< 0.001
	2 hours	< 0.001		0.064	0.005
	4 hours	< 0.001	0.064		0.502
High-light, 16 °C		Start	2 hours	4 hours	6 hours
	Start		0.180	0.025	0.042
	2 hours	0.180		0.659	0.816
	4 hours	0.025	0.659		0.991
High-light, 30 °C		Start	2 hours	4 hours	6 hours
	Start		0.885	0.972	0.856
	2 hours	0.885		0.991	0.458
	4 hours	0.972	0.991		0.624

Table A6.7.5: Post hoc tests using Tukey HSD, as assumptions of sphericity were assumed, comparing the difference in mean ($n=4 \pm \text{SE}$) of NPQ_{max} at 6 hours between all the treatments using an ANOVA analysis. Significance is assumed when $p < 0.05$.

	L8	L16	L30	H8	H16	H30
L8		< 0.001	< 0.001	0.958	< 0.001	< 0.001
L16	< 0.001		1.000	< 0.001	0.996	0.981
L30	< 0.001	1.000		< 0.001	1.000	0.924
H8	0.958	< 0.001	< 0.001		< 0.001	< 0.001
H16	< 0.001	0.996	1.000	< 0.001		0.843
H30	< 0.001	0.981	0.924	< 0.001	0.843	

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