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Chronic effects of herbicide exposure on photosynthesis, symbiosis and reproduction of reef building corals

Thesis submitted by

Neal Edward Cantin

in April 2008

For the degree of Doctor of Philosophy in Marine Biology

within the school of Marine and Tropical Biology,

James Cook University, Townsville, Queensland

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Statement on the Contribution of Others

This thesis includes collaborative work with my supervisors Dr. Andrew Negri (Australian Institute of Marine Science) and Prof. Bette Willis (James Cook University) and Dr. Madeleine van Oppen (Australian Institute of Marine Science). While undertaking these collaborations, I was responsible for the project concept and design, data collection, analysis and interpretation and the final synthesis of the results in a form suitable for publication. My collaborators provided intellectual support, financial support, technical instruction and editorial assistance.

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Abstract

The herbicide, diuron, is found at levels equivalent to 1 μg l^{-1} within sediments in the Great Barrier Reef lagoon, where it potentially reduces photosynthesis and carbon fixation within *Symbiodinium*, the dinoflagellate symbiont associated with reef corals. Little is known about the potential of diuron to reduce energy acquisition and change energy allocation strategies to reproduction in corals. The objective of this study was to examine the importance of carbon-based energy (carbohydrates) derived from photosynthesis for gametogenesis, gamete viability and larval quality of corals following the first long-term, experimental exposures to diuron and to investigate the influence of symbiont type on energy provisioning to host tissues under normal conditions and in the presence of diuron. This is the first study to investigate the chronic sub-lethal effects of herbicide-induced photoinhibition on coral symbionts and the subsequent flow on effects to the fitness of the coral host.

Two broadcast spawning corals, *Acropora tenuis* and *A. valida,* and a brooding coral, *Pocillopora damicornis,* were exposed to 0 (control), 1.0 (low) and 10 (moderate) μg l^{-1} diuron treatments for 2 to 3 months prior to spawning or planulation. Diuron caused rapid and consistent declines in effective quantum yields of approximately 20% at 1.0 μg l^{-1} and 75% at 10 μg l^{-1} in each species compared to controls (Chapter 2). Total lipid content (coral tissue, oocytes and planulae) was reduced by 2.5- to 5-fold for the three species in the presence of diuron, indicating significant use of storage lipid to meet nutritional demands under conditions of chronic photoinhibition. Polyp fecundity in *A. tenuis* was not impacted, however it was reduced by 6-fold in *A. valida,* and both *A. valida* and *P. damicornis* were unable to spawn or planulate following long-term exposures to 10 μg l^{-1} diuron.

Maternal provisioning of lipids, pigments and antioxidants to coral eggs that lack *Symbiodinium* provides energy and protection essential for the development, survival and dispersal of coral larvae. For corals that were able to spawn or planulate following 2-3 month experimental exposures to diuron (i.e. *A. valida* and *P. damicornis* in the 0 and 1 μg l^{-1} treatments; *A tenuis* in the 0, and 10 μg l^{-1} treatments), gamete fertilisation was not affected (Chapter 3). Larvae from each of these species also successfully metamorphosed into juvenile corals following parental exposures to the above diuron treatments. Although gametes were viable, gamete quality was reduced in *A. valida*

following even low exposures to diuron. Peridinin, the major carotenoid pigment identified in *A. valida* eggs, was 10-fold lower in eggs derived from corals exposed to 1.0 µg l^{-1} diuron compared with tank controls. The tank controls in turn contained 5-fold less peridinin than field controls. In contrast, no difference in vitamin E (α – tocopherol) was detected in any of the treatments. Peridinin in combination with xanthophylls may enhance the capacity of buoyant coral eggs to absorb potentially harmful high-energy photosynthetically active radiation (PAR, 400 - 530 nm) that is not absorbed by mycosporine-like amino acids (MAAs) and vitamin E. All of these compounds are likely to work synergistically to protect eggs from oxidative damage.

Algal endosymbionts of the genus *Symbiodinium* play a key role in fulfilling the nutritional requirements of reef building corals, however comparisons of photosynthetic capacity among different *Symbiodinium* types *in hospite* within the same coral species have only recently become possible. A sensitive quantitative PCR assay was developed for *Symbiodinium* spp. (Chapter 4) based upon chloroplast (cp) large subunit (23S) ribosomal DNA sequences, to detect low level background strains of *Symbiodinium* spp. It was then applied to verify symbiont assemblages within juvenile colonies of *Acropora millepora* that had been experimentally infected with two different symbiont types (Chapter 5). Using experimentally infected C1- and D-juveniles of *A. millepora*, relative electron transport ($rETR_{MAX}$) of PSII, was found to be 87% greater in *Symbiodinium* C1 than in *Symbiodinium* D *in hospite* in the control treatment, resulting in a doubling of ^{14}C photosynthate incorporation (energy) into juvenile tissues of *A. millepora* (Chapter 5). *Symbiodinium* C1 corals, however, lost this competitive advantage in the presence of diuron, due to inhibition of rapid electron transport. There was no observable difference in phytotoxicity of diuron between genetically distinct symbionts *in situ*. The finding that genetically distinct *Symbiondinium* spp. are not functionally equivalent, highlights the importance of symbiont identity in the nutritional physiology of the coral-algal holobiont.

These results provide evidence of a link between reduced energy acquisition due to diuron exposure causing significant PSII photoinhibition and reduced reproductive output in zooxanthellate corals. Energy allocated to reproduction was directed towards maintaining and releasing fewer eggs and larvae, while ensuring the full developmental viability of these progeny. Along with diuron, other herbicides such as atrazine and Irgarol 1051 that are designed to target the PSII in the same manner as diuron, are commonly found entering the marine environment, which could create an additive

effect on the chronic impacts induced by diuron exposure within the natural environment. The observed reductions in reproductive development (*A. valida*) and reproductive output (*A. valida* and *P. damicornis*) caused by the inhibition of energy acquisition from photosynthesis following long-term diuron exposure, highlights the importance of carbon-based energy from photosynthesis for coral reproduction and provides further evidence of physiological trade-offs that can result following events that limit the availability of energetic resources to individual coral colonies.

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2. From Chapter 4 (in part): Mieog, J.C., van Oppen, M.J.H., Cantin, N.E., Stam, W.T. and Olsen, J.L. (2007) Real-time PCR reveals a high incidence of *Symbiodinium* clade D at low levels in four scleractinian corals across the Great Barrier Reef: implications for symbiont shuffling. Coral Reefs **26**:449-457.

3. From Chapter 5: Cantin, N.E., van Oppen, M.J.H, Willis, B.L., Mieog, J.C. and Negri, A.P. (2008) Juvenile corals acquire more carbon from high-performance algal symbionts. Coral Reefs: *in review*.

4. From Chapter 3: Cantin, N.E., Willis, B.L. and Negri, A.P. (2008) Quality of coral gametes following chronic parental herbicide exposure. Marine Ecology Progress Series: to be submitted (*in prep.*).

Chapter 1: General Introduction

The health of coral reef ecosystems worldwide is in decline as a result of climate change (Hoegh-Guldberg 1999, Hughes et al. 2003, Hoegh-Guldberg et al. 2007), increased disease (Harvell et al. 2002), overfishing (Hughes 1994) and human land use (Haynes & Michalek-Wagner 2000, Haynes et al. 2000a). Within Australia, extensive modification of land-use within catchments adjacent to the Great Barrier Reef has resulted in over 80% of the land area of Queensland being used to support agricultural production (Wachenfeld et al. 1998). As a consequence, run-off from land-based agricultural activities is affecting water quality within the Great Barrier Reef Marine Park (GBRMP) by increasing nutrient levels, sedimentation and turbidity, and by transporting pesticides and herbicides into the marine environment (Haynes & Michalek-Wagner 2000, Haynes et al. 2000a, Fabricius 2005, Schaffelke et al. 2005). Toxicants entering the GBRMP that are of particular concern are PSII herbicides, such as diuron and atrazine, which can have immediately measurable physiological impacts on corals (Jones et al. 2003). High application rates of diuron combined with a field half life ranging from 13-250 days and evidence that measurable quantities of diuron are being transported to nearshore reef environments make diuron the key herbicide of concern (Haynes et al. 2000a, Jones et al. 2003, Mitchell et al. 2005, Shaw & Müller 2005).

The energy budget of photosynthetic corals is dependent upon sources of carbon fixed from light induced photosynthesis and/or acquired through heterotrophic feeding (particulate carbon and nutrients, N and P, Fig. 1.1A,B) (Anthony & Fabricius 2000, Grottoli et al. 2006). Availability of surplus energy (energy remaining following primary cell repair and maintenance, Fig. 1.1C) is often limited and must be divided among several competing biological functions, including reproduction and gamete development, tissue and skeletal growth, and colony regeneration (Fig. 1.1D; (Stearns 1992, Rinkevich 1996). Each of these biological functions is energetically costly, thus there will be trade-offs in resource allocation between them and any event that reduces energy acquisition may create temporary or permanent shifts in energy allocation within the coral colony (Rinkevich 1996). PSII herbicides inhibit the electron transport chain of symbionts within adult and juvenile corals, reducing the efficiency of photosynthesis

within minutes at concentrations as low as 0.3 µg l^{-1} (Jones & Kerswell 2003, Jones et al. 2003, Negri et al. 2005). Consequently, nearshore corals exposed to PSII herbicides that inhibit carbon based energy from photosynthesis (Fig. 1.1A; Owen et al. 2003) may experience depleted energy reserves that are insufficient to meet the demands of all competing biological functions (Fig. 1.1D), with significant implications for key physiological processes such as reproduction. Phytotoxic effects of diuron at low concentrations (0.3 µg l^{-1}) on photosynthetic organisms like *Symbiodinium* have been well documented (Jones & Kerswell 2003, Jones et al. 2003, Owen et al. 2003), however, currently there is no biochemical evidence of direct diuron toxicity on invertebrates at low but environmentally relevant concentrations (0.3-10 µg l^{-1}). High concentrations of diuron (100 µg l^{-1}) caused no impact on the fertilisation, metamorphosis and health of juvenile corals during their aposymbiotic (lacking symbionts) life history stages (Negri et al. 2005, Watanabe et al. 2006). This was not unexpected since the coral host lacks a direct PSII binding site for diuron to inhibit energy acquisition and the early aposymbiotic stages of juvenile corals are short for most broadcast spawning species (Harrison & Wallace 1990), which are supported by energy stored in the form of lipid (Arai et al. 1993, Harii et al. 2002). However, no study to date has investigated the potential effects of chronic photoinhibition on adult corals with established algal endosymbionts, particularly throughout the eight month, energy-dependent period of gamete and larval production.

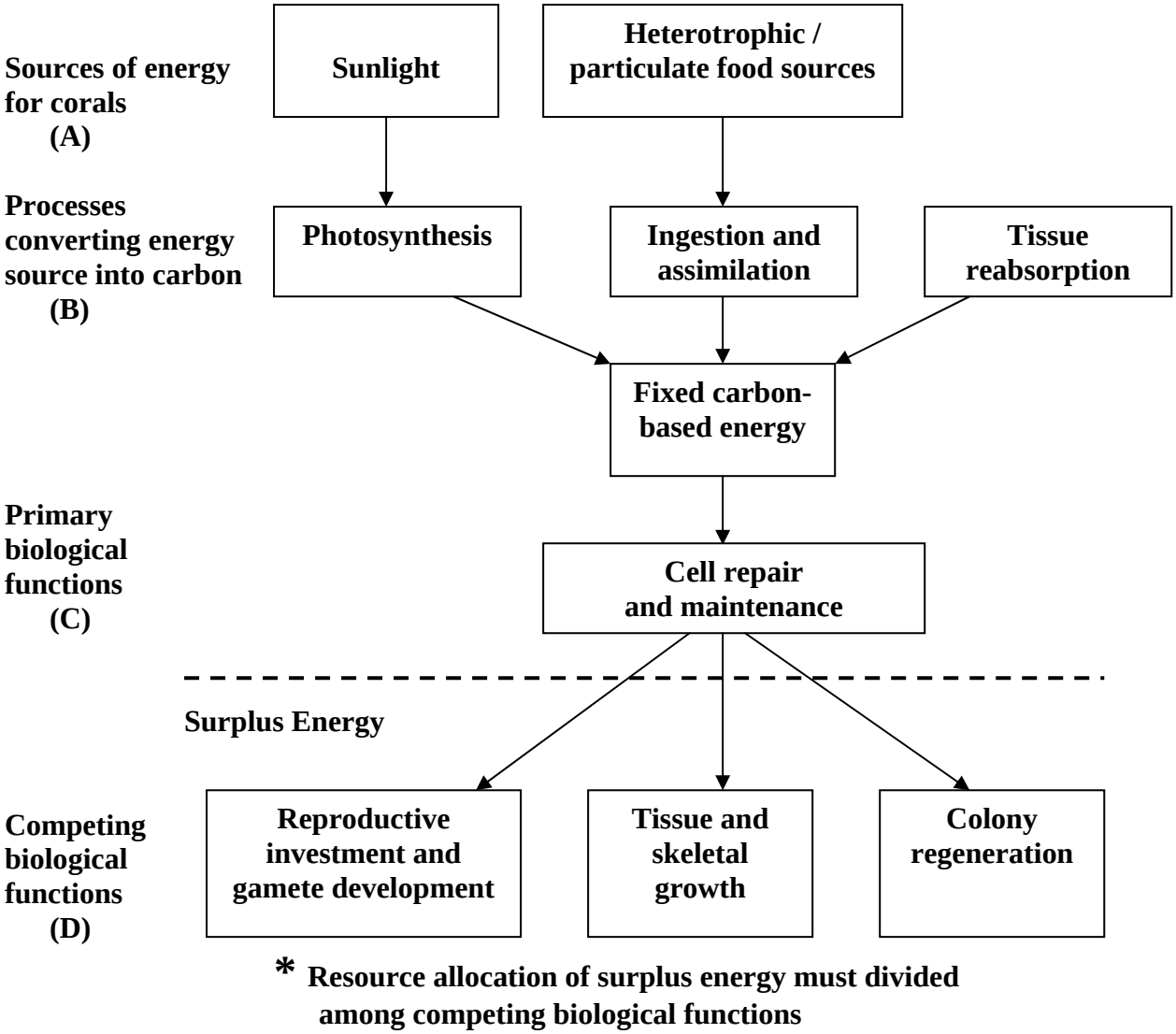

Fig. 1.1: Hierarchical allocation of energy among primary and competing biological functions for organisms with associated algal endosymbionts such as zooxanthellate corals.

Physiological trade-offs can result from allocation of energy between two or more processes that compete directly with one another for limited resources within a single individual (Stearns 1992). Modular colonial organisms, like zooxanthellate corals, may possess a level of colony integration which allows colonies to continually reprioritize resource allocation among individual units in response to stress towards specific regions of the colony, as well as among competing biological processes (Oren et al. 2001). Optimizing energy allocation to reproduction during different stages of the animal's life is important to maximize the fitness of the individual (Kozlowski & Wiegert 1986, Rinkevich 1996). Although biological functions such as maintenance, repair, growth and reproduction are all dependent on a common resource pool, current understanding of interactions between these functions remains relatively limited for corals because it is

difficult to identify how energy resources are channeled between the 'competing' biological functions and how energy trade-offs may be activated (Rinkevich 1996). Sexual and asexual reproduction in corals is highly sensitive to resource depletion and appears to be a good indicator of energy limitation and the trade-offs associated with allocation of energy among colony growth and reproductive effort (Loya et al. 2004). Although no study has identified how energy resources are specifically channeled among competing biological functions, several field studies have found significant reductions in reproductive activity (both fecundity and reproductive output) during events that require colony regeneration (Kojis & Quinn 1985, Rinkevich & Loya 1989, Van Veghel & Bak 1994) or deplete energy from photosynthesis throughout depth distributions (Villinski 2003) or during coral bleaching events (Michalek-Wagner & Willis 2001a). The effectiveness with which the herbicide diuron inhibits electron transport within the PSII of coral symbionts, thus reducing the quantity of carbon fixed from photosynthesis (Owen et al. 2003), provides an excellent tool to manipulate resource availability within coral colonies and assess the effects of energy limitation on competing biological functions, without significantly damaging the animal host.

Early researchers assumed that all symbiotic zooxanthellae associated with marine hosts belonged to a single pandemic species, *Symbiodinium microadriaticum*, (Freudenthal 1962, Taylor 1974), the corollary being that there would be limited physiological variability in photosynthetic capacity and stress tolerance of the coral holobiont as a direct result of symbiont type. However, the concept of low symbiont diversity (Taylor 1974), has since given way to recognition of the existence of wide genetic diversity within the symbiotic dinoflagellate genus *Symbiodinium*, based on studies using morphological, biochemical, physiological and molecular techniques (LaJeunnesse 2001). The genus *Symbiodinium* has been divided into eight distinct phylogenetic clades (A-H), five of which are known to associate with corals (A-D, F) (Rowan & Powers 1991, Carlos et al. 1999, LaJeunnesse 2001, Pochon et al. 2001, Santos et al. 2002, Pochon et al. 2006). Variations in symbiont associations have been observed within coral species across broad depth and geographic ranges (Rowan & Knowlton 1995, van Oppen et al. 2001, Iglesias-Prieto et al. 2004, LaJeunnesse et al. 2004, Coffroth & Santos 2005, van Oppen et al. 2005b). Such differences in partner combinations across environmental and latitudinal gradients could reflect the establishment of symbioses with *Symbiodinium* types having different sensitivities to irradiance, thermal and herbicide induced stress, and/or differences in host specificity

(van Oppen et al. 2001, LaJeunnesse et al. 2004). These findings of genetic variability within and among coral endosymbionts underscore the need for further study of the extent of physiological differences among distinct *Symbiodinium* clades, in order to determine the potential for new host-symbiont partnerships that are better able to cope with predicted changes in physical-environmental conditions and contamination stress.

Aims and thesis outline

This thesis investigates the importance of symbiotic photosynthesis for coral reproduction and investment of energy into gametogenesis. Relevant concentrations of the photosystem II (PSII) inhibiting herbicide, diuron (3,(3,4-dichlorophenyl)-1,1-dimethylurea), were used to test the key hypothesis that *the acquisition of carbon based energy translocated from symbiotic Symbiodinium* spp. *into the coral host is crucial for coral reproduction and for successful gamete development.* The primary aims of this thesis were to: **(1)** compare the impact of reduced photosynthetic productivity (following diuron-induced inhibition of PSII electron transport) on the investment of energy rich lipids into coral reproduction within two broadcast spawning and an internal brooding species of reef building corals during the final 2-3 months of gamete development, **(2)** assess the viability and quality of gametes produced by energy deficient coral colonies, and **(3)** examine the influence of genetically distinct symbionts (*Symbiodinium* spp.) on the physiology and fitness of the coral holobiont.

Chapter 2 compares the reproductive success of two broadcast spawning species and one internal brooding species that experienced increasing levels of electron transport inhibition, thereby reducing the availability of energy to competing biological functions. By analyzing the development of coral gametes prior to spawning, the impact of reduced photosynthetic efficiency on coral reproduction could be used as an indicator of a physiological trade-off between biological processes that directly compete for energy investment.

Chapter 3 examines the effects of long-term electron transport inhibition on reproductive coral colonies by quantifying parental provisioning of resources into eggs and evaluating the viability of the gametes produced. In order to fully understand the effects of energy deficiencies on gamete quality it is crucial to determine if the eggs that

are produced are fully capable of successful fertilization and can undergo metamorphosis into juvenile corals.

Chapter 4 develops a highly sensitive quantitative real-time polymerase chain reaction (qPCR) assay based upon chloroplast 23S-rDNA (cp23S-rDNA) to detect the presence of multiple symbiont types within a host colony. Through the development of these sensitive techniques we can explore the ongoing question of whether or not a single coral species can host multiple algal genotypes, either sequentially or simultaneously.

Finally, **Chapter 5** investigates the photophysiology of two dominant symbionts on the Great Barrier Reef, *Symbiodinium* C1 and *Symbiodinium* D, to determine if genetically distinct symbionts are functionally equivalent in terms of their nutritional interactions with their reef building coral hosts. The experiments were conducted under ambient conditions and in the presence of environmentally relevant concentrations of the PSII inhibitor diuron. The results from this chapter provide significant evidence to support the topical debate, that symbiont type strongly influences the physiological performance of the coral holobiont.

Chapter 2: Photoinhibition from chronic herbicide exposures reduces reproductive output of reef-building corals

This Chapter is Published as:

Cantin, N.E., Negri, A.P. and Willis, B.L. (2007) Photoinhibition from chronic herbicide exposure reduces reproductive output of reef-building corals. Marine Ecology Progress Series **344**: 81-93.

2.1. INTRODUCTION

Recent reports highlight the deleterious impacts that pesticides may have on inshore flora and fauna of the Great Barrier Reef (GBR) (Haynes et al. 2000a, Haynes et al. 2000b, Jones et al. 2003a, Markey et al. 2007). The photosystem II (PSII) herbicide diuron (3,(3,4-dichlorophenyl)-1,1-dimethylurea) is commonly applied to control weeds in catchments flowing into the GBR, with approximately 197 tonnes applied annually in the sugar cane industry alone (Hamilton & Haydon 1996). Peak application of diuron occurs during the wet season (November to January), increasing the potential delivery of diuron into the GBR Marine Park (GBRMP) (Haynes et al. 2000a, Mitchell et al. 2005) and coinciding with the final stages of gametogenesis and larval development for reef corals on the GBR. Diuron concentrations measured from instantaneous samples within GBR catchments have been recorded as high as 8.5 μg l^{-1} in the mouth of the Pioneer River (Simpson 2002, Mitchell et al. 2005), 10 μg kg^{-1} in subtidal sediments and 1.7 μg kg^{-1} in intertidal seagrasses of the GBR lagoon (Haynes et al. 2000a). Low persistent concentrations of diuron within some inshore habitats of the GBRMP are likely, although more extensive sampling is required to determine the duration of elevated levels (Haynes et al. 2000b, Mitchell et al. 2005). The moderately high solubility of diuron (42 mg l^{-1}) combined with high application rates have led to concern that diuron may be one of the most harmful agrochemical pollutants to GBR organisms and ecosystems (Haynes et al. 2000a).

Diuron inhibits the PSII electron transport chain of symbiotic *Symbiodinium* spp. (zooxanthellae) in adult corals within hours, at concentrations as low as 0.3 μg l^{-1} (Jones & Kerswell 2003, Jones et al. 2003). The incorporation of carbon from photosynthesis within isolated *Symbiodinium* spp. has also been shown to decrease by 30% at 2 μg l^{-1} and by 60-70% at 10 μg l^{-1} (Owen et al. 2003). Photosynthetic performance of symbiotic juvenile corals is affected at similar concentrations and typically results in

bleaching (loss of *Symbiodinium* spp. and/or photosynthetic pigments) after medium term (4 day) diuron exposures (Negri et al. 2005). Diuron has no known direct impact on the cellular function of the invertebrate host. Juveniles of *Acropora tenuis* without symbionts (i.e. aposymbiotic) exposed to concentrations of diuron as high as 100 μg l^{-1} for 10 days exhibited no morphological abnormalities such as tissue detachment or death (Watanabe et al. 2006). The fertilisation of gametes and the metamorphosis of aposymbiotic *A. millepora* larvae were similarly unaffected by high diuron concentrations (Negri et al. 2005). While there is clear evidence that low to medium concentrations (0.2 to 10 μg l^{-1}) of diuron have acute, deleterious impacts on *Symbiodinium* spp. photosynthesis and carbohydrate production (Jones & Kerswell 2003, Jones et al. 2003, Owen et al. 2003, Negri et al. 2005), nothing is known about potential chronic impacts arising from long-term sub-lethal exposures.

In plants and algae, diuron causes photoinhibition within chloroplasts by competing with plastoquinone for the Q_B binding site on the D_1 protein within PSII. Thus, diuron blocks light-induced electron transport from water to the Q_B site, reducing the efficiency of photosynthetic processes and interfering with repair mechanisms involved in the synthesis of new D1 protein (Oettmeier 1992, Jones et al. 2003). Short-term studies on the toxicity of diuron to reef corals have shown that it is a fast-acting inhibitor of photosynthesis and demonstrate that exposure to 1 μg l^{-1} causes secondary damage to PS II reaction centres in illuminated samples (Jones & Kerswell 2003, Jones et al. 2003, Negri et al. 2005). Ultimately, diuron-induced photoinhibition disrupts the light dependent reactions of photosynthesis by reducing the linear electron transport through PSII that leads to the formation of ATP and NADPH (Oettmeier 1992, Jones et al. 2003). These two end products are essential substrates for the reduction of carbon dioxide to form carbohydrates and glycerol within the light-independent reactions of photosynthesis (Oettmeier 1992, Finazzi & Forti 2004, Jones 2005). Since diuron reduces the fixation of carbon within symbionts (Owen et al. 2003), the translocation of photosynthetic products (carbohydrates and glycerol) into the coral host from symbiont photosynthesis will also be reduced following chronic diuron exposure.

The energy budget of an organism is finely balanced, so reductions in energy acquisition typically impact the amount of energy available for growth and reproduction (Stearns 1992). Reports of decreased reproductive output and larval development for corals with reduced densities of *Symbiodinium* spp., either due to temperature-induced bleaching (Michalek-Wagner & Willis 2001a) or nutrient enrichment (Loya et al.

2004), are consistent with reduced energy acquisition having flow-on effects for gametogenesis in corals. Both gametogenesis (production of oocytes and sperm) and larval development require protracted energy investment to produce eggs and larvae that are high in carbon-rich lipids (Arai et al. 1993). Heterotrophy and autotrophy are both critical to the energy budget of the coral holobiont, although evidence that the production of lipid is predominantly stimulated by the presence of light for photosynthesis (Crossland et al. 1980, Anthony & Fabricius 2000) highlights the importance of photosynthetically derived energy for gamete and larval development in corals. Moreover, it has been shown that heterotrophy may free up photosynthetically derived resources for reproduction. For example, the immediate metabolic requirements of symbiotic corals that were fed brine shrimp were met from heterotrophy, which meant that they stored more of the energy derived from photosynthesis as lipids (Szmant-Froelich 1981). The critical importance of photosynthetically derived carbohydrates for coral nutrition (Muscatine et al. 1984, Rinkevich 1989, Anthony & Fabricius 2000), particularly throughout the eight month, energy-dependent period of gamete and larval production (Harrison & Wallace 1990), suggests that gamete and larval development are likely to be sensitive indicators of reductions in photosynthetically derived carbon, such as those potentially caused by chronic diuron exposure.

Total lipid quantities within coral tissues provide an indication of reproductive status (Ward 1995b, Leuzinger et al. 2003), both for broadcast spawning species of *Acropora* and *Montipora*, whose eggs contain 60-72% lipid (Arai et al. 1993), and for brooding species like *Pocillopora damicornis,* whose larvae contain 70% lipid by dry weight (Richmond 1987). It has been demonstrated that lipid content decreases following planulation of brooded larvae from tissues of *P. damicornis* colonies (Ward 1995b). Furthermore, Leuzinger et al. (2003) concluded that lipids were the only component of coral tissue (cf. proteins and carbohydrates) to significantly decrease as a result of broadcast spawning. Thus, investment of energy into oogenesis, spermatogenesis and tissue repair following spawning may be estimated by a combination of fecundity measurements (egg number and size) and total lipid content of coral tissue before and after spawning (Leuzinger et al. 2003). The present study examines long-term effects of herbicide-induced photoinhibition on symbiotic broadcast spawning and brooding corals during the final stages of their gametogenic cycles. In particular, how reductions in energy (i.e. in photosynthetically derived carbohydrates available for translocation to

the coral host) caused by chronic diuron exposures affect coral reproduction, including gametogenesis and reproductive output was investigated.

2.2. METHODS

Diuron exposure: Reproductively mature colonies of *Acropora tenuis* and *A. valida* (20-30 cm in diameter) were collected from Nelly Bay, Magnetic Island, Australia (Lat. 19˚10'S; Long. 146˚52'E) on August 10-25, 2003 (*A. tenuis*), and August 3, 2004 (*A. valida*) approximately 10 and 13 weeks, respectively, before the predicted nights of spawning. Reproductively mature colonies of *Pocillopora damicornis* (15-25 cm in diameter) were collected from Horseshoe Bay, Magnetic Island (Lat. 19°06' S; Long. 146°51'E) on February 20, 2004, 4 weeks before predicted planulation. Twenty four colonies of each species were transported to outdoor aquaria at the Australian Institute of Marine Science (AIMS), Townsville for experimental diuron exposures. An additional 8 colonies of each species were re-attached at their sites of collection (3 to 4 m depth) as reef controls and later collected and transported to AIMS 3 to 4 days prior to spawning.

Corals were exposed to 3 experimental treatments: 0 μg l^{-1} (tank controls), a 1.0 μg l^{-1} low-dose diuron treatment (an ecologically relevant concentration that reduces effective quantum yields by 20%) (Jones et al. 2003) and a 10 μg l^{-1} moderate experimental-dose diuron treatment (selected because it reduces effective quantum yields by 80%) (Jones et al. 2003). Corals were exposed to these treatments for 2 to 3 months prior to either spawning (*Acropora tenuis* for 53 days, *A. valida* for 90 days) or planulation (*P. damicornis* for 67 days). Exposure times were selected to ensure that photosynthesis was affected for the final 2 to 3 months of the typical 8 to 9 month gametogenic cycle (Harrison & Wallace 1990). Each treatment was performed outdoors in 500 l tanks with unfiltered oceanic seawater that was settled in a header tank before entering the experimental tanks at a flow rate of 3 l min^{-1}. Seawater temperatures in the tanks ranged from 26°C in the spring and automn, up to 29°C in the summer months, closely paralleling local patterns in seasonal seawater temperatures. Natural food sources (plankton, suspended particulate matter, detritus and sediment) were present in the seawater and available to experimental corals throughout their exposures to diuron, providing ample nitrogen and phosphorous sources for protein synthesis. Tanks were shaded to 20% of full sunlight intensity (maximum irradiance of 350 μmol quanta $m^{-1}s^{-}$

^{1}), which is similar to the light conditions at a depth of 3 m. Corals were monitored for partial colony mortality, as indicated by tissue loss exposing bare skeleton on some branches or parts thereof, or full colony mortality (full loss of tissue on every branch of the colony).

For each of the three treatments two tanks were used and four large reproductive colonies of each species were maintained within each tank during the 3 separate exposure experiments. A multichannel peristaltic pump was used to deliver a stock solution of laboratory grade diuron (98%) (Sigma Pharmaceuticals). Nominal diuron concentrations were verified in two representative tanks per treatment by four water samples collected at 3-week intervals during the first *Acropora tenuis* experiment. Diuron concentrations were measured as described in Negri et al. (2005). Mean (± SE) diuron concentrations within the replicate tanks of each treatment were 0.91 ± 0.05 and 8.8 ± 0.2 μg l^{-1}, respectively. Seawater from the diuron tanks was diluted 100 fold in the aquarium/stormwater outflow and pumped into a 1.5 ha artificial wetland/evaporation pond for disposal. During the predicted weeks of spawning (i.e. the 7 days following the October full moons in 2003 and 2004), *A. tenuis* and *A. valida* tanks were monitored for spawning activity between dusk and 23:00 h. For 2 weeks during the March and April new moons, outflows from the *P. damicornis* tanks were run through a 100 μm mesh plankton trap to collect released planulae and track reproductive output.

Effects of parental diuron exposure: photosynthetic efficiency: The photosynthetic efficiency (maximum quantum yield and effective quantum yield) of *Symbiodinium* spp. within host tissues of the three coral species was estimated from chlorophyll fluorescence measurements taken with a pulse amplitude modulated (PAM) chlorophyll fluorometer (DIVING-PAM, Walz, Germany, with Out Gain set to 4 and Measuring Intensity set to 8). Measurements were taken from each colony on a plane perpendicular to branches by placing a 2 mm fibre-optic probe gently against the surface of the coral tissue. Minimum fluorescence (F in illuminated samples or F_0 in dark-adapted samples) was determined by applying a weak pulse-modulated red measuring light (0.15 μmol quanta m^{-2} s^{-1}). The maximum fluorescence (F_m' in illuminated samples or F_m in dark-adapted samples) was then measured, following application of a saturating pulse of actinic light (> 3000 μmol quanta m^{-2} s^{-1}). Both the effective quantum yield of light adapted symbionts, ($[F_m' - F] / F_m' = \Delta F / F_m'$) and the maximum quantum yield of dark adapted symbionts ($[F_m - F_0] = F_v / F_m$) were

measured. Twice a week, six replicate measurements were taken from random sectors of each colony, but standardized so that they were always taken about halfway down branches that were at least 5 cm from colony margins to avoid areas of new growth. Measurements were taken at 15:00 to 15:30 h to obtain $\Delta F / F_m'$ and at 1 to 1.5 hours after sunset to obtain F_v / F_m. Measurements were not taken on branches that had suffered partial or full mortality. A more detailed description on the use of PAM fluorometry to monitor photoinhibition in *Symbiodinium* spp. can be found in Jones et al. (2003).

Density of *Symbiodinium* spp.: Immediately following the conclusion of the experimental diuron exposure, three branches were collected from each colony. All branch samples were collected ~5 cm from colony margins. Coral tissues were stripped with a jet of 0.2 µm filtered seawater (~12 ml) and an airbrush (Szmant & Gassman 1990). Tissue slurries were homogenized for 15 s and the volume of the homogenate recorded. Densities of *Symbiodinium* spp. were determined with a haemocytometer. Total protein within the slurries was determined using BIO-RAD DC Protein Assay kits (Bio-Rad Laboratories, Hercules CA, USA) as per the manufacturer's instructions. Densities of *Symbiodinium* cells were normalized to total protein (number of cells per mg protein) because total protein has been shown to remain constant before and after spawning in a range of coral species (Leuzinger et al. 2003) and has also been used to normalize coral tissue biomass in previous studies (Edmunds & Gates 2002).

Photosynthetic pigment composition: Photosynthetic pigments (chlorophylls and carotenoids) were extracted from 3 additional branches collected from each colony of *Acropora valida* immediately following the conclusion of the experimental exposures. All branch samples were collected ~5 cm from colony margins. Each branch was placed in a 15 ml centrifuge tube with 4 ml of pre-chilled isopropanol:methanol (50:50, v/v). Each tube was vortexed for 30 s and stored in the dark on ice for 30 min to protect carotenoids from degradation. The supernatant was then removed and the process repeated (a third extract revealed that the combined efficiency of the first 2 extracts was >95%). The combined supernatants were then centrifuged at 2300 x g for 2 min prior to analysis. High performance liquid chromatography (HPLC) analysis was performed on a Waters 600 HPLC, combined with a Waters PDA 996 photodiodearray detector. Photosynthetic pigments were separated using a 5 µm, 250 x 4.6 mm Alltima

octadecylsilane (ODS) column (Alltech) with a mobile phase gradient (1.0 ml min^{-1}). Percentages of the solvents A, B and C, respectively, were as follows: 0 min: 100, 0, 0%; 0-4 min gradient to 0, 100, 0%; 4-15 min gradient to 0, 80, 20 %; 15-25 min gradient to 0, 20, 80%; 25-30 min hold at 0, 20, 80 %; 30-31 min gradient to 0, 100,0 %; 31-40 min gradient to 100, 0, 0%. Solvent A was methanol : 0.5 M ammonium acetate (aq.; pH 7.2, 80:20 v/v), solvent B was acetonitrile : water (90:10 v/v) and solvent C was 100% ethyl acetate (HPLC grade). Peridinin, diadinoxanthin, diatoxanthin and chloropylls *a* and c_2 (chl *a* and chl c_2) standards were obtained from the International Agency for ^{14}C Determination (Denmark) and β-carotene standard was obtained from Sigma-Aldrich. The identified peaks (91% of area on the chromatogram at 440 nm) were confirmed by comparison of retention times, absorption spectra and spiking with the above standards. The dinoxanthin peak (Fig. 2.5A, 24 min, an additional 3% of the area on the chromatogram) was identified based upon its absorption spectra and by calculating retention time ratios from the diadinoxanthin standard and published data (Wright & Jeffrey 1997). Coral tissue was lost during the pigment extraction procedure so pigment concentrations were normalized to skeletal surface area (cm^{-2}) using the wax weight method (Stimson 1987).

Reproductive investment. Total lipid content: Two branches were sampled from each colony at three stages throughout the study: (1) before exposure to diuron, (2) just prior to either spawning (i.e. 8 and 12 weeks after starting experimental exposures for *Acropora tenuis* and *A. valida*, respectively) or planulation (i.e. 4 and 8 weeks after the start of exposure for *Pocillopora damicornis*), and (3) following either spawning (i.e. within 3 d of spawning) or final planulation (i.e. 1 day after no larvae were captured in outflow nets following the second planulation event). All branches were collected ~5 cm from colony margins. Samples were frozen in liquid nitrogen and stored at –80°C prior to processing. Branches were ground into a homogeneous powder of coral tissue and skeleton with a frozen mortar and pestle and the tissue was freeze-dried prior to total lipid extraction. Total lipids were extracted from 0.5 g of tissue powder with dichloromethane:methanol (2:1 v/v) following the method described by Harland et al. (1992). Since coral branches were ground into a fine homogeneous powder total lipid content was standardized against total protein (Edmunds & Gates 2002).

Fecundity of broadcast spawners: Branches from the broadcast spawning species *Acropora tenuis* and *A. valida* were sampled (~5 cm from colony margins) at the same time as the branches used for lipid analysis and fixed in a solution of 10% formaldehyde in seawater for 24 hrs. Branches were then decalcified in a solution of 6% formic acid and 2% formaldehyde in distilled water. Four polyps, located at least 2 cm from the tip of branches to avoid sterile zones, were dissected from each colony under a dissecting microscope and the number of eggs per polyp counted. Individual eggs were separated from the mesentery and photographed. Mature eggs of *Acropora* spp. are irregular in shape so the circumference of each egg was traced using Optimas (Media Cybernetics, Silver Spring MD, USA) and the planar surface area calculated as a proxy for egg size.

Fecundity of brooding coral: One branch per colony (n = 6 for each treatment) was randomly sampled from the internal brooder *Pocillopora damicornis,* as described above for lipid analysis and fixed and decalcified as described above for broadcast spawning corals. Each branch was dissected longitudinally to examine the aboral sections of the polyps where developing larvae were visible. The number of polyps containing planulae was expressed as a percentage of the total number of polyps examined per branch (n = 560 ± 61 polyps per branch dissection). The total number of reproductive polyps included: (1) young early stage eggs with a central nucleus, (2) mature late stage eggs with nucleus located peripherally, (3) planula larvae without mesenteries and (4) planula larvae with mesenteries. Stages 3 and 4 indicate that successful fertilisation has occurred. Only 1 branch of the 36 dissected contained unfertilised eggs. Individual planulae were separated from the polyp and the size and surface areas calculated as for eggs of broadcast spawners.

Data analysis. Changes in mean photosynthetic yields (i.e. both effective and maximum quantum yields as measured by PAM fluorometry) were compared among treatments using repeated-measures ANOVA ($\alpha = 0.05$, all stats reported in text throughout the thesis as $F_{\text{d.f. factor, d. f. error}} = x$, $p = y$), with sampling occasions as the repeated measure over time (Zar 1996). For both measures of photosynthetic efficiency, only yield values obtained during the diuron exposure were used in the analysis (i.e. pre-exposure and recovery values were not included). Mean photosynthetic pigment concentrations and lipid composition ratios were compared

across the 4 diuron treatments with a 1-way ANOVA ($\alpha = 0.05$). Data not conforming to the assumptions of normality were log transformed prior to analyses. *Symbiodinium* spp. density, total lipid content and all fecundity measurements were compared for each species using 1-Way ANOVAs ($\alpha = 0.05$). Tukey's honestly significant difference (HSD) test was used to identify significantly different means. All statistical analyses were performed on SPSS 11.0.

2.3. RESULTS

PAM chlorophyll fluorescence measurements

Mean maximum effective quantum yields ($\Delta F / F_{m'}$) of the light-adapted symbionts within control colonies of *Acropora tenuis*, *A. valida* and *P. damicornis* (0 μg l^{-1} diuron tanks) were 0.67 ± 0.01, 0.61 ± 0.01 and 0.69 ± 0.01 (± SE), respectively, throughout the entire exposure period (Fig. 2.1A). In comparison, long-term exposures to nominal concentrations of 1.0 and 10 μg l^{-1} diuron caused significant reductions in the mean $\Delta F / F_{m'}$ of the light adapted symbionts for all three species and at both concentrations tested (*A. tenuis*; $F_{(2,14)} = 560$, $p < 0.01$, *A. valida*; $F_{(2,21)} = 696$, $p < 0.01$ and *P. damicornis*; $F_{(2,15)} = 842$, $p < 0.01$) (Fig. 2.1A). PSII photoinhibition occurred rapidly (within the first 24 hrs) and $\Delta F / F_{m'}$ remained depressed during the entire exposure period for each species (Fig. 2.2; *A. tenuis* data shown). Mean $\Delta F / F_{m'}$ values for all 3 species were reduced to approximately 80 and 25% that of the controls for the 1.0 and 10 μg l^{-1} diuron treatments respectively (Fig. 2.1A). The $\Delta F / F_{m'}$ values for each species returned to control levels after 3 to 5 days recovery in uncontaminated seawater (Fig. 2.2A; *A. tenuis*).

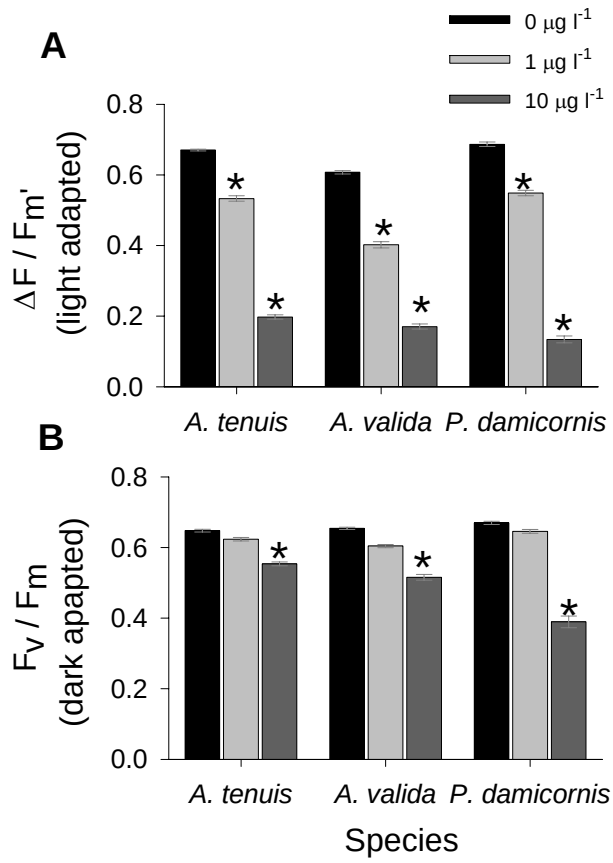

Fig. 2.1: *Symbiodinium* spp. *in hospite* in *Acropora tenuis, A. valida* and *Pocillopora damicornis.* Photosynthetic efficiencies of *Symbiodinium* spp. among corals exposed to constant flow-through concentrations of diuron (0, 1.0 and 10 µg l^{-1}) for 2-3 months prior to reproductive events: (**A**) mean $\Delta F / F_m'$ effective quantum yield (light adapted); and (**B**) mean F_v / F_m maximum quantum yield (dark adapted). Data are means ± SE, n = 6 colonies. * Means significantly different from the 0 µg l^{-1} control ($p < 0.05$).

The mean maximum quantum yields (F_v / F_m) from dark-adapted colonies of *Acropora tenuis*, *A. valida* and *P. damicornis* were identical (0.65 ± 0.01) in the 0 µg l^{-1} tanks containing uncontaminated seawater (Fig. 2.1B). F_v / F_m of all 3 species were significantly reduced during the 10 µg l^{-1} exposures: *A. tenuis* to 85% ($F_{(2,15)} = 55.8$, $p < 0.01$), *A. valida* to 80% ($F_{(2,21)} = 53.4$, $p < 0.01$) and *P. damicornis* to 58% of the control ($F_{(2,15)} = 74.4$, $p < 0.01$) (Fig. 2.1B). Reductions in maximum quantum yields were recorded for each species throughout the experimental exposures, indicating chronic photoinhibition (Jones & Kerswell 2003). F_v / F_m for *A. valida* exposed to 10 µg l^{-1} diuron declined gradually over time, dropping to as low as 80% of the control value, on day 41 of exposure. In contrast, F_v / F_m for *A. tenuis* colonies in the 10 µg l^{-1} diuron treatment dropped to 85% of the control values on day 6 of exposure and then remained consistent throughout 7.5 weeks of treatment. Symbionts in all surviving adult coral

colonies recovered rapidly, exhibiting F_v / F_m values similar to control colonies at the end of the diuron exposure (Fig. 2.2B).

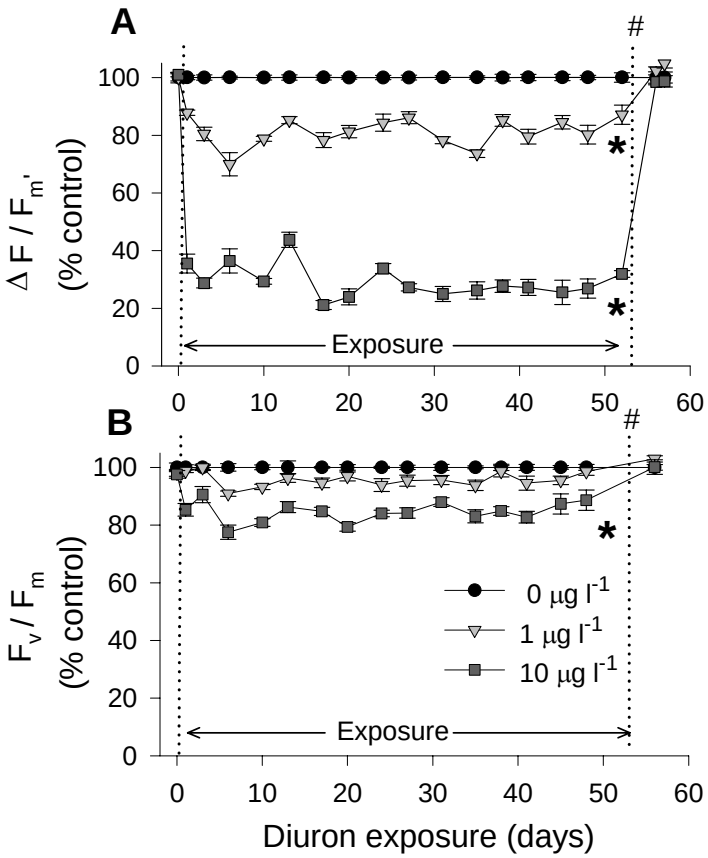

Fig. 2.2: *Symbiodinium* spp. *in hospite* in *Acropora tenuis.* Photosynthetic efficiencies (as a percent of control values) of *Symbiodinium* spp. associated with *A. tenuis* during a 53 day exposure to diuron and a 3 day recovery in uncontaminated seawater: (**A**) mean ΔF / F_m' effective quantum yield (light adapted); and (**B**) mean F_v / F_m maximum quantum yield (dark adapted). Data are means ± SE, n = 6 colonies.
* Means significantly different from the 0 μg l^{-1} control ($p < 0.05$); # = spawning event.

Bleaching and mortality of experimental corals

All colonies of *Acropora tenuis* and *Pocillopora damicornis* survived the 1.0 and 10 μg l^{-1} diuron treatments. All colonies of *A. valida* survived except for two of the eight colonies in the 10 μg l^{-1} treatment, which died following 6 to 7 weeks of diuron exposure. The remaining colonies of *A. valida* in the 10 μg l^{-1} treatment tanks sustained partial mortality. No partial mortality was observed for the other two species in any of the treatments.

Bleaching was not observed for *Acropora tenuis* following long-term photoinhibition caused by 1.0 and 10 μg l^{-1} diuron exposures. These macroscopic

observations were corroborated by the consistent *Symbiodinium* spp. densities found in comparisons among treatments (Fig. 2.3, $F_{(3,20)} = 1.2$, $p = 0.3$). All colonies of *A. valida* in the 10 μg l^{-1} treatment exhibited patchy bleaching (visible to the naked eye) with some branches fully pigmented, some with patches of bleached tissue and others being completely devoid of pigmentation (Fig. 2.4). Severe bleaching preceded the partial and complete mortality described above. Nevertheless, densities of *Symbiodinium* spp. in the unbleached and patchily bleached branchlets of *A. valida* in the 10 μg l^{-1} treatment were not significantly lower than in branches of controls ($F_{(3,21)} = 1.3$, $p = 0.3$) (Fig. 2.3). Severe bleaching and loss of *Symbiodinium* spp. was observed for the *P. damicornis* colonies exposed to 10 μg l^{-1} diuron after 25 days of exposure, and the colonies remained bleached throughout the second planulation event in April ($F_{(2,15)} = 6.0$, $p = 0.01$) (Figs. 2.3 and 2.4B, 0 μg l^{-1}; Fig. 2.4D, 10 μg l^{-1}).

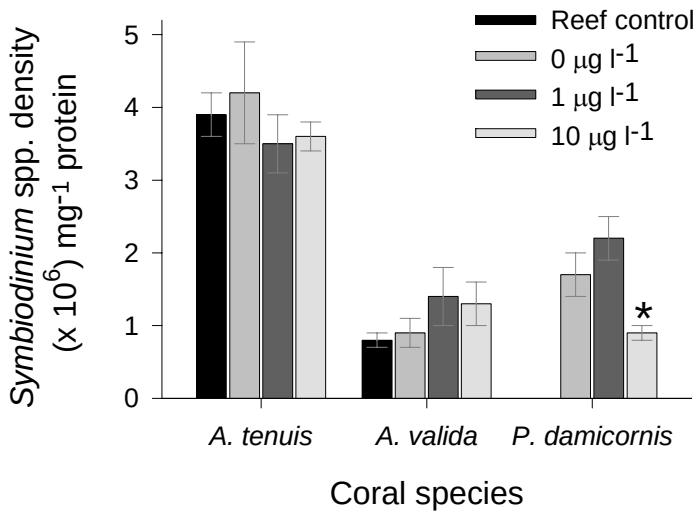

Fig. 2.3: *Symbiodinium* spp. density in *A. tenuis*, *A. valida* and *P. damicornis*. Comparison of *Symbiodinium* spp. densities among reef coral colonies following 2-3 month exposure to four diuron treatments (reef control, 0, 1.0 and 10 μg l^{-1}). Data are means ± SE, n = 6 colonies per treatment. * Means significantly different from the 0 μg l^{-1} control ($p < 0.05$).

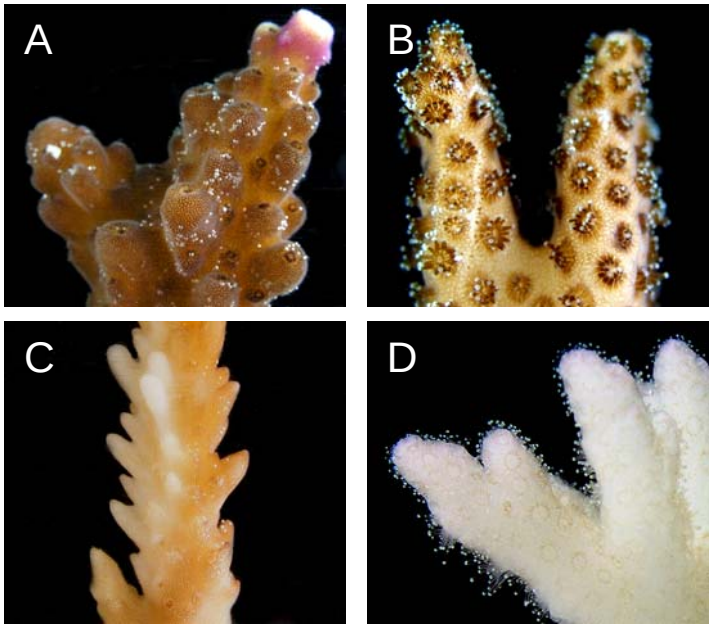

Fig. 2.4: *Acropora valida* and *Pocillopora damicornis*. Images of normally pigmented and bleached corals in control (0 μg l^{-1}) versus 10 μg l^{-1} diuron treatments: (**A**) *A. valida* in 0 μg l^{-1}; (**B**) *P. damicornis* in 0 μg l^{-1}; (**C**) *A. valida* in 10 μg l^{-1} and (**D**) *P. damicornis* in 10 μg l^{-1} diuron.

Photosynthetic pigment composition

Major light harvesting pigments identified from the *Symbiodinium* spp. within tissues collected from *Acropora valida* tank controls included chlorophyll *a* (44 mol % of total pigments), chlorophyll c_2 (12 mol %) and peridinin (35 mol %) (Fig. 2.5). Diadinoxanthin (7 mol %) was the dominant pigment identified that is involved with oxidative stress and non-photochemical quenching (NPQ). Minor peaks of the NPQ pigments, dinoxanthin (1.5 mol %), diatoxanthin (0.15 mol %) and β-β carotene (1.5 x 10^{-4} mol %) were also found (Fig. 2.5A). Total concentrations of the light harvesting pigments chl *a,* c_2 and peridinin present in branches of *A. valida* within the 10 μg l^{-1} treatment were significantly reduced to 35% of concentrations found for the other three treatments ($F_{(3,8)}$ = 8.0, p = 0.008) (Fig. 2.5B). Carotenoids involved with NPQ and oxidative stress (diadinoxanthin, dinoxanthin, diatoxanthin and β-β carotene) were also significantly reduced to 36% of the other 3 treatments (Fig. 2.5B, $F_{(3,8)}$ = 7.8, p = 0.009) when exposed to 10 μg l^{-1} diuron. Although the total concentrations of these pigments were reduced following diuron exposure, the profiles (ratios) of the light harvesting pigments : carotenoids were not affected (Fig. 2.5B). The mean ratio of peridinin : chl

a remained unchanged at 0.82 ± 0.04 ($F_{(3,8)}$ = 1.2, p = 0.4) as did the mean ratio of carotenoids (NPQ) : chl *a* at 0.22 ± 0.01 ($F_{(3,8)}$ = 1.3, p = 0.3).

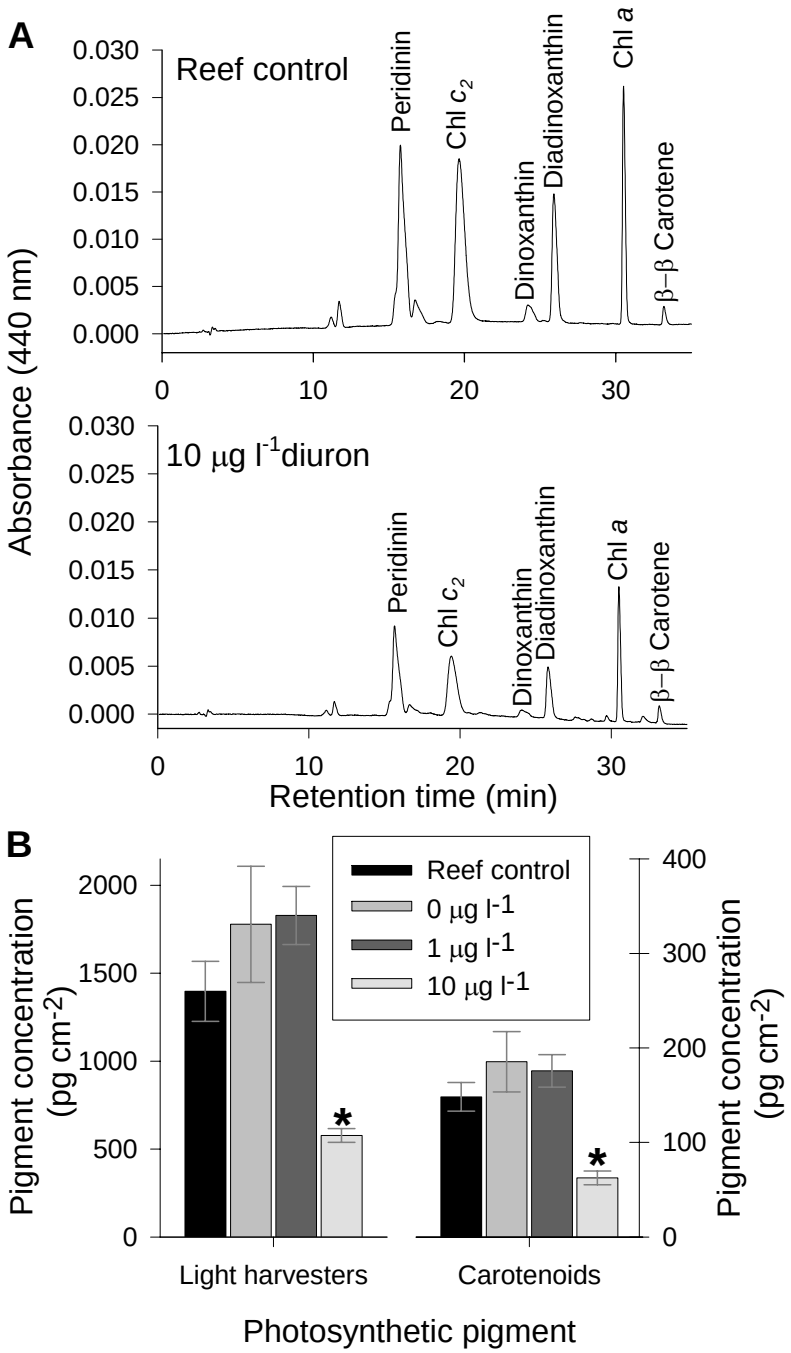

Fig. 2.5: *Acropora valida*. Photosynthetic pigments extracted from *A. valida* tissues following long-term diuron induced photoinhibition: (**A**) HPLC chromatogram of a pigment extract from *A. valida* whole tissue, (**B**) Concentration of chlorophyll-a, peridinin and xanthophyll carotenoids (diadinoxanthin, diatoxanthin and diandinochromes) in pg cm^{-2}. Data are mean ± SE, n = 3 branches per treatment.
* Means significantly different from the 0 μg l^{-1} control ($p < 0.05$).

Reproductive investment: total lipid content

Total lipid content of the two broadcast spawning species, *Acropora tenuis* and *A. valida* did not change significantly within tissues of the reef control and 0 μg l^{-1} control colonies during the 2 to 3 months leading up to spawning (*A. tenuis* reef control: $F_{(2,17)} = 2.6$, $p = 0.1$ (Fig. 2.6A); *A. valida* reef control: $F_{(2, 17)} = 0.44$, $p = 0.6$ (Fig. 2.6B)). In contrast, exposure to 10 μg l^{-1} diuron resulted in significant reductions in total (parent + egg) lipid content within the tissues of both broadcast spawning species prior to spawning; total lipids dropped in *A. tenuis* to approximately 40% of reef control values in *A. tenuis* ($F_{(3,20)} = 4.8$, $p = 0.01$) and to 15% of reef control values in *A. valida* ($F_{(3,20)} = 5.1$, $p = 0.009$). In *A. valida*, diuron concentration as low as 1.0 μg l^{-1} also reduced lipid content to ~25% of the reef control colonies prior to spawning (Fig. 2.6B, $F_{(2,15)} = 6.9$, $p = 0.007$). This decline in lipid content may be attributable to a drop in parental lipid storage since there was no further decline in lipid following spawning within the 1.0 μg l^{-1} treatment (Fig. 2.6B, $p = 0.6$). In the internal brooder, *P. damicornis*, lipid content decreased significantly within the first 3.5 weeks of exposure to 10 μg l^{-1} of diuron leading up to the first planulation event. Lipid content remained significantly reduced in comparison to the other two treatments throughout the second month of exposure leading up to the second planulation event (Fig. 2.6C, $F_{(3,20)} = 23.5$, $p < 0.001$).

Following spawning, lipid content declined further in the colonies of *A. tenuis* (coral tissue only) in the 10 μg l^{-1} diuron treatment, consistent with the release of eggs (Fig. 2.6A). The 0 and 1.0 μg l^{-1} colonies of *A. tenuis* spawned unexpectedly early, 2 or 3 nights before the full moon in October, but the precise timing of each spawning was not able to be recorded. Because the timing of spawning was unknown for this species, after-spawning total lipid values for these two treatments were omitted from the *A. tenuis* results (Fig. 2.6A). *Pocillopora damicornis* colonies exposed to 10 μg l^{-1} also exhibited a significant drop in lipid content, from 1.3 ± 0.2 to 0.35 ± 0.04 mg lipid mg $protein^{-1}$ within 20 days, as measured from the sample taken before the first planulation event and to the sample taken before the second planulation (Fig. 2.6C, Tukey HSD $p = 0.005$). Total lipid remained at this low level until after the second planulation event. Reductions in lipid content was only observed in colonies of *P. damicornis* exposed to 10 μg l^{-1} diuron, with total lipid content remaining consistent during the 3 month experiment for the tank control (0 μg l^{-1}) and the 1.0 μg l^{-1} treatment colonies (Fig. 2.6C).

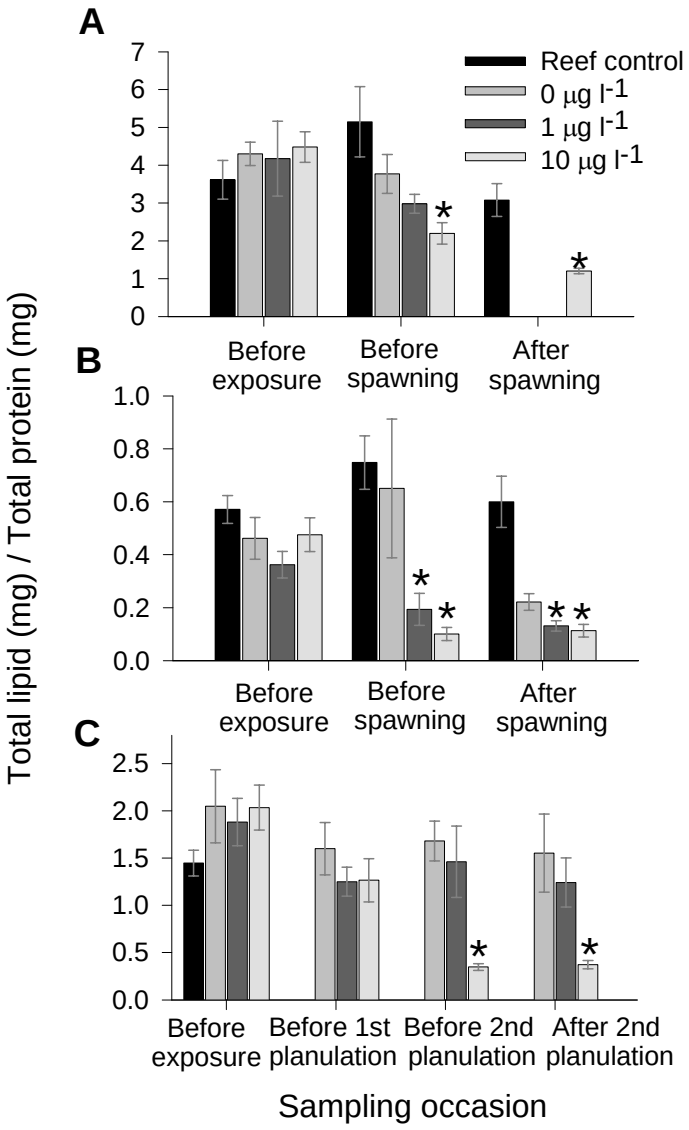

Fig. 2.6: *Acropora tenuis, A. valida* and *Pocillopora damicornis*. Comparison of total lipids (mg per mg protein) throughout long-term exposures to 0, 1.0 and 10 µg l^{-1} diuron within whole tissue of (**A**) *Acropora tenuis*, a broadcast spawner; (**B**) *Acropora valida*, a broadcast spawner; (**C**) *Pocillopora damicornis*, an internal brooder. Data are means ± SE, n= 6 colonies per treatment. * Means significantly different from the 0 µg l^{-1} control ($p < 0.05$).

Reproductive investment: fecundity of broadcast spawners

Reproductive activity following exposure to diuron also differed dramatically between the two broadcast spawning species. Colonies of *A. tenuis* exposed to 10 µg l^{-1} diuron successfully spawned gametes and there was no significant reduction in either the average number of eggs per polyp ($F_{(3,20)} = 1.2$, $p = 0.3$) or the average egg size produced ($F_{(3,20)} = 0.68$, $p = 0.6$) (Table 2.1). In contrast, very few polyps of *A. valida* exposed to similar levels of photoinhibition in the 10 µg l^{-1} diuron treatment contained

eggs, resulting in significantly reduced polyp fecundity ($F_{(3,12)} = 7.6$, $p = 0.004$) (Table 2.1). Although eggs of *A. valida* that were present in the 10 μg l^{-1} treatment did not differ in size in comparison to eggs in the other 3 treatments (Table 2.1, $F_{(3,17)} = 0.8$, $p = 0.5$), no spawning (reproductive output) was observed. Less severe photoinhibition by 1.0 μg l^{-1} diuron did not affect fecundity or egg size of either species, suggesting that the reduction in total lipid content observed for branches of *A. valida* in this treatment (Figure 2.6B) occurred within the somatic tissue and not the reproductive tissue of this species (Table 2.1).

Reproductive investment: fecundity of internal brooder

Approximately 150 to 200 planula larvae of *Pocillopora damicornis* were collected daily from most exposure tanks during two separate planulation events, which each lasted approximately 7-10 days each following the new moons in both March and April. All of the colonies (n = 8) within the 10 μg l^{-1} treatment group were bleached and failed to release larvae during the second planulation event in April following 53 days of severe photoinhibition. Dissections of branches indicated that the size of fully developed planulae did not vary significantly among diuron treatments ($F_{(2,3)} = 0.9$, $p = 0.5$) (Table 2.1). Moreover, no unfertilised oocytes were detected, indicating diuron did not negatively affect the process of internal fertilisation. In contrast, the average number of reproductive polyps per branch varied widely. Colonies in the 1.0 μg l^{-1} treatment contained a significantly greater number of reproductive polyps than colonies from the other two treatments sampled before the 2nd planulation ($F_{(2,15)} = 3.8$, $p = 0.04$) (Table 2.1). Although the severely bleached colonies in the 10 μg l^{-1} treatment did not release larvae during the second planulation event in April, 33% of the sampled branches still contained reproductive polyps, indicating that the host colony may have been storing the developed larvae as a possible energy source.

Table 2.1: *Acropora tenuis, A. valida* and *Pocillopora damicornis*. (**A**) Mean polyp fecundity (no. of eggs polyp^{-1}) and mean egg planar surface area (μm^2) of the broadcast spawners *A. tenuis* and *A. valida* and (**B**) mean branch fecundity (% reproductive polyps branch^{-1}) and mean planula planar surface area (μm^2) of the internal brooder *P. damicornis*. Branches were sampled before the spawning event (*A. tenuis* and *A. valida*) and before the first and second planulation events (*P. damicornis*). Data are means ± SE. * Means significantly different from 0 μg l^{-1}control ($p < 0.05$).

(A) Broadcast Spawners	**Polyp fecundity (egg # polyp^{-1}; n = 4 polyps)**	**Mean egg size (μm^2)**
Acropora tenuis		(n = 630 eggs total)
Reef Control	7.2 ± 0.8	183 ± 8
0 μg l^{-1}	7.3 ± 0.7	198 ± 12
1 μg l^{-1}	5.6 ± 1.2	162 ± 35
10 μg l^{-1}	5.9 ± 0.6	201 ± 20
Acropora valida		(n = 353 eggs total)
Reef Control	6.3 ± 1.3	241 ± 15
0 μg l^{-1}	5.7 ± 0.8	236 ± 23
1 μg l^{-1}	5.6 ± 0.5	245 ± 7
10 μg l^{-1}	1.0 ± 1.4*****	227 ± 28
(B) Internal Brooder	**Branch fecundity (% reproductive polyps branch^{-1}; n = 6 branches)**	**Planula size (μm^2)**
Pocillopora damicornis		
March (1st planulation)		(n = 131 planulae total)
0 μg l^{-1}	2.1 ± 0.9	155 ± 7
1 μg l^{-1}	1.1 ± 0.7	218 ± 11
10 μg l^{-1}	0.9 ± 0.4	177 ± 11
April (2nd planulation)		(n = 124 planulae total)
0 μg l^{-1}	0.2 ± 0.2	203 ± 7
1 μg l^{-1}	4.1 ± 1.7*****	285 ± 14
10 μg l^{-1}	1.7 ± 1.1	313 ± 15

2.4. DISCUSSION

These results demonstrate that photoinhibition caused by exposure to moderate levels of diuron (10 μg l^{-1}) in the final months of gametogenesis or larval development reduces the reproductive output of corals, and in some cases, may inhibit reproduction completely. The broadcast spawning coral, *Acropora valida,* and the brooding coral, *Pocillopora damicornis,* were both sensitive to energy deficiencies caused by diuron-induced photoinhibition. These species ultimately failed to release gametes or larvae following experimental exposures to 10 μg l^{-1} diuron throughout the final 13 weeks of gametogenesis or 9.5 weeks of larval development, respectively. In contrast, identical concentrations of diuron had negligible impact on *A. tenuis*, which spawned successfully following exposure throughout the final 7.5 weeks of gametogenesis. For the more sensitive *A. valida,* exposure to 10 μg l^{-1}diuron resulted in partial mortality (loss of tissue on some branches) in 100% and full mortality in 25% of experimental colonies (n = 8 colonies). This is the only documented case of coral mortality resulting from photoinhibition at moderate (10 μg l^{-1}) diuron concentrations.

The inhibition of reproduction in colonies of *Acropora valida* and *Pocillopora damicornis* and reduced lipid (energy storage) in all three species (including *A. tenuis*) exposed to 10 μg l^{-1} diuron were likely caused by significant reductions in photosynthetic energy available for allocation to reproductive processes. Diuron induces rapid inhibition of electron transport through the PSII (Jones et al. 2003) and significantly reduces carbon fixation in *Symbiodinium* spp. (Owen et al. 2003). Consistent reductions in effective quantum yields (mean $\Delta F/F_m'$) to between 19 % (*P. damicornis*) and 28 % (*A. tenuis* and *A. valida*) of controls in the 10 μg l^{-1} diuron treatments imply dramatic reductions in carbon fixation over the exposure periods. Although reductions in effective quantum yields were not as great in the 1.0 μg l^{-1} diuron treatment (yields were reduced to 67 % of control values for *A. valida* and 80 % of controls for *A. tenuis* and *P. damicornis*), they were also likely to have affected carbon fixation. Previous studies have documented similar rapid decreases in $\Delta F/F_m'$ values for adult (Jones & Kerswell 2003) and juvenile (Negri et al. 2005) corals exposed to low (1 μg l^{-1}) and moderate (10 μg l^{-1}) concentrations of diuron, although exposure periods were much shorter (a few days). Partial compensation for chronic photoinhibition at PSII by state transitions may allow a switch from linear (electrons flow in series from PSII into PSI) to cyclic electron flow around PSI that is independent

of electron transport from PSII (Finazzi & Forti 2004). However, since there is good quantitative agreement between the reduction in photosynthetic efficiency at 10 μg l^{-1} (Jones et al. 2003) and reduced fixation of carbon within isolated *Symbiodinium* spp. (Owen et al. 2003), it is more likely that photoinhibition in PSII reduces carbohydrate synthesis (hence energy from photosynthates available to the host) in a relatively direct manner.

Significant decreases in maximum quantum yields (F_v/F_m) compared to controls for all three species in the presence of diuron indicate chronic photoinhibition resulting from secondary damage to the PSII reaction centres (Osmond et al. 1999). The largest reductions in F_v/F_m (by 42%) were observed for *Pocillopora damicornis* in the 10 μg l^{-1} diuron treatment and concomitantly, this species suffered the most extensive bleaching (loss of pigments or symbionts). In contrast, mean F_v/F_m for *Acropora tenuis* was reduced by only 15 % compared to controls, and colonies did not bleach, further indicating that chronic photoinhibition was not as severe for this species. *A. valida* exhibited intermediate (21%) declines in F_v/F_m and in some cases host mortality followed. Chronic photoinhibition is a long-lasting process caused by the degradation and reduced turnover of the reaction centre D1 protein because the binding of diuron inhibits the synthesis of proteins (Arg238) specifically associated with the herbicide binding niche of the PSII (Oettmeier 1992, Osmond et al. 1999, Jones 2004). Chronic photoinhibition can lead to bleaching, as was observed for both *P. damicornis* and *A. valida* in the present study, either because of dissociation of the coral-algal symbiotic relationship and/or reductions in photosynthetic pigment content. It has been suggested that long-term diuron exposures would cause irreversible damage to PSII and would result in a 'sublethal' bleaching response (Jones et al. 2003). Although *A. tenuis* exposed to 10 μg l^{-1} diuron suffered chronic photoinhibition, its symbiosis with *Symbiodinium* remained intact, suggesting that this partnership is the most resistant of the three species. This suggests that not all coral species will suffer from the sublethal bleaching response following long-term diuron exposure at 10 μg l^{-1} and that the photochemical efficiency [maximum quantum yield (F_v/F_m)] can recover to healthy levels (compared to the control colonies) following 3 to 5 days of recovery in fresh seawater. Comparing F_v/F_m values from dark adapted symbionts as an indicator of recovery must be used cautiously however, since *A. valida* and *Pocillopora damicornis* were both visibly bleached and not physiologically healthy but exhibited recovered F_v/F_m values when compared to the control symbionts. This indicates that the remaining

symbionts within the host tissues had recovered and the PAM fluorometer is measuring the photochemical efficiency from recovered symbionts, however the *A. valida* and *P. damicornis* colonies within the 10 μg l^{-1} treatment were not physiologically recovered after 5 days of fresh seawater flow.

Reduced photosynthetic efficiency as a result of diuron exposure is likely to constitute a major energy constraint for corals, because the algal partner translocates up to 95% of its photosynthates (carbohydrates) to the coral host (Muscatine et al. 1984). Owen et al. (2003) demonstrated that exposure of *Symbiodinium* spp. to 10 μg l^{-1} diuron reduces photosynthetic carbon fixation by 60 to 70%. In these experiments, exposure of *Acropora valida* to 10 μg l^{-1} diuron resulted in an 85% reduction in polyp fecundity. Although the sizes of remaining eggs did not differ from those of controls, the corals were unable to spawn, possibly because the eggs were not fully mature. Reductions in the number rather than the size of eggs suggest that *A. valida* may have reabsorbed some oocytes to counter energy deficiencies. Michalek-Wagner and Willis (2001a) also reported that colonies of the soft coral *Lobophytum compactum,* which had been moderately bleached midway through their oogenic cycle, contained nearly 45% fewer mature eggs per polyp than non-bleached colonies as a result of nutritional limitations from the loss of photosynthetic symbionts. This strategy of sacrificing egg numbers to enable the development of a few viable eggs may be common among stressed corals (Yamazato et al. 1981, Michalek-Wagner & Willis 2001a). In the brooding coral *P. damicornis*, normal-sized planulae were observed within dissected polyps and larvae were successfully released during the first planulation event. Normal-sized planulae were also observed within the dissected polyps of the severely bleached *P. damicornis* colonies exposed to 10 μg l^{-1} diuron (Fig. 2.4D); however, these larvae were not released during the second planulation event. Energy deficiencies experienced by *P. damicornis* resulting from reduced photosynthetic production are likely to have interfered with the development of larvae, potentially inhibiting or delaying their release. It is possible that diuron also has a direct non-target effect on unknown biochemical pathways of the coral host, however there is currently no biochemical evidence of direct diuron toxicity on invertebrates at the low to moderate concentrations used in the present study. Furthermore, the lack of impact from high (100 μg l^{-1}) concentrations of diuron on the fertilisation, metamorphosis and health of juvenile corals during their aposymbiotic life history stages (Negri et al. 2005, Watanabe et al. 2006) supports the assumption that diuron does not directly impact on the hosts

physiological performance. *A. valida* and *P. damicornis* appear to be highly dependent on energy from photosynthesis for reproduction. Under the conditions of severe photoinhibition caused by diuron, these corals are likely to have preferentially allocated energy towards critical metabolic activities at the expense of reproduction.

In contrast to the decreased reproductive output found for *Acropora valida* and *Pocillopora damicornis* exposed to diuron, reductions in the numbers of eggs were not observed in *A. tenuis*. Furthermore, *A. tenuis* colonies exposed to 10 μg l^{-1} diuron successfully spawned, indicating that diuron-induced photoinhibition did not negatively impact on the reproductive development of this species. It is possible that *A. tenuis* may not be as dependent on autotrophy as *A. valida* and *P. damicornis*, potentially countering deficiencies in energy from photosynthates (induced by extended periods of photoinhibition) through heterotrophic feeding (Anthony & Fabricius 2000, Anthony 2006, Grottoli et al. 2006). Heterotrophy could have involved plankton, suspended particulate matter or detritus in the experimental treatments, and corals are known to vary in their ability to utilise heterotrophic feeding to enhance tissue growth (Anthony & Fabricius 2000). Thus, there is likely to be variation in the impacts of photoinhibition according to a species' ability to increase energy acquisition from heterotrophy to offset reductions in photosynthetic carbohydrates. It is also possible that the 2 to 4 week longer periods of exposure to diuron experienced by the other 2 species may correspond to a minimum period of photoinhibition required to produce an impact on reproduction. Although the energetic demands of spermatogenesis within corals is largely unknown, *A. tenuis* did suffer significant photoinhibition throughout most of the spermatogenic cycle, yet spawning was not inhibited. Moreover, *A. tenuis* was exposed to the diuron treatments for 1.5 weeks longer than the 6 week period that resulted in bleaching in *A. valida*. In combination, these results suggest that *A. tenuis* may be better able to meet its nutritional requirements from other sources. Alternatively, some of the variation in diuron-induced photoinhibition among the three study species could be related to differences in energy translocated from the *Symbiodinium* clades they host. *Acropora tenuis* predominantly hosts clade C1 (van Oppen et al. 2001), whereas *A. valida* harbours a mix of clades D and C at the Magnetic Island site where experimental colonies were collected (Ulstrup & van Oppen 2003). Little et al. (2004) also demonstrated that genetically distinct *Symbiodinium* spp. provide differential benefits to the physiological performance of the coral host, as shown by two-fold greater growth

rates of juvenile *Acropora* spp. hosting clade C compared to clade D. Carbon fixation experiments comparing the translocation of radiolabelled photosynthates into the tissues of corals hosting different *Symbiodinium* types, provide a promising model for exploring the physiological links between the symbiont and the coral host, and would help to unravel the differing species-level responses to diuron in the present study. Such studies would also help to further validate postulated links between chronic diuron-induced photoinhibition (this chapter), reduced photosynthate production in *Symbiodinium* spp. (Owen et al. 2003) and predicted reductions in photosynthate transfer to the host, which best explain the reduced reproductive investment and success in this study.

The significant reductions in total lipid prior to reproductive events involving the release of eggs or larvae in all corals exposed to 10 μg l^{-1} diuron indicate that moderate levels of diuron reduce lipid synthesis. Moreover, the reduced lipid content of *Acropora valida* prior to spawning in the 1.0 μg l^{-1} treatment indicates that even low concentrations of diuron can have an impact on lipid synthesis. Protein synthesis is dependent upon heterotrophy for nitrogen and phosphorus sources, whereas lipid synthesis is primarily stimulated by light and carbon from photosynthesis (Crossland et al. 1980, Anthony & Fabricius 2000). Therefore, reductions in total lipid in elevated diuron treatments prior to spawning or planulation suggest that this herbicide, reduced energy reserves available for investment into reproduction as a result of diminished photosynthetic productivity. The reductions in mean lipid content between samples collected immediately before and after spawning (reductions of 40 % for *A. tenuis* field control and 66 % for *A. valida* tank controls) are consistent with the release of eggs during spawning (c.f. the 70% reduction in the overall lipid content of *A. tenuis* tissues due to spawning; Leuzinger et al. 2003). The lack of a significant decline in lipid content in *Pocillopora damicornis* after planulation may reflect the low proportion of polyps that contained planulae, with these low numbers making an insignificant contribution to total lipids in comparison to the high lipid content of coral somatic tissue (Stimson 1987, Ward 1995a). Nevertheless, the reduced lipid content of all three species exposed to 10 μg l^{-1} diuron indicates that deficiencies in stored energy reserves occurred for each of the species tested. Energy deficiencies are interpreted as being greatest for *A. valida* and *P. damicornis* in accordance with the greater reductions in the number of eggs and larvae found for these two species.

The relative concentrations of xanthophyll carotenoids, diadinoxanthin and diatoxanthin, were of particular interest because of their potential to protect photosynthetic cells from damage created by diuron-induced photoinhibition. PSII herbicides such as diuron cause a build up of electrons within PSII, and the resulting triplet chlorophyll state (^{3}chl) can lead to the formation of free radicals and toxic oxygen species that can destroy the integrity of chlorophylls, disconnecting them from the antennae systems and protective carotenoids, thus further reducing the ability of symbiotic algae to capture light and produce carbohydrates (Osmond et al. 1999, Jones 2004). In light reactions, the xanthophyll cycle functions photoprotectively by converting diadinoxanthin to diatoxanthin, which dissipates excess absorbed light energy as heat (Ambarsari et al. 1997, Brown et al. 1999). In the present study, the ratio of diatoxanthin:diadinoxanthin was consistently low, even in bleached colonies of *Acropora valida* in the 10 μg l^{-1} diuron treatment. This indicates that either the conversion of diatoxanthin back to the protective diadinoxanthin was rapid or that the light levels during the time of sampling (Ambarsari et al. 1997) and the level of diuron-induced photoinhibition (Jones & Kerswell 2003) were not severe enough to significantly affect the balance within the xanthophyll pool. There was also no apparent increase in peridinin, the light harvesting carotenoid that may be used to offset decreases in chl *a* and c_2, as a strategy to maintain photosystem efficiency under oxidative stress. The consistent pigment ratios across all treatments (Fig. 2.5) indicate that any visual loss of pigmentation in the colonies within the 10 μg l^{-1} diuron treatments (Fig. 2.4c) was not likely to have resulted from pigment degradation but was instead related to expulsion of complete algal cells, eventhough the symbiont densities were not significantly reduced.

In summary, all three coral species displayed significant and consistent reductions in photosynthetic efficiency and total lipid content when subjected to long-term diuron exposures. The species *Acropora valida* and *P. damicornis* were most affected, exhibiting severe bleaching, diminished concentrations of photosynthetic pigments (*A. valida*), and decreased reproductive output. It is important to consider that additional stressors, such as increased turbidity, often accompany elevated herbicide and pesticide contamination in runoff events, potentially adding to the pressures on coral photosynthesis (Fabricius 2005, Harrington et al. 2005). These results, that herbicide-induced chronic photoinhibition and reduced symbiont densities decrease the amount of

energy available for reproduction and can inhibit reproductive output completely, highlight the importance of photosynthesis for coral reproduction. Elevated levels of diuron, at concentrations as low as 1 $\mu g\ l^{-1}$ and sustained throughout gametogenesis, are likely to reduce the replenishment of nearshore coral populations for corals that are highly dependent upon energy derived from photosynthesis.

Chapter 3: Quality of coral gametes following chronic parental herbicide exposure

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3.1. INTRODUCTION

Most reef building corals on the Great Barrier Reef (GBR) broadcast spawn their gametes, which develop into pelagic, lecithotrophic (non-feeding) larvae (Harrison & Wallace 1990). Larvae are mostly azooxanthellate (lack the symbiont, *Symbiodinium* spp.) and are heavily dependent upon maternal provisioning for energy and resources to develop, undergo metamorphosis and successfully recruit into populations as juvenile corals (Richmond 1987, Ben-David-Zaslow & Benayahu 1998). Eggs and larvae of broadcast spawning *Acropora* spp. contain a high proportion of lipid [60-70%, wax esters and triglycerides (Arai et al. 1993, Harii et al. 2002)] which: (I) adds buoyancy to increase fertilisation efficiency by concentrating gametes near the surface of the water (Harrison & Wallace 1990); (II) is crucial for medium to long distance dispersal of coral larvae; and (III) provides energy to enable larval development and metamorphosis (Richmond 1987, Villinski et al. 2002). Azooxanthellate eggs of *Acropora spp.* have a greater dependence on maternally derived resources (and higher lipid content) than eggs and larvae that are released with an established *Symbiodinium* association (zooxanthellate) (Ben-David-Zaslow & Benayahu 1996, 2000). Adult corals invest energy (as lipid) acquired from photosynthesis into reproductive tissue when their cellular energy budget is in surplus (Szmant-Froelich 1981, Rinkevich 1989, Ward 1995). The quality of eggs produced by reproductive coral colonies has been shown to drastically decrease (reduced lipid, protein, carotenoids, MAA's and fertilisation) in response to periods of stress which limit energy and resources available to the host colony (Michalek-Wagner & Willis 2001b, Loya et al. 2004). To understand the effects of stress on coral gamete quality it is critical to determine if eggs are capable of successful fertilisation and development into healthy larvae that can undergo metamorphosis into juvenile corals (Loya et al. 2004).

The parental provisioning of photoprotective biochemicals such as mycosporine-like amino acids (MAA's) (Gleason & Wellington 1995, Michalek-Wagner & Willis 2001b, Yakovleva & Baird 2005) and carotenoids (Tsushima et al. 1995, Michalek-Wagner &

Willis 2001b) to eggs has been well documented in marine invertebrates. The likely function of MAA's is to protect buoyant coral eggs from oxidative stress by absorbing harmful UVA and UVB radiation (310-360 nm) (Gleason & Wellington 1995, Dunlap & Shick 1998, Wellington & Fitt 2003). Pigments within *Symbiodinium* spp. have two primary functions: (I) harvesting of light energy and transferring it into the photosynthetic reaction centre of PSII to drive the primary reactions of photosynthesis (Iglesias-Prieto et al. 1992, Croce et al. 1999); and (II) photoprotection against solar irradiance through dissipation of excess excitation energy by the xanthophyll cycle [interconversion of diadinoxanthin into diatoxanthin and quenching of triplet chlorophyll (Demers et al. 1991, Brown et al. 1999, Croce et al. 1999)]. Pigments within the light harvesting complex (LHC) in *Symbiodinium* spp. include chlorophylls *a, c*$_2$ and the carotenoid peridinin while the photoprotective xanthophyll pool consists primarily of diadinoxanthin (DD) and diatoxanthin (DT) (Iglesias-Prieto et al. 1991, Venn et al. 2006, Cantin et al. 2007). Peridinin and the algal-derived, antioxidant α-tocopherol (vitamin E), which actively scavenges reactive oxygen species (Lesser 2006), may also form part of the oxidative protection mechanism in coral eggs. Quantifying the parental provisioning of protective resources such as carotenoids and vitamin E within spawned coral eggs is therefore a useful indicator of egg quality.

The photosystem II (PSII) herbicide diuron has one of the highest application rates on farms in catchments that flow into the GBR (Hamilton & Haydon 1996). Several recent studies have revealed the deleterious impacts of PSII herbicides on the photosynthetic efficiency of *Symbiodinium* spp. living symbiotically within the tissues of reef building corals (Jones & Kerswell 2003, Jones et al. 2003, Owen et al. 2003, Cantin et al. 2007). Diuron is often applied during times of high farm runoff and river flow and has been detected at higher concentrations in sediment (Haynes et al. 2000, White et al. 2002) and water samples (Bengtson Nash et al. 2005, Kapernick et al. 2007) than other herbicides. Diuron competes with plastoquinone for the Q_b binding site on the D_1 within the PSII and can inhibit electron transport within symbiotic *Symbiodinium* spp. *in hospite,* at concentrations as low as 0.3 μg l^{-1} (Jones & Kerswell 2003, Jones et al. 2003). This inhibition of light-induced electron transport from water through the Q_B site, reduces the efficiency of photosynthetic processes and interferes with repair mechanisms involved in the synthesis of new D1 protein (Oettmeier 1992, Jones et al. 2003). Diuron has also been shown to decrease the incorporation of carbon from photosynthesis within isolated *Symbiodinium* spp. by 30% at 2 μg l^{-1} and by 60-

70% at 10 μg l^{-1} (Owen et al. 2003). Long-term exposures to diuron can lead to chronic impacts caused by reduced photosynthate production, reducing the fecundity and reproductive output of broadcast spawning and internal brooding corals (Chapter 2; Cantin et al. 2007). Since corals have a strong dependence on light and photosynthesis for effective lipid production (Crossland et al. 1980, Anthony & Fabricius 2000) and maternal investment into gametogenesis, chronic parental diuron exposure could have significant impacts on the quality and competency of the gametes produced.

This chapter explores the effects of long-term parental exposure to diuron on the quality of gametes produced by two broadcast spawning corals that release azooxanthellate eggs and one brooding coral that releases zooxanthellate larvae. In particular, the effects of chronic electron transport inhibition in PSII of *Symbiodinium* spp. during late stage gametogenesis on gamete fertilisation, larval metamorphosis, and the provisioning of carotenoid pigments and vitamin E into eggs were examined.

3.2. METHODS

Diuron exposure: Reproductively mature broadcast spawning colonies of *Acropora tenuis* and *A. valida* (20-30 cm in diameter) were collected from Nelly Bay, Magnetic Island, Australia (19˚10'S; 146˚52'E) on August 10-25, 2003 (*A. tenuis*), and August 3, 2004 (*A. valida*), approximately 10 and 13 weeks, respectively, before the predicted nights of spawning. Reproductively mature internal brooding colonies of *Pocillopora damicornis* (15-25 cm in diameter) were collected from Horseshoe Bay, Magnetic Island (19°06' S; 146°51'E) on February 20, 2004, 4 weeks before predicted planulation. Twenty-four colonies of each species were transported to outdoor aquaria at the Australian Institute of Marine Science (AIMS), Townsville and maintained as previously described (Chapter 2; Cantin et al. 2007).

Parental coral colonies were exposed to three experimental treatments of the PSII inhibiting herbicide, diuron: 0 μg l^{-1} diuron (tank controls), a 1.0 μg l^{-1} low-dose diuron treatment (an ecologically relevant concentration that reduces effective quantum yields by 20% (Jones et al. 2003); and a 10 μg l^{-1} moderate-dose diuron treatment (selected because it reduces effective quantum yields by 80% (Jones et al. 2003). Corals were exposed to these treatments for 2-3 months prior to either spawning (*Acropora tenuis*: for 53 days; *A. valida*: for 90 days) or planulation (*Pocillopora damicornis*: for 67 days). Exposure times were selected to ensure that photosynthesis was affected for the final 2-3 months of the typical 8-9 month oogenic cycle (for detailed parental exposure

conditions see Chapter 2; Cantin et al. 2007) and the lipid content within each adult colony was determined on the initial day of collection from the reef prior to diuron exposure as a baseline indicator of lipid investment into oogenesis (Chapter 2, Fig. 2.6). For *A. tenuis,* mean diuron concentrations within replicate tanks were 0.91 ± 0.05 and 8.8 ± 0.2 μg l^{-1} (± S.E.) for the 0, 1.0 and 10.0 μg l^{-1} treatments respectively. During the predicted weeks of spawning (i.e. the 7 days following the October full moons in 2003 and 2004), *A. tenuis* and *A. valida* colonies were monitored for spawning activity between dusk and 23:00. Some of the *A. tenuis* corals maintained in the 0 and 1 μg l^{-1} tanks at AIMS spawned earlier than expected (more than two nights before the full moon) and therefore gametes from these colonies could not be collected. The released gametes in the form of bouyant egg-sperm bundles from each treatment were collected in separate 250 ml containers from the water surface by gentle suction. Gametes from all of the colonies that spawned within each treatment group were cross-fertilised in a 50 l plastic tank for 1 hour, keeping each set of gametes from the different diuron treatments separate. The eggs which formed a monolayer on the water surface, were gently transferred into 250 l tanks for primary rearing (n=1, tank of stock larval culture per parental diuron treatment) within a temperature control room which had the air and water temperatures set at 28°C. At 36 hours post fertilisation the tanks flow-through seawater was turned on and raised until competent for metamorphosis assays. For each metamorphosis assay 100-200 larvae were sampled from the stock larvae cultures using a PVC pipe fitted with plankton mesh. Four reef control colonies and three colonies exposed to the 10 μg l^{-1} treatment spawned 2 nights before the full moon and these gametes were used in the analysis. For 2 weeks during the March and April new moons, outflows from the *P. damicornis* tanks were run through a 100 μm mesh plankton trap to collect released planulae and track reproductive output. *P. damicornis* larvae collected were not raised in culture, but were introduced into the metamorphosis assays on the first morning following planulation.

Fertilisation assays: Gametes were collected from the two broadcast spawning species *Acropora tenuis* and *A. valida* as described in Negri and Heyward (2001). Collection jars containing the buoyant egg-sperm bundles were gently agitated to separate the eggs and sperm into two layers. The eggs were then isolated from the sperm by gentle suction and washed two times in 0.45 μm filtered, sperm-free seawater. Concentrated sperm solutions were collected from each treatment and diluted for use in each assay.

Actual sperm concentrations in these stocks were measured using a haemocytometer (ranged from 5.0 -7.4 x 10^7).

Fertilisation assays were conducted in sterile six-well polystyrene cell culture plates (12 ml wells) following the methods used by Negri and Heyward (2001). Each well contained 100-200 eggs, 1 ml of 10^7 sperm solution and uncontaminated filtered seawater (diuron-free to test parental diuron exposure only) to a final volume of 10 ml. Six replicate wells were used for gametes from each treatment. Gametes were maintained and monitored at 28ºC for 3 h, by which time fertilisation and early cell cleavage had reached a maximum (Heyward 1988). Further development of the eggs was stopped by the addition of 300 µl of fixative (10 g l^{-1} sodium β-glycerophosphate, 4% formaldehyde buffered to pH 7.0) and then stored at 28ºC. The eggs and embryos were assessed for fertilisation using a dissecting microscope.

Metamorphosis assays: *Acropora tenuis*, *A. valida* and *Pocillopora damicornis* larvae were induced to settle for the metamorphosis assays with crustose coralline algae (CCA) and/or a concentrated CCA extract (Negri and Heyward, 2001; Markey et al., 2007). Assays were performed in sterile six well polystyrene cell culture plates (12 ml wells) and maintained at 28ºC in a temperature control room, under the same conditions as stock larval cultures. Larvae (15 - 20) were added to each well containing a 5 × 5 mm CCA chip of *Hydrolithon onkodes* or 10 µl CCA extract [prepared as per Heyward and Negri (1999)] and 0.45 µm filtered seawater made up to a final volume of 10 ml. Six replicate wells were used for larvae from each treatment and species. Assays to test the competency of larvae to settle and metamorphose were conducted from day 4 through day 13 post fertilisation for *A. tenuis* larvae, on day 6 and day 9 post fertilisation only for *A. valida* larvae (because of the limited number of larvae released by the 1 µg l^{-1} parent colonies), and on the morning after planulation from the internal brooding species *P. damicornis*. Early metamorphosis was defined as the stage at which larvae changed from either free swimming or loosely attached pear-shaped forms to firmly attached disc-shaped structures with obvious mesenteries radiating from the oral pore (Heyward & Negri 1999). Percent metamorphosis was determined after 18 h by direct counting of both free swimming larvae and attached polyps in each well under a dissecting microscope.

Egg pigment composition: Approximately 2000 eggs were sampled in the dark (to protect the pigments within the eggs from light-induced degradation) from each *A. valida* colony that spawned. Seawater was removed and the eggs were immediately

frozen in liquid nitrogen and stored in the -80ºC freezer until analysis. Each egg sample was weighed and 2.0 ml of pre-chilled isopropanol:methanol (50:50, v/v) was added to the eggs in microfuge tubes. Eggs were sonicated (probe sonicator, 30 W) on ice for 30 s and stored in the dark on ice for 30 min. The eggs were centrifuged (10,000 x g) for 2 min and the supernatant removed and stored on ice. This process was repeated (a third extract revealed that the combined efficiency of the first two extracts was >95%) and supernatants were combined for pigment analysis. High performance liquid chromatography (HPLC) analysis was performed as previously described (Chapter 2; Cantin et al. 2007). Peridinin, diadinoxanthin, and diatoxanthin standards were obtained from the International Agency for ^{14}C Determination (Denmark). β-β carotene and vitamin E (α-tocopherol) standards were obtained from Sigma-Aldrich Co. (USA). The identified peaks (85-90 % of area on the chromatogram at 440 nm) were confirmed by comparison of retention times, absorption spectra and spiking with the above standards. A standard curve was produced to determine the total number of eggs per mass within each sample in order to standardize the pigment concentrations on a per egg basis (Appendix Fig. A1.1).

Data Analysis: The number of fertilised eggs and metamorphosed larvae were converted into percentages and the percentage values were arcsine transformed for statistical analysis (Zar 1996). Fertilisation success, peak metamorphosis success (day 9 for *A. tenuis* and *A. valida*, day 1 for *P. damicornis*) and carotenoid pigment concentrations were compared across each diuron treatment with a 1-way ANOVA ($\alpha = 0.05$). Competency of *A. tenuis* larvae throughout development (day 4-13) was tested using a 2-factor ANOVA with diuron treatment (reef control and 10 μg l^{-1}) and age of development as the 2 factors. Tukey's honestly significant difference (HSD) was used to identify significantly different means for all of the data. All statistical analyses were performed using Statistica 6.0 (StatSoft Inc.). Mean and standard error (S.E.) values were calculated for each treatment and plotted using Sigmaplot 2001 (Jandel Scientific).

3.4. RESULTS

Fertilisation Success

Mean fertilisation success was high for gametes collected from reef control colonies of the two broadcast spawning species, *Acropora tenuis* (92.0 ± 0.6%) and *A. valida* (97.7 ± 0.9%) (Fig.3.1). Parental exposure to 10 μg l^{-1} diuron for 53 days, which caused severe inhibition of PSII electron transport and 80% reduction in ΔF/Fm' (Chapter 2,

Cantin et al. 2007), did not negatively impact on the fertilisation success of gametes released by *A. tenuis* (Fig. 3.1, 1-way ANOVA: $F_{(1,16)} = 1.16$, $p = 0.3$). No *A. tenuis* gametes were collected from the 0 or 1.0 µg l^{-1} diuron exposures, as colonies in these treatments spawned earlier than expected. Parental exposure of *A. valida* to 10 µg l^{-1} diuron for 81 days completely inhibited successful spawning (Chapter 2, Cantin et al. 2007), thus there were no gametes available from this treatment. Exposure to 1 µg l^{-1} diuron during gamete development did not negatively impact on the fertilisation success of *A. valida* gametes compared to reef controls (Fig. 3.1, 1-way ANOVA: $F_{(2,15)} = 0.10$, $p = 0.001$, post-hoc Tukey's HSD test: $p = 0.9$ reef control vs. 1 µg l^{-1}).

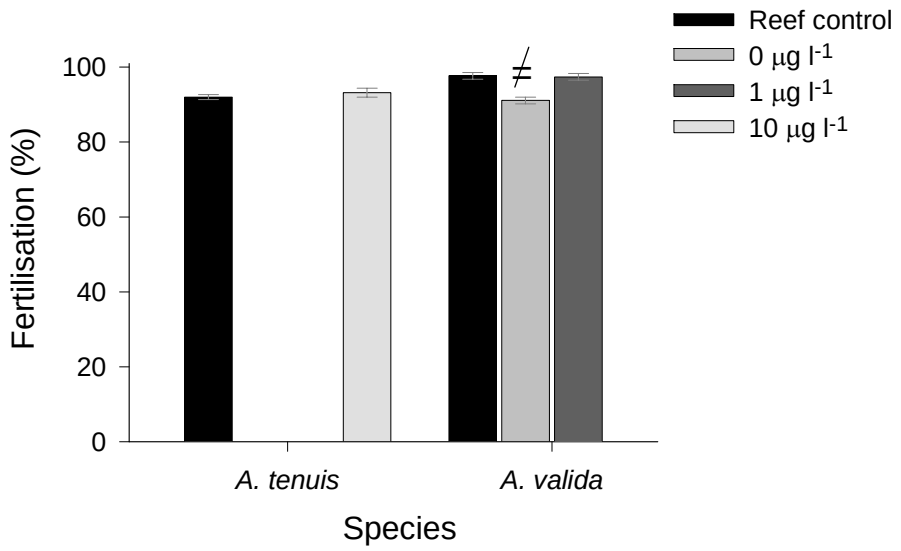

Fig. 3.1: Fertilisation success (%) of gametes collected from broadcast spawning corals, *Acropora tenuis* and *A. valida* following 53 day and 81 day parental exposures to the herbicide diuron (0, 1 and 10 µg l^{-1}). Mean ± 1 S.E. of 6 replicate assays. ≠ indicates significant difference ($p<0.05$) for treatments compared to reef control.

Larval Metamorphosis

Acropora tenuis larvae raised from reef controls and from colonies exposed to10 µg l^{-1} diuron exhibited high levels of metamorphosis competency following 4 days of development (61 ± 6 % and 63 ± 6% of larvae metamorphosed respectively), peaking on day 9 (79 ± 5 % and 96 ± 1 % metamorphosed respectively). Interestingly, there was significantly greater larval metamorphosis in the 10 µg l^{-1} diuron treatment than for the reef controls (1-way ANOVA: $F_{(1,10)} = 6.4$, $p = 0.03$, Fig. 3.3). Larvae remained highly competent (68 ± 10 % metamorphosis for reef controls) following 13 days of development (Fig. 3.2). *A. tenuis* larvae raised from colonies exposed to 10 µg l^{-1} diuron continued to display significantly higher metamorphosis competency than reef

control larvae throughout the 13 day study, with metamorphosis levels exceeding 80% between days 5 and 13 of development (2-Factor ANOVA: diuron: $F_{(1,100)} = 40.5$, $p<0.001$, larval age: $F_{(9,100)} = 4.4$, $p<0.001$, diuron*larval age $F_{(9,100)} = 0.95$, $p = 0.5$, Fig. 3.2). Similarly, after 9 days of development, metamorphosis success was significantly greater for *A. valida* larvae collected from the 0 and 1 μg l^{-1} colonies than from reef controls (1-way ANOVA: $F_{(2,15)} = 4.1$, $p = 0.04$), although metamorphosis success was still high for reef control larvae (71%, Fig. 3.3). *Pocillopora damicornis* larvae from all 3 treatments collected following the first planulation event (25 days of 1 and 10 μg l^{-1} diuron exposure), exhibited equivalent metamorphosis success within 18 h exposure to CCA chips (~40%, Fig. 3.3, 1-way ANOVA: $F_{(2,15)} = 0.8$, $p = 0.5$). The *P. damicornis* colonies within the 10 μg l^{-1} treatment were severely bleached before the 2nd planulation event and failed to release larvae after 53 days of diuron exposure (Chapter 2, Cantin et al. 2007), however larvae collected during the 2nd planulation event from the 1 μg l^{-1} colonies continued to successfully metamorphose (Fig. 3.3, 1-way ANOVA: $F_{(2,15)} = 0.4$, $p = 0.6$). Zooxanthellate larvae of *P. damicornis* from colonies within the 10 μg l^{-1} diuron treatment, which experienced 80% reduction in ΔF/Fm' (Chapter 2, Cantin et al. 2007), were visibly bleached during the first planulation event (Fig. 3.4), and no larvae were released from colonies in this treatment following the second planulation event a month later.

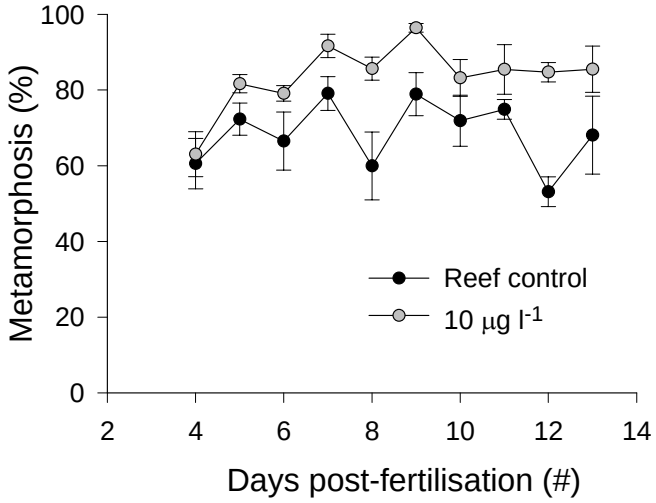

Fig. 3.2: Competency of *Acropora tenuis* larval metamorphosis from day 4-13 post fertilisation following 53 days of parental diuron exposure (10 μg l^{-1}) Mean ± 1 S.E. of 6 replicate assays.

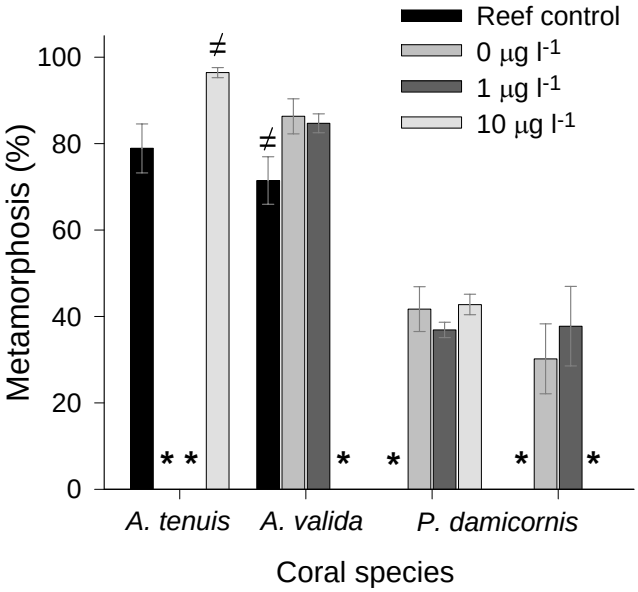

Fig. 3.3: Peak larval metamorphosis for *Acropora tenuis* (9 days old), *A. valida* (9 days old Reef Control and 1 µg l^{-1}, 11 days old 0 µg l^{-1}) and *Pocillopora damicornis* (1 day old) planulation event 1 (March new moon) and planulation event 2 (April new moon) following parental diuron exposure (0, 1 and 10 µg l^{-1}). Mean ± 1 S.E. of 6 replicate assays. * indicates larvae were not collected from that treatment. ≠ indicates significant difference ($p<0.05$) for treatments compared to reef control (*A. tenuis*) and for treatments compared to 0 µg l^{-1} (*A. valida*).

Fig. 3.4: Larvae released from the internal brooder, *Pocillopora damicornis* exhibiting maternally derived symbionts within the control treatment (0 µg l^{-1}) and bleached larvae being released following 23 days of parental diuron exposure (10 µg l^{-1}). Larval length range = 0.9 -1.2 mm.

Pigment composition

The major pigments identified within the azooxanthellate eggs collected from reef control colonies of *A. valida* included the carotenoids peridinin (43 mol % of total pigments) + *cis*-peridinin (33 mol %), diatoxanthin + diadinochromes I & II (23 mol %) and β – carotenes (1 mol %) (Fig. 3.5A). Eggs collected from the 1 µg l^{-1} diuron treatment contained 10 fold less peridinin than the eggs collected from the colonies within the 0 µg l^{-1} treatment (Table 3.1, 1-way ANOVA: $F_{(2,6)} = 7.97$, $p = 0.02$). Total xanthophylls (DT + DD) were also significantly reduced by 10 fold within the eggs collected from the 1 µg l^{-1} colonies compared to the eggs collected from the 0 µg l^{-1} colonies (Table 3.1, 1-way ANOVA: $F_{(2,6)} = 15.96$, $p = 0.003$). Reef control eggs contained 5 fold more total peridinin and xanthophyll pigments (DT + DD) than the

tank control eggs (Table 3.1, 1-way ANOVA: $F_{(2,6)} = 7.97$, $p = 0.02$ and $F_{(2,6)} = 15.96$, $p = 0.003$ respectively). β - carotenes were not detected within the 1 μg l^{-1} and there was no observable difference in the concentration of β – carotenes within the eggs collected from the reef and 0 μg l^{-1} colonies (Table 3.1, $F_{(2,6)} = 2.1$, $p = 0.2$). Large amounts of vitamin E (α – tocopherol) were also detected within *A. valida* eggs and did not differ significantly among eggs collected from reef control, 0 and 1 μg l^{-1} colonies (Table 3.1, $F_{(2,6)}=0.95$, $p = 0.4$). The four major carotenoid pigments extracted from healthy azooxanthellate eggs, like those collected from the reef control, absorb high energy visible wavelengths of light within the range of 400 – 540 nm (Fig. 3.5B).

Table 3.1: Photosynthetic pigments and vitamin E (α- tocopherol) concentrations (μg/egg) detected within azooxanthellate (lacking photosynthetic symbionts) eggs (n = 2275 ± 170 eggs per extraction) collected from *Acropora valida* colonies (n = 3) and analysed using HPLC. Peridinin = peridinin + *cis*-peridinin and xanthophylls = diatoxanthin + diadinochromes. * indicates significant statistical difference (Tukey HSD, $p < 0.05$) between treatment eggs compared to the reef control eggs, N.D.= pigment not detected.

Diuron treatment	Peridinin (μg/egg)	Xanthophylls (μg/egg)	β-carotenes (μg/egg)	Vitamin E (μg/egg)
Reef control	2.07 ± 0.5	0.62 ± 0.09	0.02 ± 0.01	3.18 ± 1.6
0 μg l^{-1}	0.44 ± 0.3	0.13 ± 0.1 *	0.005 ± 0.004	4.28 ± 0.5
1 μg l^{-1}	0.04 ± 0.02 *	0.014 ± 0.005 *	N. D.	5.83 ± 1.7

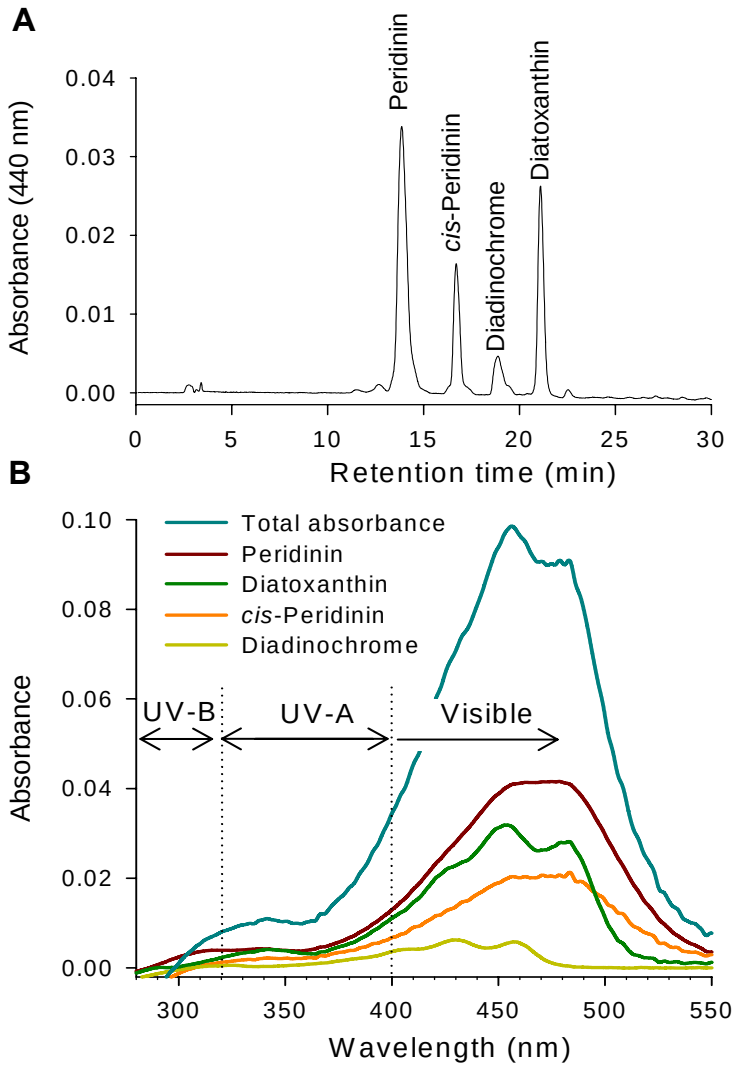

Fig. 3.5: (**A**) Typical chromatogram of photosynthetic pigments identified within the azooxanthellate eggs from an *Acropora valida* reef control colony sampled immediately after spawning, (**B**) total additive absorption of light by the photosynthetic pigments present within the azooxanthellate *A. valida* eggs.

3.5. DISCUSSION

Gametes and larvae from corals chronically exposed to the PSII inhibitor diuron leading up to broadcast spawning and planulation were able to undergo normal levels of fertilisation and metamorphosis. This indicates that diuron had no major or direct deleterious effect on gamete and larval quality despite strong evidence of reduced

reproductive output in the same parental corals (Chapter 2, Cantin et al., 2007) and bleaching in *Pocillopora damicornis* larvae within adult colonies. There were however differences in the partitioning of photoprotective pigments from the parental colonies to the eggs of *Acropora valida*. This did not affect the viability of the developing larvae under experimental conditions suggesting that pigment content is not a direct indicator of gamete viability in corals. However, reductions in pigment concentrations in the buoyant embryos and larvae developing in the field may reduce protection from oxidative stress (and subsequent survival) of coral gametes from high energy visible light during the initial 5-7 days that free swimming larvae spend within the pelagic environment.

The experimental fertilisation success of gametes from broadcast spawning species is typically very high (>85%) in the absence of external stressors (Michalek-Wagner & Willis 2001a, Negri & Heyward 2001). The azooxanthellate gametes produced by the broadcast spawning corals *Acropora tenuis* and *A. valida* were as viable as controls, exhibiting high levels of fertilisation despite 2-3 month parental exposures to the herbicide diuron (1 and 10 $\mu g\ l^{-1}$) during the gametogenenic cycle (Chapter 2, Cantin et al., 2007). The fertilisation of *Acropora spp.* has been directly inhibited by exposure to the metals Cu, Zn and Cd and the organometallic antifoulant tributlytin (TBT) (Heyward 1988, Reichelt-Brushett & Harrison 1999, Negri & Heyward 2001); however, direct exposure of *A. millepora* gametes (lacking symbionts) and *Montipora aequituberculata* (containing parentally derived symbionts) to diuron concentrations as high as 1000 $\mu g\ l^{-1}$ did not inhibit fertilisation (Negri et al. 2005). This lack of sensitivity of coral fertilisation to direct herbicide exposure is likely to be due to the mechanistic specificity of PSII inhibitors (blockage of electron transport in PSII) and the lack of direct influence that symbionts have in the fertilisation process (Negri et al. 2005). Gametes from severely bleached soft corals, *Lobophytum compactum* (caused by high irradiance and temperature) were smaller and not able to undergo fertilisation (Michalek-Wagner & Willis 2001a). This may indicate that the energetics of corals required for successful fertilisation was less affected by the chronic herbicide exposure used in the present study than the severely bleached soft corals. Successful fertilisation is a key indicator of gamete quality (Loya et al. 2004) and results from the present study demonstrate that, although the energy deficiencies from chronic diuron exposures reduced spawning and reproductive output in *A. valida* and *P. damicornis* (Chapter 2,

Cantin et al., 2007), that the key functionality of gametes in these broadcast spawning and brooding corals was not affected.

Marine invertebrates that release non-feeding lecithotropic eggs, maternally contribute large amounts of stored energy, which is required for larvae to develop and become mobile in order to successfully recruit as juveniles into parental populations (Heyward 1987, Arai et al. 1993, Hoegh-Guldberg & Emlet 1997). *Acropora tenuis* larvae, which had developed from gametes released by colonies exposed to 10 µg l^{-1} diuron, exhibited consistently greater levels of metamorphosis than larvae from reef control colonies throughout the 4 – 13 days of larval development tested. It is possible that conditions within the aquarium enabled the development of high quality (competency) larvae due to subtle differences in nutrition or light profiles, despite the 80% reduction in photosynthetic efficiency ($\Delta F / F_m'$) due to 10 µg l^{-1} diuron exposure (Chapter 2, Cantin et al. 2007). Larvae from all diuron treatments for both broadcast spawning species (*A. tenuis* and *A. valida*) exhibited peak metamorphosis after 9 days of development. Consistent larval competency profiles suggests that parental exposure of *A. valida* to low diuron concentrations (1 µg l^{-1}, reducing $\Delta F / F_m'$ by 20-25%) and *A. tenuis* to medium diuron concentrations (10 µg l^{-1}, reducing $\Delta F / F_m'$ by 80%) of diuron throughout the last 2-3 months of gametogenesis (Chapter 2, Cantin et al., 2007) does not have a negative effect on the energetics or biochemical signaling pathways of competent (4 – 13 d old) larvae.

The availability of energy from lipids in azooxanthellate larvae or from zooxanthellae in larvae containing symbionts is an important factor controlling the dispersal, competency and metamorphosis success of coral larvae (Richmond 1987, Arai et al. 1993, Ben-David-Zaslow & Benayahu 1996, 1998, Harii et al. 2002). Lipid synthesis by adult corals is primarily stimulated by light and the carbon acquired from photosynthesis (Crossland et al. 1980, Anthony & Fabricius 2000). Diuron exposure inhibits photosynthesis and significantly reduces the total lipid content within reproductive adult corals (Cantin et al. 2007). Photoinhibition of *Symbiodinium* spp. by 10 µg l^{-1} diuron exposures also significantly reduces carbon fixation by coral symbionts (Owen et al. 2003) and translocation of photosynthates to the host coral tissue in juvenile *A. millepora* colonies (Chapter 5). The energy budget of reproductive corals is likely to have been affected by diuron-induced photoinhibition in the present study. Observed competency of azooxanthellate larvae that were developed from energy deficient colonies (*A. valida* exposed to 1 µg l^{-1} diuron) suggests that diuron induced

photoinhibition in parental colonies primarily affected energy reserves within the adult colonies and reproductive output but had little negative impact on the lipid energy reserves and the metamorphosis success of their released offspring.

Pocillopora damicornis releases fully developed larvae, which contain maternally derived *Symbiodinium* spp. and are competent to metamorphose once they are released (Richmond 1987). The presence of visibly bleached coral larvae from parent colonies exposed to 10 μg l^{-1} diuron (first planulation after 23 days of exposure) has not been previously reported and indicates reduced maternal provisioning of symbionts to their offspring. However, these visibly bleached larvae exhibited similar levels of metamorphosis success when compared to larvae of tank control colonies (0 μg l^{-1} diuron). Diuron at a concentration of 10 μg l^{-1} prevented the second planulation event (after 53 days of exposure), and branch dissections confirmed that planulae larvae were held within the polyps of the bleached colonies (Cantin et al. 2007). Ben-David Zaslow and Benayahu (1998) suggest that planulae released from several brooding species of soft coral exhibit a precompetent period of 1-3 days prior to peak metamorphosis. Since the goal of the present study was to determine the effects of reduced parental provisioning of photosynthates following long term exposure to diuron (not to understand peak competency), the larvae needed to be tested for competency immediately upon release. Had the metamorphosis assays been performed over several days, the effects on competency may also have been masked since larvae may derive further energy from their symbionts. Furthermore, metamorphosis of *P. damicornis* larvae is not directly sensitive to diuron at high concentrations (1000 μg l^{-1}), indicating that larvae have enough stored energy to undergo metamorphosis and that active photosynthesis is not required for metamorphosis in *P. damicornis* larvae (Negri et al. 2005). This study also indicates zooxanthellate larvae produced under conditions of limiting photosynthetic potential are able to complete successful metamorphosis, despite signs of reduced parental provisioning of symbionts (visible bleaching).

In addition to the competency of coral larvae (fertilisation and metamorphosis success), biochemical parameters (carotenoids, MAA's, lipid content) are important indicators of maternal contributions to the quality of gametes produced (Michalek-Wagner & Willis 2001b). In this study, the concentration of carotenoid pigments invested within *A. valida* eggs decreased by an order of magnitude following chronic parental exposure to diuron at 1 μg l^{-1} compared with tank controls. It is critical to compare carotenoid pigments between herbicide treatments only under identical

conditions of illumination, since pigment profiles and concentrations can change rapidly in response to intensity and spectral variations (Brown et al. 1999). Both the tank control and diuron – treated *A. valida* experienced identical illumination conditions for 3 months (filtered, natural sunlight, maximum irradiance of 350 μmol quanta $m^{-2}s^{-1}$, 1% UV radiation), therefore the 10-fold difference in pigment concentration in eggs between treatments is most likely due to the diuron exposure of parental colonies. Peridinin concentrations were similarly reduced by 90% within eggs collected from the soft coral *Lobophytum compactum*, in response to severe temperature and solar radiation induced bleaching and following the breakdown of the symbiotic algal-coral host relationship (Michalek-Wagner & Willis 2001b).

Reduced provisioning of pigments from adult *A. valida* exposed to 1 μg l^{-1} diuron may have resulted directly from lower parental resources available within parental colonies suffering from chronic inhibition of photosynthesis [33% lower $\Delta F / F_m'$ (Cantin et al. 2007)] and consequently lower rates of carbon fixation (Owen et al. 2003 and Chapter 5). It is also possible that pigment provisioning is not related to parental energetics (and photosynthate transfer), but is more directly dependent on the ability of parental symbionts to provide reproductive tissue with protective biochemicals and that this may be directly affected by diuron. The light-harvesting and xanthophyll pigments of adult *A. valida* were reduced by 65% and 64% respectively following 10 μg l^{-1} diuron but no significant affect on pigment content was observed in adults exposed to 1 μg l^{-1} diuron (Chapter 2, Cantin et al., 2007). As expected, the reef controls contained significantly different concentrations of pigments to tank treatments and this is almost certainly due to differences in light histories and exposure to UV radiation between the aquarium tanks and the reef environment.

During the first 2-3 days of development, lipid rich coral eggs and larvae are buoyant (Harrison & Wallace 1990, Arai et al. 1993) and therefore vulnerable to the damaging effects of UV radiation and light which can induce the formation of oxygen radicals [singlet oxygen ($^1O_2$), superoxide (O_2^-) and hydrogen peroxide (H_2O_2)] that can disrupt protein synthesis, damage cell membranes and degrade lipids (Lesser 1996, Shick et al. 1996, Mobley & Gleason 2003). MAA's are crucial UV screening compounds within corals that have been shown to increase larval survivorship during UVA + UVB radiation exposure (Gleason & Wellington 1995, Wellington & Fitt 2003) and fluorescent pigments (FP's) have also been shown to supplement the photoprotective properties of MAA's by dissipating UV radiation and can dissipate

excess light energy from PAR through fluorescence and light scattering (Salih et al. 2000). The presence and role of carotenoids and vitamin E within coral tissues lacking symbionts has received less attention. The principal role of vitamin E within plants is scavenging of free radicals to reduce lipid peroxidation (oxidative degradation of lipids) (Havaux & Niyogi 1999, Havaux et al. 2005). The detection of carotenoids and Vitamin E within coral eggs within this study indicates parental provisioning of resources acquired from *Symbiodinium* spp. within the coral host, since animals must acquire these compounds from plant sources either through diet or symbiosis (Lesser 2006). Provisioning of vitamin E was not affected by parental diuron exposure, indicating that none of the corals were experiencing significant oxidative stress. The role peridinin in coral eggs and larvae has not been explored and diatoxanthin, the second-most abundant pigment in *A. valida* eggs and vitamin E has not been described in coral eggs previously. Within photosynthetic dinoflagellates, peridinin extends the range of absorption for light harvesting (Iglesias-Prieto et al. 1991). Peridinin has also been shown to have singlet oxygen quenching potential within the planktonic dinoflagellate *Gonyaulax polyedra* (Pinto et al. 2000) and reduces lipid oxidation by enhancing the structural rigidity of lipid membranes, reducing the permeability of cellular membranes to lipoperoxidation promoting compounds (Barros et al. 2001). When vitamin E quenches superoxid (O_2^-) radicals an α-tocopheroxyl radical is formed, carotenoids are capable of regenerating the antioxidant α-tocopherol form of vitamin E, thus providing a synergistic level of protection against $^1O_2$ radical damage (Bohm et al. 1997, Havaux et al. 2005). Since peridinin (including *cis*-peridinin) was found to be the dominant carotenoid (75 %) within the non-photosynthetic, azooxanthellate coral eggs and larvae, peridinin synergistically with vitamin E likely enhances the protective effects of MAA's and FP's by: (I) efficient absorption of high energy PAR light wavelengths (400-550nm) to minimize oxygen radical formation and (II) active quenching of oxygen within coral tissues to reduce lipid degradation.

The specific function of peridinin, vitamin E and the presence of diatoxanthin rather than diadinoxanthin within coral eggs and larvae will be examined in future research. Coral tissues hosting photosynthetic symbionts are typically hyperoxic (150% oxygen saturation) in the light (Shick 1990, Gardella & Edmunds 1999), whereas the oxygen microenvironment within the tissue of coral eggs lacking symbionts will likely be significantly less saturated. Changes in thylakoid lumen pH and acidification caused by oxygenic photosynthesis are believed to catalyze the de-epoxidation of diadinoxanthin

to diatoxanthin inducing the NPQ cycle (Lavaud et al. 2004, Warner & Berry-Lowe 2006). Thus, the absence of diadinoxanthin within the coral eggs suggests that diatoxanthin within coral eggs likely acts more as an antioxidant rather than forming an active NPQ cycle with diadinoxanthin. Peridinin in combination with the xanthophylls may provide buoyant coral eggs with the ability to absorb potentially harmful high-energy visible light wavelengths in the region of PAR (400 - 530 nm) (Fig. 3.5B) that is not absorbed by MAAs and enhance antioxidant properties of vitamin E, reducing lipid degradation.

Diuron is predominantly used as a herbicide within catchments along the GBR and enters the marine environment, particularly during runoff events during the wet season (Haynes et al. 2000). Early rainfall events can coincide with late stage coral gametogenesis and spawning. Diuron is also an active ingredient within antifouling paint formulations and can be deposited on coral reefs following recreational and commercial ship groundings (Negri & Heyward 2001, Jones et al. 2003). Although diuron has been found in marine sediments and water samples during high flow events (Haynes et al. 2000a, Bengtson Nash et al. 2005a, Mitchell et al. 2005), not enough data exists to determine the risks that diuron and other PSII herbicide pose to gamete development and quality within the GBR marine park. The results from this chapter show however, that gametes successfully released from corals severely affected by chronic diuron exposures are likely to remain viable and competent to metamorphose into juvenile corals, it is the quantity rather than the quality of gametes that was affected. The results also show that maternal provisioning of photoprotective pigments is reduced in *A. valida* following chronic parental exposure to only 1 $\mu g\ l^{-1}$ diuron. The experiments were conducted under benign UV conditions and the reduced level of photoprotection had no affect on larval metamorphosis. However, this may not be the case in the field, where high UV stress over the first 5 - 7 days of larval development prior to metamorphosis and the establishment of symbiosis, may impact upon survival and recruitment of pelagic coral larvae.

Chapter 4: Development of a real-time PCR assay that quantifies the chloroplast large subunit 23S ribosomal DNA (cp23S-rDNA) of *Symbiodinium*

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4.1. INTRODUCTION

The symbiotic relationship formed between reef building corals and the photosynthetic symbiont of the genus *Symbiodinium* is an integral component of modern coral reef ecosystems (Trench 1993, van Oppen 2007). The diverse genus *Symbiodinium*, has been divided into eight distinct phylogenetic clades (A-H) (Rowan & Powers 1991, Carlos et al. 1999, LaJeunnesse 2001, Pochon et al. 2001, Santos et al. 2002, Pochon et al. 2006) and individual coral colonies have been shown to associate with multiple symbiont types simultaneously (van Oppen et al. 2001, Ulstrup & van Oppen 2003). Developing our understanding of *Symbiodinium* phylogenetics and accurately identifying the symbiont within the coral-symbiont association is crucial in order to answer important questions related to the physiological interactions of these dinoflagellates with their invertebrate host (Santos et al. 2002). The importance of *Symbiodinium* spp. to the nutritional demands of the coral host is well established, and it has been shown that *Symbiodinium* provides photosynthetically fixed carbon compounds capable of meeting the host's respiratory carbon requirements (Muscatine et al. 1984, Edmunds & Davies 1986), recycle nitrogen within the symbiotic relationship (Muscatine & Porter 1977, Falkowski et al. 1984) and enhances lipid production and skeletal calcification (Muscatine 1967, Chalker & Taylor 1978, Crossland et al. 1980, Dennison & Barnes 1988, Anthony & Fabricius 2000). Unfortunately, these studies did not provide information in terms of the functional variation within the diverse *Symbiodinium* genus because the phylogenetic position of the *Symbiodinium* isolates used in these studies were unknown (Loram et al. 2007).

Traditional and most commonly used molecular techniques for symbiont detection, which include denaturing gradient gel electrophoresis (LaJeunnesse 2002), single strand conformational polymorphism (Fabricius et al. 2004), and restriction fragment length

polymorphism of nuclear ribosomal DNA (nrDNA) (Diekmann et al. 2002) and DNA fingerprinting (Goulet & Coffroth 2003) are not able to reliably detect potentially low level background strains below 5-10% of the total symbiont population (Mieog et al. 2007). A real-time PCR method was recently developed that quantifies the nuclear ribosomal DNA (nrDNA) Internal Transcribed Spacer 1 (ITS1) of *Symbiodinium* C versus *Symbiodinium* D within a single sample (Mieog et al. 2007). This method is at least 100 times more sensitive than previously used techniques and is able to detect the relative abundance of clades over eight orders of magnitude, which provides a powerful technique to determine the prevalence of low level, background *Symbiodinium* types within an individual corals symbiont assemblage (Mieog et al. 2007). While the nrDNA-ITS marker continues to be one of the main markers in use for *Symbiodinium* identification, it has many unfavourable properties associated with its multi-copy nature, such as intragenomic rDNA variation (Mieog et al. 2007). The aim of this chapter was to develop a real-time quantitative PCR assay that quantifies the chloroplast large subunit 23S ribosomal DNA (cp23S-rDNA) of *Symbiodinium* C1 versus *Symbiodinium* D within a single sample. Through the development of sensitive molecular assays the phylogenetic position of genetically distinct *Symbiodinium* spp. within individual coral colonies (Chapter 5) can be accurately determined to enhance the relevance of outcomes from physiological experiments testing the significance of symbiont type within invertebrate symbiotic relationships. Additionally this study explores the potential for cp23S-rDNA as a single copy marker for *Symbiodinium*. To test the sensitivity of the cp23S-rDNA, the symbiont assemblage within *Acropora valida* colonies, previously shown to host multiple symbiont types simultaneously (Ulstrup & van Oppen 2003), will be quantified following chronic diuron exposure (colonies from Chapter 2).

4.2. METHODS

Extraction, amplification and sequencing of *Symbiodinium* cp23S-rDNA: To identify cp23S-rDNA sequences of local Great Barrier Reef *Symbiodinium* strains, small branches were collected from colonies of *Acropora tenuis* harboring *Symbiodinium* C1 (from Magnetic Island, Great Barrier Reef (GBR)) and *Acropora millepora* harboring *Symbiodinium* D (collected from Magnetic Island), *Symbiodinium* C2 (from Davies Reef), and *Symbiodinium* C2 or D (from Keppel Islands, GBR) and

fixed in absolute ethanol. DNA was extracted following a previously published DNA isolation method (Wilson et al. 2002). An approximately 0.7 kb region of *Symbiodinium* cp23S-rDNA was PCR amplified using the primer pair 23S1 (5′-GGCTGTAACTATAACGGTCC-3′) and 23S2 (5′-CCATCGTATTGAACCCAGC-3′) (Santos et al. 2002). PCRs were performed in 25 µl volumes containing 2 mM MgCl, 200 µM dNTPs, 1 x DNA Polymerase PCR buffer, 0.02 units *Taq* polymerase (Fisher Biotec, Australia), 40 pmol of each primer and 50-70 ng of template DNA. Reactions were carried out in a Corbett Research PC-960G Gradient Thermal Cycler (Corbett Research, NSW Australia) under the following conditions: initial denaturing period of 1 min at 95°C, 35 cycles consisting of 95°C for 45 s, 55°C for 45 s and 72°C for 1 min, followed by a final extension period at 72°C for 7 min. PCR products were precipitated by adding 0.3M ammonium acetate and 100% ethanol, and then centrifuged at 1,530 x g in a bench top centrifuge at 4°C for 20 min. The products were cloned using the TOPO TA Cloning kit (Invitrogen, Victoria Australia) according to the manufacturer's directions. Colony PCR was conducted using the universal M13 plasmid primers (Table 1, M13F and M13R). The products were then purified with an ethanol precipitation and centrifuged (at 1,530 x g for 2 min) through a Sephadex G-50 resin column and then sequenced at the Macrogen Sequencing Service (Macrogen Inc., South Korea).

Table 4.1. Universal M13 plasmid primers for PCR and cp23S-rDNA primers for qPCR.

Primers	Sequence
M13 universal plasmid forward	5′-GTAAAACGACGGCCAG-3′
M13 universal plasmid reverse	5′-CAGGAAACAGCTATGAC-3′
cp23S C1 forward	5′-GGGATAAAACTTGGGTAACATTC-3′
cp23S C1 reverse	5′-CCAATTAAACAGTGGTCTTAGGAG-3′
cp23S D forward	5′-AACCCCCGATTGGCCTAG-3′
cp23S D reverse	5′-CTTGATTGGGCCATTAAGCA-3′

Symbiodinium *cp23S-rDNA real-time quantitative PCR assay*: To determine the relative abundance of symbiont types hosted by GBR coral colonies, the local GBR cp23S-rDNA *Symbiodinium* sequences were added to an existing alignment (Santos et al. 2002) to design *Symbiodinium* C1 (GenBank Accession No. EF140804) - and D (GenBank Accession No. EF140808) specific primers for the real time quantitative PCR (qPCR) assay (Table 1). Relative symbiont abundance within the *A. valida*

colonies was determined using the primer pairs in Table 1, with an expected PCR product length of 100 bp and 103 bp for the *Symbiodinium* C1 and *Symbiodinium* D reactions respectively. Each DNA sample was analyzed in duplicate with a *Symbiodinium* C1 specific reaction, a *Symbiodinium* D specific reaction, and a quantified standard with DNA isolated from 100,000 cells of each *Symbiodinium* type, along with no-template controls. qPCR reactions were performed in 20 µl volumes containing 10.0 µl Platinum SYBR Green qPCR Supermix UDG (Invitrogen, Victoria Australia), 1.0 µl of 4 µM C1/D specific FP, 1.0 µl of 4 µM C1/D specific RP, 6.0 µl MilliQ water and 2.0 µl DNA template. Reactions were carried out in the Rotor-Gene RG-3000A thermal cycler (Corbett Research, NSW Australia) under the following conditions: an initial denaturing period of 2 min at 50°C and 2 min at 95°C, 40 cycles of 15 s at 95°C and 30 s at 60°C. To check for the formation of primer dimers and to verify *Symbiodinium* type primer specificity, a melt curve was generated at the end of the run by starting at 60°C for 30 s and raising the temperature by 1°C every 5 s until 95°C was reached. Fluorescence measurements were made after each 60°C step during cycling and after each temperature step of the melt curve. Serial dilutions over a range of 5 logs were run to ensure both the *Symbiodinium* C1 and D reactions had similar efficiencies. Relative abundances (as a percentage of total copies per reaction) were determined from the calculated concentration (copies per reaction) and the melt curve analysis were both conducted within Rotor-Gene Analysis Software v. 6.0 (Corbett Research, NSW Australia) for each *Symbiodinium* type per reaction in comparison to the 100,000 copy standard after importing the standard curve from the serial dilution series (Fig. 4.2).

***Acropora valida* symbiont composition:** Small branches from each diuron treatment [reef controls, 0 (tank controls), 1 and 10 µg l^{-1}] were sampled from the Magnetic Island, *Acropora valida* colonies (n = 6 per diuron treatment) that were used in Chapter 2 and fixed in absolute ethanol. DNA was extracted following a previously published DNA isolation method (Wilson et al. 2002). The extracted DNA was then used to validate the sensitivity of the cp23S-rDNA qPCR assay to detect background strains within a single coral host and to determine the symbiont composition of the *A. valida* colonies following bleaching and 90 days of chronic diuron exposure (Chapter 2). Relative symbiont assemblages were compared across the 4 diuron treatments with a one-way ANOVA ($\alpha = 0.05$) and Tukey's HSD post hoc analysis ($\alpha = 0.05$). Percentage data was transformed using the arcsine transformation, to better approximate the

assumption of a normal distribution (Zar 1996). Statistica v. 6.0 (StatSoft, Inc. Oklahoma, USA) was used for all statistical analyses.

4.3. RESULTS

Specificity, efficiency and copy number variability of the cp23S-rDNA real time qPCR assay

An initial PCR amplification of cloned type C1 and D cp23S PCR products (i.e., using plasmids as template) verified that the cp23S clade C1 and D reactions were specific to their respective clades. Melt curve analysis at the end of each qPCR run also indicated the specificity of each symbiont specific reaction based upon the melting point of each *Symbiodinium* clades product sequence and confirmed the absence of primer dimers as indicated by the lack of non specific peaks in the melt curve (Fig. 4.1). The fluorescent threshold was set at 300 relative fluorescent units in all Icycler runs to enable direct quantitative comparisons between symbiont types. Clade C1 and D reactions with identical plasmid concentrations differed in their threshold cycle (C_T) values by less than 0.2, which allowed for the direct comparison of C_T (C1) and C_T (D) (Mieog et al. 2007).

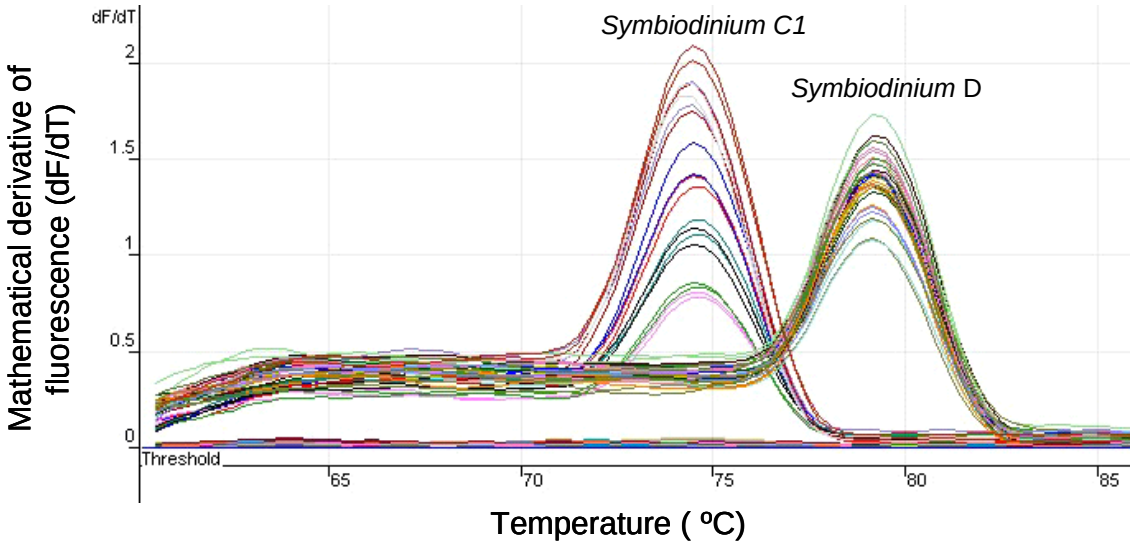

Fig. 4.1. A typical melt curve analysis conducted at the end of each cp23S-rDNA qPCR run for samples with *Symbiodinium* C1, which melts at 74 ºC and *Symbiodinium* D, which melts at 78 ºC.

In order to calculate the relative abundances of the two *Symbiodinium* types using the differences in C_T values, near equal PCR efficiencies were required (Mieog et al. 2007). PCR efficiency is 100% when the product is doubled every cycle. Using a serial

dilution series of a *Symbiodinium* C1 and D sample, a C_T range of 8-26 for each clade was obtained (Fig. 4.2). The ΔC_T was plotted against the Log of the template concentrations for each clade and showed that the PCR efficiencies were 92% (*Symbiodinium* C1) and 95% (*Symbiodinium* D) and are well matched, allowing for equal comparison of the two clade specific reactions (Fig. 4.2). The duplicate C_T for the 10^3 copies *Symbiodinium* D diluted sample (Fig. 4.2B) were considered outliers from the standard curve and dropped from the analysis. Based on the bulk analysis of 100,000 isolated cells per colony, cell average cp23S copy number for *Symbiodinium* C1 was 19 ± 4 and 10 ± 2 for *Symbiodinium* D (3 colonies extracted in triplicate, mean ± SE). Therefore the D/C copy number ratio based upon the cp23S qPCR assay is estimated to be 1.9.

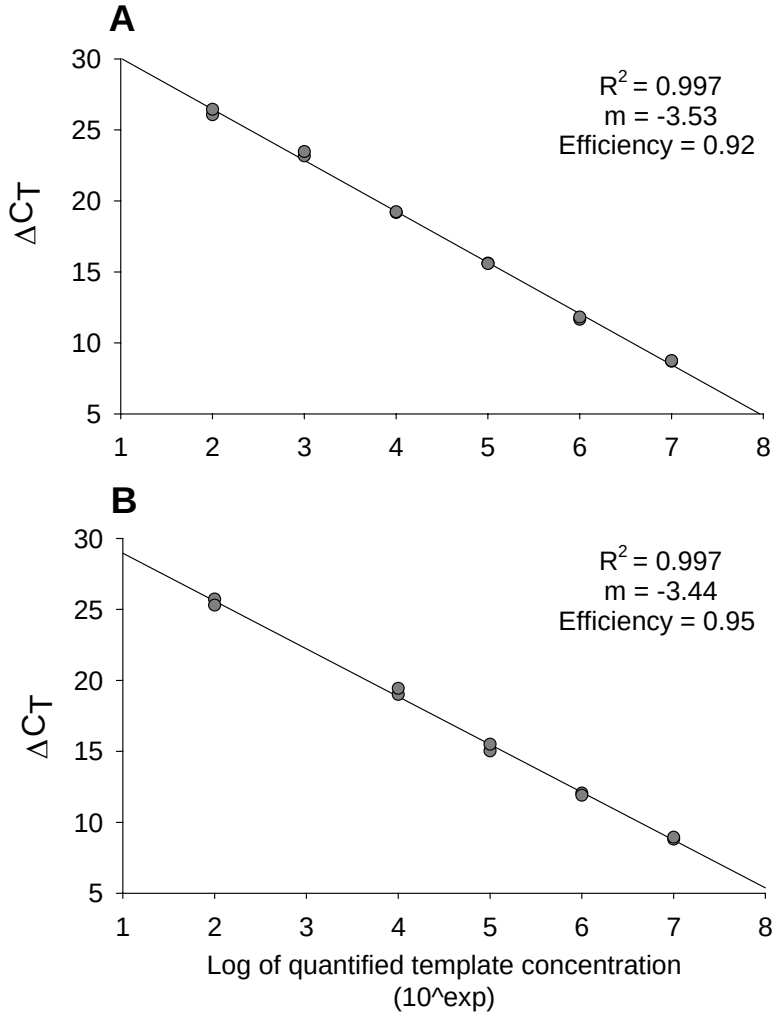

Fig. 4.2. Standard curve to test the efficiency of the cp23S qPCR assay developed from a serial dilution series of quantified known concentrations of (**A**) *Symbiodinium* C1 and (**B**) *Symbiodinium* D PCR template.

Validation of cp23S-rDNA real time qPCR assay with *Acropora valida* symbionts

Acropora valida freshly collected from Magnetic Island (Reef Control) hosted both *Symbiodinium* D and C1 with a ratio of approximately (2:1) (60 ± 11% *Symbiodinium* D) (Fig. 4.3). Statistical analysis of mean relative abundances (% *Symbiodinium* D) showed a significant effect of treatment on the relative symbiont assemblage within *A. valida* (one-way ANOVA, $F_{(3,44)} = 5.1$, $p = 0.004$). *Symbiodinium* D was significantly more dominant (97 ± 1%, Tukey's HSD: $p = 0.006$) in tank control colonies and in colonies exposed to 1 μg l^{-1} diuron for 90 d (98 ± 1%, Tukey's HSD: $p = 0.01$) than the relative assemblage within the reef control colonies (Fig. 4.3). Bleached colonies exposed to 10 μg l^{-1} diuron were also dominated by *Symbiodinium* D but there was more variability between colonies (82 ± 11% *Symbiodinium* D). Despite a consistent dominance of *Symbiodinium* D in *A. valida*, *Symbiodinium* C1 was detected as a minor component (at least 4%) within 11 of the 24 colonies sampled.

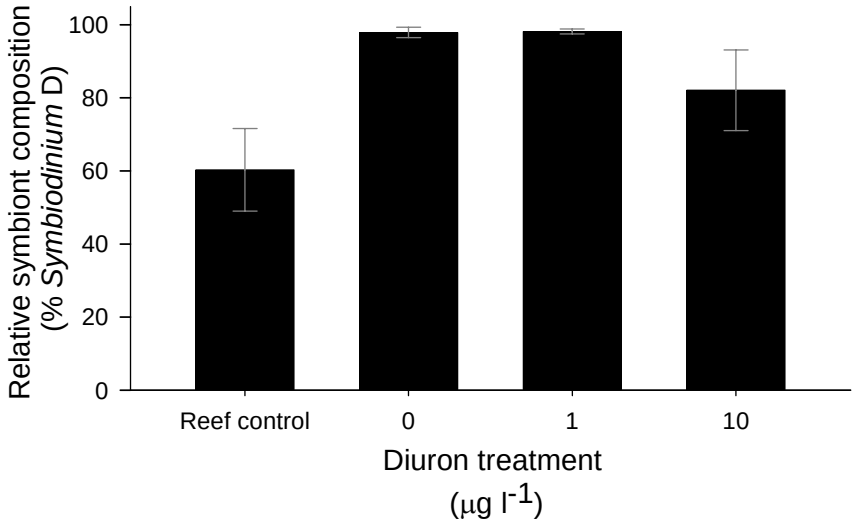

Fig. 4.3. Relative composition of endosymbionts *Symbiodinium* D and C1 hosted by *Acropora valida* colonies (mean % ± SE of *Symbiodinium* D) from Magnetic Island, Great Barrier Reef, following 90 days of exposure to the herbicide diuron [reef controls, 0 (tank control), 1 and 10 μg l^{-1}].

4.4. DISCUSSION

The real-time PCR assay developed here was a clade specific, sensitive and accurate assay capable of detecting and quantifying relative abundances of both the dominant symbiont and low level background strains of *Symbiodinium* C1 and D within a single hosts tissue based upon a less commonly used DNA marker from chloroplast 23S-

rDNA. The detection of the mixed assemblages within *A. valida* indicates the cp23S-rDNA real-time assay is a suitable technique to detect multiple strains of *Symbiodinium* within a single host colony. The accuracy of this cp23S-rDNA real-time PCR assay was also validated against the ITS1 real-time PCR assay (Mieog et al. 2007) and showed a high level of agreement of the 2 markers on identical samples, indicating that the measured background clade signals were not due to intragenomic variation or PCR artifacts (Mieog et al. 2007).

The symbiont assemblages of *Acropora valida* colonies collected from the reef just prior to DNA sampling were dominated by *Symbiodinium* D but also contained a considerable proportion of *Symbiodinium* C1 symbionts. The simultaneous presence of multiple *Symbiodinium* strains within *A. valida* from Magnetic Island is consistent with previous studies that have also shown *Symbiodinium* C and D within single individual colonies of *A. valida* (Ulstrup & van Oppen 2003). The *A. valida* colonies maintained within the aquariums, under filtered natural sunlight on the other hand contained almost exclusively *Symbiodinium* D. It is possible that the symbiont populations may have shuffled in response to different light conditions, since the abundance of *Symbiodinium* D vs *Symbiodinium* C within *A. valida* has been shown to be strongly correlated with the ambient light regime (Ulstrup & van Oppen 2003). This observation is consistent with the differences in egg pigmentation between tank controls and reef controls reported in Chapter 3. A shift to greater dominance by *Symbiodinium* D, may be an indication of the hosts preference for a 'shade tolerant' symbiont type that is more photosynthetically efficient under lower irradiance conditions (Ulstrup & van Oppen 2003) and real time PCR analysis of cp23S-rDNA at the start of the experiment may have confirmed this shift.

Nuclear ribosomal DNA, such as the ITS1 marker, is by far the most commonly used DNA marker used in *Symbiodinium* research to date, however additional genetic markers like the cp23S-rDNA marker have been applied to studies of *Symbiodinium* diversity (Santos et al. 2002, Pochon et al. 2006). The nrDNA-ITS1 marker has some unfavorable properties associated with its multi-copy nature (Mieog et al. 2007). nrDNA copy number per genome ranges from ~ 40 - > 20,000 in most higher plants and animals (Appels & Honeycutt 1986) and changes in nrDNA copy number sometimes occur within a single generation or between cells within an individual (Walbot & Cullis 1985, Rogers & Bendich 1987). Representatives of up to three distinct clades (based on nrDNA) were isolated within single *Symbiodinium* cells taken from multiple coral

hosts, indicating high levels of intragenomic variation in the nrDNA-ITS1 gene region (van Oppen & Gates 2006). The multi-copy nature of the nrDNA-ITS1 marker could have significant implications regarding the accuracy of converting ITS1 copy numbers into an estimate of the number of symbiont cells, based upon single-cell analysis (Mieog et al. 2007). On the other hand, average ITS1 copy number estimates for *Symbiodinium* C and D have been shown to be remarkably stable for each clade when they are obtained from "bulked cell" analysis of 100,000 cells, indicating that cell-to-cell variation resulting from intragenomic variation has only minor impacts on the results from a real-time PCR assay when enough cells are analyzed, minimum 500 copies (Mieog et al. 2007). A 3 fold difference in average copy number was determined from the "bulked cell" analysis between clades C and D with the ITS1 marker (Mieog et al. 2007). A 1.9 fold difference was observed between C and D using the cp23S gene region, indicating a 1/3 reduction in the potential intragenomic variation between clades as a result of copy number when using the cp23S-rDNA marker and comparing background clade abundance estimates.

The development of novel single-copy markers for *Symbiodinium* should remain a research priority for studies assessing symbiont diversity and identity below the level of clades, to avoid the potential of low copy variants increasing within a single genome and replacing currently dominant rDNA types. The application of this real-time PCR assay based upon cp23S-rDNA provides an additional gene marker that can be used to effectively identify *Symbiodinium* C1 and D within a single coral host. A high level of agreement was shown to exist in the identification of *Symbiodinium* C and D clades between the ITS1 and this cp23S-rDNA real-time PCR assay. This assay was implemented in Chapter 5 to accurately identify the symbiont assemblage within juvenile *Acropora millepora* colonies in order to explore the functional variation that exists with respect to the nutritional demands and the physiological performance of the coral host as a result of the genetic diversity of the genus *Symbiodinium*.

Chapter 5: Juvenile corals can acquire more carbon from high-performance algal symbionts

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5.1. INTRODUCTION

Zooxanthellae (symbiotic dinoflagellates of the genus *Symbiodinium*) are critical to the survival of reef-building corals, providing a major source of energy from photosynthesis for cell maintenance, growth and reproduction of their coral hosts (Crossland et al. 1980, Muscatine et al. 1984). Molecular techniques using 28S, ITS1, ITS2, 23S and COX1 markers have uncovered a large diversity describing eight major groupings or clades within the genus *Symbiodinium* (van Oppen et al. 2001; LaJeunesse 2002; Santos et al. 2002; Baker 2003; Pochon et al. 2004; Rowan 2004; Takabayashi et al. 2004; Coffroth and Santos 2005; Apprill and Gates 2007, Chapter 4). A review by Goulet (2006) revealed that most hard and soft corals reportedly may contain only one *Symbiodinium* type. However, sensitive molecular detection methods have recently revealed a considerable number of anthozoans can harbour several algal types simultaneously (Ulstrup and van Oppen 2003; Apprill and Gates 2007; Loram et al. 2007; Mieog et al. 2007). Differences in photobiology are known to exist between *Symbiodinium* types, especially under conditions of thermal or irradiance stress (Rowan 2004; Goulet et al. 2005; Berkelmans and van Oppen 2006). As ocean warming associated with climate change is predicted to cause an increase in the frequency and intensity of coral bleaching events, assessing the performance of distinct coral-zooxanthella associations under conditions of photosystem stress will enhance our understanding of the future health of coral reefs (Little et al. 2004).

Physiological traits of the coral host are at least partly shaped by the dominant symbiont type present within its tissues. For instance, the genetic type of symbiont within juveniles of *Acropora millepora* and *A. tenuis* has been linked to a 2-3 fold increase in growth rate within the first 6 months of development on the reef (Little et al. 2004). Furthermore, adult *A. millepora* corals on the Great Barrier Reef (GBR) have been shown to acquire a 1-1.5 ºC increase in thermal tolerance by shuffling the

dominant symbiont type present within coral tissues (Berkelmans & van Oppen 2006). Likewise, in Guam, colonies of *Pocillopora* spp. associating with *Symbiodinium* D exhibited greater tolerance to thermal stress compared with corals associating with *Symbiodinium* C (Rowan 2004). Capacity for photoacclimation and tolerance to high irradiance stress has also been linked to the genetic type of *Symbiodinium* spp., both in culture and within multiple coral host species (Robison & Warner 2006, Warner et al. 2006). A recent study shows that symbiont type can affect the incorporation of algal-derived photosynthetic carbon (^{14}C) into host tissues of an anemone (Loram et al. 2007), further supporting the notion that symbiont type can affect growth and resilience to stress. Anemones hosting *Symbiodinium* type A obtained a greater proportion of photosynthetically fixed carbon (energy) into animal lipids and amino acid pools than those hosting type B. Total fixation rates were elevated at high temperatures for type A anemones and were depressed for type B anemones, demonstrating that these two distinct *Symbiodinium* types are not functionally equivalent. The ability of corals to associate with a diverse range of symbiont types (van Oppen et al. 2001; Baker 2003; Rowan 2004) may provide ecological advantages to the host colony, enabling it to colonize a variety of reef habitats and survive a changing global climate.

Acropora millepora is typical of most broadcast spawning corals, acquiring symbionts from the environment just prior to or following larval metamorphosis. It associates with *Symbiodinium* D on reefs surrounding Magnetic Island, a nearshore island in the central section of the GBR. However, it commonly harbours *Symbiodinium* C1, C2 and C3 on inner-, mid- and outer-shelf reefs (van Oppen et al. 2005a). On the GBR, *Symbiodinium* D has a predominantly inshore distribution and hence experiences high temperatures, turbidity and pollution events relatively frequently. Its rarity on mid- or outer-shelf reefs is possibly due to higher light intensities common at these sites (van Oppen et al. 2005a). Here I compared the effect of hosting *Symbiodinium* C1 and D on the translocation of carbon based energy (measured as tissue ^{14}C incorporation) to the coral host. The relative electron tranport rates of photosystem II ($rETR_{MAX}$) of these symbiont types was also measured *in hospite* as a secondary measure of photosynthetic performance. The experiments were performed under controlled light and temperature conditions using 9 month old *A. millepora* juveniles that had a common parentage and had been experimentally infected with either *Symbiodinium* C1 or D immediately following metamorphosis. The experimental design therefore controlled for both environmental and host influences on symbiont physiology (Coles & Brown 2003,

Rowan 2004). The juvenile corals were also exposed to the herbicide diuron, an environmentally relevant contaminant that acts by inhibiting photosynthesis by blocking electron transport and causes damage to photosystem II (PSII) (Vandermeulen et al. 1972, Jones et al. 2003). Unlike high temperature and irradiation stresses, diuron exposure influences the symbiont's performance without directly affecting the coral host (Schreiber et al. 1997; Negri et al. 2005; Cantin et al. 2007), therefore enabling the effects of photoinhibition to be distinguished between symbiont types.

5.2. METHODS

Juvenile inoculation and treatments: Gametes were collected following spawning from 8 (hermaphroditic) colonies of *Acropora millepora* and mixed for fertilization. Larvae were raised in filtered seawater (1 μm). On day 4 following spawning, when larvae were first observed to exhibit settlement behaviour, preconditioned (for 16 weeks on the reef at Magnetic Island), autoclaved (to kill any *Symbiodinium* spp. present on the tiles) terracotta tiles were placed on the bottom of two 500 l tanks as settlement surfaces. *Symbiodinium* strains C1 (GenBank Accession No. AF380555) and D (GenBank Accession No. EU024793) based on ITS1 were selected for experimental infection of juveniles because both associate with *A. millepora* on inshore reefs of the GBR (van Oppen et al. 2005). *Symbiodinium* C1 and D strains were obtained from adult colonies of *A. tenuis* and *A. millepora* at Magnetic Island, respectively, by airbrushing the coral tissue and isolating the *Symbiodinium* cells from the coral-algal slurry by centrifugation (5 min. at 350 x g). These isolated symbionts were offered to and acquired by larvae and newly settled juveniles at 10^8 cells/tank after 3 and 5 days following spawning. Infection of the coral juveniles was confirmed by microscopic observation of squash preps indicating the presence of symbionts within the tentacles of each juvenile. *Symbiodinium* genotypes were confirmed following infection prior to field deployment by single-stranded conformation polymorphism (SSCP) and potential presence of unexpected background strains was tested using quantitative PCR at the end of the experiment (see below). The juvenile corals were allowed to develop for a further 2 weeks in the laboratory, after which the tiles were attached vertically to racks on a fringing reef (Nelly Bay, Magnetic Island) in a zone where *A. millepora* is common, and were randomly arranged to minimize any effects of partial shading during this grow-out period. The juveniles were collected 9 months later and acclimated

horizontally under identical natural illumination (75% shading, max 350 µmol photons m^{-2} s^{-1}) in an outdoor flow-through aquarium for 2 days prior to experimental testing. In this set-up, juveniles experimentally infected with *Symbiodinium* C1 or D originated from crosses involving the same parent corals, thereby minimizing potential host genetic differences that may influence the physiology of the holobiont (host-symbiont partnership). Nine month old juveniles were randomly distributed within 3 glass tanks (4 l filtered sea water, 0.25 µm) per diuron (3,(3,4-dichlorophenyl)-1,1-dimethylurea, photosystem II inhibitor) treatment [0 (control), 1 and 10 µg l^{-1}; n = 9 juveniles per diuron treatment and symbiont type; 10 hrs diuron exposure]. Tanks were placed under metal halide lamps exposing the corals to a constant illumination of 180-200 µmol photons $m^{-2}s^{-1}$, similar to light levels the corals were exposed to on a daily basis in the reef habitat during development.

Pulse amplitude modulation (PAM) fluorometry: All fluorescence measurements were taken with a Diving-PAM (Walz, Germany) by holding the 2 mm fibre-optic probe at a consistent distance of 2 mm directly above each juvenile coral using the manufacturer-supplied leaf clip. Rapid light curves (RLCs) can be used to assess the current capacity of PSII as a function of irradiance under increasing levels of light intensity (PAR) (Schreiber et al. 1997, Ralph and Gademann 2005). RLCs are constructed by plotting the effective quantum yield as measured with a PAM fluorometer against PAR. Cardinal points calculated from a RLC such as the maximum relative electron transport rate of PSII ($rETR_{MAX}$) reflect the present state of photosynthesis (of PSII) and are strongly dependent on the immediate light pre-history of the sample (Schreiber, 2004). These measurements differ from traditional photosynthesis-irradiance (*P-E*) curves derived from gas exchange measurements which effectively describe how the entire photosynthesis apparatus acclimates to different light intensities and are less dependent on light pre-history. RLC measurements calculated within this study were used comparatively among the different symbiont and diuron treatments to reflect the current photosynthetic performance under the experimental light conditions.

After 2 hrs of light exposure, corals were placed in darkness for a short time (2 min dark adaptation) to allow substantial re-oxidation of the primary electron acceptor (Q_A) (Schreiber 2004) and minimize the total time required for RLC measurements. RLC measurements were performed in the dark on three juveniles for each symbiont

type and diuron treatment (n=3), taking a total of 90 min. Each tank was immediately returned to the light regime following each RLC. The RLC's were measured using a pre-installed software routine, where the actinic measuring light was incremented over 8 light steps (0, 44, 72, 116, 147, 222, 283, 428 and 653 μmol photons m^{-2} s^{-1}), each with a duration of 10 s. The relative electron transport rate (rETR) (Equation 1) obtained from RLCs provides a reliable approximation of relative electron flow through PSII when absorbance between samples is identical (Genty et al. 1989). In the present experiments this was assumed since all juveniles: (i) had similar colony heights (2-4mm), (ii) received the same irradiance, (iii) contained the same quantity of symbionts (see below) and pigment concentrations in control treatments (see Results section), and (iv) were tested using the same host coral species therfore the corallite shapes were identical, thus reducing the influence of light scattering based on coral skeleton morphologies.

Relative electron transport rate of PSII (rETR) = $\Delta F/F_{m'}$ x PAR (1)

Minimum fluorescence (F in illuminated samples or F_o in dark-adapted samples) was determined by applying a weak pulse-modulated red measuring light (0.15 μmol photons m^{-2} s^{-1}). The maximum fluorescence (F_m' in illuminated samples or F_m in dark-adapted samples) was then measured following application of a saturating pulse of actinic light (> 3000 μmol photons m^{-2} s^{-1}). The maximum quantum yield (F_v/F_m, Equation 2) is the proportion of light absorbed by chlorophyll (in PSII) used for photochemistry in the dark adapted organisms, when all reaction centers are open (Genty et al. 1989). Maximum quantum yield (F_v/F_m) values were obtained from dark adapted symbionts (n=3 juvenile corals) following each RLC and an additional 10 minute dark adaptation.

Maximum quantum yield of PSII (F_v/F_m) = $(F_m - F_o)/F_m$ (2)

Radio-labelled 14C incorporation: Following the fluorescence measurements, the volume of filtered seawater was reduced to 1 l and 1 ml of $NaH^{14}CO_3$ (specific activity 74 MBq ml^{-1}, Amersham Biosciences, USA) was added to each tank. The water level was subsequently raised to 2 l to ensure equal distribution of radiolabel throughout the tank. ^{14}C incubation was carried out for 6 hrs under constant illumination (180-200

μmol photons $m^{-2}s^{-1}$). At the end of the incubation, each tile was removed from the experimental light exposure and rinsed twice with fresh filtered sea water for 5 min to remove unincorporated ^{14}C from the surface of coral tissues. Each juvenile (n = 9) was removed from the terracotta tile with a scalpel, snap frozen in liquid nitrogen and stored at –80° C until analysis. The time frame used to rinse each juvenile coral at lower light intensity (approx 15-30 μmol photons $m^{-2}s^{-1}$ for 10-15 min.) prior to freezing may have influenced the xanthophyll pigment ratios [conversion of diatoxanthin back to diadinoxanthin in the absence of light stress (Brown et al. 1999)], however this was not likely to influence the total xanthophyll pool. Tissue from each juvenile *A. millepora* (n = 9) was removed by airbrushing and the host tissue was separated from the symbionts by centrifugation (490 x g). Host tissue samples (100 μl) were acidified with 0.1M HCl (100 μl) prior to scintillation counting, to remove unincorporated ^{14}C. Host tissue samples were counted on a 1450 Microbeta Plus scintillation counter (Perkin Elmer) for 2 min to determine disintegrations per minute (dpm) and the amount of ^{14}C incorporation. *Symbiodinium* cells were resuspended in 10% formalin and cell densities were determined with a haemocytometer. Mean cell densities were not different between symbiont types: *Symbiodinium* C1 = 5.5 ± 0.4 (SE) x 10^5 cells /cm^2 and Symbiodinium D = 4.6 ± 0.8 x 10^5 cells /cm^2, p = 0.20]. Digital images of the juveniles were taken from a standardized height on a tripod and the perimeter of each juvenile coral was then traced using image analysis software (Optimas, Media Cybernetics, Silver Spring, MD, USA) and surface area of each juvenile coral was then calculated using Optimas (Negri et al. 2005). The radiolabelled photosynthate incorporation was expressed as radioactivity per unit area of juvenile per dinoflagellate cell (dpm $zoox^{-1}cm^{-2}$) from the calculated surface area measurements to standardize for the variations in the size of individual juveniles and the *Symbiodinium* densities they host.

Symbiodinium cp23S-rDNA real-time quantitative PCR assay: To determine the relative abundance of symbiont types within *Acropora millepora* juveniles immediately prior to ^{14}C studies, a sub-sample of *Symbiodinium* cells from each tissue slurry (above) was taken and fixed in absolute ethanol. Symbiont DNA was extracted following a previously published DNA isolation method (Wilson et al. 2002). Relative symbiont abundance within the *A. millepora* juveniles was determined using the cp23S-rDNA real-time PCR assay developed in Chapter 4. Relative abundances (as a percentage of total copies per reaction) were determined from the calculated concentration (copies per

reaction) within Rotor-Gene Analysis Software v. 6.0 (Corbett Research, NSW Australia) for each *Symbiodinium* type per reaction in comparison to the 100,000 copy standard after importing the standard curve from the serial dilution series (Chapter 4, Fig. 4.2).

Pigment analysis by HPLC: Chlorophylls and carotenoids were extracted sequentially by sonication (Cole Parmer Ultrasonic Processor, Extech Equipment, Victoria, Australia) in 100% acetone from *Symbiodinium* cells that were separated from the host tissue (n=8 juvenile corals). High performance liquid chromatography (HPLC) was used to analyse the extracts on a Waters 600 HPLC, combined with a Waters PDA 996 photodiodearray detector, on a 3 μm, 50 x 4.6 mm Phenomonex C-18 Gemini 110Å column (Phenomonex, NSW, Australia). A two solvent gradient with a flow rate of 1 ml min^{-1} was used to separate the pigments with a run time of 18 min. Percentages of the solvents A and B respectively, as follows: 0 min: 75, 25 %; 0 to 5 min linear gradient to: 0, 100 %; 5 to 10 min hold at: 0, 100%; 10 to 11 min linear gradient to: 75, 25 %; 11 to 18 min hold at 75, 25 %. Solvent A was 70:30 v/v methanol:28 mM tetrabutyl ammonium acetate (TBAA, 1.0M aq. Sigma-Aldrich, Australia) and solvent B was 50:50 v/v methanol:acetone. Chlorophyll *a, c_2*, peridinin and diadinoxanthin standards were obtained from the International Agency for ^{14}C Determination (DHI, Denmark). The peaks reported were identified by comparison of retention times and absorption spectra with standards and published data (Wright & Jeffrey 1997).

Data analysis: Two-way ANOVA ($\alpha = 0.05$) was used to test the effect of *Symbiodinium* type and diuron concentration on $rETR_{MAX}$, F_v/F_m, ^{14}C incorporation and total pigment concentrations. Fishers LSD post hoc test ($p < 0.05$) was used to identify statistical differences between treatments. Data were tested for assumptions of normality and homogeneity of variances and transformations were not required. All figures and curve-fitting to determine the characteristic parameters of the rapid light curves (Ralph et al. 2002) were created using Sigmaplot 2001 for Windows (v. 7.1, SPSS Inc.). Statistica v. 6.0 (StatSoft, Inc. Oklahoma, USA) was used for all statistical analyses. Rotor-Gene Analysis Software v. 6.0 (Corbett Research, NSW Australia) was used for all real-time qPCR analysis.

5.3. RESULTS

Quantitative PCR assays confirmed that the juvenile *Acropora millepora* colonies were dominated by the *Symbiodinium* types that they were exposed to during experimental infections after nine months of growth on the reef at Magnetic Island, GBR. Juveniles exposed to *Symbiodinium* C1 were estimated to contain 98 – 100% relative abundance C1 (n = 27), while juveniles exposed to *Symbiodinium* D contained 94 – 100% relative abundance D (n = 27).

Comparison of rapid light curves (RLCs, Fig. 5.1A, B) indicated that *Symbiodinium* C1 had an 87% greater relative electron transport rate through PSII ($rETR_{MAX}$, Fig. 5.2A) than that of *Symbiodinium* D when associated with the same coral species in the absence of diuron (Two-way ANOVA, symbiont type: $F_{(1,12)}$=6.85, p=0.02, diuron: $F_{(2,12)}$, p<0.001, symbiont*diuron interaction: $F_{(2,12)}$=2.07, p=0.2). RLCs also revealed that the minimum saturating irradiance (E_K) was not significantly different between types [E_K type C1 = 80 ± 16 (SE), type D = 52 ± 13, $F_{(1,12)}$=2.38, p=0.15], suggesting that the photosynthetic characteristics of the two genetically distinct symbionts were similarly acclimatized to low-light conditions (Fig. 5.1 A,B). This is not surprising since the juveniles were raised for 9 months in the frequently turbid inshore waters of Magnetic Island. Exposure of the juvenile colonies to the electron transport inhibitor diuron significantly reduced the $rETR_{MAX}$ in both *Symbiodinium* C1 and D (Fig. 5.1 and 5.2A). At 10 μg l^{-1} diuron, the $rETR_{MAX}$ was reduced by 86% in *Symbiodinium* C1 and by 71% in *Symbiodinium* D under the same experimental conditions (Fig. 5.2A). The maximum quantum yields (F_v/F_m) in dark adapted corals were identical for each symbiont type in the absence of diuron (Fig. 3, Two-way ANOVA, symbiont type: $F_{(1,102)}$=0.05 p=0.8, diuron: $F_{(2,102)}$=103.76, p<0.001, symbiont*diuron interaction: $F_{(2,102)}$=0.61, p=0.5), indicating similar efficiencies of excitation energy capture by PSII for each symbiont (Genty et al. 1989). Diuron exposure caused similar reductions in F_v/F_m in both symbionts (Fig. 5.3), indicating equivalent damage to the D1 protein of PSII in both symbiont types (Schreiber 2004).

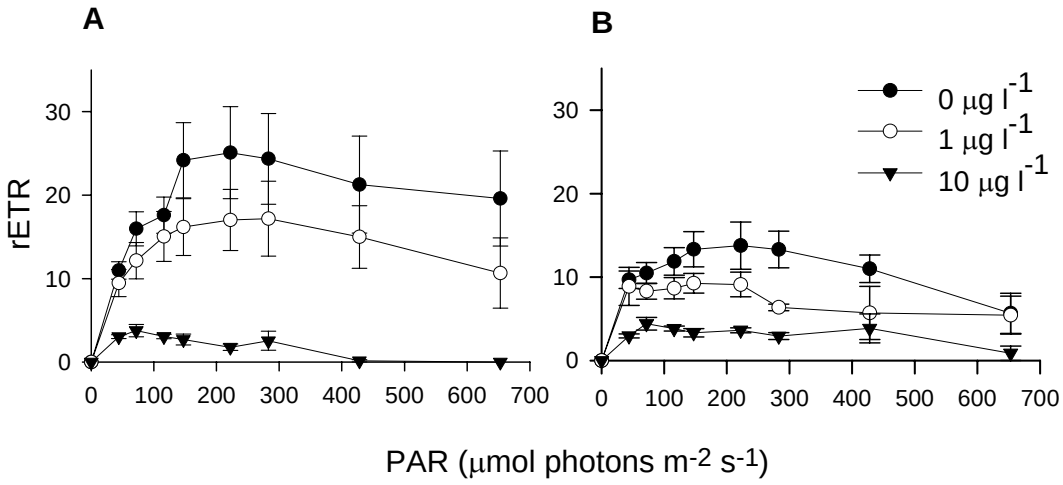

Fig. 5.1: Rapid light curves from juvenile colonies of *Acropora millepora*. Relative electron transport rate (rETR) as a function of photosynthetically active radiation (PAR, μmol photons m^{-2} s^{-1}) derived from colonies hosting (**A**) *Symbiodinium* C1 and (**B**) *Symbiodinium* D exposed to 3 diuron treatments (0, 1 and 10 μg l^{-1}). n = 3 juveniles, mean ± SE.

Incorporation of radiolabelled photosynthate (^{14}C, energy) into the host tissue was 121% greater within juvenile *A. millepora* corals hosting *Symbiodinium* C1 than the colonies associated with *Symbiodinium* D in control treatments at 200 μmol photons m^{-2} s^{-1} (0 μg l^{-1}, Fig. 2B, Two-way ANOVA, symbiont type: $F_{(1,48)}$=6.69, p=0.02, diuron: $F_{(2,48)}$=4.11, p=0.01, symbiont*diuron interaction: $F_{(2,48)}$=0.84, p=0.4). Exposure to the electron transport inhibitor diuron for 10 hours reduced the incorporation of photosynthates into tissues of corals hosting both *Symbiodinium* C1 and D (Fig. 5.2B). Corals hosting *Symbiodinium* C1 exhibited a 58% drop in photosynthate accumulation when exposed to 10 μg l^{-1} diuron compared to corals hosting *Symbiodinium* D, whose photosynthate level was reduced by 42% (Fig. 5.2B).

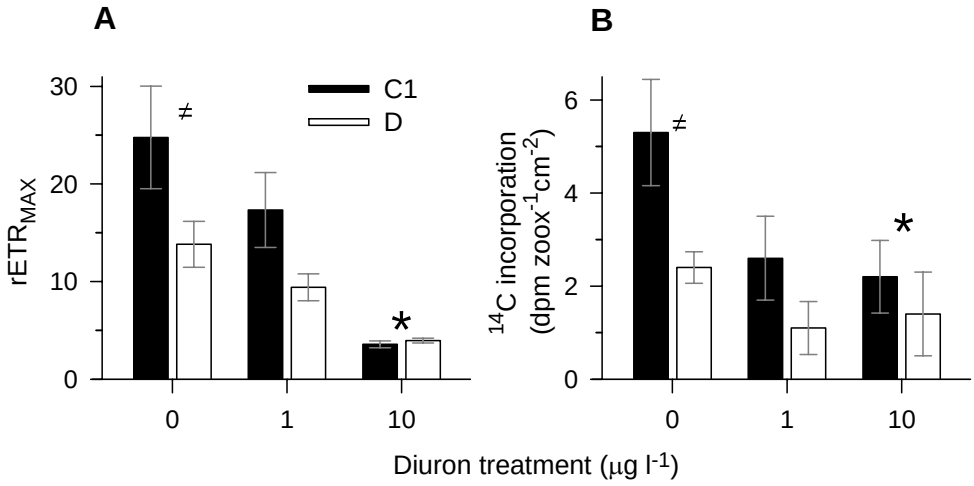

Fig. 5.2: Photosynthetic capacity of symbionts and photosynthate incorporation into juvenile corals exposed to 3 diuron treatments (0, 1 and 10 µg l^{-1}). (**A**) Photosynthetic capacity of PSII in *Symbiodinium* C1 and D hosted by *Acropora millepora* juveniles as measured by relative maximum electron transport ($rETR_{max}$) derived from rapid light curve. n = 3 juveniles (mean ± SE). (**B**) Photosynthate incorporation into juvenile *A. millepora* colonies hosting *Symbiodinium* C1 and *Symbiodinium* D as measured by uptake of ^{14}C into host tissue n = 9 juveniles, mean ± SE. Asterisks (*) indicate significant differences ($p<0.05$) between diuron treatments compared to the control (0 µg l^{-1}) and inequalities (≠) indicate significant differences between *Symbiodinium* C1 and D.

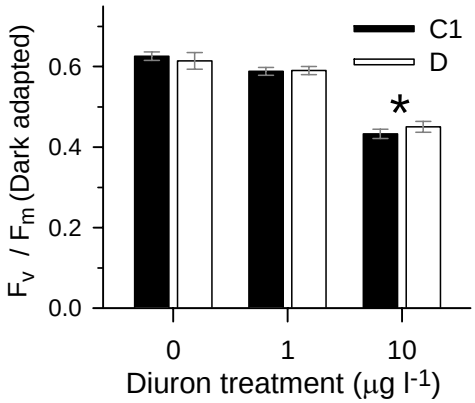

Fig. 5.3: Quantum yields of *Symbiodinium* C1 and D. Maximum quantum yields (F_v/F_m, mean ± SE, n=3 juveniles), for dark adapted *Acropora millepora* juveniles hosting *Symbiodinium* C1 and *Symbiodinium* D exposed to 3 diuron treatments (0, 1 and 10 µg l^{-1}). Asterisks (*) indicate significant differences ($p<0.05$) between diuron treatments compared to the control (0 µg l^{-1}) and inequalities (≠) indicate differences between *Symbiodinium* C1 and D.

The pigments, chlorophyll *a*, c_2 and peridinin, which constitute the major light harvesting complex (LHC) within dinoflagellates, were detected with a molar ratio of 1:0.3:0.5 within the control juveniles. Diadinoxanthin was the major xanthophyll

carotenoid within coral symbionts, along with low concentrations of diatoxanthin. No differences in light harvesting and xanthophyll pigments were evident between *Symbiodinium* types in the absence of diuron (Figs. 5.4A and 5.4B) (Two-way ANOVA LHC symbiont type: $F_{(1,42)}$=0.25, p=0.6, diuron: $F_{(2,42)}$=3.14 p=0.05, symbiont*diuron interaction: $F_{(2,42)}$=2.54, p=0.09, Fishers LSD, p=0.04. Two-way ANOVA xanthophylls, symbiont type: $F_{(1,42)}$=0.52, p=0.5, diuron: $F_{(2,42)}$=2.76, p=0.07, symbiont*diuron interaction: $F_{(2,42)}$=2.91 p=0.07, Fishers LSD, p = 0.02). Severe inhibition of PSII electron transport (10 μg l^{-1} diuron treatment) for 10 h resulted in significant increases in both the total light harvesting and total xanthophyll pigments (by 108% and 114% respectively) in *Symbiodinium* C1, whereas pigment concentrations did not change in type D symbionts (Fig. 5.4). While the total light harvesting and xanthophyll pools increased for *Symbiodinium* C1, no changes in pigment ratios were observed for either symbiont type at any of the diuron concentrations.

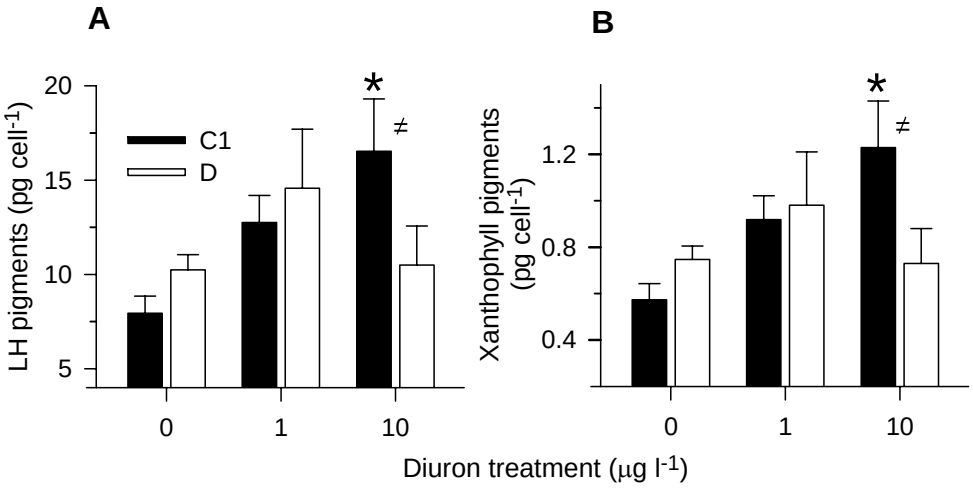

Fig. 5.4: Pigment concentrations of *Symbiodinium* C1 and D. Pigments of *Symbiodinium* C1 and D within juveniles of *Acropora millepora* exposed to 3 different diuron treatments (0, 1 and 10 μg l^{-1}). (**A**) Concentrations of total light harvesting pigments (LH), comprising chlorophyll a and c_2 and peridinin (pg $cell^{-1}$). (**B**) Concentrations of xanthophyll pigments, diadinoxanthin and diatoxanthin (pg $cell^{-1}$). n = 8 juveniles (mean ± SE). Asterisks (*) indicate significant differences ($p<0.05$) between diuron treatment compared to the control (0 μg l^{-1}) and inequalities (≠) indicate differences between *Symbiodinium* C1 and D.

5.4. DISCUSSION

Distinctive patterns in rates of photosynthate (^{14}C) incorporation and RLCs revealed major physiological differences between *Symbiodinium* C1 and D when associated with the same host species. *Symbiodinium* C1 exhibited a 121% greater capacity for translocation of photosynthates to *Acropora millepora* juveniles along with an 87% greater relative electron transport rate through photosystem II under identical environmental conditions. Using two measures of photosynthetic performance in combination, ^{14}C photosynthate incorporation and relative electron transport of PSII ($rETR_{MAX}$), indicates that the genetic identity of *Symbiodinium* spp. can influence the nutritional benefits provided through photosynthesis to the host colony under some PAR (light intensity) conditions. *Acropora tenuis* and *A. millepora* juveniles in a previous study exhibited 2-3 times faster growth rates when associated with *Symbiodinium* C1 compared to those associated with *Symbiodinium* D (Little et al. 2004) at the same field site where juveniles were reared in the present study. The enhanced growth rate previously found within C1 juveniles (Little et al. 2004) may result from enhanced translocation of photosynthates by colonies hosting *Symbiodinium* C1 as demonstrated here. The differences in carbon based energy transfer between symbiont types provides a competitive advantage to corals associating with *Symbiodinium* C1, particularly during their early life histories, when greater energy investment into rapid tissue and skeletal growth can prevent overgrowth of juveniles by competitors and mortality from grazers (Hughes & Jackson 1985).

The large differences in photosynthate incorporation into host tissue and photosynthetic performance observed between symbiont types (in coral hosts having the same parentage) highlights the functional influence of symbionts on the nutritional physiology of corals. *Symbiodinium* type has also been shown to affect photosynthate transfer within the sea anemone, *Condylactis gigantea* (Loram et al. 2007). Differential photosynthate transfer was observed under elevated temperature conditions (from 25^{o}C up to 30°C), with total incorporation ^{14}C and proportion of ^{14}C translocated to the host tissue decreasing for anemones hosting *Symbiodinium* type B and increasing for type A symbionts (Loram et al. 2007). Differences in photosynthetically fixed ^{14}C incorporation into lipids and low molecular weight amino acids within the host tissue of anemones was significantly greater for those hosting type A symbionts than B symbionts (Loram et al. 2007). The efficiency of photosynthate transfer to the host and

specific molecular allocation of fixed carbon have not been compared between symbiont types in corals, but are likely to contribute to the large (121%) differences in total photosynthate incorporation observed in the present study. Differences in the performance of *Symbiodinium* types are likely to be widespread in anthozoan endosymbiosis and an important feature in the nutritional economy of reef corals.

Although both ^{14}C uptake and $rETR_{MAX}$ were significantly higher for *Symbiodinium* C1 corals, the actual relationship between carbon fixation and symbiont performance is not likely to be simple. For instance, the self-shading of symbionts may lead to an artificially high estimation of rETR compared with oxygen evolution in PSII (Hoogenboom et al., 2006). In the present study this effect should be consistent between symbiont types as the heights, corallite morphology, symbiont numbers and pigment concentrations in juvenile corals were consistent between control treatments. Electron transport through PSII as measured using the PAM fluorometer and whole-organism photosynthesis (O_2 or CO_2 flux) as measured using respirometry are not likely to be equivalent over a wide range of PAR (Ulstrup et a. 2006). Hoogenboom et al. (2006) demonstrated that the saturation of O_2 evolution in corals can occur at lower PAR than rETR saturation. This indicates non-assimilatory electron flow through PSII, further complicating the expected relationship between rETR and carbon fixation in corals. ^{14}C uptake in the present study was only measured in the host tissue and not in the symbiont and translocation of ^{14}C to the host tissue may be different between the symbiont types. Additional respirometry and ^{14}C fixation (and translocation) experiments over a wider range of PAR, in conjunction with detailed quenching analysis, are required to fully appreciate the complex relationship between the photosynthetic performance of different symbiont types and energetic benefit to the coral host tissue.

The reduction in ^{14}C incorporation into coral tissue exposed to diuron confirms strong links between relative electron transport and photosynthate incorporation for both symbiont types. The reduction of $rETR_{MAX}$ in both symbiont types by 10 $\mu g\ l^{-1}$ diuron to indistinguishable levels indicates that, under these conditions of severe electron transport inhibition and at this level of PAR, C1 colonies would receive the same ^{14}C allocation from their symbionts as D-colonies. However, *Symbiodinium* C1 suffered a greater proportional drop since, in the absence of diuron, its $rETR_{MAX}$ was 85% greater than was observed for *Symbiodinium* D (Fig. 5.2A). The relative reduction in ^{14}C incorporation into host tissues in the presence of diuron was also greater for C1

colonies (Fig. 5.2B). Therefore, C1 colonies may lose their potential for more rapid growth and any competitive advantage over D-colonies at the juvenile stage under stressful conditions that limit electron transport. While future experiments under a full range of irradiance conditions are required to confirm differences in physiology between *Symbiodinium* C1 and D, the present results reveal how PSII herbicide exposures might affect corals differently, depending on the symbiont types they harbour.

The identical maximum quantum yields (F_v/F_m) in dark adapted samples for each symbiont type in the absence of diuron (Fig. 5.3) indicates similar efficiencies of excitation energy capture by PSII for each symbiont type (Genty et al. 1989). This result is consistent with other reports, which show similar F_v/F_m for coral-inhabiting *Symbiodinium* when measured in the absence of stress (Rowan 2004; Berkelmans and van Oppen 2006; Robison and Warner 2006; Warner et al. 2006). Reductions in F_v/F_m at 10 μg l^{-1} diuron were similar for both symbiont types tested in this study, indicating equivalent levels of damage to the D1 protein of PSII (Genty et al. 1989). Marine and freshwater algal species in culture have displayed inter-species differences in sensitivity to low diuron concentrations within toxicity tests (Bengtson Nash et al. 2005b). It is plausible that longer exposure to diuron and exposures at higher irradiances might reveal similar differences in diuron sensitivity between symbiont types, analogous to the PSII damage observed during longer exposure experiments to thermal stress. For example far greater reductions in F_v/F_m were observed in C2 symbionts in adult *Acropora millepora* exposed to elevated seawater temperatures than for the more thermally tolerant D symbionts in the same species, indicating greater damage to the D1 protein of C2 symbionts (Berkelmans & van Oppen 2006). *Pocillopora* spp. hosting *Symbiodinium* C exhibited identical F_v/F_m values and higher levels of photosynthesis (measured as oxygen flux) at 28°C than *Symbiodinium* D, but suffered a greater decline in photosynthetic performance and more damage to PSII at elevated temperatures (Rowan 2004). However, relationships between thermal tolerance, photoacclimation and growth are not always consistent. For instance, thermally tolerant *Symbiodinium* F2 and A1 exhibited opposite trends in F_v/F_m and growth at elevated temperatures in culture (Robison & Warner 2006).

The characteristics of photosynthesis are often limited by three processes: (i) enzymatic capacity of RuBisCo at light saturating intensities, (ii) the capacity of light harvesting and electron transport at subsaturating light and (iii) the capacity to

metabolize the products of photosynthesis (ATP and NADPH) (Sage 1990). No differences in light harvesting and xanthophyll pigments were evident between *Symbiodinium* types in the absence of diuron (Fig. 5.4). Thus, the higher $rETR_{MAX}$ of *Symbiodinium* C1 in control treatments did not result from higher concentrations of light harvesting pigments that would enhance the capability of photon capture at the beginning of the electron transport chain. To fully determine the cause of the enhanced electron transport within *Symbiodinium* C1, future studies should be conducted to determine which photosynthetic processes limit the photosynthetic performance of *Symbiodinium* D. Severe inhibition of PSII electron transport (10 μg l^{-1} diuron treatment) for 10 h resulted in significant increases in both the light harvesting and xanthophyll pigments (by 108% and 114% respectively) in *Symbiodinium* C1, whereas pigment concentrations did not change in type D symbionts (Fig. 5.4). Under these diuron exposures, a large proportion of the PSII reaction centres are inactive due to photoinhibition and damage of the D1 protein (Jones et al. 2003). Xanthophyll carotenoids are used for non-photochemical quenching (NPQ), preventing oxidative damage from singlet oxygen species and reducing damage caused by high light and herbicide exposure (Muller et al. 2001). It is possible that rapid pigment biosynthesis was stimulated in *Symbiodinium* C1 in an attempt to compensate for reduced electron transport caused by diuron. This type of rapid pigment biosynthesis was reported for high light acclimated green alga *Dunaliella salina* following a 12 h transition to low illumination (Masuda et al. 2002). Experiments that subject *Symbiodinium* C1 to both low and high irradiances may confirm this mechanism in corals. However, this apparent upregulation of pigments was unable to compensate for reduced rETR, since electron transport was reduced under these conditions to the same level as for *Symbiodinium* D.

This study identifies the potential energetic consequences to the coral host of association with genetically distinct types of the algal endosymbiont, *Symbiodinium*, that differ intrinsically in their photophysiology. It was demonstrated that the photosynthetic performance, as measured by photosynthate incorporation (carbon based energy) and PSII relative electron transport, was significantly enhanced within *Symbiodinium* C1 compared to *Symbiodinium* D, which possibly could explain the influence that symbiont type has previously been shown to have on juvenile coral growth rates (Little et al. 2004) and therefore symbiont type can provide a competitive advantage to the coral host. These results underscore the importance of carbon based energy sources from photosynthesis and the important influence that distinct

Symbiodinium partners may have in determining coral physiology and performance. I suggest that a physiological trade-off between stress (thermal, irradiance or contamination) tolerance and photosynthetic performance underlies the growth advantage gained by corals when associated with *Symbiodinium* C1 in early life history (Little et al. 2004). As the community structure of coral reefs shift in response to global climate change and water quality impacts (Hughes et al. 2003), opportunistic corals harbouring symbionts that enable maximum rates of growth may similarly gain a competitive advantage. Shifts in reef communities to coral assemblages dominated by *Symbiodinium* D, a symbiont commonly associated with high-temperature tolerance, have been recorded in Pacific Panama, the Persian (Arabian) Gulf and Kenya following episodes of severe, high temperature bleaching (Baker et al. 2004). While such shifts may increase the survival of corals under warming conditions (Berkelmans & van Oppen 2006), decreases in sea temperatures between bleaching events may favour corals that harbour more photosynthetically active *Symbiodinium* types such as C1 (ITS1, used in this study) and enhance the growth rate of coral colonies that maintain stable symbiotic relationships that have been observed following bleaching events (Thornhill et al. 2006). Further comparisons between other *Symbiodinium* types may reveal even more extreme differences in photosynthetic performance. These findings reveal underlying photophysiological differences between genetically distinct algal endosymbionts that advance our understanding of the dynamic relationship between the coral host and its symbiotic partner.

Chapter 6: Concluding Discussion

Carbon based energy from symbiont photosynthesis stimulates calcification for skeletal growth and lipid production in reef-building corals (Crossland et al. 1980, Anthony & Fabricius 2000) however, the extent to which coral species rely on this source of energy for reproduction is not well understood. Even less is known about the vulnerability of coral reproduction to the effects of potentially harmful contaminants that inhibit photosynthesis of coral symbionts. Diuron represents the herbicide of greatest environmental concern within the Great Barrier Reef lagoon based upon its high application rates, persistence in the soil, its stability within the marine environment and its detection at concentrations capable of producing physiological effects in marine autotrophs (Haynes et al. 2000a). This study revealed significant differences in energy delivery to host corals between genetically distinct symbiont types and that this difference in symbiont performance was reduced in the presence of diuron. The negative impacts of the photo-inhibitor diuron on reproductive development and output in the corals *Acropora valida* and *Pocillopora damicornis* found in this study highlight the importance of carbon-based energy from photosynthesis for coral reproduction. Although this study did not assess the impacts of reduced autotrophic input on other aspects of the corals' energy budgets, it is possible that energy was so limiting that even the reduced reproductive output measured occurred at the expense of other biological functions, like growth and regeneration. Further studies, which measure growth as well as reproduction, are required to characterize the nature of physiological trade-offs that enable both limited and successful reproduction in the presence of stressors that decrease the availability of autotrophic energetic resources to corals.

6.1. Implications of resource limitation for corals

Coral reefs are experiencing unparalleled levels of anthropogenic stress from a variety of sources including climate change (Hoegh-Guldberg 1999, Hughes et al. 2003, Berkelmans 2004, Hoegh-Guldberg et al. 2007), high UV and solar irradiance (Fitt & Warner 1995, Lesser 1996, 1997) and reduced water quality (Fabricius et al. 2005), which are causing an unprecedented rate of decline in the health of coral reefs (Hughes et al. 2003, Pandolfi et al. 2003, Bruno & Selig 2007, Hoegh-Guldberg et al. 2007). This decline in the health of coral reefs may partly result from nutritional limitations

during stressful events that disrupt the otherwise productive relationship between the coral host and its symbiotic algae, *Symbiodinium* (Szmant & Gassman 1990, Michalek-Wagner & Willis 2001a, Lesser 2004). Coral bleaching (the loss of *Symbiodinium* spp. from coral host tissues) is the most common "biological signal" of coral stress and is widely associated with both global climate change and anthropogenic stress (Hoegh-Guldberg 1999, Hughes 2000, Lesser 2004). Loss of algal symbionts constitutes a significant nutritional constraint for the coral host, since the algal partner translocates up to 95% of its photosynthates to the invertebrate host (Muscatine et al. 1984). As a consequence of energy deficiencies, the host may decrease lipid production and energy storage (Fitt et al. 1993), thereby decreasing calcification, growth and reproduction (Szmant & Gassman 1990). During prolonged periods of bleaching, gametogenesis may be suspended altogether (Michalek-Wagner & Willis 2001a) and ultimately corals will die (Hoegh-Guldberg 1999). Given predictions of rising levels of stress on corals reefs in the coming century (Hoegh-Guldberg et al. 2007), developing our understanding of energy allocation to reproduction in corals under stressful conditions is becoming increasingly important.

Diuron exposure poses a significant nutritional constraint to photosynthetic corals and provides a useful tool for examining energy allocation strategies in corals under chronic photoinhibition. Diuron induces rapid inhibition of electron transport through the PSII of *Symbiodinium* (Jones & Kerswell 2003, Jones et al. 2003), which significantly reduces carbon fixation (Owen et al. 2003) and the amount of carbon (energy) that is translocated to the coral host (Chapter 5). The chronic inhibition of photosynthesis observed for up to three months prior to each spawning or planulation event for the three species tested (Chapter 2), created a persistent reduction in photosynthates that are crucial for the energy budget of the coral host. This reduction in photosynthates is likely to have resulted in a shift in the allocation of available energy among several important competing biological functions (see Fig. 1.1), impacting energy stores (total lipid) within all three species and reducing the reproductive development (*A. valida*) and output of *A. valida* and *P. damicornis* (Chapter 2). Diuron causes chronic photoinhibition by inhibiting the synthesis of proteins (Arg238) directly associated with the herbicide binding niche of the PSII, which in turn causes degradation and diminished repair of the reaction centre D1 protein (Oettmeier 1992, Jones 2004). This damage is likely to have caused the severe bleaching observed for 2 of the 3 species (*A. valida* and *P. damicornis*) exposed to 10 µg l^{-1} diuron (Chapter 2).

All three of the study species were found to be at least partly dependent on efficient photosynthesis to supply sufficient energy for lipid production and successful completion of oogenesis. These results provide evidence that diuron exposure creates nutritional deficiencies within the coral host that limit energy available for reproduction.

Availability of energy resources is often limited in the marine environment (Rinkevich 1996) and, during conditions of environmental stress that further drain energy reserves, the allocation of energy among competing biological functions becomes critical to the long-term persistence of the species. The contribution of photosynthates to the nutritional requirements of corals is considered a key to the success of corals as modern reef builders (Muscatine et al. 1984, Rinkevich 1989), but corals are capable of drawing on alternative resources to meet the energetic demands of competing biological functions. Heterotrophy is an essential contributor of nitrogen, phosphorus, carbon and other nutrients to the metabolic requirements of the coral host (Sebens et al. 1996, Anthony & Fabricius 2000, Grottoli et al. 2006), especially in nearshore, turbid environments where the rates of photosynthesis decrease due to reduced light intensity (Anthony & Fabricius 2000). Successful reproduction observed in *A. tenuis* colonies that were severely photosynthetically inhibited (80%, Chapter 2) may indicate alternative energy acquisition from heterotrophic feeding (Anthony & Fabricius 2000, Grottoli et al. 2006) or reabsorption of energy from lipid stores (Szmant & Gassman 1990, Michalek-Wagner & Willis 2001a). Diversity in coral morphology (i.e. polyp size and growth form) potentially corresponds to a range of dependence upon autotrophic resources from photosynthesis, as demonstrated for Caribbean coral species, which are hypothesized to partition resources along an autotrophic - heterotrophic continuum as polyp size increases (Porter 1976). Of the three species tested, *P. damicornis* had the smallest polyp size and showed the greatest sensitivity to reduced energy from photosynthesis following chronic diuron exposure (Chapter 2). However, *A. tenuis* and *A. valida* have similar polyp and growth form morphologies, thus the differential impacts of diuron on their capacity to successfully reproduce were not expected (Chapter 2). Although this study did not directly measure the effects of diuron exposure on other competing biological functions such as skeletal and tissue growth, colonies of *A. tenuis* may have compensated for energetic deficiencies by allocating resources away from growth or other processes to ensure successful reproduction. Further work is required to examine the broad range of trophic strategies

used by corals that span the range of dependence on autotrophy versus heterotrophy and link the role of these strategies to the allocation of energy among competing biological functions during events that limit the availability of nutritional resources. The reproductive success of *A. tenuis* following long-term photosynthate deficiencies highlights the potential importance of energy reserves and heterotrophic feeding capacity to the resilience of photosynthetically stressed corals and demonstrates that morphology is not the only predictor of reliance upon autotrophic resources.

Disturbances that hinder reproductive processes related to the production of eggs in sessile marine organisms may have fundamental impacts on the distribution and abundance of recruits, as evidenced by links between the fecundity of adult species of *Acropora* and large scale spatial and temporal patterns in coral recruitment (Hughes et al. 2000). In this study, moderate concentrations of the agricultural herbicide diuron reduced the photosynthetic performance of symbionts within adult corals and inhibited successful reproductive development of adult coral colonies, notably causing a significant reduction in the number of eggs produced (*Acropora valida*, Chapter 2). Although the eggs developed by *A. valida* were not reduced in size (10 µg l^{-1} treatment, Chapter 2), they were not released at the time of spawning. The suppression of spawning, along with the reduction in fecundity and/or resabsorption of eggs may be an attempt to counter photosynthetic energy deficiencies and could be a common survival strategy among energy-depleted corals (Yamazato et al. 1981, Michalek-Wagner & Willis 2001a). Despite evidence of reduced egg quality (lower pigments, Chapter 4) and reduced numbers of gametes released by *A. valida* following exposure to low to moderate concentrations of diuron (Cantin et al., 2007; Chapter 2), all of the gametes released by the three species of corals tested remained fully viable, exhibiting normal fertilisation success and normal levels of metamorphosis into juvenile corals (Chapter 3). These results suggest that spawning only occurs with fully developed and viable gametes. Chronic contamination of nearshore coral reefs with PSII inhibitors like diuron and increased turbidity following flood plume events can act synergistically to reduce the photosynthetic efficiency of symbiotic corals, which may chronically reduce the fecundity of local coral breeding stocks and could have the potential to cause reduced recruitment to areas that are in most need of replenishment due to prior anthropogenic disturbances (Hughes et al. 2000).

6.2. Functional consequences of molecular diversity in *Symbiodinium*

Knowledge of the functional consequences of the wide molecular diversity observed recently within the genus *Symbiodinium* is important to our understanding of the symbiotic relationship between the dinoflagellate alga, *Symbiodinium* and its invertebrate cnidarian host. Although differences in physiological traits are known to exist between genetically distinct *Symbiodinium* types, which can influence photosynthate transfer into anemone host tissues (Loram et al. 2007), the growth rate of coral hosts (Little et al. 2004) and response of corals to thermal and irradiance stress (Rowan 2004, Goulet et al. 2005, Berkelmans & van Oppen 2006), the mechanisms underlying these differences are poorly understood. My results suggest that genetically distinct *Symbiodinium* clades are not functionally equivalent in terms of their physiological performance and nutritional interactions with coral hosts. In the present study, juvenile corals from common parentage were infected with genetically distinct *Symbiodinium* types (Little et al. 2004), controlling for both environmental and host influences that may impact upon symbiont physiology (Coles & Brown 2003, Rowan 2004). *Acropora millepora* juveniles hosting *Symbiodinium* C1 had a greater capacity to transport electrons through photosystem II ($rETR_{MAX}$) than juveniles hosting *Symbiodinium* D. As a result of this greater capacity for electron transport, corals hosting *Symbiodinium* C1 incorporated 121% more radiolabelled carbon (^{14}C) based energy into their tissues (Chapter 5). *Symbiodinium* C1 suffered greater proportional reductions in $rETR_{MAX}$ and ^{14}C translocation in the presence of diuron and attempted to compensate by increasing the cellular pigment concentrations. Ultimately, these results provide evidence that genetically distinct symbionts are not functionally equivalent *in hospite* and that *Symbiodinium* type exerts a profound influence upon the nutritional economy of the coral host.

Considerable debate exists as to whether or not coral species can host multiple algal genotypes, either sequentially or simultaneously (Kinzie et al. 2001, Goulet 2006) and it has been suggested that most coral species host only a single symbiont type (Goulet 2006). Extremely sensitive quantitative PCR assays have recently been developed for *Symbiondinium* spp. using the ITS1 region of nrDNA (Mieog et al. 2007) and chloroplast 23S-rDNA (Chapter 4 & 5), which provide important tools for testing alternative hypotheses about the likelihood of multiple algal genotypes in coral endosymbioses. Using these tools, a recent study demonstrated that 78% of colonies

tested on the Great Barrier Reef possess low background levels of secondary symbiont clades (Mieog et al. 2007), suggesting that the proportion of corals that host multiple *Symbiodinium* types simultaneously is considerably greater than previously reported. The presence of multiple *Symbiodinium* strains simultaneously within a coral symbiosis suggests the possibility of symbiont shuffling from one dominant symbiont type to another that may be better suited to environmental conditions and thus enhance the stress tolerance and physiological performance of the coral host (Buddemeier & Fautin 1993, Mieog et al. 2007). The development of chloroplast 23S-rDNA markers (Chapter 4) enabled identification of *Symbiodinium* types within *A. millepora* juveniles, which were then used to demonstrate differences in physiological performance between these symbionts *in situ* (Chapter 5). Now that sensitive molecular techniques exist to detect multiple symbiont types simultaneously within a single coral host (Mieog et al. 2007), further research should focus on: (i) exploring whether symbiont shuffling occurs within coral populations before, during and after environmental stress events, (ii) how this shuffling is regulated by the coral host and (iii) whether symbiont shuffling successfully enhances the performance and/or resilience of the coral under conditions of stress.

This study has highlighted the importance of photosynthesis for the physiological performance of corals by measuring the effects of chronic diuron-induced energy deficiencies on energy storage, gametogenesis and reproductive output of photosynthetic corals. The coral species tested demonstrated diminished reproductive success following prolonged periods of reduced energy acquisition from photosynthesis. Future investigations into physiological trade-offs that occur as a result of differential resource allocation during conditions that limit resource availability need to also address the likely effects of symbiont type. Genetically distinct *Symbiodinium* spp. are not functionally equivalent and symbiont type can have a strong influence on the nutritional economy of the coral-algal symbiotic association. In this study, photosynthate production was compared for two symbiont types that are commonly found on the Great Barrier Reef. Future comparisons between other *Symbiodinium* types may reveal even more extreme differences in physiological properties within the coral-algal endosymbioses. The relationships between *Symbiodinium* and host corals are complex. Further long-term experiments need to be performed to better understand the flexibility, vulnerability and performance of coral-algal symbiosis and subsequent

effects on reproductive success and colony growth under the conditions of stress that corals are likely to face over the coming century.

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Appendix 1

Egg sampling for HPLC analysis (Chapter 3):

To standardize the number of eggs used in each pigment extraction (Chapter 3), additional samples were counted (50, 100, 250, 500, 2 000 eggs in triplicate samples) under a dissecting microscope. Seawater was removed and the eggs were frozen in liquid nitrogen, similar to the egg samples used in Chapter 3. Each frozen egg mass of known egg number was then weighed on a four place balance. A standard curve (Fig. A1.1) was produced to determine the total number of eggs per mass within each sample in order to standardize the pigment concentrations within each extraction on a per egg basis (Chapter 3).

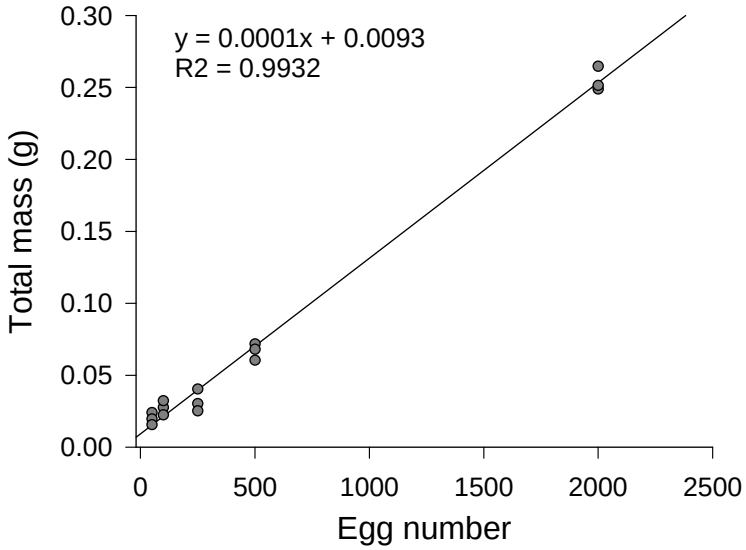

Fig. A1.1: Relationship between the number of *Acropora valida* eggs sampled and total mass of sample to determine the number of eggs used in pigment analysis in Chapter 3. n = 3 samples for each egg number counted and weighed. Regression: y = 0.0001x + 0.0093, R^2 = 0.993.