ABSTRACT

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The symbiosis between unicellular phototrophic dinoflagellates (zooxanthellae of the genus *Symbiodinium*) and reef building corals is critical to the survival of reefs as conditions warm with climate change. The symbiosis is highly sensitive to thermal stress as temperatures only 1-2 °C above the corals normal range can cause bleaching severe enough to kill the coral. This sensitivity makes reef corals highly vulnerable to temperature increase. Recent advances in the molecular identification of the *Symbiodinium* genotypes that are hosted by scleractinian corals have elucidated much finer scale population dynamics than previous studies have determined. Both experimental and field studies have revealed that *Symbiodinium* genotypes within clades are characterised by photophysiological differences. For instance, bleaching and changing to thermally tolerant *Symbiodinium* type D, can give *Acropora millepora* an extra 1.0-1.5 °C of heat tolerance. During bleaching stress, the natural temperature-induced loss of symbionts that normally occurs in summer on the reef is magnified by severe heat stress, causing the corals to expel the resident population of symbionts. If the coral survives, bleaching can represent an opportunity for physiologically unique symbiont genotypes to become predominant, thereby altering the physical

characteristics of the host coral to better suit the new environment. However, the characteristics that make the symbiont more thermally tolerant also have the potential to affect host physiological characteristics that are dependent on photosynthesis (such as energy reserves, growth, and reproduction), in much the same way that heat tolerance has been demonstrated to affect the productivity of other phototrophic organisms. In order to gain a better understanding of some of the physiological trade-offs that might accompany symbiont change, this study examines some aspects of key aspects of the physiology and photobiology of a common and abundant reef-building coral *A. millepora* in the Keppel region of the southern Great Barrier Reef that typically hosts symbionts with contrasting thermal tolerance. The growth, energy reserves and reproduction of colonies hosting thermally sensitive type C2 are compared to those hosting predominantly thermally tolerant type D *Symbiodinium* before, during and after a natural bleaching event. Under normal conditions in the field before the bleaching, *A. millepora* colonies with type D symbionts are ecologically less beneficial compared to C2 symbionts due to 38% lower growth, 23% lower stored lipids and 33% smaller eggs. The basis of this disadvantage is 41% lower relative maximum rate of electron transport through the photosystems ($rETR_{max}$), 38% lower light utilisation efficiency (α) and 33% lower maximum excitation pressure over photosystem II (Φ_{max}) of type D symbionts. Following the bleaching, there was a 71% shift from type C2 to type D and another, potentially thermally tolerant type C1. Such dramatic shifts, while currently mostly transitory (Thornhill, D. *et al.* 2006), may be the precursor for more permanent symbiont changes that come with incremental increases in sea surface temperatures. Regardless of the apparent ecological disadvantages of hosting type D symbionts, all colonies, irrespective of symbiont

genotype, and in spite of recovering their zooxanthellae complements, were significantly affected by the stress of the bleaching itself for up to nine months, and possibly longer after the event. These results provide important insights into some of the physiological trade-offs that acclimation to warmer conditions might hold for reef corals. Some corals may have some capacity for acclimation to climate change but the advantages of increased thermal tolerance may be far out-weighed by the detrimental effects of the conditions that caused the change originally.

PHYSIOLOGICAL TRADE-OFFS TO THERMAL ACCLIMATION BY SYMBIONT COMMUNITY CHANGE IN THE REEF CORAL *ACROPORA MILLEPORA*

by

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The work contained in this thesis has not been previously submitted either in whole or in part for a degree at Central Queensland University or any other tertiary institution. To the best of my knowledge and belief, the material presented in this thesis is original except where due reference is made in text.

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GENERAL INTRODUCTION

The impact of climate change on earth's ecosystems has emerged as a major global issue in the last 30 years. Climate change, as a result of increasing CO_2 gas emissions from air pollution, has contributed to a 0.6-0.9 ºC rise in average air and water temperatures and an increase in El Niño/Niña events (Jones, P. *et al.* 2007). Global mean air temperatures are predicted to rise a further 2.0–4.5 ºC over the next century (Meehl *et al.* 2007) and Australia's regional temperatures are predicted to increase even further, which will make coral bleaching an annual event by 2030 (A1FI scenario) (Done *et al.* 2003). Of all the earth's ecosystems, reefs are the most endangered by climate changes as corals are extremely sensitive to temperature change (Hoegh-Guldberg, O. *et al.* 2007; Hughes *et al.* 2003). Degradation of reefs from temperature-induced bleaching can lead to decreases in biodiversity and the abundance of marine life (Woodford 2004). Recently, the possibility that some corals may be able to cope with warmer conditions by changing their endosymbiotic (zooxanthellae) partners has raised the hope that at least some reefs may survive climate change (Buddemeier, R.W *et al.* 2004; Buddemeier, Robert W & Fautin 1993). However, which coral species have this capacity, how widespread the phenomenon of symbiont change is, and what the ecological effects of such acclimation are, remains in question. As such, the fitness of corals in the context of the photobiology of their autotrophic partners is of key concern to coral scientists and reef managers alike.

Reef building or 'scleractinian' corals can associate with diverse communities of zooxanthellae consisting of several clades made up of numerous subclades, types or

strains (LaJeunesse, T C 2002; Rodriguez-Lanetty 2001; Rowan & Knowlton 1995; van Oppen, M. J. H. *et al.* 2001). These are now known to be ecologically and biogeographically distinct genetic groups (Baker, A. C. 2003; Thornhill, D. *et al.* 2006). Recently, new methods of molecular identification have elucidated the presence of previously enigmatic genotypes other than the predominant (more abundant, but not necessarily stronger) symbiont population in some reef corals (Mieog, J. C. *et al.* 2009; Mieog, J C *et al.* 2007). Much is yet to be discovered of the photophysiological differences between *Symbiodinium* genotypes, which exist perhaps even below the level of 'types' (Reimer *et al.* 2006). For instance, in one study, a single zooxanthellar genotype was shown to exist in two photo-physiologically distinct sub-populations within the same colony (Ulstrup, K. *et al.* 2006). The differential use of light by symbiont genotypes has allowed hermatypic corals to occupy habitat niches that would otherwise be ecologically disadvantageous (Iglesias-Prieto, R. *et al.* 2004). However, the dependence of both partners on photosynthesis in the algae also sets constraints on the association as the process is highly sensitive to both light and temperature (Stambler 2004).

Corals live in a narrow envelope of environmental conditions and even slight changes such as a rise of 1.0-1.5°C above normal water temperature (Berkelmans, Ray 2006) and light (Hoegh-Guldberg, O. & Jones 1999) can affect them significantly. Surpassing these conditions can result in a breakdown in the symbiosis between coral and its algal partners (zooxanthellae of the genus *Symbiodinium*). As water temperature rises, the ability of the algal symbiont to photosynthesise is eroded causing

the polyps to reject the algae. The loss of the algal symbiont leaves the white coral skeleton visible through the translucent tissue of the polyp. The detachment and loss of the zooxanthellae can eventually result in the death of the coral (coral bleaching, Douglas 2003). If water temperatures return to normal soon enough the coral can survive and re-establish its symbiont population. The temporary loss of the coral's normal zooxanthellae represents an opportunity for less predominant symbiont genotypes that are able to cope with the warm conditions to populate the now vacant tissues. Hosting a complement of different algal partners can ascribe a different set of physiological characteristics to the coral host. For instance colonies of *Acropora millepora* have been shown to gain an extra 1.0–1.5 °C of heat tolerance after losing their normal type C2 symbionts in favour of thermally tolerant type D symbionts (Berkelmans, Ray & van Oppen 2006). However, the stress of heat that causes bleaching in the first place has detrimental impacts on the long term health of reef corals (Glynn 1993; Michalek-Wagner & Willis 2000).

The scleractinian corals are the main species on tropical reefs because their calcium carbonate skeletons form the foundation of the reef structure (Barnes & Taylor 1973). Zooxanthellae live in symbiosis within the tissues of scleractinian coral polyps, supplying a large proportion of their energetic requirements by photosynthesis and nitrogen recycling (Muscatine & Porter 1977). The provision of these translocates by the algae has allowed corals to exploit tropical waters relatively barren of nutrients because they can rely less on energy derived from heterotrophy and almost entirely on autotrophy by the zooxanthellae if there is adequate light (Falkowski *et al.* 1984).

Photosynthetically fixed carbon from the zooxanthellae forms an important energy source for reef-building corals that is used for reproduction (Edmunds & Davies 1986; Muscatine, McCloskey & Loya 1985), tissue growth (Davies 1984; Muscatine, McCloskey & Loya 1985), skeletal growth (calcification) (Pearse & Muscatine 1971), cell repair (Fine, Oren & Loya 2002) and host respiration (Muscatine 1990). In turn, the algae use CO_2 from host respiration for photosynthesis, respiration and cell division (Muscatine 1990), to gain nutrients (Trench, R. K. 1979) and as a habitat in the coral tissue where they are protected from excess light and damaging UV and where incident light is enhanced for photosynthesis (Salih *et al.* 2000). Processes that affect photosynthesis have the potential to influence the physiological fitness of the entire coral + algal association (holobiont).

Acclimatisation to climate change could come at a physiological cost to coral fitness as the mechanisms for thermal tolerance in the symbiont are likely to involve aspects of the photosynthetic processes. Although the exact mechanisms for increased thermal tolerance in *Symbiodinium* are still unclear, it is possible that the same mechanisms that exist in other heat tolerant phototrophs also operate in zooxanthellae. For example, a study by Tchernov *et al.* (2004) has shown that thylakoid membrane stability is implicated in the differential heat sensitivity of symbiont genotypes. Thylakoid membrane stability has also been proposed as a key component of heat tolerance in unicellular freshwater algae (Sato *et al.* 1996) and cress plants (Hugly *et al.* 1989). Although there are fundamental differences in photosynthesis and in the chloroplast structure of plants, algae and zooxanthellae, it seems likely that thylakoid

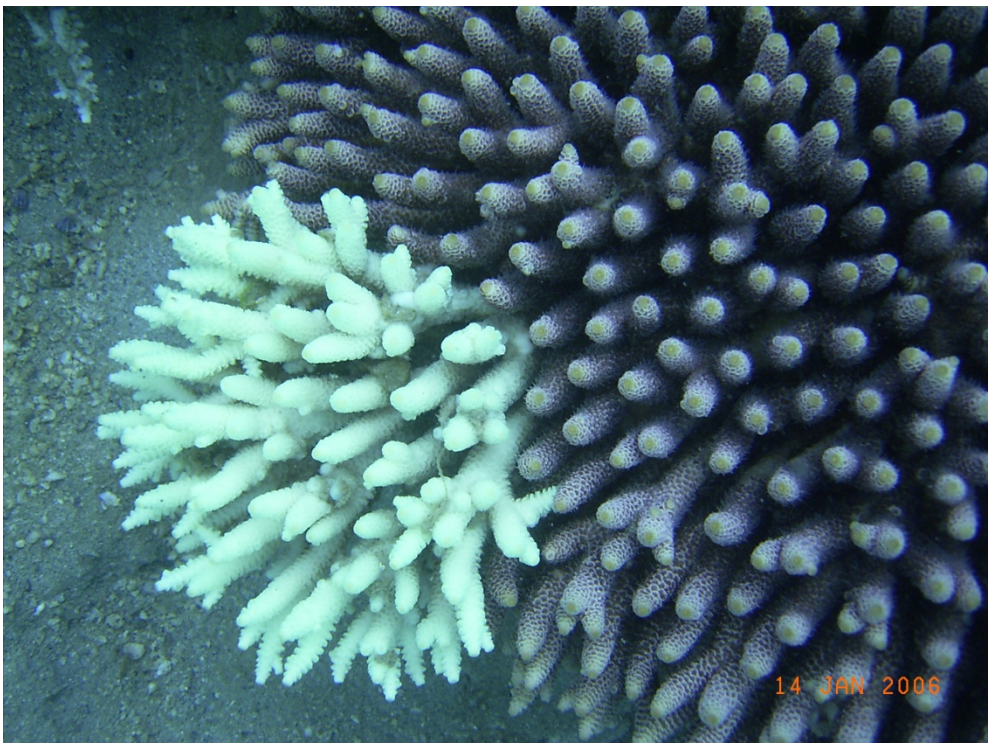
membrane stability underwrites thermal tolerance in them all. In algae (Sato *et al.* 1996) and plants (Hugly *et al.* 1989; Laing, Greer & Schnell 1995), the flip side of heat tolerance is reduced growth. In thermally tolerant symbiont types, the reversible down-regulation of photosystem II (PSII) by photoinhibition also plays a part in reducing overall photosynthetic capacity (Rowan 2004a). This connection between algal thermal tolerance and photosynthesis suggests that the photophysiological basis for thermal tolerance in the symbiont will also result in an energetic cost to the coral/algal association. This idea is supported by the results of a study by Loram *et al.* (2007) which has shown differential carbon fixation in a cnidarian host with thermally tolerant symbiont types under temperature stress. Even further evidence is provided by Little *et al.* (2004) which has shown reduced growth in juvenile *A. millepora* and *A. tennuis* that hosted more thermally tolerant *Symbiodinium* type D. However to date, no studies have shown a direct connection between the photosynthetic function of symbionts of contrasting thermal tolerance and a reduction in the physiological fitness of adult corals under normal conditions in the field. Such explicit information could shed light on some of the physiological costs of wholesale symbiont changes as conditions warm with climate change.

In this study, five key physiological characteristics of one of the most important structural reef-building corals *A. millepora* in the southern Great Barrier Reef were studied to compare the effects of hosting *Symbiodinium* genotypes of contrasting thermal tolerance. The field component of the study was conducted on a shallow reef flat at Miall Island in the southern, inshore Great Barrier Reef. Miall Island (23°09′ S,

150°54′ E), is one of 15 islands in the Keppel Island group. *A. millepora* colonies at Miall Island normally associate with *Symbiodinium* nuclear ribosomal inter-transcribed spacer deoxyribonucleic acid region 1 (nrDNA-ITS1) types C2, D and C1 or a combination of these types, making it an ideal species for a comparative study of physiology in relation to predominant symbiont type. Tagged colonies on the reef flat at Miall Island were sampled before, during and after the bleaching event to investigate the dynamics of the symbiont community (Chapter 1). Photobiology using rapid light curves was used to investigate the link between thermal tolerance and photosynthetic processes (Chapter 2), and growth (Chapter 3), energy stores and reproduction (Chapter 4) were investigated under stable environmental conditions and following a natural bleaching event in February 2006. A novel method based on 3D image analysis was developed to accurately assess the surface area of the coral branches collected on each sampling occasion because of the importance of standardising biochemical parameters to some ecologically meaningful parameter (Chapter 5). Because shallow water reef building corals derive almost all their energy from photosynthesis (Falkowski *et al.* 1984) a shift in symbiont community to more thermally tolerant type D in colonies of *A. millepora* at this site was expected to result in changes to physiological characteristics such as skeletal growth, reproduction and macromolecular composition that depend on the photosynthetic processes investigated in Chapter 2.

Chapter 1

A COMMUNITY CHANGE IN THE ALGAL ENDOSYMBIONTS OF A SCLERACTINIAN CORAL FOLLOWING A NATURAL BLEACHING EVENT: FIELD EVIDENCE OF ACCLIMATISATION



A stark landscape of 100% bleached *Acropora millepora* colonies predominated by *Symbiodinium* type C2 and unbleached colonies with type D symbionts at Miall Island reef in the Keppel region of the southern Great Barrier Reef during the January/February 2006 summer bleaching confirmed the differential bleaching susceptibility of colonies with these symbiont types. Photograph courtesy of Alison Jones.

ABSTRACT

The symbiosis between reef-building corals and their algal endosymbionts (zooxanthellae of the genus *Symbiodinium*) is highly sensitive to temperature stress, which makes coral reefs vulnerable to climate change. Thermal tolerance in corals is known to be substantially linked to the type of zooxanthellae they harbour and, when multiple types are present, the relative abundance of types can be experimentally manipulated to increase the thermal limits of individual corals. Although the potential exists for this to translate into substantial thermal acclimatisation of coral communities, to date there is no evidence to show that this takes place under natural conditions. In this study, field evidence of a dramatic change in the symbiont community of *A. millepora*, a common and widespread Indo-Pacific hard coral species, was documented after a natural bleaching event in early 2006 in the Keppel Islands (Great Barrier Reef). Before bleaching, 93.5% (n = 460) of the randomly sampled and tagged colonies predominantly harboured the thermally sensitive *Symbiodinium* type C2, while the remainder harboured a tolerant *Symbiodinium* type belonging to clade D or mixtures of C2 and D. After bleaching, 71% of the surviving tagged colonies that were initially C2 predominant changed to D or C1 predominance. Colonies that were originally C2 predominant suffered high mortality (37%) compared with D-predominant colonies (8%). It is estimated that just over 18% of the original *A. millepora* population survived unchanged leaving 29% of the population C2 and 71% D or C1 predominant six months after the bleaching event. This change in the symbiont community structure, while it persists, is likely to have substantially increased the thermal

tolerance of this coral population. Understanding the processes that underpin the temporal changes in symbiont communities is the key to assessing the acclimatisation potential of reef corals.

INTRODUCTION

Coral reefs owe their success to the symbiosis between reef-building corals and intracellular, phototrophic dinoflagellates of the genus *Symbiodinium* (zooxanthellae) that supply up to 95% of the coral host's energy requirements (Muscatine 1990). Under stressful environmental conditions, such as abnormally high water temperatures in combination with high light, this symbiosis can break down and the algae are lost in a process known as 'bleaching'. Such conditions have occurred on reefs globally (Hoegh-Guldberg, O. 1999; Wilkinson 2004) and are predicted to become more frequent as a result of global warming (Donner *et al.* 2005; Hoegh-Guldberg, O. *et al.* 2007). Therefore, coral bleaching is considered one of the biggest threats to coral reefs (Marshall, P. & Schuttenberg 2006).

Nuclear ribosomal and chloroplast DNA markers show that the genus *Symbiodinium* is highly diverse. The genus is currently divided into eight distinct clades (categorized as A–H), each containing multiple subclades, strains or types (Coffroth & Santos 2005; Pochon *et al.* 2006; Stat, M, Carter & Hoegh-Guldberg 2006). This level of genetic diversity appears to be matched by appreciable levels of physiological diversity within and between clades. For instance, symbiont types differ in their photosynthetic response to light (Iglesias-Prieto, R. *et al.* 2004) and temperature stress (Robinson &

Warner 2006). Reef-building corals can form associations with members of six of the eight *Symbiodinium* clades (A–D, F and G; reviewed by Baker 2003) and some of these associations seem to be more flexible than others (van Oppen, Madelaine J H, Little & Willis 2004). *Symbiodinium* C is the most common symbiont type in *Acropora* corals on the Great Barrier Reef (LaJeunesse, T CBhagooli *et al.* 2004; Smith, C. 2004; van Oppen, M. J. H. *et al.* 2001) and certain types within this clade have been shown to be particularly sensitive to heat stress (Berkelmans, Ray & van Oppen 2006). *Symbiodinium* clade D is common in *Acropora* corals on shallow and inshore reefs and has been shown to be relatively tolerant to high temperatures (Baker, Andrew C *et al.* 2004; Fabricius *et al.* 2004; Glynn *et al.* 2001; van Oppen, M. J. H. *et al.* 2005).

Some corals are known to harbour multiple types within a single colony (Rowan & Knowlton 1995; Rowan *et al.* 1997; Ulstrup & van Oppen 2003), which may allow for changes in the relative abundances of each symbiont type under influence of the environment (symbiont 'shuffling'). One way in which this change can occur is by the predominant, thermally sensitive symbiont population being replaced by a population of thermally tolerant symbionts that arise from the presence of less abundant 'background' symbionts (Baker, A. C. 2003). As a result, the entire coral colony becomes more thermally tolerant. This acclimatisation mechanism has been shown to occur in at least one population of *A. millepora* on the Great Barrier Reef after transplantation to a different thermal environment (Berkelmans, Ray & van Oppen 2006). However, only a few species of coral have been shown to shuffle their symbiont

communities (Goulet 2007; Goulet & Coffroth 2003; Thornhill, D. J. *et al.* 2003) and the longest symbiont monitoring study to date indicates stability rather than wholesale changes in symbiont communities (Thornhill, D. *et al.* 2006). Symbiont 'switching', i.e. the acquisition of new symbionts from the surrounding environment (Baker, A. C. 2003), may be another way by which corals can achieve a functional change in their predominant symbiont population, but so far this has not been demonstrated in scleractinian corals. Bleaching is predicted to become more frequent as a consequence of climate change (Dunbar *et al.* 1994; Hoegh-Guldberg, O. 1999; Hoegh-Guldberg, O. *et al.* 2007). Shuffling to more heat-resistant symbiont types may be an important acclimatisation mechanism, but it must operate at the scale of populations and communities if reefs are to acclimatise and become more resistant to subsequent events (Buddemeier, R.W *et al.* 2004; Buddemeier, Robert W & Fautin 1993). To date, this has not been shown to occur in a natural setting.

In this study, the *Symbiodinium* community is characterised in an inshore population of *A. millepora* and compared to the *Symbiodinium* community in the same tagged colonies before and after a natural bleaching that took place in 2006. This is the first field study that follows changes in *Symbiodinium* genotypes in specific colonies over three years that includes a natural bleaching event. A dramatic shift in the symbiont community within this host population occurs as a result of the disturbance, which is likely to have increased its thermal tolerance. If this shift is sustained and is community wide, the reefs in this area are likely to have substantially increased their capacity to withstand the next bleaching event.

MATERIAL AND METHODS

Study site

The study site is a reef flat adjacent to Miall Island (23°09′ S, 150°54′ E), which is one of 15 islands in the Keppel Island group, in the southern inshore Great Barrier Reef. Miall Island, like many of the islands in this group, has an extensive reef flats on its leeward shore with an average coral cover of approximately 50%, dominated by colonies of the corymbose, Indo-Pacific stony coral *A. millepora* (van Woesik & Done 1997). The region suffered moderate to severe mass bleaching (more than 60% corals bleached) in February 2002 (Berkelmans, Ray *et al.* 2004) and severe bleaching in January/February 2006 (89% corals bleached; R. Berkelmans & A. M. Jones 2006, unpublished data).

Coral sampling

To determine the *Symbiodinium* community composition before the bleaching, 460 colonies were tagged on the reef flat at Miall Island between September 2004 and March 2005. A small (2–3 cm) branch was sampled from the central area of each colony and placed in a labelled bag for subsequent storage in 100% ethanol. Symbiont changes were monitored in a subset of 79 tagged colonies that survived the bleaching three and six months (May and August, respectively) after the bleaching event in January/February 2006. The subset of 79 colonies was chosen haphazardly from surviving colonies and comprised 58 with predominantly C2-type (no background types detected), 15 with predominantly D-type (no background types detected) and 6 with both C2 and D types present. To minimise confounding of temporal trends in

symbiont community by intra-colony variation in symbiont types, sampling took place from the same area within each colony on each sampling occasion and ensured that only the tips of branches were used for DNA extraction. Mortality in the *A. millepora* population was assessed six months after the bleaching event in August 2006 by visually estimating the percentage of live and dead coral tissue on 159 haphazardly chosen tagged colonies using pre-bleaching photos of each colony as a reference.

Genotyping and sequencing

DNA was extracted from coral tissue based on the method of Wilson *et al.* (2002). A combination of single-stranded conformation polymorphism (SSCP) analysis, cloning and DNA sequencing was used for symbiont identification. The ITS1 region was amplified as described by van Oppen *et al.* (2001). SSCP analysis was used to identify the predominant symbiont type in each colony and estimate the relative abundance of *Symbiodinium* types within each sample when more than one type was identified. Relative abundances of less than 5–10% are not detected using SSCP (Fabricius *et al.* 2004). SSCP bands that were faint compared with another more intense band in the same sample were identified as background and predominant types, respectively. The presence of two equally intense bands in the same sample was interpreted as the colony hosting equal amounts of each type. Fabricius *et al.* (2004) found that this was a reliable method for estimating the relative abundance of different *Symbiodinium* types. SSCP profiles were assigned to symbiont type by comparing to reference samples of known identity and by cloning and sequencing in the case of novel SSCP profiles.

STATISTICAL ANALYSIS

Counts of colonies of *A. millepora* before the bleaching were analysed using a Pearson's chi-squared contingency table to compare the frequencies of colonies with different combinations of predominant symbiont types C2, C1 and clade D with the null hypothesis that there were no differences in the observed and expected cell frequencies.

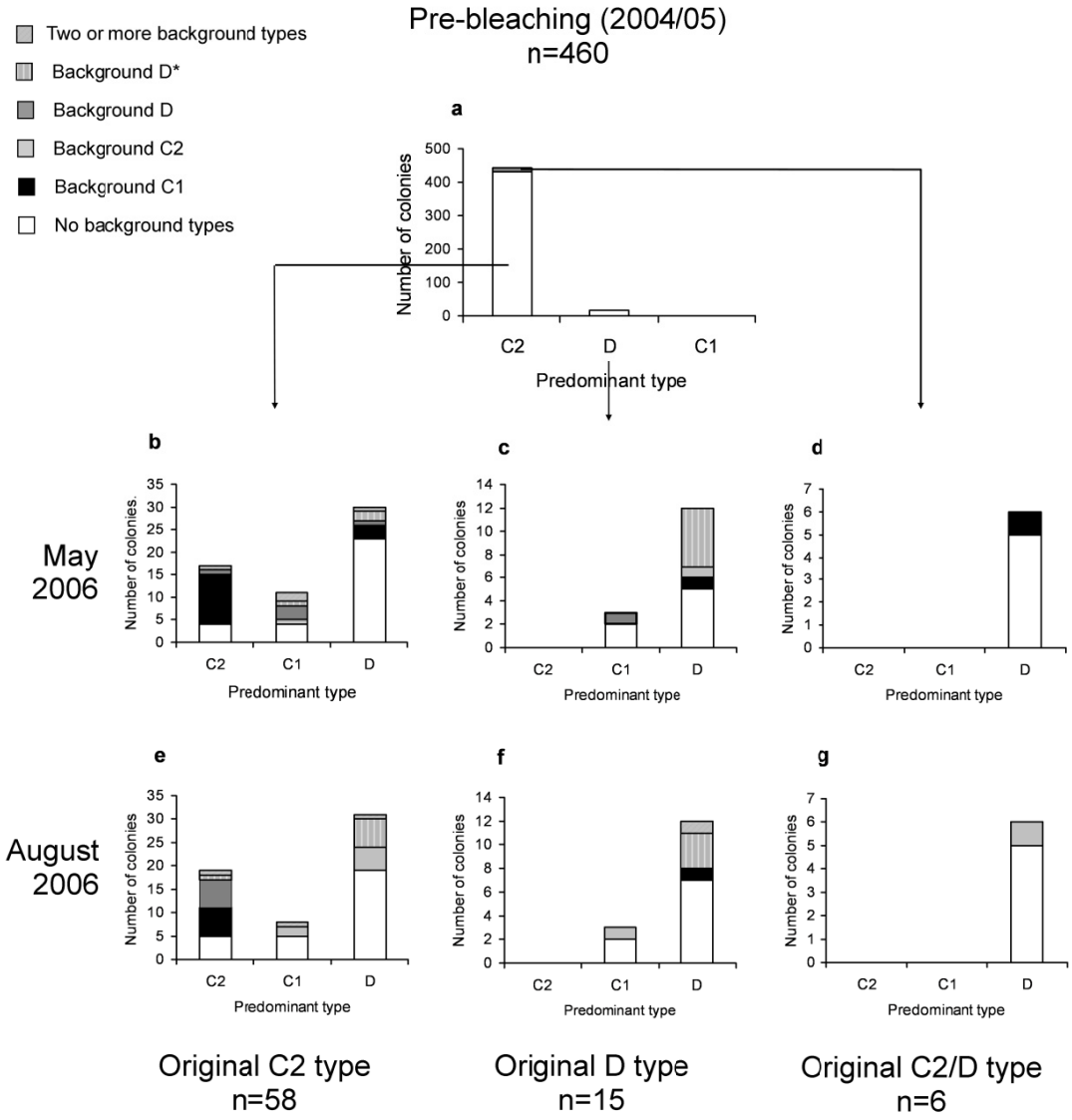
In addition, two separate multinomial loglinear regressions were used to test for significant changes in the (i) predominant-and (ii) low-level background symbiont types in the subset of 79 colonies three and six months after bleaching with the null hypotheses that there were no differences in the log ratios of the observed and expected cell frequencies. Predominant symbiont types (C2, D and C1) and background types (C2, D, C1, D, multiple types and no background types) before bleaching and three and six months after bleaching were fixed factors in the analyses, and cases were weighted by the number of colonies of each type. The parameter estimates derived from the multinomial loglinear regressions were used to show the nature of any significant changes. All statistical analyses were performed with SPSS v. 15.0.

RESULTS

Symbiont diversity at Miall Island before and after bleaching

Before the bleaching in 2006, *A. millepora* at Miall reef associated predominantly with *Symbiodinium* type C2 (93.5%, sensu van Oppen, M. J. H. *et al.* 2001) and to a much lesser extent with *Symbiodinium* clade D (3.5%) or mixtures of C2 and D (3.0%; $\chi_1^2 = 398$, $p < 0.05$, $n = 460$, Figure 1.1 a). Cloning and sequencing of five clade D and six clade C ITS1 PCR products (370 bp in length) showed that these differed by 1–6 bp within clades, which were assumed represent intragenomic variants. By late February 2006 when bleaching was at its most intense, the relative difference in bleaching susceptibility between corals predominated by C2 and D was clearly evident, with the former bleaching white and the latter normally pigmented. Tagged corals harbouring a mix of *Symbiodinium* C2 and D were mostly pale in appearance.

Figure 1.1. There was a significant shift in the predominant symbionts harboured by *Acropora millepora* colonies at Miall Island (Keppel Islands, southern Great Barrier Reef) during the study. (a) Prior to a natural bleaching in 2006 colonies harboured predominantly type C2 symbionts (n = 460), but three months after bleaching in May 2006 (b-d, n = 79), and six months after bleaching in August 2006 (e-g, n = 79) there was a significant shift to type D and C1 symbionts and most colonies harboured multiple symbiont types.



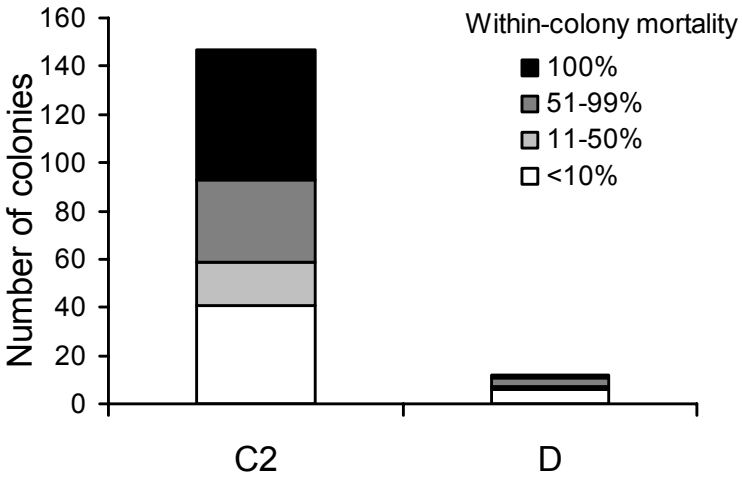
In May 2006, three months after the bleaching, a major shift to thermally tolerant type D and C1 symbiont communities occurred in the surviving colonies. Of 58 C2-predominant colonies monitored post-bleaching, 30 became predominant in type D symbionts (most without detectable levels of C2 symbionts; Figure 1.1 b). Type C1 was not detected in any of the SSCP gels of samples just before the bleaching. By May 2006, however, C1 became the predominant type in 11 out of the 58 colonies and was clearly evident as a background type in 11 out of 17 colonies that remained C2 predominant. C2-predominant colonies without other detectable background symbiont types (lower detection limit 5–10%) made up only four out of the 58 colonies three months after bleaching. Of the 15 original D-predominant colonies monitored post-bleaching, 12 retained their D predominance while three changed to C1 predominance. All six colonies that initially hosted C2 with background clade D became D predominant by May 2006. A variant of D which was called D* was not apparent in any colonies prior to bleaching but was detectable at low levels in 10% of colonies after bleaching (Figure 1.1 b, c). The appearance of previously undetected C1 and D* led to an increased diversity of symbiont types three months after bleaching.

By August 2006, six months after bleaching, the proportion of predominant symbiont types in each of the three initial groups of colonies (C2 or D predominant and C2 with D) remained stable, but there were substantial changes in the mix of background types. In the group that changed from C2 to D predominance, none had detectable background levels of C2 in May but C2 reappeared in five colonies in August. In addition, the other two groups also showed a slight increase in the background

occurrence of C2 in August, possibly suggesting the start of a drift back to pre-bleaching C2 predominance. By contrast, more colonies had C1 in May compared with August while the abundance of D* increased from May to August (Figure 1.1 b, e). The loglinear regressions showed that a significant change occurred in the predominant symbiont types of the colonies at Miall Island. The C2-predominant colonies were more likely to have changed to clade D predominance than to have remained unchanged or changed to C1 predominance in both May and August ($Z = -15.0$, $p < 0.05$, $df = 10$). Type C2 colonies were more likely to occur with clade D than any other type or combination of types (C1, C2, D or D) in May ($Z = 29.7$, $p < 0.05$, $df = 70$) and August ($Z = 34.4$, $p < 0.05$, $df = 70$).

While the symbiont community change in surviving colonies was dramatic [71% changed predominance from C2 to D or C1 (n = 79)], selective mortality also played a substantial role in shifting the symbiont community in the coral population. Of 159 colonies monitored for survival, 147 were initially C2 predominant and of these, 54 colonies suffered 100% mortality and a further 34 suffered more than 50% partial mortality (Figure 1.2). Only one of 15 colonies that were initially D predominant died. The difference in mortality between clades was statistically significant ($p < 0.05$, $\chi_1^2 = 4.1$), confirming their differential thermal tolerance.

Figure 1.2. A summary of the partial mortality in tagged *Acropora millepora* colonies at Miall Island (n=159). Thirty seven percent of colonies with type C2 *Symbiodinium* suffered 100% mortality compared to only 8% of clade D colonies ($\chi 12 = 4.1$, $p < 0.05$, $df = 1$). D = colonies with clade D. C2 = colonies with type C2.



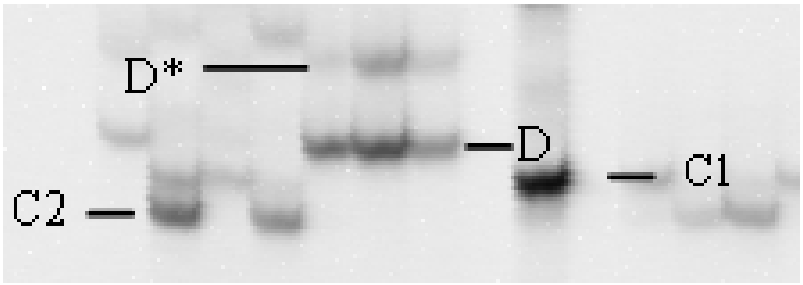
In terms of relative contribution to symbiont community change, selective mortality accounted for 37% of the change while altered symbiont-type predominance accounted for 42% of the change (n = 159). Just over 20% of the original C2-predominant population survived and maintained C2 as their predominant symbiont (cf. 93.5% prior to bleaching).

Phylogenetic analysis of *Symbiodinium* ITS1

Following amplification of the ITS1 region and SSCP analysis, four different bands were observed (Figure 1.3), and the ITS1 region of 8 representative samples were cloned and/or sequenced. Amplification products of about 370 bp length were obtained for all samples. Sequences were compared and aligned to closely matching sequences in Genbank using BioEdit (version 7.0) and CLUSTER W (version 1.7, Thompson,

Higgins & Gibson 1994). Primer sequences were removed from the alignments before analysis. Phylogenetic analyses were conducted using the Maximum Parsimony (MP) method (Saitou & Nei 1987) in PAUP (Version 4.0b10 for 32-bit Microsoft Windows). Either exhaustive or branch-and-bound searches were conducted, with gaps treated as a fifth base. Support for MP clusters was tested by bootstrap analysis with 1000 replicates (heuristic search). Type C and type D ITS1 sequences were analysed separately because the ITS region is un-alignable between these clades.

Figure 1.3. Several SSCP variants of *Symbiodinium* types C and D were found before and after bleaching in *Acropora millepora* colonies from the Keppels. Cloning and sequencing of one of these (D*) revealed a difference of only one base pair from other type D sequences. Clade C variants that matched C1 and C2 had either none or 3 to 6 base pair differences.

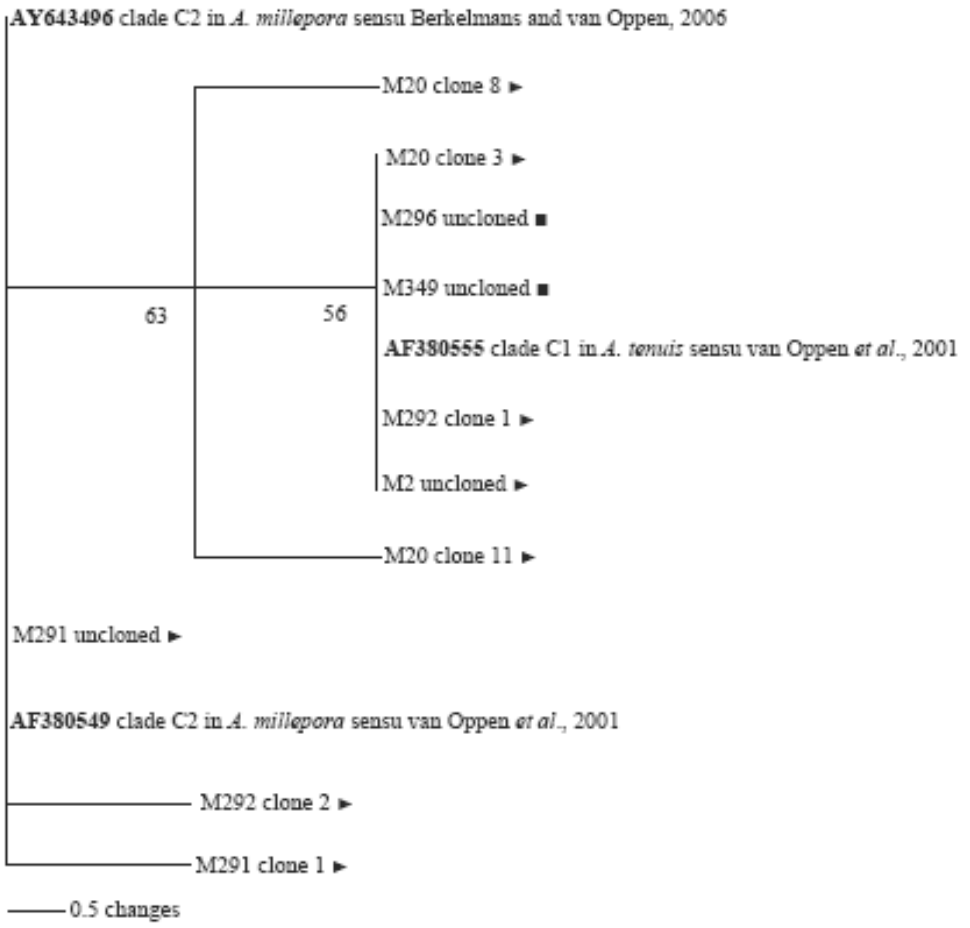


The *Symbiodinium* ITS1 clade C alignment consisted of 253 alignment positions and 13 taxa (Taxa for which the ITS1 sequences were obtained for this study are shown in Table 1.1). Six positions were variable, two of which were parsimony informative. The *Symbiodinium* ITS1 clade D alignment consisted of 8 taxa and 329 characters, six of which were variable but not parsimony-informative. The clade D sequences obtained from the Keppel island *A. millepora* colonies are identical or very similar to clade D

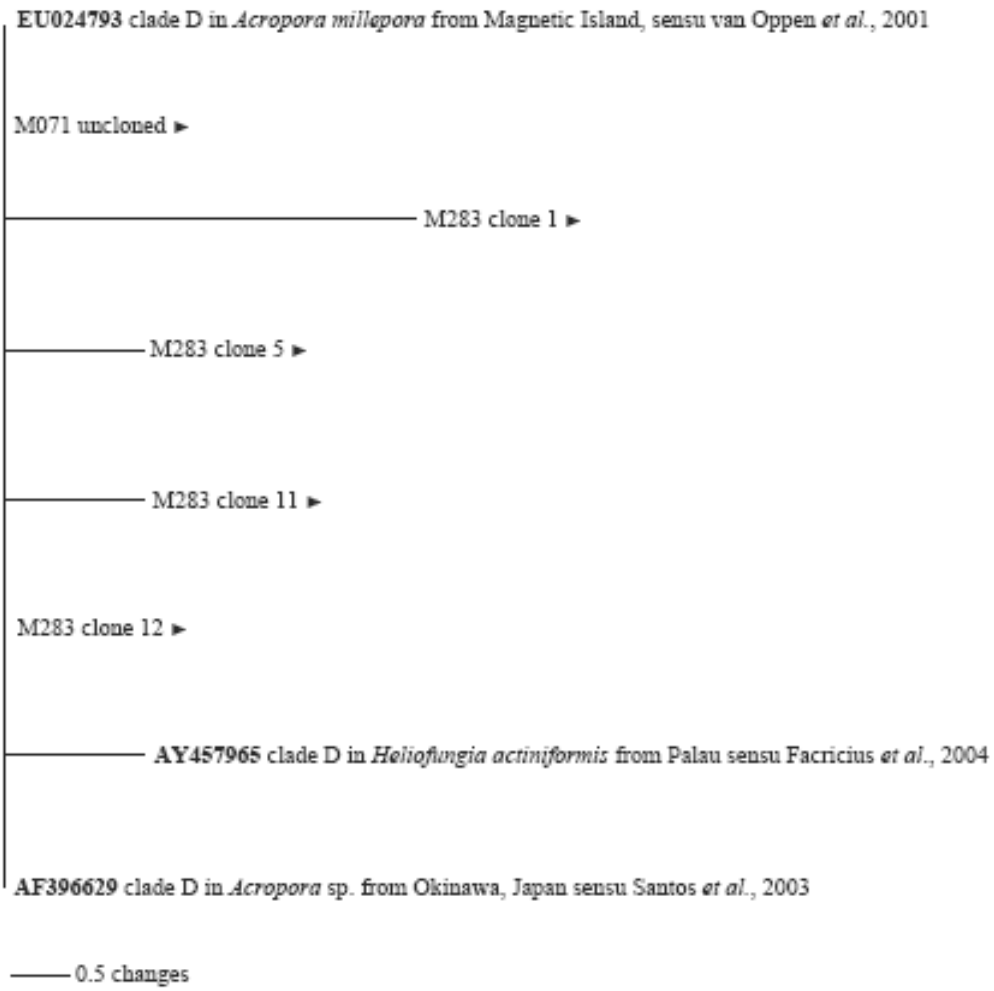
obtained from *A. millepora* at other Great Barrier Reef locations (van Oppen, M. J. H. *et al.* 2001, accession number EU024793). Phylogenetic analysis distinguished the two subclades C1 and C2 within clade C (Berkelmans, Ray & van Oppen 2006; van Oppen, M. J. H. *et al.* 2001) as well as a few other sequence variants (Figure 1.4 a). There was one clade D variant (named type D*) that appeared on SSCP in a number of samples before the bleaching (Figure 1.3). Sequence analysis of the clone matching the D* SSCP profile revealed a single base pair change from the most common type D variant (Figure 1.4 b).

Figure 1.4. a-c. Phylogenetic relationships derived by Maximum Parsimony analysis of (a) the clade C ITS1 and (b) clade D ITS1 and (c) clade C full length ITS regions of *Symbiodinium* sequences from *Acropora millepora* colonies in the Keppel region and matches from GenBank. (Trees are branch-length informative and numbers at nodes represent bootstrap support values based on 1000 replicates). Two major clades were identified using this method, C and D (van Oppen, M. J. H. *et al.* 2001). Standards with Genbank accession numbers used in SSCP analysis are shown in boldface. Branch lengths represent phylogenetic distances. Host names are shown in italics. Subclades are labelled on the right. The symbol ► denotes samples taken before the bleaching and ■ denotes samples taken after the bleaching in 2006. M=Miall Island.

(a) Rooted *Symbiodinium* ITS1 type C (MP) phylogenetic relationships (1000 replicates) showing branch lengths to scale (Mega 3).



(b). Rooted *Symbiodinium* ITS1 type D (NJ) phylogenetic relationships (1000 replicates). No type D samples were sequenced after the bleaching in January 2006. Branch lengths indicate the strength of the relationships. *denotes type D* which had a higher SSCP band compared to all other clade D sequences.



(c) Rooted Symbiodinium full length nrDNA-ITS type C sequence (MP) consensus phylogenetic relationships (1000 replicates). Two equally (and most) parsimonious trees are shown.

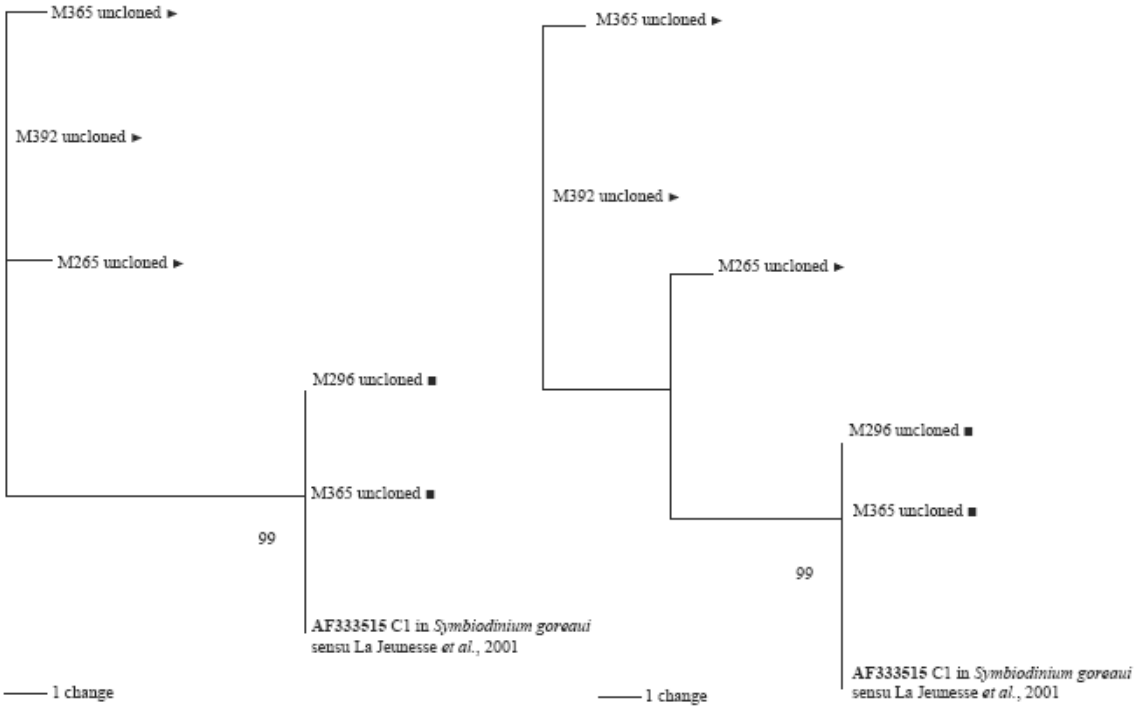


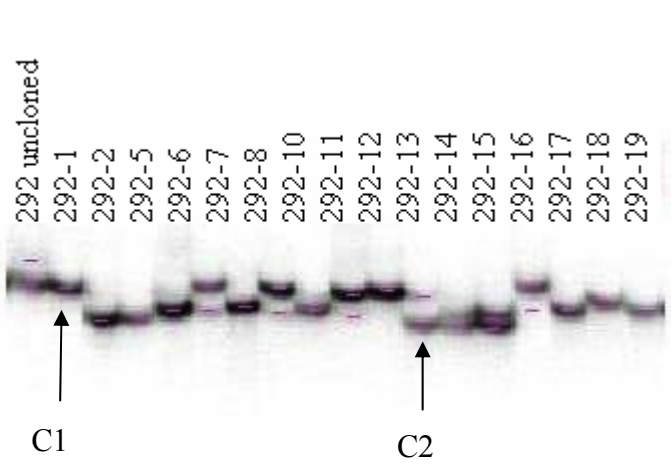
Table 1.1. Samples from which ITS1 and full ITS DNA sequences were obtained in this study via either direct sequencing of the PCR product ('direct sequence') or cloning of the PCR product followed by sequencing of a number of clones ('cloned'). Phylogenetic analysis of the ITS1 and full length ITS regions of a subset of the samples confirmed the presence of clade D and type C1 and C2 *Symbiodinium*. Sequenced samples were used as references in subsequent SSCP analysis to confirm the predominant symbiont type of the colonies in this study. Note, samples are classified by a prefix that represents the reef name (e.g. M= Miall Island, K=Keppels) and the colony tag number, and suffixed with a dash and a number if cloned.

Inter-transcribed Spacer Region 1 (ITS1)		Full length Inter-transcribed Spacer Region (ITS, comprising ITS1, 5.8S and ITS2)		
Type C		Clade D	Type C	
Before bleaching	After bleaching	Before bleaching	Before bleaching	After bleaching
cloned	**cloned**	**cloned**	**direct sequence**	**direct sequence**
M20-3 EU189440	M292-1 EU189447	M283-1 EU189451	M365 EU189438	M365 EU189435
M20-8 EU189441	M292-2 EU189448	M283-5 EU189452	M392 EU189436	M296 EU189439
M20-11 EU189442	M296-1 EU189449	M283-11 EU189453	M265 EU189437	
	direct sequence	M283-12 EU189454		
	M291 EU189443	M283-13 EU189455		
	M296 EU189444	**direct sequence**		
	M349 EU189445	M071 EU189450		
	M2 EU189446			

Cloning of samples that appeared to harbour one symbiont type (according to SSCP) in some samples revealed low-abundance background types that had missed detection (Figure 1.5). For instance, nineteen clones of sample 292, harbouring only C1

according to the uncloned sample SSCP gel image, were sequenced and multiple variants of C1 and one C2 clone were detected. Only samples that contained clear, sharp SSCP gel image bands of type C2 or clade D or equal bands of both were chosen for direct sequencing (see below).

Figure 1.5. SSCP gel image showing diversity of symbiont types within a single sample after recovery from bleaching. The wide band of the uncloned sample 292 (far left) suggests multiple type C1 variants. Cloning and sequencing revealed 4 different sequences, unexpectedly including C2. The sample was taken after the colony bleached and then recovered. The SSCP image of the sample taken before bleaching showed a sharp C2 band with no obvious evidence of other background symbiont types. Greater than 90% of the colony (mostly the tips of branches) had mortality in August 2006, six months after bleaching.



Comparison of phylotypes with other studies

Comparisons of the *Symbiodinium* clade C type nomenclature between studies is often hampered by the use of different parts of the nrDNA region. To overcome this, the entire *Symbiodinium* nrDNA-ITS region (comprising ITS1, 5.8S and ITS2) of a small

subset of five uncloned type C1 and C2 (from ITS1 genotyping) samples from the tagged colonies was amplified (three sampled before bleaching and two sampled after bleaching) and sequenced as described by Baillie *et al.*(2000). Forward primer, 'zITSf': 5'- CCG GTG AAT TAT TCG GAC TGA CGC AGT GCT-3' (Hunter, Morden & Smith 1997) and reverse primer 'zITSr': 5'- TCC TCC GCT TAT TGA TAT GC-3' (White, T. J. *et al.* 1990) were used to obtain approximately 650 base pair PCR products (Table 1.1). Sequences were compared and aligned to previously sequenced ITS1 samples and closely matching sequences in Genbank using BioEdit (version 7.0) and CLUSTER W (version 1.7, Thompson, Higgins & Gibson 1994).

The uncloned full-length ITS sequences were assigned to type C1 or C2 based on their match to the ITS1 region of the previously sequenced representative ITS1 region samples (Table 1.1) and the SSCP gel profile. The sequences were similar but not identical to the LaJeunesse ITS2 sequence regions of *Symbiodinium* type C1 e & f (Genbank accession numbers AY258489, AY258490) found in *Fungia scutaria* (Hawaii LaJeunesse, T CThornhill *et al.* 2004), C1b (AY239363) and C1c (AY239364) from the Great Barrier Reef (2003) and to the entire ITS sequence region of *Symbiodinium goreaui* type C1 (AF333515 LaJeunesse, Todd C. 2001). The alignment of the full length ITS sequences from this study and the LaJeunesse C1 sequence (AF333515) consisted of six taxa and 542 alignment positions. Nine alignment positions were variable, 7 of which were parsimony informative. Phylogenetic analysis of the six full-length ITS sequences obtained from before and

after the bleaching and the *Symbiodinium goreaui* C1 sequence (AF333515) confirmed the assignment to types C1 and C2 (Figure 1.4 c).

Twenty sequences have been submitted to Genbank under the accession numbers EU189435 - EU189455 (Table 1.1). All except one cloned and one uncloned ITS1 sequence had at least one or two base pair variations from previous Genbank entries. All sequences used as references, standards and matches from Genbank are shown in Table 1.2.

Table 1.2. Additional *Symbiodinium* types for which full ITS and ITS1 sequences were referred to in this study.

Species	Clade	Host	Location	Citation	Accession No.
Symbiodinium sp.	C1	*A. tenuis*	Magnetic Island	(van Oppen, M. J. H. *et al.* 2001)	AF380555
Symbiodinium sp.	C2	*A. millepora*	Orpheus Is.	(van Oppen, M. J. H. *et al.* 2001)	AF380549
Symbiodinium sp.	D	*A. millepora*	Magnetic Island	(van Oppen, M. J. H. *et al.* 2001)	EU024793
Symbiodinium sp.	C	*A. millepora*	Keppel Is.	(Berkelmans, Ray 2006)	AY643496
Symbiodinium sp.	D	Various spp.	Palau	(Fabricius *et al.* 2004)	AY457965
S. goreaui	C1	*Rhodactis lucida*	Carribean, Jamaica	(LaJeunesse, Todd C. 2001)	AF333515
Symbiodinium sp	C1b, c	Various spp.	Heron Is.	(LaJeunesse, T. C. *et al.* 2003)	AY239363/4
Symbiodinium sp	C1e	Various spp.	Hawaii	(LaJeunesse, T CThornhill *et al.* 2004)	AY258489
Symbiodinium sp.	C1f	*Fungia scutaria*	Hawaii	(LaJeunesse, T CThornhill *et al.* 2004)	AY258490

DISCUSSION

This study has shown field evidence of a dramatic shift in the symbiont community in a reef-building coral as a result of bleaching. The balance of the symbiosis shifted from a predominant association between *A. millepora* and *Symbiodinium* type C2 to a predominance of type D and to a lesser extent to predominance of type C1. This shift resulted partly from a change of symbionts within coral colonies that survived the bleaching event (42%) and partly from selective mortality of the more bleaching-sensitive C2-predominant colonies (37%). While these numbers are event, population and location specific, they do confirm that several interrelated processes play a role in shaping reef symbiont communities after bleaching episodes (Baker, A. C. 2003). It is proposed that symbiont shuffling is a more likely explanation for the observed shift in symbiont communities than switching (i.e. de novo uptake) because (i) all 14 colonies that harboured low levels of D-type symbionts prior to the bleaching event survived and changed from C2 to D predominance, (ii) SSCP analysis is known to lack the sensitivity to detect symbiont types at a relative abundance of less than 5–10% and (iii) cloning and sequencing a subset of samples before bleaching revealed D and C1 below the detection limits of SSCP, the presence of which predicted their appearance after bleaching if shuffling was the mechanism of change. This is supported by the observation of novel symbiont types three and six months after the bleaching. Although de novo uptake cannot be ruled out, mathematical modelling of the recovery of symbiont populations after bleaching suggests that such rapid changes are more

easily explained by upward and downward regulations of existing symbiont populations (Jones, R. J. & Yellowlees 1997).

As a direct result of the shift in symbiont community, the Miall Island *A. millepora* population is likely to have become more thermo-tolerant. This conclusion is based on the experimental evidence of Berkelmans & van Oppen (2006) who found that differences in thermal tolerance in *A. millepora* from the same area is driven by symbiont type rather than the host coral. Furthermore, a shift from bleaching-sensitive type C2 to clade D increased the thermal tolerance of this species by 1–1.5°C. These findings are supported by our observation of differential bleaching susceptibility between C2- and D-predominant colonies during the 2006 bleaching event. *A. millepora* colonies that host predominantly C1-type symbionts are also more thermally tolerant than their counterparts with C2. Unbleached colonies of the staghorn coral *Acropora formosa* sampled in February 2006 at Miall Island harboured predominantly C1 symbionts whereas white-bleached colonies of this species hosted C2. These observations, together with the high occurrence of C1 in *Acropid* corals (van Oppen, M. J. H. *et al.* 2001) at one of the most thermo-tolerant reefs on the Great Barrier Reef (Berkelmans, R. 2002), suggest that C1 may confer thermal tolerance to some species, just like D-type symbionts. Given the direct experimental evidence of increased thermal tolerance of *A. millepora* with D-type symbionts and the circumstantial evidence of similar thermal tolerance in this species with C1-type symbionts, the symbiont community change documented in this study is therefore likely to have resulted in increased thermal resistance for the majority of the *A. millepora* population.

If the symbiont community drifts back to C2 predominance, the increased thermal tolerance will be lost. A drift back to pre-bleaching symbiont types was suggested for *Montastraea annularis* in the Florida Keys (Thornhill, D. *et al.* 2006), and there are signs of a similar drift back to pre-bleaching C2 predominance in this study six months after bleaching.

Our results strongly support the re-interpreted adaptive bleaching hypothesis of Buddemeier *et al.* (2004), which postulates that a continuum of changing environmental states stimulates the loss of bleaching-sensitive symbionts in favour of symbionts that make the new holobiont more thermally tolerant. However, such a change may come at a physiological cost such as loss of photosynthetic efficiency (Rowan 2004a) leading to lower energy reserves (Hoogenboom, Anthony & Connolly 2006; Loram, Trapido-Rosenthal & Douglas 2007) and slower growth (Little, van Oppen & Willis 2004). Our field observations provide the first extensive colony-specific documentation and quantification of temporal symbiont community change in the field in response to temperature stress, suggesting a population-wide acclimatisation to increased water temperatures. If this shift is sustained and extends to other species, the reefs in this area are likely to have substantially increased their capacity to withstand the next bleaching event. However, at this stage, it is unknown whether the increased thermal tolerance, even if it persists, will necessarily translate into increased reef resilience, particularly if growth and carbonate accretion are depressed to levels whereby bioerosion outweighs net accretion.

This study highlights the importance of improving our understanding of multi-clade symbiotic partnerships (Baker, A. C. & Romanski 2007). Our results show an increase in the diversity of symbionts after bleaching together with a considerable change in the make-up of the symbiont community within individual colonies over time scales as short as three months. This increase in the diversity and variation of symbionts has not been previously shown following a bleaching event. Most studies that have followed the *Symbiodinium* community during bleaching (Baker, Andrew C *et al.* 2004; Glynn *et al.* 2001; Guzman & Cortes 2001; Van Woesik, Irikawa & Loya 2004) have not used molecular techniques sensitive enough to detect the low-density symbiont genotypes and genetic variations of rDNA types (Apprill, A. M. & Gates 2007). A recent study has shown that the majority of scleractinian corals are likely to harbour symbiont types at levels that are undetectable using electrophoretic genetic techniques (Mieog, J C *et al.* 2007), suggesting that symbiont flexibility may also be more common than previously thought. Subtle seasonal and spatial shifts in symbiont populations that occur as a result of even minor changes in environmental variables such as temperature and light may underwrite the more permanent, climate-driven shifts following dramatic bleaching events (Thornhill, D. *et al.* 2006). Smith (2005) found that four months before a major bleaching event in early 2002, 20 out of 20 *A. millepora* colonies at Miall Island were predominant in type C2, while van Oppen *et al.* (2005) found that five months after the 2002 bleaching event, 6 out of 19 were predominant in type D. Although the sample sizes in these studies are small, these results suggest that the *A. millepora* symbiont community underwent a similar shift towards clade D predominance as a result of the 2002 bleaching event and then drifted back to C2 predominance 4 years later just prior to the 2006 bleaching event. This

poses the question of why some coral populations retain thermally tolerant symbionts while others revert back to former sensitive types. Baird *et al.* (2007) hypothesize that symbiont community shuffling to clade D may persist only as a result of enduring changes in environmental conditions, e.g. repeated warm summers. This may be evident at Magnetic Island, where temperatures exceed 30.5°C during most summers (Berkelmans, R. 2002) and *A. millepora* have harboured exclusively clade D symbionts over many years (Berkelmans, Ray & van Oppen 2006; van Oppen, M. J. H. *et al.* 2001). Conditions similar to those currently occurring at warm reefs such as Magnetic Island have been projected to occur on in the southern Great Barrier Reef by 2020–2030 (Done *et al.* 2003). Understanding the role of these backgrounds symbionts and the process and conditions under which they are up-and down-regulated is the key to assessing the acclimatisation potential of coral reefs and their ability to withstand future thermal stress events in an era of climate change.

SUMMARY

Some reef corals may be able to cope with warmer conditions by changing their predominant symbiont type. Hosting a more thermally tolerant symbiont type, like *Symbiodinium* type D has been shown to increase the tolerance of the host coral, *A. millepora*, by 1.0–1.5 °C (Berkelmans, Ray & van Oppen 2006). This study shows that this species can increase its thermal tolerance by a combination of symbiont shuffling and selective mortality of type C2 colonies, resulting in a 71% shift from C2 to D and C1 (another thermo-tolerant type Abrego *et al.* 2008). However, the shift was transitory and there were signs of reversion to C2 in colonies six months after

bleaching, making the acclimation temporary. If more coral species host these thermally tolerant symbionts at levels that have previously been undetectable (Mieog, J. C. *et al.* 2009; Mieog, J C *et al.* 2007; Mieog, J C *et al.* 2008), and these changes become permanent with incremental changes in temperature, at least some corals may be able to acclimatise to climate change (Buddemeier, R.W *et al.* 2004; Buddemeier, Robert W & Fautin 1993).

Chapter 2

PHYSIOLOGICAL RESPONSES OF *ACROPORA MILLEPORA* WITH C2 AND D SYMBIONTS: PHOTOSYNTHESIS

***Acropora millepora* colonies in the Keppel Islands of the southern Great Barrier Reef associate with *Symbiodinium* type C2, D or a combination of both types. The image shows a fragment of an *A. millepora* colony used in a study of rapid light curves to investigate the differential photokinetics of branches with C2 and D symbionts. Photograph courtesy of Alison Jones.**

ABSTRACT

Many reef building corals may be able to acclimatise to climate change by shuffling the levels of more thermally tolerant *Symbiodinium* (zooxanthellae) types within their tissues to gain increased tolerance to warmer conditions. The exact mechanisms for increased heat tolerance in the symbiont are still unclear but, just as it does in plants, it is likely to involve aspects of the photosystems. Regardless of the mechanism, like the plants, increased thermal tolerance could also come at a physiological cost to fitness in corals. In order to shed light on potential trade-offs of thermal tolerance in corals, the photokinetics of a common reef-building coral, *A. millepora,* with symbionts of contrasting thermal tolerance, was investigated using pulse amplitude modulation (PAM) fluorometry. This species naturally hosts either thermally sensitive type C2, thermally tolerant type D or a mixture of both types of *Symbiodinium* in the inshore Keppel region of the southern Great Barrier Reef. The results show that corals with predominantly type D symbionts had a 41% lower relative maximum rate of electron transport through the photosystems ($rETR_{max}$), 38% lower light utilisation efficiency (α) and 33% lower maximum excitation pressure over photosystem II (Φ_{max}) than corals with type C2 symbionts. Whilst the relationship between electron transport and carbon fixation is not straightforward and Rapid Light Curves do not take into consideration the contribution of symbiont respiration, carbon uptake, differences in the optic properties of host tissues and photosynthates translocation to the host, these results suggest a link between increased thermal tolerance and reduced photosystem II capacity in the symbiont. Since reef building corals obtain a large proportion of their

energy from their symbionts, *A. millepora* that acclimatise by changing from type C2 to type D may be trading off increased thermal tolerance for considerably reduced PSII competence; even in the absence of heat stress. This is likely to result in lower net photosynthetic energy production, manifesting as reduced growth and reproductive output.

INTRODUCTION

Reef-building (scleractinian) corals host numerous types of single-celled zooxanthellae (algae) within their tissues which provide their hosts with most of their energy requirements. There are currently six clades of zooxanthellae (*Symbiodinium)*, which have been identified using nuclear ribosomal and chloroplast DNA (A-D, F, G) (reviewed by Baker, A. C. 2003). Recent studies have shown that corals may change the proportions of their symbiont complement in response to changes in their environment (Jones, A. M. *et al.* 2008). Hosting a thermally tolerant type can decrease susceptibility to bleaching by increasing the host's tolerance to heat stress (Berkelmans, Ray & van Oppen 2006). The exact mechanisms that infer increased thermal tolerance to the coral are still not well understood but may be linked to symbiont photosynthetic function (Tchernov *et al.* 2004). If this is the case, improving stress tolerance by changing *Symbiodinium* type may lead to trade-offs which could ultimately have implications for the robustness, productivity and regenerative capacity of coral reefs (Hoogenboom, Anthony & Connolly 2006; Loram, Trapido-Rosenthal & Douglas 2007).

One of the potential costs for increased thermal tolerance may arise in the amount of energy translocated from the symbiont to the host coral. Zooxanthellae provide their hosts with most of their energy requirements through photosynthesis (Muscatine 1990; Trench, R. K. 1971). Photosynthetically active radiation (PAR) is converted into either photochemical energy, dissipated as heat (non-photochemical energy) or re-emitted as chlorophyll fluorescence by the algae. Diversion of photon energy through photosystem I (PSI) and photosystem II (PSII) results in the production of adenosine-5'-triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) which are used as an energy source by the zooxanthellae and to fix carbon into glycerol and triglyceride molecules via the Calvin Cycle. These initial simple molecules are then translocated to the coral polyp and incorporated into more complex molecules or used immediately by both symbionts and host in cell metabolism (Bachar *et al.* 2007). Photon energy that is not used in ATP/NADPH production is dissipated via non-photochemical pathways in order to prevent excess light from damaging the algal photosystems. Differences in the proportions of these photosynthetic processes have been suggested to be an expression of the genetic diversity of the symbionts (Iglesias-Prieto, R & Trench 1994). As zooxanthellae vary in their photosynthetic response to temperature (Rowan 2004a) and in the composition of lipids in the chloroplast membranes (Tchernov *et al.* 2004), variations in the amounts of light absorbed by the photosystems or in the rate of electron transport through PSII can influence the critical point at which photosynthesis becomes toxic during temperature stress. Similar mechanisms operate in heat tolerant plants (Hugly *et al.* 1989), freshwater algae (Sato *et al.* 1996) and zooxanthellae (Iglesias-Prieto, R *et al.* 1992; Tchernov *et al.* 2004) which have all been shown to have altered thylakoid membrane

composition compared to heat-sensitive types. In plants, increased thermal tolerance has been linked with downstream consequences in photosynthesis such as reduced electron transport and photochemical energy conversion (even in the absence of temperature stress). The downstream consequences of symbiont thermal tolerance has been studied in corals under temperature stress (Rowan 2004a) but it is symbiont effects on the productivity under normal temperatures (for instance following a bleaching event) that is of concern as corals acclimatise to warmer conditions.

There are a number of mechanisms utilised by corals and zooxanthellae to avoid damage to the symbiont photosystems under stress. Excess irradiance can be dissipated by the symbionts by reducing the flow of excess electrons and by the constant repair of proteins (Takahashi & Murata 2005). The photosynthetic pathways can also be protected by down-regulation of PSII (Bhagooli & Hidaka 2003; Hoegh-Guldberg, O. & Jones 1999; Iglesias-Prieto, R *et al.* 1992; Rowan 2004a), by decreasing the rate of electron transport (Jones, R. J. *et al.* 1998; Ohad *et al.* 1994; Warner, Mark E., Fitt & Schmidt 1999), or by diverting excess electrons into non-assimilatory pathways (Osmond & Grace 1995; Schreiber & Neubauer 1990). Thermally tolerant types of zooxanthellae have been shown to have more stable thylakoid membrane composition under temperature stress (Iglesias-Prieto, R *et al.* 1992; Tchernov *et al.* 2004) which seems to mirror the mechanisms observed in freshwater unicellular algae (Sato *et al.* 1996) and plants (Ristic, Bukovnik & Vara Prasad 2007). The O_2/CO_2 specificity of Rubisco enzymes in the Calvin Cycle has also been suggested as a possible mechanism for increased algal thermal tolerance

(Whitney & Andrews 1998). Irrespective of the exact mechanisms, some of these photoprotective processes have been shown to have a net energetic cost in corals (Dubinsky *et al.* 1984; Hoogenboom, Anthony & Connolly 2006) and sea anemones (Loram, Trapido-Rosenthal & Douglas 2007), just as they do in algae (Sato *et al.* 1996) and plants (Laing, Greer & Schnell 1995). Given the link between PSII function and holobiont productivity (Muscatine 1990; Trench, R. 1993) then it is likely that the photosynthetic performance of corals with symbionts of contrasting thermal tolerance is likely to vary just it does for plants of contrasting thermal tolerance.

In this study, intact symbioses of the reef-building coral, *A. millepora*, with predominantly thermally tolerant *Symbiodinium* type D are compared to those harbouring thermally sensitive type C2 using Rapid Light Curves (RLC's). Corals are capable of maintaining photosynthetic activity over a wide range of light conditions on the reef (Franklin *et al.* 1996). RLC's measure chlorophyll fluorescence emission at steadily increasing irradiance and are a useful tool for measuring the light saturation state of electron transport through PSII as well as the overall photosynthetic performance. Chlorophyll fluorescence measurements can be described by a simple equation which can be applied to estimate the relative rate of electron transport, light capture efficiency and saturating light intensity (Genty, Briantais & Baker 1989). These characteristics can provide information about the photosynthetic capacity of the coral under a given light-adapted state. Absolute assumptions about the state of the PSII reaction centres, oxidation of the electron transport chain or relaxation of photoprotective mechanisms cannot be made using RLC's as there are clearly

assumptions about the amount of light reaching PSII in individual samples, the optical properties of the host tissue and oxygen dependent electron flow. However, RLC's provide a reasonable approximation of the photosynthesis-irradiance state of corals in a given light adaptation state as measurements of chlorophyll fluorescence are made over a relatively short time span (1-2 min) and after only brief (10 s) exposure to light without inducing steady state photosynthesis. Whilst it is recognised that RLC's represent the excitation pressure over PSII rather than net photosynthetic activity (measurement of which traditionally includes gas exchange measurements) , and that the relationship between electron transport rate and carbon fixation is complex, RLC measurement on colonies of the same species with similar tissue characteristics, ambient conditions and light prehistory can help in part to explain the measured differences in energy reserves, reproduction and growth that are reported for this species in Chapters 3 and 4.

MATERIALS AND METHODS

Collection and maintenance of corals

Colonies of *A. millepora* were collected from Miall Island (23°09′S 150°54′E) in the Keppel Island group (southern inshore Great Barrier Reef) in August 2006. Five C2 and five D colonies of approximately 15-20 cm diameter were collected in August 2006 and transported in flow-through tanks 650 km by ship to the Australian Institute of Marine Science laboratories in Townsville. The predominant *Symbiodinium* type in each colony was identified using the ITS1 region of algal nrDNA as described in

Chapter 1 and Appendix I. Only colonies with intense bands representing either type C2 (EU189443) or type D (EU1894505) symbionts were chosen for the study.

The corals were acclimatised in a shaded, flow-through outdoor aquarium facility for six weeks prior to the experiment. Light at the aquarium facility varied diurnally with a typical midday underwater irradiance of ~250 μmole photons m^{-2} s^{-1} and water temperatures were ~26 °C (similar to the ambient conditions at Miall Island at the time of collection). Five replicate branches (explants) of ~3-4 cm length were removed from each colony just prior to conducting the fluorescence assays.

Experimental protocol

Dark-adapted F_v/F_m measurements were performed between 9 am and 11 am, immediately followed by Rapid Light Curves (RLC's) with a 10 min period of recovery to compare the response of corals with type C2 and type D symbionts. RLC's were performed after a period of light exposure in the morning to allow oxidation of the plastoquinone (PQ) pool followed by ~20 min in a darkened room to allow partial relaxation of PSII.

All fluorescence measurements were made using an imaging pulse-amplitude-modulated fluorometer (Imaging-PAM, Heinz-Walz, Germany) with a built-in light sensor and a standard measuring head. An LED-ring-array provided a pulse-modulated blue excitation light and actinic illumination for stepped irradiances, measuring light and the saturating pulses (470 nm). Measurements were made

simultaneously on single explants from 10 individual colonies placed side-by-side in a clear glass vessel (325 ml volume) filled with atmosphere-equilibrated filtered seawater. Five replicate RLC's were measured separately for each branch from the 10 colonies using the experimental design shown in Figure 2.1. Dark-adapted quenching analyses and RLC's were measured for each set of 10 explants simultaneously on a single randomly-chosen 5 mm diameter area midway along the branch (Figure 2.1). The data were visualised using the WinControl software (Walz GmbH, Effeltrich, Germany). RLC parameters were calculated by the equations of Genty *et al.* (1989) and Maxwell and Johnson (2000) (Table 2.1).

Figure 2.1. PAR-absorptivity images of 10 *Acropora millepora* branches measured during RLC's. Explants with predominantly C2 symbionts had significantly higher PAR-absorptivity (light green) than those with D symbionts due to their higher level of algal cell chlorophyll *a*.

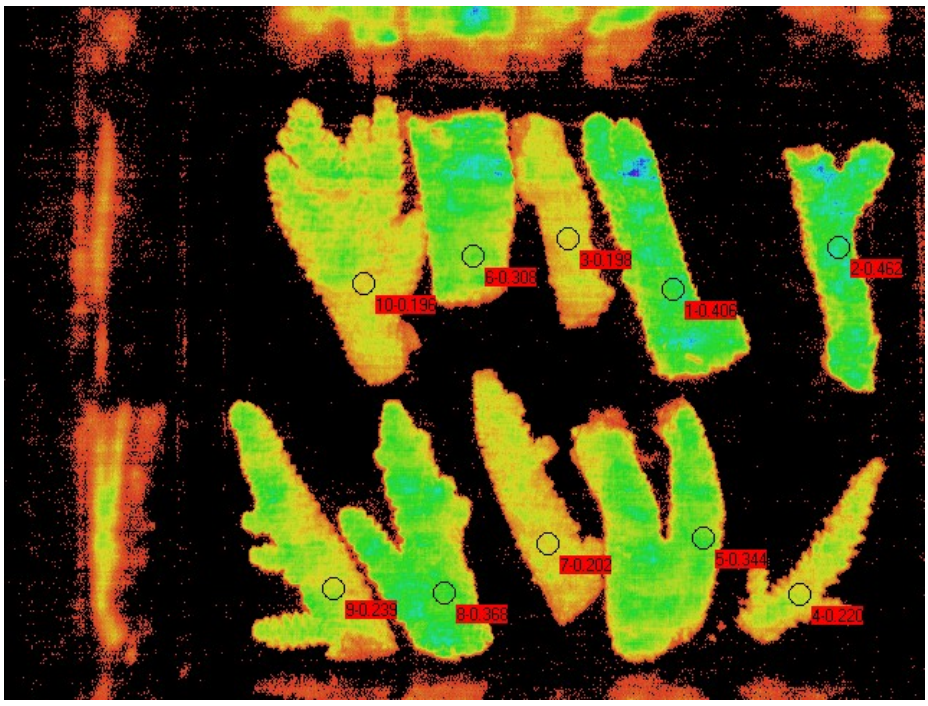


Figure 2.2 Experimental protocol for RLC's performed using an imaging Pulse Amplitude Modulated fluorometer on five replicate branches from each of 10 colonies of *Acropora millepora*.

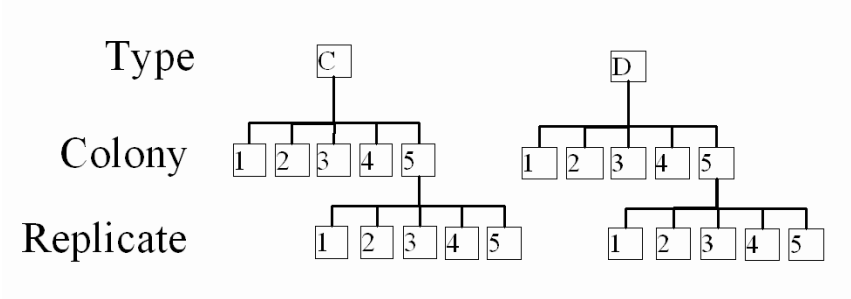


Table 2.1. Parameters and equations used in the study and quenching analyses. F = Chlorophyll fluorescence above 710 nm.

Photochemical quenching parameters		
Φ_{PSII}	Effective quantum yield (light – dark)	$F / F_m' = F_m' - F / F_m'$
Fv/Fm (Φ_{max})	Maximum quantum yield of PSII (dark - light)	$F_v / F_m = F_m - F_0 / F_m$
Rapid Light Curve parameters		
rETR	relative electron transport rate	Φ_{PSII} x PAR
$rETR_{max}$	maximum relative electron transport rate	$rETR_{max} = P_s (\alpha / [\alpha + \beta]) (\beta / [\alpha + \beta])^{\beta/\alpha}$
α	slope of the initial light limited region of the RLC = light capture efficienc	$\alpha = rETR_a - rETR_b / PAR_a - PAR_b$ a and b = separate points on the initial linear part of the curve
E_k	maximum saturating irradiance	$E_k = ETR_{max} / \alpha$
β	slope of the RLC during photoinhibition when PSII declines	$\beta = 0$ in the absence of photoinhibition

Maximum and minimum fluorescence measurements (F_v/F_m)

Dark-adapted quenching analyses were initially performed to determine the minimum and maximum fluorescence (F_0 & F_m). These parameters represent the minimum and maximum potential of PSII. F_0 represents the proportion of open PSII reaction centres when the photosystems are inactive and F_m represents the proportion of closed reaction centres after dark-acclimation. Dark adaptation allows opening of the PSII reaction centres, oxidation of the electron transport chain, relaxation of the xanthophyll cycle and depletion of the trans-thylakoid proton gradient. F_0 is the fluorescence measured using a weak measuring light without inducing photosynthesis. F_m is the fluorescence measured after a saturating pulse of light (> 10,000 μmole photons m^{-2} s^{-1}, 0.4–0.8 s) which closes all PSII reaction centres

Explants were removed from colonies in the aquarium where ambient light levels were ~250 μmole photons m^{-2} s^{-1} and placed in a darkened room for ~20 min after which an initial 1 s saturating pulse of light was applied. Damping and Gain were set at 2 and the measuring light (ML) was set at 1 μmole photons m^{-2} s^{-1}. Fluorescence measurements were made before (F_0) and during (F_m) the saturating pulse of light. The ratio of variable (F_m - F_0) to maximum fluorescence (F_m), F_v/F_m, was used as an indicator of the maximum potential quantum yield (Table 1.1). All subsequent fluorescence measurements were compared to these values for F_v/F_m and used these instruments settings.

Rapid Light Curves

To explore the photosynthetic response of explants with type C2 and D symbionts to increasing light levels, RLC's were performed by applying a series of 1 s saturating pulses under 20 increasing 10 s actinic irradiance steps (0-1801 µmole photons m^{-2} s^{-1}, Table 2.2). Measurements of fluorescence are made before (F) and during (F_m') the saturating light pulse.

Table 2.2. Twenty stepped irradiances (including the initial dark-adapted measurement when PAR = 0) were used during the Rapid Light Curves performed on *Acropora millepora* explants with C2 and D symbionts. PAR = photosynthetically active irradiance in µmole photons m^{-2} s^{-1}.

t	PAR
1	0
2	1
3	11
4	36
5	51
6	76
7	101
8	151
9	351
10	451
11	551
12	701
13	801
14	901
15	1001
16	1201
17	1301
18	1501
19	1601
20	1801

The ratio of open to closed PSII reaction centres at each irradiance step (Φ_{PSII}) was calculated using the equation in Table 2.1. Since no widely accepted absorption coefficient has been developed for corals which have complex light absorption

characteristics (Enriquez, Méndez & Iglesias-Prieto 2005; Genty, Briantais & Baker 1989), only relative electron transport rates (rETR's) were derived from the RLC measurements by multiplying Φ_{PSII} x PAR. rETR is a relative measure of the rate of electron transport through the photosystems and is not linked to the light absorption characteristics of the coral (Schreiber 2004) which has been shown to vary with tissue type (Ralph, P. J. *et al.* 2002), tissue age (Durako & Kuss 1994) or symbiont genotype (Dubinsky *et al.* 1984).

To compare the potential for C2 and D corals to relax after light exposure, measurements of Φ_{PSII} were continued for 10 min in darkness after the final RLC irradiance step of 1801 μmole photons m^{-2} s^{-1}.

Curve fitting

To quantitatively compare several descriptive parameters of the RLC (α, E_k and $rETR_{max}$) a double exponential decay function (Platt, Gallegos & Harrison 1980) was fitted to the data using SigmaPlot 2000 (version 6.0 SPSS Inc.). An unweighted curve was fitted by regression analysis using the settings described in Ralph and Gademann (2005).

Chlorophyll *a* content and zooxanthellae densities

To determine the contributions of the photosynthetic pigment chlorophyll *a* and zooxanthellae densities to differences in photosynthetic function, explants were snap-frozen at -80 °C immediately after completion of the fluorometry measurements and stored at -20 °C until processed. Frozen branches were stripped of tissue using an air

gun and the resultant slurry was macerated with a tissue homogenizer for 20 s. The homogenate volume was recorded and a 9 ml aliquot was drawn off and preserved with 1 ml of formalin (32% $w.w^{-1}$). Zooxanthellae counts were made on eight independent drops (0.0001 mm^3) from each sample using the New, Improved Nuebauer haemocytomer (Brown, B. E. *et al.* 2002; Jones, R. J. *et al.* 1998; Perez, Cook & Brooks 2001) under a compound light microscope. Zooxanthellae numbers were standardised to coral tissue surface area using the 3D digital image analysis method described in (Jones, A. *et al.* 2008) (O'Donnell, Jones, Noel method) and in Appendix II.

A separate 10 ml aliquot was drawn from the remaining tissue homogenate and the algal pellet was separated from the host tissue by centrifugation (3000 g for 5 min) at 4 °C. Chlorophyll was extracted overnight from the algal pellet using 100% methanol at 4 °C (Berkelmans, Ray & van Oppen 2006; Brown, B E *et al.* 2002). The first 10 samples were extracted three times to determine the extraction efficiency. Absorbance at 668 nm and 635 nm was measured with a spectrophotometer (Hitachi U-3200). Total branch chlorophyll *a* was calculated from the equation of Jeffrey and Haxo (1968) after adjustment for extraction efficiency and standardised to algal cells.

STATISTICAL ANALYSIS

Rapid Light Curves-derived parameters

The parameters derived from the RLC's, $rETR_{max}$, α, $E_{k,}$ and Φ_{max} and data for algal chlorophyll *a* content were analysed with a multivariate single factor ANOVA using symbiont type (two levels) as the fixed predictor variable. Data for each colony were averaged over the five replicates (Figure 2.2). Values of $rETR_{max}$, α and Φ_{max} were normalised to algal cells (10^6) by dividing the actual value by the mean zooxanthellae number per cm^2 of explant surface area to allow for differences in zooxanthellae densities. Values for E_k were determined using the equation (Genty, Briantais & Baker 1989): $E_k = rETRmax/ \alpha$. Unstandardised residuals and standardised predicted values were used to verify the assumptions of normality and homogeneity of variances were verified using Levene's Test.

Relaxation kinetics

To determine the effects of symbiont type on the relaxation kinetics of effective quantum yield of *A. millepora* explants during the RLC's, the averaged data for Φ_{PSII} from the RLC's for each colony were analysed with a one-factor ANOVA with symbiont type as the fixed, predictor variable (two levels). Unstandardised residuals and standardised predicted values were used to verify the assumptions of normality and homogeneity of variances were verified using Levene's Test. Data were natural log transformed before the analysis to satisfy the assumption of homogeneity of variances. All statistical analyses were performed using SPSS version 15.0.

RESULTS

Rapid Light Curves-derived parameters

The rapid light curves (RLC's) followed the classic shape of P-I curves, increasing to a maximum and then declining as irradiance increased (Figure 2.3). Explants with type D symbionts had lower values than those with C2 symbionts for three of the key parameters that describe photosynthesis: relative maximum rate of electron transport ($rETR_{max}$), light capture efficiency (α) and maximum dark-adapted quantum yield (F_v/F_m). These results suggest that *A. millepora* with type D *Symbiodinium* may have lower overall PSII performance compared to type C2 symbionts at the same light levels (Table 2.3, 2.4).

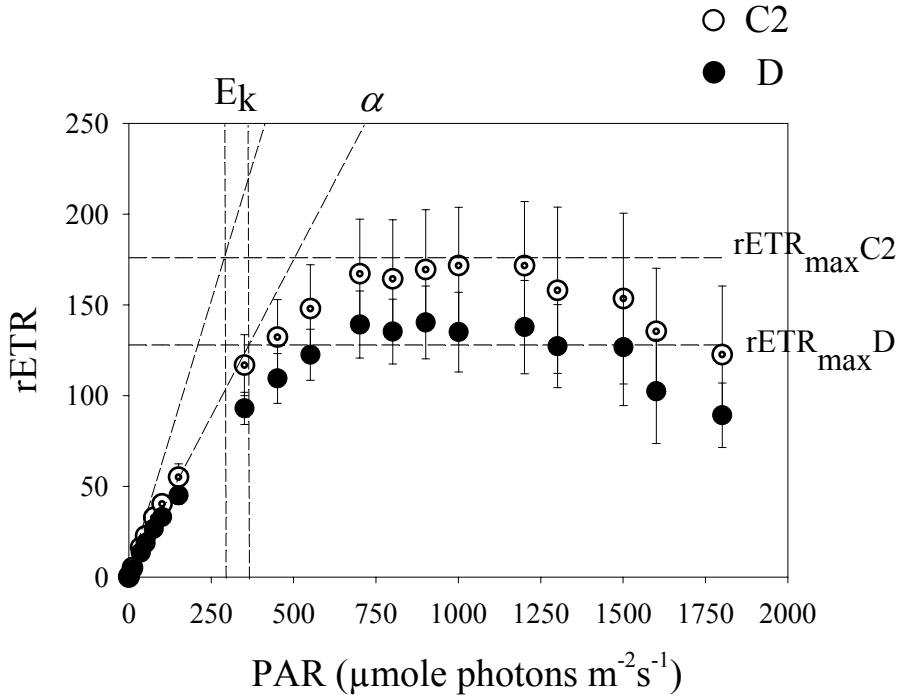
Table 2.3. ANOVA table showing the significant effect of predominant symbiont type (C2 or D) present in *Acropora millepora* explants on RLC parameters derived using regression analysis.

		SS	df	Mean Square	F	Sig.
$rETR_{max}$	Between Groups	13074.39	1	13074.39	6.4	0.036
	Within Groups	16418.29	8	2052.29		
	Total	29492.68	9			
α	Between Groups	0.16	1	0.16	9.5	0.015
	Within Groups	0.13	8	0.02		
	Total	0.29	9			
E_k	Between Groups	358.57	1	358.57	0.3	0.601
	Within Groups	9695.29	8	1211.91		
	Total	10053.86	9			
F_v/F_m	Between Groups	0.08	1	0.08	6.4	0.036
	Within Groups	0.10	8	0.01		
	Total	0.19	9			
Chlorophyll *a*	Between Groups	153.98	1	153.98	7.4	0.026
	Within Groups	165.72	8	20.72		
	Total	319.70	9			

Table 2.4. Table showing the values for five of the parameters that describe photosynthesis of *Acropora millepora* explants with C2 and D symbionts. $rETR_{max}$, α and E_k were derived using regression analysis of RLC measurements. F_v/F_m represents the maximum dark-adapted quantum yield of photosystem II. $rETR_{max}$, α, F_v/F_m and chlorophyll *a* content were normalised to zooxanthellae cells.

Parameter	Type	Mean	S.D.	N	units
$rETR_{max}$	C2	175	60	5	
	D	103	21	5	
α	C2	0.66	0.17	5	µmole photons m^{-2} s^{-1} $cell^{-1}$
	D	0.41	0.06	5	µmole photons m^{-2} s^{-1} $cell^{-1}$
E_k	C2	261	39	5	µmole photons m^{-2} s^{-1}
	D	249	30	5	µmole photons m^{-2} s^{-1}
F_v/F_m	C2	0.55	0.14	5	
	D	0.37	0.08	5	
Chlorophyll *a*	C2	33.7	4.1	5	µg x 10^{-6} $cell^{-1}$
	D	25.9	5.0	5	µg x 10^{-6} $cell^{-1}$

Figure 2.3. Rapid Light Curves for explants of *Acropora millepora* with C2 and D symbionts. Corals were dark-adapted for ~20 min prior to the measurements. Previously determined F_0 and F_m (dark-adapted) were used for calculation of effective quantum yield (Φ_{PSII}) which was multiplied by the photosynthetically active radiation (PAR) to determine the relative electron transport rate (rETR), standardised to zooxanthellae density (algal cells cm^{-2}) and plotted against PAR (µmole photons m^{-2} s^{-1}). Curves represent the average of five replicate RLC's measured on explants from *A. millepora* colonies with *Symbiodinium* type C2 (n = 5) or D (n = 5). Hairline curves represent fitted non-linear regression curves (r^2 C2 = 0.96, r^2 D = 0.94). Symbols represent the mean and error bars represent ± S.D.

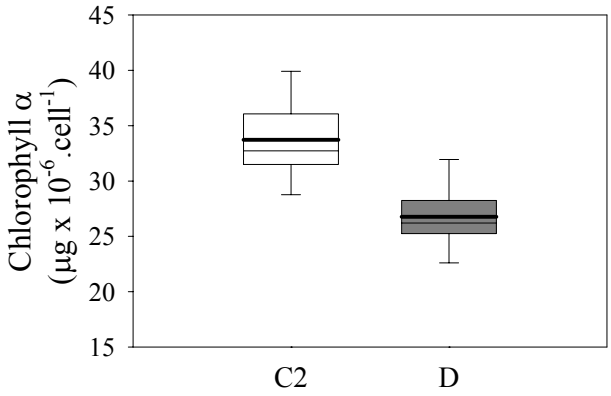


The relative maximum rate of electron transport ($rETR_{max}$) through the photosystems varied significantly with predominant symbiont type (Figure 2.3). $rETR_{max}$ for type D explants was 41% lower than for explants harbouring C2 symbionts ($p < 0.05$).

Maximum quantum yield (Φ_{max}) varied significantly with predominant symbiont type. Type D explants had 33% lower Φ_{max} than C2 explants ($p < 0.05$). Values were low as data were normalised to zooxanthellae densities.

A. millepora explants with type D symbionts appeared to capture light less effectively than type C2 explants. Type D explants had a 38% less acute slope (α) in the light-limited region of the RLC which represents the light-harvesting capacity of the antennae matrix (Figure 2.3). One possible reason for this difference in light capture efficiency is that type D symbionts had 23% lower chlorophyll *a* content compared to type C2 symbionts ($p < 0.05$, Figure 2.4). Chlorophyll *a* content is a measure of the amount of light-harvesting pigments that capture incident photons of light. When normalised to algal chlorophyll *a* rather than zooxanthellae, there was no significant difference in the slopes of type C2 and D curves. The differences in light capture efficiency between type D and C2 explants were therefore most likely due to differences in algal cell chlorophyll *a* content.

Figure 2.4. Boxplot showing the significantly lower algal chlorophyll *a* content of explants of *Acropora millepora* with type D symbionts compared to those with type C2 symbionts. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



Type D explants reached saturating irradiance at a slightly lower PAR than C2 explants (Figure 2.3, Table 2.3, 2.4, ns). E_k is a measure of the level of irradiance at which energy used in photosynthesis is diverted into non-photochemical energy and variations in E_k can be an indication of differences in the tolerance of symbionts to light. Contrary to suggestions in other studies (Ulstrup & van Oppen 2003) that type C *Symbiodinium* prefer high light and type D are low irradiance adapted, these results suggest that there was little significant difference in the light tolerance of the *A. millepora* colonies from Miall Island.

Relaxation kinetics

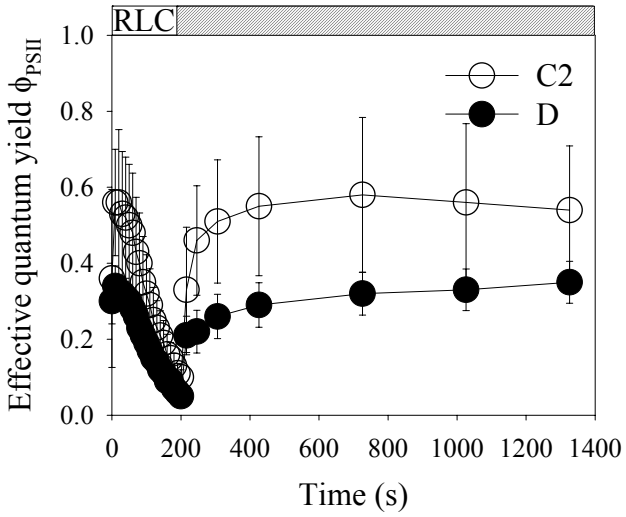
There were significant differences in the relaxation kinetics of type C2 and D corals following the RLC's (Figure 2.5, Table 2.5). At the beginning of the RLC (PAR = 0)

the excitation pressure over PSII for type C2 explants was 61% higher than that for type D explants ($p < 0.05$). Values for Φ_{PSII} are low because data were normalised to zooxanthellae densities.

Table 2.5. The results of a one-way ANOVA showing the significant effect of symbiont type (C2 or D) on the effective quantum yield (Φ_{PSII}) of *Acropora millepora* explants (averaged over 5 colonies).

	SS	df	Mean Square	F	Sig.
Between Groups	2.44	1	2.44	7.4	0.010
Within Groups	11.85	36	0.33		
Total	14.29	37			

Figure 2.5. Rapid light curve (RLC) showing the effective quantum yield and recovery over time (shaded section) for explants of *Acropora millepora* harbouring *Symbiodinium* type C2 or type D. Following the RLC (200 s), corals were allowed to recover in darkness with a saturating pulse and fluorescence measurements at 20, 30, 60, 120, 300 and 600 s to measure recovery. Symbols and error bars represent mean ± S.D.



DISCUSSION

There are significant differences in the chlorophyll fluorescence kinetics of *A. millepora* with type C2 and D symbionts, even in the absence of heat stress. Rapid light curves show that corals with thermally tolerant type D symbionts have lower relative maximum rate of electron transport through PSII, lower dark-adapted maximum quantum yield and lower light absorption capacity compared to those with C2 symbionts. These results present a strong argument for a link between heat tolerance and reduced photosynthetic activity in *A. millepora* with type D symbionts that could explain the lower growth, lipid stores and reproduction that were demonstrated in Chapters 3 and 4. If this is the case, the ecological advantage of hosting a more stress tolerant symbiont type is offset by reduced phototrophic benefits to the host.

The three key processes that describe the light dependent reactions of PSII in the chloroplasts were found to differ significantly between corals with type D and C2 *Symbiodinium*. rETR is an approximation of the rate of electron transport at a given light intensity which is independent of the light absorption and the distribution of photon energy between PSI and PSII. Electron transfer is coupled with the translocation of protons across the thylakoid membrane, which generates electrochemical potential. Under steady state conditions this proton gradient can drive ATP synthesis. The value of $rETR_{max}$ represents the maximum efficiency of electron transfer at saturating light intensity. Lower values for $rETR_{max}$ suggest that the saturation state of electron transport in type D corals is less than that in type C2 corals,

with potential consequences for the establishment of a proton gradient across the thylakoid membranes and ATP synthesis.

The initial slope of the RLC curve, α, is synonymous with that of the traditional P-I curve which represents the efficiency of the antennae matrix to utilise incident light energy. Chlorophyll *a* concentrations could contribute to variations in α but the initial slope of the RLC could also be affected by host tissue optics (Apprill, A., Bidigare & Gates 2007), the number of PSII reaction centres or PQ molecules (Honti 2007) or the size and number of the photosynthetic units that capture incident light (Ekelund *et al.* 2008; Nygård & Ekelund 2006). The differences in the initial slope of the RLC for C2 and D corals are likely to be largely a result of differences in light absorption capacity caused by lower chlorophyll *a* content. Reducing the amount of light entering the photosystems would also act to protect against damage during temperature stress by reducing the excitation pressure over PSII. Regardless of the underlying causes, lower light absorption contributes to the reduced physiological benefit to the host coral demonstrated in Chapters 3 and 4 by restricting the amount of light available for photosynthesis.

F_v/F_m describes the maximum potential excitation pressure of the PSII reaction centres following relaxation during a period of dark adaptation. Lower values for F_v/F_m can indicate photodamage, lower light absorption capacity or a greater number of closed reaction centres. In corals, interpretations of absolute values of F_v/F_m are complicated by dark reduction of the PQ pool. Previous studies have shown that in corals, host

respiration during dark adaptation induces a state of hypoxia in the tissues (Hill, R. & Ralph 2005; Hill, Ross & Ralph 2006, 2008; Jones, R. J. & Hoegh-Guldberg 2001; Ulstrup, Hill & Ralph 2005). Rather than opening PSII reaction centres and oxidising PQ as occurs in plants, full dark adaptation without far red illumination serves to reduce effective quantum yield during the initial period of light exposure until enough PQ molecules become available to re-start the PSII reactions. As a result, direct interpretation of the RLC is made more complex following long periods of dark adaptation. However, a comparison of the photokinetics of corals with symbionts of contrasting thermal tolerance is possible if the period of dark adaptation is short (~20 min) and consistent for all samples and the RLC's are performed after a period of light exposure to allow oxidation of the PQ pool. The lower F_v/F_m for type D corals in this study is likely to reflect the lower potential capacity of PSII reaction centres but reduced light absorption is also likely to be a contributing factor.

The parameters derived from RLC's are not entirely synonymous with those derived from traditional P-I curves which incorporate gas exchange processes deep within the coral tissue layers whereas fluorescence yield originates superficially from the tissue surface. It can be argued that when absolute measures of quantitative rates of rETR, E_k and α are not required, these correlate well with the level of photosynthesis in corals with symbionts of contrasting thermal tolerance (Rascher, Liebig & Lüttge 2000; Schreiber *et al.* 1997; White, A. J. & Critchley 1999) and are superior to single measures of light-adapted quantum fluorescence yield F_v/F_m' (Schreiber 2004). While there are limitations to making assumptions about the absolute values derived from

RLC measurements in different coral species under different conditions, the *A. millepora* colonies used in this study are considered analogous experimental units. This species is extremely abundant in the Keppels, and colonies can harbour either predominantly type C2 or D symbionts or a mixture of both types. The colonies used in the study occurred side by side on the reef flat at Miall Island and were exposed to the same levels of light and ambient water temperatures. Factors such as host tissue optical properties (Apprill, A., Bidigare & Gates 2007), the amount of photosynthates exported to the host (Stat, Michael, Morris & Gates 2008), photoprotective mechanisms (Rowan 2004a) and oxygen dependent electron flow (Ulstrup, K. *et al.* 2006) all potentially complicate interpretation of absolute photosynthetic rates. However, the similarity of the colonies with type C2 and D symbionts makes it plausible that the differences in $rETR_{max}$, α and F_v/F_m represent real differences in photosynthetic efficiency.

The results of this study show that increased heat tolerance is correlated with reduced photosynthetic activity. This is consistent with other studies of heat tolerance in *Symbiodinium*. Tchernov *et al.* (2004) found that isolated and *in-hospite* thermally tolerant symbionts maintained the structural integrity of the thylakoid membranes and the capacity for relaxation of the proton gradient across the membranes and the maintenance of normal photochemical energy conversion at temperatures that would normally affect photosynthesis (Jones, R. J. *et al.* 1998). By reducing the saturation state of the thylakoid membranes, the temperature at which destabilisation can occur during heat stress is increased although separation of the layers interrupts the

photosynthetic reactions (Gournaris, Barber & Harwood 1986) and reduces electron transport (Hugly *et al.* 1989). Decreased membrane appression effectively reduces the length of the thylakoid membrane along which a proton gradient can be established. Increased fatty acid saturation also protects the thylakoid membranes against attack by reactive oxygen species (Murakami *et al.* 2000). The characteristic of thermal tolerance in *Symbiodinium* type D could be a result of natural selection for stability of the chloroplast thylakoid membranes, probably at the level of the biosynthesis of lipids (Tchernov *et al.* 2004) or of the enzymes that control the proportions of saturated fatty acids (Hazel 1995). Just as it does in plants, increased thermal tolerance could reduce the capacity for the algal photosystems to establish a proton gradient across the membranes and therefore to the electron carrying capacity of PSII.

The underlying mechanisms that reduce photosynthetic activity in heat tolerant *Symbiodinium* may be similar to those proposed for heat tolerant plants, involving the composition of the lipids and stability of the thylakoid membranes. Studies of plants show that saturation of the thylakoid lipids by catalytic hydrogenation (increased polyunsaturation) increases the stability of the membranes during temperature stress (Hugly *et al.* 1989; Thomas *et al.* 1986). While there are differences between the structure of the thylakoid membranes in the chloroplasts of plants and zooxanthellae (Smillie 1976), there are also many parallels. Correlations have been made between heat tolerance and thylakoid membrane structure in algae (Sato *et al.* 1996) and plants (Hugly *et al.* 1989; Vigh *et al.* 1985) and it is evident in these studies that thermal tolerance was linked to ~15-35% lower rate of electron transport through PSII. Other

plant studies suggest that the mechanism for heat tolerance involves gradual adaptation of the fatty acids during temperature stress. When grown in the presence of heat and/or light stress the membranes became more fluid; making them more heat tolerant (Nishiyama *et al.* 1993; Papina, Meziane & van Woesik 2007). While there are parallels between thermal tolerance accompanying thylakoid membrane composition in plants and zooxanthellae, acquiring heat tolerance by acclimation of the membranes during heat stress would not explain why type D symbionts retain their heat tolerance even after dividing in the absence of high temperature (i.e. under normal temperature conditions).

Diminished relative maximum rate of electron transport in relation to light levels may be an important mechanism by which *Symbiodinium* type D, and other thermo-tolerant types (e.g. C1, Abrego *et al.* 2008), cope with light during heat stress. Variations in $rETR_{max}$ reflect species differences in unicellular algae (Dimier *et al.* 2007) but so far have not been compared in the *Symbiodinium* types harboured by reef corals. During temperature stress, a lower rate of electron transport would limit the rate of production of excess electrons and reactive oxygen species that are likely to incur damage to the photosystems. A low rate of electron transport under normal conditions may therefore also be the mechanism by which type D symbionts cope with light during temperature stress.

Corals rely heavily on translocated photosynthates for their energy requirements. The lower Φ_{PSII} in type D symbionts in this study strongly suggests down-regulation of

photosynthesis or closed PSII reaction centres compared to C2 symbionts. Effective quantum yield (Φ_{PSII}) is indirectly related to the amount of carbon fixed in the light-independent reactions of photosynthesis (Calvin Cycle). The lower Φ_{PSII} of type D symbionts may lead to reduced carbon fixation. This is supported by a study by Loram *et al.* (2007) who found that thermally tolerant *Symbiodinium* A fix less photosynthetic carbon than thermally-sensitive type B. A more recent study of isolated type A symbionts suggests that host factors may play an important role in regulating the export of photosynthates by the symbionts. Tchernov *et al.* (2004) also found that thermally tolerant symbionts had lower photochemical energy conversion (qP) than thermally sensitive symbionts. qP is a measure of the rate of transfer of electrons from the primary electron acceptor, Q_A, to the secondary quinone, Q_B which induces a proton gradient across the thylakoid membranes. This gradient and the supply of ATP and NADPH help to drive the fixation of carbon through the Calvin Cycle. As a result, reductions in qP contribute to lower carbon fixation. qP can also be affected by diversion of excitation pressure to alternate pathways (e.g. photoprotection or the Mehler cycle) preventing damage to the photosystems from excess electrons as temperature increases, but again, at a net cost to carbon fixation (Jones, R. J. *et al.* 1998; Polle 1996). The down-regulation of photosynthesis may be a mechanism which allows thermally tolerant symbionts to maintain normal (although lower) photosynthetic rates without damage to the photosystems during temperature and light stress.

The differences in photosynthetic function of C2 and D predominant *A. millepora* are unlikely to be the result of transient photoinhibitory or photoacclimatory responses (Dubinsky *et al.* 1984). These are temporal responses whereby light is diverted by the coral to avoid damage to the photosystems from excitation pressure. Photoinhibition is a protective response which operates at a time scale of minutes to hours (Brown, B. E. *et al.* 1999; Lesser, M. P. & Gorbunov 2001) whereas photoacclimatory light/shade acclimation through zooxanthellae and pigment regulation takes a number of weeks (Falkowski, Dubinsky & Muscatine 1993). Due to these photoacclimatory responses, a study by Fitt *et al.* (2000) failed to find a significant pattern between photosynthesis-irradiance curves and the genetic identity of *Symbiodinium* that were isolated from their hosts. The *A. millepora* in this study were sampled from the same part of the reef flat at similar depths and were exposed to the same levels of light and temperature before the study. Under these conditions, photoacclimatory effects on photosynthesis are the same for both C2 and D corals, making a comparison possible. In addition, the differential symbiont densities of type D and C2 corals were accounted for by normalising the measurements to algal cells or pigment concentrations where appropriate. Therefore the differential response of type D and C2 corals to increasing light demonstrate intrinsic genetic photophysiological differences between these two holobionts.

Although the exact link between reduced photokinetics and increased thermal tolerance remains elusive, what is clear is that reef corals that adapt to elevated

temperatures by symbiont shuffling will be significantly compromised because of the trade-off between increased thermal tolerance and reduced photosynthesis.

SUMMARY

Acclimation to climate change is likely to result in trade-offs to coral fitness as the mechanisms for heat tolerance in their endosymbiotic zooxanthellae, which supply their hosts with most of their energy demands, appear to depress photosynthetic function. There is evidence from the differential photokinetics of corals with symbionts of contrasting thermal sensitivity that increased heat tolerance in *Symbiodinium* is achieved by similar mechanisms to those found in heat tolerant freshwater algae (Sato *et al.* 1996) and plants (Hugly *et al.* 1989). These probably involve the composition of the lipids in the thylakoid membranes of the chloroplasts, resulting in stability during temperature stress, which allows for normal (although lower) rates of photosynthetic energy fixation. Regardless of the exact mechanism, the consequences of increased thermal stability are significantly lower electron transport and light absorption capacity in the symbionts that provide most of the coral's energetic requirements.

Chapter 3

PHYSIOLOGICAL RESPONSES OF *ACROPORA MILLEPORA* WITH C2 AND D SYMBIONTS: GROWTH AND BLEACHING

Weighing *Acropora millepora* colonies during a study of growth at Miall Island in the Keppel Islands region of the southern Great Barrier Reef. Colonies were kept in their natural environment tied on racks raised slightly off the sea floor. Buoyant weighing took place a few hundred metres from the site of the growth study. Colonies were weighed using digital scales suspended above a bin of seawater. A plastic wire rack was suspended underneath the scales using a custom designed balance. The AIMS ship, Lady Basten, can be seen moored in the distance to the west of Miall Island. Photograph courtesy of Eric Matson, AIMS.

ABSTRACT

One of the principle ways in which reef building corals are likely to cope with a warmer climate is by changing to more thermally tolerant *Symbiodinium* (zooxanthellae) genotypes. However hosting a more heat-tolerant genotype is likely to be accompanied by tradeoffs in the physiology of the corals. To better understand these tradeoffs, one of the key measures of physiological performance (growth), was investigated in the Indo-Pacific reef-building coral *A. millepora*. In the Keppel Islands in the southern Great Barrier Reef this species is naturally found harbouring ITS1 *Symbiodinium* type C2 or type D as its dominant symbiont. Colonies of this species at Miall Island have been shown to shuffle to type D following bleaching. The growth rate of *A. millepora* hosting symbionts with contrasting thermal sensitivity was investigated in both the laboratory and the field. Under controlled laboratory conditions the buoyant weight of C2 and D explants was measured at two temperatures (23 °C and 29 °C) over a period of four weeks. In addition, whole colonies of C2- and D-predominant *A. millepora* were kept on racks in their native environment at Miall Island in the Keppel Islands and their buoyant weight was measured every three months over a period of two years. In the laboratory, explants with type D symbionts grew 29% slower than those type C2 symbionts. In their natural environment type D colonies grew 38% slower than C2-predominant colonies. These results give an indication of the magnitude of trade-offs likely to be experienced by this species as they acclimatise to warmer conditions by changing to more thermally tolerant *Symbiodinium* type D. These differences are substantially smaller than the 200 –

300% differences found by Little *et al.* (2004) for juveniles of this species and highlight the fact that considerable work is still required in order to predict the ecological impact of warmer conditions on surviving corals as reefs acclimatise to warmer conditions. However, irrespective of symbiont genotype, the medium-term growth (up to 18 months) of corals was affected to an even greater degree by the stress of a bleaching event which reduced growth by up to 50% compared to pre-bleaching growth rates. The co-related processes of symbiont change and acute thermal stress are likely to act in concert on coral growth as they acclimatise to warmer conditions and may compromise the regeneration capacity of reefs.

INTRODUCTION

Coral reefs are generally thought to be highly vulnerable to climate change. Recent research however, indicates that scleractinian (reef-building) corals may have considerable scope for acclimatisation to warmer conditions (Baker, Andrew C *et al.* 2004; Rowan 2004a; van Oppen, Madelaine J H, Little & Willis 2004). One of the principle processes through which corals could acclimatise is through symbiont 'shuffling'. Symbiont (zooxanthellae) shuffling occurs when the relative dominance of thermally-sensitive symbiont genotypes changes in favour of thermally tolerant types (Berkelmans, Ray & van Oppen 2006; Chen *et al.* 2005; Ulstrup, K. E. *et al.* 2006; van Oppen, Madelaine J H, Little & Willis 2004). Symbiont change on reefs essentially involves a community shift in symbionts at the level of species that increases the thermal tolerance of the entire coral population (Baker, Andrew C *et al.* 2004). For instance, changing to thermally tolerant *Symbiodinium* type D in one study was found

to increase thermal tolerance between 1.0-1.5 °C in a common Indo-Pacific coral species, *A. millepora* (Berkelmans, Ray & van Oppen 2006). It is possible that many other coral species may also host low levels of thermally tolerant types and may be able to acclimatise to warmer conditions by symbiont shuffling (Mieog, J C *et al.* 2007). However, reef corals depend almost entirely for their energy needs on photosynthesis by the algal symbionts. Changing symbiont communities could affect the physiological characteristics of the coral that rely on energy from algal photosynthesis. Field studies have yet to demonstrate how widespread the phenomenon of shuffling is, whether all corals have the ability to shuffle symbiont types, or what ecological benefits may result from 'new' host-symbiont combinations.

It is likely that the phenotypic differences between *Symbiodinium* genotypes may affect physiological characteristics of reef corals that rely on energy from photosynthesis. Corals rely heavily on their symbionts for their energy requirements through the translocation of photosynthetically fixed carbon, which has been estimated to be as high as ~95% of the total energy requirement (Bachar *et al.* 2007; Davies 1984; Muscatine 1990). In zooxanthellate corals, some of this energy is used to drive carbonate accretion (Barnes & Chalker 1990; Pearse & Muscatine 1971; Vago, E., Gill & Collingwood 1997). Deposition of $CaCO_3$ is the process by which reef-builders form their hard skeletons. Processes that affect photosynthesis have the potential to have a simultaneous effect on host calcification (Barnes & Chalker 1990; Gladfelter 1984). In Chapter 2, *A. millepora* with thermally tolerant type D zooxanthellae (identified using the ITS1 region of rDNA) were shown to have ~20-30% diminished

photosynthetic competence compared to those with thermally-sensitive type C2 symbionts. Because photosynthesis is directly related to the amount of energy available to the host for calcification, this is likely to reflect in differences in skeletal growth rate. A 200 – 300% difference in skeletal growth was demonstrated in juvenile colonies of *A. millepora* and *A. tennui*s with ITS1 type C1 and D zooxanthellae (Little, van Oppen & Willis 2004). Both of these zooxanthellae types are thermally tolerant (Chapter 2, Abrego *et al.* 2008). If these growth differences extend to, and are of similar magnitude in adult corals, then this has implications for skeletal growth in corals that acclimatise to climate change by shuffling to type D symbionts.

The growth rate of reef-building corals is a key factor in the regeneration capacity of tropical reefs. The hard skeletons of reef-building corals form the framework of reefs, providing food and habitat niches for other marine organisms (Henry & Hart 2005). Factors that affect calcium carbonate accretion in these structural species, such as *Pocillopora* and *Acropora*, also influence the habitat and food supply of other marine organisms. Following disturbance, such as coral bleaching, the growth rate of these species is the most important factor in reef recovery (Smith, L. D., Gilmour & Heyward 2008; Wakeford, Done & Johnson 2008). Frequent disturbance has been shown to result in phase shifts to macro-algal and soft coral-dominated communities (Done 1992) and as such, the growth rate of hard coral species is important in preventing these phase shifts. If changing symbiont genotype following bleaching affects calcification rate of these species then this could influence the community structure on coral reefs. In the context of the annual bleaching events that are

predicted to occur on reefs within the next 30-50 years (Done *et al.* 2003; Donner *et al.* 2005), symbiont community shifts to more thermally tolerant genotypes like *Symbiodinium* type D that could potentially affect coral growth, may compromise the competitiveness and survival of hard-coral dominated reef communities.

This study investigates one of the most important elements of reef resilience to climate change, namely growth. The coral species, *A. millepora* was chosen for this study as this is an abundant and dominant reef-builder on the leeward shores of islands in the Keppel region of the Great Barrier Reef (van Woesik 1991). Colonies on these reefs naturally host either thermally-sensitive type C2, thermally-tolerant type D *Symbiodinium* or a combination of both types. The growth rate of colonies with either C2 or D symbionts was measured in two studies. One study took place in the field on the reef slope at Miall Island in the Keppel region. To support the results of the field study, the second study took place under controlled laboratory conditions at two temperatures (23 °C and 29°C). These temperatures represent the average stressful summer and non-stressful spring/autumn temperature ranges for corals at this site. The explants used in the laboratory experiment were sourced from the same reef flat at Miall Island. The field experiment was repeated opportunistically following a natural bleaching event in February 2006 to further investigate the complication of bleaching on the growth differences between C2 and D corals. The results indicate that the growth rate of *A. millepora* is significantly affected in hosts containing the thermally tolerant symbiont type compared to the sensitive type but that bleaching confounds any benefits of symbiont type.

MATERIALS AND METHODS

Laboratory growth experiment

Collection and maintenance of corals

In March 2005, 16 colonies of the Indo-Pacific stony coral *A. millepora*, Ehrenberg, 1834, with known *Symbiodinium* type C2 or type D were transplanted from the Keppel Islands region (a cool, clear southern inshore section of the Great Barrier Reef) to Magnetic Island (central Great Barrier Reef, ~800 km north of Keppel). Corals were kept for a period of three months at Magnetic Island to allow recovery from transplantation prior to the experiment. Corals were kept on wire mesh racks at approximately the same depth that they were collected.

In May 2005, the colonies were removed from the racks at Magnetic Island and transported to the Australian Institute of Marine Science (AIMS) where they were kept in outdoor aquariums for approximately three weeks in tanks of filtered and aerated seawater before the growth experiment.

Experimental protocol

Six explants were cut from each of the 16 colonies (9 colonies with rDNA ITS1 type C2 and 7 colonies with ITS1 type D *Symbiodinium*) and distributed randomly and equally between three tanks (treatment replicates) within each of two temperature treatments (23 °C and 29 °C). Seawater in the tanks was heated to the target

temperatures [23 °C and 29 °C ± 1 °C, (mean ± S.D.), averaged and logged at 1 min intervals] and kept aerated and circulated by water pumps flowing at ~1000 l h^{-1}. Coral explants were fixed to plastic stands with a cyanoacrylate-based adhesive (Loctite 454™ super glue gel) and then placed on elevated rotisseries. Light was provided by 10 x 400 W metal halide lamps (10,000 °K colour temperature, BLV Germany) with a spectral quality suitable for coral photosynthesis. Each rotisserie was turned 180° twice daily to ensure even exposure to light and water flow. Corals were gradually acclimated for 10 days to light conditions in the tanks at the treatment temperatures. For the duration of the experiment, corals were supplied with 3.5 hours of shaded light (30-36 µmole photons m^{-2} s^{-1}) followed by 5 hours of un-shaded light (87-107 µmole photons m^{-2} s^{-1}), followed by another 3.5 hours of shaded light and 12 hours darkness each day to approximate their natural diurnal light cycle.

To monitor the health of explants with respect to the laboratory conditions, the dark-adapted maximum quantum yield of each explant was determined every second day by measuring F_v/F_m with a mini-PAM fluorometer (Heinz Walz, Germany) at the same time each morning after 8 hours of darkness. Reductions in F_v/F_m indicate photo-damage and/or photo-inhibition (Hoegh-Guldberg, O. & Jones 1999; Jones, R. J., Kildea & Hoegh-Guldberg 1999; Jones, R. J. *et al.* 2000; Krause & Weis 1991; Ralph, P. J., Gademann & Larkum 2001; Ralph, P. J. *et al.* 1999; Warner, M. E., Fitt & Schmidt 1996). Measurements were made with the tip of the fibre-optic probe touching the base of the explant surface on a vertical plane. Light measurements were

taken with a Diving-PAM fluorometer (Heinz Walz, Germany) photosynthetically active radiation (PAR) sensor 5 cm underwater just above the coral explants.

The predominant *Symbiodinium* type in the colonies was verified just before the start of the experiment using SSCP analysis of the Inter-transcribed Spacer Region 1 (ITS1) of algal nuclear ribosomal DNA as described in Chapter 1 and Appendix I. Only colonies with intense SSCP bands representing type C2 and type D (EU189443, EU1894505) were chosen for the study although the presence of other types below 5% abundance is not ruled out (Fabricius *et al.* 2004).

Buoyant weight determination

Coral explants were weighed to three decimal places at the end of each week for 4 weeks to determine equivalent skeletal buoyant weight using the methods described in Jokiel *et al.* (1978).

Zooxanthellae densities and pigments

To determine the influence of zooxanthellae densities and algal pigment concentrations on coral growth, explants were snap-frozen in liquid nitrogen and stored at -20 °C immediately following the experiment. Zooxanthellae densities and chlorophyll *a* and c_2 content of each coral explant were determined using the methods described in Chapter 3 and Appendix II.

Field study

In March 2004, 43 pieces (15-20 cm) of *A. millepora* colonies of known genotype from the Keppel region were cut from larger colonies from the reef flat and pruned to approximately similar sizes (Elahi & Edmunds 2007). Due to the low abundance of type D colonies at Miall Island when the study began, thirty six C2 colonies and only five D colonies were included in the first of two field growth experiments. Initial buoyant weight measurements were made on the coral colonies in March 2004. Buoyant weight measurements (to the nearest gram) were performed by carefully transporting the colonies underwater to the weighing equipment a few 100 m from the study site. Colonies were carefully transported back to the study site after the buoyant weight measurements were completed and secured with plastic cable ties onto wire racks 75 cm above the sea bed at a depth of 3-4 m. Buoyant weight measurements were repeated seasonally every three months for a total of 9 months at the end of autumn (March to June 2005), winter (June to September 2005) and spring (September to December 2005).

The field growth experiment was repeated for another 12 months commencing in August 2006, six months after a bleaching event in February 2006. Only seven of the original set of C2 colonies survived the bleaching and recovered with their original C2 symbionts at the start of the second growth study. These seven C2 colonies and 15 type D colonies were initially placed on the growth racks for the second study. Colonies were weighed every three months at the end of spring (August to November 2006), summer (November to January 2007), autumn (January to May 2007) and

winter (May to August 2007). However, by the end of the study, nearly all of the 22 colonies on the racks had undergone some change in symbiont proportions; gaining C2, D, or another thermally tolerant type, C1. The dynamic nature of the symbiont community after bleaching made a comparison of the growth rate of colonies as a function of symbiont genotypes difficult. Nevertheless, results from the second field experiment are included because they provide an insight into the overall growth performance of *A. millepora* pre-and post bleaching.

STATISTICAL ANALYSIS

Laboratory study

The weekly growth rates of the explants in the laboratory study were expressed as a percentage of the initial buoyant weight of the explant. To examine overall growth (buoyant weight in week four – buoyant weight in week one), measurements were analysed with a nested ANOVA using type (fixed, two levels), temperature (fixed, two levels), tank (random, three levels, nested within temperature) as factors in the model. There were no significant differences between growth in the treatment tanks and data were averaged across the three tanks and the model re-run with an orthogonal ANOVA model using the fixed factors symbiont type and temperature. Unstandardised predicted values and standardised residuals were used to check the assumptions of normality. Levene's test was used to verify homogeneity of variances.

To examine trends over time, the weekly growth rates were analysed with a repeated-measures ANOVA using type and temperature as fixed factors and week (four levels) as the repeated measure in the analysis. Weekly growth was expressed as the daily buoyant weight gain at the end of each week of the study as a percentage of the initial buoyant weight of the explant at the start of the study. Mauchly's test for sphericity was used to verify the assumptions of sphericity (Huynh & Feldt 1970) and Box's M test was used to verify the homogeneity of covariance matrices (Anderson 1958). Unstandardised predicted values and standardised residuals were used to check the assumptions of normality. Levene's test was used to verify homogeneity of variances. Simple pair-wise comparisons were performed to further investigate significant differences among type and temperature treatment groups using Sidak's adjustment for multiple comparisons (Weinberg & Abramowitz 2002).

To examine their influence on explant growth, data for zooxanthellae densities and chlorophyll *a* and c_2 concentrations in the laboratory experiment were analysed with multivariate ANOVA's using type (two levels) and temperature (two levels) as fixed factors in the models and tank (three levels) as a random factor nested within temperature. Unstandardised predicted values and standardised residuals were used to check the assumptions of normality. Levene's test was used to verify homogeneity of variances. Zooxanthellae densities and chlorophyll *a* and c_2 values were aggregated across all three treatment tanks. Zooxanthellae densities and algal cell chlorophyll *a* and c_2 concentrations were examined with an orthogonal multivariate ANOVA using temperature (two levels) and type (two levels) as fixed factors in the analysis.

Field study

To examine growth variation of *A. millepora* colonies with respect to symbiont type in the first field study, before the bleaching, a one-factor ANOVA was performed on the weekly growth rates using symbiont type as the fixed, predictor variable (two levels). Growth for each colony was expressed as the weekly buoyant weight gain as a percentage of the initial buoyant weight of the colony at the start of the study. The assumptions of normality were verified using plots of the unstandardised predicted values by the standardised residuals and Levene's test was used to verify the homogeneity of variances. Data were natural-log transformed to improve the normality of the distribution.

To examine the seasonal variations in growth of *A. millepora* colonies with different symbionts types in the first field experiment, data for the weekly buoyant weight gain during each three-month season were analysed with a repeated-measures ANOVA using symbiont type as the fixed variable (two levels) and season (three levels) as the repeated measure in the model. The growth rate was expressed as the weekly buoyant weight gain of each colony in the study over the three month season as a percentage of the initial buoyant weight of the colony. The model residuals were examined to verify the validity of the assumptions of normality and Levene's test was used to verify the homogeneity of variances. Data were natural-log transformed to improve the normality of the distribution.

To examine the variation in seasonal growth of *A. millepora* colonies as a result of the bleaching event in early 2006, the weekly growth rates for each season in the two studies were analysed with ANOVA using the fixed factor bleaching (before or after bleaching), and the random factor season (three levels) as predictor variables. The growth rate was expressed as the weekly buoyant weight gain as a percentage of the initial buoyant weight of the colony. The assumptions of normality were verified using plots of the unstandardised predicted values by the standardised residuals. Levene's test was used to verify the homogeneity of variances. Data were natural-log transformed to improve the normality of the distribution. Simple pair-wise comparisons were performed to further investigate significant differences in growth using Sidak's adjustment for multiple comparisons (Weinberg & Abramowitz 2002). All statistical tests were completed using SPSS Version 15.0.

RESULTS

Laboratory study

The growth rate of *A. millepora* explants in the laboratory experiment varied significantly with the predominant symbiont genotype. There was no significant effect of treatment temperature on explant growth. The weekly buoyant weight gain of explants with type D symbionts, averaged over the temperature treatments for the entire four weeks of the study, was 29% less than that gained by C2 explants ($p < 0.05$, Figure 3.1, Table 3.1, 3.2). There was no significant interaction between symbiont type and treatment temperature.

Table 3.1. ANOVA result showing the significant effects of the predictor variable symbiont type (C2, D) on the weekly buoyant weight gain of *Acropora millepora* explants during a four-week laboratory study. There was no significant effect of treatment temperature and no interaction between type and temperature.

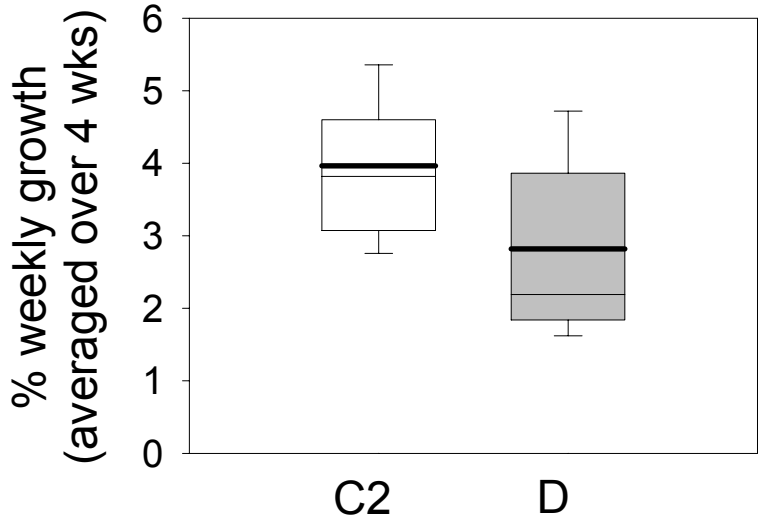
	SS	df	Mean Square	F	Sig.
Corrected Model	47.69[a]	3	15.90	8.3	0.000
Intercept	1449.72	1	1449.72	760.1	0.000
type	41.17	1	41.17	21.6	0.000
temperature	4.30	1	4.30	2.3	0.136
type * temp	3.01	1	3.01	1.6	0.211
Error	236.51	124	1.91		
Total	1819.63	128			
Corrected Total	284.20	127			

a. $R^2 = 0.168$ (Adjusted $R^2 = 0.148$)

Table 3.2. Table showing the mean and standard deviation of growth rates, zooxanthellae densities and pigment content of *Acropora millepora* explants with C2 and D symbionts in a laboratory study of growth. Growth was calculated by averaging the weekly buoyant weight gain for each of the four weeks over the entire four week study (expressed as a percentage of the initial buoyant weight of the explant at the beginning of the study).

	Predominant symbiont type/temperature treatment	Mean	S.D.	N	units
Laboratory growth	C2	3.96	0.98	9	% week^{-1}
	D	2.82	1.28	7	% week^{-1}
Zooxanthellae	C2	3.07	0.85	18	cells x 10^6 cm^{-2}
	D	2.38	0.86	14	cells x 10^6 cm^{-2}
	23°C	3.09	0.99	16	cells x 10^6 cm^{-2}
	29°C	2.44	0.72	16	cells x 10^6 cm^{-2}
Chlorophyll *a*	C2	26.21	4.85	18	µg x 10^6 cell^{-1}
	D	21.96	5.74	14	µg x 10^6 cell^{-1}
	23°C	22.16	6.21	16	µg x 10^6 cell^{-1}
	29°C	26.54	3.98	16	µg x 10^6 cell^{-1}
Chlorophyll c_2	C2	23.98	3.26	18	µg x 10^6 cell^{-1}
	D	19.90	4.44	14	µg x 10^6 cell^{-1}
	23°C	20.29	4.38	16	µg x 10^6 cell^{-1}
	29°C	24.10	3.31	16	µg x 10^6 cell^{-1}

Figure 3.1. Average weekly growth rate of *Acropora millepora* explants with type C2 symbionts was significantly higher than that of explants with type D symbionts during a laboratory experiment of buoyant weight gain. Boxes represent the buoyant weight gained over a week expressed as a percentage of the initial buoyant weight of the explant. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



In the laboratory study, there was significant variation in weekly growth rate of *A. millepora* explants and a significant interaction between week and treatment temperature (Figure 3.2, Table 3.3). At 23 °C, the growth rate of all explants was significantly lower in week one compared to weeks two, three ($p < 0.05$), and four ($p < 0.05$, Figure 3.2 a, b). At 23 °C, the growth rate of type C2 explants was significantly higher than that of type D explants in week three ($p < 0.05$) and week four ($p < 0.05$).

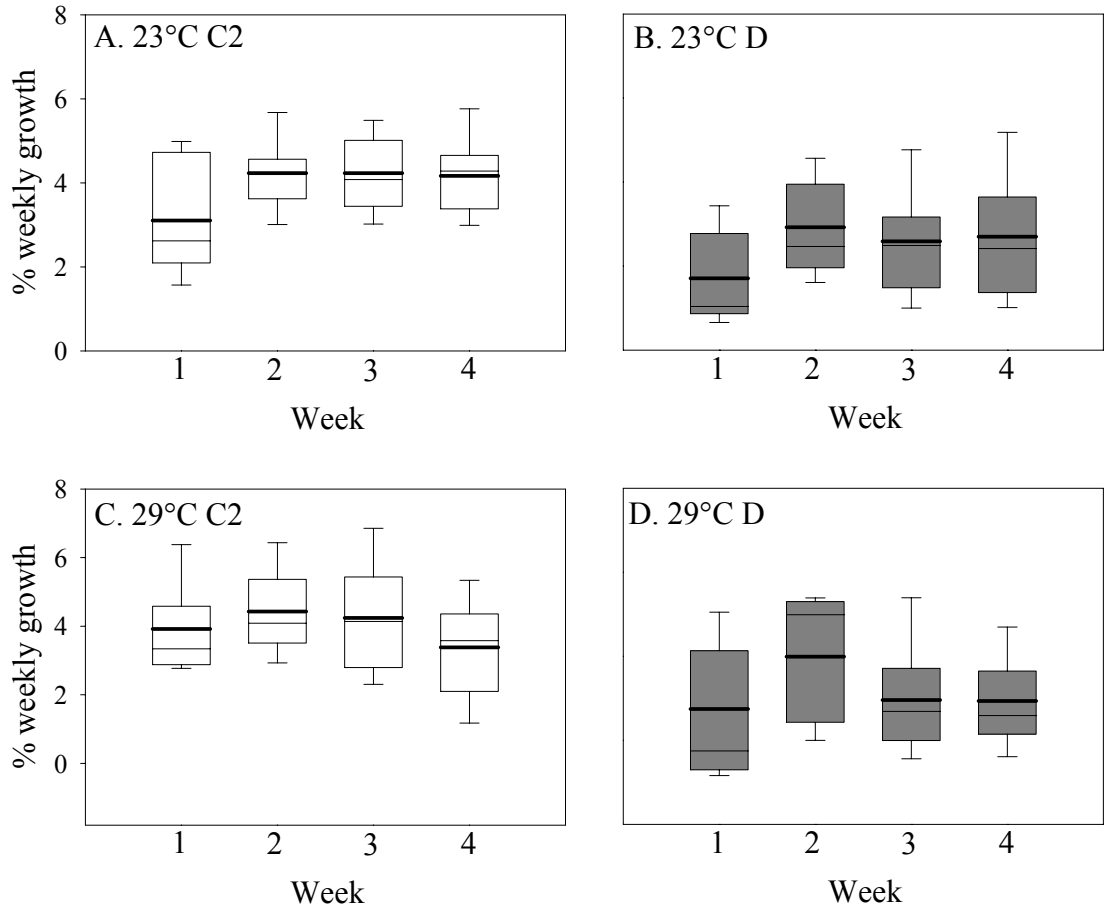
At 29 °C, the growth rate of all explants initially increased 24% between week one and week two but decreased 25% between week two and week four ($p < 0.05$, Figure 3.2 c,

d). There was no significant interaction between symbiont type and week and between symbiont type, week and treatment temperature (Table 3.3).

Table 3.3. Table showing the Greenhouse-Geisser corrected significant within-subjects effects of week (1-4) and the significant interaction between week and treatment temperature (temp, 23 °C and 29 °C) in a repeated-measures ANOVA of the weekly growth of *Acropora millepora* explants.

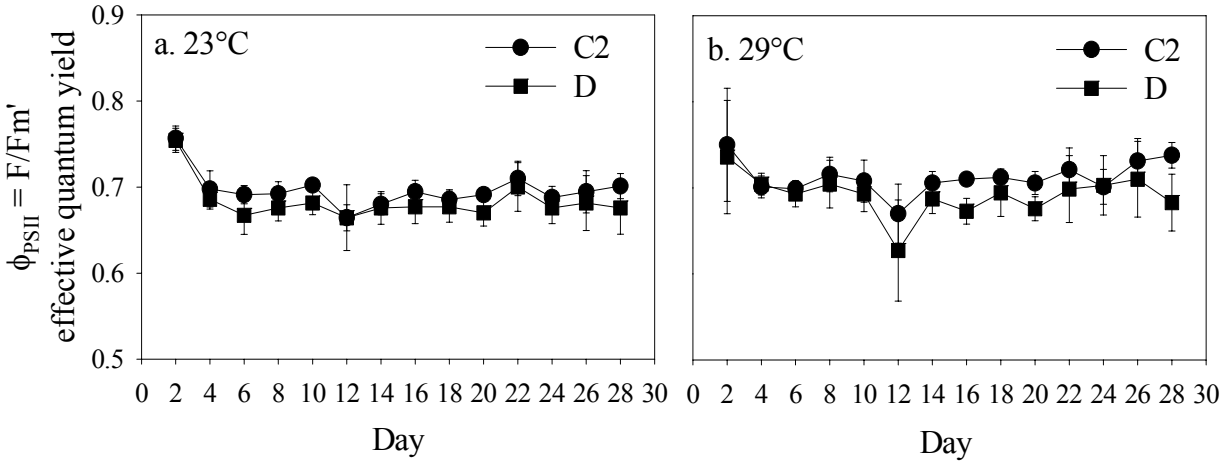
	SS	df	Mean Square	F	Sig.
week	17.18	3	5.96	12.7	0.000
week * type	1.83	3	0.63	1.4	0.263
week * temp	6.51	3	2.26	4.8	0.004
week * type * temp	0.88	3	0.31	0.7	0.576
Error (Week)	37.75	81	0.47		

Figure 3.2. In a laboratory study, the weekly growth rate of *Acropora millepora* explants varied significantly between the weeks of a four week study. (a) The weekly growth rate of type C2 explants at 23 °C. (b) The weekly growth rate of D explants at 23 °C. (c) The weekly growth rate of C2 explants at 29 °C. (d) The weekly growth rate of D explants at 29 °C. Boxes represent the weekly buoyant weight gain expressed as a percentage of the initial buoyant weight of the explant. Thick lines within the boxplots represent the mean and thin lines represent the median. Error bars above and below the boxes represent the 95th and 5th percentiles. Box boundaries represent the 75th and 25th percentiles. Missing and out of range values are not shown.



Despite the diminishing growth rate in week four at 29 °C, there was no indication of a significant reduction in F_v/F_m (Figure 3.3 a, b).

Figure 3.3. Photosynthesis measured by dark-adapted quantum yield (F_v/F_m arbitrary units) of *Acropora millepora* explants with type D and C2 symbionts did not show signs of heat stress at 29 °C in a laboratory growth experiment. (a) F_v/F_m at 23 °C and (b) F_v/F_m at 29 °C. Symbols represent the mean effective quantum yield for C2 (n=9) and D (n=7) explants. Error bars represent ± S.D.



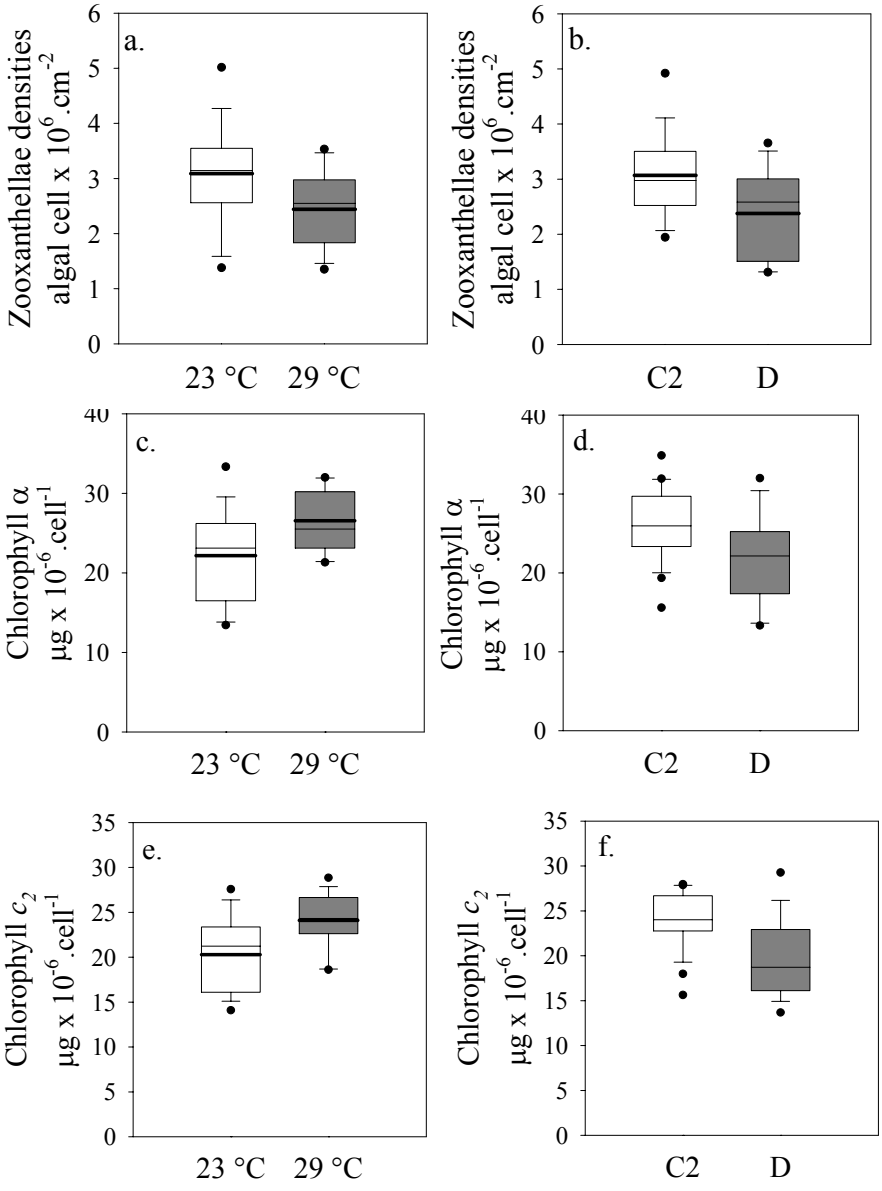
At the end of the laboratory study, zooxanthellae densities and algal cell chlorophyll *a* and c_2 all varied significantly with the predominant symbiont genotype and with treatment temperature but there were no significant interactions between the two factors for any of the three variables (Figure 3.4 a-f, Table 3.1. 3.4). Densities for type D explants (averaged across temperature treatments) were 22% lower than densities for C2 explants ($p < 0.05$). Zooxanthellae densities at 29 °C were 21% lower than densities at 23 °C ($p < 0.05$).

Table 3.4. Results of a multivariate ANOVA of zooxanthellae densities and algal chlorophyll *a* and c_2 for *Acropora millepora* explants in a laboratory study of growth showing the significant effect of symbiont type (C2, D) and temperature (23 °C and 29 °C). There were no significant interactions between the effects of temperature and type for any of the three variables.

	Dependent Variable	SS	df	Mean Square	F	Sig.
Corrected Model	Zooxanthellae	7.218[a]	3	2.41	3.6	0.025
	Chlorophyll *a*	348.151[b]	3	116.05	5.2	0.005
	Chlorophyll c_2	262.354[c]	3	87.45	8.0	0.001
Intercept	Zooxanthellae	233.38	1	233.38	349.9	0.000
	Chlorophyll *a*	18275.94	1	18275.94	822.0	0.000
	Chlorophyll c_2	15162.09	1	15162.09	1387.3	0.000
type	Zooxanthellae	3.77	1	3.77	5.7	0.025
	Chlorophyll *a*	142.50	1	142.50	6.4	0.017
	Chlorophyll c_2	130.76	1	130.76	12.0	0.002
temp	Zooxanthellae	3.21	1	3.21	4.8	0.037
	Chlorophyll *a*	174.25	1	174.25	7.8	0.009
	Chlorophyll c_2	125.34	1	125.34	11.5	0.002
type * temp	Zooxanthellae	0.07	1	0.07	0.1	0.753
	Chlorophyll *a*	51.99	1	51.99	2.3	0.137
	Chlorophyll c_2	15.07	1	15.07	1.4	0.250
Error	Zooxanthellae	18.67	28	0.67		
	Chlorophyll *a*	622.57	28	22.23		
	Chlorophyll c_2	306.02	28	10.93		
Total	Zooxanthellae	270.57	32			
	Chlorophyll *a*	19948.86	32			
	Chlorophyll c_2	16330.80	32			
Corrected Total	Zooxanthellae	25.89	31			
	Chlorophyll *a*	970.72	31			
	Chlorophyll c_2	568.38	31			

a. R^2 =0.279 (Adjusted R^2 = 0.202)
b. R^2 =0.359 (Adjusted R^2 = 0.290)
c. R^2 =0.462 (Adjusted R^2 =0.404)

Figure 3.4. Boxplots showing the significant effects of (a, c, e) treatment temperature on zooxanthellae densities and algal chlorophyll *a* and c_2 pigments, and (b, d, f) symbiont type on zooxanthellae densities and algal chlorophyll a and c_2, in *Acropora millepora* explants during a laboratory growth experiment. White boxes represent 23 °C or explants with C2 symbionts and dark grey boxes represent 29 °C or explants with D symbionts. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



Algal chlorophyll *a* and c_2 concentrations of *A. millepora* explants varied significantly with both symbiont type and temperature treatment during the laboratory study (Table 3.4, Figure 3.4 c-f). The algal cell chlorophyll *a* content of type D explants was 16% lower than for type C2 explants ($p < 0.05$). Algal cell chlorophyll c_2 for type D explants was 17% lower than for type C2 explants ($p < 0.05$). At 29 °C, the mean algal chlorophyll *a* was 20% higher than concentrations at 23 °C ($p < 0.05$). At 29 °C algal chlorophyll c_2 was 19% higher than at 23 °C ($p < 0.05$).

Table 3.5. Mean zooxanthellae densities and algal chlorophyll *a* and c_2 concentrations for type C2 and D explants of *Acropora millepora* at 23 °C and 29 °C temperature treatments during a laboratory study of buoyant weight.

a. Symbiont type	C2			D		
	Mean	N	S.D.	Mean	N	S.D.
Zooxanthellae density cells x 10^6 cm^{-2}	3.07	18	0.85	2.38	14	0.86
Chlorophyll *a* µg x 10^6 $cell^{-1}$	26.21	18	4.85	21.96	14	5.74
Chlorophyll c_2 µg x 10^6 $cell^{-1}$	23.98	18	3.26	19.90	14	4.44

b. Temperature treatment	23 °C			29 °C		
	Mean	N	S.D.	Mean	N	S.D.
Zooxanthellae density cells x 10^6 cm^{-2}	3.09	16	0.99	2.44	16	0.72
Chlorophyll *a* µg x 10^6 $cell^{-1}$	22.16	16	6.21	26.54	16	3.98
Chlorophyll c_2 µg x 10^6 $cell^{-1}$	20.29	16	4.38	24.10	16	3.31

Field study

Before bleaching

In the first field growth experiment (before the bleaching event) the weekly growth rate of *A. millepora* colonies varied significantly with symbiont type (Figure 3.5, Table 3.6, 3.7). The growth rate of D colonies was 38% lower than that of C2 colonies ($p < 0.05$).

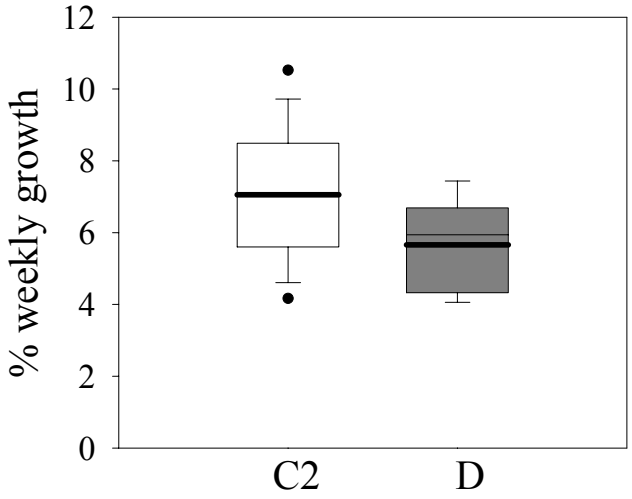
Table 3.6. Table showing the mean and standard deviation of weekly growth rates of *Acropora millepora* colonies with C2 and D symbionts in a field study of growth before a bleaching event. Growth was calculated by expressing the weekly buoyant weight gain as a percentage of the initial buoyant weight of the colony at the beginning of the study.

Predominant symbiont type	Mean	S.D.	N	units
C2	2.19	0.96	36	% $week^{-1}$
D	1.36	0.20	5	% $week^{-1}$

Table 3.7. The results of a one-way ANOVA of weekly growth of *Acropora millepora* colonies before a bleaching event showing the significant effect of symbiont type (C2 or D). Data were natural log transformed to improve the normality of the distribution.

	SS	df	Mean Square	F	Sig.
Corrected Model	0.71[a]	1	0.71	4.9	0.032
Intercept	4.43	1	4.43	30.6	0.000
type	0.71	1	0.71	4.9	0.032
Error	5.65	39	0.15		
Total	23.94	41			
Corrected Total	6.37	40			
a. $R^2 = 0.112$ (Adjusted $R^2 = 0.089$)					

Figure 3.5. Boxplots showing the significantly higher weekly growth rate of *Acropora millepora* colonies with type C2 symbionts compared to colonies with type D symbionts before a bleaching event. Boxes represent the weekly buoyant weight gain as a percentage of the initial buoyant weight of the colony. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



The growth rate of *A. millepora* explants also varied significantly with season in the year before the bleaching (Figure 3.6, Table 3.8. 3.9). Daily growth rate was higher in spring and autumn than in winter. Growth rates were 27% higher in spring than in autumn ($p < 0.05$) and 71% higher in spring than in winter ($p < 0.0.5$). Daily growth rate was 34% higher in autumn than in winter ($p < 0.05$). There was no interaction between type and season.

Figure 3.6. The daily growth rate of colonies of *Acropora millepora* varied significantly with season in the year before a bleaching event. Boxes represent the daily buoyant weight gain expressed as a percentage of the initial buoyant weight of the colony. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.

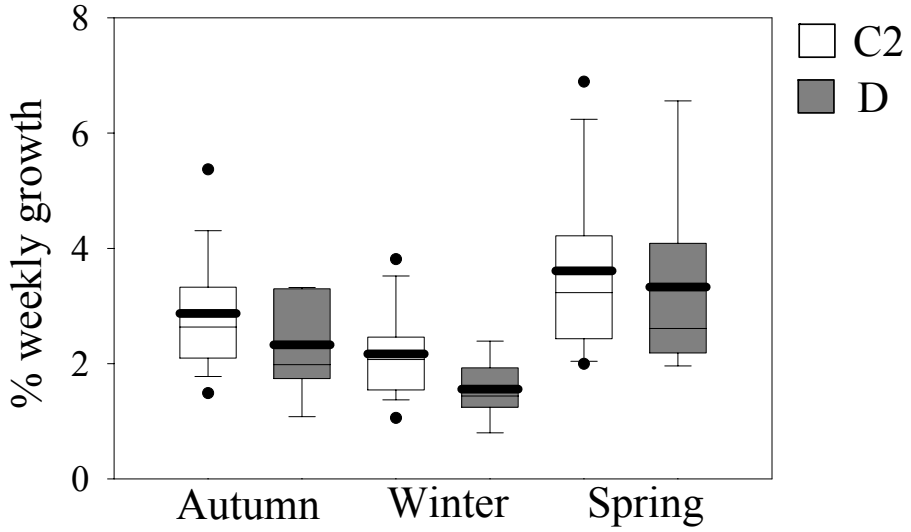


Table 3.8. Table showing the means and standard deviations of seasonal weekly growth rates of *Acropora millepora* colonies in a field study before a bleaching event. Growth was calculated by expressing the weekly buoyant weight gain as a percentage of the initial buoyant weight of the colony at the beginning of the study.

Season	Mean	S.D.	N	units
Mar - Jun (autumn)	2.80	1.09	41	% week^{-1}
Jun - Sept (winter)	2.09	0.83	40	% week^{-1}
Sept - Dec (spring)	3.57	1.54	40	% week^{-1}

Table 3.9. The results of a repeated-measures ANOVA showing the Greenhouse-Geisser corrected significant within-subjects effects of season (spring, autumn and winter) on the % weekly buoyant weight gain of colonies of *Acropora millepora* in the year before a bleaching event.

	SS	df	Mean Square	F	Sig.
season	3.21	2	1.61	34.6	0.000
season * type	0.11	2	0.06	1.2	0.311
Error(season)	3.43	74	0.05		

Bleaching effects on field growth

The bleaching event in early 2006 severely affected *A. millepora* growth rates irrespective of symbiont genotype (which correlated with bleaching severity). In the second experiment after the bleaching event, colonies gained only half of the weekly buoyant weight gain of colonies before the bleaching ($p < 0.05$, Figure 3.7, Table 3.10, 3.11).

Table 3.10. Table showing the mean and standard deviation of weekly growth rates of *Acropora millepora* colonies in a field study of growth before and after a bleaching event. Growth was calculated by expressing the weekly buoyant weight gain as a percentage of the initial buoyant weight of the colony at the beginning of the study.

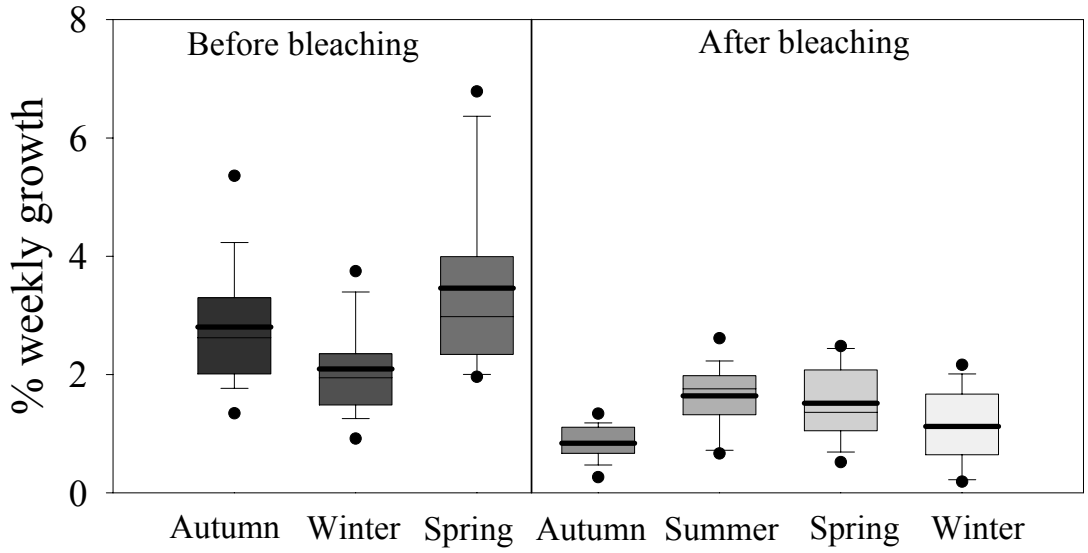
Overall growth rates		Mean	S.D.	N	units
	Before bleaching	2.79	1.37	122	% week^{-1}
	After bleaching	1.28	0.64	100	% week^{-1}
Symbiont type		Mean	S.D.	N	units
C2	C2 before bleaching	2.85	1.36	107	% week^{-1}
	C2 after bleaching	0.84	0.35	15	% week^{-1}
D	D before bleaching	2.40	1.39	15	% week^{-1}
	D after bleaching	1.35	0.58	27	% week^{-1}
Seasonal growth rates		Mean	S.D.	N	units
Before bleaching	Autumn before bleaching	2.80	1.09	41	% week^{-1}
	Spring before bleaching	3.46	1.69	41	% week^{-1}
	Winter before bleaching	2.09	0.83	40	% week^{-1}
After bleaching	Autumn after bleaching	1.52	0.65	25	% week^{-1}
	Summer after bleaching	1.64	0.61	25	% week^{-1}
	Spring after bleaching	0.84	0.30	25	% week^{-1}
	Winter after bleaching	1.12	0.64	25	% week^{-1}

Table 3.11. The results of a univariate ANOVA showing the significant effects of bleaching (before or after bleaching) and season (spring, autumn, winter) and the significant interaction between the two variables on the daily buoyant weight gain of *Acropora millepora* colonies during a two year study.

		SS	df	Mean Square	F	Sig.
Corrected Model	Hypothesis	52.57[a]	6	8.76	39.5	0.000
Intercept	Error	56.25	1	56.25	253.5	0.000
season	Hypothesis	7.23	3	2.41	10.9	0.000
bleaching	Error	41.96	1	41.96	189.1	0.000
season * bleaching	Hypothesis	5.84	2	2.92	13.2	0.000
Error	Error	47.48	214	0.22		
Total	Hypothesis	168.16	221			
Corrected Total	Error	100.05	220			
a. $R^2 = 0.525$ (Adjusted $R^2 = 0.512$)						

Because of the low number of type C2 colonies left by the end of the second field experiment and the shifting background population of symbiont types it was impossible to conduct a robust statistical analysis of differential effects of hosting type C2 or D symbionts. However, the overall daily growth rate of all the colonies in the second experiment (after bleaching) was 47% lower than the lowest growth rate (that of type D colonies) in the first experiment (Table 3.10)

Figure 3.7. The weekly growth rate of *Acropora millepora* colonies was severely affected by a bleaching event in January/February 2006. Boxes represent the weekly buoyant weight gain expressed as a percentage of the initial buoyant weight of the colony. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



The growth of *A. millepora* colonies followed a similar seasonal pattern as colonies in the first study before the bleaching. The highest growth rate in 2006 was in spring, six months after the bleaching event, 76% lower than the spring 2005 before the bleaching ($p < 0.05$). Growth rate in autumn 2006 (12 months after the bleaching) was 46% lower than in autumn 2005 before bleaching ($p < 0.05$). The winter 2006 growth rate (nearly 18 months after the bleaching), was 47% lower than the growth rate in the winter 2005 before the bleaching ($p < 0.05$). The highest growth rates of *A. millepora*

colonies after the bleaching event were in spring and the lowest growth rates were in autumn and winter.

DISCUSSION

The growth rate of reef-building corals is likely to be significantly compromised by two separate and independent processes with acclimation to a warmer climate. Firstly, growth will be compromised by symbiont genotype as corals shuffle to more thermally tolerant types, trading-off reduced growth for increased resistance. The second process is the significant affect of the bleaching stress itself on coral growth. A significant community shift from thermally sensitive type C2 to thermally tolerant types D and C1 symbionts occurred in *A. millepora* colonies at Miall Island in the southern Great Barrier Reef following severe bleaching in 2006 (Chapter 1). Before the bleaching, colonies with predominantly type D symbionts had significantly lower growth rate than colonies with type C2 symbionts. Under normal conditions, this shift is likely to have caused ~38% lower growth in surviving colonies with significant levels of type D symbionts. However, the affects of the bleaching event itself far out-weighed the effects of symbiont genotype, reducing growth in all colonies by 56% of the growth rate in the year before the bleaching. Even in corals with type D symbionts, that were apparently unaffected by heat stress at the peak of the bleaching event, growth was significantly reduced for at least 18 month after the bleaching event. It is unknown how long this stress event continued to impact coral growth as the experiment was terminated before there was any sign of recovery to pre-bleaching levels.

Studies of scleractinian corals have previously found that bleaching can effect coral growth up to a year after bleaching. Baird and Marshall (2002) found considerable variation in the growth (percentage gain in colony area) of severely bleached *A. millepora* colonies, independent of the severity of the bleaching. While moderately bleached colonies grew ~20%, severely bleached colonies either remained the same size or shrank over a six week period following a natural bleaching event. Mendes and Woodley (2002) compared skeletal extension rates in *Montastraea annularis* after a bleaching event in 1996 and found that severely bleached colonies had ~80% lower skeletal extension rates during the bleaching but rates had recovered within a year of the event. Mendes and Woodley *et al.* concluded that bleaching had no long-term effect on the skeletal extension rates of *Montastraea annularis*. In contrast, Leder *et al.* (1991) found that both bleached and unbleached *Montastraea annularis* had between 66% and 98% reduced skeletal extension rates after recovery in the year following a bleaching. While it is difficult to compare buoyant weight gain to skeletal extension or colony area, these studies confirm that severe bleaching can have a debilitating effect on coral growth rate up to a year and possibly longer following recovery. The relatively minor differences in growth of *A. millepora* that were related to symbiont identity were therefore far outweighed by the larger and longer-term effect of heat stress. Following the 2006 bleaching at Miall Island, there was also evidence of a shift back to thermally sensitive C2 symbionts in the colonies within 18 months of the bleaching (Jones, A. M. *et al.* 2008). In this case, the effects of bleaching are therefore expected to persist even longer than symbiont community change, suggesting that the growth differences that are a result of symbiont identity are transitory and relatively minor in comparison to the longer term effects of heat damage to cells and

photosystems. However, if climate change causes repeated anomalously warm summers, type D symbionts could become more widespread and permanent on reefs, resulting in the additive effects of these two processes acting to depress coral growth possibly for several years.

In terms of symbiont effects on growth, one possible explanation for the lower growth of type D compared to type C2 *A. millepora* under normal conditions may lie in the photokinetics of the symbionts. In Chapter 2, rapid light curves (RLC's) were used to show that *A. millepora* branches with type D symbionts have 41% lower maximum relative rate of electron transport through PSII and 38% lower light absorption capacity compared to type C2 corals. There was also a 33% anomaly in dark-adapted F_v/F_m between corals with type D and C2 symbionts. While the exact correlation between reduced photosynthetic function and increased heat tolerance is still unclear, one of the key mechanisms involves the composition and fluidity of the thylakoid membranes that house the photosystems (Chuartzman *et al.* 2008; Tchernov *et al.* 2004). Because these mechanisms in plants (Hugly *et al.* 1989; Vigh *et al.* 1985) and micro algae (Sato *et al.* 1996) are linked to reduced growth, it is highly likely that the lower electron transport rate and light absorption capacity of type D photosystems may be the cause for the 38% lower growth in the field under normal conditions. Lower photosynthetic function may help type D symbionts to cope with excess electrons and reactive oxygen species during heat stress, thereby maintaining normal photosynthetic function. However, the growth differences found in the field and laboratory in this study and

those of Little *et al.* (2004) suggest that heat tolerance comes at a cost to growth rates even at normal temperatures.

Corals with thermally tolerant symbionts like *Symbiodinium* type D, may still suffer the effects of heat stress, which can override the positive effects of retaining the symbionts during the bleaching. There are a number of possible explanations for the reduced growth rate of corals with type D symbionts that did not show signs of bleaching. The first of these is that the photosynthetically fixed carbon from intact type D symbionts is not made available to their hosts for skeletal growth. This concept of type D symbionts as 'greedy' partners under stressful conditions (Mark Warner, personal communication) may explain why *A. millepora* colonies with predominantly type D symbionts, in spite of appearing unbleached, had significantly lower growth in the year after the bleaching event. It is possible that surviving stress tolerant symbiont genotypes retain a greater portion of their photosynthetically fixed carbon following stress, thereby ensuring their survival (and that of their hosts) but effectively starving the coral. This stems from the fact that a greater proportion of autotrophic carbon, fixed in the Calvin Cycle during photosynthesis, may be diverted to symbiont and coral cell metabolism. The overall reduction in organic carbon affects the coral, which is supported by the lower calcification rates seen in bleached corals. This would not occur under non-stressful conditions (i.e. before the bleaching) during which photokinetics remain a more likely explanation for the observed growth differences. A second explanation may be that in spite of a normal (although lower) rate of algal photosynthesis, *A. millepora* with type D symbionts had increased respiration as a

result of the warmer conditions during the summer bleaching. During summer, the respiratory demand of reef corals has been shown to more than double, increasing the metabolic demands and thereby reducing the amount of energy available for skeletal growth (Fitt *et al.* 2000). The increased respiratory demand would have occurred in both bleached and unbleached corals, resulting in reductions in growth in both C2 and D corals, irrespective of symbiont losses. The third explanation may be that in spite or retaining their symbionts, corals with type D symbionts had reduced photosynthesis. At high temperatures (e.g. 32 °C), type D *Symbiodinium* has been shown to undergo photoinhibition (protective and reversible) which mimics a 30% decrease in habitat irradiance (Rowan 2004a). Diversion of photon energy via photoprotective processes, reduced capacity to absorb incident light through difference in host tissue optical properties (Apprill, A., Bidigare & Gates 2007), lower symbiont numbers or pigment concentrations or reduced electron transport could be mechanisms to cope with heat stress on the photosystems as temperature can damage the algal cell's capacity to repair proteins (Takahashi *et al.* 2004). Finally, type D symbionts may export lower amounts of photosynthates in the presence of host factor compared to the more dominant symbiont type C2 (Stat, Michael, Morris & Gates 2008). It is highly likely that a combination of these mechanisms may cause the loss of skeletal growth in type D symbionts. What is clear is that in spite of increasing the heat tolerance of *A. millepora*, hosting thermally tolerant type D *Symbiodinium* does not protect the coral from the effects of the bleaching itself.

The growth differences of adult *A. millepora* with symbionts of contrasting thermal tolerance were not as dramatic as those found for juveniles of this species. Little *et al.* (2004) found a 200-300% growth difference in juvenile *A. millepora* with type D compared to C1 symbionts. Three factors may be responsible for the dissimilarity in growth differences in the two studies. The first two factors may be the effect of isometric scaling with the size of the subject (Leuzinger, Anthony & Willis 2003) and the age of the coral tissues (Elahi & Edmunds 2007). As tissues age, cell senescence causes a reduction in the proportion of the coral's energy allocated to growth and an increase in the energy allocated to reproduction. This means that the growth of older, larger colonies will partition less of their resources into growth than smaller, younger colonies. Coral recruits, such as those investigated in the study by Little *et al.* (2004), invest all their energy in tissue and skeletal growth in the absence of any reproductive effort. The third rationale may lie in the identity of the symbionts compared in each study. Little *et al.* (2004) compared *A. millepora* juveniles with type D to those with C1, whereas in the present study, adult corals with type D symbionts were compared to those with type C2 symbionts. The growth differences between adult *A. millepora* and juveniles of this species (Little, van Oppen & Willis 2004) may assume more parity in the context of these factors. A further factor may lie in the effects of environmental variables which can influence growth rates in the field (Cruz-Piñón, Carricart-Ganivet & Espinoza-Avalos 2003) as Little *et al.* (2004) studied growth at Magnetic Island whereas the present study took place at Miall Island, 800 km south of Magnetic Island (along-shelf distance). Irrespective of these factors, the range of growth values found between and within studies (Baird & Marshall 2002) indicate that further studies are

required before it will be possible to fully quantify the effects of symbiont genotypes on growth in the field as corals acclimatise to climate change.

Some of the growth differences in *A. millepora* explants in the laboratory study can be explained by the lower symbiont densities of type D explants. However, as other studies of reef corals have failed to find a correlation between *Symbiodinium* genotype and zooxanthellae densities (Chapter two and Chapter four, Chen *et al.* 2005), a significant proportion of the growth anomaly (~13% at 23 °C) can be attributed solely to symbiont genotype. This was confirmed by re-analysing the laboratory growth data after standardising the percentage weekly growth rate for all explants by zooxanthellae densities (Table 3.12). The model results were consistent with the unstandardised growth data, confirming the link between symbiont identity and growth.

Table 3.12. The results of a repeated-measures ANOVA showing the significant within-subjects effects (sphericity assumed) of symbiont type (C2, D) and temperature (23 °C or 29 °C) on the weekly percentage buoyant weight gain of *Acropora millepora* explants after standardising to zooxanthellae densities.

	SS	df	Mean Square	F	Sig.
Week	3.02	3	1.01	11.8	0.000
week * type	0.69	3	0.23	2.7	0.050
week * temp	0.96	3	0.32	3.8	0.014
week * type * temp	0.15	3	0.05	0.6	0.626
Error (week)	7.16	84	0.09		

In the field, there was evidence of a seasonal effect on coral growth. The growth of *A. millepora* colonies peaked during spring and autumn in the year before the bleaching event and in spring the year after the bleaching. Similar seasonal growth patterns were

demonstrated in a study of *A. formosa* at Davies Reef (Great Barrier Reef) by Oliver *et al.* (1983). Zooxanthellae numbers and chlorophyll levels in reef corals typically reach their annual maxima during spring and autumn when temperatures are less stressful (Fagoonee *et al.* 1999) resulting in an increase the amount of energy available to drive calcification. During the stress of summer temperature and light conditions in the field, extra demand is placed on the energy reserves by up to a two-fold increase in the coral's respiratory metabolism (Fitt *et al.* 2000; Muller-Parker 1987a). Symbiont densities and photosynthetic function are also depressed by heat and light stress in the field during summer (Jones, R. J. *et al.* 1998). Temperatures at Miall Island regularly exceed 29°C in summer and may fall as low as 16°C in winter (http://www.aims.gov.au/pages/facilities/adc/seatemps.html). These temperatures fall outside the optimal range 26°C to 29°C for calcification in scleractinian corals (Clausen & Roth 1975; Coles & Jokiel 1978; Jokiel & Coles 1990). However, seasonal changes in light and temperature swamp the relatively minor differences in coral growth that can be attributed to symbiont genotype. In the absence of heat stress in the field (i.e. in winter) there was a significant effect of symbiont genotype on coral growth, but in summer the difference was probably overwhelmed by the increased respiratory demand and reduced zooxanthellae densities that accompany increased temperature and light, acting to reduce the amount of energy available for calcification (Barnes & Chalker 1990).

The relative differences in growth rate of *A. millepora* in the field and the laboratory (nearly double) are likely to be a result of a complex interaction of influences such as

light, morphology and changes in heterotrophic feeding behaviour on calcification rates. Corals actively feed on zooplankton and particulate matter to supplement photosynthetically derived carbon in the field (Porter 1976). A study by Anthony *et al.* (2000) has shown that some corals can switch from autotrophy to heterotrophy under certain conditions. Theoretically at least, increased heterotrophy in the field, where zooplankton and particulate matter are available, should reduce incorporation of the heavier carbon isotope ^{13}C into the coral skeleton because zooplankton and particulate matter are lower in $\delta^{13}C$ (ratio ^{13}C:^{12}C relative to Vienna Peedee Belemnite Limestone Standard) than seawater (Grottoli & Wellington 1999). Increased autotrophy increases skeletal buoyant weight because the carbon pool becomes enriched with the ^{13}C isotope as a result of calcification, thereby increasing skeletal $\delta^{13}C$ (Grottoli 2000; Grottoli & Wellington 1999). In the field, corals are expected to have lower rates of buoyant weight gain compared to the laboratory, where heterotrophy is partially reduced because they are supplied with filtered seawater which has comparatively low $\delta^{13}C$ due to the absence of zooplankton. This shift between heterotrophy and autotrophy may help to explain the ~20% lower growth of *A. millepora* colonies in the field (before the bleaching) compared to laboratory growth. However, the light levels at Miall Island (~400 μmole photons m^{-2} s^{-1}, average midday irradiance over three days in autumn 2006) were four times higher than the light levels in the laboratory experiment (~100 μmole photons m^{-2} s^{-1}). As light enhances calcification rate in reef corals (Reynaud-Vaganay *et al.* 2001) this should have increased the growth rate of corals in the field relative to those in the laboratory. There are two possible explanations for this apparent paradox. The first is that high light levels during the daylight hours in the field at Miall may have inhibited photosynthesis, thereby limiting

the enhancing effects of high light on calcification in the (Hoogenboom, Anthony & Connolly 2006). The second explanation may be that the explants in the laboratory experiment were of simpler morphology than the more complex colonies used in the field experiment which could act to increase the amount of light reaching the tissue surface (Helmuth, Timmerman & Sebens 1997) or even alter the light environment within particular polyps (Kühl *et al.* 1995). The more complex morphology of the *A. millepora* colonies used in the field experiment could result in shading of some polyps from light. Two other factors may act to reduce calcification in the field in spite of higher light levels. The first of these is the availability of nitrogen as a stimulus for algal cell growth. Increases in ambient nitrogen have been shown to enhance calcification by increasing the density of zooxanthellae in tissues (Grottoli 2002). However, zooxanthellae densities of the laboratory explants ranged between 2.5–4.0 cells cm^{-2} (23 °C) which correlate well with those measured in the field at Miall during autumn under similar temperature conditions (Chapter 4). Therefore the most likely explanation for the ~17% lower weekly growth rate of corals in the field compared to those in the laboratory at 23 °C is that the field rates were an average of the seasonal rates. Growth rates in the field incorporate both high winter growth rates and lower summer growth rates. When compared to the weekly growth rate of *A. millepora* colonies in spring (when temperatures were most similar to those in the laboratory), laboratory growth rates at 23 °C assume greater parity to field rates [3.52 ± 1.57 cf. 3.42 ± 1.34 % $week^{-1}$ respectively, (mean ± S.D.)].

This study has provided some insights into the processes that affect the growth rate of reef corals and their magnitude. Some of these are likely to have implications to the future resilience and regeneration capacity of reefs. However more work is required to determine how applicable these effects are to other coral/algal associations and localities. The results of the field studies suggest that symbiont genotype will affect the growth rate of some reef corals, and that this will be compounded by the long-term effects of severe heat stress on these corals if they survive. Predictions of annual bleaching events within the next 30-50 years could result in more frequent disturbances which have the potential to shift the community composition of some reefs from hard-coral to macro-algae and soft coral-dominated communities (Done 1992). Some of the most structurally important scleractinian corals may be able to acclimatise to gradually warmer waters by hosting thermally tolerant symbionts, (Mieog, J. C. *et al.* 2009; Mieog, J C *et al.* 2007) but, the pressures of annual heat stress, ocean acidification and permanent symbiont changes on growth may act synergistically in compromising the competitiveness of these species to recover and compete between events.

SUMMARY

The growth rate of reef-building corals is likely to be significantly compromised by acclimation to warmer conditions by two parallel processes. In the first, the growth rate of reef coral that can host multiple symbiont types will be affected by changing to more thermally tolerant types like *Symbiodinium* D. In the second process, the growth rate of reef corals that survive bleaching will be severely affected by the bleaching event itself that caused the symbiont change in the first place. These two process may be additive if temperature increases at the rate of current predictions (Meehl *et al.* 2007), causing bleaching to become an annual event (Donner *et al.* 2005; Hoegh-Guldberg, O. *et al.* 2007), and symbiont changes become permanent and widespread. The results of this study provide some insights into one of the trade-offs of coral acclimation to climate change and may help to predict how annual bleaching events will impact the capacity of reef corals that survive to recover in between events.

Chapter 4

PHYSIOLOGICAL RESPONSES OF *ACROPORA MILLEPORA:* MACROMOLECULAR COMPOSITION, REPRODUCTION AND BLEACHING

A photograph showing a severely bleached *Acropora millepora* colony at Miall Island in the Keppel Islands region of the southern Great Barrier Reef. Colonies initially showed brilliant colourful 'fluorescent' pigments before losing these after two weeks at >29 °C in February 2006. Photography courtesy of the Courier Mail.

ABSTRACT

Some reef corals have the capacity to acclimatise to climate change by shuffling the levels of symbiont types in their tissues. Whole coral communities can become less susceptible to temperature stress by hosting significant levels of zooxanthellae like type D *Symbiodinium*. However, the mechanisms for thermal tolerance in the symbiont may have detrimental effects on the physiology of the host coral, which could result in compromising the fitness and regeneration capacity of the holobiont (coral + zooxanthellae + bacteria). This study investigates four key indicators of reef health in colonies of *A. millepora* in the Keppel Islands region of the Great Barrier Reef that normally host thermally tolerant *Symbiodinium* ITS1 type D or thermally sensitive type C2 or a combination of both types. Colonies of this species at Miall Island were shown to 'shuffle' temporarily to type D and C1 following a natural bleaching event in 2006 at this site. Seasonal levels of protein, carbohydrates, lipid and reproductive output were investigated in the year before and after the bleaching event. Zooxanthellae densities and chlorophyll levels were investigated concurrently to determine their influence on these physical characteristics. Under normal conditions, thermally tolerant type D colonies had 23% lower lipids than thermally-sensitive C2 colonies. Before the annual spawning type D colonies also had 33% smaller eggs than C2 colonies; making them significantly less fecund. Proteins and carbohydrates varied seasonally but did not vary with symbiont type. However, irrespective of symbiont genotype, medium-term (up to 12 months) the fitness of corals was more adversely affected by the stress of the bleaching itself which reduced

lipids and reproduction by ~50% compared to pre-bleaching levels for up to 9 months, and possibly longer. These results provide insights into some of the potential trade-offs to coral fitness that are likely to be experienced by this species as they acclimatise to warmer conditions and suggest that the processes of symbiont change and acute thermal stress are likely to act in concert as reefs acclimatise to warmer conditions, compromising lipid stores and reproduction.

INTRODUCTION

Reef-building corals are vulnerable to climate change as they live in a narrow thermal range and are extremely sensitive to changes in temperature. An increase of only 1-2 °C above the coral's normal temperature, in combination with light, can cause expulsion of the zooxanthellae that live within the tissues (coral bleaching. Berkelmans, Ray & Willis 1999; Jokiel & Coles 1990). Temperatures that exceed the tolerance range of corals are expected to occur regularly on reefs within the next century as a result of climate change (Meehl *et al.* 2007). Corals must adapt or acclimatise at a rate of 0.1-0.5 °C every decade if they are to keep pace with the predicted rates of warming (Brown *et al.* 2002; Brown, B E *et al.* 1999; Donner *et al.* 2005). One way in which corals can increase their upper thermal limit is by hosting more thermally tolerant types like *Symbiodinium* D (Berkelmans, Ray & van Oppen 2006). Hosting significant levels of algal partners may give them a better chance of survival in a warmer environment (Buddemeier, R.W *et al.* 2004; Buddemeier, Robert W & Fautin 1993). Recent studies have revealed previously undetectable levels (<5–10%) of these thermally tolerant symbionts types (Jones, A. M. *et al.* 2008; Mieog, J.

C. *et al.* 2009; Mieog, J C *et al.* 2007; Mieog, J C *et al.* 2008), raising the possibility that many more corals may be able to survive to climate change by symbiont shuffling. However, so far, symbiont changes have been transitory and it remains to be seen whether these changes will become permanent with incremental increases in sea temperatures. However, it raises the question: what effects will symbiont change have on the holobiont (coral + zooxanthellae + bacteria) physiology?

Host physiological characteristics that rely on photosynthesis are likely to be affected by changes to the predominant symbiont genotype present in the coral's tissues. Shallow water reef corals rely almost entirely on energy derived from photosynthesis by their algal endosymbionts (Davies 1984; Muscatine 1990). Photochemical energy is used to fix carbon to produce low molecular weight carbon compounds (glycerol and triglycerides) that are translocated to the coral (Whitehead & Douglas 2003). In the host, these simple compounds are incorporated into more complex molecules like lipids, proteins and carbohydrates (Davies 1991). The lipids are used as energy store for tissue growth (Spencer-Davies 1991), skeletal growth (Pearse & Muscatine 1971) and reproduction (Edmunds & Davies 1986). Proteins and phospholipids are used in the structure of cell walls and in tissue growth while carbohydrates are used as a short-term energy source for cell metabolism (Mews 1980). The diverse morphological (Schoenberg & Trench 1980) and functional differences (Loram, Trapido-Rosenthal & Douglas 2007; Rowan 2004a) that are gradually being elucidated by studies of *Symbiodinium* genotypes suggest that the proportions of these compounds, or at the very least, the amounts that are translocated to the host coral, may vary with the

genotype of the symbiont. A study by Loram *et al.* (2007) has provided evidence in support of host physiological differences with respect to symbiont genotype that may possibly relate to differential symbiont thermal tolerance in the sea anemone *Condylactis gigantea*. In this species thermally tolerant type A symbionts fix less carbon during photosynthesis than thermally sensitive type B symbionts. The amounts of photosynthetically fixed carbon that are incorporated into lipids and amino acids in the host also varied with symbiont genotype. While this study has shown differences in the amount of photosynthetically fixed carbon translocated to sea anemones that relate to symbiont genotype, to date, no studies have shown significant differences in adult reef corals with symbionts of contrasting thermal tolerance.

Although the exact link between thermal tolerance and photosynthesis is still unclear, various studies have shown that the composition of the fatty acids that constitute the chloroplast thylakoid membranes, which house the photosystems, and the fluidity and structural integrity of the thylakoid membranes and synthesis of repair proteins are implicated in plant thermal tolerance (Chuartzman *et al.* 2008; Critchley 1990; Hugly *et al.* 1989; Öquist *et al.* 1992; Ristic *et al.* 2008). Studies by Tchernov *et al.* (2004) and Takahashi *et al.* (2008) strongly suggests that some of these same attributes also correlate with thermally tolerant symbiont types. The most significant consequence of these for corals is that they have also been shown to reduce the amount of energy available for plant growth (Hugly *et al.* 1989) and metabolism, thereby potentially reducing the physical fitness of the organism or in the case of corals, in the holobiont. This link between improved stress tolerance and reduced fitness is also underlined by

the observation that stress tolerant plants partition more of their available energy reserves to coping with the adverse conditions surrounding them, thereby reducing their fitness (Wahid *et al.* 2007). If this model can be extrapolated to the coral-algal symbiosis then increased symbiont thermal tolerance may reduce the amount of energy available to the host from the symbionts by either inhibiting photosynthesis or by retaining a greater portion of their energy reserves to cope with adversity. Regardless of the exact physical mechanism for increased thermal tolerance, there is little doubt that thermal tolerance involves aspects of photosynthesis and as such, has the potential to influence physiological processes in the host that rely on energy derived from autotrophy.

Various mechanisms for thermal tolerance have implications for the energy translocation to the host. One such mechanism, inhibition of photosynthesis, has been reported to be enhanced in type D symbionts in *Pocillopora damicornis* in response to thermal stress (32 °C, Rowan 2004a), potentially reducing autotrophy (similar in magnitude to a 30% reduction in light). By diverting energy to heat (non-photochemical quenching or NPQ), the photosystems are protected from excess excitation pressure. Down-regulation of photosynthesis can also be achieved in corals by reducing the number and pigment content of algal cells [although this process can have the effect of increasing stress on the remaining symbionts due to greater internal light fields from scattering due to the skeleton, thereby counteracting photoprotection (Enriquez, Méndez & Iglesias-Prieto 2005)]. These mechanisms for photoprotection occur seasonally in some reef corals in response to light (Falkowski & Dubinsky 1981;

Lesser, M P *et al.* 1990) and heat stress (Fitt *et al.* 2000; Glynn *et al.* 1993; Hoegh-Guldberg, C. & Smith 1989a; Stimson, J 1997). In a comparison of host corals with symbionts of contrasting thermal tolerance, it is essential to consider these photoprotective mechanisms to separate acclimatory responses from intrinsic genotypic differences. In addition, while these studies shed light on coral and symbiont photoprotection under temperature stress they fail to show intrinsic differences in photosynthesis that are linked to the genetic identity of the symbiont under normal conditions. That is, other than the direct effects of warmer conditions on photosynthesis, what happens to the fitness of corals if they permanently adopt thermally tolerant symbionts as a result of incremental temperature rise?

Factors that affect the physiology of reef corals also affect the capacity of reefs to regenerate following acute disturbance. The reef-building corals form the physical structure of tropical reefs, providing habitat and food for a variety of other marine organisms (Henry & Hart 2005). Regeneration following disturbance is, in some part, provisional upon the recruitment of these structurally important coral species (Connell, Hughes & Wallace 1997; Wakeford, Done & Johnson 2008). Factors that affect the reproduction of reef-building corals can impinge on the regeneration capacity of the entire reef following disturbance (Henry & Hart 2005). During regeneration, resources, such as lipids, that would normally be used for reproduction are diverted to repair and regrowth (Baird & Marshall 2002; Omori & Hatta 2001; Tamelander 2002). Lower reproductive effort as a result of disturbance can result in lower recruitment and lower growth of juvenile corals (Tamelander 2002). The situation is potentially

compounded if the coral recovers with a more thermally tolerant, but less photosynthetically efficient symbiont type (Little, van Oppen & Willis 2004). Acclimation to climate change by symbiont shuffling could therefore further compound the effects of a disturbance event such as coral bleaching on the reef regeneration process.

A. millepora, which is a common reef-builder in the Keppel Islands region of the southern Great Barrier Reef, has been shown to shift from thermally sensitive C2 to thermally tolerant type D and C1 *Symbiodinium* following bleaching (Jones, A. M. *et al.* 2008). While the shift is expected to have increased the resistance of this species to temperature stress at this site by 1.0-1.5 °C if it remains permanent (Berkelmans, Ray & van Oppen 2006), it is possible that this change has come at a physical cost to coral fitness. This study investigates four key physiological/biochemical attributes of adult colonies of *A. millepora* which naturally host type C2 and occasionally type D or a combination of both types (identified using the ITS1 region of nrDNA). In the first part of the study, lipids, proteins, carbohydrates, and reproductive output of tagged colonies of known predominant genotype were measured seasonally in the year before a natural bleaching event to investigate the link between symbiont thermal tolerance and coral fitness. The bleaching event in early 2006 provided an opportunity to investigate the effects of this event on the same attributes during the recovery of corals following bleaching. Zooxanthellae densities and chlorophyll *a* and c_2 content were measured simultaneously with lipids in both parts of the study (where possible) to

differentiate the effects of these parameters from those of symbiont genotype on coral physiology.

MATERIALS AND METHODS

Study site

Branches of *A. millepora* were collected from Miall Island (23°09'S 150°54'E) in the Keppel Island group (southern inshore Great Barrier Reef). A severe summer bleaching in February 2006 caused significant mortality of colonies on reef flats and slopes in the region (Chapter 1). Colonies were assigned to bleaching status in February 2006 as bleached white, unbleached and normally pigmented, or partially bleached. Before the bleaching event, colonies of *A. millepora* at this site naturally hosted *Symbiodinium* type C2 with the occasional occurrence of type D, or mixtures of both C2 and D. Following the bleaching event there was a 71% shift in the symbiont community from C2 to D and another potentially thermally tolerant type, C1 (Jones, A. M. *et al.* 2008).

Coral sampling

The initial experimental design (sampling the same 30 colonies with equal numbers of C2 and D colonies) was modified during the study because so few predominantly type D colonies were found during the initial sampling, some colonies changed predominant type after recovering from bleaching and because some of the original 16 colonies with C2 symbionts died as a result of the bleaching. Details of the numbers of colonies sampled on each occasion are shown in Table 4.1.

At each sampling occasion two branches were removed from roughly the same part of each colony. A small piece of one branch was placed in 100% ethanol for genotyping and the remaining samples were snap-frozen in liquid nitrogen and stored at -80 °C until processed. One branch was used for the analysis of lipids, protein and carbohydrate and the second branch for the determination of chlorophyll *a* and c_2 content and zooxanthellae densities.

Two branches were removed from each of the tagged colonies before and after spawning in November 2005 and before spawning in November 2006 (9 months after the February 2006 bleaching event). The spawning analysis was confounded by the bleaching event which prevented sampling after spawning in November 2006. One branch was snap-frozen in liquid nitrogen and stored at -80 °C for the analysis of lipids, and the second branch was stored in 10% formaldehyde and seawater and used for the determination of reproductive output (egg number and egg volume).

Table 4.1. Numbers of *Acropora millepora* colonies sampled during a seasonal study of bioenergetics and reproduction before and after a bleaching event in February 2006. Colonies with C2, D or both C2 and D symbionts were chosen for the study to determine the effects of predominant symbiont type, bleaching status and/or season on host physiology. B = bleached, UB = unbleached, PB = partially bleached

		Lipids, proteins or carbohydrates			Zooxanthellae densities			
Before bleaching	**Total**	C2	D	C2/D	Total	C2	D	C2/D
March 2005 (summer)	28	12	12	4	26	11	11	4
July 2005 (winter)	28	12	12	4	28	11	11	4
Nov 2005 (pre-spawning)	30	12	13	5				
Nov 2005 (post-spawning)	30	12	13	5				
Jan 2006 (pre-bleaching)	32	12	12	8	32	12	12	8
After bleaching	**Total**	B	UB	PB	Total	B	UB	PB
Feb 2006 (bleaching)	32	12	12	8	32	12	12	8
May 2006 (autumn)	25	11	10	4	30	12	12	6
Aug 2006 (winter)	32	12	12	8	30	12	11	7
Nov 2006 (pre-spawning)	30	12	13	5				

Before bleaching	**Chlorophyll content**				**Eggs and lipids**			
	Total	C2	D	C2/D	Total	C2	D	C2/D
March 2005 (summer)	28	12	12	4				
July 2005 (winter)	28	12	124					
Nov 2005 (pre-spawning)					30	12	13	5
Nov 2005 (post-spawning)					30	12	13	5
Jan 2006 (pre-bleaching)	32	12	12	8				
After bleaching	**Total**	B	UB	PB	Total	B	UB	PB
Feb 2006 (bleaching)	32	12	12	8				
May 2006 (autumn)	30	12	12	6				
Aug 2006 (winter)	30	12	11	7				
Nov 2006 (pre-spawning)					30	12	13	5

Sample preparation

Analyses of protein, carbohydrate and lipid were performed on dried, ground whole coral tissue samples including zooxanthellae and skeleton. The samples were ground under liquid nitrogen (Brown, B E *et al.* 2002) to produce a fine homogenous powder and placed in acetone-washed and pre-weighed 25 ml sintered glass vials. The vials containing the frozen ground samples were freeze-dried and weighed to four decimal places and the total dry tissue weight (DWT) recorded. Sub-samples of known weight (approx 0.1-0.5 mg) were removed for separate analysis of total soluble protein and total carbohydrate and the remaining sample was used for lipid analysis. Lipid, protein and carbohydrate measurements were standardised to coral tissue surface area using the 3D digital image analysis methods described in Jones *et al.* (2008).

Lipid analysis

The total lipid content of each branch was determined using two consecutive chloroform: methanol extractions following the technique described by Folch *et al.* (1957) and Harland *et al.* (1992). The organic phase was washed once with 0.88% KCl and three times with methanol: water (1:1), evaporated and dried in an oven at 60 °C. The lipid content was expressed as the difference between the weight of the pan, and the weight of the pan plus sample, multiplied by the proportion of the total DWT. Total lipids were calculated by multiplying the lipid content per DWT of the subsample by the total dry weight of tissue of the branch (Appendix III).

Protein analysis

Total protein was determined by the Folin phenol reagent colourimetric detection and quantification method described by Lowry *et al.* (1951) using bovine serum albumin (BSA, Sigma) as a standard (Appendix IV). Following 1:2 dilution (V/V) of the digested samples with 0.5M NaOH, a 5 µl sub sample of each digest and standard was assayed for protein in triplicate using a DC protein assay kit (Bio-Rad laboratories, Australia). Absorbance was measured at 690 nm in a spectrometer (Perkin Elmer Wallace 1420 Victor^2) and analysed using Perkin Elmer Workout (Applications Data Management software version 1.5). Protein concentration in the microassay plate cell was estimated using the regression coefficient of the standard calibration curve. The protein concentration of the tissue was calculated by multiplying the cell concentration by the NaOH dilution factor divided by the DWT. The total protein in the branch was calculated by multiplying the protein concentration per DWT by the total DWT in the branch.

Carbohydrate analysis

Total carbohydrate was quantified using the phenol-sulfuric acid method for the colourimetric determination and quantification of total carbohydrates described in Dubois *et al.* (1956) using D-glucose as standard. A subsample (230 µl) of the digested samples and standards were transferred to a 96-well microassay plate in triplicate and the absorbance measured at 490 nm in a spectrophotometer (Perkin Elmer Wallac 1420 Victor^2). Carbohydrate concentration in the microassay plate cell was estimated using the regression coefficient of the calibration curve (Perkin Elmer

Workout Applications Data Management software version 1.5) and the carbohydrate concentration of the coral tissue was calculated by multiplying the carbohydrate concentration of the cell by the dilution factor of the sample in the acid, divided by the DWT. To determine the total carbohydrate in the branch the carbohydrate concentration per DWT was multiplied by the total DWT in the branch (Appendix V).

Symbiodinium identification

DNA was extracted from the coral tissue using a technique based on the method of Wilson *et al.* (2002) and described in Chapter 1 and Appendix I. Predominant symbiont type was identified in each colony of *A. millepora* using Single Stranded Conformational Polymorphism (SSCP) analysis of the amplified Internal Transcribed Spacer 1 Region (ITS1). SSCP gel bands were compared to sequenced reference standards (C2 EU189443 and D EU1894505). Only colonies with intense type C2 or type D SSCP bands or equally intense C2 and D bands in the same sample were chosen for the study. Intense single bands matching C2 or D were considered monomorphic symbioses for the purposes of the study and two equally intense bands matching C2 and D were considered mixed symbioses, although the presence of other genotypes below the relative abundance of <5-10% in the branches used in this study are not ruled out (Fabricius *et al.* 2004).

Zooxanthellae counts

Frozen branches were stripped of tissue using an air gun and the resultant slurry was macerated with a tissue homogenizer for 20 s. The homogenate volume was recorded and a 9 ml aliquot was drawn off and preserved with 1 ml of formalin (32% $w.w^{-1}$).

Zooxanthellae counts were made on eight independent drops from each sample using the New, Improved Nuebauer haemocytometer (Brown, B. E. *et al.* 2002; Jones, R. J. *et al.* 1998; Perez, Cook & Brooks 2001) under a light microscope. Zooxanthellae numbers were standardised to coral tissue surface area determined using the 3D image analysis method described in Jones *et al.* (Jones, A. *et al.* 2008).

Chlorophyll content

A separate 10 ml aliquot was drawn from the remaining tissue homogenate and the algae were separated from the host tissue by centrifugation (3000 g for 5 min) at 4 °C. Chlorophyll was extracted overnight from the algal pellet using 100% methanol at 4 °C (Berkelmans, Ray & van Oppen 2006; Brown, B E *et al.* 2002). The first ten samples were extracted three times to determine the extraction efficiency. Absorbance at 668 nm and 635 nm was read with a spectrophotometer (Hitachi U-3200). Total branch chlorophylls *a* and c_2 were calculated from the equations of Jeffrey and Haxo (1968) after adjustment for extraction efficiency. Chlorophyll *a* and c_2 content were standardised to algal cells.

Reproductive output

Branches that had been stored in 10% formaldehyde and seawater for the determination of reproductive output were decalcified in 15 ml Falcon tubes using 6% hydrochloric acid and 2% formaldehyde in reverse osmosis (RO) purified water. The decalcifying solution was changed twice over three days until no skeletal material remained. Samples were then washed in RO water and stored in 5% formaldehyde and RO water in 15 ml Falcon tubes.

Six polyps were dissected from each decalcified coral tissue sample under a stereo dissecting microscope. Individual polyps were then dissected under a light microscope with a calibrated eyepiece micrometer (Oliver, J. 1979; Wallace 1985) to expose the mesenteries. Each increment of the micrometer scale represented 0.25 mm. For each polyp, the total number and the size of each egg and sperm bundle (hereafter referred to as eggs) was recorded. Maximum 'length' and 'width' measurements were taken for each egg (Ward 1995; Ward & Harrison 2000). The volume of each egg was calculated as the width multiplied by the length because eggs have a uniform depth of ~1mm (Ward & Harrison 2000).

To compare the lipids allocated to spawning in branches with different symbiont types, total lipids before and after spawning in November 2005 and before spawning in November 2006 were determined using the extraction method outlined above.

STATISTICAL ANALYSIS

Lipids, carbohydrates and protein

To investigate the effects of predominant symbiont genotype on the lipids, protein and carbohydrate content of *A. millepora* branches in the year before the bleaching, data were analysed with a multivariate repeated-measure two-way orthogonal ANOVA using symbiont type as the fixed factor (three levels) and season as the repeated measure (two levels) in the model. Lipid and carbohydrate data were initially

normalised to tissue surface area for the analyses. However, as the protein content (tissue biomass) of branches was significantly lower in summer than in winter, lipids and carbohydrates were re-analysed after normalisation to protein to isolate the effect of symbiont genotype from any seasonal effect on tissue depth (Edmunds & Gates 2002).

Zooxanthellae densities and chlorophyll pigments

Densities of zooxanthellae and chlorophyll concentrations have been shown to vary seasonally in reef corals (Fitt *et al.* 2000). So two separate repeated-measure ANOVA's were performed to determine whether these variables influenced the seasonal lipid, protein and carbohydrate content or varied with symbiont genotype in *A. millepora* colonies in the year before the bleaching event. No chlorophyll data were available for summer (March 2005) and so in the first analysis, zooxanthellae densities were analysed with a repeated-measure ANOVA using symbiont type as the fixed factor (three levels) and season as the repeated measure (two levels) in the model. In the second analysis, data for chlorophyll *a* and c_2 were analysed with a single factor multivariate ANOVA using symbiont type (three levels) as the fixed, predictor variable in the model.

Reproductive output

To investigate the effects of symbiont genotype on the reproductive output of *A. millepora*, the egg volume and egg number for six polyps were averaged for each colony. The egg data and lipids allocated (lipid allocated = lipids before spawning – lipids after spawning), were analysed with a multivariate ANOVA using symbiont type

(fixed, three levels) as the predictor variable in the model. Pre- and post-spawning lipids were analysed with a separate repeated-measure multivariate ANOVA using symbiont type as the fixed, predictor variable. Data for egg number and egg volume were natural log-transformed before the final analysis to meet the assumptions of the model.

Bleaching effects on zooxanthellae densities and chlorophyll pigments

To investigate the effects of month and bleaching severity on the photosynthetic pigments of *A. millepora* after the bleaching, data for algal cell chlorophyll *a* and c_2 content were analysed with a repeated-measure multivariate ANOVA using bleaching status (three levels) as the fixed, predictor variable and month as the repeated measure (four levels).

Bleaching effects on macromolecular composition

To investigate the differences in lipid content of *A. millepora* branches for each of the eight sampling occasion of the study, data were analysed with ANOVA using month (eight levels, March, July and November 2005, and January, February, May, August and November 2006) as the single fixed, predictor variable in the analysis.

To investigate the effects of the bleaching severity (which correlated with predominant symbiont genotype) on the lipid levels in the year after the bleaching, data for each of the four sampling occasions January, February, May and August were examined with a repeated-measure ANOVA using bleaching status (three levels, bleached = C2,

unbleached = D and partially bleached = C2/D) as the fixed, predictor variable, and season as the repeated measure in the model (four levels).

To investigate the seasonal loss of lipids in colonies which originally (before the bleaching) had predominantly C2 or D symbionts, lipids in summer (March 2005 and May 2006) and winter (July 2005 and August 2006) before and after the bleaching event, were analysed with a separate repeated-measure ANOVA using bleaching status (two levels, C2 = bleached, D = unbleached) (which correlated with predominant symbiont type before the bleaching) and season before or after bleaching as the repeated-measures (four levels) in the model.

Bleaching effects on reproduction

To investigate the effects of the bleaching on the reproductive output of colonies of *A. millepora* with different symbiont types, data for egg number, egg volume and pre-spawning lipids were analysed with a multivariate ANOVA using bleaching status (three levels which correlated with predominant symbiont type before the bleaching where C2 = bleached, D = unbleached and C/D = partially bleached) and year (two levels, 2005 and 2006, before and after the bleaching event) as fixed predictor variables in the model. Data for egg number and egg volume were natural log-transformed to meet the assumptions of the model.

In all the analyses scatterplots of the unstandardised residuals and standardised predicted values were used to verify the normality of the data and Levene's tests were

used to verify the homogeneity of variances. Wherever significant interactions between predictor variables were found, simple, pair-wise comparisons were performed to further investigate significant differences in the macromolecular composition of *A. millepora* colonies using Sidak's adjustment for multiple comparisons (Weinberg & Abramowitz 2002). All statistical analyses in the study were performed using SPSS version 15.0.

RESULTS

Lipids

In the year before the bleaching event, the lipid content varied significantly with the predominant symbiont genotype present in *A. millepora* branches (Table 4.2, 4.3, Figure 4.1). The lipid content of branches with type D symbionts was 23% lower than those with C2 symbionts ($p < 0.05$). Branches with both C2 and D symbionts had 25% lower lipids than that of C2 branches ($p < 0.05$).

Table 4.2. Means and standard deviations of the macromolecular composition of *Acropora millepora* colonies with C2, D and C2/D symbionts in summer and winter in the year before a bleaching event.

Season	Measure	Type	Mean	S.D.	N	units
Summer	lipids	C2	3.05	1.47	12	mg cm^{-2}
		D	2.35	0.86	12	mg cm^{-2}
		C2/D	2.28	1.39	4	mg cm^{-2}
	proteins	C2	1.80	0.62	12	mg cm^{-2}
		D	1.95	0.62	12	mg cm^{-2}
		C2/D	2.32	0.68	4	mg cm^{-2}
	carbohydrates	C2	0.23	0.09	12	μg x 10^{-6} cm^{-2}
		D	0.27	0.10	12	μg x 10^{-6} cm^{-2}
		C2/D	0.31	0.11	4	μg x 10^{-6} cm^{-2}
Winter	lipids	C2	3.82	1.40	12	mg cm^{-2}
		D	2.70	0.82	12	mg cm^{-2}
		C2/D	2.43	0.51	4	mg cm^{-2}
	proteins	C2	2.93	0.49	12	mg cm^{-2}
		D	2.92	0.54	12	mg cm^{-2}
		C2/D	2.95	0.34	4	mg cm^{-2}
	carbohydrates	C2	0.63	0.14	12	μg x 10^{-6} cm^{-2}
		D	0.63	0.07	12	μg x 10^{-6} cm^{-2}
		C2/D	0.70	0.17	4	μg x 10^{-6} cm^{-2}

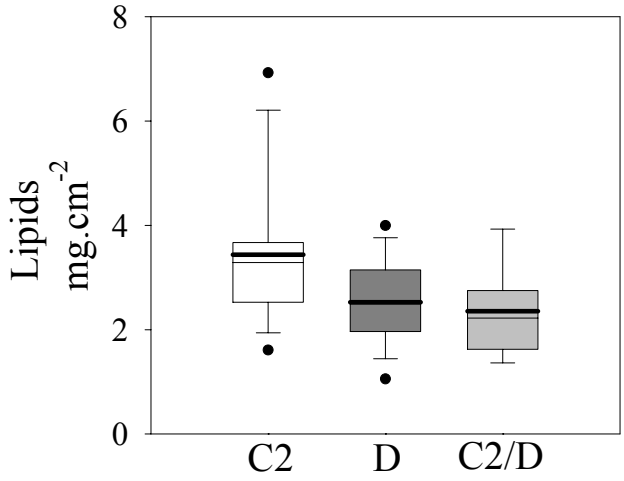
Table 4.3. The results of a multivariate repeated-measure ANOVA showing the significant between-subjects effect of predominant symbiont type on the lipid content (normalised to surface area) of *Acropora millepora* colonies sampled in summer and winter in the year before a bleaching event.

	Measure	SS	df	Mean Square	F	Sig.
Intercept	lipid	331.84	1	331.84	196.6	0.000
	protein	265.43	1	265.43	990.5	0.000
	carbohydrate	9.19	1	9.19	693.0	0.000
type	lipid	12.69	2	6.35	3.8	0.037
	protein	0.43	2	0.21	0.8	0.461
	carbohydrate	0.04	2	0.02	1.3	0.290
Error	lipid	42.21	25	1.69		
	protein	6.70	25	0.27		
	carbohydrate	0.33	25	0.01		

Table 4.4. The results of a multivariate repeated-measure ANOVA showing the significant within-subjects effects of season on the protein and carbohydrate content (normalised to surface area) of *Acropora millepora* colonies sampled in the year before a bleaching event. There was no significant effect of season on lipid content. Significance values are calculated assuming sphericity of the data. There were no significant interactions between type and season for any of the three variables.

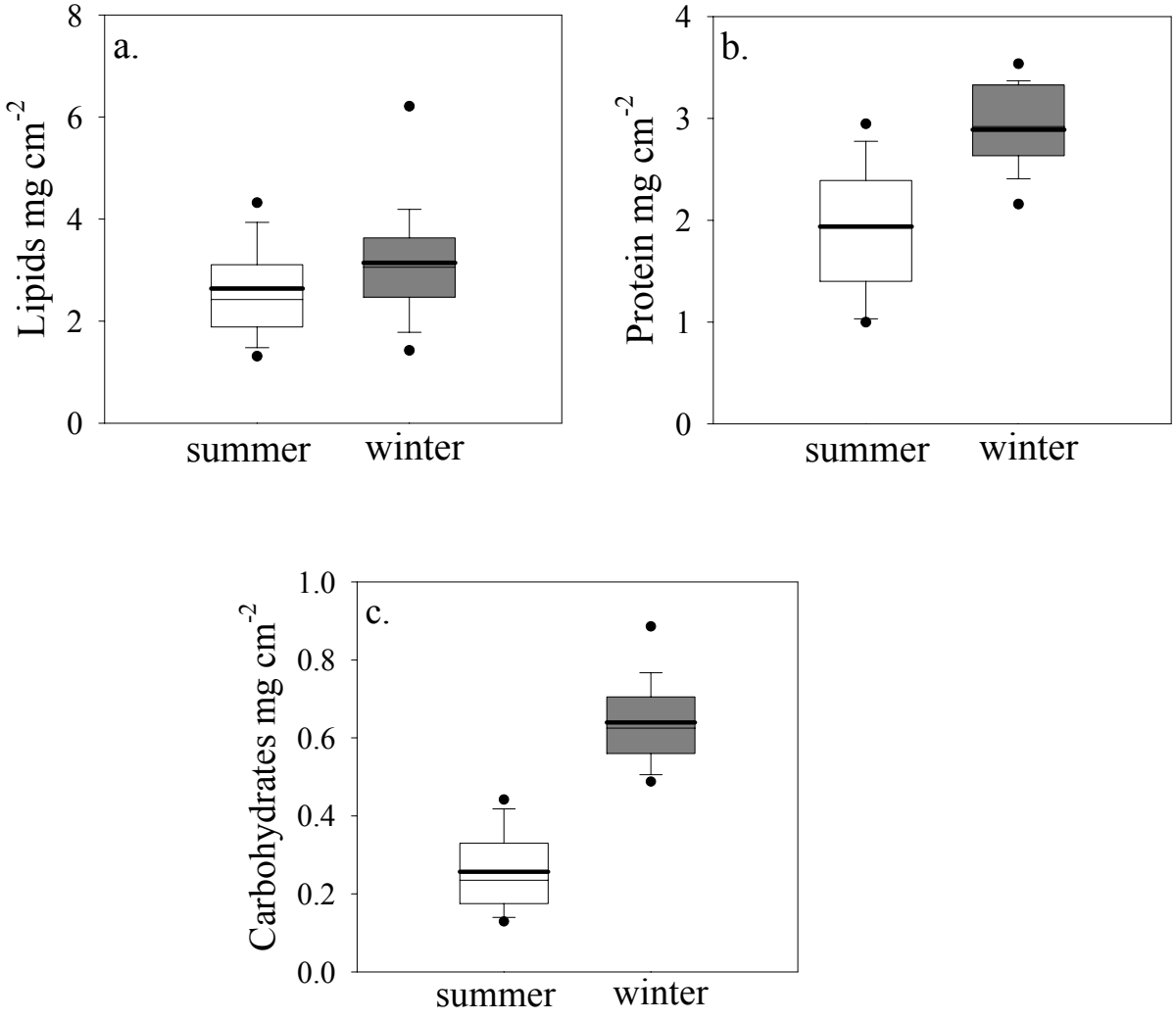
	Measure	SS	df	Mean Square	F	Sig.
Season	Lipid	1.95	1	1.95	1.9	0.177
	Protein	9.00	1	9.00	24.1	0.000
	Carbohydrate	1.61	1	1.61	148.4	0.000
season * type	Lipid	0.83	2	0.42	0.4	0.667
	Protein	0.37	2	0.19	0.5	0.612
	Carbohydrate	0.01	2	0.00	0.3	0.751
Error (season)	Lipid	25.24	25	1.01		
	Protein	9.35	25	0.37		
	Carbohydrate	0.27	25	0.01		

Figure 4.1. Boxplots showing the significant effect of symbiont genotype on the lipid content of branches of *Acropora millepora* before a bleaching. White boxes represent corals with type C2 symbionts, dark grey boxes represent corals with type D symbionts and light grey boxes represent corals with type C2/D symbionts. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



The lipid content of *A. millepora* branches (normalised to coral branch surface area) did not vary significantly with season in the year before the bleaching (Table 4.2, 4.4, Figure 4.2 a). The lipid content of branches in summer was 16% lower than lipid content in winter. There was no significant interaction between symbiont genotype and season in the lipid content of branches sampled before the bleaching.

Figure 4.2. Boxplots showing the effect of season (summer or winter) on (a) the lipid (ns), (b) protein ($p < 0.05$) and (c) carbohydrate content ($p < 0.05$) of branches of *Acropora millepora* sampled before a bleaching. White boxes represent branches sampled in summer, dark grey boxes represent branches sampled in winter. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



Carbohydrates and proteins

There were no significant differences in the carbohydrate or protein content of *A. millepora* branches with respect to predominant symbiont genotype before the bleaching (Table 4.2, 4.3). However, there was a significant effect of season on both the carbohydrate and protein content of the corals in the study (Table 4.4). Branches had significantly greater tissue biomass (protein) in the cooler months than in summer (Figure 4.2 b). The protein content of branches collected in summer (March 2005) 38% lower than for branches collected from the same colonies in winter (July 2005) ($p < 0.05$). The carbohydrate content of branches collected in summer (March 2005) was 60% lower compared to those collected in winter (July 2005) ($p < 0.05$, Figure 4.2 c). Protein content was highly predictive of carbohydrate content but more so in winter than summer (model II $r^2 = 0.79$, $p < 0.05$). There was no significant interaction between symbiont type and season on the carbohydrate or protein content of *A. millepora* branches.

Normalisation to tissue biomass (protein) rather than to surface area had a significant effect on the results of the repeated-measure ANOVA of lipid content of *A. millepora* branches. As in the previous analysis, the lipid content varied significantly with both symbiont genotype and season and there was no significant interaction between the two variables (Table 4.5, 4.6, 4.7, Figure 4.3 a-b). However, in contrast to the previous analysis, lipids were significantly lower in winter than in summer when normalised to tissue biomass rather than the opposite trend (normalised to surface area). The lipid content of branches in summer was 25% higher than the lipid content of branches in

winter ($p < 0.05$). The mean lipid content of type C2 branches was 59% higher than the lipid content of type D branches ($p < 0.05$) and 76% higher than that of C2/D branches ($p < 0.05$). Normalisation to protein did not have a significant effect on the analysis of carbohydrates. Carbohydrate content (normalised to tissue biomass) was 38% lower in summer than in winter in the year before the bleaching event ($p < 0.05$, Figure 4.3 c). There was no significant difference in the amount of tissue gained between summer and winter for branches with respect to symbiont type. These results demonstrate that the tissue biomass of all corals, irrespective of symbiont type is significantly higher in the cooler, winter months. When comparing seasonal variations in biochemical parameters, this necessitates normalisation to protein rather than surface area, the latter producing more variable results because of seasonal variations in tissue depth.

Table 4.5. Means and standard deviations of the macromolecular composition of *Acropora millepora* colonies with C2, D and C2/D symbionts (normalised to protein) in summer and winter in the year before a bleaching event.

Season	Measure		Mean	S.D.	N	units
summer	lipids	C2	1.79	0.65	12	mg mg^{-1}
		D	1.28	0.47	12	mg mg^{-1}
		C2/D	1.07	0.75	4	mg mg^{-1}
	carbohydrates	C2	0.13	0.03	12	µg x 10^{-6} mg^{-1}
		D	0.14	0.02	12	µg x 10^{-6} mg^{-1}
		C2/D	0.13	0.01	4	µg x 10^{-6} mg^{-1}
winter	lipids	C2	1.36	0.65	12	mg mg^{-1}
		D	0.93	0.30	12	mg mg^{-1}
		C2/D	0.83	0.22	4	mg mg^{-1}
	carbohydrates	C2	0.22	0.05	12	µg x 10^{-6} mg^{-1}
		D	0.22	0.03	12	µg x 10^{-6} mg^{-1}
		C2/D	0.24	0.03	4	µg x 10^{-6} mg^{-1}

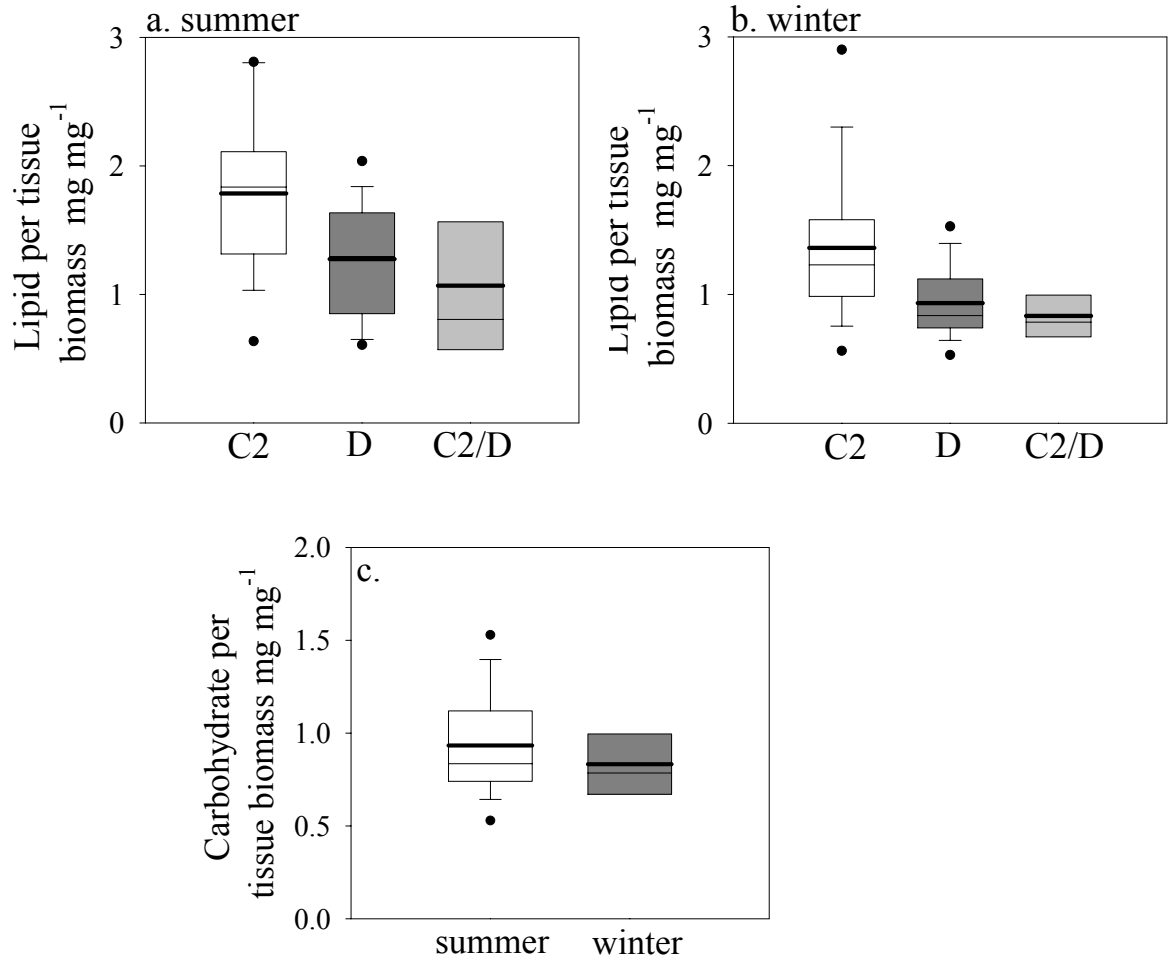
Table 4.6. The results of a multivariate repeated-measure ANOVA showing the significant between-subjects effect of predominant symbiont type on the lipid content (normalised to surface area) of *Acropora millepora* colonies sampled in summer and winter in the year before a bleaching event. There was no significant effect of type on the carbohydrate content.

	Measure	SS	df	Mean Square	F	Sig.
Intercept	lipid	63.21	1	63.21	174.9	0.000
	carbohydrate	1.38	1	1.38	1114.9	0.000
type	lipid	3.69	2	1.85	5.1	0.014
	carbohydrate	0.00	2	0.00	0.2	0.808
Error	lipid	9.04	25	0.36		
	carbohydrate	0.03	25	0.00		

Table 4.7. The results of a multivariate repeated-measure ANOVA showing the significant within-subjects effect of season on the lipid and carbohydrate content (normalised to protein) of *Acropora millepora* colonies with type C2, D or C2/D symbionts sampled in the year before a bleaching event. Significance values are calculated assuming sphericity of the data. There were no significant interactions between type and season.

	Measure	SS	df	Mean Square	F	Sig.
Season	Lipid	1.21	1	1.21	5.6	0.026
	Carbohydrate	0.09	1	0.09	82.7	0.000
Season * type	Lipid	0.06	2	0.03	0.1	0.874
	Carbohydrate	0.00	2	0.00	0.4	0.703
Error (Season)	Lipid	5.42	25	0.22		
	Carbohydrate	0.03	25	0.00		

Figure 4.3. Boxplots showing the effects of symbiont type on lipid content in (a) summer and (b) winter, and, (c) season on the carbohydrate content (normalised to tissue biomass and not to branch surface area as in the previous figures) of *Acropora millepora* branches. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



Zooxanthellae densities and chlorophyll pigments

The zooxanthellae densities of *A. millepora* branches varied significantly with season in the year before the bleaching (Figure 4.4 a, Table 4.8, 4.9). In summer, branches had algal cell densities 37% lower than densities in winter ($p < 0.05$). There was no

significant effect of symbiont type and no significant interaction between symbiont type and season. The zooxanthellae in type D branches were <13% less dense than those in type C2 branches but this difference was not statistically significant.

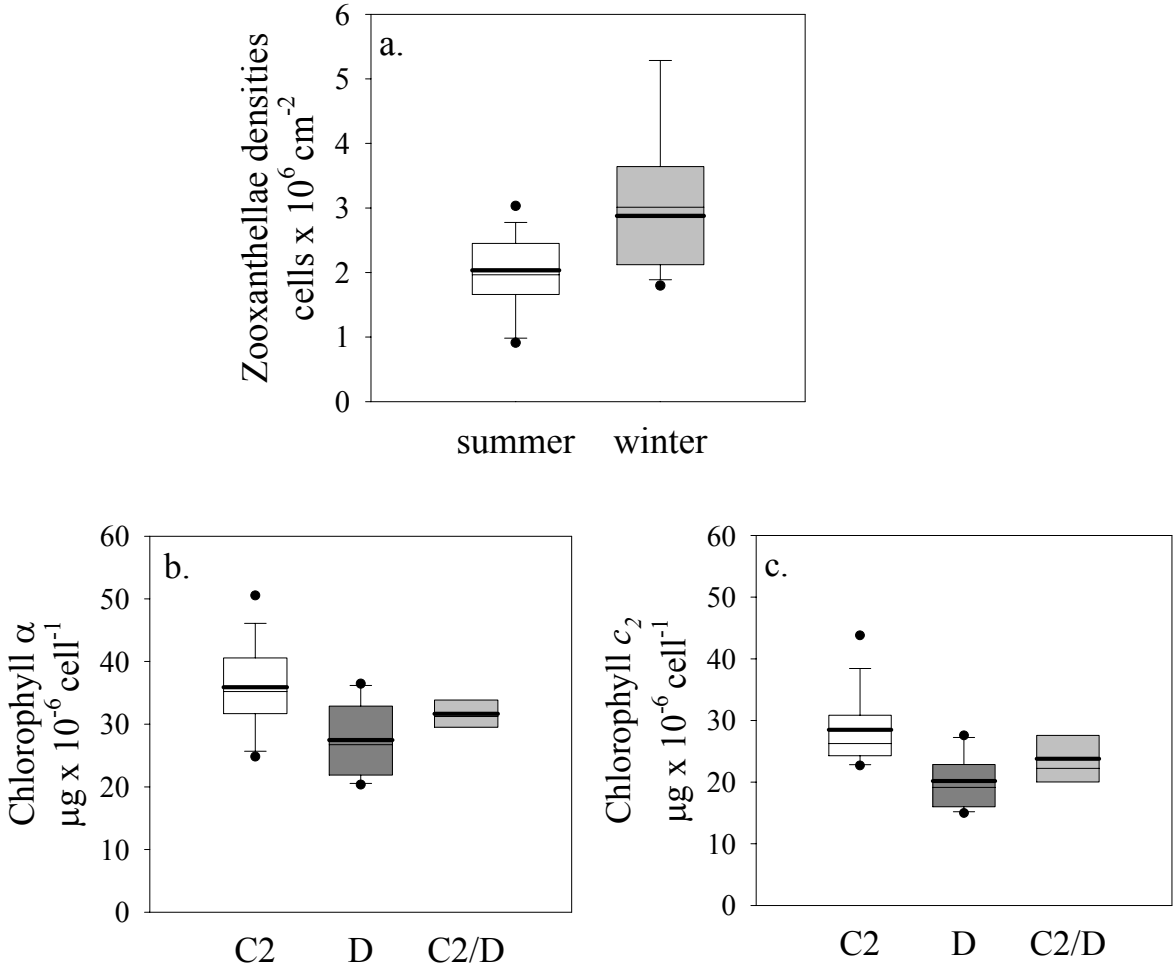
Table 4.8. Means and standard deviations of the zooxanthellae densities of *Acropora millepora* colonies with C2, D and C2/D symbionts in summer and winter in the year before a bleaching event.

Season	Measure	Type	Mean	S.D.	N	units
Summer	Zooxanthellae	C2	2.08	0.66	11	cells x 10^6 cm^{-2}
		D	1.82	0.58	11	cells x 10^6 cm^{-2}
		C2/D	2.49	0.72	4	cells x 10^6 cm^{-2}
Winter	Zooxanthellae	C2	2.62	1.09	11	cells x 10^6 cm^{-2}
		D	3.89	0.85	11	cells x 10^6 cm^{-2}
		C2/D	3.02	1.51	4	cells x 10^6 cm^{-2}
	Chlorophyll *a*	C2	35.88	7.49	12	µg x 10^{-6} cm^{-2}
		D	27.47	6.14	12	µg x 10^{-6} cm^{-2}
		C2/D	31.68	3.26	4	µg x 10^{-6} cm^{-2}
	Chlorophyll c_2	C2	28.50	6.44	12	µg x 10^{-6} cm^{-2}
		D	20.19	4.42	12	µg x 10^{-6} cm^{-2}
		C2/D	23.80	5.21	4	µg x 10^{-6} cm^{-2}

Table 4.9. Results of a repeated-measure ANOVA showing the significant within-subjects effect of season (summer or winter) on the zooxanthellae densities of *Acropora millepora* branches. Significance values are calculated assuming sphericity of the data. There was no significant interaction between season and symbiont type.

	SS	df	Mean Square	F	Sig.
Season	11.42	1	11.42	8.4	0.008
season * type	7.42	2	3.71	2.7	0.085
Error (season)	31.09	23	1.35		

Figure 4.4. Boxplots showing (a) the significantly higher zooxanthellae densities of *Acropora millepora* branches in collected in winter compared to the densities of branches collected in summer ($p < 0.05$) and (b) significantly higher chlorophyll *a* content of C2 symbionts compared to type D symbionts ($p < 0.05$). (c) A boxplot showing the significantly higher chlorophyll c_2 content of *A. millepora* branches with C2 and D symbionts ($p < 0.05$). Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



Chlorophyll *a* content varied significantly with the predominant symbiont genotype present in *A. millepora* branches in the year before bleaching (Figure 4.4 b, Table 4.8, 4.10). Type D zooxanthellae had 23% lower chlorophyll *a* concentrations than type C2 zooxanthellae ($p < 0.05$). The algal chlorophyll *a* concentrations in branches with both C2 and D symbionts was intermediate between concentrations found in monomorphic symbioses (ns). There was no significant difference in *a*: c_2 ratio of corals with different symbiont types.

Table 4.10. The results of a multivariate ANOVA showing the significant effect of symbiont type on the chlorophyll *a* and c_2 of branches of *Acropora millepora* in winter before a bleaching.

	Dependent Variable	SS	df	Mean Square	F	Sig.
Corrected Model	Chlorophyll *a*	424.26[a]	2	212.13	5.0	0.015
	Chlorophyll c_2	415.56[b]	2	207.78	6.9	0.004
Intercept	Chlorophyll *a*	21675.49	1	21675.49	509.6	0.000
	Chlorophyll c_2	12614.23	1	12614.23	419.3	0.000
type	Chlorophyll *a*	424.26	2	212.13	5.0	0.015
	Chlorophyll c_2	415.56	2	207.78	6.9	0.004
Error	Chlorophyll *a*	1063.38	25	42.54		
	Chlorophyll c_2	752.09	25	30.08		
Total	Chlorophyll *a*	29586.81	28			
	Chlorophyll c_2	17660.79	28			
Corrected Total	Chlorophyll *a*	1487.64	27			
	Chlorophyll c_2	1167.66	27			

a. $R^2 = 0.285$ (Adjusted $R^2 = 0.228$)
b. $R^2 = 0.356$ (Adjusted $R^2 = 0.304$)

Algal chlorophyll c_2 concentrations also varied significantly with predominant symbiont type in the year before the bleaching (Figure 4.4 c, Table 4.8, 4.10). Chlorophyll c_2 in type C2 zooxanthellae in winter before the bleaching (July 2005) was 29% higher than that in type D zooxanthellae ($p < 0.05$). The algal chlorophyll c_2

concentrations in branches with both C2 and D symbionts were intermediate between concentrations found in monomorphic symbioses (ns).

Reproductive output

In the year before the bleaching the reproductive output varied significantly with predominant symbiont genotype in *A. millepora* branches one week before the annual spawning (November 2005, Figure 4.5 a, b, Table 4.11, 4.12). The egg volume of polyps from branches with predominantly type D symbionts was 33% smaller than the egg volume in type C2 polyps ($p < 0.05$). The egg volume in C2 polyps was also 22% higher than that in polyps from branches with a mixture of C2 and D symbionts (ns, Figure 4.5 a). The mean number of egg bundles for all colonies in the study was 6.8 ± 3.0 eggs per polyp. The polyps dissected from C2 branches had the highest number of egg bundles; 18% higher than the number in polyps from type D branches (ns, Figure 4.5 b). C2/D branches had highly variable egg numbers ranging from 10.8 to 1.3. All the polyps dissected from C2 branches contained egg bundles whereas; two of the type D branches and one of the C2/D branches contained no eggs in any polyps.

Table 4.11. Means and standard deviations of the reproductive output (egg number, egg volume and total lipid content) of *Acropora millepora* colonies with C2, D and C2/D symbionts in summer and winter in the year before a bleaching event.

Measure		Mean	S.D.	N	units
Egg number	C2	7.49	1.49	12	
	D	6.13	3.75	13	
	C2/D	7.07	3.97	5	
Egg volume	C2	0.09	0.01	12	mm^3
	D	0.06	0.04	13	mm^3
	C2/D	0.07	0.02	5	mm^3
Lipids before	C2	4.63	1.46	12	mg cm^{-2}
	D	3.19	1.44	13	mg cm^{-2}
	C2/D	4.31	2.21	5	mg cm^{-2}
Lipids before	C2	3.09	1.66	12	mg cm^{-2}
	D	2.06	1.06	13	mg cm^{-2}
	C2/D	1.96	0.37	5	mg cm^{-2}

Figure 4.5. Boxplots showing (a) the significantly higher volume of egg bundles in the polyps of *Acropora millepora* with predominantly type C2 symbionts compared to those with type D symbionts ($p < 0.05$), or polyps with both type C2 and D symbionts (ns) and, (b) no significant differences in the numbers of eggs per polyp. White boxes represent the polyps from C2 branches, dark grey boxes represent polyps from type D branches and light grey boxes represent the polyps from C2/D branches. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.

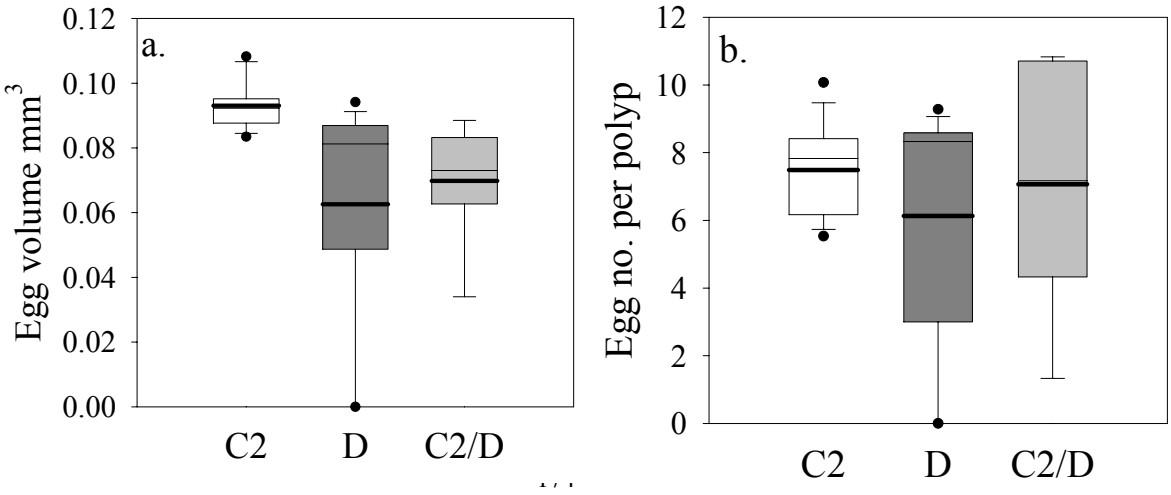


Table 4.12. ANOVA table showing the significant effect of predominant symbiont type (C2, D or C2/D) on eggs bundle volume in polyps of *Acropora millepora* before the annual summer spawning. All the egg bundles in six polyps from each branch were counted and measured (width x length) and then a mean egg volume and mean egg number was determined for each of the 30 colonies in the study. Lipids were calculated as the lipid content of branches before spawning minus the lipid content after spawning.

Source	Dependent Variable	S.S.	df	Mean Square	F	Sig.
Corrected Model	Egg number	0.346[a]	2	0.17	1.0	0.369
	Egg volume	0.4035[b]	2	0.20	5.9	0.008
	Lipids	2.34[c]	2	1.17	0.3	0.715
Intercept	Egg number	87.21	1	87.21	524.8	0.000
	Egg volume	150.92	1	150.92	4450.2	0.000
	Lipids	81.52	1	81.52	23.8	0.000
type	Egg number	0.35	2	0.17	1.0	0.369
	Egg volume	0.40	2	0.20	5.9	0.008
	Lipids	2.34	2	1.17	0.3	0.715
Error	Egg number	3.99	24	0.17		
	Egg volume	0.81	24	0.03		
	Lipids	82.34	24	3.43		
Total	Egg number	108.87	27			
	Egg volume	168.75	27			
	Lipids	167.36	27			
Corrected Total	Egg number	4.33	26			
	Egg volume	1.22	26			
	Lipids	84.67	26			

a. $R^2 = 0.080$ (Adjusted $R^2 = 0.003$)
b. $R^2 = 0.331$ (Adjusted $R^2 = 0.276$)
c. $R^2 = 0.028$ (Adjusted $R^2 = -0.053$)

The lipid content of *A. millepora* branches did not vary significantly with predominant symbiont type either before, or after, the annual spawning in November 2005 (Table 4.13). Thirty eight percent of stored lipids were allocated to spawning. Lipid allocation to spawning did not vary with respect to symbiont genotype (Table 4.12).

Table 4.13. The results of a repeated-measure ANOVA of the lipid content of *Acropora millepora* branches sampled before and after the annual spawning in November 2005.

	SS	df	Mean Square	F	Sig.
Spawning	34.76	1	34.76	19.2	0.000
spawning * type	2.70	2	1.35	0.7	0.485
Error(spawning)	48.93	27	1.81		

Bleaching effects

The onset of the bleaching occurred in by January 2006 when the *A. millepora* colonies in the study had begun to appear pale or 'fluorescent'. The peak of the bleaching occurred in February 2006 when water temperatures remained at >29 °C for over two weeks (Ray Berkelmans, unpublished data). As a result of heat stress, all 12 C2 colonies bleached white due to major losses of zooxanthellae. All 11 type D colonies appeared unaffected by the heat stress, retaining their normal colouring. All 9 colonies with mixed symbioses appeared partially bleached (pale brown/mottled).

Bleaching effects on zooxanthellae densities

In the year of the bleaching, the densities of zooxanthellae in branches of *A. millepora* varied significantly with monthly sampling occasion, and there was a significant interaction between the two variables (Figure 4.6, Table 4.14, 4.15). All colonies,

irrespective of symbiont genotype, had significantly lower zooxanthellae densities in January 2006 compared to densities in winter the year before (July 2005). Between January and February 2006, type C2 colonies had lost 72% of their zooxanthellae ($p < 0.05$, Figure 4.6 a) and whereas colonies with type D symbionts had gained 4% more zooxanthellae ($p < 0.05$, Figure 4.6 b) and colonies with a mixture of symbiont types C2 and D had gained 20% more zooxanthellae ($p < 0.05$, Figure 4.6 c). Bleached C2 colonies regained 79% of their zooxanthellae between February and May ($p > 0.001$) and a further 15% between May and August, six months after the bleaching ($p < 0.05$).

In February 2006, type C2 colonies had 81% less symbionts than unbleached type D colonies ($p < 0.05$) and 80% less then partially bleached C2/D colonies ($p < 0.05$). All 12 of the C2 colonies survived the bleaching.

Table 4.14. Means and standard deviations of the zooxanthellae densities of *Acropora millepora* colonies with type C2, D and C2/D symbionts in the year after a bleaching event.

Month	Bleaching status	Mean	S.D.	N	units
January	Bleached	1.43	0.44	12	cells x 10^6 cm^{-2}
	Unbleached	1.98	0.80	12	cells x 10^6 cm^{-2}
	Partially bleached	1.42	0.53	8	cells x 10^6 cm^{-2}
February	Bleached	0.40	0.51	12	cells x 10^6 cm^{-2}
	Unbleached	2.13	0.88	12	cells x 10^6 cm^{-2}
	Partially bleached	2.02	0.84	8	cells x 10^6 cm^{-2}
May	Bleached	1.93	0.57	12	cells x 10^6 cm^{-2}
	Unbleached	1.97	0.55	12	cells x 10^6 cm^{-2}
	Partially bleached	2.12	0.90	6	cells x 10^6 cm^{-2}
August	Bleached	2.22	0.86	12	cells x 10^6 cm^{-2}
	Unbleached	2.60	0.97	11	cells x 10^6 cm^{-2}
	Partially bleached	2.53	0.44	7	cells x 10^6 cm^{-2}

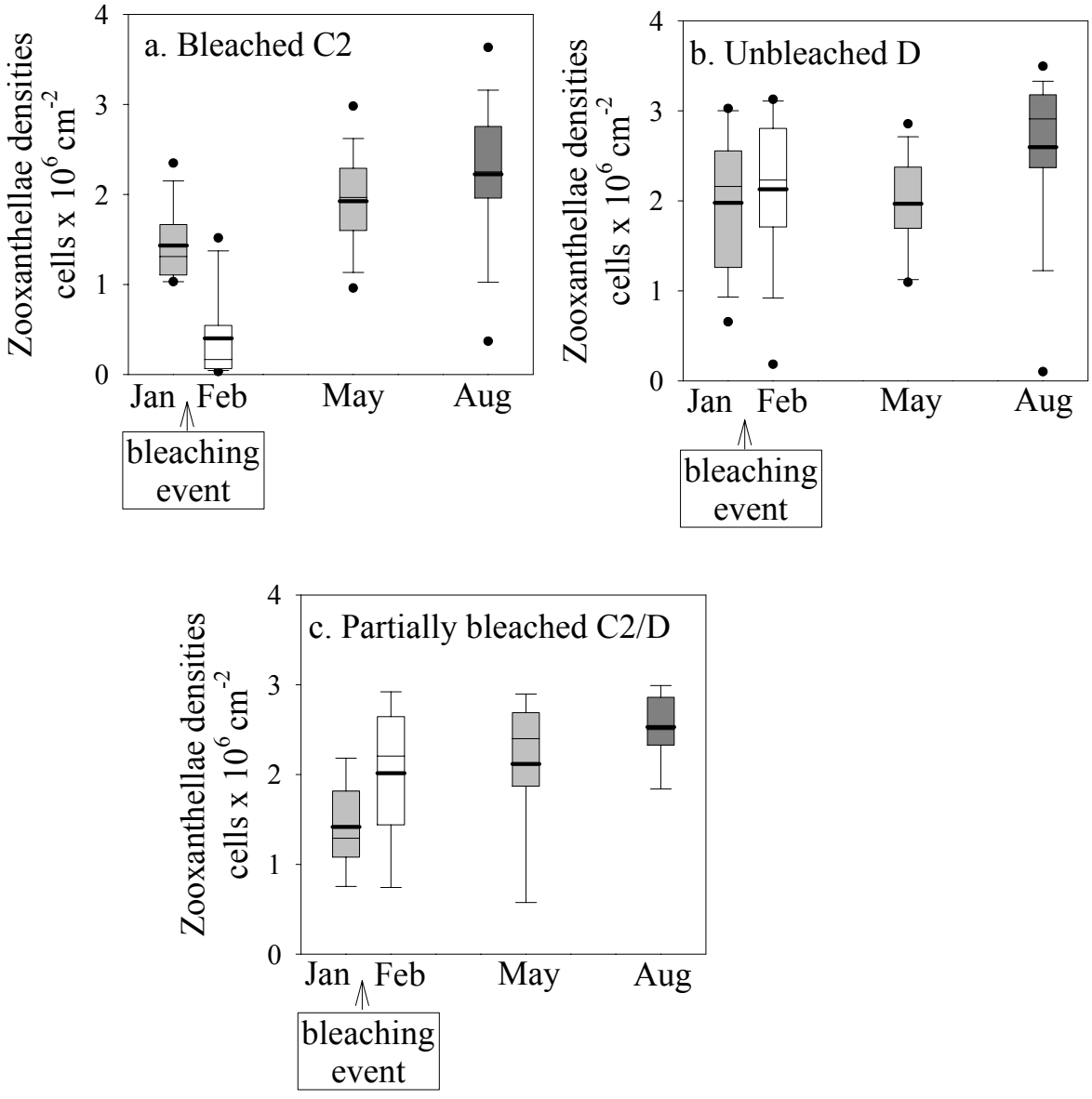
Table 4.15. Results of repeated-measure multivariate ANOVA showing the significant effect of month (Jan, Feb, May, Aug) on the zooxanthellae densities and algal chlorophyll *a* and c_2 content of *Acropora millepora* colonies after a bleaching event in February 2006. Significance values for chlorophyll *a* and c_2 are adjusted using the Greenhouse-Geisser epsilon for sphericity, significance values for zooxanthellae densities are calculated assuming sphericity of the data. There was also a significant interaction between bleaching status and month. All C2 colonies bleached white whereas all type D colonies were unbleached and type C2/D colonies were partially bleached.

	Measure	SS	df	Mean Square	F	Sig.
Month	Chlorophyll *a*	0.03	2	0.01	25.4	0.000
	Chlorophyll c_2	0.25	2	0.11	18.5	0.000
	Zooxanthellae density	14.12	3	4.71	8.8	0.000
Month * Bleaching	Chlorophyll *a*	0.00	5	0.00	0.6	0.650
	Chlorophyll c_2	0.02	4	0.01	0.9	0.506
	Zooxanthellae density	10.43	6	1.74	3.2	0.007
Error (Month)	Chlorophyll *a*	0.03	57	0.00		
	Chlorophyll c_2	0.33	54	0.01		
	Zooxanthellae density	40.23	75	0.54		

Table 4.16. Means and standard deviations of the algal chlorophyll *a* and c_2 content of *Acropora millepora* colonies with C2, D and C2/D symbionts in summer and winter in the year after a bleaching event.

Measure	Month	Bleaching status	Mean	S.D.	N	units
Chlorophyll *a*	January	Bleached	72.51	9.03	12	µg x 10^{-6} cm^{-2}
		Unbleached	55.31	33.86	12	µg x 10^{-6} cm^{-2}
		Partially bleached	76.38	28.42	8	µg x 10^{-6} cm^{-2}
	February	Bleached	36.54	25.48	12	µg x 10^{-6} cm^{-2}
		Unbleached	27.75	8.59	12	µg x 10^{-6} cm^{-2}
		Partially bleached	32.65	7.92	8	µg x 10^{-6} cm^{-2}
	May	Bleached	28.48	9.06	12	µg x 10^{-6} cm^{-2}
		Unbleached	24.34	7.97	12	µg x 10^{-6} cm^{-2}
		Partially bleached	20.28	5.11	6	µg x 10^{-6} cm^{-2}
	August	Bleached	53.05	24.94	12	µg x 10^{-6} cm^{-2}
		Unbleached	40.19	15.50	11	µg x 10^{-6} cm^{-2}
		Partially bleached	36.83	13.11	7	µg x 10^{-6} cm^{-2}
Chlorophyll c_2	January	Bleached	145.10	113.58	12	µg x 10^{-6} cm^{-2}
		Unbleached	100.13	41.62	11	µg x 10^{-6} cm^{-2}
		Partially bleached	79.04	34.87	7	µg x 10^{-6} cm^{-2}
	February	Bleached	49.71	33.61	12	µg x 10^{-6} cm^{-2}
		Unbleached	27.40	11.06	12	µg x 10^{-6} cm^{-2}
		Partially bleached	32.71	16.95	8	µg x 10^{-6} cm^{-2}
	May	Bleached	159.09	55.41	12	µg x 10^{-6} cm^{-2}
		Unbleached	125.03	38.82	12	µg x 10^{-6} cm^{-2}
		Partially bleached	99.33	11.33	6	µg x 10^{-6} cm^{-2}
	August	Bleached	145.10	113.58	12	µg x 10^{-6} cm^{-2}
		Unbleached	100.13	41.62	11	µg x 10^{-6} cm^{-2}
		Partially bleached	79.04	34.87	7	µg x 10^{-6} cm^{-2}

Figure 4.6. Boxplots showing significant zooxanthellae losses from (a) type C2 branches, (b) compared to no significant losses from type D branches and (c) no significant losses from type C2/D branches of *Acropora millepora* in February 2006, at the peak of a bleaching event. Most C2 colonies had recovered to pre-bleaching, algal cell densities by August 2006; six months after the bleaching. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.

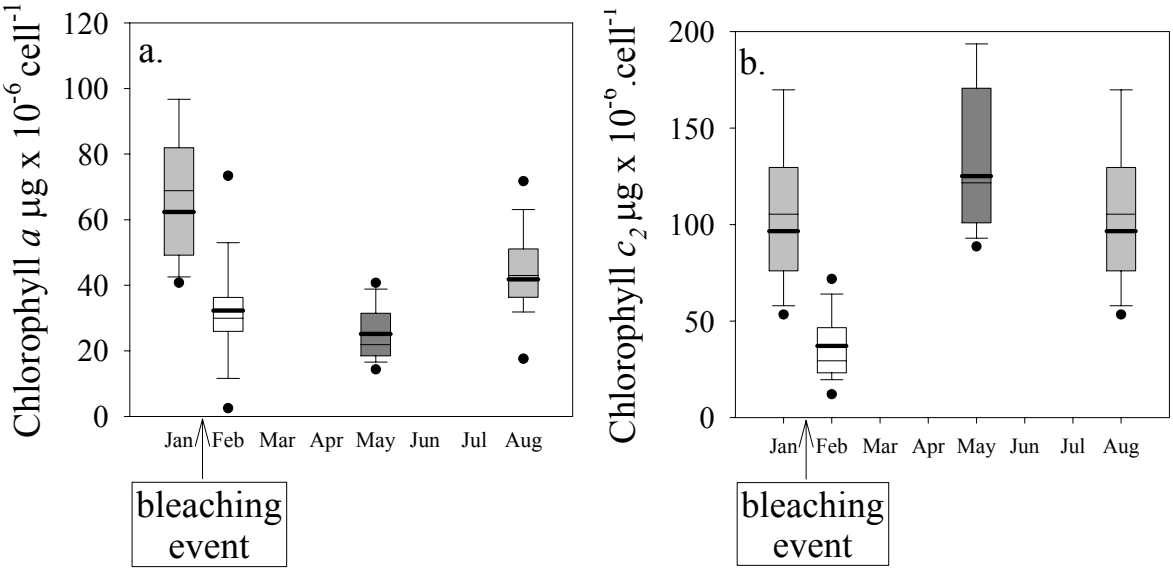


Bleaching effects on algal chlorophyll levels

In the year following the bleaching, the levels of chlorophyll *a* in algal cells of *A. millepora* branches varied significantly with respect to monthly sampling occasion (Figure 4.7 a, Table 4.14, 4.16). In February 2006, at the peak of the bleaching, algal chlorophyll *a* levels had fallen to less than half the levels at the onset of the bleaching in January 2006 ($p < 0.05$). Chlorophyll *a* levels were at their lowest in May 2006, three months after the bleaching ($p < 0.05$) but had begun to recover by August 2006 ($p < 0.05$).

Algal chlorophyll c_2 levels varied significantly with monthly sampling occasion (Figure 4.7 b, Table 4.14, 4.16). By January 2006 at the onset of the bleaching, algal chlorophyll c_2 levels had increased >three fold compared to levels in July 2005. However, within a month, at the peak of the bleaching in February 2006, and irrespective of their predominant symbiont genotype, zooxanthellae densities in colonies had fallen by 66% ($p < 0.05$). Three months after the bleaching, algal chlorophyll c_2 levels had risen to higher than pre-bleaching levels ($p < 0.05$), and remained high up to six months after the bleaching ($p < 0.05$). In spite of a lack of significant interaction between month and bleaching status in the statistical analysis, in February 2006, the zooxanthellae left in completely bleached type C2 colonies still had higher algal chlorophyll c_2 levels than the zooxanthellae in unbleached type D colonies ($p < 0.05$).

Figure 4.7. Boxplots showing the significant effect of month on (a) algal chlorophyll *a* and (b) algal chlorophyll c_2 in branches of *Acropora millepora* sampled after a bleaching in February 2006. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



Bleaching effects on lipids

The bleaching event had a profound effect on the lipids of *A. millepora* colonies irrespective of symbiont genotype compared to lipid levels in the year before the event (Figure 4.8, Table 4.17, 4.18). In the year after the bleaching lipids reached their lowest levels of the year in May 2006, three months after the peak of the bleaching in February 2006 ($p < 0.05$). Bleached colonies (irrespective of post-bleaching symbiont genotype) regained 41% of their lipids by August 2006, six months after the bleaching ($p < 0.05$). Lipid levels did not revert to their pre-bleaching levels during the study.

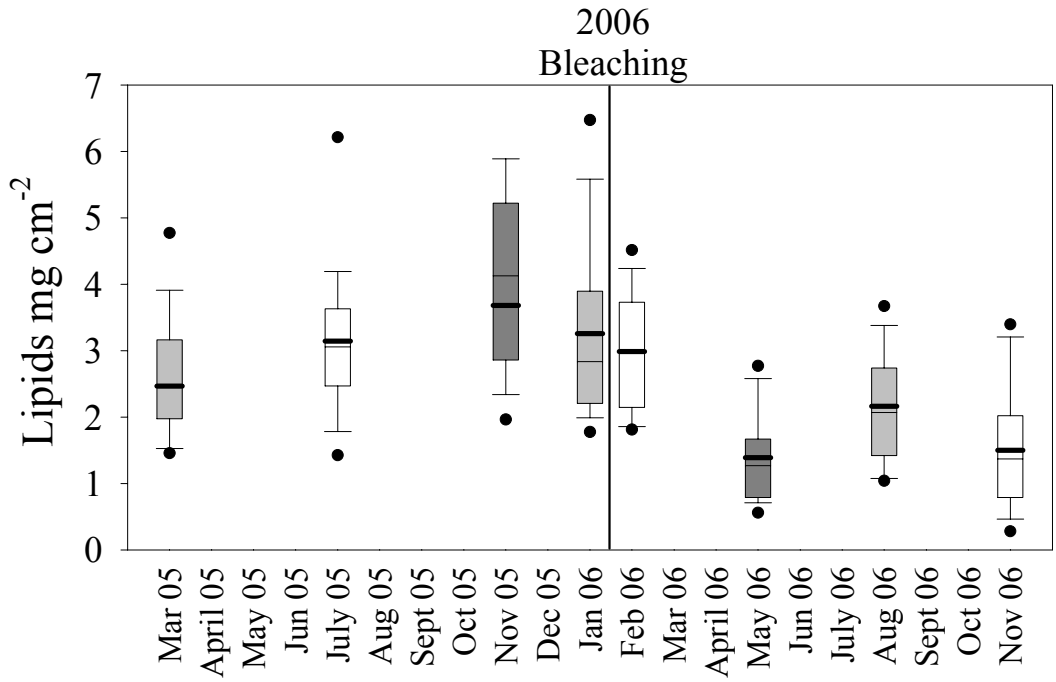
Table 4.17. Means and standard deviations of the lipid content of *Acropora millepora* colonies with C2, D and C2/D symbionts in the year after a bleaching event.

Month	Bleaching status	Mean	S.D.	N	units
January	Bleached	3.68	1.56	12	mg cm^{-2}
	Unbleached	3.14	1.46	12	mg cm^{-2}
	Partially bleached	2.79	1.06	8	mg cm^{-2}
February	Bleached	2.90	0.78	12	mg cm^{-2}
	Unbleached	3.33	0.93	12	mg cm^{-2}
	Partially bleached	2.61	1.01	8	mg cm^{-2}
May	Bleached	1.01	0.61	11	mg cm^{-2}
	Unbleached	1.65	0.66	10	mg cm^{-2}
	Partially bleached	1.79	0.82	4	mg cm^{-2}
August	Bleached	1.73	0.69	12	mg cm^{-2}
	Unbleached	2.37	0.84	11	mg cm^{-2}
	Partially bleached	2.58	1.33	7	mg cm^{-2}

Table 4.18. The results of a single factor ANOVA showing the significant effect of month (Mar, July and Nov 2005, and Jan, Feb, May, Aug and Nov 2006) on the lipid content of *Acropora millepora* branches during a two year study.

	S.S	df	Mean Square	F	Sig.
Corrected Model	113.37[a]	7	16.20	8.5	0.000
Intercept	1925.51	1	1925.51	1013.2	0.000
Month	113.37	7	16.20	8.5	0.000
Error	486.50	256	1.90		
Total	2683.21	264			
Corrected Total	599.87	263			
a. $R^2 = 0.189$ (Adjusted $R^2 = 0.167$)					

Figure 4.8. Boxplots showing the signification variation in lipid levels of *Acropora millepora* branches in the year before and after a natural bleaching event. Lipids reached their lowest levels in May 2006, three months after the bleaching but levels had significantly recovered by August 2006, six months after the bleaching. Boxplots in November 2005 and 2006 denote pre-spawning lipids. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



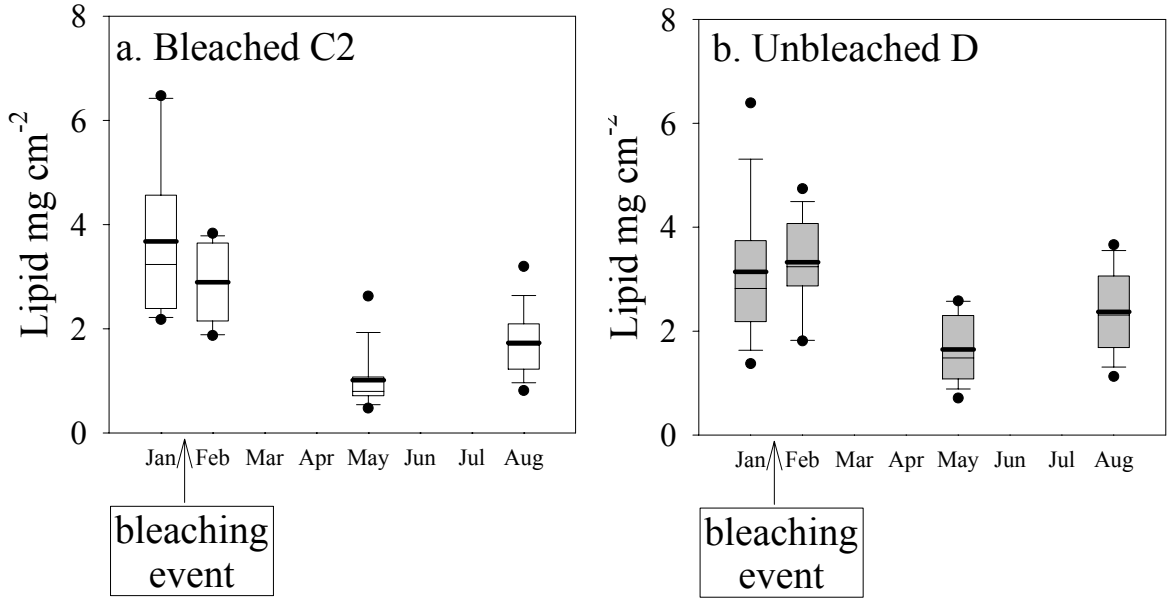
Only the lipid content of bleached type C2 colonies decreased significantly between February and May (Figure 4.9 a, Table 4.19). In January 2006, the lipid levels of bleached C2 colonies were 15% higher than lipids in type D colonies (ns) which reflected the trend observed in summer before the bleaching (March 2005). In February 2006 at the peak of the bleaching, lipids in bleached C2 colonies were 13% lower than the levels in January (ns). However, three months after the peak of the bleaching, in May 2006, the lipid levels of bleached type C2 colonies had fallen to

65% of February levels ($p < 0.05$). Unbleached type D (Figure 4.9 b) and partly bleached C2/D (not shown) colonies had also lost half their lipid content between February and May (ns).

Table 4.19. The results of a repeated-measure ANOVA showing the significant effect of month (Jan, Feb, May, Aug) on the lipid content of *Acropora millepora* branches in the year after a bleaching event. There was no significant interaction between month and bleaching severity (which correlated with predominant symbiont type before the bleaching).

	SS	df	Mean Square	F	Sig.
Month	34.74	2	20.32	13.1	0.000
Month * Bleaching	6.56	3	1.92	1.2	0.311
Error (Month)	55.55	36	1.55		

Figure 4.9. Boxplots showing the loss of significant quantities of lipids from branches of (a) bleached C2 (b) unbleached type D colonies of *Acropora millepora* in the year after a bleaching event (type C2/D colonies are not shown). Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



Seasonal lipid losses

There were significant lipid losses between summer in the year before bleaching and spring in the year after bleaching and between winter before and after bleaching (Table 4.11, Figure 4.20, 4.21). Lipids were 52% lower in spring after the bleaching than in summer before the bleaching ($p < 0.05$) and 41% lower in winter in the year after the bleaching compared to winter before the bleaching ($p < 0.05$). There was also a significant interaction between bleaching severity and the seasonal lipid content of branches collected before and after the bleaching. The lipid content of bleached C2 colonies was 67% lower in spring (May 2006), three months after the bleaching, than in summer (March 2005) before the bleaching ($p < 0.05$) in spite of the trend for higher lipids in cooler months in 2005. The lipids of bleached C2 colonies were 55% lower in winter (August 2006), six months after bleaching than in winter (July 2005) before the bleaching ($p < 0.05$). In contrast, after the bleaching unbleached type D colonies had only 30% lower spring lipids (ns) and 12% lower winter lipids (ns) compared to the levels in the year before bleaching.

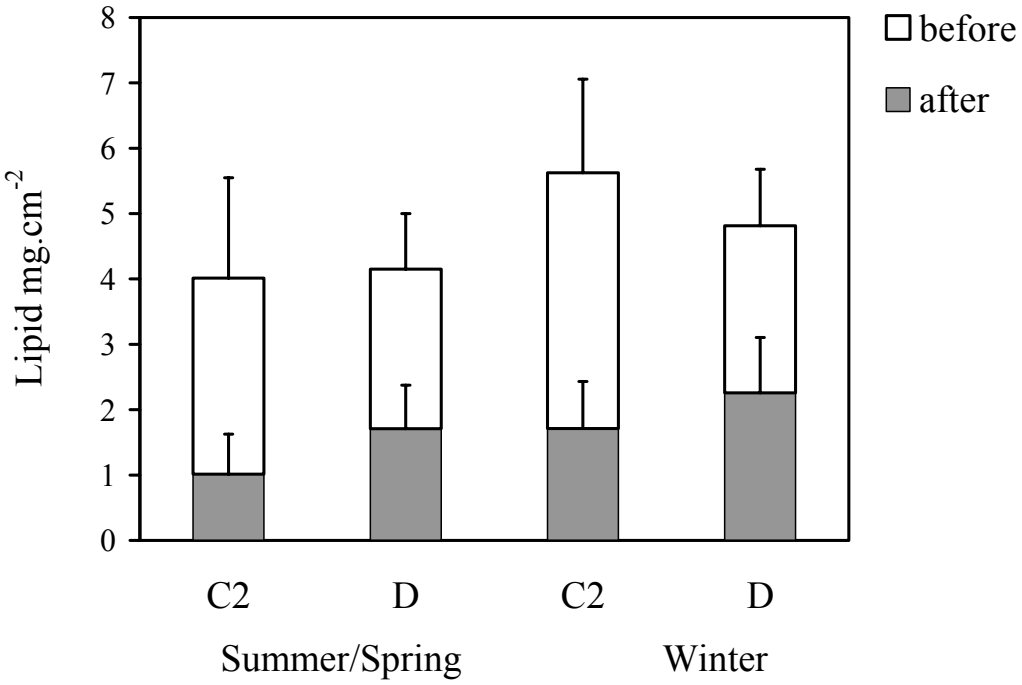
Table 4.20. Means and standard deviations of the seasonal lipid content of *Acropora millepora* colonies in the year after a bleaching event. Bleached colonies all contained C2 symbionts and unbleached colonies all contained D symbionts before the bleaching.

Year	Season	Bleaching severity	Mean	S.D.	N	units
Before bleaching	Summer	Bleached C2	3.05	1.47	12	mg cm^{-2}
		Unbleached D	2.35	0.86	12	mg cm^{-2}
	Winter	Bleached C2	3.82	1.40	11	mg cm^{-2}
		Unbleached D	2.70	0.82	10	mg cm^{-2}
After bleaching	Spring	Bleached C2	1.01	0.61	12	mg cm^{-2}
		Unbleached D	1.65	0.66	12	mg cm^{-2}
	Winter	Bleached C2	1.73	0.69	12	mg cm^{-2}
		Unbleached D	2.37	0.84	11	mg cm^{-2}

Table 4.21. The results of a repeated-measures ANOVA showing the significant effects of season (summer before, spring after, winter before and winter after bleaching) and bleaching severity (bleached, unbleached) and the significant interaction between the two variables on the lipid content of branches of *Acropora millepora*. Only bleached C2 and unbleached D colonies were used in the analysis.

	SS	df	Mean Square	F	Sig.
Season	40.24	2	18.69	14.8	0.000
Season * Bleaching	13.94	2	6.47	5.1	0.009
Error (Season)	48.92	39	1.26		

Figure 4.10. Stacked bar chart showing the significant losses of lipid from bleached type C2 and non-significant losses from unbleached type D colonies of *Acropora millepora* in spring and summer, and winter before and after a natural bleaching event in 2006. White bars represent the mean lipid content of branches sampled before the bleaching (2005) and grey bars represent branches sampled after bleaching (2006). Error bars represent ± S.D.



Bleaching effects on reproduction

The lipid stores and egg number in polyps of *A. millepora* colonies, irrespective of predominant symbiont type, were severely affected by the bleaching (Figure 4.12, Table 4.22, 4.23). Just before the annual spawning in November 2005, *A. millepora* branches had 62% more lipids than pre-spawning levels after the bleaching ($p < 0.05$). The number of eggs in the polyps of *A. millepora* branches in the year after bleaching was 46% less than in the year before the bleaching (Figure 4.11 b, $p < 0.05$). There was no significant effect of bleaching on the egg volume in *A. millepora* polyps (Figure 4.11 c). The significant interaction between symbiont type and year (Table 4.23) was a result of the significant differences in egg volume of *A. millepora* colonies with C2 and D symbionts before the bleaching (Figure 4.5 a, $p < 0.05$) but did not reflect differences in egg volume as a result of bleaching severity (simple pairwise comparisons using Sidak's adjustment for multiple comparisons).

Table 4.22. Means and standard deviations of the reproductive output (egg number, egg volume and total lipid content) of *Acropora millepora* colonies in the year after a bleaching event. Bleached colonies all contained C2 symbionts, unbleached colonies all contained D symbionts and partially bleached colonies contained both symbiont types before the bleaching. Only lipid content before the spawning is shown because a rain event killed all the colonies in the study.

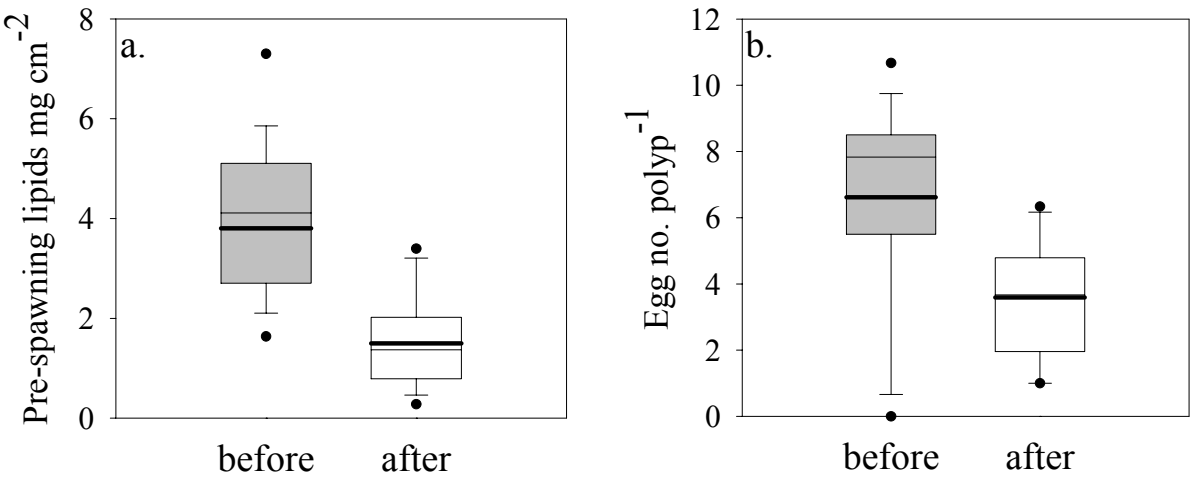
Measure	Bleaching status	Mean	S.D.	N	units
Egg number	Bleached	3.70	1.85	12	
	Unbleached	3.82	1.57	13	
	Partially bleached	2.97	2.15	5	
Egg volume	Bleached	0.08	0.01	12	mm^3
	Unbleached	0.08	0.01	13	mm^3
	Partially bleached	0.08	0.01	5	mm^3
Lipids before	Bleached	1.48	1.11	12	mg cm^{-2}
	Unbleached	1.50	0.55	13	mg cm^{-2}
	Partially bleached	1.53	1.15	5	mg cm^{-2}

Table 4.23. The results of a multivariate ANOVA showing the significant effect of year (before or after bleaching) on the lipids and egg number and the significant interaction between year and predominant symbiont type (which correlated with bleaching severity (type, C2 = bleached, D = unbleached and C2/D = partially bleached) on the volume of egg bundles in polyps of *Acropora. millepora*.

	Dependent Variable	SS	df	Mean Square	F	Sig.
Corrected Model	Lipids	78.10[a]	5	15.62	8.3	0.000
	Egg number	141.00[b]	5	28.19	4.0	0.004
	Egg volume	0.01[c]	5	0.00	2.7	0.033
Intercept	Lipids	376.27	1	376.27	200.0	0.000
	Egg number	1175.97	1	1175.97	166.6	0.000
	Egg volume	0.27	1	0.27	572.0	0.000
type	Lipids	6.91	2	3.45	1.8	0.171
	Egg number	8.44	2	4.22	0.6	0.554
	Egg volume	0.00	2	0.00	1.9	0.161
year	Lipids	58.53	1	58.53	31.1	0.000
	Egg number	116.37	1	116.37	16.5	0.000
	Egg volume	0.00	1	0.00	0.5	0.474
type * year	Lipids	4.67	2	2.34	1.2	0.299
	Egg number	11.69	2	5.85	0.8	0.443
	Egg volume	0.00	2	0.00	3.5	0.037
Error	Lipids	86.56	46	1.88		
	Egg number	324.78	46	7.06		
	Egg volume	0.02	46	0.00		
Total	Lipids	633.26	52			
	Egg number	1948.44	52			
	Egg volume	0.34	52			
Corrected Total	Lipids	164.66	51			
	Egg number	465.74	51			
	Egg volume	0.03	51			

a. $R^2 = 0.474$ (Adjusted $R^2 = 0.417$)
b. $R^2 = 0.303$ (Adjusted $R^2 = 0.227$)
c. $R^2 = 0.225$ (Adjusted $R^2 = 0.141$)

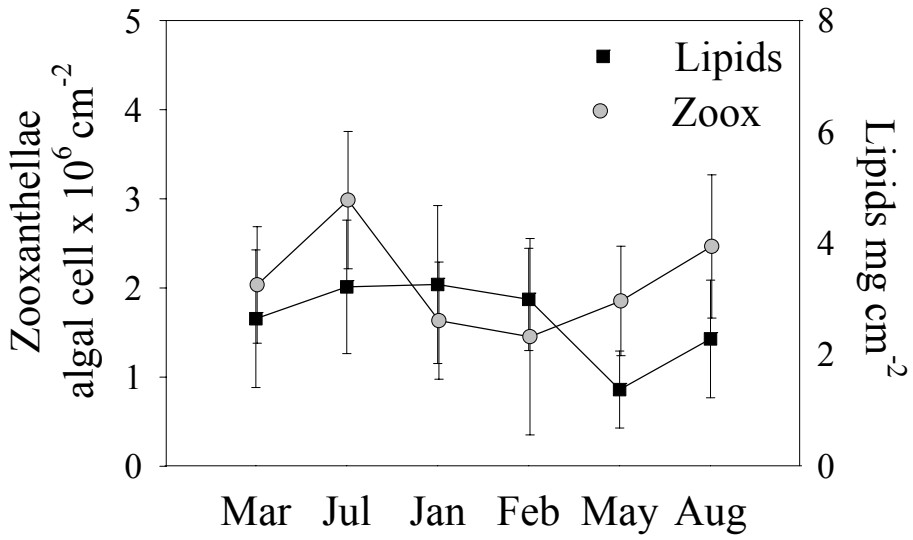
Figure 4.11. Boxplots showing the effects of bleaching on the (a) total branch lipids and (b) egg number in polyps of *Acropora millepora* colonies before the annual spawning in the year before and after a bleaching event in 2006. Before = Pre-spawning in November 2005, After = Pre-spawning in November 2006, 9 months after the bleaching event. White boxes represent branches sampled before the bleaching and grey boxes represent branches sampled after the bleaching. Box boundaries represent the 75th and 25th percentiles. Thick lines within the boxplots represent the mean and thin lines represent the median. Whisker bars above and below the boxes represent the 95th and 5th percentiles. Missing and out of range values are not shown. Dots represent data that fall outside the confidence limits.



Correlation between lipids and zooxanthellae densities

Symbiont densities were positively correlated with lipid levels in *A. millepora* colonies (Figure 4.12).

Figure 4.12. Scatter and line plot showing the three monthly lag of the lipid stores of *Acropora millepora* colonies and zooxanthellae densities in the year from March 2005 to August 2006, before and after a natural summer bleaching event in February 2006.



DISCUSSION

The energy stores and reproduction of some reef-building corals are likely to be significantly compromised with acclimatisation to climate change. Shuffling symbiont type from *Symbiodinium* type C2 to type D may result in greater resistance to heat stress (Berkelmans, Ray & van Oppen 2006) but the trade-off will be reduced energy for the holobiont (coral + zooxanthellae + bacteria). The results of this study show that corals that host thermally tolerant type D symbionts have lower lipid stores than those with thermally sensitive type C2 symbionts under normal conditions on the reef. Corals with type D symbionts also contained lower levels of storage lipids in the lead up to the annual mass spawning than C2 corals; leading to lower gamete size (egg volume). A significant *Symbiodinium* community shift from thermally sensitive type C2 to thermally tolerant types D and C1 symbionts occurred in *A. millepora* colonies at Miall Island following severe bleaching in 2006 (Chapter 1). In contrast to a study by Michalek-Wagner *et al.* (2000; 2001) , the effects of symbiont genotype and bleaching severity on lipids and gamete production in *A. millepora* colonies were masked by the much greater effects of heat stress on all corals, irrespective of symbiont type. Lipid stores were reduced by 40% for up to nine months compared to the year before bleaching. Nine months after the bleaching, pre-spawning lipids, and egg size and number were 50-60% lower as a result of the bleaching. These results compare well with the differences in pre-spawning lipids and egg number and size observed for the soft coral *Lobophytum compactum* (Michalek-Wagner & Willis 2000, 2001) 8 months after a bleaching event. Michalek-Wagner *et al.* found that severe bleaching (i.e. the

loss of 93% of zooxanthellae) caused a 41% reduction of pre-spawning lipids, 59% reduction in egg size, and a five month delay in egg development. Eight out of 10 severely bleached corals failed to spawn 8 months after the bleaching. Although the positive correlation between lipid losses and zooxanthellae losses were mirrored in the study by Michalek-Wagner *et al.*, unbleached corals were not significantly affected. In contrast, even *A. millepora* with type D symbionts, that appeared unaffected by heat stress at the peak of the event, had significantly reduced lipid stores for at least 9 months following bleaching. The experiment was terminated before there were signs of recovery; however, the study by Michalek-Wagner *et al.* suggests reproductive output may have recovered by the next spawning (i.e. 20 months after the bleaching).

One possible explanation for the lower lipids and reproduction to *A. millepora* with type D symbionts may lie in the photobiology of the symbionts. In Chapter 2, rapid light curves (RLC's) were used to show that *A. millepora* branches with type D symbionts have 41% lower maximum relative rate of electron transport through PSII and 38% lower light absorption capacity compared to type C2 corals. The ATP and NADPH (adenosine-5'-triphosphate and nicotinamide adenine dinucleotide phosphate-oxidase) produced during photosynthesis are used as chemical energy to drive carbon fixation through the Calvin Cycle. The simple carbon molecules that are produced are then incorporated into more complex carbohydrate, amino acid and lipid molecules. These photokinetics are similar to that of freshwater micro algae (Sato *et al.* 1996) and heat tolerant plants (Hugly *et al.* 1989; Vigh *et al.* 1985). Reduced photosynthetic activity may be either a consequence or a cause for increased thermal tolerance in the

symbionts. While the exact link between symbiont photosynthesis and holobiont thermal tolerance is still unclear, it seems likely that thermal tolerance comes at a physiological cost to the holobiont as it does in heat tolerant algae and higher plants.

As a result of reduced photosynthetic activity, type D symbionts would subsequently fix less carbon that type C2 symbionts, resulting in lower levels of these carbon molecules to their host coral (Battey & Patton 1984; Patton & Burris 1983). These results support those of Loram *et al.* (2007) who demonstrated that, in the sea anemone *Condylactis gigantea,* thermally tolerant type A symbionts fix less carbon than thermally sensitive type B symbionts. Loram *et al.* (2007) also showed that in symbioses with type A, more photosynthetically fixed carbon was incorporated into lipids and amino acids than in type B symbioses, suggesting that the lipid stores of thermally tolerant symbioses are more important to the host than thermally sensitive symbioses. This aspect of Loram's study is not supported by this study as corals with type D symbionts had lower lipids than corals with type C2 symbionts under normal conditions on the reef, there were no differences in total protein, and both C2 and D corals had reduced lipid stores after the bleaching. To confound the issue, there is now evidence that the symbiosis between *Symbiodinium* type A and its coral host is more parasitic than symbiotic (Stat, Michael, Morris & Gates 2008). The host characteristics can confound direct inferences between whole colony macromolecular composition and symbiont thermal tolerance. The photosynthetic mechanisms for increased thermal tolerance in type D symbionts may be the underlying cause of the lower lipid stores in the intact symbiosis under normal conditions, but host factors may

also play a part in reducing the amount of photosynthetically fixed carbon available to the coral host.

Lower algal chlorophyll *a* pigment may be partly responsible for the differences between type C2 and D lipids in the year before the bleaching. Algal chlorophyll *a* content was 22% lower in type D corals, exactly matching the 23% lower lipids. As chlorophyll *a* is the light harvesting pigment of PSI and PSII, the lower levels could result in lower light absorption, leading to reduced photosynthetic activity and carbon fixation. In Chapter 2, it was suggested that lower light absorption is linked to the increased thermal tolerance of type D symbionts. There was a 23% anomaly in the algal chlorophyll *a* levels for type D and C2 corals in Chapter 2 These results confirm this link and the suggested implication that type D corals would have reduced lipids as a trade-off for increased thermal tolerance, partly due to lower light absorption capacity.

The lower storage lipids of type D predominant colonies are linked to the significantly lower reproductive output compared to C2 corals. Although the relationship between storage lipids and gonad development is not straightforward, there is evidence that a major proportion of the lipids produced throughout the 9 months of gonad development are used for egg and sperm development (Rossi *et al.* 2006; Stimson, J. S. 1987). Broadcast spawning reef corals release bundles of eggs and sperm in the beginning of spring, just after winter when corals have the highest symbiont densities and photosynthetic pigments (chlorophyll a and c2, Fitt *et al.* 2000). During these

cooler months algal densities and photosynthesis are at an annual maximum and host respiration is lower, resulting in increases in the production of triglycerides and lipids. It is during these months that a proportion of the lipids and triacylglycerides produced by the symbionts are diverted to the development of the egg and sperm for reproduction (Grottoli, Rodrigues & Juarez 2004; Oku, H. *et al.* 2002; Yamashiro *et al.* 1999). Michalek-Wagner *et al.* (2000; Michalek-Wagner & Willis 2001) observed that egg production and lipids were positively correlated with symbiont densities, confirming the suggested link between reduced symbiont densities in *A. millepora* with type D symbionts and lower lipids. Since type D colonies have lower levels of storage lipids in the months before spawning it is reasonable to expect that they would have less available to divert to reproduction at spawning although there is no evidence that they retain a greater proportion of their lipids than C2 colonies.

Corals that have both *Symbiodinium* type C2 and type D have physiological characteristics that are either intermediate between the monomorphic symbioses or similar to type D symbioses. This study shows that hosting type C2 symbionts are more physiologically beneficial to the coral than type D symbionts at non-stressful temperatures. One might expect that in polymorphic symbioses the less effective type D symbionts would eventually be excluded from the association by natural selection. However, when temperatures increase above the coral's normal range, hosting type D symbionts gives the coral an ecological advantage (Berkelmans, Ray & van Oppen 2006). Although hosting only type C2 maximises the energy budget of the coral under normal conditions, if temperatures increase, the host already has levels of the more

thermally tolerant type D symbionts that can proliferate and become more predominant. While hosting mixed symbiont types reduces the energy budget of the coral under optimal conditions, it also acts as insurance if environmental conditions change (Belda-Baillie, Baillie & Maruyama 2002; Douglas 1998).

In spite of the shortfalls in storage lipids and reproductive output in thermally tolerant corals with type D symbionts, there were no significant differences in soluble proteins. The products of algal photosynthesis and nitrogen recycling (Lewis, C. L. 1989) also include glucose (carbohydrates, Mews 1980), phospholipids, sterols and amino acids (Falkowski, Dubinsky & Muscatine 1993; Lewis, D. H. & Smith 1971) the latter which are transferred to the host for the synthesis of proteins (Muscatine & Lenhoff 1965). The carbon products that are derived from algal photosynthesis are usually nitrogen-poor (Davies 1984; Falkowski *et al.* 1984). Proteins, phospholipids and sterols are used as structural materials in the cell wall and as such, total soluble protein is a rough measure of tissue biomass (Edmunds & Gates 2002). However, the host coral also produces some of the amino acids essential for synthesising protein itself by externally acquired nitrogen (Ben-David-Zaslow & Benayahu 2000; Burris 1984; Falkowski, Dubinsky & Muscatine 1993; Hoegh-Guldberg, O. & Williamson 1999; Titlyanov *et al.* 1998). This alternate and more plentiful source of protein may explain the lack of differences in the total soluble proteins of type C2 and D corals. This is also supported by a study by Bachar *et al.* (2007) of sea anemones which may suggest that the autotrophically derived carbon from the symbionts in reef corals is also

diverted to lipids but not to the more complex materials such as proteins and membranes.

Differences in the amounts of short term carbohydrate stores that are related to symbiont genotype may be difficult to quantify in reef corals because of their transient nature. Photosynthetic carbon fixation in the Calvin Cycle initially produces simple carbon compounds (triacylglycerides) which are used to synthesise more complex carbohydrates. These carbohydrates are quickly incorporated into more lipids, phospholipids and sterols or used as an energy source for algal respiration. Measuring the total carbohydrate content of branches with different symbiont types is complicated by the transitory and temporary nature of such low molecular weight compounds and the speed at which these are continuously metabolised in respiration or incorporated into more complex molecules (Whitehead & Douglas 2003). Therefore, although there may be significant differences in the precursor compounds of protein and carbohydrates due to differences in the photosynthetic differences of C2 and D symbionts, these are difficult to quantify without first separating coral and zooxanthellae.

Previous studies of reef corals have shown significantly detrimental effects of bleaching on lipid stores, irrespective of bleaching severity. During bleaching, decreases in algal symbiont densities and photosynthetic pigments result in a net loss of photosynthesis (Lesser, M. P. 1997; Warner, M. E. *et al.* 2002). Until the symbionts re-populate the coral's tissues, some corals may have to survive on their stored energy

reserves. Without these the coral would effectively starve. As the tissue biomass of some corals has been shown to decrease after summer when zooxanthellae numbers and pigments are at their lowest levels, the structural proteins of cell walls represent a potential source of energy following bleaching (Fitt *et al.* 2000). In addition, small quantities of carbohydrates may also be consumed for short-term energy requirements (Fitt *et al.* 1993). However, by far the primary source of energy is the stored lipids which comprise up to 40% of the dry weight of the coral's tissues (Harland, A D *et al.* 1993; Stimson, J. S. 1987; Yamashiro *et al.* 1999). As corals use the stored reserves for metabolism and cellular repair, the lipid, protein and carbohydrate levels are depleted. Grottoli *et al.* (2004) found that bleaching reduced lipids, proteins and carbohydrates by 39-73% for up to 5 months. It is evident that the *A. millepora* colonies in this study used their stored lipids (and possibly carbohydrates and proteins) as an energy source for at least three months following the bleaching. In a study of bleached *Pocillopora damicornis, Montipora verrucosa and Porites lobata,* Spencer-Davies (1991) showed that lipid stores can last from one to several months. Although some bleached corals can feed heterotrophically to replace photosynthetic carbon, it is evident from the partial recovery of lipid levels three months after recovery of zooxanthellae densities that the *A. millepora* colonies with C2 symbionts were unable to replenish their lipid stores completely through heterotrophy. However, by August, six months after the bleaching there were signs of recovery of the lipid stores, but not to pre-bleaching levels. This is evidence that at least in the short term; *A. millepora* at this site relies heavily on photosynthetically fixed carbon from their endosymbionts and may not be able to make the full transition to heterotrophy to supplement their energy requirements by feeding on

zooplankton and particulate matter following bleaching. This supports the suggestion of Grottoli *et al.* (2004) that some branching corals may be unable to make a full transition from autotrophy to heterotrophy (Anthony, K. & Fabricius 2000).

The energetic and reproductive advantages of hosting type C2 *Symbiodinium* over type D colonies were lost as a result of the detrimental effects of bleaching on all colonies, irrespective of symbiont genotype. The clear difference in bleaching resistance of C2 and D colonies was demonstrated in Chapter 1 when all *A. millepora* colonies with predominantly C2 symbionts bleached white and some colonies later died. The preferential loss of symbionts from C2 colonies resulted in decreases in stored lipids three months after the bleaching (May 2006). This is consistent with the results of other studies that have shown decreases in the total lipids (Grottoli, Rodrigues & Juarez 2004) and reproductive output (Baird & Marshall 2002; Mendes & Woodley 2002; Szmant & Gassman 1990) of reef corals as a result of bleaching. The three month lag between symbiont loss and reduced lipid stores is also consistent with a study by Fitt *et al.* (2000) who found that peaks or troughs in tissue biomass also lagged three months behind those of zooxanthellae densities. The temporary loss of photosynthesis causes the coral to starve, possibly metabolising stored lipids and proteins for survival. However, all colonies, irrespective of symbiont type lost significant amounts of stored lipids as a result of the bleaching. Type D colonies, in spite of not suffering significant zooxanthellae losses at the peak of the bleaching, also had lower lipid stores up to 9 months after the bleaching. One possible explanation for

this may lie in the concept of the 'greedy' algal partners (Mark Warner, personal communication). Thermally tolerant symbionts may retain a proportion of their photosynthetically fixed carbon during stressful conditions in order to proliferate and re-populate the space left by expelled thermally sensitive symbionts. This ensures their own survival (and that of their hosts) but effectively starves the coral. Partitioning resources to cope with adversity is a recognised mechanism for stress tolerance in plants (Wahid *et al.* 2007). While this seems an unlikely cause for differential lipid stores and reproduction under normal conditions, it is possible that it plays some part in the loss of lipid stores and reproductive output from unbleached colonies following bleaching.

The zooxanthellae pigments in *A. millepora* followed exactly the same patterns as a study by Fitt *et al.* (2000) in the Bahamas, increasing to a maximum in the cooler, winter months (July 2005 and August 2006) and decreasing to a minimum when temperatures and light levels were at their highest levels in summer and symbiont numbers are lowest. Other studies have also confirmed this negative correlation between zooxanthellae densities and temperature (Glynn *et al.* 1993; Hoegh-Guldberg, C. & Smith 1989b; Lesser, M P *et al.* 1990) and light levels (Falkowski & Dubinsky 1981; Stimson, J 1997) in a range of reef corals. In the same way that the tissue biomass of the corals in the study by Fitt *et al.* (2000) peaked three months after zooxanthellae densities peaked, the lipids in the *A. millepora* colonies also peaked and then declined within the three months of zooxanthellae in both years. This is evidence that, to some extent, the lipid stores of *A. millepora* colonies are dependent on the

presence of the symbionts (Oku, Hirosuke, Yamashiro & Onaga 2003) as low symbiont densities were also accompanied by lower tissue biomass (protein) and carbohydrate levels in the summer months.

The lipid stores of reef corals can be affected by a number of other factors, such as host respiration, host factors, photodamage and photoinhibition. Type D colonies lost similar amounts of their lipid stores to C2 colonies which lost almost all their zooxanthellae. If lipid stores are totally dependent on photosynthesis then unbleached type D colonies should retain their normal lipid content after the bleaching. Increased host respiratory demands could contribute to lower lipid, protein and carbohydrate stores during summer months when host cell metabolism increases as a result of increased temperature (Fitt *et al.* 2000; Muller-Parker 1987a). As the respiration rate of reef corals can double during summer, this places an extra demand on the stored energy reserves. This could also help to explain the lower lipid content of *A. millepora* colonies that appeared unaffected by bleaching. If the host's respiratory demands remained high throughout the bleaching (and possibly longer as a result of the stress), just as in summer, lipids could be metabolised faster than the rate of repopulation of the zooxanthellae. The second possibility is that the zooxanthellae photosystems were not coping with high temperatures in combination with light (Jones, R. J. *et al.* 1998). Reversible uncoupling of normal photosynthetic function can occur as a result of higher than normal temperatures in combination with high light, resulting in a net decrease in photosynthesis, even in corals with thermally tolerant type D symbionts (Hill, Ross & Ralph 2006; Rowan 2004a). The net effect is a loss of autotrophic

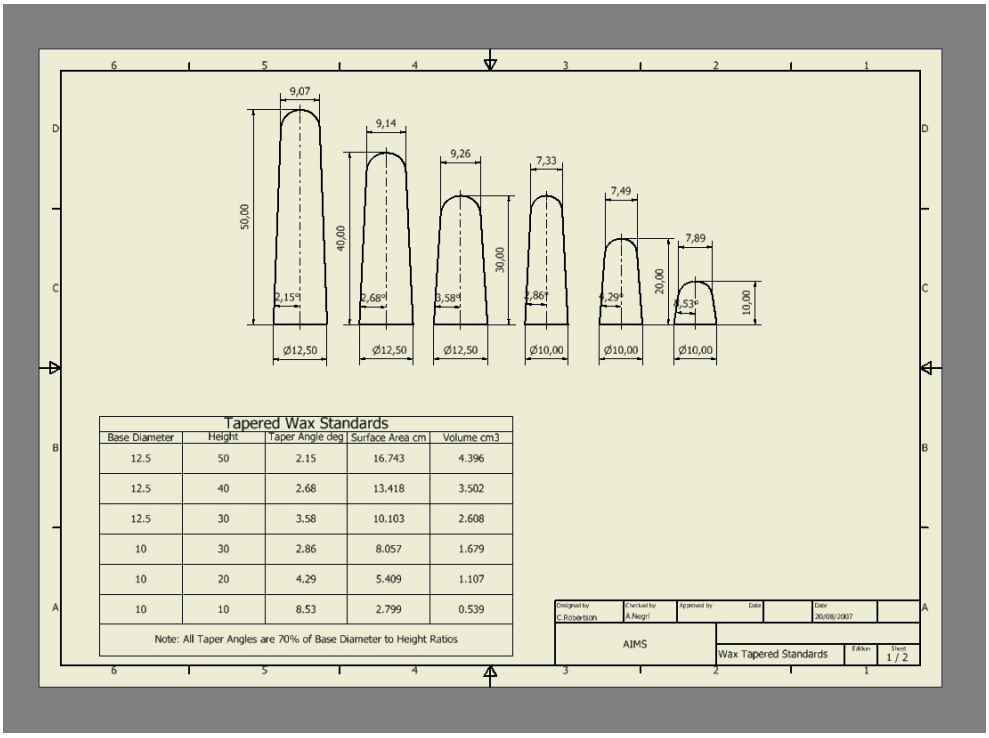
capacity (Hoogenboom, Anthony & Connolly 2006), possibly leading to reduced lipid stores as these are used for metabolism by the coral. This is supported by Loram *et al.* (2007) who found reduced carbon fixation rate but increased translocation rates in sea anemones with thermally tolerant type B symbionts. Another potential explanation for the decreased in lipids in type D corals is that the triacylglycerides produced by photosynthesis are not made available to the coral during temperature stress. This is the concept of the 'greedy' partner (Mark Warner, personal communication) whereby the symbionts retain a proportion of their photosynthetically fixed carbon for survival and repopulation. This is not supported by Loram *et al.* (2007) who found increased translocation to the host by thermally tolerant type B symbionts. It remains unclear exactly what factors contributed to the lower lipid stores of type D colonies in the year following the bleaching. What is clear is that symbiont thermal tolerance does not protect coral from the detrimental but sub-lethal effects of the heat stress itself; especially in terms of bioenergetics.

SUMMARY

Symbiont community shifts to more thermally tolerant types like *Symbiodinium* type D are likely to compound the detrimental effects of the bleaching event itself, further compromising coral fitness as conditions warm with climate change. Predicted increases in sea temperatures as a result of global warming (Meehl *et al.* 2007) suggest that within the next 50 years temperatures will be at levels high enough to cause widespread annual bleaching (Donner *et al.* 2005; Hoegh-Guldberg, O. *et al.* 2007) that may result in entire reefs undergoing permanent changes to more thermally tolerant types. This study provides some insights into two of the physiological trade-offs for corals as they acclimatise to warmer conditions; lipid stores and reproduction. If entire communities of reef-building corals bleach and recover with predominantly thermally tolerant symbionts and these shifts become permanent (e.g. Magnetic Island Berkelmans, Ray & van Oppen 2006), then the trade-offs of depressed lipids and reproduction that come with symbiont change are likely to further compound the detrimental impacts of the heat stress event that originally caused the change.

Chapter 5

PHYSIOLOGICAL RESPONSES OF *ACROPORA MILLEPORA*: A 3D MODELING METHOD TO CALCULATE THE SURFACE AREAS OF CORAL BRANCHES



Engineering drawings of the tapered models used to verify a 3D image analysis method of determining the surface area of small coral branches. The cones were designed using Autodesk Inventor® (http://usa.autodesk.com) with known surface areas and prepared using an automated lathe (Mazak, 15 M) to a tolerance of 10 µm. Photograph courtesy of Andrew Negri, AIMS.

ABSTRACT

The quantification of physiological and biochemical parameters in coral branches require normalisation to a stable factor, such as the tissue biomass or surface area. Three dimensional (3D) animation software (Gmax®) was evaluated for estimating the surface area of simple coral branches. The software was highly predictive of the known surface areas of small (20–60 mm long) plastic rods and cones ($r^2 = 0.99$), and of small (30–60 mm) *A. millepora* branches ($r^2 = 0.98$) whose surface area had been obtained using the traditional wax-weight method. Two normalisation parameters, 3D modeled surface area and tissue biomass (measured as protein), were then compared for *A. millepora* branches collected in summer and winter. In winter, protein and surface area were correlated ($r^2 = 0.61$), but not in summer, indicating that choice of normalising parameter will influence the outcome of experimental analyses.

INTRODUCTION

Branches and fragments of corals are often sampled from adult colonies in the field to assess physiological and biochemical responses to stresses, such as elevated sea-surface temperatures. Coral branches are also used in manipulative aquarium experiments to examine the impacts of individual stressors under controlled conditions. Symbiont density, pigment concentration, and lipid content are key measures of fitness and response to environmental change in scleractinian corals, but they need to be normalised to some attribute of the sample to account for the varying quantities of material used among samples. Surface area and tissue biomass are frequently used in

normalisation but the research hypothesis requires careful consideration before deciding on the most appropriate option (Edmunds & Gates 2002). Tissue biomass of coral branches and fragments are often estimated by measuring protein content as a proxy (Edmunds & Gates 2002), while popular techniques for estimating surface area include wax-weight (Stimson, J. & Kinzie 1991), latex wrapping (Meyer & Schultz 1985), dye dipping (Hoegh-Guldberg, O. 1988), and foil wrapping (Marsh 1970). Although valid, these surface area techniques are often awkward and may not be suitable when branches or fragments need to be destroyed to extract the protein, lipid or other biochemical components within the sample (Brown, B E *et al.* 2002; Cantin, Negri & Willis 2007; Leuzinger, Anthony & Willis 2003).

The surface areas of small (relatively flat) juvenile corals have been estimated using 2D image analysis software (Negri *et al.* 2005). Image analysis by 3 dimensional (3D) reconstructions has been used as a non-invasive method for measuring the surface areas and growth of branching (Kruszyński, Kaandorp & van Liere 2007; Vago, R. *et al.* 1994) or foliate corals (Rahav *et al.* 1991), and the surface area of non-branching massive corals (Bythell, Pan & Lee 2001; Cocito *et al.* 2003). X-ray computed tomography equipment is able to handle complex morphologies, even accounting for cavities in samples (Kruszyński, Kaandorp & van Liere 2007), but the equipment is not available to most researchers and is unlikely to be required for coral branches with simple morphologies. The fine-scale morphologies (calcite and septal levels) of corals can also be accurately quantified by applying fractal methods to microscopy images of corals and their sections (Martin-Garin *et al.* 2007). Although reliable, these techniques

can be time consuming and complex, often requiring multiple images taken at specific angles and accurate camera calibration (Bythell, Pan & Lee 2001). An alternative method applicable to determine the surface area of small and simple coral branches is to model the surface area using digital images combined with simple 3D animation software. Two or three images are taken on a grid of preset dimensions for reference and calibration. The software uses circular or elliptical splines (curves that join two or more points) placed around the image of a coral branch. The diameter, angle, and position of each spline are manually adjusted to the dimensions of the coral image and a simple text program (script) computes the surface area. Imaging does not require complex camera equipment and can be very rapid, avoiding deterioration of the coral tissue. In this study, the surface areas of simple coral branches were calculated using 3D animation software (Gmax®) and validated against plastic models of known surface area. The surface areas generated were compared with those obtained using the commonly used wax-weight method (Stimson, J. & Kinzie 1991). The tissue biomass (measured as soluble protein) of *A. millepora* branches were then measured and compared to 3D modeled surface areas to examine the seasonal consistency between these common normalising parameters.

MATERIALS AND METHODS

Surface area methods

Surface area estimates by 3D animation modeling (Figure 5.1) were initially validated against simple plastic rods and cones of known surface area and against the wax-weight method (Stimson, J. & Kinzie 1991) for plastic rods and simple coral branches.

PVC rods (Length = 28–60 mm, Diameter = 10 mm; see insert, Figure 5.2 a-c) were accurately prepared and their dimensions verified using callipers. Cones were prepared mimicking simple coral branches (10–12 mm base diameter and 20–50 mm length), tapering linearly to 70% of the base diameter with curved tips (see insert, Figure 5.2 b). The cones were designed using Autodesk Inventor® (http://usa.autodesk.com) with known surface areas and prepared using an automated lathe (Mazak, 15 M) to a tolerance of 10 μm. Five 26–45 mm (length) branches of the Indo-Pacific corymbose scleractinian coral *A. millepora* (Ehrenberg 1834, see insert Figure 5.2 d) were also prepared for surface area determination by stripping the tissue for the sample in a sealed plastic bag containing 2 ml of filtered seawater using compressed air.

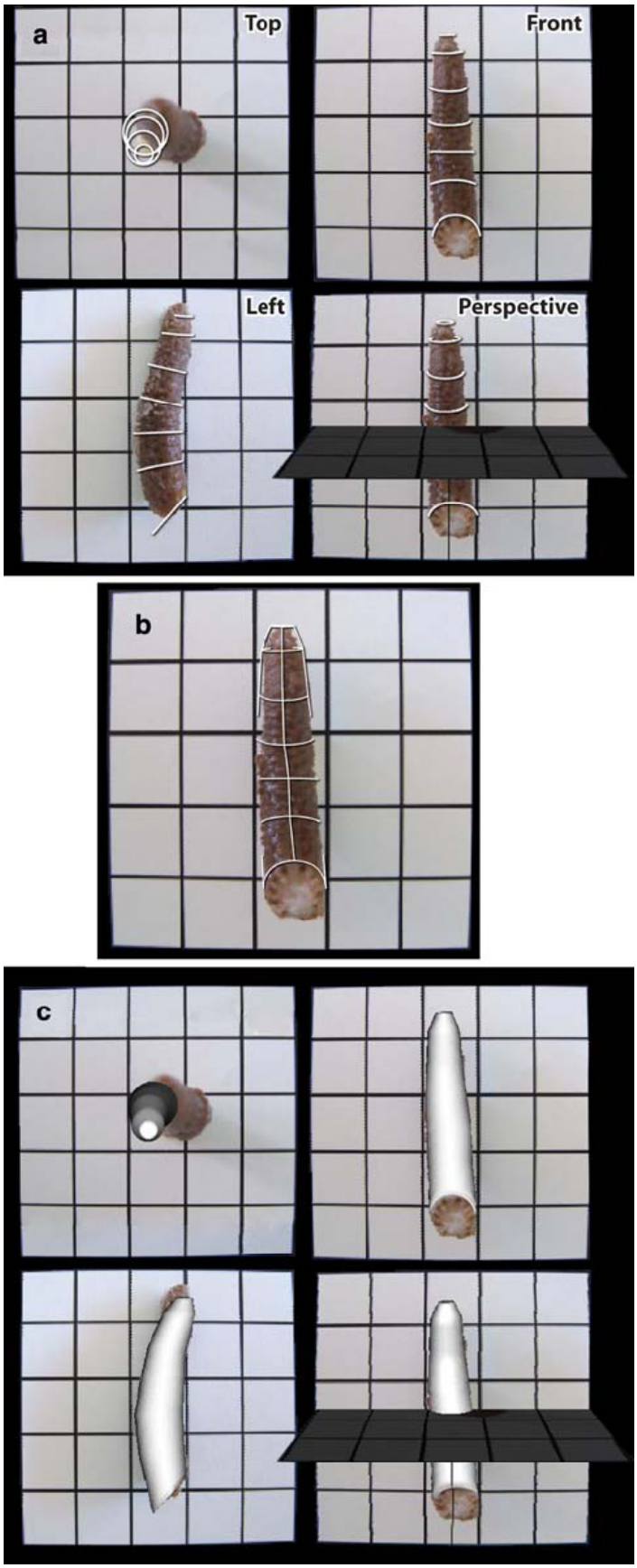


Figure 5.1. Gmax® surface area method. (a) Coral branch with 5 x 5 cm grid in each of the four views (1 cm^2). One image of the 'front', one of the 'side' (90° from the first view) and one image of the 'top' of the coral branch were used to manually arrange digital splines (curves that join two or more points) around the branch by manual dragging and rotation. (b) Circles or ellipses were added and then converted to editable splines forming a virtual net over the branch. (c) The net was modified to show the cross section and surface and then these were joined with an opaque (white) net of microscopic triangular surfaces. Finally, the Maxscript subprogram 'surfacearea.ms' was executed to calculate the surface area of the entire net of triangles.

Two images were taken of each subject on a 5 x 5cm grid (1 cm^2) at a distance of 15 cm from the subject (Figure 5.1 a) using a digital camera on a tripod. A third image could be taken from above (top) but this was optional. The first image was the 'front' perspective of the sample and the second image or 'left' perspective was obtained after turning the sample 90° in a clockwise direction. Circles or ellipses were added to the image using the freeware program Gmax (which is now available as part of Autodesk 3Ds Max, http://usa.autodesk.com) (Figure 5.1 a). The circles and ellipses were then converted to 'splines' forming a virtual net over the branch (Figure 5.1 b). The net was modified to show the cross section and surface and then the edges of the net were joined with an opaque (white) net of microscopic triangular surfaces (Figure 5.1 c). Finally, the Maxscript subprogram 'surfacearea.ms' (downloadable from www. aims.gov.au/gmaxsurfacearea) was executed to calculate the surface area of the entire net of triangles. The wax-weight surface area method was performed as per Stimson and Kinzie (1991).

Protein analysis of field samples

Branches from *A. millepora* colonies at Miall Island, southern Great Barrier Reef (23° 090′ S, 150° 540′ E) were removed by levering them off with a narrow blade in March 2005 (late summer, n = 22) and July 2005 (winter, n = 28). After collection, samples were snap-frozen in liquid nitrogen and stored at -80°C until processed. The surface area of each sample was determined using Gmax® 3D digital image analysis as described above. Protein analyses were performed on dried, ground whole coral tissue samples including zooxanthellae and skeleton. The samples were ground under liquid

nitrogen (Brown, B. E. *et al.* 2002) to produce a fine homogenous powder and placed in acetone-washed and pre-weighed sintered glass vials (25 ml). The vials containing the frozen ground samples were freeze–dried and accurately reweighed. The total dry weight (DWT) was recorded as the difference between the weight of the vial and the weight of the sample plus vial. A subsample of known weight (~0.1–0.5 mg) was removed for analysis of total soluble protein. Total protein was determined by the Folin phenol reagent colorimetric detection and quantification of total protein method by Lowry *et al.* (1951) using bovine serum albumin (BSA, Sigma) as the standard (Appendix IV).

STATISTICAL ANALYSIS

Surface area methods and relationships between protein and surface area were tested using model II regressions (Riggs, Guarnieri & Addelman 1978; Webb *et al.* 1981). A paired sample t-test was used to compare the ratio of protein/3D image-derived surface area in 18 branches collected from the same *A. millepora* colonies collected in both summer and winter.

RESULTS AND DISCUSSION

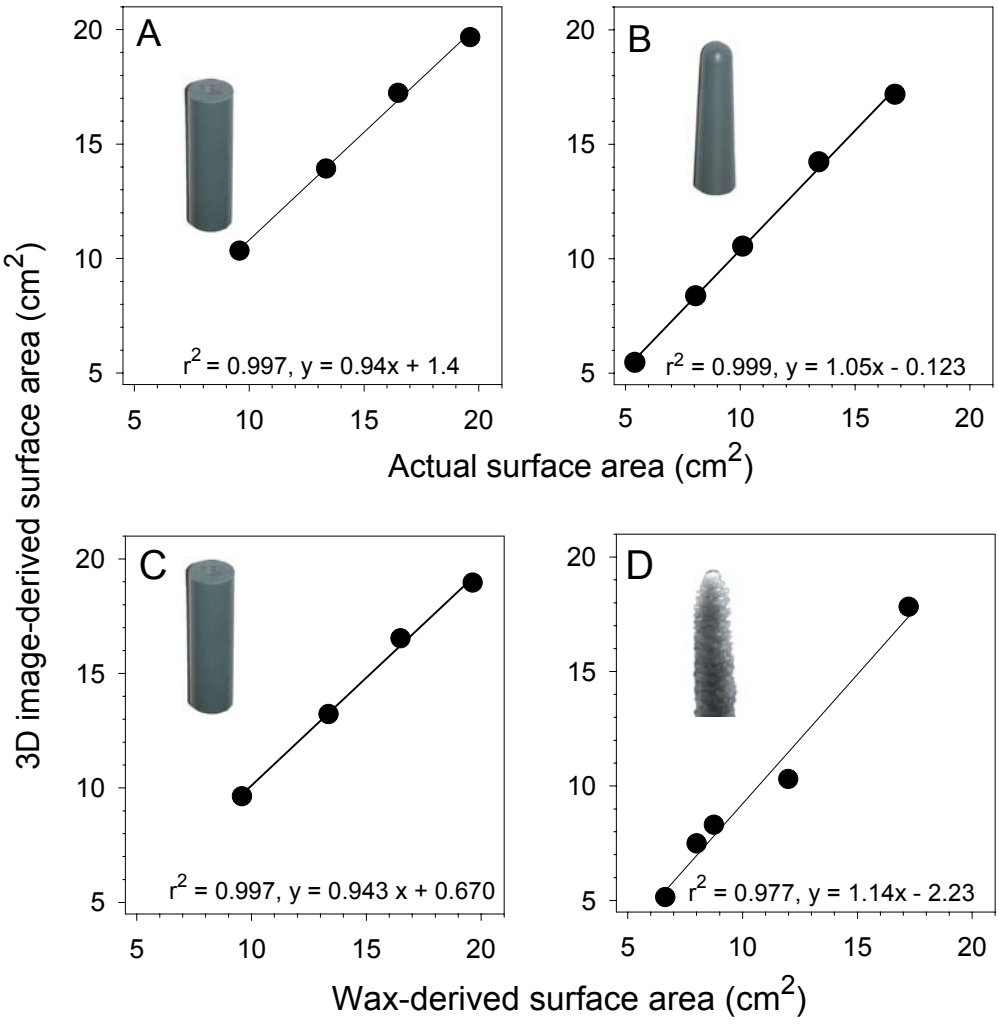
The relationships between the 3D image-derived surface areas and known surface areas of small plastic rods and cones with curved tips were both well correlated (r^2 = 0.997, and 0.999, respectively) and linear (Figure 5.2 a, b). These results indicate that 3D modeling provides accurate estimates of surface area for small and simple objects of similar size and shape to simple coral branches. The 3D image-derived surface areas

also correlated well with wax-weight surface area estimates for cylinders ($r^2 = 0.997$) and small coral branches ($r^2 = 0.977$) (Figure 5.2 c, d), indicating that the modeling works equally well for corals with the rugosity of *A. millepora* as it does for smooth surfaces. Finally, a comparison between the 3D modeled surface areas and biomass, a second normalisation parameter, revealed a good linear correlation ($r^2 = 0.61$, $p < 0.05$) in winter samples, but this relationship broke down in branches collected in summer; this has important implications for the choice of normalisation parameters.

Surface area estimations by 3D modeling are applicable to both live specimens from aquarium experiments and frozen samples from the field, since all required images (2–3) of the coral branches could be taken very rapidly (<15 s). The 3D modeling and surface area calculation by Gmax was also relatively rapid after some practice (5–10 min per sample). The program used was freeware and many similar 3D modeling programs are commercially available and are used by recreational modelers of all ages. In addition, no expensive or unusual hardware is required. Although the simple coral branches used in this study are easy to collect from many species, and are often used in manipulative experiments, some applications might require the use of branches or fragments with more complex morphologies. This does not pose any technical problems for 3D surface area modeling. No further photographs are required for more complex morphologies (such as two or three subbranches); however, the fitting of circles and ellipses to samples with multiple branches will be more time consuming and, as for most surface area methods, may introduce greater uncertainty. For very

complex morphologies (e.g., branching, fused or overlapping branches), a subset of branches could be modeled separately and the surface areas combined.

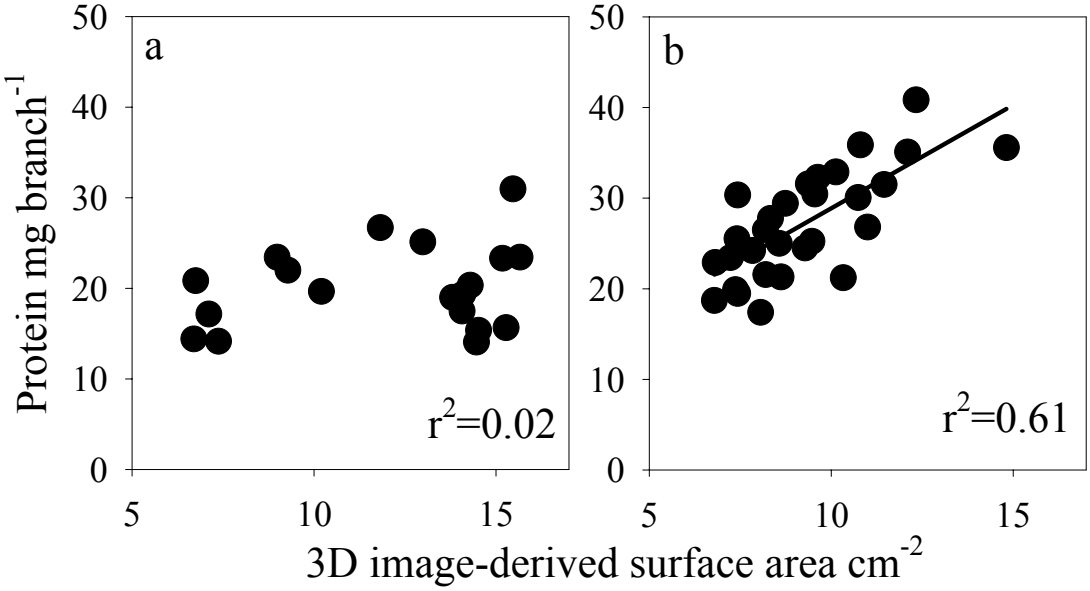
Figure 5.2. Linear regressions between actual and 3D image analysis-derived surface areas of (a) PVC cylinders and (b) curved PVC cones, and between wax-weight-derived surface areas and image analysis-derived surface area of (c) PVC cylinders and (d) simple branches of *Acropora millepora*. All regressions were significant: $p < 0.05$ in all cases.



Tissue biomass (measured as protein content) is commonly used as an alternative to surface area as a normalising parameter for quantifying biochemical and physiological measurements in corals (Edmunds & Gates 2002). The protein content of branches collected in winter was 3.1 ± 0.5 mg cm^{-2} (mean ± S.D.), 72% higher than for branches collected from the same colonies in summer with a protein content of 1.8 ± 0.6 mg cm^{-2} ($t = -4.26$, $p < 0.05$). This indicates significantly greater biomass per 3D modeled surface area in the colder months. Similar seasonal patterns in total tissue biomass (measured by ash-free dry weight) have been observed for multiple coral species (Fitt *et al.* 2000). The key drivers of the patterns in tissue biomass are seasonal variations in the intensity of light and temperature on the physiology of the animal host. Seawater temperatures steadily increase from the spring into the late summer and autumn, which has the potential to nearly double the coral's respiratory metabolism, resulting in a significant decrease in the energy reserves within coral tissue (Fitt *et al.* 2000; Jokiel & Coles 1990). During the cooler winter months reduced host respiration places less demand upon energy reserves, resulting in increases in the incorporation of excess glycerol and triacylglycerides into cell-wall proteins (Spencer-Davies 1991), thus increasing the total tissue biomass within a coral colony (Fitt *et al.* 2000). Symbiont densities and their photosynthetic capacities usually reach a maximum during cooler winter months (Fagoonee *et al.* 1999) prior to the observed spring peak in tissue biomass, suggesting a functional relationship between these parameters which results in the observed peak in energy acquisition by the host in cooler months (Fitt *et al.* 2000; Warner, M. E. *et al.* 2002).

The protein content of branches collected in winter exhibited a good linear relationship with 3D modeled surface area (r^2 = 0.61, Figure 5.3 b), indicating both parameters would be suitable for normalising the quantity of other biochemical measures such as total lipid during this season. However, this relationship was not evident in branches collected over summer (Figure 5.3 a). It is possible that the rapid summer metabolic rates (Fitt *et al.* 2000; Jokiel & Coles 1990) and the temporary dispersal of symbiont densities may vary to a greater extent between coral colonies with different symbiont types and between subpopulations of zooxanthellae in each polyp (Apprill, A. M. & Gates 2007). This might result in greater variability in biomass per unit area than in winter. The seasonal cycle of tissue biomass in corals and its greater variability in summer, highlight the importance of matching normalisation parameter with experimental hypothesis (Edmunds & Gates 2002). For instance, normalisation of lipid content or symbiont density to surface area may be preferred for seasonal comparisons, since variation in tissue biomass over the same period may complicate the interpretation of the measurements. Alternatively, the quantification of antioxidant enzyme and stress protein production in corals is better normalised to soluble protein (Brown, B. E. *et al.* 2002), as these cellular responses are more functionally relevant to biomass. Taking two or three rapid digital images of coral branches for later 3D modeled surface area measurements is an ideal way to either replace standard surface area measurements and/or to complement biomass normalisation in live or snap-frozen samples.

Figure 5.3. Relationships between biomass (soluble protein) and 3D modeled surface area of *Acropora millepora* branches collected in (a) summer and (b) winter.



GENERAL DISCUSSION

Acclimation to climate change is likely to reduce the physiological fitness of reef corals that change their predominant symbiont communities to host thermally tolerant types like *Symbiodinium* type D following bleaching. Under normal conditions on the reef, hosting type D symbionts was shown to reduce the lipid stores, growth and reproduction of *A. millepora* colonies ~30% compared to those that host type C2 symbionts. This discrepancy in fitness is linked to the increased thermal tolerance of type D symbionts which had ~30-40% lower photosynthetic activity than C2 symbionts. This depressed photosynthetic function was evidenced during their photokinetic response to increasing light as lower electron transport, lower light absorption capacity and reduced maximum PSII potential. However, the magnitude of the effects on coral fitness that originated from symbiont identity and photokinetics was small in comparison to the adverse effects of the bleaching itself which caused the symbiont community change in the first place. Bleaching reduced coral fitness irrespective of symbiont type by up to 50-60%, leading to reduced growth, lipid stores and reproduction for nine months, and possibly longer. Although some corals may be able to acclimatise to the warmer conditions that are predicted to occur as a result of climate change by changing their predominant symbionts, the mechanisms that underwrite the symbionts ability to cope with heat stress and the very conditions that instigate the changes, will act synergistically in reducing coral fitness.

A. millepora colonies can change their *Symbiodinium* communities from thermally sensitive type C2 to more thermally tolerant type D and C1 following bleaching. A

71% shift in symbiont community at Miall Island occurred by a number of interrelated processes, partly as a result of bleached colonies surviving and recovering with type D and C1 symbionts (42%) and partly as a result of selective mortality of the more bleaching-sensitive C2-predominant (37%) colonies (Baker, A. C. 2003). The change is most likely to have occurred by 'shuffling' (i.e. up-regulation of symbionts already in the coral tissue) because cloning and sequencing revealed the presence of low levels of D and C1 symbionts in colonies before the bleaching (Jones, A. M. *et al.* 2008) and because shuffling is more easily explained by mathematical models (Jones, R J 1997). The presence of low level, 'background' C1 and D before the bleaching strongly supports the shuffling theory as this predicted their appearance in colonies after the preferential loss of C2 symbionts. The observations of strikingly differential thermal tolerance between C2, and D and C1 colonies during the 2006 bleaching, together with the experimental evidence of Berkelmans and van Oppen (2006) who found that differences in thermal tolerance in *A. millepora* from the same area are driven by symbiont type rather than the host coral, suggest that the symbiont community shift, if permanent, is likely to have increased the thermal tolerance of the *A. millepora* community by 1.0-1.5 °C (Berkelmans, Ray & van Oppen 2006). However, signs of a drift back to C2 symbionts within six months after the bleaching suggest that the change is only temporary as the population will eventually drift back to pre-bleaching symbiont types (Thornhill, D. *et al.* 2006).

The mechanisms for thermal tolerance in type D symbionts appear to be linked to the depression of photosynthesis and consequently to the reduction in host processes that

depend on photosynthate translocation. Although the exact mechanisms for thermal tolerance in type D *Symbiodinium* are still unclear, it seems likely that they are similar to those found in heat tolerant micro algae and plants. The most likely of these similarities may be the stability and composition of the thylakoid membranes of the chloroplasts. Studies by Hugly *et al.* (1989) and Vigh *et al.* (1985) have shown that altered membrane structure can result in a lower rate of electron transport through PSII. This correlates with the relative maximum rate of electron transport in type D symbionts. Reduced chlorophyll *a* content led to lower light absorption capacity in Hugly's plant mutants which corresponds with that of type D symbionts in this study. These anomalies in photosynthetic function also led to reduced growth rates in heat tolerant algae (Sato *et al.* 1996) and plants (Hugly *et al.* 1989; Laing, Greer & Schnell 1995) in a similar way that hosting type D symbionts leads to reduced growth in *A. millepora*. There was also evidence of the role of reversible photoinhibition in the heat tolerance of type D symbionts. Rowan (2004a) found increased photoinhibition *Pocillopora verrucosa* and *P. damicornis* with type D symbionts compared to those with type C symbionts during heat stress. Rowan concluded that photoinhibition of photosynthesis operated to reduce the excitation pressure of light on PSII during heat stress resulting in an effective depression of autotrophy. This may have been the cause of reduced growth and lipid stores in the *A. millepora* colonies with type D symbionts which survived with no apparent effect after the bleaching event. Regardless of the mechanism of thermal tolerance, it is clear that increased thermal tolerance under normal conditions comes at a cost, namely reduction in the translocation of photosynthetically fixed carbon from the symbiont to the coral host.

Symbiont community change comes at a physiological cost to the host due to the depression of photosynthesis leading to lower energy reserves and slower growth. The 41% lower electron transport capacity, 33% lower maximum quantum yield and 38% lower light capture efficiency capacity found in *A. millepora* corals with type D explants in Chapter 2 are the underlying cause of the reduced physiological fitness of the *A. millepora* colonies demonstrated in Chapters 3 and 4 of this study. Depressed photosynthesis translated to ~30% lower daily growth rate (measured by calcification), 23% lower stored lipids and 22% lower reproductive output for type D corals under normal conditions in the field. To date, studies of the photo-physiology of thermally tolerant symbiont types have shown reduced carbon fixation at temperatures above 30 °C (Loram, Trapido-Rosenthal & Douglas 2007; Rowan 2004a) and under irradiance stress (Hoogenboom, Anthony & Connolly 2006) but no studies have linked the effects of symbiont thermal tolerance to adult coral fitness under normal conditions in the field. Storage lipids in corals are closely linked to the autotrophic fixation of carbon through the Calvin Cycle following photosynthesis by the algal symbionts (Harland, A. *et al.* 1991; Kellog & Patton 1983; Oku, Hirosuke, Yamashiro & Onaga 2003; Yamashiro *et al.* 1999). a great proportion of these lipids are eventually utilised in the production of eggs and sperm for reproduction (Rossi *et al.* 2006; Stimson, J. S. 1987). The lower electron transport capacity found in D explants in the laboratory would result in D symbionts producing less chemical energy in the form of ATP and NADPH molecules during photosynthesis for the purpose of fixing carbon into glycerol and triacylglycerides (Crossland *et al.* 1980; Falkowski, Dubinsky & Muscatine 1993). Type D symbionts would subsequently translocate less stored glycogen and lipid in the form of fat droplets to their host coral (Battey & Patton 1984; Lewis, D. H. & Smith

1971; Patton, Abraham & Benson 1977; Patton & Burris 1983; Trench, R. K. 1971) resulting in less lipids and triacylglycerides available for storage and the production of eggs and sperm during reproduction. Calcification in reef-building corals is strongly correlated with the level of light and symbiont photosynthesis (Barnes & Chalker 1990; Pearse & Muscatine 1971; Vago, E., Gill & Collingwood 1997). Although the exact mechanism is still unclear, it is likely that depressed photosynthetic function would also depress calcification. A study by Little *et al.* (2004) was the first to show the link between symbiont thermal tolerance and reduced growth in juvenile corals. Although the growth differences in Little's study were 10 to 20 times greater than the differences found between adult with C2 and D symbionts, both studies clearly show that increased thermal tolerance comes at a significant cost to coral growth. Therefore the underlying cause for the physiological disparity between corals with thermally sensitive and thermally tolerant symbionts is depressed photosynthesis leading to reduced photochemical energy production and lower carbon fixation.

During summer, when water temperatures and light levels are high, the physiological differences between *A. millepora* with type C2 and D *Symbiodinium* are less evident. Temperature- and light-induced reductions in zooxanthellae densities (Fitt *et al.* 2000) and uncoupling of photosynthetic function (Hill, Ross & Ralph 2006; Rowan 2004a; Warner, M. E. *et al.* 2002) during summer are characteristic of reef corals during summer months. Combined with an increase in host metabolism and respiratory demand on energy supply from the symbionts (Fitt *et al.* 2000; Muller-Parker 1987b), the net result is reduced holobiont productivity (Hoogenboom, Anthony & Connolly

2006). Prolonged temperatures, similar to those found during a typical summer at Miall Island (www.aims.gov.au/pages/facilities/adc/seatemps.html), therefore reduce net energy production in all corals, irrespective of symbiont type. These factors negate the enhancing effects of temperature on lipid synthesis through increased cell metabolism and calcification (Marshall, A. T. & Clode 2004; Yap, Montebon & Dizon 1994). The net effect is that the differences in growth and energy reserves of C2 and D colonies of *A. millepora* are less evident during the summer months on the reef flat.

Levelling of the physiological differences also occurs following temperature-induced bleaching which had a differential effect on C2 and D corals. Although bleaching may present an opportunity for corals to acclimatise to environmental stress by shuffling to host algal partners that are more suited to the environment, increased thermal tolerance as a result of symbiont community change, even if it persists, will not necessarily translate into increased resilience. Bleaching had a detrimental effect on the growth and energy reserves of *A. millepora* up to 9 months after the event and irrespective of symbiont genotype or bleaching status. The explanation for this apparent anomaly may be the direct effects of heat stress on the photosystems of type D *Symbiodinium*. In spite of retaining their symbionts, type D colonies had reduced calcification and depleted their lipid stores following the event. This seems incongruous as studies have shown that corals of this species with type D symbionts can cope with temperatures 1.0–1.5 °C above their normal temperature range (Berkelmans, Ray & van Oppen 2006). A number of explanations are offered for this anomaly. The first is the effects of photoinhibition which has been shown to depress autotrophy in a similar way to

reduced light levels (Rowan 2004a). The second is that host respiratory demands increase as a result of stress, reducing the energy available for growth, lipid storage and reproduction. The third is the concept of the 'greedy' partners proffered by Mark Warner (personal communication) whereby stress tolerant symbionts may retain a proportion of their photosynthetically fixed carbon following a stress event in order to proliferate as space becomes available. Regardless of the reason or reasons for the seemingly enigmatic reduction in coral fitness in spite of a lack of bleaching response, it is clear that bleaching reduces coral fitness regardless of symbiont genotype. The adoption of thermally tolerant symbiont types, combined with the depressive effects of prolonged temperature on photosynthesis may therefore act synergistically in reducing coral fitness following bleaching.

Corals that have both *Symbiodinium* type C2 and type D have physiological characteristics that are either equal to or intermediate between the monomorphic symbioses which may be ideal in the context of climate change-induced temperature rise. This study shows that hosting type C2 symbionts are more beneficial to the host than type D and C1 symbionts. Rather than being excluded from the symbiosis by natural selection, type D and C1 *Symbiodinium* remain present at levels below the detection of most electrophoretic methods. However, when temperatures increase above the coral's normal range, the presence of type D and C1 symbionts gives the coral an ecological advantage because the levels of these more thermally tolerant types can be up-regulated to re-populate the coral tissue thereby maintaining photosynthetic translocation to the host. Corals that host predominantly C2 symbionts but have

background type D and C1 symbionts have the ecological benefits of hosting both. Type C2 maximizes the energy budget of the coral under normal conditions but if temperatures increase, the host already has low levels of the more thermally tolerant type D and C1 symbionts that can proliferate and become more predominant. Hosting mixed symbiont types does not reduce the effectiveness of either type but results in physiological characteristics that are intermediate between the effects of hosting only one and acts as insurance if environmental conditions change (Belda-Baillie, Baillie & Maruyama 2002; Douglas 1998).

The results show an increase in the diversity of symbionts after bleaching together with a considerable change in the make-up of the symbiont community within individual colonies over time scales as short as three months. These results suggest that the population of *A. millepora* at Miall Island can acclimatise to temperatures up to 1.0-1.5°C above their normal range. Baird *et al.* (2007) hypothesised that symbiont community shuffling to type D may persist only as a result of enduring changes in environmental conditions, e.g. repeated warm summers. This may be evident at Magnetic Island (lat. 19.2°S, long. 146.9°E), where temperatures regularly exceed 30.5 °C during most summers (Berkelmans, R. 2002) and *A. millepora* have harboured exclusively type D symbionts over many years (Berkelmans, Ray & van Oppen 2006; van Oppen, M. J. H. *et al.* 2001). Conditions similar to those currently occurring at warm reefs such as Magnetic Island have been projected to occur on in the southern Great Barrier Reef by 2020–2030 (Done *et al.* 2003). Symbiont community shifts to more thermally tolerant types like *Symbiodinium* type D may be an adaptive trait of

reef corals that helps them cope with changes in their environment (Buddemeier, R.W *et al.* 2004). But rather than ensuring coral resilience to bleaching, the change acts in synergy with the direct effects of heat exposure on the association, reducing coral fitness irrespective of symbiont type. Predicted increases in sea temperatures as a result of global warming (Meehl *et al.* 2007) suggest that within the next 50 years temperatures will be at levels high enough to cause widespread annual bleaching (Donner *et al.* 2005; Hoegh-Guldberg, O. *et al.* 2007). If these temperature changes are gradual and incremental then this may result in entire reefs changing permanently to more thermally tolerant types like *Symbiodinium* type D. Although type D corals may cope better with the next bleaching event, a year is obviously not long enough for complete recovery from the direct effects of heat stress before the next bleaching event. If entire communities of reef-building corals bleach and recover permanently with predominantly type D symbionts, then rather than ensuring resilience to climate changes, the trade-off of depressed symbiont photosynthesis that accompanies increased heat tolerance may mean that surviving corals are less able to recover fully between annual summer bleaching events.

THESIS SUMMARY

Acropora millepora colonies at Miall Island host *Symbiodinium* ITS1 type C2, C1 and D within single colonies before the bleaching in 2006. All symbiont types had multiple ITS1 variants as identified by cloning and sequencing a subset of the samples in the study.

Under normal conditions, *A. millepora* colonies form a stable association with type C2 *Symbiodinium* however occasional colonies host type D and type C1 is present in some colonies below 5–10%. The symbiont community was stable before the bleaching in February 2006.

Following a natural bleaching event in February 2006, there was a major shift to thermally tolerant *Symbiodinium* types D and C1 in the *A. millepora* colonies at Miall Island. All colonies that hosted type C2 symbionts bleached white in February 2006. C2 colonies suffered higher mortality (37%) than type D colonies (8%) six months after the bleaching. The population changed from 93% type C2 before the bleaching to 71% type D and C1 after the bleaching.

A. millepora corals with type D symbionts have 41% lower relative maximum electron transport rate, 38% lower photon capture efficiency, 33% lower dark-adapted F_v/F_m and 23% lower algal chlorophyll *a* content than type C2 corals.

A. millepora corals with type D symbionts have 38% lower growth rate in the field and 29% lower growth rate in the laboratory compared to C2 corals. However, irrespective of symbiont type, prolonged heat stress significantly affected growth rates by ~50% compared to pre-bleaching rates.

A. millepora corals with type D symbionts have 23% lower storage lipids which translated to 33% lower reproductive effort compared to C2 corals. However, irrespective of symbiont type, prolonged heat stress significantly affected lipid stores and reproduction by up to ~50% compared to pre-bleaching rates.

APPENDIX I *SYMBIODINIUM* IDENTIFICATION

DNA preparation

This method of DNA extraction is based on the techniques used in the preparation of DNA from animal tissues by Wilson (2002).

Preparation of grinding buffer

Final concentration	Stock to add for 20 ml
100 mM Tris pH 9.0	2 ml 1.0 M
100 mM EDTA	4 ml 0.5 M
1% SDS	2 ml 10% (add last)
100 mM NaCl	400 ul 5.0 M
Milli Q filtered water	11.6 ml

Prepare grinding buffer, add 750 µl to 1.5 ml Eppendorf tubes

Scrape off small piece of ethanol-preserved coral with chirurgical blade, dry on paper towel, add to 1.5 ml tube

Vortex, incubate in a water bath or oven 2-3 hours to overnight at 65°C

Remove samples from the incubator and add 187.5 µl of 5 M KOAc to each tube (giving a final concentration of 1 M).

Mix well and incubate on ice 10 mins.

Spin samples for 10 mins at maximum speed in bench top centrifuge at room temperature.

Carefully transfer supernatant without debris to clean and labelled 1.5 ml Eppendorf tubes.

Add 600 µl isopropanol to each tube to precipitate the DNA. Mix gently and let stand 5 mins at room temperature.

Spin samples for 15mins at room temperature at maximum speed.

Carefully remove supernatant by decanting (pellet may not stick well). Blot on paper towel.

Add 150 µl of 70% ethanol to each tube to the DNA pellets. After adding the ethanol mix gently and spin at maximum speed in bench top centrifuge at room temperature for 5 minutes.

Remove supernatant with extreme care - use a pipette and if pellet is removed too, re-spin.

Air dry the pellets for 5 minutes (don't over dry).

Resuspend pellet in up to 500 µl of 0.1 M Tris pH 9. Leave to dissolve > 3 h at room temperature or overnight at 4 °C. Store at -20 °C.

Polymerase Chain Reaction (PCR) amplification

This method for PCR of nrDNA ITS1 is based on the technique described in van Oppen *et al.* (2001) except that a fluorescently labelled (TET) labelled forward primer is used in the reaction for detection on the GelScan 2000 (Corbett Research). PCR reagents were supplied by Fisher Biotech (Australia).

Forward primer TET labelled 10 nm	1.0 µl
Reverse primer 10 nM	1.0 µl
dNTP	2.5 µl
reaction buffer	2.5 µl
$MgCl_2$	2.0 µl
Taq DNA polymerase	0.1 µl
Milli-Q purified water	14.9 ml

The PCR profile was initial denatured at 94 ºC for 3 minutes followed by 30 cycles of 30 s at 94 ºC, 30 seconds at 59 ºC, 30 s at 72 ºC and a final five minute phase at 72 ºC to elongate the strands. The resultant product was checked on a 1% agarose gel to estimate dilution factors for SSCP analysis.

The *Symbiodinium* specific forward primer was labelled with 5'tetrachloro-flourescein phosphoramidite (TET) for detection on the GelScan 2000-system (Corbett Research, Australia) using single-stranded conformational polymorphism (SSCP) analysis.

Single Stranded Conformational Polymorphism analysis

PCR product (1-3 µl) was mixed with 1-30 µl (depending on the yield) formamide gel-loading dye (Sambrook, Fritsch & Maniatis 1989).

Samples were denatured for 3 min at 95 ºC and snap-cooled on ice.

Each sample (1 µl) was loaded onto a 4% non-denaturing Tris-borate-EDTA (TBE)-polyacrylamide gel (20 cm).

Approximately 10 ml solution was used for each gel (1.5 ml 40% acrylamide 37:1 bisacrylamide to acrylamide), 12.225 ml milli-Q H_2O, 0.9 ml 10 M TBE (Amresco), 0.375 ml of 80% glycerol) .N,N,N',N'-Tetramethyle- thylenediamine (TEMED) (30

µl) and 75 µl of 10% ammonium persulfate were added to start polymerisation prior to pouring the gel.

Gels were run on the Gelscan 2000 (Corbett Research) for approximately 40 min (1,200 volts, 22 ºC) with 0.6 X TBE buffer.

SSCP profiles were compared with reference samples of known ITS1 sequence for clade C2 and D run on the same gel (Fabricius *et al.* 2004).

Cloning and Sequencing

This method for cloning is based on the method outlined in the literature provided with the TOPO TA Cloning® Kit for Sequencing with One Shot® TOP10 Chemically competent *Escheria coli*

For cloning, non-fluorescently labeled (no TET label on the forward primer) PCR products were cleaned using microCLEAN and the purified products were cloned using a TOPO® TA cloning kit (Invitrogen).

Positive clones (white colonies on the Agar LB plate) were sampled with a pipette tip, placed in 30 µl milliQ water and PCR amplified using fluorescently-labelled (TET) forward primer for detection using SSCP analysis.

For sequencing, the cloned product was PCR amplified using non-fluorescently-labelled forward primer and the amplified products were cleaned by adding 3 µl ExoSAP-IT and run on a Corbett Research thermal cycler at 37 °C for 20 mins then 80 °C for 15 mins.

Samples were transferred to 1.5 ml Eppendorf tubes, dried in a rotary evaporator and sent for sequencing (Macrogen) with *Symbiodinium* specific forward and reverse primers.

APPENDIX II ZOOXANTHELLAE COUNTS AND CHLOROPHYLL DETERMINATION

Zooxanthellae counts

The method for tissue stripping and zooxanthellae counts is based on the methods of Jones *et al.* (1998), Perez *et al.* (2001), Ambarsari *et al.* (1997) and Rowan (2004b).

Place the coral branch in a plastic bag containing ~ 10 ml filtered seawater (FSW)

Seal the bag except for a corner and insert the tip of an air gun

Move the jet of water over the coral surface until the tissue is blasted off with the air and water pressure.

Rinse the solution into a 25 ml Falcon tube and repeat the blasting and rinsing twice until all the visible algal cells are removed.

Homogenise the blastate with a tissue homogeniser for 20 s until the zooxanthellae are released from the coral tissue.

Rinse the tip of the homogeniser with FSW and transfer the blastate to a measuring cylinder

Make up the volume to 40 ml (or to the nearest ml) and record the blastate volume.

Remove 9 ml of the blastate to a 15 ml Falcon tube using a graduated pipette and add 1 ml formalin (32% w w^{-1}) to preserve the sample.

Count zooxanthellae in eight independent drops using the New, Improved Nuebauer haemocytomer and a light microscope.

Zooxanthellae counts are averaged across the 8 drops normalised to the surface area of the coral branch

Chlorophyll determination

This method for chlorophyll determination is based on methods by Brown (2002), Berkelmans (2006) and Jeffrey and Haxo (1968) using 100% methanol.

Remove a separate 10 ml subsample from the remaining tissue homogenate using a graduated pipette into a 15 ml Falcon tube,

Store immediately at <4 °C in the dark.

Centrifuge the sample at 3000 rpm for 5 mins to separate the algae from the host tissue.

Discard the supernatant.

Resuspend the pellet in 2 ml 100% methanol.

Extract at <4 °C overnight (12-24 h).

Centrifuge the sample to remove debris from the methanol phase.

Repeat the extraction two more times for the first 10 samples to estimate the extraction efficiency.

Read absorbance at 668 and 635 nm in a spectrophotometer using 100% methanol as a blank

Extinctions (E) were measured at the red maxima of the two chlorophylls *a* and c_2 and the chlorophyll concentration calculated from the equations of Jeffrey and Haxo (1968) where E = extinction in litres $gram^{-1}$ cm^{-1} (1 cm^2 cuvette):

chl *a* = 13.8 x E_{668} - 1.3 x E_{635}

chl *c* = -14.1 x E_{668} + 67.3 x E_{635}

Chlorophyll was adjusted for extraction efficiency by multiplying the concentration by the reciprocal of the % extraction efficiency.

APPENDIX III LIPID EXTRACTION

This technique for the extraction of total lipids from coral tissue samples was modified by Anthony (2005) from methods by Harland (1992) and Folch (1957).

The coral fragments were snap frozen with liquid nitrogen and stored at -80 °C before processing.

Finely grind fragments under liquid nitrogen in a steel mortar and pestle and transfer quantitatively to pre-weighed acetone washed 20 ml glass scintillation vials.

Freeze-dry vials with tissue 1-3 hours.

Accurately weigh freeze dried tissue and vial.

Add 10 ml of fresh chloroform: methanol (2:1, v/v, 3.33 ml: 1.67 ml). Cap and vortex, and let stand at 4 °C for 24 h (use 166.7 ml chloroform and 88.3 ml methanol to make 250 ml).

Transfer the slurry (without the ground skeleton) while filtering through a Whatman GF/C filter (use a glass funnel) in to an acetone-washed 25 ml glass test tube with suction arm.

Extract residual lipid from the ground skeleton over one 4 h rinse in 5 ml fresh chloroform: methanol.

Cap, vortex, and store at 4 °C during extractions.

Filter the residual extract into the initial 25 ml test tube containing the initial extract, and wash the filter in 2 ml fresh chloroform: methanol to ensure a high transfer into the suspension.

Add 5 ml of 0.88% KCl (0.88 g/ 100 ml) in distilled water to the filtered extract and let stand for >30 min at 4 °C.

Shake very gently in the side arm test tube.

Remove the upper aqueous phase gently with a pipette, and wash the organic phase three times with 10 ml methanol: water (1:1, v/v, 5ml: 5ml) shaking each wash gently.

Let each wash stand for >30 min at 4 °C before removing the aqueous top layer carefully with a 5 ml glass pipette.

Dry, weigh and label a batch of acetone-washed aluminium pans on a 5 decimal place balance.

Transfer the remaining washed organic phase into the pre weighed, acetone washed, labelled aluminium pans and leave in a fume cabinet ~1 h.

Leave in 60 °C oven overnight or 3-4 h (treat all samples the same).

Remove from the oven and let cool for $\sim^{1}/_{2}$ h.

Weigh each pan and sample on a 5 decimal place balance.

APPENDIX IV PROTEIN DETERMINATION

This method for protein determination is based on the Folin phenol reagent colourimetric detection and quantification of total protein by Lowry *et al.* (1951) and is compared to a known standard of bovine serum albumin (BSA). The method is modified for the microassay detection of low concentrations of protein in coral tissue using the Bio-Rad DC protein assay kit (Bio-Rad laboratories, Australia) †.

Prepare a series of 5 standards ranging from 0.0 to 2.0 mg/ml using Sigma BSA standard (2.0 mg/ml) with 0.5 M NaOH buffer. Use some spare samples to pick by eye the range of concentrations of samples. Once the range has been determined, the most appropriate range of concentrations of the BSA standards can be chosen.

Dilution series of BSA protein standards from stock solution (2.0 mg ml^{-1})

Standard #	Protein standard µl	NaOH buffer µl	Protein concentration mg ml-1
1	0	100	0
2	25	75	0.5
3	50	50	1.0
4	75	25	1.5
5	100	0	2.0

Accurately weigh (4 decimal places) ~2 g of ground, freeze dried tissue into a 96 deep-well plate.

Cover with aluminium foil.

Digest the sample with 1.5 ml NaOH at 90 °C for 1 h and let cool.

† Craig Humphries, Australian Institute of Marine Science

Spin the tubes at low speed (4000 rpm) for 30 s in a benchtop centrifuge to separate the cell debris from the solution.

If necessary, dilute the samples with 0.5 M NaOH so that the concentrations appear within the range of the BSA standards.

Pipette a 5 µl subsample of each sample and standard to a clean, dry 96-well microassay plate in triplicate.

Assay for protein using the DC protein assay kit (Bio-Rad)

Add 25 µl reagent A into each well.

Add 200 µl reagent B into each well.

Gently agitate the plate for 5 s to mix the reagents. If bubbles form, pop them with a clean, dry pipette tip. Be careful to avoid cross contamination of sample wells.

After 15 mins, read absorbance at 690 nm on a spectrophotometer.

Protein concentration in the microassay plate cell can be estimated using the regression coefficient of the calibration curve. The protein concentration of the sample was calculated by the formula:

mg protein/mg tissue = (protein concentration in the microassay plate cell) x (1/dilution factor) x (volume of NaOH) x (1/DWT)

The total protein in the branch was calculated by multiplying the protein concentration by the total dry weight of tissue (DWT) in the branch. The total protein in the branch was divided by the surface area of the branch and expressed as protein per unit surface area (mg cm^{-2}).

APPENDIX V CARBOHYDRATE DETERMINATION

Total carbohydrates were quantified using the phenol-sulfuric acid method for the colourimetric determination and quantification of total carbohydrates described in Dubois *et al.* (1956) using D-glucose as standard.

Prepare 20 ml of a 200 μg ml^{-1} stock solution of D-glucose and RO water (4000 μg of d-glucose and 20 ml of water).

Prepare a series of standard dilutions of D-glucose by dilution of the stock solution with RO water as outlined in the table below.

Dilutions for the preparation of standards for carbohydrate analyses from a 200 μg ml^{-1} stock solution of D-glucose

Dilution ID	Volume of RO water	Volume of d-glucose solution	Final d-glucose conc.
A	0 ml	4 ml from stock	200 μg ml^{-1}
B	0.5 ml	3.5 ml from stock	175 μg ml^{-1}
C	1 ml	3 ml from stock	150 μg ml^{-1}
D	1.5 ml	2.5 ml from stock	125 μg ml^{-1}
E	2 ml	2 ml from stock	100 μg ml^{-1}
F	2.5 ml	1.5 ml from stock	75 μg ml^{-1}
G	3 ml	1 ml from stock	50 μg ml^{-1}
H	3.5 ml	0.5 ml from stock	25 μg ml^{-1}
I	4 ml	0.5 ml from dilution H	5 μg ml^{-1}
J	4 ml	0 ml	0 μg ml^{-1}

Remove 1 ml from each standard dilution to its own 15 ml Falcon tube.

Rinse the ground, freeze-dried tissue of known weight with 10 ml RO water into separate 15 ml Falcon tubes and homogenise for 20 s.

Remove a 1 ml subsample from each tube to its own 15 ml Falcon tube.

Add 1 ml of 5% phenol to each standard and sample.

Add 5 ml of 100% sulfuric acid in a direct stream towards the liquid surface and not down the side of the tube (the addition of sulfuric acid will cause fizzing and give-off heat – wear safety glasses and conduct the reaction inside the fume hood) using an automatic liquid dispenser.

Let stand for 10 mins.

Place in a water bath for 20 mins at 30 ºC.

Let stand for 4 h in the fume hood before measuring the absorbance.

Measure the absorbance of each standard and subsample at 490 nm.

Prepare a standard curve by plotting the average absorbance for each glucose standard against its concentration in μg ml^{-1}.

Use the regression curve to determine the carbohydrate concentration of each subsample. The carbohydrate concentration per tissue surface area of each sample is calculated by the formula:

μg carbohydrate per mg tissue = (carbohydrate concentration) x (diluents volume/volume subsample) x (1/DWT)

The total carbohydrate was calculated by multiplying the carbohydrate per mg tissue by the total DWT in the branch. The carbohydrate per surface area was determined by dividing the total carbohydrate in the branch by the area of the branch (μg cm^{-2}).

SAMPLE CALCULATIONS

Zooxanthellae densities						
Average zooxanthellae in the haemocytometer cell (0.0001 mm^3)	Zooxanthellae per ml = average zooxanthellae in cell x vol haemocytometer (0.0001 mm^3) x dilution factor of formalin (10/9)	Volume blastate	Zooxanthellae per ml x blastate volume ml	Surface area branch	zooxanthellae per surface area of the branch	Zooxanthellae per branch surface area
cells	$cell.ml^{-1}$	ml	cells	cm^2	cm^2	cell cm^{-2}
31	344,444.44	40	13,777,778	9.83	135,405,079	135.41
Chlorophyll concentrations						
Extinction = litres gm^{-1} cm^{-1}	Extinction = litres gm^{-1} cm^{-1}	chl *a* = 13.8*E_{668} - 1.3*E_{635} * 100/93.78	chl c_2 = -14.1E_{668} +67.3E_{635} * 100/83.16	Total zooxanthellae per ml	Chlorophyll *a* per zooxanthellae	Chlorophyll c_2 per zooxanthellae
(1 cm cell)	(1 cm cell)	$\mu g.ml^{-1}$	$\mu g\ ml^{-1}$		x 10^6	x 10^6
Pigm @ 668 nm first extraction	Pigm @ 635 nm first extraction	Extraction efficiency 93.78%	Extraction efficiency 83.16%	cells	$\mu g\ cell^{-1}$	$\mu g\ cell^{-1}$
1.2227	0.4416	16.26112424	18.49362757	344,444.44	47.21	53.69
Lipid concentration						
Lipid in aluminum plate	DWT used for lipid analysis	Total lipid per gram freeze dried tissue = mg lipid* (1/DWT)	Total DWT from branch	Lipid per DWT * total DWT	Surface area	Lipid per surface area tissue
mg	mg	$mg\ g^{-1}$	mg	mg	cm^2	$mg\ cm^{-2}$
22.8	1067.8	0.02	2654.8	56.69	15.65	3.62

Protein concentration						
protein concentration in the microplate assay cell	DWT used for protein analysis	Total DWT from branch	Total protein per g freeze dried tissue = (conc)* (dilution factor* (vol NaOH/1)*(1/DWT)	mg protein per DWT (g) * total DWT(g)	Surface area	Total soluble protein per surface area
mg ml^{-1}	g	g	mg g^{-1}	mg	cm^2	mg cm^{-2}
3.646	1.0678	2.6548	25.209	66.924	15.66	4.27
Carbohydrate concentration						
Concentration d-glucose equivalents in the cell	DWT used in carbohydrate analysis	Total carbohydrate per g of tissue = (carbohydrate concentration in the cell)* (vol diluent/vol subsample 10/1) * (1/DWT)	Total DWT from branch	Carbohydrate per DWT * total DWT	Surface area branch	Carbohydrate per surface area
g ml^{-1}	g	µg mg^{-1}	g	µg	cm^2	µg cm^{-2}
200.46	0.6176	3245.73	2.6548	8616.75	15.66	550.38

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