Stress resistance in an extreme environment: Lessons learnt from a temperate symbiotic sea anemone
Stress resistance in an extreme environment: Lessons learnt from a temperate symbiotic sea anemone

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Supervisor’s Statement

I can affirm that, to the best of my knowledge, Milena Palka has carried out the research for her Masters thesis according to the requirements of the VUW Statutes as set out in the Calendar.

Milena has been solely supervised by myself. I have provided advice about methodology, research, resources and analysis.

Milena carried out her research project independently and therefore the thesis represents her own work.

Your name:

Simon K. Davy

28th January 2010
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And so begins the next chapter of life…
Abstract

Coral bleaching, the loss of symbiotic dinoflagellates (zooxanthellae) or their photosynthetic pigments in response to environmental stress, is of huge global concern. In contrast to tropical corals, which are highly sensitive to fluctuations in environmental parameters such as temperature, light and salinity, zooxanthellate invertebrates in temperate waters rarely bleach despite highly variable conditions. In this study, we tested the effects of salinity with combined effects of light and temperature stress on the photophysiology and stability of the temperate symbiotic sea anemone, Anthopleura aureoradiata, through chlorophyll fluorescence. In the field it was demonstrated that A. aureoradiata was resilient to abiotic fluctuations of considerable magnitude in the intertidal zone. Salinity was revealed to range naturally between a winter low of 30 and summer high of 40 ppt in an elevated tide pool with no measurable effects on the photophysiology of A. aureoradiata residing within. In a controlled environment, only extreme high and low salinities had an effect on the zooxanthellar photosystem, with a wide range of tolerance between 15-50 ppt dependent on the levels of temperature and light. Both high and low light, and temperature, also impacted upon photophysiology. Moreover, each of these variables independently, as well as combined, exacerbated the impact of salinity stress. In addition, the duration of exposure played an important role in the survival of this symbiosis, with only 48-96 h exposure to the extreme salinities of 5, 10, 55 and 60 ppt inducing irreversible photosynthetic failure, bleaching and death. Thus, the data supports the idea that this anemone-zooxanthellar symbiosis is highly resilient to considerable amounts of abiotic stress, a likely a function of the robust photophysiology of its zooxanthellae. This resilience to bleaching suggests that A. aureoradiata and its zooxanthallae have evolved a combination of powerful defensive
mechanisms to help aid against the heterogenous environment from which they come. I will present an overview of these osmoregulatory mechanisms, photoacclimatory strategies and behaviours that this symbiosis likely deploys in order to combat environmentally realistic ranges in abiotic factors. Further studies would be necessary to deduce whether it is the host or zooxanthellae which are responsible for the breakdown of this symbiosis.
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Introduction

1.1  Symbiosis

In 1879, Heinrich Anton de Bary defined symbiosis as “the living together of unlike organisms”, a definition that has yet to reach a consensus across the scientific community (Smith and Douglas 1987). This broad definition incorporates 3 categories: mutualism, parasitism and commensalism (Goff 1982; Saffo 1993; Wilkinson 2001). Mutualism specifically refers to a relationship that is beneficial to both parties, parasitism describes an interaction where one partner experiences a fitness benefit while the other is disadvantaged, and commensalism is experienced when one partner benefits but there is no negative effect on the other (Saffo 1992; Douglas 1994; Wilkinson 2001). Rather than these three interactions being referred to as exclusive categories, they are best considered as part of a continuum, whereby broad spectra of relationships exist and do not necessarily remain static (Goff 1982). Typically, symbiotic participants are of two considerably different sizes, of which the smaller is called the ‘symbiont’
while the larger organism is referred to as the ‘host’ (Smith and Douglas 1987; Douglas 1994). If the symbiont and/or host cannot live without its partner, the relationship is considered obligate rather than facultative (Smith and Douglas 1987; Moran 2006).

Symbiosis is arguably one of the most important driving forces of evolution, complementing Darwin’s theory of natural selection (Sagan and Margulis 1986; Moran 2006). It can be grouped as either ecto- (living on) or endosymbiosis (living within) in which case there is also a distinction between intra- versus extracellular endosymbiosis (Smith and Douglas 1987). Endosymbiosis, being more common than ectosymbiosis, is believed to be the major underlying mechanism involved in the evolution of eukaryotic organisms, whereby early prokaryotic cells ingested bacteria through endophagocytosis, ultimately giving way to organelles such as mitochondria and chloroplasts (Margulis 1970; Smith and Douglas 1987). It is through subsequent endosymbioses that modern eukaryotes have gained access to a plethora of novel metabolic capabilities, namely photosynthesis, chemosynthesis, nitrogen fixation, luminescence, amino acid and vitamin synthesis, among others (Douglas 1994).

### 1.2 Cnidarian-dinoflagellate symbioses

In the marine environment, symbiotic associations commonly occur between unicellular microalgae and invertebrates across numerous phyla including the Protozoa, Urochordata, Platyhelminthes, Porifera, Mollusca, and Cnidaria (Smith and Douglas 1987; Trench 1993). These predominantly endosymbiotic microalgae are represented within the following six divisions: diatoms, prasinophytes, rhodophytes (red algae), cyanobacteria (blue/green algae or zoocyanellae), chlorophytes (green algae or
zoochlorellae) and dinoflagellates (golden/yellow-brown algae or zooxanthellae) (Trench 1993).

Such heterotrophic-autotrophic associations typically offer a competitive advantage in a nutrient-poor environment through the mutual transfer of otherwise inaccessible metabolites (LaJeunesse 2002; Yellowlees et al. 2008). The most abundant marine animal hosts for these photosynthetic symbioses by far are the Cnidaria (Douglas 1994), with copious representatives of corals, anemones, jellyfish, and others distributed in both tropical and temperate waters. The photosynthetic algae most frequently found within the tissues of these invertebrates, most notably the anthozoans (e.g. corals, sea anemones), are dinoflagellates predominantly from the genus *Symbiodinium* (Douglas 1994; Rowan 1998; Coffroth and Santos 2005). However, several other genera of symbiotic dinoflagellates have been described, including: *Amphidinium, Aureodinium, Gloeodinium, Gymnodinium, Gyrodinium, Prorocentrum, Pyrocystis* and *Scrippsiella* (Banaszak et al. 1993; Trench 1993; Wakefield et al. 2000). These cnidarian-dinoflagellate symbioses show varying degrees of specificity, modes of establishment and levels of stability, yet are globally widespread and ecologically important.

### 1.2.1 Zooxanthellalar diversity and distribution

*Symbiodinium* spp. of the family Symbiodiniaceae (Fensome et al. 1993) are also commonly referred to as ‘zooxanthellae’, a term first used as a genus (*Zooxanthella*) by Karl Brandt in 1881 when he discovered the yellow-brown alga’s endosymbiotic residence within numerous marine animals (Figure 1.1; Blank and Trench 1986; Smith and Douglas 1987; Coffroth and Santos 2005). Freudenthal (1962) established the genus
Symbiodinium with a single type species *S. microadriaticum*, however both *Gymnodinium microadriaticum* (Taylor 1971) and *Zooxanthella microadriaticum* (Loeblich and Sherley 1979) have been arguably used as alternative classifications until the early 1980s, thus rendering a rather confusing taxonomic history (Blank and Trench 1985; Blank and Trench 1986; Coffroth and Santos 2005).

**Figure 1.1.** Micrograph of *Symbiodinium* sp. shown dividing within the tentacles of a well-fed anemone, *Aiptasia pallida*, kept under 2 light regimes: 1) 50 µmol photons/m²/sec and 2) 5 µmol photons/m²/sec. 3) *Symbiodinium* sp. within a tentacle of *A. pallida* which was not fed for 22 days and kept at 50 µmol photons/m²/sec. Ch = chloroplast, M = mitochondrion, N = nucleus, Va = vacuole. Scale bar = 1 um. Modified from Muller-Parker et al. (1996).
There are currently 11 accepted species of *Symbiodinium*, another two that are suspected to have been incorrectly identified as *Gymnodinium*, and predicted hundreds that are yet to be discovered (Trench 1993; Baker 2003). As historical reliance on morphological characters is abandoned and new molecular techniques are being developed and applied, it is now proposed that the genus *Symbiodinium* in fact consists of several major subgenera or lineages, also known as clades, each containing multiple species (Blank and Trench 1985; Baker 2003; Coffroth and Santos 2005). Thus far, eight genetically distinct clades (A-H) have been described (Baker 2003; Coffroth and Santos 2005). The differing morphologies, biochemistries, and physiologies of the species within these clades appear to reflect in their variable ecological ranges and resilience to environmental stresses (Trench 1993; Mostafavi et al. 2007). Some clades are endemic to remote regions while others can be distributed across great geographic ranges (Baker 2003). Some species are considered “specialists” and only reside within a single or small group of closely related hosts, while others are broadly distributed across numerous host taxa and are thus considered “generalists” (Baker 2003). There is evidence of a host-symbiont recognition system, whereby the pairings between host and zooxanthellae are conserved over space and time (Coffroth and Santos 2005). However, specific environmental conditions ultimately allow for the coupling of certain hosts with either a single symbiont or a dynamic multi-species community (Trench 1993; Rowan et al. 1997; LaJeunesse 2002; Baker 2003; Coffroth and Santos 2005). Examples of parameters which elicit changes in these relationships include depth, irradiance, temperature, seasonal variation, latitude and longitude gradients, and host ontogeny (Rowan 1998; Baker 2003; Coffroth and Santos 2005). Furthermore, some hosts are capable of switching from one clade to another after considerable environmental fluctuations and this is explained more thoroughly in sections 1.2.3 and 1.3.1.
1.2.2 Location and metabolic interactions

It is the significant alteration to the metabolism of both parties, to facilitate nutrient coordination, that has allowed for symbiosis to evolve (Yellowlees et al. 2008). Endosymbiotic dinoflagellates generally occur as coccoid cells within their hosts and only produce gymnodinioid motile cells when in culture, although the flagellated motile cells can also occur internally for some species (Blank and Trench 1985). In most cases, the algae reside intracellularly within the gastodermal tissue, where they are contained by a host-derived vacuole, called a ‘symbiosome’, which is generated during endocytosis (Douglas 1994; Yellowlees et al. 2008). Typically, there is only one alga per host cell (Muscatine et al. 1998). Symbiosomes can be composed of a single or numerous membranes that effectively form a boundary layer between the alga and the host cell’s cytoplasm, creating a regulated internal environment (Douglas 1994; Trautman et al. 2002). Symbiosome function is virtually unknown, although its role as a critical interface for cell-to-cell communication and nutrient (eg. CO$_2$, NH$_3$, and PO$_4^{3-}$) transfer to the alga has been recognized (Rands et al. 1993; Wakefield and Kempf 2001; Yellowlees et al. 2008). Thus, this cellular arrangement not only provides a means for symbiont recognition but it is also believed to allocate some control to the host over the alga’s proliferation and growth (Douglas 1994; Yellowlees et al. 2008).

Because *Symbiodinium* spp. are photoautotrophs, they are capable of harnessing and converting light energy into essential carbohydrates, with the excess then translocated to and utilized by the host (Smith and Douglas 1987). The photosynthetically-fixed carbon, also referred to as “photosynthate”, is transferred in the form of glycerol, glucose, lipid, or other small molecular weight metabolites and can account from anywhere between 10-90% of the total energy captured by the symbiont.
host (Smith and Douglas 1987; Douglas 1994; Whitehead and Douglas 2003). What stimulates this transfer has been subject to much debate, but the presence of a ‘host release factor’ (HRF) in the form of free amino acids (FAAs), mycosporine-like amino acids (MAAs) or both, present in host tissue at sufficient concentrations, is believed to play an important role (Trench 1971; Wang and Douglas 1997; Gates et al. 1999; Cook and Davy 2001). Although the donated photosynthate is usually supplemented with host feeding, its contribution is substantial, particularly in shallow waters (Douglas 1994). Minor alterations to this balance via shading or zooxanthellar loss can negatively affect host metabolism; inhibiting growth (Wellington 1982), reproduction (Clayton 1983) and calcification rates (Pearse and Muscatine 1971). In addition, there is also evidence for the conservation and recycling of metabolites such as nitrogen (Douglas 1994; Wang and Douglas 1998; Roberts et al. 1999; Lipschultz and Cook 2002) and phosphorus (Muller-Parker et al. 1990), which further facilitates the existence of these mutualistic symbionts in oligotrophic seas (Muscatine and Porter 1977; Goodson et al. 2001; Yellowlees et al. 2008). Nitrogen recycling refers to the process by which zooxanthellae assimilate wastes from the host’s metabolic activities, such as ammonia and phosphate, into useful amino acids and releases them back to the host (Wang and Douglas 1998). Comparatively, nitrogen conservation is accomplished when there is a net reduction in ammonium production by the host due to the receipt of photosynthate from their zooxanthellae, which is believed to increase ammonium-assimilating enzyme activity (Wang and Douglas 1998).

Symbiont populations can reach densities upwards of several million or more per square centimeter of host tissue; thus they survive at numbers otherwise unsustainable in a nutrient poor external environment (LaJeunesse 2002). With such a substantial presence within their host tissue, it is no surprise that they make such a
significant contribution to the productivity, survival and global success of cnidarian-algal relationships (Muscatine and Porter 1977).

1.2.3 Acquisition and expulsion of symbionts

Symbionts can be passed onto a new generation of hosts either through vertical or horizontal transmission (Moran 2006; Yellowlees et al. 2008). Vertical transmission, also known as maternal or closed transmission, involves the inheritance of symbionts directly from the host parent either via asexual or sexual processes (Douglas 1994; Schwarz et al. 2002; Yellowlees et al. 2008). Horizontal or open transmission appears to be the more common of the two types for cnidarians (~85%) and occurs when zooxanthellae are taken up from the external environment (Douglas 1994; Schwarz et al. 2002; Yellowlees et al. 2008). Species experiencing closed transmission are believed to harbour a lower diversity of zooxanthellae but have the assurance of gaining a compatible symbiont (Douglas 1994) as compared to those with open transmission, which potentially have access to new symbiont strains with every generation, thus permitting flexibility in the partnership (Buddemeier and Fautin 1993; Baker 2003; van Oppen 2004). During open transmission, particles are indiscriminately ingested but selectively digested, where vacuoles containing appropriate phagocytosed zooxanthallae avoid the host’s intracellular digestive function and instead are incorporated into the endodermal cells (Trench 1979; Colley and Trench 1983; Schwarz et al. 2002). Chemical cues are believed to bring the partners together (Pasternak et al. 2006), however it is the ability to block the fusion of endosomes or lysosomes to the vacuoles containing algae (phagosomes) that allows for the establishment and persistence of a healthy symbiosis (Trench 1979; Thornhill et al. 2006).
Despite open transmission being demonstrated both in the laboratory and in the field (Coffroth et al. 2001; Kinzie et al. 2001; Weis et al. 2001), very few free-living zooxanthellae have ever been found in the water column (Wilcox 1998; Carlos et al. 1999; Gou 2003; Littman et al. 2008); it appears that macroalgal beds, fish faeces and sediment maybe more likely locations (Muller-Parker 1984; Littman et al. 2008; Porto et al. 2008). This theoretical pool of free-living zooxanthellae is suspected to be a natural collection of regularly ejected symbionts, but little is known about their density, distribution and temporal variation (Hoegh-Guldberg et al. 1987; Kinzie et al. 2001).

Densities of symbionts within anthozoans have been shown to fluctuate under both normal (seasonal) and stressful (abnormal) conditions (Smith and Douglas 1987; Fagoonee et al. 1999; Douglas 2003). Seasonal changes in symbiont densities have been shown to be directly correlated with changes in host tissue biomass (Fitt et al. 2000). Because the host tissue provides limited space for the algae, routine housekeeping is believed to occur when the densities are at their maximum (Shick 1991; Dimond and Carrington 2008). Thus, pre-mitotic (before algal cell division) mechanisms such as the suppression of growth/division by controlling nutrients have been proposed as the major contributors to symbiont population regulation, with additional important supplementation received from post-mitotic mechanisms such as expulsion (via the mouth) and even lysis (selective culling) (Douglas 1994; Baghdasarian and Muscatine 2000; Dimond and Carrington 2008). These intrinsic mechanisms are believed to be essential in maintaining a stable symbiosis, however the exact cellular processes are still not well understood (Baghdasarian and Muscatine 2000).
1.3 Bleaching: causes and consequences

1.3.1 Triggers and ecological implications

Although they can be seasonally plastic, cnidarian-zooxanthella symbioses are generally stable, primarily in the tropics (Fagoonee et al. 1999; Dimond and Carrington 2008). During unfavourable conditions, either anthropogenic or natural, this stability can be compromised to a point of breakdown in a phenomenon known as ‘bleaching’. This process describes the whitening appearance of cnidarian hosts, particularly scleractinian corals, during which the host experiences a massive release of the symbiotic algae or their pigments as a response to stress (Brown 1997; Douglas 2003). This debris is discharged into the environment through the mouth, either as loose cells or accumulated pellets (Gates et al. 1992). Scientifically, bleaching is quantified through zooxanthelllar numbers remaining or expelled, and the assessment of their physiological status (Glynn 1996; Brown 1997). The fate of expelled zooxanthellae remains unresolved, however several studies have shown that the released algae can be both healthy-looking and photosynthetically viable (Ralph et al. 2001; Dunn et al. 2002; Bhagooli and Hidaka 2004; Ralph et al. 2005a). The polyps, on the other hand, are rendered colourless and in both a physiologically and nutritionally vulnerable state (Gates et al. 1992; Glynn 1996; Shenkar et al. 2005). Losing such a substantial proportion of zooxanthellae can induce mechanical damage of the host tissue, as well as reduce growth and reproduction, increase susceptibility to disease and cause death (Glynn 1996; Hoegh-Guldberg 1999).

It is still unknown exactly how and why bleaching occurs, but researchers have identified high and low temperature, solar irradiance, darkness, sedimentation, turbidity,
starvation, subaerial exposure, xenobiotics and changes in salinity as the major stressors inducing zooxanthellar expulsion (reviewed in Glynn 1996; Brown 1997; Douglas 2003). All of these factors can act at a local scale, but generally it is only the influence of abnormally high temperatures associated with El Nino-Southern Oscillation (ENSO) events that causes widespread destruction (Glynn 1984; Wilkinson et al. 1999). Several of these factors can also have synergistic relationships, particularly the interaction between temperature and light (Figure 1.2; Fitt et al. 2001). The processes invoking a bleaching response vary accordingly with these environmental triggers, as well as duration of exposure (Fitt et al. 2001; Douglas 2003; Dunn et al. 2004) and at present there are 5 host cellular mechanisms known which lead to zooxanthellar degeneration and/or release: 1) exocytosis; 2) host cell apoptosis (programmed cell death); 3) host cell necrosis; 4) in situ degradation; and 5) host cell detachment (Gates et al. 1992; Brown 1997; Weis 2008). Of these processes, it is the in situ degradation and apoptosis which are the most commonly reported cellular mechanisms of coral bleaching (Weis 2008). Both these dissociations are most often caused by the buildup of reactive oxygen species (ROS) due to damage to both photosynthetic and mitochondrial membranes and a breakdown of several ROS coping mechanisms, which are discussed more thoroughly in section 1.3.3 (Weis 2008). Nitric oxide (NO) may also play a pivotal role by acting as both a cytotoxic and a signaling molecule between partners (Perez and Weis 2006; Weis 2008). Ultimately, bleaching is believed to be a function of a host innate immune response to a compromised symbiont (Weis 2008). Unless these symbionts re-populate the coral in a limited period of time after expulsion (typically ≤ 4 weeks), the coral will likely die as a result of starvation (Reimer 1971; Glynn 1996; Douglas 2003). Coral reefs are found circumglobally in shallow tropical waters and in areas of extremely high productivity and biodiversity. Many of the organisms found here live in close
association with the scleractinian corals and thus when bleaching events occur, it can be devastating for the entire ecosystem (Glynn 1996; Hoegh-Guldberg 1999).

Figure 1.2. A hypothetical illustration of the synergistic relationship between temperature and light with regards to coral bleaching. Taken from Fitt et al. (2001).

It is still unclear as to which partner initiates the symbiotic breakdown, but it has been proposed that bleaching is not merely pathological, but also an adaptive mechanism to environmental change whereby the host may be provided with an opportunity to be repopulated with a different type of algae, conferring greater stress resistance in the future (Buddemeier and Fautin 1993; Ware et al. 1996). These zooxanthellae can originate from residual populations within the host (‘shuffling’), or can be taken up from the environment (‘switching’) with specificity adjusting in accordance to the environmental conditions (Baker 2003; Fautin and Buddemeier 2004; Lewis and Coffroth 2004; Berkelmans and van Oppen 2006). As it has been shown by several studies, different symbiotic algae exhibit varying sensitivity in growth and photophysiology, even within a single clade of *Symbiodinium* (Kinzie et al. 2001; Baker
2003; Rowan 2004; Warner et al. 2006). For instance, clade A zooxanthellae appear to favour high light and a wide range of temperatures while those of clade C and B prefer low light and are less heat tolerant (Iglesias-Prieto and Trench 1997; Goulet et al. 2005). But in addition, although clade A distribution in the tropics is restricted to shallow waters (Rowan and Knowlton 1995; LaJeunesse 2001), when occurring in the temperate environment it is harboured by hosts at far greater depths (Davy et al. 1996). Clade D is also unusual because even though it is distributed widely throughout the tropics, it is not the dominant symbiont in any particular host (Baker 2003). It appears to be a transition mode between the A-types of the shallows and the deeper C-types, as well as occurring sporadically at extreme depths (Baker 2003). Jones et al. (2008) demonstrated that clade D may indeed be favored in conditions where other symbionts are poorly suited by documenting a dramatic shift in symbiont communities on the Great Barrier Reef. After a natural bleaching event, surviving colonies of Acropora millepora switched from predominantly harbouring Symbiodinium type C2 to either clade D or C1 (Jones et al. 2008). The switch was then made back to C2 within the next 4 years. On the other hand, Venn et al. (2008b) demonstrated that bleaching was not required in order for a symbiotic reef-dwelling anemone Condylactis gigantea to change the prevalent symbiont in its tissue between clade A and B. With an ever present threat of global climate change, such examples of recombination highlight the importance of improving our understanding of the tolerances and flexibility of symbiont-host partnerships and their physiological responses to a myriad of conditions (Baker 2003; Baird et al. 2007). Furthermore, with the frequency and severity of bleaching becoming drastically more notable in the last 25 years, opinions tend to favour this event as fundamentally unnatural with the long-term fate of the organisms involved remaining tentative (Hoegh-Guldberg 1999; Douglas 2003).
Nonetheless, before we can understand the signaling cascades that can trigger bleaching, these recombinant dynamics and the potential for adaptation to change more thoroughly, we must examine the affects of bleach-inducing environmental stress on the most fundamental property of an algal-cnidarian symbiosis, that is photosynthesis.

1.3.2 Photophysiology

Photosynthesis occurs in two stages: a light-dependent reaction (light reaction) and a light-independent reaction (dark reaction), during which light is captured and turned into high-energy molecules which then chemically reduce CO$_2$ to make carbohydrate precursors. During the light reaction, which occurs in the thylakoid membrane of the chloroplast, chlorophyll pigments (or other types) located in the light-harvesting antenna complexes of photosystem II (PSII) absorb photons in exchange for electrons, producing O$_2$ in the process (Falkowski and Raven 2007). The transportation of these electrons down an electron transport chain (ETC) to photosystem I (PSI) and beyond exists as two forms of reactions: cyclic and non-cyclic photophosphorylation. The non-cyclic reaction proceeds through photosystem I, creating a proton gradient across the chloroplast membrane needed for simultaneous ATP synthesis and ultimately (by using more light energy) reduces NADP$^+$ to NADPH (Falkowski and Raven 2007). The cyclic reaction is similar, also involving ATP production but the electrons are instead fed back to photosystem I rather than be used to produce NADPH. Finally the dark reaction, or Calvin-Benson Cycle, involves the enzyme RuBisCO fixing CO$_2$ that is captured from the environment (in the case of zooxanthellae from their animal host), using ATP and NADPH to release the required sugars (Falkowski and Raven 2007).
Of the total light spectrum, only photons falling within the 400-740 nm range (48.7%) are usable as photosynthetically active radiation (PAR), with PSII and PSI most efficient at 680 and 700 nm, respectively (Zhu et al. 2008). Another ~10% of the PAR can be reflected due to the imperfect absorbance capability of chlorophyll (Zhu et al. 2008). Of the light that is absorbed, only 30-50% is successfully captured (Falkowski and Raven 2007; Skillman 2008). Because of these limitations and ongoing respiration, the maximum conversion efficiency of solar energy to biomass in terrestrial photosynthesis occurs between 4.6-6% of the captured PAR (Zhu et al. 2008). Chlorophyll is the primary pigment used by most terrestrial plants, however accessory pigments can also include carotenes, phycocyanins and xanthophylls (Spector 1984). In zooxanthellae, carotenoids are more numerous than chlorophylls, thus giving these algae their golden-brown colour (Spector 1984). This and other structural modifications may allow a higher efficiency at which light is absorbed and transferred in these aquatic algae (Taylor 1987).

Photosynthetic rate, on the other hand, is influenced mainly by light intensity/wavelength, temperature and the concentration of CO₂ in the atmosphere (Kramer 1981; Falkowski and Raven 2007). A typical photosynthesis versus irradiance (P-I) curve demonstrates that, as irradiance increases, providing temperature remains constant, so does the light-dependent reaction rate (Figure 1.3; Hoegh-Guldberg 1999; Falkowski and Raven 2007). It has three distinct regions: the light limited, the light-saturated, and the photoinhibited region (Ralph and Gademann 2005). At the light-limited region, the efficiency between photosynthesis and irradiance, denoted α, experiences a linearly proportional increase until it plateaus (P_max) at the minimum level of light-saturation (E_k) whereby it is believed that the dark reaction becomes the rate limiting factor (Figure 1.3; Ralph and Gademann 2005; Falkowski and Raven 2007). If
irradiance continues to increase, then the photosystem will sometimes enter a state of photoinhibition (Ralph and Gademann 2005). Photosynthesis is a sensitive indicator of stress, thus the parameters $\alpha$, $P_{\text{max}}$, and $E_k$ are particularly important and are often used by researchers to measure subtle alterations in photosynthetic activity (Ralph and Gademann 2005). Temperature, on the other hand, affects enzyme activity, thus as temperature becomes optimal so does the overall photosynthetic rate (Falkowski and Raven 2007). Above or below this optimum, the rate decreases until reaching a complete stop. The relationship with $\text{CO}_2$ concentration is similar to that with irradiance, whereby photosynthetic rate increases as carbon incorporation becomes easier until limitation occurs by another process (Kramer 1981).

Figure 1.3. A typical photosynthesis versus irradiance (P-I) curve. In the dark, there is a net consumption of oxygen ($C_o$) as a result of respiration ($R$). Photosynthetic rate increases linearly ($\alpha$) with irradiance intensity until saturation of irradiance ($E_k$) is reached and the rate plateaus at its maximum ($P_{\text{max}}$). Past this optimum irradiance, a decline in photosynthetic rate often occurs ($\beta$). Taken from Falkowski and Raven (2007).

Abbreviation definitions:

- $R$ – dark respiration
- $C_o$ – oxygen consumption
- $\alpha$ – efficiency between photosynthesis and irradiance, which is a linearly proportional increase in photosynthetic rate when light is limited (also known as light utilization efficiency)
- $P_{\text{max}}$ – light-saturated (maximum) photosynthetic rate
- $\beta$ – decline in photosynthetic rate which often occurs in the photoinhibited region, analogous to initial slope $\alpha$
- $E_k$ – minimum saturating irradiance, given as the intercept between $\alpha$ and $P_{\text{max}}$
Given these low predicted efficiencies and limitation of photosynthetic rate by various environmental factors, it is no surprise that a major underlying mechanism of bleaching appears to be damage to the zooxanthellar photosynthetic apparatus (Venn et al. 2008a). When temperature and light exceed the natural thresholds of the photosystem, harmful by-products can build up leading to photohibition, as is discussed more thoroughly in the next section (Hoegh-Guldberg 1999; Baker et al. 2008; Weis 2008). It is important to understand this breakdown and its relationship to algal expulsion if we are to accurately predict the future of the world’s reefs.

1.3.3 Photophysiological mechanisms of bleaching

The bleaching and destruction of coral reefs due to environmental stress is a major issue perplexing researchers today. Many abiotic factors have a negative impact on corals and their close relatives. Photosynthesis provides obvious nutritional benefits to the animal host, but it also comes with major physiological risks and a profound effect on distribution due to its intimate relationship with light and temperature. It has been shown by several studies that a high dose of these environmental factors, often acting synergistically (Figure 1.2), can compromise the efficiency of PSII, exceeding its photosynthetic capacity and upsetting the balance between light collection and use (Lesser and Shick 1989; Warner et al. 1996; Jones et al. 1998; Venn et al. 2008a). If various host and symbiont photoprotective mechanisms are overwhelmed, for example non-photochemical quenching (NPQ) or the activity of enzymes such as catalase, ascorbate peroxidase and superoxide dismutase (SOD), then the apparatus enters a state of photoinhibition whereby there is a decline in functional reaction centres due to the inevitable over-production and build-up of potentially harmful ROS (Figure 1.4; Warner
et al. 1996; Fitt et al. 2001; Franklin et al. 2004; Weis 2008). In the case of thermal bleaching, it is also the production of heat-shock proteins (HSPs) which is overwhelmed, ultimately leading to the same photophysiological result as that of high light (Dunn et al. 2004; Takahashi et al. 2004; Smith et al. 2005; Weis 2008). ROS can damage protein function (particularly the D1 protein), membrane integrity (such as that of the thylakoid), nucleic acids and other vital processes (e.g. Calvin Cycle), causing subsequent oxidative stress in both symbiont and host tissue and most likely leading to bleaching via *in situ* degradation of symbionts or apoptosis of host cells (Figure 1.4; Warner et al. 1999; Venn et al. 2008a; Weis 2008).

Figure 1.4. The 3 possible impacts (I, II, and III) of temperature-irradiance stress on the photosystem of *in situ* zooxanthellae. I) Degradation of the D1 protein at PSII, II) energetic coupling of the thylakoid membrane and III) impairment of the Calvin Cycle can all lead to bleaching through oxidative stress. Taken from Venn et al. (2008a).
By similar mechanisms, both low light and low temperature can also hinder the photophysiology and thus survival, growth and distribution of these photosynthetic symbioses (Saxby et al. 2003; Rodolfo-Metalpa et al. 2008). In addition, ultraviolet radiation (UVR) has shorter wavelengths of high-energy photons such as UV-A (320-400 nm), which are capable of driving the seawater warming cycle, increasing the potential of molecular damage to proteins, membrane lipids, and DNA and RNA bonds of both symbiotic partners and have also been found to inhibit photosynthetic efficiency (Hannack et al. 1997; Fitt et al. 2001; Lesser and Farrell 2004). If not adequately defended against by enzymes or protective UV-absorbing compounds, namely mycosporine and mycosporine-like amino acids (MAAs) which are also known for their role in active oxygen scavenging and stimulating photosynthate release from zooxanthellae, bleaching will result (Shick et al. 1991; Banaszak et al. 1995; Fitt et al. 2001; Lesser and Farrell 2004). Ultimately, any abnormal abiotic stress which can lower photosynthetic efficiency will disrupt algal photopysiology and lead to an excess of available photon energy causing photoinhibition (Shick et al. 1991; Fitt et al. 2001). Photoinhibition can be a precursor to bleaching and although it is reversible after short-term exposure to abiotic anomalies (dynamic photoinhibition), persistent oxidative stress can inflict permanent photosynthetic damage (chronic photoinhibition) and/or death (Warner et al. 1996; Fitt et al. 2001; Venn et al. 2008a).

Since most corals live at their upper thermal limits (Ralph et al. 2001) and an increase of 1.2°C in sea temperature is to be expected by 2100 (Bijlsma et al. 1995), it is particularly important to develop an understanding of their tolerance and ability to adapt to this and a variety of environmental scenarios. Outside the tropical latitudes of 30° N and 30° S, temperatures are generally too low for scleractinian corals to retain their physiological tolerances and their calcification capabilities (Jacques et al. 1983;
Furthermore, corals are typically found within the first 100 m of tropical oceans, beyond which light transmission is limited to insufficient levels for their algae (Hoegh-Guldberg 1999). Light attenuation is also affected in areas of high sedimentation and turbidity such as the mouths of rivers, lagoons or certain anthropogenic sources (Hoegh-Guldberg 1999; Fitt et al. 2001). Thus the predicted sea level rise of 95 cm in the next 100 years is cause for concern, as local boundaries will be shifted and newly formed territories may not be ideal (Pittock 1999). Many of these predicted scenarios will involve multiple factors changing, with the potential for exacerbating affects. Therefore, it is vital to gather as much information about the processes, ranges, and photophysiological tolerances governing the cnidarian-algae symbioses exposed to various stressors. Bleaching is not always caused by direct photosystem dysfunction within the zooxanthellae and there exists a challenge in discerning the actual steps leading to symbiotic breakdown in many witnessed cases (Fitt et al. 2001; Ralph et al. 2001).

With most of the attention on temperature and irradiance, one environmental factor that has not been looked at in great detail in terms of its relationship to photophysiology and bleaching, is salinity.

1.3.4 Salinity ranges and bleaching

Coral reefs are most common between the latitudes of 20° north and south of the Equator and generally exist in areas that are stable in regards to fluctuating salinity (Coles and Jokiel 1992). The anthozoan cnidarians inhabiting coral reefs have long been considered strict stenohaline osmoconformers, with little or no ability to osmoregulate,
and typically reside in regular strength seawater of 34-36 ppt (Kleypas et al. 1999). Any fluctuation in the osmolarity of the external environment is followed closely by the water within their coelenterons. In order to remain iso-osmotic with external changes, the animals must rapidly modify internal levels of amino acids, ions and proteins. If this change in solute levels exceeds that of physiological tolerance, then the organism faces metabolic disruption of cellular electrochemical processes, enzyme activity, and nerve conduction (Mayfield and Gates 2007). Having overcome these challenges, many anthozoan species are now known to successfully tolerate a natural salinity range of 32-40 ppt (Muthiga and Szmant 1987; Hoegh-Guldberg 1999) and even reside in areas which experience both cyclic and stochastic salinity fluctuations of varying magnitude and duration (see Coles and Jokiel 1992 for a review). Thus, reef distribution limits are currently believed to lie between 20.7-42 ppt (Coles and Jokiel 1992; Kleypas et al. 1999).

Anthozoan distribution becomes selective near coastal regions such as estuaries and tide pools (Muthiga and Szmant 1987; Hoegh-Guldberg 1999). In these nearshore areas, rapid changes in salinity between ±5-10 ppt are not uncommon (Muthiga and Szmant 1987). Both in the tropics and temperate environments, coastal reductions in salinity cause much more localized bleaching than the extensively documented effects of global sea temperature anomalies (Brown 1997; Rogers and Davis 2006). This may be due in part to the sporadic nature of salinity decreases when compared to seasonal fluctuations in temperature and light. Natural bleaching episodes by reduced salinity have been reported after major storms and hurricanes (Goreau 1964; Egana and DiSalvo 1982; van Woesik et al. 1995). At the opposite end of the spectrum, prolonged drought and evaporation of tide pools can cause high salinities (Muthiga and Szmant 1987).
Rapidly progressive and extreme salinity stress (outside the normal daily and seasonal fluctuations) has been shown to induce bleaching and/or death, however recent studies have also revealed that some species of symbiotic anthozoans have a certain level of tolerance (Muthiga and Szmant 1987). Many are easily capable of withstanding slow and gradual changes (over days) upwards of 10 ppt from their acclimated salinity with some even tolerating ranges of 17.5-52.5 ppt (Muthiga and Szmant 1987; Hoegh-Guldberg and Smith 1989; Manzello and Lirman 2003). In corals experiencing salinities outside their threshold for acclimation, a number of researchers have found there to be a measurable decline in photosynthetic and respiration rates proportional to the salinity change (Muthiga and Szmant 1987; Moberg et al. 1997; Ferrier-Pagès et al. 1999; Porter et al. 1999; Kerswell and Jones 2003; Manzello and Lirman 2003). If the duration and/or magnitude of salinity stress is substantial, then bleaching and even death can occur (Muthiga and Szmant 1987; Porter et al. 1999; Manzello and Lirman 2003; Mayfield and Gates 2007). Mechanisms involved in salinity-induced bleaching remain unresolved, however host cell swelling and rupture typically associated with loss of water volume regulation (Engebretson and Martin 1994; van Woesik et al. 1995; Mayfield and Gates 2007), as well as the release of zooxanthellae-containing host cells have been reported (Titlyanov et al. 2000). However, some cases of bleaching due to reduced salinities may not be characterized by the actual expulsion of zooxanthellae but instead the sloughing of dead host tissue that causes superficially similar symptoms (Hoegh-Guldberg 1999). Additionally, salinity stress has been demonstrated to act synergistically with both temperature and light (Coles and Jokiel 1978; Porter et al. 1999; Sakami 2000).

Because salinity can be a contributing factor to the bleaching of symbiotic anthozoans, it is important to explore the mechanisms behind this loss more thoroughly
and to assess what kind of damage to the photophysiology of the zooxanthellae, if any, is being committed. Furthermore, a tropical versus temperate comparison in bleaching susceptibility is needed to gain a better understanding of how the global symbiotic assemblages may be altered in the face of climate change.

1.3.5 Tropical versus temperate symbioses

Environmental parameters can vary across a local scale, however there are also broad spatial and temporal changes which differ between temperate versus tropical sites. By reviewing the environmental parameters from two such distinct locations (Washington, USA and Discovery Bay, Jamaica), Muller-Parker and Davy (2001) summarized the notable differences between sites as well as seasons for irradiance, temperature, several inorganic nutrients, and chlorophyll \( a \) (Table 1). They illustrated that tropical seas experience higher irradiances and temperatures but lower nutrient and chlorophyll \( a \) levels than do temperate seas. Alternatively, temperate seas experience marked seasonal cycles in all environmental parameters compared to those of tropical seas. As salinity was not compared by the authors at these two sites, Table 1 was modified to demonstrate the lowest hypo- and highest hypersaline conditions that have been documented in both temperate and tropical environments across the world (Rawlinson 1934; Cloud 1952; Goreau 1964; Robblee et al. 1989; Coles 2003; Z. Haws, VUW pers. comm. of a tide pool in New Zealand). Comparatively, tropical latitudes tend to experience more seasonal variation in rainfall whilst in temperate areas it is more uniformly spread throughout the year (Houghton and Woodwell 1989). Although values are similar, the occurrence of this distinct wet and dry season in most tropical regions, may be particularly important in creating the more pronounced high and low salinities.
seen in this table. In addition, tropical wet seasons are closely correlated with summer, while it is the winter which typically experiences more rainfall in the temperate environment (Houghton and Woodwell 1989).

Table 1. The environmental parameters from a representative temperate (Washington, USA) and tropical (Discovery Bay, Jamaica) site for comparison, with the addition of salinity values from across the world. Modified from Muller-Parker and Davy (2001). Values of salinity were taken from literature as follows: a) Rawlinson 1934; b) Coles 2003; c) Robblee et al. 1989; d) Z. Haws, VUW pers. comm. of a tide pool in New Zealand; e) Goreau 1964; f) Cloud 1952.

<table>
<thead>
<tr>
<th></th>
<th>Temperate site</th>
<th>Tropical site</th>
</tr>
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<tbody>
<tr>
<td>Salinity (ppt)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>winter</td>
<td>13(a)</td>
<td>50-52(b,c)</td>
</tr>
<tr>
<td>summer</td>
<td>45(d)</td>
<td>3-4(e,f)</td>
</tr>
<tr>
<td>Maximum surface irradiance (µmol·m⁻²·s⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>winter</td>
<td>548</td>
<td>2100</td>
</tr>
<tr>
<td>summer</td>
<td>1891</td>
<td>2015</td>
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<tr>
<td>Temperature (°C)</td>
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<tr>
<td>winter</td>
<td>7.5</td>
<td>26.5</td>
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<tr>
<td>summer</td>
<td>11.7</td>
<td>29</td>
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<tr>
<td>Inorganic nutrients (µM)</td>
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<td></td>
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<tr>
<td>Nitrate + Nitrite</td>
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<td></td>
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<tr>
<td>winter</td>
<td>32.0</td>
<td>0.39</td>
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<tr>
<td>summer</td>
<td>16.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Ammonium</td>
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<td></td>
</tr>
<tr>
<td>winter</td>
<td>0.9</td>
<td>2.6</td>
</tr>
<tr>
<td>summer</td>
<td>2.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Phosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>winter</td>
<td>3.1</td>
<td>2.3</td>
</tr>
<tr>
<td>summer</td>
<td>2.3</td>
<td>0.2</td>
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<tr>
<td>Chlorophyll α (µg·L⁻¹)</td>
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<td></td>
</tr>
<tr>
<td>winter</td>
<td>0.29</td>
<td>0.08</td>
</tr>
<tr>
<td>summer</td>
<td>4.34</td>
<td>0.12</td>
</tr>
</tbody>
</table>

At higher latitudes, light availability is reduced by more frequent turbidity (which also reduces the penetration depth of UV), a shorter day-length on average and lower irradiance intensity with a shallower angle of entry into the water (Muller-Parker and Davy 2001; Harriott and Banks 2002). Light attenuation is further increased in the winter period by storm-induced run-off. These differences in irradiance also strongly
contribute to the overall lower maximum temperatures experienced further from the
Equator. Although this maximum is lower, the temperature shift across seasons
experienced by temperate zooxanthellate cnidarians is significant (Muller-Parker and
Davy 2001). Furthermore, intertidal anemones can be exposed to a water temperature
change of 10°C or more within a single summer day (Jensen and Muller-Parker 1994).
Dingman (1998) documented that the internal body temperature of A. elegantissima
reached 28°C during air exposure on a summer day and 6°C at low tide in winter. Such
broad ranges suggest that temperate anemones, their symbionts or both are potentially
more temperature-tolerant then their tropical equivalents which have been shown to
bleach with only minor temperature alteration (Gates et al. 1992; Muller-Parker and
Davy 2001; Dunn et al. 2004). In the temperate winter, inorganic nutrient
concentrations are higher but light intensity and duration is lower, thus limiting
plankton growth (and hence seawater chlorophyll a) which the anemones feed on as
well as their capacity to photosynthesize (Muller-Parker and Davy 2001). The reverse is
ture for summer months. Zooxanthellae are nitrogen-limited in tropical waters but this
does not appear to be the case in temperate symbionts (Davy et al. 2006). Nutrient-rich
waters, such as those in temperate regions also generally favour the presence of
macroalgal competitors over corals, which can experience considerable growth
retardation (Johannes et al. 1983; Miller and Hay 1996; Harriott and Banks 2002).

In contrast to the obligate symbioses found in most tropical species of
anthozoans, temperate anemones are more often facultatively associated with
zooxanthellae (Miller and Hay 1996; Dimond and Carrington 2008). Failure to acquire
symbionts would most likely be a lethal situation for tropical hosts, particularly
scleractinian corals which rely on these algae to maintain growth and calcification rates
(Dimond and Carrington 2007), whereas many aposymbiotic temperate host species
(found in low-light environments such as caves or buried in the sand) can grow and propagate, but at a lesser rate than their symbiotic counterparts (Schwarz et al. 2002; Dimond and Carrington 2007). Light-saturated photosynthetic rates of zooxanthellae from these two regions are similar (possibly attributed to low-light adaptation), however temperate hosts receive a substantially lessened supply of C from their symbionts in the light-reduced winter months (Muller-Parker and Davy 2001; Verde and Mccloskey 2007). Photosynthetic efficiency ($\alpha$) is also lower which is counter intuitive given that photoadaptation to lower irradiance might result in a higher value, but may possibly be attributed the greater attenuation of light by the bulky tissue of temperate anemones (Muller-Parker and Davy 2001). The lower densities of zooxanthellae found in most temperate anemones in general and the absence of specialized “auxiliary” structures when compared to tropical species suggests a greater reliance on food rather than light, but data are limited to only a few comparisons as symbiotic representatives in the temperate region are scarce (Muller-Parker and Davy 2001). Contrary to expectation, temperate zooxanthellae densities persist through unfavourable conditions such as over winter, and in some cases even double (Squire 2000; Muller-Parker and Davy 2001). For symbiotic anemones living in particularly stressful areas like the intertidal zone, this stability is remarkable and implies a greater tolerance of this relationship to fluctuations in environmental parameters.

In temperate oceans, such as those surrounding New Zealand, non-reef building corals and their close relatives the sea anemones are locally dominant and ecologically important members of coastal communities (compared to the tropics), but relatively little is known about their susceptibility to environmental stress. For example, the effects of salinity on temperate versus tropical systems have not been well documented. Both environments are subject to fluctuations in salinity, particularly in the intertidal
zone where rain and evaporation have the greatest influence on shallow or isolated bodies of water. What is observed in New Zealand is that localized bleaching events due to salinity changes are practically non-existent, which is unexpected considering the heavy rains that occur on a frequent basis in many part of the country. However, given their previously documented exposure to temperature and light fluctuations, any increased tolerance to stress observed in temperate symbionts is likely due to their algae’s physiological adaptation to a more heterogeneous environment (Rodriguez-Lanetty et al. 2001). Understanding the adaptive mechanisms employed by temperate symbiotic organisms will help to shed light on how some tropical symbioses are also more tolerant of environmental stress than are others.

### 1.4 Anthopleura aureoradiata

*Anthopleura aureoradiata* (Figure 1.5) is a small intertidal symbiotic anemone endemic to the temperate coastal waters of New Zealand. Its range extends throughout numerous tide pools and mudflats from Cape Reinga in the far north to Stewart Island in the south. *A. aureoradiata* measures approximately 1 cm across its oral disc and 1.5-2 cm in height and is often found in the cracks and crevices of tide pools or attached to buried cockles on mudflats. It is also not uncommon to find debris (e.g. tiny shell fragments) held on the body column, conceivably for added protection.

Zooxanthellae are densely packed within the mesenterial tissue of *A. aureoradiata* and are particularly abundant in the tentacles and oral disc, giving the anemone its olive-brown coloration (Figure 1.5). These symbionts are approximately 5 μm in diameter, classified under the genus *Symbiodinium* and are categorized as belonging to clade A of this genus (Phillips 2006). *A. aureoradiata* reproduces
asexually, brooding the young within its body cavity for a variable length of time before expelling them; both environmental stress and physical manipulation can act as a trigger for release (M. Palka, pers. obs.). These young are ejected with zooxanthellae already present in their tissue, suggesting that the symbiosis is established early on through maternal inheritance (Davy and Turner 2003).

Figure 1.5. The study organism, Anthopleura aureoradiata, with its associated zooxanthellae (Symbiodinium sp.). A. A. aureoradiata measuring ~1 cm across its oral disc. B. A tentacle squash from A. aureoradiata illustrating the dense arrangement of zooxanthellae, symbiotic dinoflagellates from clade A, located within the endodermal tissue. C. Zooxanthellae measuring ~5 µm in diameter. D. Zooxanthellae viewed using a fluorescence microscope, which highlights chlorophyll autofluorescence.

1.5 Research aims

The sea anemone, A. aureoradiata was chosen as a model organism for the study of temperate symbioses due to its abundance across coastal communities of New Zealand and apparent tolerance to environmental perturbations. It resides on shallow mudflats
and in rock pools where it is exposed to considerable salinity, light, and temperature fluctuations, yet is never observed to bleach. Understanding this natural range of tolerance against bleaching and the photophysiological reactions of its zooxanthellae to abiotic stressors could be an important step in deciphering the basis of the robustness of this symbiosis to environmental fluctuations.

This project had 2 overall aims:

1) To determine the primary impact of salinity, and compounding impacts of light and temperature stress on the photophysiology and stability of the *A. aureoradiata*-Symbiodinium symbiosis.

2) To ascertain a maximum duration of exposure to these environmental stressors before recovery of photosynthetic function and/or host survival is no longer feasible.

Because *A. aureoradiata* resides on shallow mudflats and in rockpools, where it is likely to be exposed to considerable environmental fluctuations yet is never observed to bleach, it was predicted that the resistance of *A. aureoradiata* to environmental changeability is a function of the robust photophysiology of its zooxanthellae. If this symbiosis does indeed have an increased tolerance to environmental perturbations, then it will be much more resistant to zooxanthellar expulsion, thus retaining its photosynthetic capacity.

It was hypothesized that:

1) Loss of photosynthetic function and bleaching will only occur at extreme (high & low) levels of salinity.
2) If this is true, then extremes of temperature and light will have an exacerbating effect on salinity stress in the same manner that light exacerbates thermal bleaching.

3) A possible threshold value (e.g. photoinhibition) may exist for each environmental stressor (salinity, light and temperature), both independent and combined, before zooxanthellar expulsion occurs.

4) Recovery of photosynthetic function will only occur after short periods of exposure to stress (e.g. 24 h). This period of tolerance will decrease while the recovery time will escalate with increasing stressor levels.

These results will advance knowledge and understanding of the environmental tolerance and range of the temperate sea anemone, *A. aureoradiata*, particularly evaluating the effects of fluctuating salinity on the photophysiology of its zooxanthellae.
Materials and Methods

2.1 Study organism and location

This study investigated *Anthopleura aureoradiata* from two habitats in the Wellington region of New Zealand, a tide pool at Kau Bay (Figure 2.1) and a mudflat at Pauatahanui Inlet (Figure 2.2). These sites were located in Wellington harbour and 25 km north of Wellington respectively.

In rocky tide pools of the low- to mid-midlittoral zones at Kau Bay, these anemones were found wedged into narrow crevices and/or crowded into areas partially shaded from the sun. The location of some individuals across this shore height made them susceptible to aerial exposure. In addition, sand and debris was often seen attached to anemones that were fully exposed to light.

Across the low- to mid-intertidal mudflat zones at Pauatahanui Inlet, *A. aureoradiata* was found just under the surface of the substratum attached primarily to buried, and typically living, New Zealand cockles (*Austrovenus stutchburyi*) but also
occasionally at the bases of sea grass and algae. At high tide, or in areas where water remains pooled during low tide, *A. aureoradiata* was seen with its tentacles emerged onto the surface of the sediment. During unfavourable conditions such as low tide or periods of high irradiance, the animal had its tentacles retracted. Typically, only one anemone was found in association with a cockle, but upwards of 6 were discovered on some and the distribution of *A. aureoradiata* across the mudflat in general was patchy. Reasons for this lack of uniformity in distribution have yet to be quantified but substrate texture and depth of anoxic layers may play a role. The anemone-cockle relationship is considered to be a non-obligate mutualistic symbiosis, whereby the former gains hard surface in a malleable and potentially damaging environment while the latter benefits from significantly depressed parasite loads, as the anemone consumes trematode cercariae from the seawater (Mouritsen and Poulin 2003).
Figure 2.1. An overview of the field site and location of the tide pool containing *Anthopleura aureoradiata*. A. The rocky inter-tidal field site, Kau Bay, located at 41°17’20 S and 174°50’02 E in Wellington, New Zealand. B. A map of New Zealand indicating the field site location. C. The close up of the indicated area from map B highlighted further by the arrow. D. A close up of the specified rock mass from map A at Kau Bay showing the water level during high and low tide as well as the location of the tide pool indicated by the arrow. E. A close up of the area indicated by the arrow in map D highlighting the exact position of the tide pool. F. *A. aureoradiata* residing within the tide pool studied.
Figure 2.2. An overview of the collection sites and location of the study organisms, *Anthopleura aureoradiata*. A. The collection site Pauatahanui Inlet and associated mudflats, located at 41°05'55 S and 174°52'21 E in Porirua, New Zealand. B. A map of New Zealand indicating the location of the collection site. C. A close up of the indicated area from map B highlighting the site location further with an arrow. D. A mudflat in Pauatahanui Inlet from which *A. aureoradiata* was collected. E. *A. aureoradiata* attached to the cockle, *Austrovenus stutchburyi*, found buried just beneath the surface of the mudflat.
2.2 Photophysiology of *Anthopleura aureoradiata* in an isolated tide pool

2.2.1 Tide pool description

Anemones from a single high-shore tide pool, located at Kau Bay, Wellington, New Zealand were investigated during this part of the study (Figure 2.1). This tide pool was of particular interest due to its unique elevation on a large rock mass, meaning that at high tide it remained separate from the incoming seawater. Fresh seawater was only splashed into the pool when high tide was coupled with episodes of strong wind. This occurred periodically, as did heavy rainfall and long hot days, thus weekly salinity fluctuations in range of 34 ±2 ppt were expected, with extreme values of up to 45 ppt previously observed (Z. Haws, pers. comm.). In addition to salinity, temperature and light also fluctuated daily and were expected to influence the photophysiology of the anemones inhabiting this tide pool.

Measuring 75 cm H × 45 cm W x 16 cm D with a 35 L volume, this tide pool was home to approximately 15-20 *A. aureoradiata* individuals of various sizes, with no other anemone species present. *A. aureoradiata* was not present in any of the other proximal tide pools, however they were found in the low- to mid-midlittoral zone approximately 900 m NW along the coast. This distance coupled with lack of good weather and battery limitation of some instruments prevented a direct comparison with the low- to mid-shore tide pools which experience little change in salinity levels.
2.2.2  Survey protocol

This survey was designed to investigate the natural fluctuations in salinity, temperature, and irradiance over time and record their effect on the photophysiology of the anemone-zooxanthella symbiosis.

Measurements were taken as often as possible over 9 months on days when both weather and tide allowed but always at solar noon (sun at its highest point in the sky). Additionally, consistent irradiance was required both during the survey and up to 3 hours before to allow for evenness of photoacclimation, thus only days with either completely clear or overcast skies were assessable. Days with light rain and gentle wave splash were also included.

A pocket refractometer (PAL-06S, ATAGO®, U.S.A.) and a standard alcohol thermometer were used to record salinity and temperature, respectively. The photosynthetic capacity of up to 5 anemones measuring 1.5 ±0.5 cm in basal disc diameter at various locations and depths in the tide pool (avoiding shaded individuals) were investigated from point measurements with a fiber-optical probe using a Diving Pulse-Amplitude Modulated (D-PAM, refer to section 2.2.3) underwater fluorometer (Heinz Walz GmbH, Germany). Care was taken to ensure the probe was always held at the same distance (0.5 cm) above the oral disc of the anemone during the readings (Logan et al. 2007). Observations on anemone position (open or closed tentacles) were also noted and irradiance was read at each anemone location using a light meter (LI-1000, LI-COR®, U.S.A.).
2.2.3 D-PAM settings

Since bleaching is believed to be triggered by photosystem dysfunction, traditional quantifications of symbiont/pigment densities and coral tissue biomass only provide an estimate of its severity and do not offer any insight into the steps leading up to the breakdown. D-PAM is a compact, portable and fully submersible chlorophyll fluorometer that investigates photosynthetic function through fluorescence using a rapid and non-invasive technique applicable in situ (Logan et al. 2007). It was employed to generate rapid light curves (RLCs) for all field readings in this study. The Photosynthetically Active Radiation (PAR) list selected was as follows: 0, 488, 619, 816, 1063, 1524, 2034, 2804, and 4449 µmol photons/m²/sec. The following D-PAM settings were applied during the survey:

<table>
<thead>
<tr>
<th>Setting</th>
<th>Value</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>saturation width</td>
<td>0.80s</td>
</tr>
<tr>
<td>actinic light intensity</td>
<td>8</td>
</tr>
<tr>
<td>actinic light width</td>
<td>0:30s</td>
</tr>
<tr>
<td>gain</td>
<td>5</td>
</tr>
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<td>damping</td>
<td>2</td>
</tr>
<tr>
<td>light curve width</td>
<td>0:20s</td>
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<tr>
<td>light curve intensity</td>
<td>5</td>
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<tr>
<td>ind. width</td>
<td>0:20s</td>
</tr>
</tbody>
</table>

RLCs provide an in-depth assessment of the photosynthetic capacity of an organism via light saturation (Ralph and Gademann 2005). RLCs are similar to photosynthesis versus irradiance (P-I) curves (Figure 1.3) but have relative electron transport rate (rETR) as the y-axis (Ralph and Gademann 2005; Platt et al. 1980). Thus, photosynthetic efficiency ($\alpha$), minimum saturating irradiance ($E_k$) and maximum relative electron transport rate ($rETR_{max}$) are derivable parameters from RLCs and can be used to quantify the probable state of photosynthesis, through photosystem II (PSII)
function, unlike P-I curves that show the optimal state without taking into consideration light history (see previous section 1.3.3 for a review of definitions; Ralph and Gademann 2005). D-PAM also measures minimum light-adapted fluorescence (F_o’), maximum light-adapted fluorescence (F_m’), and variable fluorescence (F_v or F_m’-F_o’), which are used to calculate the light-adapted maximum quantum yield of PSII (F_v’/F_m’) (Ryan et al. 2004; Ralph and Gademann 2005). F_v’/F_m’ is indicative of the current light acclimation state and is not equivalent to a traditional dark-adapted value (F_v/F_m) which allows for the relaxation of the non-photochemical quenching (NPQ) coefficient (Fitt et al. 2001; Ryan et al. 2004; Ralph et al. 2005b). However both F_v’/F_m’ and F_v/F_m give a reasonably accurate indication of the relative abundance of photosynthetically healthy (functioning PSII) zooxanthellae in a mixed (healthy and damaged) population (Fitt et al. 2001; Bhagooli and Hidaka 2004). Data were accessed and manipulated via the computer using WinControl® v2.08 (Walz GmbH, Germany). The RLCs were fitted to curves using the empirical equation “PlatPlus” (Platt et al. 1980) through the “Regression Wizard” in Sigmaplot® v8 (Jandel Scientific, San Rafael, CA, U.S.A.).

2.3 Laboratory experiments

2.3.1 Specimen collection, housing and care

Individuals of Anthopleura aureoradiata no smaller than 3 mm in diameter across the pedal disc (when closed) were collected from the mudflats of Pauatahanui Inlet (Figure 2.2) and transported back to the laboratory in a water-filled container.

In the lab, anemones were placed into large glass bowls holding 1.5 L of 1-µm-filtered sea water (FSW) (Figure 2.3) and housed in an incubator (Precision
Environmental Chamber 180 RH, Contherm Scientific Ltd., New Zealand) at a constant salinity (34 ppt), temperature (17 ±1°C) and light (12:12 at 100 ±10 µmol photons/m²/sec) regime. Brine shrimp nauplii were fed 3-4 times a week followed by an equal amount of water changes. Anemones were acclimated to these conditions for at least 2 weeks prior to each experiment.

2.3.2 Impact of a gradient of salinity on the photophysiology of *Anthopleura aureoradiata*

This experiment was designed to assess the effect of a range of salinities on the photosynthetic health of the anemone-zooxanthella symbiosis in a controlled environment. The possible exacerbating effects of light and temperature were also investigated.

Anemones were randomly selected and transferred into 6 × 24-well plates (18 individuals/plate, totaling 108 individuals/trial) filled with 34 ppt 1-µm-FSW (Figure 2.3). The lidless plates were then placed back into the incubator at the same temperature and light regime (see section 2.3.1) to acclimate for 3 days prior to trial commencement. During the first 48 hrs of settlement, it was assured that anemones were centered and upright. A water change was also performed daily to prevent any increase in salinity due to evaporation. Anemones were not fed both during this acclimation and the experiment.

A 15-L water bath connected to a heater/circulator and immersion cooler (Haake IP30 Typ003-5009 and Haake EK20 Typ002-4269 respectively, Thermo Scientific Inc., Germany) was assembled so that 6 of the total 12 well-plates needed to complete a full set of light treatments (see below) could be suspended at the water surface (Figure 2.3).
A light bank of 12 × 50 W halogen lamps was mounted 40 cm above the plates with a light diffuser inserted at a height of 22 cm. This created a continuous light source of 450 ±20 µmol photons/m²/sec across the water bath surface. In addition, 10 of the total 12 well-plate lids were fitted with varying combinations of neutral density filter of grades 50%, 25% and 12.5% in order to create the following light treatments: 1, 20, 45, 100, and 200 µmol photons/m²/sec within the plates. The remaining 2 lids were left clear, giving a light level of 420 µmol photons/m²/sec. Excess heat created by the halogen lights was controlled using fans.

Treatment seawater at salinities 5-60 ppt, in 5 ppt increments was produced by mixing 1 µm-FSW with either distilled water or previously evaporated super-saline seawater. The solutions were then filtered through a 0.45 µm Millipore filter (Millipore Corporation, U.S.A.) using a mobile phase filtration apparatus and kept in the dark at the appropriate temperatures to inhibit algal growth. Trials were conducted consecutively at either 6, 18, or 30°C, as measured within the wells using a digital thermometer. Trials run at 6°C required the addition of 95% ethylene glycol into the water bath (final concentration 28% ethylene glycol in water) in order to prevent excessive ice buildup on the coil of the chilling unit.

At T₀, the photosynthetic parameters α, Eₖ, rETRₘₐₓ and Fᵥ/Fₘ were measured using Imaging Pulse-Amplitude Modulated (I-PAM, refer to section 2.3.4) chlorophyll fluorometry (Heinz Walz GmbH, Germany) before the treatment salinities were applied. The light curve was performed for each anemone only after the plate was placed into the dark I-PAM chamber for 2 min to ensure that Fᵥ/Fₘ was dark-adapted (indicative of low F₀ values; Fitt et al. 2001; Ryan et al. 2004). Only 6 different treatment salinity levels were used in each plate at one time and each salinity level was repeated 3 times (1 well/row; 3 rows total) within a plate (Figure 2.3), thus the total 12 salinity levels were
spread across 2 separate plates (5, 10, 15, 20, 25, 30 and 35, 40, 45, 50, 55, 60). Plates were then fitted with the aforementioned lids (see above for light treatments), immediately placed in the water bath and I-PAM readings were taken at hours T1, T3, T6, T24, T48, T72, and T96. Plates were positioned to ensure maximum consistency across the 3 light treatments. A water change was done daily, after completion of I-PAM readings.

This experiment required 6 trials and 648 anemones to complete all the possible salinity, light and temperature combinations with a sample size of n=3. The organization of these factors both within and across trials was to combat any potential effects from the plates, wells or position in the water bath. Finally, the whole experiment was repeated to enhance the sample size, giving a total of 12 trails and 1296 anemones (n=6). Refer to Table 2.1 for the summary of treatments and experimental set-up.
Figure 2.3. The experimental setup used for the salinity gradient experiment. **A.** *Anthopleura aureoradiata* was housed in 1.5 L glass bowls after collection. **B.** *A. aureoradiata* was acclimated to the 24-well plates in control salinity prior to the experiment in an incubator at constant temperature (17 ±1°C) and light (12:12 at 100 ±10 µmol photons/m²/sec). **C.** Experimental set up included a temperature regulated 35-L water bath in which 6 plates were fitted with a combination of neutral density filter (see example of light treatment arrangements) placed beneath a light bank of 12 × 50 W halogen lamps, a diffuser, and a fan to disperse heat. Each light treatment had 2 plates with low and high treatment salinities (see example of salinity arrangement).
Table 2.1. A complete summary of all treatments for the salinity gradient experiment. A total of 12 trials were run with 3 temperatures, 6 light levels, and 12 salinities spread over 6 × 24-well plates/trial (n=6).

<table>
<thead>
<tr>
<th>Trial</th>
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<th>Light (µmol photons/m²/sec)</th>
<th>Salinity (ppt)</th>
<th>Plate</th>
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<tr>
<td>1</td>
<td>18</td>
<td>45</td>
<td>5-30</td>
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<td></td>
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<td>420</td>
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<td>35-60</td>
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<td>3</td>
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<td>Repeat of trial 6</td>
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<td>12</td>
<td>Repeat of trial 10</td>
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2.3.3 Impact of variable duration to extreme salinity exposure on the photophysiological recovery of *Anthopleura aureoradiata*

This experiment was designed to investigate the maximum length of time the anemones could be exposed to a range of extreme salinities before a decline in photosynthetic health became irreparable. The possible differences in response due to light or dark exposure were also explored.

Anemones were chosen randomly and placed into 4 × clear 24-well plates and 4 × dark 24-well plates completely sealed with black electrical tape (20 individuals/plate, 160 individuals/trial). They were covered with 34 ppt 1-µm-FSW and allowed to acclimate in the incubator at constant temperature (17 ±1°C) and light (12:12 at 85 ±5 µmol photons/m²/sec) for 3 days. During the first 48 hrs of settlement, it was assured that anemones were centered and upright. One water change was also performed daily to prevent any increase in salinity due to evaporation. Anemones were not fed both during this acclimation and the experiment.

The initial I-PAM reading, $T_0$, was taken in the same manner as described in section 2.3.2, with a dark-adaptation of 2 min. The water was then replaced to the appropriate treatment salinities and the plates were fitted with the clear or sealed lids. A total of 8 treatment salinities in addition to the control (35 ppt) were used: 5, 10, 15, 20, 45, 50, 55, and 60 ppt. The controls, were placed in 4 of the wells, while all other treatments were placed in pairs into 16 wells in a randomized fashion, leaving the remaining 4 wells empty. All 8 plates were placed back into the incubator, and left undisturbed between the I-PAM readings (Figure 2.4). Exposures ran for 24, 48, 72, and 96 hrs for all treatments, and the protocol for I-PAM readings and water changes followed that of the previous experiment (refer to section 2.3.2.). At the end of each
experimental time period, all the treatment salinities in a pair of plates (light and dark) were changed to 35 ppt (recovery) and the anemones were monitored with I-PAM for an additional 96 hrs with measurements taken every 24 hrs after this point.

This experiment required 1 trial and 80 anemones to complete all the possible salinity and light combinations with a sample size of n=2. The organization of the salinities and light treatments within the trial was to combat any potential effects from the plates or wells. The whole experiment was repeated to enhance the sample size, giving a total of 2 trails and 160 anemones (n=4). Table 2.2 gives a summary of treatments.

Figure 2.4. The experimental setup used for the variable duration and recovery experiment *Anthopleura aureoradiata* was placed into the wells of 8 × 24-well plates contained inside an incubator. The values demonstrate one possible arrangement of both control (35 ppt) and treatment (5, 10, 15, 20, 45, 50, 55, and 60 ppt) salinities. Half the plates were sealed with black duct tape so that no light was transmittable. The light source (indicated by the arrow) was centered over the clear plates giving a range of 85 ±5 µmol photons/m²/sec at the plate’s surface.
Table 2.2. A complete summary of all treatments for the variable duration and recovery experiment. A total of 2 trials were run with 1 temperature, 2 light levels, and 9 salinities spread over 8 × 24-well plates/trial. 4 wells in each plate remained empty. Exposure (E) lasted 24, 48, 72, or 96 hrs with recovery (R) following for 96 hrs.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Temperature (°C)</th>
<th>Light (µmol photons/m²/sec)</th>
<th>Salinity (ppt)</th>
<th>Duration (hrs)</th>
<th>Plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td></td>
<td>5, 10, 15, 20 = 2 wells/plate</td>
<td>E = 24 R = 96 I</td>
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<td></td>
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<td>35 = 4 wells/plate</td>
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<td>45, 50, 55, 60 = 2 wells/plate</td>
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<td>Repeat of trial 1</td>
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<td>0</td>
<td></td>
<td>5, 10, 15, 20 = 2 wells/plate</td>
<td>E = 24 R = 96 V</td>
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<td>35 = 4 wells/plate</td>
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<td>45, 50, 55, 60 = 2 wells/plate</td>
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</table>

2.3.4 I-PAM settings

I-PAM is a high-resolution chlorophyll fluorometer that investigates photosynthetic function through fluorescence using a rapid and non-invasive technique. It was employed to generate rapid light curves (RLCs) for all laboratory readings in this study. The Photosynthetically Active Radiation (PAR) list selected was as follows: 0, 81, 146, 231, 281, 336, 396, 461, 531, 611, and 701 µmol photons/m²/sec. The following I-PAM settings were applied during all laboratory experiments:
The parameters extracted from these readings were $\alpha$, $E_k$, rETR$_{\text{max}}$, $F_o$, $F_m$, $F_v$ and $F_v/F_m$ (see section 2.2.3 and for review of RLCs and their parameters). ImagingWin® v2.30 (Walz GmbH, Germany) was used via a personal computer to both manipulate the I-PAM unit during the experiment and access the data post-reading. The RLCs were fitted to curves using an exponential decay function (PlatPlus) through the “Regression Wizard” in Sigmaplot® v8 (Jandel Scientific, San Rafael, CA, U.S.A.).

2.4 Data analysis

2.4.1 Field data

To test whether there is a significant effect of the environmental parameters (salinity, temperature and light) on $F_v'/F_m'$, $\alpha$, ETR$_{\text{max}}$, and $E_k$, a multiple linear regression was performed for each variable using Minitab® v14 (Minitab Inc., State College, PA, U.S.A.). The Bonferroni procedure was applied for all analyses giving an adjusted critical p-value of 0.0125.
2.4.2 Laboratory data

All statistical tests on laboratory data were performed using either R© v2.7.2 (R Development Core Team, Auckland, New Zealand) or PERMANOVA© v6.0 (Anderson 2001; Anderson 2005, Auckland, New Zealand). Using R©, a repeated-measures ANOVA via the linear mixed effects (lme) function was supplemented with univariate non-parametric permutations tests to examine combined stress effects (salinity, light, temperature and time) on dark-adapted photosynthetic capacity (Fv/Fm). Supplementation with non-parametric permutation tests was necessary when a test for normality had failed, however only 99 runs were performed due to the large size of the data set. Results from these permutation tests did coincide with the p-values calculated during the repeated measures ANOVA, thus significance was accepted and additional permutation tests were deemed unnecessary. Multivariate analysis of the effects on α, ETRmax, and Ik, was performed solely using PERMANOVA©. PERMANOVA© was also used to analyze the exposure and recovery effects (salinity, light versus dark and time) on Fv/Fm as well as α, ETRmax, and Ik.
Results

3.1 Photophysiology of *Anthopleura aureoradiata* in an isolated tide pool

Investigation of a high-shore tide pool at Kau Bay, New Zealand, revealed large fluctuations in salinity, temperature and light across a 9-month monitoring period (Table 3.1). Collection of data was limited by the strict criteria imposed on the weather in order to allow a comparison between surveying days. Salinity varied the least with seasonal minimum and maximum values as follows: summer (Dec-Jan-Feb) = 33-40 ppt, fall (Mar-Apr-May) = 33-37 ppt and winter (June-July-Aug) = 30-36 ppt. At solar noon, temperature ranged between 18-28.5 °C in the summer, 17.5-25 °C in the fall and 10.5-16 °C in the winter. Light varied both seasonally and directly with the amount of cloud cover, with solar noon values of 553-1675 μmol photons/m²/sec during summer, 371-1288 μmol photons/m²/sec in the fall, and 17-789 μmol photons/m²/sec over winter. Unfortunately no readings were attained during spring as equipment was unavailable.
Individuals of *Anthopleura aureoradiata* remained numerous (15-20) throughout the monitored period and were typically found in the same location of the tide pool across most of the dates investigated.

<table>
<thead>
<tr>
<th>Date</th>
<th>Season</th>
<th>Salinity (ppt)</th>
<th>Temperature (°C)</th>
<th>Cloud cover</th>
<th>Average Light (µmol photons/m²/sec)</th>
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Table 3.1. Natural ranges in salinity, temperature and light in a single high-shore tide pool located at Kau Bay, Wellington, New Zealand. The survey was taken at solar noon on days restricted by low tide or weak wind as well as consistent irradiance (completely clear or overcast skies) up to 3 hours prior to readings. Season is represented by summer (Su), fall (F) and winter (W). No readings were taken during spring.

The light-adapted maximum quantum yield of PSII (F'v'/F'm') in the anemones ranged between 0.137-0.654. In Table 3.2, all effects of salinity, light and temperature on the photosynthetic parameters are summarized. Multiple linear regression analysis revealed a significant negative correlation between F'v'/F'm' and light intensity (p=0.012). The full regression model accounted for 31% of the variability in F'v'/F'm'.
There was no significant effect of salinity or temperature on $Fv'/Fm'$ in this survey ($p>0.0125$). Furthermore, neither photosynthetic efficiency ($\alpha$) nor minimum saturation irradiance ($E_k$) were significantly affected by salinity, temperature or light ($p>0.0125$). However, the regression model revealed a significant positive correlation between maximum relative electron transport rate ($rETR_{max}$) and both salinity and temperature ($p=0.003$ and $p=0.001$ respectively). The full regression model accounted for 13% (Figure 3.1b) and 45% (Figure 3.1c) of the variability in $rETR_{max}$ respectively.

Refer to Appendix A for all graphs illustrating the effects of salinity, temperature and light on $Fv'/Fm'$, $\alpha$, $rETR_{max}$, and $E_k$ in the field.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>SE Coefficient</th>
<th>T</th>
<th>P</th>
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</thead>
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<td>$Fv'/Fm'$</td>
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</tr>
<tr>
<td>Constant</td>
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<td>0.158</td>
<td>3.70</td>
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<td>0.001**</td>
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<td>Light</td>
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<td>1.94</td>
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<tr>
<td>Light</td>
<td>-0.000</td>
<td>0.000</td>
<td>-1.22</td>
<td>&gt;0.0125</td>
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<tr>
<td>$rETR_{max}$</td>
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<td>Constant</td>
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<td>7.330</td>
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<td>Light</td>
<td>0.107</td>
<td>0.055</td>
<td>1.94</td>
<td>&gt;0.0125</td>
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Table 3.2. Multiple linear regression analysis summarizing the field effects of salinity, temperature and light on $Fv'/Fm'$, $\alpha$, $rETR_{max}$, and $E_k$. The Bonferroni procedure was applied for all multiple linear regressions giving an adjusted p-value threshold of 0.0125. Significance is indicated by * ($p\leq0.0125$), ** ($p\leq0.009$) and *** ($p<0.001$).
Figure 3.1. (A) The significant relationship between maximum quantum yield of PSII 
\((F_{v'/F_{m'}})\) versus light \((p=0.012)\). The regression model accounted for 31% of the variability 
in \(F_{v'/F_{m'}}\). (B,C) The significant relationships between maximum relative electron transport 
rate \((rETR_{\text{max}})\) versus salinity \((p=0.003)\) and temperature \((p=0.001)\) respectively. The 
regression model accounted for 13% and 45% of the variability in \(rETR_{\text{max}}\) respectively.
3.2 Photophysiological stress response of *Anthopleura* *aureoradiata* to a gradient of salinity

In this experiment, anemones were exposed to a combination of salinity, light and temperature stress in order to investigate the effect of these abiotic factors on the photosynthetic health of the anemone-zooxanthella symbiosis in a controlled laboratory environment.

Dark-adapted maximum quantum yields of PSII (F \(_{v}/F_{m}\)) for the zooxanthellae of 1296 individuals of *Anthopleura aureoradiata* at T\(_{0}\) averaged at 0.442 ± 0.059 (mean ± SD). As the experiments progressed towards 96 h, the extreme values of salinity (5, and 60 ppt) had a significant effect on F \(_{v}/F_{m}\) over time (PERMANOVA F \([11,1080]\) 61.4883, p<0.001) at temperature of 18 °C and light of 45 μmol photons/m\(^2\)/sec. Both low and high temperature (6 and 30 °C) and high light (100, 200 and 420 μmol photons/m\(^2\)/sec) exacerbated the salinity effect on F \(_{v}/F_{m}\) (PERMANOVA F \([2,1080]\) 828.4397 and F \([5,1080]\) 374.8900 respectively, p<0.001) and the decline in F \(_{v}/F_{m}\) was proportional to the magnitude of the stresses, a trend made most obvious at 96 h (Figures 3.2, 3.3, and 3.4). There were 2-way interactions between salinity:temperature (PERMANOVA F \([22,1080]\) 9.8151, p<0.001) and temperature:light (PERMANOVA F \([10,1080]\) 68.9776, p<0.001), but not salinity:light (PERMANOVA F \([55,1080]\) 1.0454, p=0.387). In addition, a 3-way interaction was found to exist between the factors (PERMANOVA F \([110,1080]\) 1.6325, P<0.001). Refer to Appendix B for a summary of the full statistics from the salinity gradient experiment.

Figure 3.2 depicts the resulting values of F \(_{v}/F_{m}\) for all levels of salinity, temperature and light at 96 h. At a moderate temperature of 18 °C and light of 45 μmol photons/m\(^2\)/sec, the most notable declines in F \(_{v}/F_{m}\) were detected at 5 ppt (after 1 h of
treatment) and 60 ppt (after 48 h of treatment), after which $F_v/F_m$ levels gradually decreased by 20 and 30% at 96 h, respectively. At 18 °C and 100 μmol photons/m²/sec $F_v/F_m$ values at these same salinity values decreased by 40 and 60% respectively. At 420 μmol photons/m²/sec the effect of extreme salinity on $F_v/F_m$ was magnified 3-fold, occurred more rapidly (visible within 1 h), involved changes across all salinities and progressed consistently until all photosynthetic activity ceased at 5 and 60 ppt at 96 h. Figure 3.3 further highlights this progression across 5 different salinities and 3 different light treatments at 18 °C. In contrast to high light, low light levels (1 and 20 μmol photons/m²/sec) appeared to have a short-term benefit on $F_v/F_m$ of anemones exposed to the control salinity (35 ppt), with negative impacts minimized (<30% decline) for extreme levels of salinity (5, 10, 55, and 60 ppt). Approximately 15% of all anemones at 18 °C experienced visible tissue damage and died soon after, a value which represents those anemones exposed to only the most extreme salinities. However, this value was not rigorously quantified by appropriate histological examination and variability appeared to exist between individuals of different sizes.

When exposed to a temperature of 6 °C, the effect of salinity on $F_v/F_m$ was amplified markedly, particularly in those anemones at 100, 200, and 420 μmol photons/m²/sec (Figure 3.2). $F_v/F_m$ drops of 30% for 100 μmol photons/m²/sec, 50% for 200 μmol photons/m²/sec and 75% for 420 μmol photons/m²/sec were notable within just 1 h across all salinities and complete photosystem shutdown occurred for 5 and 10 ppt at 420 μmol photons/m²/sec after just 3 hrs. The remainder of the anemones at 1, 20, and 45 μmol photons/m²/sec showed highly variable $F_v/F_m$ values, but were more photosynthetically viable at 96 h than their less shaded counterparts. The majority of anemones also closed their tentacles within the first few hours. Although their tissues
were paler, they appeared superficially intact for the duration of the trial. Mortality, however, increased to approximately 60% by the end of the experiment.

The combinations occurring at 30 °C had a similar devastating effect, with the extreme (low and high) salinities of 5, 10, 15, 55, and 60 inflicting complete shutdown of zooxanthellar photosynthetic functions between 72 and 96 h (Figure 3.2). This effect, although ultimately harsher than that witnessed at 6 °C, took longer to come about whereby a minimum of 48 h was required before the first anemones reached a F_v/F_m of zero. Furthermore, more of the light treatments were tolerated around mid-range salinities. Figure 3.4 summaries how the decline in F_v/F_m was influenced by 5 different salinities and 3 different temperatures at a single light treatment, 100 μmol photons/m²/sec. Of the 12 salinities used, 5 ppt consistently had the most affect on F_v/F_m across all temperatures and light treatments. Initially, *A. aureoradiata* appeared to thrive in the warm water whereby most individuals were fully expanded, thus appearing larger in size. As the experiment advanced, the anemones began to close (while still appearing balloon-like) and eventually turned to ‘jelly’ suggesting severe tissue damage. A mortality of 75% was observed. Refer to Appendix C for all graphs illustrating the effects of salinity, temperature and light on F_v/F_m across all time points: T_1, T_3, T_6, T_24, T_48, T_72, and T_96.

Individuals of *A. aureoradiata* exposed to sufficient stress typically closed and remained so throughout the majority of the trial. Those exposed to extreme salinities (5, 10, 15, 45, 50 and 55 ppt) readily bleached and individuals who survived tissue damage appeared aposymbiotic for months after the trial.
Figure 3.2. The effects of salinity (5-60 ppt in 5 ppt increments) on the maximum quantum yield of PSII (Fv/Fm) at 6 different light levels (1, 20, 45, 100, 200 and 420 μmol photons/m²/sec) and 3 different temperatures: (A) 6, (B) 18 and (C) 30 °C after 96 h of treatment. Only extreme values of salinity (5 and 60 ppt) had a significant effect on Fv/Fm over time (PERMANOVA F[11,1080] 61.4883, P<0.001) at a moderate temperature of 18 °C and light of 45 μmol photons/m²/sec. Both low and high temperature (6 and 30 °C) and high light (100, 200 and 420 μmol photons/m²/sec) also had exacerbating effects on salinity (PERMANOVA F[2,1080] 828.4397 and F[5,1080] 374.8900 respectively, P<0.001) and the decline in Fv/Fm was proportional to the magnitude of the stresses.
Figure 3.3. The effect of 4 extreme salinities (5, 10, 55, 60 and control 35 ppt) on the maximum quantum yield of PSII (Fv/Fm) at 3 different light levels (1, 200 and 420 μmol photons/m²/sec) and at the moderate temperature of 18 °C.

Figure 3.4. The effect of 4 extreme salinities (5, 10, 55, 60 and control 35 ppt) on the maximum quantum yield of PSII (Fv/Fm) at 3 different temperature levels (6, 18 and 30 °C) and at the moderate-high light of 100 μmol photons/m²/sec.
Light curves were created from a subset of the data to include all 3 temperatures but only 4 of the light treatments (1, 45, 100, and 420 μmol photons/m²/sec) and 7 salinities (5, 10, 15, 35, 50, 55, and 60 ppt). Photosynthetic efficiency (α) at T₀ for these light curves averaged 0.392 ± 0.069 (Figure 3.5). At T₉₆, α dropped to 0.107 ± 0.060 with all 3 factors having a significant effect over time: salinity (PERMANOVA F[6,420] 6.1520, p<0.001), temperature (PERMANOVA F[2,420] 227.2690, p<0.001) and light (PERMANOVA F[3,420] 29.5506, p<0.001). Extreme salinities had the greatest impact, with both high light, and low and high temperature exacerbating the effect. There were also 2-way interactions between salinity:temperature (PERMANOVA F[12,420] 3.4258, p<0.001) and temperature:light (PERMANOVA F[6,420] 30.4402, p<0.001), but not salinity:light (PERMANOVA F[18,420] 0.7331, p=0.781). In addition, a 3-way interaction was found to exist between the factors (PERMANOVA F[36,420] 1.5148, p=0.033).

Maximum relative electron transport rates (rETRₘₐₓ) at T₀ from the same light curves averaged 45.920 ± 8.180 (Figure 3.6). At T₉₆, average rETRₘₐₓ dropped to 11.343 ± 7.955 with all 3 factors having a significant effect: salinity (PERMANOVA F[6,420] 4.1009, p=0.003), temperature (PERMANOVA F[2,420] 234.6314, p<0.001) and light (PERMANOVA F[3,420] 18.2154, p<0.001). Extreme salinities again had the greatest impact, with both extreme light and extreme temperature values enhancing the effect. There was a 2-way interaction between temperature:light (PERMANOVA F[6,420] 6.5631, p<0.001), but not for salinity:temperature (PERMANOVA F[12,420] 1.0639, p=0.397) nor salinity:light (PERMANOVA F[18,420] 0.5106, p=0.946). There was also no 3-way interaction found to exist between the factors for this photosynthetic parameter (PERMANOVA F[36,420] 0.5386, p=0.992).

Minimum saturating irradiance (Eₖ) at T₀ averaged 120.537 ± 26.778 μmol photons/m²/sec (Figure 3.7). At T₉₆, average Eₖ dropped to 28.736 ± 18.972 μmol
photons/m²/sec with all 3 factors having a significant effect: salinity (PERMANOVA $F_{[6,420]}$ 5.0764, $p<0.001$), temperature (PERMANOVA $F_{[2,420]}$ 147.2769, $p<0.001$) and light (PERMANOVA $F_{[3,420]}$ 15.9138, $p<0.001$). Once again extreme salinities were most detrimental, with both extreme light and extreme temperature exacerbating the effect. There were also 2-way interactions between salinity:temperature (PERMANOVA $F_{[12,420]}$ 1.9045, $p=0.033$) and temperature:light (PERMANOVA $F_{[6,420]}$ 5.1984, $p<0.001$), but not salinity:light (PERMANOVA $F_{[18,420]}$ 0.6257, $p=0.877$). There was no 3-way interaction between the factors (PERMANOVA $F_{[36,420]}$ 0.9920, $p=0.992$).
Figure 3.5. A comparison between the natural ranges in photosynthetic efficiency ($\alpha$) at the start of the trials ($T_0$) to those experiencing the effects of salinity (A), light (B) and temperature (C) seen after 96 h ($T_{96}$).
Figure 3.6. A comparison between the natural ranges in maximum relative electron transport rate (rETR$_{\text{max}}$) at the start of the trials (T$_0$) to those experiencing the effects of salinity (A), light (B) and temperature (C) seen after 96 h (T$_{96}$).
Figure 3.7. A comparison between the natural ranges in minimum saturation irradiance ($E_k$) at the start of the trials ($T_0$) to those experiencing the effects of salinity ($A$), light ($B$) and temperature ($C$) seen after 96 h ($T_{96}$).
3.3 Recovery of *Anthopleura aureoradiata* from exposure to extreme salinities of variable duration

The impact of variable duration of extreme salinity exposure on the photosynthetic health of the anemone-zooxanthella symbiosis and the possible differences in response due to light or dark exposure were investigated during this experiment.

Salinity (PERMANOVA $F_{[8,216]} = 30.5508$, $p<0.001$), light (PERMANOVA $F_{[1,216]} = 63.0963$, $p<0.001$) and duration of exposure to treatment (PERMANOVA $F_{[3,216]} = 11.4468$, $p<0.001$) all had a significant effect on the recovery of $F_v/F_m$. There were also 2-way interactions between salinity:light (PERMANOVA $F_{[8,216]} = 12.2805$, $p<0.001$) and salinity:duration (PERMANOVA $F_{[24,216]} = 1.7274$, $p=0.015$). No 2-way or 3-way interactions existed between light:duration (PERMANOVA $F_{[3,216]} = 0.7733$, $p=0.527$), and salinity:light:duration (PERMANOVA $F_{[24,216]} = 0.5700$, $p=0.978$) respectively. Refer to Appendix D for a summary of the full statistics from the variably duration and recovery experiment.

For the light treatment (85 µmol photons/m²/sec), effects from extreme salinities (5 and 60 ppt) were visible within just 24 h (Figure 3.8). Anemones exposed for only 24 h experienced minimal declines of 15% in $F_v/F_m$ levels at 5, 10 and 60 ppt and were fully recovered within 48 h when returned to 35 ppt. As duration of exposure increased to 48 h, full recovery of $F_v/F_m$ activity was visible within 96 h after transfer back to 35 ppt for anemones at 10-55 ppt but not 5 and 60 ppt. This trend continued for anemones exposed for 72 and 96 h, with those treated at 5, 10, 55 and 60 ppt not recovering within the allotted monitoring time (96 h) and experiencing declines of $F_v/F_m$ between 60 and 95% respectively. The dark treatment resulted in a comparable trend, however effects appeared to be slightly less intensified with fewer of the extreme salinities having an
impact on $F_v/F_m$ values early on (Figure 3.9). For instance, at 48 h, only 5 and 60 ppt caused a 50% decline in $F_v/F_m$, compared to the 70% seen for 5, 10, 55, and 60 ppt in the light. No recovery of anemones was witnessed after 48 h of exposure to 5 and 60 ppt and 72-96 h of exposure to 5, 10, 55, and 60 ppt, either in the light or dark. Anemone tissue damage and mortality coincided with the severity of the stress, however these variables were not robustly quantified. Refer to Appendix E for all graphs illustrating the variable declines of photosynthetic efficiency ($\alpha$), maximum relative electron transport rate ($r\text{ETR}_{\text{max}}$) and minimum saturating irradiance ($E_k$) exposed to a range of salinities for both light and dark treatments and their subsequent recovery between different treatment durations (24, 48, 72 and 96 h).
Figure 3.8. The variable decline of maximum quantum yield of PSII (Fv/Fm) exposed to a range of salinities (5-60 ppt at 5 ppt increments) at 85 μmol photons/m²/sec and the subsequent recovery seen between different treatment durations (24, 48, 72 and 96 h).
Figure 3.9. The variable decline of maximum quantum yield of PSII ($F_{v}/F_{m}$) exposed to a range of salinities (5-60 ppt at 5 ppt increments) at 0 μmol photons/m²/sec and the subsequent recovery seen between different treatment durations (24, 48, 72 and 96 h).
Light curves were created from all of the data. Photosynthetic efficiency ($\alpha$) was significantly affected by salinity (PERMANOVA $F_{[8,216]}$ 6.5036, $p<0.001$), light (PERMANOVA $F_{[1,216]}$ 4.8130, $p=0.018$) and duration (PERMANOVA $F_{[3,216]}$ 4.2788, $p=0.002$). $T_0$ values averaged $0.490 \pm 0.040$. For treatments at 85 $\mu$mol photons/m²/sec, drops in $\alpha$ values mirrored those of $F_v/F_m$ in both magnitude and timing. For instance, $\alpha$ at 5 and 60 ppt experienced a decline of approximately 55% after the 24 h exposure and did not recover during the rest of the monitoring period. At 48 h and 96 h, 5, 10, 15 and 60 ppt had declined 70% and 90% decline without recovery. In the dark, the pattern noted above was lost and the values became more variable, ranging between 0-0.55 randomly throughout the duration of the trial. For the anemones treated 48 and 72 h, 60 ppt consistently caused the lowest $\alpha$ values. There were also 2-way interactions between salinity:light (PERMANOVA $F_{[8,216]}$ 3.0314, $p=0.002$), salinity:duration (PERMANOVA $F_{[24,216]}$ 1.7235, $p=0.005$) and light:duration (PERMANOVA $F_{[3,216]}$ 6.8497, $p<0.001$), but no 3-way interaction was found to exist between the factors (PERMANOVA $F_{[24,216]}$ 1.0172, $p=0.430$).

Maximum relative electron transport rates ($rETR_{max}$) were significantly affected by salinity (PERMANOVA $F_{[8,216]}$ 6.3653, $p<0.001$), light (PERMANOVA $F_{[1,216]}$ 24.2385, $p<0.001$) and duration (PERMANOVA $F_{[3,216]}$ 9.7773, $p<0.001$). At $T_0$, $rETR_{max}$ values averaged $53.590 \pm 6.059$. In the light, anemones at 5 ppt began to experience minor declines, of about 25%, up to 24 h but then recovered fully once treatment salinities were returned to 35 ppt at this time. The anemones which experienced 48 h of treatment at 5 and 60 ppt had a significant drop of about 60% and did not recover once returned to the control salinity. Anemones treated for 72 and 96 h at 5, 10, 55, and 60 ppt showed declines in $rETR_{max}$ that were proportional in magnitude to the duration of treatment, hence approximately 60 and 90% respectively, and also
showed no recovery once returned to 35 ppt. In the dark, \( \text{rETR}_{\text{max}} \) values for all salinities and durations ranged between 0-30, about 60-70\% less than \( \text{rETR}_{\text{max}} \) in the light, with the most extreme salinities causing the lowest levels and remaining so for the duration of the trial. There were also 2-way interactions between salinity:light (PERMANOVA \( F_{[8,216]} 5.3486, p<0.001 \)), salinity:duration (PERMANOVA \( F_{[24,216]} 1.7146, p=0.016 \)) and light:duration (PERMANOVA \( F_{[3,216]} 6.6174, p<0.001 \)), but no 3-way interaction was found to exist between the factors (PERMANOVA \( F_{[24,216]} 1.2863, p=0.144 \)).

Minimum saturating irradiance (\( E_k \)) was significantly affected by salinity (PERMANOVA \( F_{[8,216]} 1.3117, p=0.024 \)) and duration (PERMANOVA \( F_{[3,216]} 2.5784, p=0.028 \)) but not light (PERMANOVA \( F_{[1,216]} 0.1748, p=0.792 \)). At \( T_0 \), \( E_k \) values averaged \( 114.431 \pm 20.772 \) \( \mu \text{mol} \) photons/m\(^2\)/sec. In the light, \( E_k \) values for all salinities changed little after 24 h of treatment. However, after 48 h of treatment, \( E_k \) at 5 and 60 ppt dropped to approximately 50\%. After 72 h of treatment at 5, 10, and 60 ppt, \( E_k \) also dropped to about 60\%, while after 96 h of treatment at 5, 10, 55 and 60 ppt, \( E_k \) dropped about 80\%, though the anemones at 55 ppt fully recovered by the end of the trial. Similar but subtler trends were visible for the anemones treated in the dark, with 5 and 60 ppt producing the lowest \( E_k \) values for the 48 and 72 h treatments, and 5, 10, 55 and 60 ppt producing the lowest \( E_k \) values after 96 h of treatment. There were no 2-way interactions between salinity:light (PERMANOVA \( F_{[8,216]} 1.4663, p=0.108 \)), salinity:duration (PERMANOVA \( F_{[24,216]} 0.8110, p=0.821 \)) and light:duration (PERMANOVA \( F_{[3,216]} 1.7417, p=0.100 \)), and no 3-way interaction was found to exist between the factors (PERMANOVA \( F_{[24,216]} 0.7576, p=0.881 \)).
Discussion

In this study, it was demonstrated that the temperate symbiotic sea anemone, *Anthopleura aureoradiata*, was resilient to abiotic fluctuations of considerable magnitude in the intertidal zone. Salinity was revealed to range naturally between a winter low of 30 and summer high of 40 ppt in an elevated tide pool with no visible effects on the photophysiology of *A. aureoradiata* residing within. In a controlled environment, extremes of salinity (5, 10, 55 and 60 ppt) had a dramatic effect on zooxanthelllar photosystem health and anemone survival, with a wide range of tolerance between 15-50 ppt dependent on the levels of temperature and light. Both high and low light, and temperature, impacted upon photophysiology. Moreover, each of these variables independently, as well as combined, exacerbated the impact of salinity stress. In addition, the duration of exposure played an important role in the survival of this symbiosis, with only 48-96 h exposure to the extreme salinities of 5, 10, 55 and 60 ppt inducing irreversible photosynthetic failure and death. Here I will discuss: 1) the role of salinity on symbiotic anthozoan distribution and 2) the mechanisms behind the osmoregulatory and photophysiological tolerance of *A. aureoradiata* to salinity stress.
4.1 The role of salinity on symbiotic anthozoan distribution and health

4.1.1 Natural resilience to in situ salinity fluctuations

Habitats such as estuaries and tide pools, although often protected from rough waves and larger predators, are subject to changes in salinity through processes of evaporation, coastal runoff and seasonal rainfall that can last anywhere from hours to months. The high-shore tide pool at Kau Bay reached a summer high of 40 ppt and a winter low of 30 ppt during a 9-month monitoring period. As seen in other studies these highs and lows in salinity were preceded by periods of prolonged heavy rainfall and evaporation. Incidences of major rainfall events such as cyclones or hurricanes are not uncommon in the tropics. During these major storms, many areas are transformed into raging rivers and floodland, thus greatly influencing the coast, especially at low tide (Houghton and Woodwell. 1989). These transient effects can also last anywhere from several minutes to several weeks. Numerous cases of prolonged reduced salinities due to storm-related events have been documented thus far. For instance, Cloud (1952) noted a value of 4 ppt on a high-shore reef-flat tide pool on Onotoa Atoll in Kiribati after only a day of heavy rainfall; Moberg et al. (1997) recorded a value of 10 ppt in coral-containing tide pools on the reefs of the inner Gulf of Thailand; and Orr and Moorhouse (1933) found a salinity level of 17 ppt in a shallow tide pool of a reef flat in the Low Isles of the Great Barrier Reef. These salinities returned to normal following high tide flushing. This was also seen at Kau Bay, whereby the high and low values in salinity were only reached after 1-2 week periods of low tides of a particularly low amplitude coupled with weak
inshore winds which inhibited subsequent flushing. For instance, 40 ppt was achieved through evaporation following a week of drought and lack of sufficient wind which prevented the larger waves needed to flush this pool during high tide. Similarly, weak winds also prevented the full strength sea water from splashing into the tide pool after it had been diluted to 30 ppt by prolonged heavy rainfall days before. Salinity and temperature were typically returned to normal by subsequent tidal flushing within a few days to a week.

Despite the tide pool at Kau Bay being particularly isolated, a range of 30-40 ppt is not as impressive as that seen in some areas of the world which experience much more prolonged and frequent hypo- and hypersaline conditions. For example, a reduction of surface salinity levels to 5.4 ppt was reported by Goodbody (1961) in Jamaica following heavy rain and coastal runoff, and it was 3 months before all of the effect had worn off. Similarly, following Hurricane ‘Flora’ in 1963, Jamaican surface waters (<2.5 m) once again dropped to as little as 3 ppt immediately following the storm but values of <30 ppt persisted for over 5 weeks (Goreau 1964). On the Great Barrier Reef, Cyclone ‘Joy’ induced plumes of reduced salinity that continued for up to 3 weeks (van Woesik et al. 1995; Devlin 1998) with Cleveland Bay experiencing values of 28-32 ppt for 4 weeks (Berkelmans and Oliver 1999) and waters off Keppel Island at the peak of the flood being 7-10 ppt at the surface, 15-28 ppt at 3 m, 31-34 ppt at 6 m and 33-34 ppt at 12 m (Brodie and Mitchell 1992). Biscayne Bay, Florida, is a tropical estuarine area characterized by chronically wide fluctuations and low mean salinity values which coincide with some of the lowest values of coral density and species richness known. Here, the coral population is primarily composed of Siderastrea radians and Porites furcata (Lirman et al. 2003). Patterns measured using field probes across 2 years (1998 and 1999) revealed that the coral communities were exposed to salinities below 25 ppt.
for 188 and 156 days with a minimum daily salinity of 12 and 13 ppt, respectively (Lirman et al. 2003). Similarly, established populations of the temperate non-symbiotic sea anemone *Metridium senile* were reported in the Mersey River estuary, England, where the salinity ranges at 2 different areas were only 13-20 and 21-28 ppt, respectively (Rawlinson 1934). Thus, similar scenarios can occur in both tropical and temperate environments. Further examples of reefs which exist within such “marginal” salinity habitats (identified due to their proximity to environmental limits) were summarized by Kleypas et al. (1999). The most notable of these locations include the Gulf of Guinea, Burma and the Bay of Bengal, with minimum monthly salinity values of 20.7, 23.3 and 27.0 ppt respectively (Kleypas et al. 1999).

Alternatively, some coral reefs exist in areas that are regularly subject to elevated salinities or have naturally high ranges year round. Such values vary seasonally, annually and over decadal timescales primarily due to changes in regional precipitation, freshwater runoff, and evaporation (Robblee et al. 1989; Cronin et al. 2002). These few areas include certain parts of Western Australia, some Pacific atoll lagoons and most notably, the waters of the Middle East (Sheppard 1988; Coles and Jokiel 1992). Florida Bay, a large shallow embayment, has also seen monthly mean salinity values as high as 52 ppt, periodically even reaching 70 ppt (Robblee et al. 1989; Cronin et al. 2002). In the Arabian Gulf (Persian Gulf) and parts of the Red Sea (for example the Gulf of Aqaba and the Gulf of Suez) average salinity exceeds 40 ppt, yet reef building corals appear to thrive (Kleypas et al. 1999; Coles 2003). The Arabian Gulf, in particular, experiences hypersalinity due to restriction by the narrow opening of the Strait of Hormuz coupled with low rates of freshwater input (from the Tigris-Euphrates River) and high rates of evaporation (John et al. 1990). Thus, the open waters reach an average of 42-50 ppt with many of the smaller embayments (eg. Gulf of
Salwah, between Saudia Arabia and Qatar) peaking at values of 70 ppt (John et al. 1990; Coles 2003). Although 70 ppt is outside the range of coral development, certain coral species in the area are capable of tolerating salinities up to 50 ppt (Sheppard 1988; Coles 1993; Coles 2003). Similarly, the Red Sea contains extensive coral communities capable of continuously tolerating 40-45 ppt (Sheppard and Sheppard 1985; Piller and Kleemann 1992; Kleypas et al. 1999). The communities, although widespread, are typically less diverse than those residing in regular strength sea water and are estimated to decrease by approximately one species with every unit rise between 41-50 ppt (Sheppard 1988). Of the 10 species that Sheppard (1988) listed capable of surviving salinities in excess of 46 ppt for at least 1-3 months, only 3 species (*Siderastrea savignyana*, *Porites nodifera* and *Cyphastrea microphthalm*) could potentially continuously do so at 50 ppt off the coast of Bahrain. However, this adaptation to a more hypersaline environment by the corals of the Arabian Gulf (giving them an ambient range of 40-42 ppt and an upper tolerance range of 47-49 ppt) when compared to those from the Atlantic-Pacific (35-37 and 40-45 ppt respectively) comes at a cost to the other end of the salinity spectrum, with the lower tolerance range of corals in the Arabian versus Atlantic-Pacific being only 20-23 ppt and 15-20 ppt respectively (Coles 1993).

Pauatahanui Inlet, the collection site of the *A. aureoradiata* during this study, experiences comparatively similar values for light and temperature as those seen at Kau Bay. Summer ranges of 300-1800 µmol photons/m²/sec and 10-22 °C and winter ranges of 200-800 µmol photons/m²/sec and 5-12 °C were recorded during the same season for a different study (C. Gibbons, unpublished work). Because this large estuarine mudflat is semi-enclosed, relatively shallow (high surface-to-volume ratio) and receives input from rivers, it also has the potential for both of the hypo- and hypersaline scenarios.
occurring (from the effects of heavy rainfall or drought) at such marginal habitats as Biscayne Bay and the Arabian Gulf (Coles 2003; Lirman et al. 2003). However no such values were recorded during the periodic collection of *A. aureoradiata*. Instead salinity was always within the range of 34-36 ppt. It is my belief due to previous findings of salinity levels as high as 45 ppt at Kau Bay (Z. Haws, VUW per. comm.), that it is not inconceivable that such highs and lows in salinity are achievable given a sufficient monitoring period and associated weather conditions which were both outside the timeline of this study.

No significant negative effects of salinity were revealed within this naturally-fluctuating regime of 30-40 ppt with only light having a negative impact on the maximum quantum yield of PSII ($F_v'/F_m'$). Both salinity and temperature were positively correlated with maximum relative electron transport rate ($\text{rETR}_{\text{max}}$) possibly due to an interaction effect between these 2 factors, though the mechanisms behind this result will be covered more thoroughly in the next section 4.2.2. This substantial resilience of the algal-anthozoan symbiosis to abiotic stress is likely due to its adaptation to a more heterogeneous environment (Bates 2000), albeit more shade-adapted. The majority of other individuals of *A. aureoradiata* located on the nearby rocky shore tend to be found imbedded within cracks or in areas where they are only partially exposed to light, however most of the anemones found in this particular high-shore tide pool lacked suitable cover. Likewise, at the mudflats of Pauatahanui Inlet, *A. aureoradiata* is found buried just beneath the surface, attached to cockles or sea grass roots where it is partially in control of its exposure to light. It is estimated that this resilience could extend into a greater range of salinity (20-50 ppt) with no visible negative effect on photosynthetic health given that temperature and light remained
The reasons behind this prediction are explored more thoroughly in section 4.1.2 and 4.2.

4.1.2 Tolerance to and recovery from extreme salinities

Due to the naturally occurring salinities ranges around the world and the potential for both Pauatahanui and Kau Bay to experience more extreme scenarios given the right weather conditions, investigations of the effect of salinity on photosynthetic health were extended to include a range of environmentally realistic values from 5 to 60 ppt. Since no visible negative effects occurred between 30-40 ppt in the field, the range was increased to assess what extremes would cause photoinhibition and damage to the photosynthetic apparatus leading to bleaching and death.

Investigations in the laboratory revealed that *A. aureoradiata* has a non-lethal salinity range (0% mortality) of 15-50 ppt for 96 hrs. Only extreme values of salinity (5 and 60 ppt) had a significant effect on Fv/Fm, at a moderate temperature (18 °C) and light level (45 µmol photons/m²/sec). However, it was found that both low and high temperature (6 and 30 °C) and high light (100, 200 and 420 µmol photons/m²/sec) greatly exacerbated the effects of salinity. Similarly, Pierce and Minasian (1974) found that the euryhaline and non-symbiotic anemone *Diadumene leucolena* had a non-lethal salinity range (<50% mortality) between 6-33 ppt for 8 days with no mortality witnessed at 11 ppt, while another non-symbiotic anemone *Bunodosoma cavernata* was found to survive salinities ranging from 11-49 ppt for 2 weeks (Benson-Rodenbough and Ellington 1982) and the non-symbiotic anemone *Metridium senile* could survive a value of as low as 18.7 ppt for at least 2 weeks (Deaton and Hoffmann 1988). Comparably, the zooxanthellate anemone *Condylactis gigantea* withstood 19.8-46.5 ppt
for over 3 weeks (Bursey and Harmer 1979). In this study, $F_v/F_m$ became increasingly affected with escalating hypo- and hypersalinity, however the photosystem remained viable even after 96 hrs of treatment, so long as temperatures and light levels were close to normal. Remarkably, *A. aureoradiata* was capable of withstanding 24 hrs of exposure without adverse affects on $F_v/F_m$ for all salinities between 5-60 ppt and 48 hrs of exposure with full recovery for salinities of 10-55 ppt. At 72 and 96 hrs of exposure, only anemones in 15-50 ppt recovered fully within 96 hrs. In contrast, Kerswell and Jones (2003) found that the coral fragments of the scleractinian coral *Stylophora pistillata* were dead 1 day after exposure to salinity levels of only 15 ppt for 12 h and 10 ppt for 120 min, while Manzello and Lirman (2003) demonstrated that the coral *Porites furcata* was capable of recovering from a 2-24 hr exposure to 20-45 ppt. Light curves created from the current data revealed that all 3 factors (salinity, temperature, and light) also had a notable impact on photosynthetic efficiency ($\alpha$), maximum relative electron transport rate ($rETR_{max}$) and minimum saturating irradiance ($E_k$) which was proportional to the severity of abiotic stress administered. Although $F_v/F_m$ decreased during the treatments, the zooxanthellae remained viable at 5, 10, 55, 60 ppt while the anemone’s tissue did not and disintegrated, suggesting that the symbiont is the more robust member of this symbiosis in response to salinity stress.

All *A. aureoradiata* treated at 5 and 60 ppt and the majority of those individuals treated at 10 and 55 ppt experienced radical zooxanthellar expulsion and a subsequent mortality close to 100%. Of the anemones which survived treatment at 5 and 55 ppt, several individuals were also visibly bleached and remained so indefinitely post experiment. This result is consistent with the handful of the papers that looked at the effects of salinity stress on photophysiology and zooxanthellar expulsion in both the field and the laboratory. Goreau (1964) documented that extensive bleaching of coral
reef communities comprised of *Millepora*, *Scleractinia*, *Zoanthidea* and *Actiniaria* occurred after severe rain and flooding in Jamaica caused salinity values to drop to 3 ppt. Other natural bleaching episodes following heavy storm systems have occurred at Easter Island, whereby corals recovered their colouration within 2-3 months and the Great Barrier reef whose *Acropora* sp. experienced significant bleaching and mortality (Egana and DiSalvo 1982; van Woesik et al. 1995). Likewise, many specimens of *Anthopleura elegantissima*, a temperate intertidal sea anemone, appeared bleached (i.e. white) when located near a stream which repeatedly supplied freshwater during heavy rains; nearby neighbours located in a rocky intertidal zone away from the outflow were nearly all brown. This same anemone exposed to a hyposalinity of 8, 16 and 24 ppt in the laboratory for 7, 14 and 21 days, expelled zooxanthellae in quantities directly related with the strength and duration of the exposure (Engebretson and Martin 1994), whereas *S. pistillata* and *Seriatopora hystrix* suddenly exposed to 30 ppt for 23 days did not (Hoegh-Guldberg and Smith 1989). Kerswell and Jones (2003) found that individuals of *S. pistillata* which experienced the largest reduction in dark-adapted $F_v/F_m$ due to hyposalinity lost the most zooxanthellae, with mortality and sloughing of the coral tissues occurring only at the most extreme low-salinitities. At 17 ppt, these same coral polyps expelled only one third of their zooxanthallae (Tityanov et al. 2000).

Thus, this temperate algal-anthozoan partnership is particularly resilient when compared to published literature, capable of withstanding and recovering from ranges more closely relating to the tolerances of estuarine anemones rather than their symbiotic coral relatives. The mechanisms responsible for its tolerance as well as breakdown in response to extreme salinity stress and how this relates to the breakdown observed during thermal and photo-bleaching will be discussed in the next section. Particular
attention will be given to osmoregulatory mechanisms and the impact of salinity on photophysiology.

4.2 Mechanisms behind the osmoregulatory and photophysiological tolerance of *Anthopleura aureoradiata* to salinity stress

This study intended to investigate the photophysiological response of an intact anemone-zooxanthellar symbiosis to combined salinity-light-temperature stress, focusing on how the system breaks down photosynthetically, the duration and extent of the response, and the point at which recovery is no longer feasible. Experimentation in the laboratory revealed that this anemone-zooxanthellar symbiosis is capable of tolerating a great degree of abiotic variability. Here I will present an overview of the osmoregulatory mechanisms, photoacclimatory strategies and behaviours that this symbiosis likely deploys to combat natural ranges in abiotic factors.

4.2.1 Osmoregulation of an algal-anthozoan symbiosis

The anthozoan cnidarians inhabiting coral reefs have long been considered strict stenohaline osmoconformers, with little or no ability to osmoregulate (Kleypas et al. 1999). Any fluctuation in the osmolarity of the external environment is followed closely by the water within their coelenterons. In order to remain iso-osmotic with external changes, the animals must rapidly modify internal levels of amino acids, ions and proteins. If this change in solute levels exceeds that of physiological tolerance, then the
organism faces metabolic disruption of cellular electrochemical processes, enzyme activity, and nerve conduction (Mayfield and Gates 2007). Having overcome these challenges, *Anthopleura aureoradiata* has been shown to tolerate a wide range of salinity without any measurable effects.

The importance of maintaining an internally compatible osmotic environment has been recently reviewed (Mayfield and Gates 2007). These authors suggest that the stability seen in cnidarian-dinoflagellate associations under normal environmental conditions can be credited to effective and rapid exchanges of osmotically active compounds. Although invertebrates are generally osmoconformers, they do possess the ability to make alterations to intracellular osmolarity, dedicating a large amount of energy to these processes. The physiological mechanisms behind this type of osmoregulation are poorly understood, as are the osmotic scenarios elicited during bleaching. Osmotic stress, known as the point when osmoregulation is no longer energetically efficient, is synonymous with volume and osmolyte fluctuations that compromise cell structure and function, and typically occurs in response to desiccation and/or salinity stress. It is quite probable that the series of events that take place during the first hours following PSII damage can inflict osmotic stress on the symbiosis prior to bleaching taking place (Figure 4.1, Mayfield and Gates 2007). Once photosynthesis is impaired, photosynthate transfer is reduced and there is a depletion of necessary metabolites within the host cells. Water may therefore exit the cells and hyperosmotic stress ensues, potentially leading to cytoskeletal damage, cell adhesion protein detachment, and eventual expulsion of host cells and their zooxanthallae. If the initial trigger is significant enough to surpass homeostasis attempts, namely free amino acid (FAA) regulation (discussed below), then bleaching can occur within a few hours, although several days is a more likely timeline (Mayfield and Gates 2007).
A hypothetical set of steps has not been sequentially demonstrated, but many of the biochemical and histological symptoms associated with bleaching are consistent with osmotic stress. This is the likely scenario which caused the photoinhibition and bleaching at extreme salinities documented in this study.

Figure 4.1. A hypothetical flowchart illustrating the onset times for various cellular events as a result of multiple stressors and the possible role of osmotic stress in coral bleaching. Taken from Mayfield and Gates (2007).
Under the influence of less extreme salinities, it was likely that the cell-mediated response triggered within minutes to hours in *A. aureoradiata* was sufficient to alter volume and ionic fluctuations and restore homeostasis. The most likely strategy utilized by anthozoans and their zooxanthellae is achieved through compatible organic osmolytes (COOs), most commonly taurine (a FAA) and glycerol (a polyol), which are either synthesized or degraded in order to alter the intracellular osmolarity and thus combat damaging fluxes of water and ions. COOs tend to be simpler molecules which are easy and energetically inexpensive to catabolize from larger ones to serve as osmolytes. COOs have a further protective effect on various elements of the cell, such as the membrane and essential macromolecules (Mayfield and Gates 2007). In the case of hyperosmotic stress, COOs are rapidly synthesized in order to prevent unnecessary ion gain or water loss. FAAs and glycerol most likely accumulated in *A. aureoradiata* in proportion to the increase in salinity (Mayfield and Gates 2007). In addition, several studies have also noted proportional increases of FAAs in unicellular algae exposed to higher salinities (Blackwell and Gilmour 1991; Rani 2007), thus these osmoprotectants may not only play a role in host cells, but also the cells of the zooxanthellae residing within. Under hypoosmotic conditions, the opposite is shown to be true, and COOs are depleted rapidly to prevent water uptake and important ion loss (Herrera et al. 1989; Deaton and Hoffmann 1988; Mayfield and Gates 2007). Similarly, unicellular algae have also been found to decrease glycerol concentrations (Marengo et al. 1985; Chitlaru and Pick 1991), suggesting the same may be true for zooxanthellae. In addition, anemones have also been shown to secrete mucus (Bursey and Harmer 1979) and this was consistent with the findings of this study. This response is believed to help reduce the osmotic influx of water, also achieved by certain behavioural mechanisms observed
with *A. aureoradiata*, such as inverting the oral disc and tentacles in response to both high and low salinities.

Osmoregulation is a constant cellular activity of multi-layered biological cascades which can be fine-tuned with changes to the abiotic environment. There is not a lot of literature dealing with the mechanisms involved in the breakdown of a symbiosis during osmotic stress, however we do know that any factor which exceeds these regulatory thresholds has the potential to interfere with metabolic processes leading to cytoskeleton disruption, cell adhesion dysfunction, pH shifts, ionic imbalances, increased respiration, and/or formation of ROS (Mayfield and Gates 2007), all of which have been documented during bleaching.

### 4.2.2 Impact of salinity on photophysiology

Figure 4.1 illustrates a myriad of abiotic factors that can lead to damage of photosystem II. This study shows that osmotic stress can indeed lead to loss of function for a temperate symbiotic sea anemone. In addition the combined effects of light and temperature exacerbated the impact immensely.

In photosystem II, it is the pumping of H$^+$ ions into the thylakoid that drives the conversion of ADP+P into ATP. If the scenario in Figure 4.1 is true, then the disruption of ion flow across the zooxanthellar thylakoid membrane during severe osmotic stress will interrupt photosynthesis. This will in turn favour a buildup of ROS and consequently lead to cellular damage and photosystem breakdown. ROS can damage protein function (particularly the D1 protein), membrane integrity (such as that of the thylakoid), nucleic acids and other vital processes (eg. Calvin Cycle). As with osmoregulation, several protective pathways exist to prevent damage to the light-
harvesting antennae of photosystem II. Such photoprotective defenses include xanthophylls cycle pigments with act to dissipate excess photon energy as heat through non-photochemical quenching (NPQ); down-regulation of reaction centres; anti-oxidant enzymes; p-carotene production; and mycosporine-like amino acids; in addition to behavioural responses such as retraction will help keep the zooxanthellae photoactive (Brown et al. 2000). If these defenses are overwhelmed with sufficient stress, the resulting affect will be chronic photoinhibition. *A. aureoradiata* only experienced significant photodamage at salinity extremes of 5, 10, 55 and 60 ppt. When coupled with high and low values of temperature and light, this damage was magnified notably, resulting in loss of photosynthetic function, bleaching and death. All photosynthetic parameters measured (\(F_v/F_m\), \(\alpha\), \(\text{rETR}_{\text{max}}\) and \(E_k\)), suffered decreases that increased proportional at hypo- and hypersalinites. This is consistent with numerous studies examining the effects of temperature and light on symbiotic photosynthetic damage and bleaching (Warner et al. 1999; Brown et al 2000; Saxby et al. 2003; Smith et al. 2005). This resilience to bleaching suggests that *A. aureoradiata* and its zooxanthallae have evolved a combination of powerful defensive mechanisms to help aid against the heterogenous environment from which they come. Furthermore, it was demonstrated that bleaching due to salinity stress was a sublethal response, whereby the remaining symbiosis had the ability to recover photosynthetically within days of the exposure.

### 4.2.3 Resilience of *Symbiodinium* of clade A

Zooxanthellae from clade A are indeed known to possess a highly flexible photosynthetic apparatus and tend to favour variable conditions. Their ability to mediate light-harvesting complexes and enrich xanthophyll content allows them to
photosynthesize effectively under low light and to take advantage of strong pulses of light (Iglesias-Prieto and Trench 1997). It is possible that the low tolerance to high light exhibited during this study was a direct cause of the duration at which it was continuously kept on. With no opportunity for photosynthetic ‘rest’, the protective mechanisms were quickly overwhelmed resulting in damage to the photosynthetic apparatus and loss of function. Alternatively, they may have acclimated to the low light conditions within the incubator prior to the experiment. It was also noted that anemones collected from Kau Bay were much darker than those located at Pauatahanui Inlet, either as a result of increased zooxanthellar densities or animal pigments. Clade A is also known for its preference toward cooler water, so it was expected to see this symbiosis breakdown more quickly in warm water especially with the added stress of high light. It is unknown as to which of the partners was responsible for the resilience toward salinity stress, however, it is suspected to be the host due to the visible tissue damage that resulted at the end of the trial. It is possible that the zooxanthellae remained photosynthetically viable at moderate temperature and light, due to the added barrier of animal tissue which encased them.

4.3 Conclusions and future directions

In this study, it was demonstrated that the temperate symbiotic sea anemone, *Anthopleura aureoradiata*, was resilient to abiotic fluctuations of considerable magnitude and duration. Only extreme high and low salinities (5, 10, 55 and 60 ppt) had an effect on the zooxanthellar photosystem, with a wide range of tolerance between 15-50 ppt dependent on the levels of temperature and light, which exacerbated the effect. In addition, the duration of exposure played an important role in the survival of this
symbiosis, with only 48-96 h exposure to the extreme salinities of 5, 10, 55 and 60 ppt inducing irreversible photosynthetic failure and death.

Future investigations in this temperate symbiosis should include a closer look into the suspected mechanisms behind this resilience. Zooxanthellar loss needs to be quantified and the experiments should be repeated on both aposymbiotic anemones and isolated zooxanthellae in culture in order to gain a better picture as to which abiotic factor is having the greatest affect on each partner. A closer look at levels of COOs, D1 reaction center proteins and ROS measured in both symbiont and host would help deduce the mechanisms behind this observed tolerance as well as the breakdown which occurs at the extremes. Acclimatization trials should also be included to investigate whether a more gradual change in salinities will result in increased tolerance to high levels for longer periods of time.

With the ever present threat of global climate change, studies involving investigations of various abiotic factors on photosynthetic health and bleaching become increasingly important. Salinity stress is associated with major storm events and long periods of drought, which are both events that are expected to increase in frequency and severity within the near future. Although corals reefs have survived greater changes over geological time, their condition may be severely compromised over the next hundred years (Hoegh-Guldberg 1999). Corals can show acclimation, and it is possible in the case of salinity that more gradual changes will be tolerated by most species.
Figure 1. The relationship observed between maximum quantum yield of PSII (Fv'/Fm') versus salinity (A), temperature (B) and light (C) of *Anthopleura aureoradiata* in an isolated tide pool. Significance noted by *. 

Appendix A
Figure 2. The relationship observed between photosynthetic efficiency ($\alpha$) versus salinity (A), temperature (B) and light (C) of *Anthopleura aureoradiata* in an isolated tide pool.
Figure 3. The relationship observed between maximum relative electron transport rate ($r\text{ETR}_{\text{max}}$) versus salinity (A), temperature (B) and light (C) of *Anthopleura aureoradiata* in an isolated tide pool. Significance noted by *. 
Figure 4. The relationship observed between minimum saturation irradiance (E_s) versus salinity (A), temperature (B) and light (C) of Anthopleura aureoradiata in an isolated tide pool.
### Appendix B

Table 1. A summary of the full statistics from the salinity gradient experiment. Significance at p<0.05.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Factors</th>
<th>Significance</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\Delta F_{v'}/F_m')</td>
<td>Salinity</td>
<td>Y</td>
<td>PERMANOVA (F_{[11,1080]}) 51.8935, p&lt;0.001</td>
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<tr>
<td></td>
<td>Temperature</td>
<td>Y</td>
<td>PERMANOVA (F_{[2,1080]}) 659.4794, p&lt;0.001</td>
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<td>Light</td>
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<td>PERMANOVA (F_{[5,1080]}) 147.3246, p&lt;0.001</td>
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<td></td>
<td>Salinity:Temperature</td>
<td>Y</td>
<td>PERMANOVA (F_{[22,1080]}) 5.5990, p&lt;0.001</td>
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<td></td>
<td>Salinity:Light</td>
<td>N</td>
<td>PERMANOVA (F_{[55,1080]}) 1.1154, p&gt;0.05</td>
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<td>Temperature:Light</td>
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<td>PERMANOVA (F_{[10,1080]}) 51.1965, p&lt;0.001</td>
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<tr>
<td></td>
<td>Salinity:Temperature:Light</td>
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<td>PERMANOVA (F_{[110,1080]}) 1.9323, p&lt;0.001</td>
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<td>(\alpha)</td>
<td>Salinity</td>
<td>Y</td>
<td>PERMANOVA (F_{[6,420]}) 6.1520, p&lt;0.001</td>
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<td>PERMANOVA (F_{[2,420]}) 227.2690, p&lt;0.001</td>
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<td>PERMANOVA (F_{[18,420]}) 0.7331, p&gt;0.05</td>
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<td>(E_k)</td>
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<td>PERMANOVA (F_{[6,420]}) 5.0764, p&lt;0.001</td>
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<td>Temperature</td>
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<tr>
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<td>Salinity:Temperature:Light</td>
<td>N</td>
<td>PERMANOVA (F_{[36,420]}) 0.5142, p&gt;0.05</td>
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Figure 6. The natural ranges of maximum quantum yield of PSII ($F_{v}/F_{m}$) at 0 h (before treatment).
Figure 7. The effects of a gradient of salinity (5-60 ppt in 5 ppt increments) on the maximum quantum yield of PSII (Fv/Fm) at 6 different light levels (1, 20, 45, 100, 200 and 420 μmol photons/m²/sec) and 3 different temperatures: (A) 6, (B) 18 and (C) 30 °C after 1 h of treatment.
Figure 8. The effects of a gradient of salinity (5-60 ppt in 5 ppt increments) on the maximum quantum yield of PSII ($F_v/F_m$) at 6 different light levels (1, 20, 45, 100, 200 and 420 μmol photons/m²/sec) and 3 different temperatures: (A) 6, (B) 18 and (C) 30 °C after 3 h of treatment.
Figure 9. The effects of a gradient of salinity (5-60 ppt in 5 ppt increments) on the maximum quantum yield of PSII ($F_v/F_m$) at 6 different light levels (1, 20, 45, 100, 200 and 420 μmol photons/m²/sec) and 3 different temperatures: (A) 6, (B) 18 and (C) 30 °C after 6 h of treatment.
Figure 10. The effects of a gradient of salinity (5-60 ppt in 5 ppt increments) on the maximum quantum yield of PSII (F\textsubscript{v}/F\textsubscript{m}) at 6 different light levels (1, 20, 45, 100, 200 and 420 μmol photons/m\textsuperscript{2}/sec) and 3 different temperatures: (A) 6, (B) 18 and (C) 30 °C after 24 h of treatment.
Figure 11. The effects of a gradient of salinity (5-60 ppt in 5 ppt increments) on the maximum quantum yield of PSII \((F_v/F_m)\) at 6 different light levels (1, 20, 45, 100, 200 and 420 \(\mu\)mol photons/m\(^2\)/sec) and 3 different temperatures: (A) 6, (B) 18 and (C) 30 °C after 48 h of treatment.
Figure 12. The effects of a gradient of salinity (5-60 ppt in 5 ppt increments) on the maximum quantum yield of PSII (Fv/Fm) at 6 different light levels (1, 20, 45, 100, 200 and 420 μmol photons/m²/sec) and 3 different temperatures: (A) 6, (B) 18 and (C) 30 °C after 72 h of treatment.
Figure 13. The effects of a gradient of salinity (5-60 ppt in 5 ppt increments) on the maximum quantum yield of PSII (Fv/Fm) at 6 different light levels (1, 20, 45, 100, 200 and 420 μmol photons/m²/sec) and 3 different temperatures: (A) 6, (B) 18 and (C) 30 °C after 96 h of treatment.
### Table 2. A summary of the full statistics from the variable duration and recovery experiment. Significance at \( p<0.05 \).

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<td>PERMANOVA ( F_{[8, 216]} ) 30.5508, ( p&lt;0.001 )</td>
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<td>PERMANOVA ( F_{[1, 216]} ) 64.0963, ( p&lt;0.001 )</td>
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<td>( \text{rETR}_{\text{max}} )</td>
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<td>( E_{k} )</td>
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<td></td>
<td>Salinity:Duration:Light</td>
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<td>PERMANOVA ( F_{[24, 216]} ) 0.8110, ( p&gt;0.05 )</td>
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Figure 14. The variable decline of photosynthetic efficiency (α) exposed to a range of salinities (5-60 ppt at 5 ppt increments) at 85 μmol photons/m²/sec and the subsequent recovery seen between different treatment durations (24, 48, 72 and 96 h).
Figure 15. The variable decline of photosynthetic efficiency ($\alpha$) exposed to a range of salinities (5-60 ppt at 5 ppt increments) at 0 $\mu$mol photons/m$^2$/sec and the subsequent recovery seen between different treatment durations (24, 48, 72 and 96 h).
Figure 16 The variable decline of maximum relative electron transport rates (rETR\textsubscript{max}) exposed to a range of salinities (5-60 ppt at 5 ppt increments) at 85 μmol photons/m²/sec and the subsequent recovery seen between different treatment durations (24, 48, 72 and 96 h).
Figure 17. The variable decline of maximum relative electron transport rates (rETR\textsubscript{max}) exposed to a range of salinities (5-60 ppt at 5 ppt increments) at 0 \( \mu \text{mol} \) photons/m\(^2\)/sec and the subsequent recovery seen between different treatment durations (24, 48, 72 and 96 h).
Figure 18. The variable decline of minimum saturating irradiance ($E_k$) exposed to a range of salinities (5-60 ppt at 5 ppt increments) at 85 $\mu$mol photons/m$^2$/sec and the subsequent recovery seen between different treatment durations (24, 48, 72 and 96 h).
Figure 19. The variable decline of minimum saturating irradiance ($E_k$) exposed to a range of salinities (5-60 ppt at 5 ppt increments) at 0 μmol photons/m$^2$/sec and the subsequent recovery seen between different treatment durations (24, 48, 72 and 96 h).
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