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The free-living *Symbiodinium* reservoir and scleractinian coral symbiont acquisition

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Abstract

The genus *Symbiodinium* (Dinophyceae, Suessiales), a group of geographically widespread marine dinoflagellates, comprises numerous ecologically and genetically distinct taxa. *Symbiodinium* spp. establish intracellular symbioses with cnidarians, infecting hosts such as jellyfish, sea anemones, octocorals and reef-building corals. The energetic demands of hosts can be met at varying levels by *Symbiodinium* through translocation of photosynthetically-fixed carbon, assisting in the formation of coral reefs. The cnidarian-dinoflagellate symbiosis is relatively well studied. In contrast, empirical data for population dynamics, distribution, and physiology of free-living *Symbiodinium* are limited. Therefore this thesis aims to characterise the dynamics between free-living *Symbiodinium* cells, hosts, and their habitat.

Free-living *Symbiodinium* cells have been identified in reef sediment, and this study investigated whether adult coral colonies are a significant source of benthic *Symbiodinium* cells. *Acropora millepora* colonies were translocated to bare patches of sediment (Heron Reef, Great Barrier Reef, Australia), and the surrounding sediment was sampled for *Symbiodinium* cells over a period of 18 months. An 8-fold increase in visually-identified cells relative to the background population was recorded from the sediment at the immediate base of coral colonies. *Symbiodinium* abundance returned to background levels after the removal of the coral. These fine-scale changes in the distribution of cells suggests that hard-coral may be an important 'passive' source of *Symbiodinium*.

The 'active' supply of *Symbiodinium* cells to the benthos was investigated through direct contact of coral tissue with sediment. Symbiont loss was induced in the coral *Pocillopora damicornis* through burial of coral branches. Peak cell abundance in the sediment occurred after four days, but the photophysiology (F_v/F_m) of the *Symbiodinium* cells was significantly impaired. By day 12, *Symbiodinium* cells were present only in low concentrations in sediment samples, and the majority of cells were substantially degraded. In this study, *Symbiodinium* appeared to survive only transiently following expulsion, with an approximate window of viability of seven days. Such a period may be sufficient for coral recruits to make contact with potential symbionts *in situ*.

Elevated temperatures induce a range of serious, deleterious effects in *Symbiodinium.* The potential amelioration of these effects was investigated, testing the hypothesis that *Symbiodinium* cells find refuge from stress when cultured within sediment. An exclusively free-living clade A (not known to form symbioses) and the symbiosis-forming type A1 were grown with or without a microhabitat of carbonate sediment at 25°C, 28°C or 31°C. The exclusively free-living clade A was physiologically superior to symbiosis-forming A1 across all measured variables and treatment combinations: it

reproduced faster within the sediment, exhibited high levels of motility and maintained a stable maximum quantum yield (F_v/F_m). In contrast, A1 exhibited dramatic declines in cell concentration and cell motility when cultured in sediment, most severely at 31°C. These data suggest that symbiosis-forming *Symbiodinium* types may live only transiently in sediment or outside coral hosts.

Most scleractinian coral species produce aposymbiotic juveniles that must acquire *Symbiodinium* from a free-living reservoir, but the nature of the source remains unclear. The final study examined whether juvenile corals derive S*ymbiodinium* cells from a benthic population, and if the added presence of adult coral (a conspecific) enhances symbiont acquisition. This question was investigated at Heron Reef with two broadcast spawning species, *Acropora millepora* and *Acropora selago,* and the brooder *Isopora palifera.* Newly-settled, aposymbiotic corals were maintained in open systems containing: sediment + adult coral fragments; adult coral fragments; sterilised sediment or seawater. For the *Acropora* species, the first instance of symbiosis was apparent by day seven of exposure to treatments. By day 12, approximately 70% of juveniles exposed to the combined treatment of sediment + adult coral had acquired *Symbiodinium*, compared with only 19% of those exposed to only seawater. Separately, exposure to adult coral or sediment produced intermediate acquisition in juveniles. By comparison, an accelerated but similar pattern was observed for *I. palifera* in that symbiotic juveniles were apparent after only four days of exposure to treatments. It thus appears that the supply of *Symbiodinium* from a symbiotic conspecific is indeed advantageous for recruiting corals, and it therefore follows that juveniles recruiting to recovering or damaged reefs devoid of specific *Symbiodinium* types (and their sources), may be seriously limited. The presence or absence of suitable free-living *Symbiodinium* types may prove to be a new aspect of coral reef health worthy of monitoring.

This thesis examined *Symbiodinium in situ, in vitro* and *in hospite*, providing new insight into the dynamics between free-living cells, potential hosts and known microhabitats. Particularly noteworthy results include the apparently transient survival of expelled *Symbiodinium* cells and how reductions in growth and cell motility during elevated temperature is type specific. Future studies should aim to determine how benthic reservoirs of *Symbiodinium* and the process of symbiont acquisition, a critical period in the coral life history, may change during the environmental conditions predicted for the future.

Declaration by author

This thesis *is composed of my original work, and contains* no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Journal articles

Starzak DE, Quinnell RG, Nitschke MR, Davy SK (2014) The influence of symbiont type on photosynthetic carbon flux in a model cnidarian–dinoflagellate symbiosis. Marine Biology 161:711-724

Nitschke MR, Davy SK, Cribb TH, Ward S (2015) The effect of elevated temperature and substrate on free-living *Symbiodinium* cultures. Coral Reefs 34:161–171

Gibbin EG, Putnam, HM, Gates RD, Nitschke, MR, Davy, SK (2015) Species-specific susceptibility to ocean acidification and cellular acidosis in reef corals is influenced by thermal sensitivity. Marine Biology

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Conference abstracts

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Nitschke MR, Davy SK, Ward S (2013) Physiological responses to thermal stress of free-living *Symbiodinium* cultured in reef sediment. Australian Coral Reef Society Annual Conference

Nitschke MR, Gibbin EM Davy SK, Ward S (2014) the effect of substrate and elevated temperature on symbiotic and free-living *Symbiodinium* cells. Marine and Freshwater Student Symposium

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Free-living, *Symbiodinium*, scleractinian, coral, symbiosis, acquisition, sediment, reservoir, coral spawning, reproduction

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0602 Ecology 40%

0606 Physiology 40%

0605 Microbiology 20%

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List of abbreviations used in the thesis

ANOVA – Analysis of variance

CCA – Crustose coralline algae

EFL – Exclusively free-living

F – Steady state fluorescence

FL – Free-living

FSW – 0.22 μm filtered seawater

F_v/F_m – Dark adapted yield of photosystem II

GBR – Great Barrier Reef

HIMB – Hawaii Institute of Marine Biology

HIRS – Heron Island Research Station

ITS – Internal transcribed spacer

MI – Mitotic index

MPB – Microphytobenthos

PAM – Pulse amplitude modulation

PAR – Photosynthetically-active radiation

PCR – Polymerase chain reaction

PSII – Photosystem II

qPCR – Quantitative polymerase chain reaction

RPM – Revolutions per minute

S1, S2, S3 – Sites 1, 2 and 3

SE – Standard error of the mean

SED – Sediment

SEDC – Sediment + coral

SFSW – Sterile 0.22 μm filtered seawater

SST – Sea surface temperature

SW – Seawater

SWC – Seawater + coral

TFL – Transiently free-living

μ - Cell specific growth rate

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Chapter 1: General introduction

1.1 Symbiosis

Symbiosis is defined as "the living together of unlike organisms" (De Bary 1879). Close interactions are categorized according to their outcomes, specifically whether participants are harmed, unaffected or advanced by the symbiosis. Examples include: parasitism, where one species is harmed (but not killed) while the other benefits (Johnson et al. 1997); amensalism, the obliteration or inhibition of a species through competition and antibiosis (Townsend et al. 2002); commensalism, an interaction where one participant benefits from the interaction while the other is unaffected (Heard 1994); and finally, mutualism, an interaction which is beneficial to both organisms (Muscatine and Porter 1977). Cooperation between organisms is ubiquitous across the biosphere and is theorized to be a significant driving force behind adaptive radiation and the diversification of niche occupancy (Moran 2006). Indeed, it is thought that endosymbioses (where one organism lives within another) between prokaryotic bacteria and the earliest of eukaryotes gave rise to key cellular organelles, namely mitochondria and chloroplasts (Timmis et al. 2004).

1.1.1 Mutualism in the marine environment

Symbioses are evident at all scales in the marine environment, from ocean-crossing mega-fauna and the symbiotic organisms that clean them of parasites (O'Shea et al. 2010), to the chemoautotrophic microbes and invertebrates that form the nutritional basis of life in the deep oceans (Cavanaugh 1994). Of these symbioses, some involve the exchange or transfer of nutrients and play an important role in the overall productivity of marine ecosystems. For example, some bivalve molluscs form associations with marine vascular plants which provide a stable habitat for sessile life. The ribbed mussel, *Geukensia demissa*, recruits to the basal region of the salt marsh cordgrass (*Spartina alterniflora*) and as a by-product of its filter feeding, deposits nutrients in the sediments of the marsh. This addition of organic matter to the benthos stimulates the growth of *S. alterniflora* both above ground and below ground, increasing the overall productivity of this ecosystem (Bertness 1984).

The exchange of nutrients between unicellular photosynthetic algae and marine invertebrates epitomises how primary productivity in an ecosystem can be amplified through symbiosis. Indeed, the paradoxical abundance of life in the oligotrophic waters of tropical regions (e.g. coral reefs) can, in part, be described as a function of tight nutrient cycling and exchange from microalgae to invertebrates, and *vice versa*. These photosynthetic symbioses have been observed across a range of taxa, including: molluscs of the genus *Tridacna* (Fitt et al. 1986); sea anemones such as *Cereus*

pedunculatus (Davy et al. 1997) and *Aiptasia pulchella* (Davy and Cook 2001); a great diversity of octocorals (Van Oppen et al. 2005); and jellyfish (Mellas et al. 2014). The most striking and well-studied of these are the endosymbioses of unicellular microalgae and scleractinian corals, where the exchange of the products of algal photosynthesis and nitrogenous animal waste ultimately forms the nutritional basis of coral reefs (Muscatine et al. 1981; Voolstra et al. 2009). Specifically, the dinoflagellate genus *Symbiodinium* (and the species that comprise this group), has been in co-evolution with scleractinian corals since the Triassic period (Stanley 2003), forming one of the most conspicuous marine symbioses of the present day.

1.1.2 The genus Symbiodinium

Dinoflagellates are alveolate protists with approximately 2000 extant species that share a close relationship to apicomplexans and ciliates (Stat et al. 2006). There are several dinoflagellate genera that can form symbioses with invertebrates and protists, including: *Amphidinium*; *Aureodinium*; *Gloeodinium*; *Gymnodinium*; *Gyrodinium*; *Prorocentrum*; *Pyrocystis*; *Scrippsiella* and *Symbiodinium* (Rowan 1998; Wakefield et al. 2000). A number of these are morphologically indistinguishable (to the eye) from *Symbiodinium*, which led early researchers to conclude the existence of a single species of zooxanthella (the golden-brown symbionts of invertebrates), *Symbiodinium* (= *Gymnodinium*) *microadriaticum* (Freudenthal, 1962). Investigations of *Symbiodinium* at the ultrastructural and molecular levels have since revealed widespread cryptic diversity within the genus.

The notable characteristics of *Symbiodinium* which have proved useful during the description of cells include: differences in chromosome number (Blank and Trench 1985; Blank 1987); isoenzyme composition (Schoenberg and Trench 1980); cell size during vegetative state (LaJeunesse et al. 2014); chloroplast number/arrangement (LaJeunesse 2001; Stat et al. 2006); mycosporine-like amino acid (MAA) production (Banaszak et al. 2000); the composition of fatty acids (Blank and Trench 1985); and photoadaptive physiology (Chang et al. 1983; Iglesias-Prieto and Trench 1994). Although these characteristics can be used in concert to aid the delineation of *Symbiodinium* species (LaJeunesse et al. 2014), *Symbiodinium* cells have the potential to exhibit considerable phenotypic plasticity (Stat et al. 2006). Morphological characteristics change depending on life phase (e.g. coccoid or mastigote), nutrient exposure, starch and lipid concentrations and the intensity of photosynthetically active radiation (PAR) which can alter cell and chloroplast size (Rowan and Powers 1991; Muller-Parker et al. 1996; Stat et al. 2006). These characteristics of *Symbiodinium* cells therefore, are often accompanied by a description of diversity and divergence at the molecular level.

At present, nine ‘clades’ of *Symbiodinium* (A-I, and multiple ‘sub-clades’ or ‘types’ within each

clade) are recognised (Pochon and Gates 2010; Pochon et al. 2014). Clade A and its constituents are thought to be the ancestral *Symbiodinium* lineage (Fig. 1.1), followed by sequentially derived clades and sub-clades: E; G1-G2; D1-D2; I; B; F2-F5; H; and C (Pochon et al. 2014). A large database of nuclear rDNA sequences of the internal transcribed spacer 2 (ITS2) gene has been generated for *Symbiodinium* and is the dominant method for the molecular typing of *Symbiodinium* (LaJeunesse 2002; LaJeunesse et al. 2003; Pochon et al. 2004). In a recent study, six concatenated genes from three different organelles (nuclear, chloroplastic and mitochondrial) have been used to create a highly-resolved phylogeny of the *Symbiodinium* lineage (Fig. 1.1). The outcome of the multi-gene phylogeny generally supports the widely used ITS2 and nr28s region for the molecular typing of *Symbiodinium* (Pochon et al. 2014).

The enigmatic features of certain *Symbiodinium* types have led to the formal description of a number of species within the genus. For example, all members of clade E (including geographically widespread isolates from New Zealand, California and Japan) are considered to be a single species, *Symbiodinium voratum, sensu* Jeong et al. (2014b). *Symbiodinium voratum* has unique characteristics relative to other *Symbiodinium* types, including motility throughout the night and a highly predatory behaviour in culture (Jeong et al. 2014a; Jeong et al. 2014b). Three recent additions to the list of formally described *Symbiodinium* spp. include: *Symbiodinium boreum, sensu* LaJeunesse et al. (2014); *Symbiodinium eurythalpos, sensu* LaJeunesse et al. (2014) and *Symbiodinium trenchii, sensu* LaJeunesse et al. (2014). The formal description of these species involved quantifying geographic range (*in hospite*), cell size and volume, and the analysis of multiple gene sequences (nrDNA, microsatellite markers, chloroplastic DNA and mitochondrial DNA). There are at least seven other formally described species in the genus, and at least eight other species that have been referred to without formal description (see LaJeunesse et al. (2012)). It is likely that many new species will be discovered, especially in view of the uncharacterised diversity of *Symbiodinium* types that exists exclusively outside of hosts (Yamashita and Koike 2013).

The life-cycles of *Symbiodinium* spp. have mainly been revealed from strains brought into permanent culture (Freudenthal 1962). *Symbiodinium* cells form a non-motile haploid vegetative cyst (Santos and Coffroth 2003) and in culture they interchange between this and a mastigote (flagellate motile) cell. The vegetative cyst is also the morphology cells assume while *in hospite*, and it is suggested that the host arrests cells (*via* an unknown mechanism) in this state (Koike et al. 2004). Two mastigote cells are formed by a single vegetative cyst undergoing division (Fitt and Trench 1983b) and mastigote cells maintain their motility throughout the light phase (although the duration of the motile phase is species/type specific (Fitt et al. 1981; Yacobovitch et al. 2004; Jeong et al. 2014b)). The mastigote cell has thecal plates with clear epicone (anterior) and hypocone (posterior) regions (Freudenthal

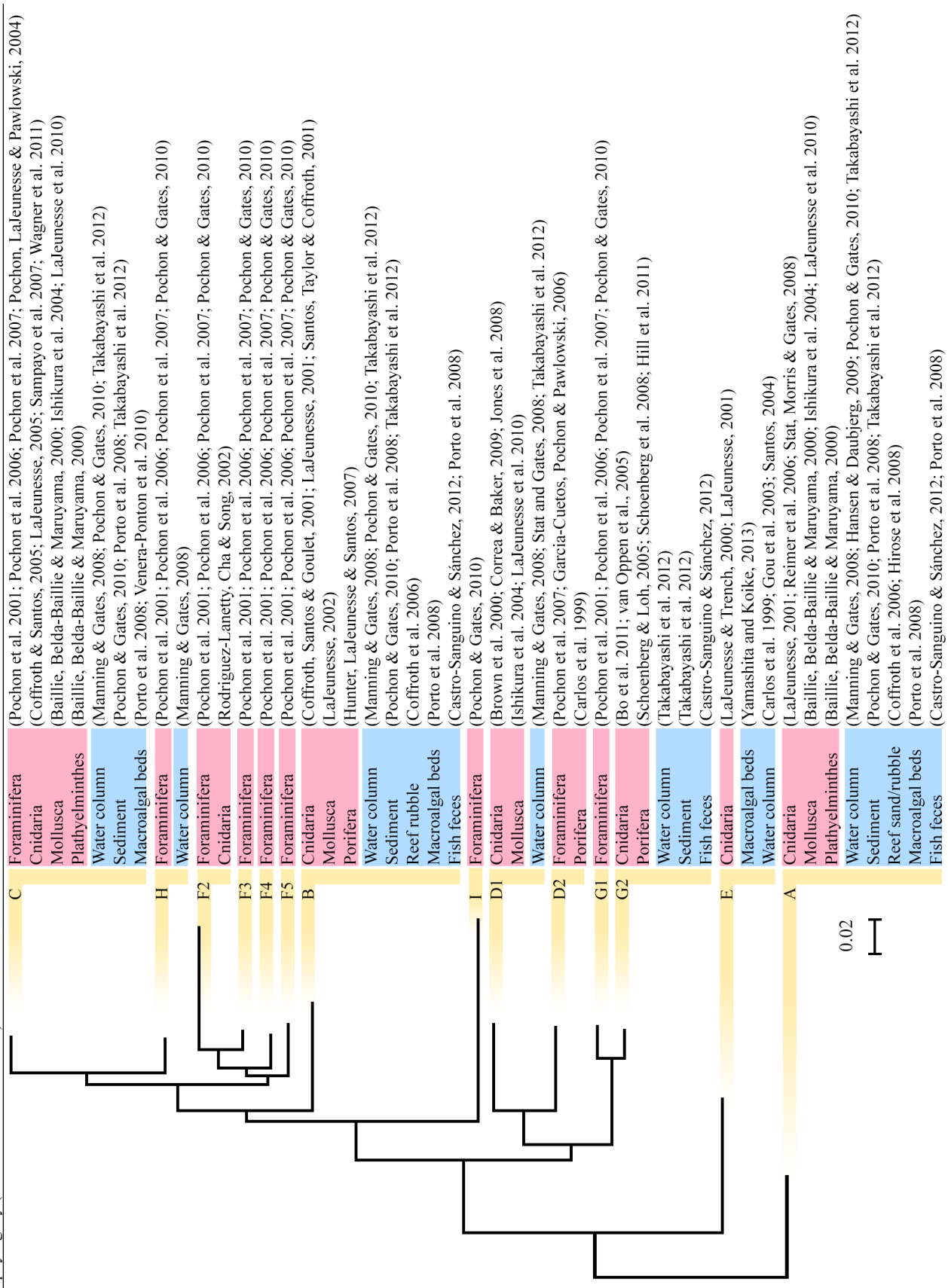

Figure 1.1 Best topology of the *Symbiodinium* phylogeny based on multi-organelle DNA analysis, recreated following Pochon et al. (2014). Host occupancy (pink) and microhabitats (blue) are listed with accompanying references. Scale bar (0.02 bp) represents number of nucleotide substitutions per site.

1962; Fitt and Trench 1983b; Fujise et al. 2014) and two flagella (longitudinal and transverse). Motile cells exhibit two types of movement. First, gyratory movement occurs where the cell rotates in either clockwise or anticlockwise motion while attached to a surface *via* the longitudinal flagellum. Secondly, forward movement occurs where the cell rapidly moves from place to place and is propelled by the longitudinal flagellum with the epicone forward (Steele 1975). During forward movement, cells also rotate along the same axis as the longitudinal flagellum. Following the cessation of motility, *Symbiodinium* cells lose their flagella and quickly become indistinguishable from other vegetative cells (Steele 1975).

1.1.3 Symbiodinium *in symbiosis*

The genus *Symbiodinium* forms intracellular partnerships with a diverse range of taxa. For cnidarian's, *Symbiodinium* cells are located in the gastrodermal cells (i.e. the tissue layer along the margin of the gastrovascular cavity) where they are bound by a series of algal and host membranes, referred to as the 'symbiosome' (Davy et al. 2012). *Symbiodinium* clades A, B, C and D are the principal symbionts of scleractinian corals (Fig. 1.1, Fig. 1.2) although C and D can also be found in symbiosis with Foraminifera (Baker 2003; Coffroth and Santos 2005; Stat et al. 2006). Clades F, G and H commonly associate with foraminiferans, while F and G can also occasionally be found within scleractinians (Rodriguez-Lanetty et al. 2002; Pochon et al. 2004; Pochon and Pawlowski 2006). Clade E forms symbioses with anemones (LaJeunesse and Trench 2000) and lastly, clade I, which is the most recently recognised clade, associates only with foraminiferans (Pochon and Gates 2010).

Symbiodinium cells can potentially meet the energy demands of cnidarian hosts through the translocation of photosynthetically-fixed carbon (Falkowski et al. 1984; Muscatine 1990), and for reef-building corals this energy subsidy is obligatory for growth. The coral affords *Symbiodinium* cells protection from predation (for example, heterotrophic dinoflagellates and ciliates prey upon *Symbiodinium* cells (Jeong et al. 2014a)) and provides access to the inorganic nutrients present in the coral's waste (Muscatine and Porter 1977; Muller-Parker and D'Elia 1997). The *Symbiodinium* cells utilize these nutrients in photosynthesis, synthesizing a complement of organic compounds (sugars, alcohols, amino acids, fatty acids and lipids) which are released to the host, thus completing the mutualism (Gordon and Leggat 2010; Burriesci et al. 2012).

Populations of *Symbiodinium* cells show considerable dynamism and may change according to the physical environment and host ontogeny. *Symbiodinium* cells can replicate at high rates within host tissues, achieving rates comparable to the exponential phase of cells in culture (Trench 1971; Schoenberg and Trench 1980). The *in hospite Symbiodinium* population eventually stabilises *via* a

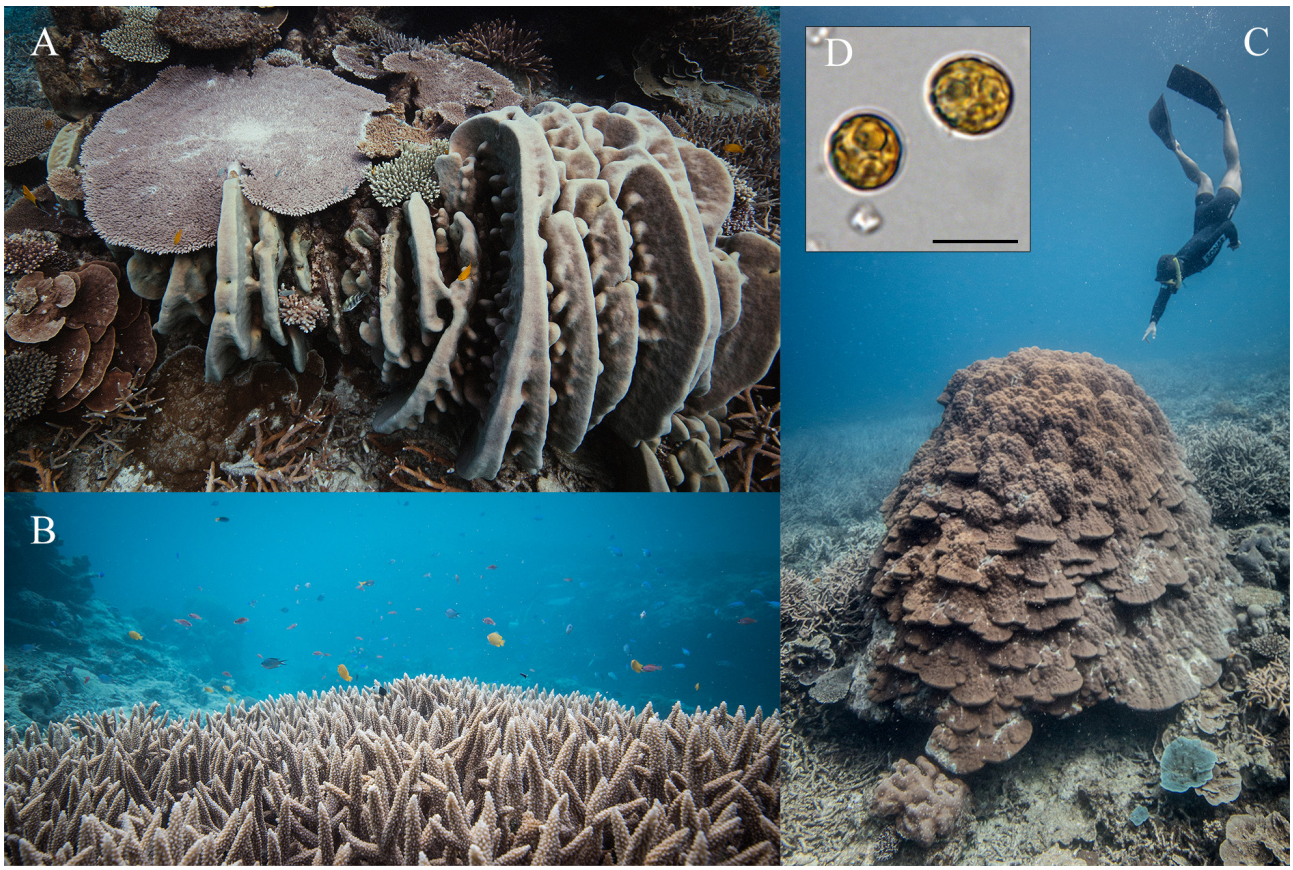

Figure 1.2 Scleractinian coral assemblages and *Symbiodinium* cells. (A) Multispecies assemblage of branching, tabulate and massive coral colonies; (B) an *Acropora* sp. colony with damselfish feeding above the branches; (C) a large *Porites* sp. colony with snorkeler for scale; and (D) *Symbiodinium* cells isolated from the coral *Acropora millepora*. Scale bar represents 10 µm.

number of mechanisms, such as: space limitation (Muscatine and Pool 1979; Jones and Yellowlees 1997); nitrogen limitation (Cook and D'elia 1987; Cook et al. 1994; Davy et al. 2006); phosphorous limitation (Jackson and Yellowlees 1990); and control of the *Symbiodinium* cell cycle (Smith and Muscatine 1999). Any further division in the symbiont population at this stage may be balanced through cellular processes, including the loss of *Symbiodinium* cells through auto-senescence and host cell apoptosis (Dunn and Weis 2009), and symbiont expulsion (Davy et al. 2012).

Symbiodinium cells are frequently removed or expelled from adult cnidarians through: thermal bleaching (Hill and Ralph 2007); expulsion of excess symbionts and algal pellets (Steele 1975; Hoegh-Guldberg et al. 1987; Stimson and Kinzie 1991); host cell detachment or exocytosis (Weis 2008); and tissue removal caused by physical abrasion or predation (Muller-Parker 1984; Castro-Sanguino and Sanchez 2012). Symbionts that are released from hosts can be photosynthetically active (Ralph et al. 2001; Ralph et al. 2005; Hill and Ralph 2007), yet we know little regarding how the different mechanisms of release affect the survival of *Symbiodinium* cells *ex hospite*. The released

cells potentially contribute to a reservoir of free-living *Symbiodinium* cells that are competent and establish symbioses with other hosts.

1.1.4 Free-living Symbiodinium

Cnidarian-*Symbiodinium* symbioses are relatively well studied. In contrast, empirical data for population dynamics, distribution, and physiology of free-living *Symbiodinium* are limited. 'Free-living' (FL) refers to either: A) living outside a host while retaining the facility of forming symbioses (Hirose et al. 2008), and this term includes 'transiently free-living' (TFL) cells recently expelled from their hosts that may not continuously persist in a free-living state (Yamashita and Koike 2013); or B) novel *Symbiodinium* types not currently known to engage in symbiosis that are permanently free-living (Takabayashi et al. 2012). These types are referred to as 'exclusively free-living' (EFL) hereafter. Although these EFL *Symbiodinium* types are not well understood, the ecology and physiology of free-living types that are ubiquitous in forming symbioses are of great interest.

There are a number of studies which have identified FL *Symbiodinium* spp. in the water column. Gou et al. (2003) isolated clade E (*S. voratum*) from the waters of Jiaozhou Bay in China. Coffroth et al. (2006) used aposymbiotic octocoral recruits from reefs in Florida (USA) as 'symbiont sampler arrays' to detect the presence of *Symbiodinium* in the water column. The clades represented in the plankton were A, B and C, present in 7%, 63% and 2% of the octocoral polyps (*Briareum* spp.) respectively. Coffroth et al. (2006) also demonstrated the initial uptake of multiple clades simultaneously, as 23% of polyps harboured clades A+B, and 5% B+C. Manning and Gates (2008) isolated *Symbiodinium* clades B, C, D, and H in seawater samples taken from above coral reefs in Kane'ohe Bay, (Oahu, Hawaii) and Puerto Morelos (Quintana Roo, Mexico). Novel types belonging to clades B and C were also uncovered in this instance. At present, it is unclear whether *Symbiodinium* cells in the water column are only temporarily in suspension or are perennial members of the phytoplankton. FL *Symbiodinium* cells are motile in culture (but are yet to be observed as motile cells *in situ*), and these examples may also represent observations of cells migrating vertically to encounter hosts.

Symbiodinium cells have also been isolated from benthic turf and macroalgae. Benthic macroalgae provide suitable habitats for free-living *Symbiodinium via* a number of physical and chemical attributes. First, they provide a large surface area and a three dimensional structure which attenuates light (Porto et al. 2008). Secondly, some macroalgae release organic compounds which stimulate microbial growth in the immediate surrounding area (Wada et al. 2007; Venera-Ponton et al. 2010) and these may facilitate the epiphytic growth of FL *Symbiodinium* cells. Studies conducted by Porto et al. (2008) and Venera-Ponton et al. (2010) demonstrated the presence of *Symbiodinium* cells on

a wide range of macroalgal species in the Caribbean (Colombia: *Lobophora variegata*; *Halimeda* spp.; *Amphiroa tribulus*) and the Pacific (Great Barrier Reef: *Laurencia intricata*; *Hypnea* spp.; *Asparagopsis taxiformis*; *Halimeda discoidea*; *Chlorodesmis fastigiata*; and *Lobophora variegata*). Venera-Ponton et al. (2010) identified macro-alga associated *Symbiodinium* types C3, C15, and C17, and these are known to form symbioses with many scleractinian corals. Although it is possible that these cells were recently ejected from nearby coral hosts and not truly epiphytically FL, the potential for a macroalgal-reservoir of *Symbiodinium* cells is worthy of further investigation.

The microphytobenthos (MPB) can be split into two general groups based upon their behaviour. 'Epipsammic' microalgae live attached to, or associated with sand grains, whereas 'epipelic' microalgae migrate vertically within the interstitial waters of sediment on a daily basis (Heil et al. 2004). A number of studies have now isolated FL *Symbiodinium* cells from sediments, however, *Symbiodinium* cells are not currently attributed to either of these categories. In culture they have been observed to clump and adhere to the glass in coccoid non-motile forms (Domotor and Delia 1986) and it is possible that they also adhere to sediment grains. Additionally, *Symbiodinium* cells have diel rhythms of motility where cells migrate vertically into culture media, and thus *Symbiodinium* cells represent an intermediary of the two general groupings.

Carlos et al. (1999) questioned whether benthic *Symbiodinium* cell isolates were actually from a permanently FL population of cells. This uncertainty arose from the possibility that many FL *Symbiodinium* cells represent recent ejections from nearby hosts. Due to the nature of *Symbiodinium* cells and the fact that they are inherently difficult to culture (Coffroth et al. 2006; Krueger and Gates 2012), studies which have attempted to identify FL types of benthic *Symbiodinium* cells *via* culturing are likely to underestimate the true diversity (Hirose et al. 2008; Reimer et al. 2010). Use of molecular techniques to isolate *Symbiodinium* cells directly from sediment samples has some success. Pochon et al. (2010) and Takabayashi et al. (2012) investigated the diversity of benthic FL *Symbiodinium* cells in Hawaii, and, surprisingly, reported only limited similarities between the FL population of cells and the local endosymbiotic types. For example, Pochon et al. (2010) did not find the locally dominant scleractinian endosymbionts (C1, C1d, C3, C15, and C31) in sediment (or water) samples. Takabayashi et al. (2012) also found low genetic diversity of FL *Symbiodinium* cells from clade C, which is otherwise known to contain many endosymbiotic types (Correa and Baker 2009). In contrast, FL isolates belonging to clade A from the same area were highly diverse in comparison with the endosymbiotic diversity known for clade A. This novel diversity may represent an ancestral guild of EFL *Symbiodinium* types (Correa and Baker 2009; Takabayashi et al. 2012). Hirose et al. (2008) and Reimer et al. (2010) also reported novel clade A types from sediment samples that may be EFL (Japan).

Studies of FL *Symbiodinium* are steadily increasing in number, however, we still know more about the genetic diversity than we do about the distribution and abundance of FL *Symbiodinium* cells. The only information regarding the abundance of FL *Symbiodinium* cells is the investigation by Littman et al. (2008). In this investigation, *Symbiodinium* cells were isolated from the calcium carbonate sediment of Lizard Island (Great Barrier Reef, Australia) at densities 15-fold greater than in the corresponding water column. Littman et al. (2008) observed variability in *Symbiodinium* cell density over spatial scales spanning eastern versus western reefs and reef flats versus reef margins. High densities were also found at the bases of coral colonies. The density range of *Symbiodinium* within the sediment was 1000-4000 cells/mL, and the principal conclusion of the study was that FL *Symbiodinium* cells are mainly demersal.

It is likely that the capacity to persist outside of hosts in a FL state varies significantly among *Symbiodinium* spp. There are numerous studies of *Symbiodinium* cells in culture, however the life history of *Symbiodinium* cells *in vitro* does not necessarily represent what is occurring in the environment. As mentioned, a substantial reservoir of FL *Symbiodinium* cells associates with the benthos, and the three dimensional structure of sediments differs dramatically from that experienced by microalgae in culture (and even within hosts). A holistic understanding of how *Symbiodinium* cells interact with microhabitats may prove useful, especially considering that the majority of cnidarian hosts must initially draw *Symbiodinium* cells from the surrounding environment.

1.2 Early life-history of scleractinian corals

1.2.1 Sexual reproduction

The order Scleractinia comprises a group of marine calcifying corals with a bipartite life cycle. The dominant phase of coral ontogeny is benthic, where individuals are either solitary or form colonies of interconnected polyps. A pelagic larval phase follows a sexual reproduction event, which is variable in mode and timing. Although corals can reproduce asexually (e.g. fragmentation, modular budding and rare asexual production of larvae), sexual reproduction is critical for the maintenance of coral populations through the dispersal of juveniles to suitable substrata and the continuation of genetic recombination. Of the 900+ species of hermatypic (reef building) scleractinian corals that are currently recognised (Veron 2000), information detailing sexual reproduction is available for approximately 444 species (Harrison 2011). Most scleractinian corals are either hermaphroditic or dioecious (gonochoric), however a number of atypical sexual systems have been documented (Fig. 1.3). Simultaneously hermaphroditic coral species develop ova and sperm within each polyp at the same time, whereas sequential hermaphrodites may split gamete development within breeding seasons,

or exhibit true sex change across breeding seasons (Harrison 2011). Hermaphroditism accounts for 70% of all scleractinian corals (for which reproduction data are available) and is therefore the dominant sexual system (Harrison 2011). In comparison with hermaphroditism, gonochoric species of coral which have truly separate sexes for the entirety of the life of the colony, are uncommon and account for approximately 26% of all scleractinian corals (Harrison 2011). Occasionally, gonochoric coral species are observed to have a low incidence of hermaphroditism. For example, gynodioecious corals (Fig. 1.3) have female and co-sexual (both male and female) polyps found on separate colonies (Chornesky and Peters 1987; Guest et al. 2012).

Not all combinations of potential sexual systems have been observed across scleractinian corals. For example, there are no corals that have been observed to be monoecious (male and female polyps within a single colony), however the coral *Diploastrea heliopora* is polygamomonoecious (Fig. 1.3), where male, female and cosexual polyps are found within single colonies (Guest et al. 2012). These rare combinations of sexuality (4% of all corals for which reproduction data are available) provide insight to the potential uncharacterised diversity of reproductive systems in the scleractinian corals, especially considering how many species are data deficient.

1.2.2 Developmental mode

Following the identification of the mass synchronised spawning of corals on the Great Barrier Reef (GBR) (Harrison et al. 1984; Babcock et al. 1986), spawning is now recognised as the most common mode of development across the scleractinian corals. Of the 428 coral species for which the mode of development has been recorded, 354 species (82.7%) are spawners (Harrison 2011). This mode of reproduction involves the release of gametes into the sea where fertilization and development occurs (Harrison 2011). The seasonal cue for the maturation of gametes is generally thought to be related to sea surface temperature (SST) (Harrison and Wallace 1990; Baird et al. 2009) and the night of spawning cued by the lunar cycle (Levy et al. 2007; Baird et al. 2009). Broadcast spawning dominates the families Acroporidae and Favidae for 150 and 76 species, respectively (Harrison 2011), and this is suggested to be the ancestral mode of reproduction (Shlesinger et al. 1998).

Some species of corals brood their embryos (‘brooders’) through to the larval stage of development where the offspring are competent (motile and ready for settlement) upon release. This mode of reproduction is suggested to be derived from spawning (Shlesinger et al. 1998). Some brooders also spawn sperm which potentially self-fertilize the eggs in the polyps of hermaphroditic brooders (Okubo et al. 2007) or outcross to other colonies of the same species (Miller and Ayre 2006). Of the coral species for which the mode of development has been recorded, 61 species (14.3%) are brooders

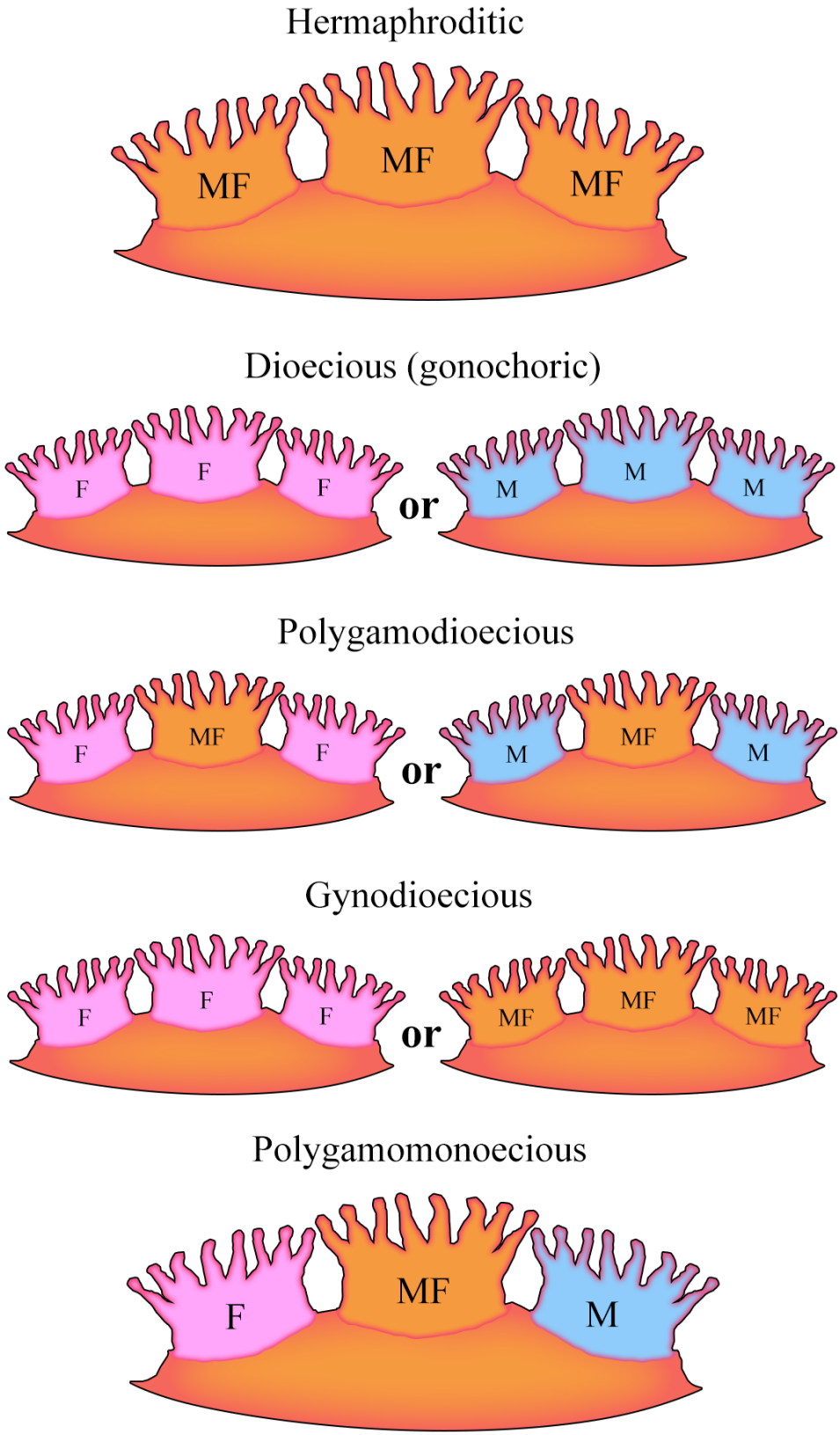

Figure 1.3 The observed sexual systems of scleractinian coral colonies. MF = simultaneous hermaphroditic polyps (orange); F = female only polyps (pink); and M = male only polyps (blue).

(Harrison 2011). Although most families in the order Scleractinia contain species that spawn and species that brood, the Pocilloporidae is the only family of corals in which brooding is more common than spawning (Harrison 2011). Additionally, there is evidence that at least 3% of coral species spawn gametes and brood larvae (Ward 1992; Sakai 1997), comprising a simultaneous mixture of the two main developmental modes.

1.2.3. Recruitment

The process of coral recruitment can be split into three general phases: A) dispersal during the planktonic stage; B) assessment of the benthic microhabitats for suitability as settlement substrate; and C) attachment to the benthos. Brooded larvae are usually competent and ready to attach to the substrate shortly or immediately after release (Harrison and Wallace 1990), whereas zygotes which form in the water column must spend between four and 10 days developing before becoming a mobile planula ready for settlement (Connolly and Baird 2010). There is high potential for the dispersal of larvae produced by both modes of reproduction. Brooded larvae from the coral *Pocillopora damicornis* can settle after as little as two hours, or after 103 days (Richmond 1987) and larvae from the broadcast spawning species *Acropora latistella, Favia pallida, Pectinia paeonia, Goniastrea aspera,* and *Montastraea magnistellata* are potentially viable in the water column for 150 days (Graham et al. 2008).

During the search for appropriate settlement habitats, coral planulae are exposed to cues which encourage metamorphosis. Crustose coralline algae (CCA) and the associated benthic microbial communities are known to provide settlement cues (Heyward and Negri 1999; Webster et al. 2004). Direct coral settlement onto the surface of CCA is common and more specifically, *Titanoderma* spp. are often the preferred settlement substrate (Harrington et al. 2004; Arnold et al. 2010; Doropoulos et al. 2012). For example, Harrington et al. (2004) demonstrated that larvae of *Acropora millepora* and *Acropora tenuis* preferred to settle on *Titanoderma prototypum* rather than five other microhabitats and this also resulted in high post-settlement survivorship. Doropoulos et al. (2012) also demonstrated that larvae of *Acropora millepora* preferred *Titanoderma* sp as a settlement microhabitat, however this pattern was severely disrupted by alterations in seawater chemistry, highlighting the sensitivity of the settlement process. Successful recruitment is further influenced by algal overgrowth and preclusion (Arnold et al. 2010), substrate depth and orientation (Babcock and Mundy 1996), light levels (Mundy and Babcock 1998) and sedimentation (Hodgson 1990b; Babcock and Davies 1991; Birrell et al. 2005). It is also during this process that many juvenile corals first encounter what will become their future complement of *Symbiodinium* cells.

1.3 Symbiont acquisition

1.3.1 Vertical transmission

The acquisition of *Symbiodinium* typically occurs during the early life-history of cnidarians. There are two pathways through which cnidarians can acquire their algal symbionts and these are largely dependent on reproductive mode (Richmond and Hunter 1990; Baird et al. 2009). The first is a host-to-host, parent to offspring pathway known as vertical transmission (Fig. 1.4). This occurs in 15-20% of all coral species for which data are available (Baird et al. 2009). This mode of symbiont transmission is more commonly observed in species that brood larvae than those which spawn (Baird et al. 2009). There is also a relatively small number of species of coral within the genera *Montipora, Anacropora, Pocillopora*, and *Porites* that produce eggs containing *Symbiodinium* cells prior to spawning. For *Montipora* spp. and *Pocillopora* spp., *Symbiodinium* cells are transferred to the oocytes five to seven days before spawning and are probably acquired through phagocytosis (Hirose et al. 2001).

The process of vertical transmission of *Symbiodinium* has previously been described as a 'closed system' (Karako et al. 2002; Van Oppen 2004; Stambler 2011), in that the *in hospite Symbiodinium* populations are reflective of previous generations. Indeed, Padilla-Gamiño et al. (2012) found this to be true of *Montipora capitata,* where the *Symbiodinium* types recovered from symbiotic eggs were represented in the adult colonies. It is thought that vertical transmission results in a transgenerational symbiosis with extended periods of stability (Thornhill et al. 2006a). This is theorized to be advantageous for recruits, given that they begin their lives with a symbiont that is adapted to the local physical environment. Exclusive symbioses that remain closed, however, potentially lack the ability to adapt to new locations and physical conditions by forming novel partnerships with more suitable *Symbiodinium* types (Byler et al. 2013; Starzak et al. 2014). Recent evidence suggests, however, that multiple symbiont acquisition strategies may be used by scleractinian coral species. Byler et al. (2013) reported depth-partitioning in the brooding coral *Stylophora pistillata*/*Symbiodinium* symbiosis. Analysis of *Symbiodinium* cells in the planulae released by corals from shallow and deep habitats reflected the types found in the parental colony (i.e. the source). Juvenile colonies sampled from the same shallow and deep habitats were found to harbour a mixture of types (differing from the adult colonies and planulae), suggesting that horizontal acquisition of *Symbiodinium* cells*,* most likely from the surrounding environment, had occurred following settlement (Byler et al. 2013). It is not known to what extent this occurs in other species or regions, however this study suggests that horizontal acquisition may be more widespread among coral taxa than previously recognised.

1.3.2 Horizontal transmission

Drawing *Symbiodinium* cells from the surrounding environment is the prevalent mode of symbiont acquisition and is observed in 80-85% of scleractinian coral species (Baird et al. 2009). Juvenile cnidarians will begin to phagocytose the algal cells following the development of the oral pore and coelenteron (Harii et al. 2009) and appear to be non-specific in regards to the type of *Symbiodinium* they ingest (Little et al. 2004; Harii et al. 2009). Investigations have largely focused on cellular responses to infection and it is thought that the successful acquisition of *Symbiodinium* is dependent on the compatibility of the host and the symbiont (Belda-Baillie et al. 2002). 'Winnowing' (Nyholm and McFall-Ngai 2004) describes the range of step-by-step cellular mechanisms that will eventually lead to: A) continuation of the symbiosis; or B) the selective removal of incompatible *Symbiodinium* cells from the host tissue. During the horizontal acquisition of *Symbiodinium* cells, cnidarians acquire symbionts through phagocytosis, resulting in a host-derived intracellular vesicle containing *Symbiodinium* cells. In view of the fact that most items that are phagocytosed are soon to become products of digestion (i.e. fusion with lysosomal vesicles), an interesting question arises as to how *Symbiodinium* cells manage to avoid this fate.

There is some evidence for pre-phagocytic selection of *Symbiodinium* cells, such as *via* cell-to-cell surface identification proteins. For example, glycoproteins, which have been observed on the outer surface of *Symbiodinium* cells (Markell et al. 1992; Logan et al. 2010; Bay et al. 2011), might interact with lectins (sugar-binding proteins) in a "lock and key" interaction (Jimbo et al. 2000; Wood-Charlson et al. 2006). Markell et al. (1992) also detected the release of water soluble polypeptides/glycoproteins from *Symbiodinium* cells in culture, suggesting the possibility of an extracellular signalling pathway between host and symbiont. Post-phagocytic winnowing mechanisms of *Symbiodinium* cells are also evident. The vesicles containing recently phagocytosed, viable *Symbiodinium* cells show none of the characteristic features of lysosomal fusion (Fitt and Trench 1983a; Chen et al. 2004) and it appears that *Symbiodinium* cells can interfere with vesicle trafficking within the host and the continuation of the normal phagosomal pathway (Chen et al. 2003; Chen et al. 2004). Through multiple infections, intra-colony populations of *Symbiodinium* cells in corals may comprise a dominant clade (by proportion) and one or more cryptic clades (Mieog et al. 2007; Silverstein et al. 2012). It has been suggested that the establishment of one *Symbiodinium* type as the dominant clade *in hospite* is in part related to competition between types. For example, Thornhill et al. (2006b) demonstrated that aposymbiotic jellyfish (*Cassiopea xamachana*) could initially acquire as many as four clades of *Symbiodinium*. This is at odds with naturally occurring individuals from the same area (Florida Bay), which are without exception, always found to harbour a single *Symbiodinium* clade. The authors suggested that this is the result of competitive exclusion by the dominant symbiont type.

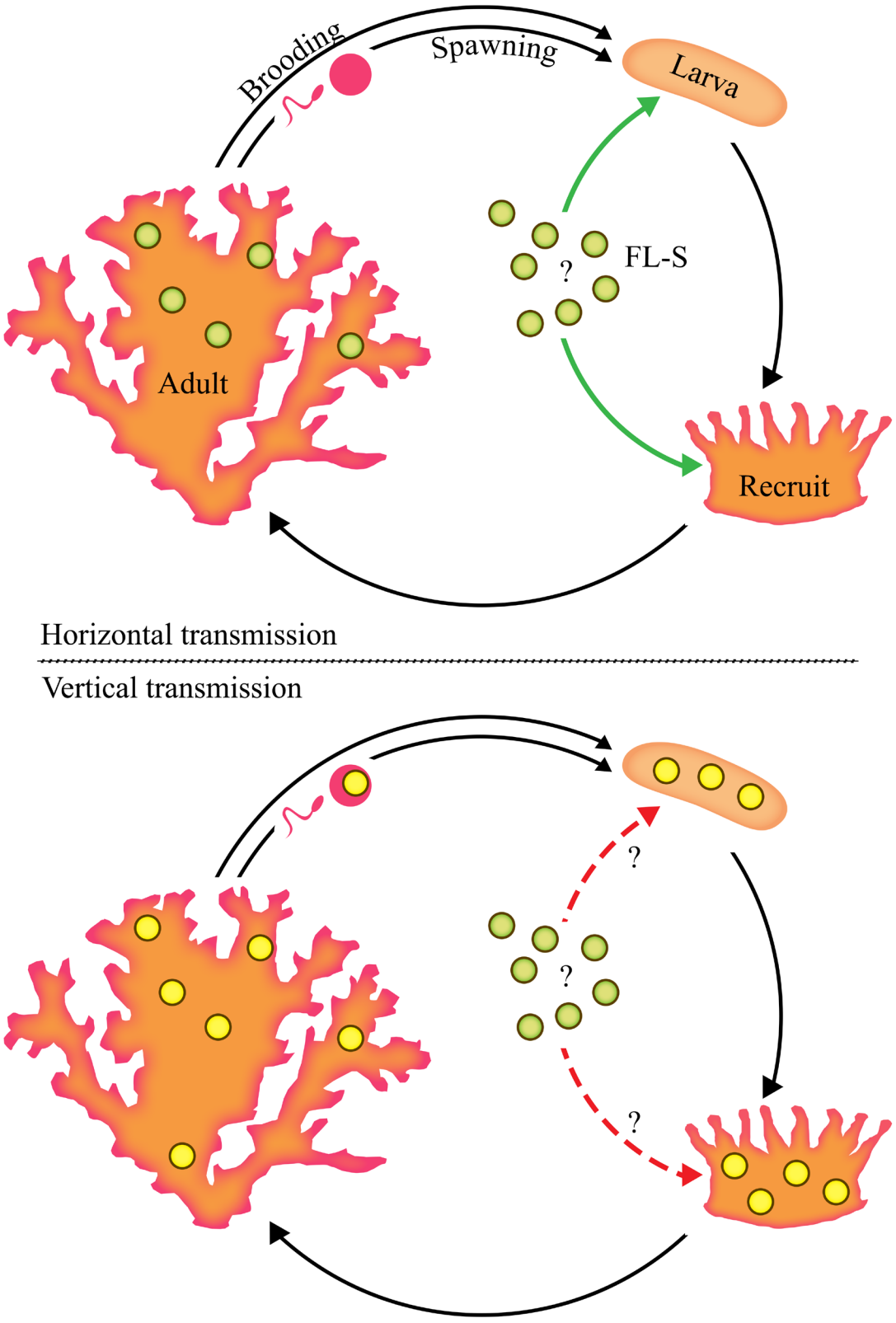

Figure 1.4 The modes of symbiont acquisition in scleractinian corals (also applies to many other cnidarians). Black arrows represent the coral life history. Horizontal transmission (upper diagram) requires the acquisition (green arrows) of free-living *Symbiodinium* (FL-S, green circles) from the surrounding environment by larvae or recruits. Vertical transmission (lower diagram) involves the passing of *Symbiodinium* cells (yellow circles) from the adult to the oocytes (pink circle). It is suggested, but unknown (?), if horizontal acquisition (red hashed arrows) also occurs in addition to maternal transmission.

Host species can discriminate between different types of *Symbiodinium* cells once they have become intracellular. For example, Rodriguez-Lanetty et al. (2004) reported that diversity at the intra-cladal level influences the strength of the symbiotic association following experimental infections with homologous and heterologous *Symbiodinium* cells of clade C. Dunn and Weis (2009) also documented the activation of caspases (enzymes involved in programmed cell death) when coral larvae (*Fungia scutaria*) were infected with a heterologous type of *Symbiodinium*. Dunn and Weis (2009) noted that after inhibiting caspase activity of the host, the heterologous symbiont density did not significantly decrease through time as it did in the controls. Intracellular symbioses fall onto a mutualism - parasitism continuum, and indeed some types of *Symbiodinium* are thought to exhibit parasitic traits (Lesser et al. 2013; Starzak et al. 2014). An innate ability to select between *Symbiodinium* types as they are encountered in the environment will afford mutualism, rather than indiscriminate infections and a potential parasitism of the host. Contrasting with vertical transmission, it is possible that recruiting corals encounter different symbiont types from those in their parents, creating new trans-generational, host-symbiont combinations (Richmond and Hunter 1990), however, little is known regarding where FL *Symbiodinium* cells occur, when they are first encountered, and whether symbiont acquisition is density dependent.

For those species that must acquire their symbionts from the environment, the availability of a FL reservoir of *Symbiodinium* is inevitably a crucial component of colony survival and maturation. Studies have demonstrated that cnidarians can acquire *Symbiodinium* cells from the water column (Coffroth et al. 2006) and sediment (Adams et al. 2009; Cumbo et al. 2013), although as previously mentioned, it is not known whether FL *Symbiodinium* cells are active members of the phytoplankton or the MPB, or if they are simply TFL cells recently expelled from hosts. Abrego et al. (2009b) demonstrated that some *Symbiodinium* types may be opportunistic and have a high degree of infectiousness. Despite relatively low abundance in the coral assemblages of experimental study sites (Magnetic Island, Orpheus Island and Davies Reef, GBR), clade D *Symbiodinium* was the main symbiont type initially acquired by juveniles of *Acropora millepora* and *Acropora tenuis* (Abrego et al. 2009b). This suggests that there may be different host-locating abilities or abundances of FL *Symbiodinium* types. The physiology and ecology of FL *Symbiodinium* cells and the early-life history of many cnidarian species are inextricably linked. As the recruitment of juvenile corals is a crucial component of reef resilience (Bellwood et al. 2004), an understanding of the process of symbiont acquisition is required to develop comprehensive metrics of coral reef health. Indeed, the distribution and physiology of FL *Symbiodinium* cells may prove to be a new aspect of coral reef health worthy of monitoring (Manning and Gates 2008).

1.4 Thesis Aims

The main aim of this thesis was to use experimental manipulations of free-living *Symbiodinium* cells to characterise the connections between known microhabitats of FL *Symbiodinium* cells and scleractinian coral hosts. Specifically, I aimed to determine:

Whether adult scleractinian corals contribute to the standing stock of free-living *Symbiodinium* cells in coral reef sediments (**chapters 2, 3, and 5**);

Whether *Symbiodinium* cells released into calcium carbonate reef sediment from coral hosts are viable and persist as FL cells (**chapter 3**); and

If exclusively free-living *Symbiodinium* types are physiologically better suited to benthic microhabitats than those that form endosymbiosis (**chapter 4**);

Whether free-living *Symbiodinium* cells find refuge in light-reduced interstitial spaces of the sediment during stress (**chapter 4**);

Whether juvenile corals acquire symbionts from microhabitats seeded with free-living *Symbiodinium* cells (**chapter 5**).

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Chapter 2: Free-living *Symbiodinium* cells at the base of translocated scleractinian coral colonies

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2.1 Abstract

Many juvenile cnidarians (including reef-building corals, jellyfish and sea-anemones) acquire intracellular symbionts from the environment *via* a process known as horizontal transmission. The varying distributions of these free-living symbionts (dinoflagellates in the genus *Symbiodinium*) may therefore be of importance during the establishment of symbiosis. Free-living *Symbiodinium* have recently been identified in reef sediment, and we explore whether adult corals seed the benthos with *Symbiodinium* cells. We hypothesized that a relatively high abundance of free-living *Symbiodinium* cells would be detected in sediments in close proximity to coral colonies. Three permanent sites of expansive bare sediment were identified on the reef flat of Heron Reef (Great Barrier Reef, Australia). At each site the surficial carbonate sediment and interstitial water was sampled *via* coring in three phases: 1) a 'baseline' phase of sampling, 2) a 'seeding' phase, following the translocation of an *A. millepora* colony to the centre of the site and 3) an 'after' phase following the returning of the site to its baseline state of bare sand. The sediment was filtered and *Symbiodinium* cells from the interstitial water manually counted. From baseline samples, a background population of 1.88 to 7.03 × 10^3 cells/mL was detected, suggesting that free-living *Symbiodinium* are widespread in coral reef sediments. Following the translocation of *A. millepora* to the centre of each site, up to an 8 fold increase in the abundance of benthic *Symbiodinium* cells was detected in sediment sampled at the immediate basal area, and periodically, at up to 25 cm from the colony. There were no detectable seasonal differences and the amplified abundance was consistent throughout the seeding period of the study. Following the removal of the coral colonies the abundances of benthic *Symbiodinium* returned to baseline levels. These results suggest that recruits that draw their symbionts from the environment (especially those with symbiont type specificity) may benefit from the presence of a conspecific adult, and the consequent exposure to a relatively larger pool of prospective symbionts.

2.2 Introduction

The genus *Symbiodinium* comprises a group of marine dinoflagellates that occur as intracellular symbionts or free-living in the environment. The formal description of *Symbiodinium* species is currently advancing (LaJeunesse et al. 2012; LaJeunesse et al. 2014) and nine 'clades' (A-I and numerous 'sub-clades' or 'types' within each clade) have also been delineated (Pochon and Gates 2010). While engaged in symbiosis, *Symbiodinium* can potentially meet the energy demands of its host through the translocation of photosynthetically-fixed carbon (Falkowski et al. 1984; Muscatine 1990). Ultimately, this process facilitates the formation of scleractinian coral reefs and the cnidarian/*Symbiodinium* symbiosis has therefore been relatively well-studied. In contrast, empirical data for the population dynamics, distribution, and physiology of free-living *Symbiodinium* are limited. A holistic understanding of how *Symbiodinium* cells interact with both the host (*in hospite*) and environment (*ex hospite*) is required, especially as the juveniles of many host species initially acquire their symbionts from the environment (Richmond and Hunter 1990; Schwarz et al. 2002; Barneah et al. 2004; Thornhill et al. 2006; Baird et al. 2009).

The microscopic algae and cyanobacteria that live on the seafloor are collectively the 'microphytobenthos' (MPB) (MacIntyre et al. 1996). In marine sediment, the MPB functions in a number of physical and biological roles, including: sediment stabilisation (Yallop et al. 1994); significant generation of primary productivity (Werner et al. 2008); acting as an important source of food for macrofauna (Miller et al. 1996); and enabling nutrient cycling between the sediment and water (MacIntyre et al. 1996; Sundbäck et al. 2000). Sediment can also serve as a holding habitat and reservoir for the resting (or dormant) stages of benthic microalgae, especially for dinoflagellates (Dale 1976; Baldwin 1987; Lewis et al. 1999). A number of studies have now identified free-living *Symbiodinium* in the sediment as a constituent of the MPB (Carlos et al. 1999; Hirose et al. 2008; Littman et al. 2008; Reimer et al. 2010; Takabayashi et al. 2012; Yamashita and Koike 2013). Additionally, symbiont acquisition during exposure to sediment was reported by Adams et al. (2009) and Cumbo et al. (2013), demonstrating that *Symbiodinium* cells are present in the sediment and are capable of forming symbioses with corals. It therefore follows that there is a need to explore the subtleties which affect symbiont acquisition, such as the fine-scale density and distribution of benthic free-living *Symbiodinium* cells.

During the development of methods for quantifying free-living *Symbiodinium*, Littman et al. (2008) reported *Symbiodinium* cells in reef sediment in abundances 15-fold greater than in the water column

(Lizard Island, Great Barrier Reef, Australia), suggesting that free-living cells are mainly demersal. Littman et al. (2008) further noted that high densities of free-living *Symbiodinium* cells were present in the sediment near the base of coral colonies. It is currently unknown if *Symbiodinium* cells isolated from the environment are only 'transiently free-living', representing recently expelled cells from a nearby host species (Yamashita and Koike 2013). Other possibilities include 'continuously free-living' populations of cells that are viable after release from hosts, or potentially even 'exclusively free-living' cells, representing *Symbiodinium* types living solely outside of hosts (LaJeunesse 2002; Takabayashi et al. 2012; Yamashita and Koike 2013; Nitschke et al. 2015). Investigating the dynamics and potential source populations of free-living *Symbiodinium* is required to begin differentiating between these categories. Spatial heterogeneity of free-living *Symbiodinium* has been suggested (Littman et al. 2008; Takabayashi et al. 2012), yet the potential drivers of the distribution of free-living *Symbiodinium* cells remain unexplored.

Within the endodermal tissue of scleractinian coral, *Symbiodinium* cells can reach densities exceeding 2 x 10^6 cells cm^{-2} (Fitt et al. 2000). *Symbiodinium* can also reach high densities in other hosts, including sea anemones (Venn et al. 2008) and jellyfish (Estes et al. 2003), reaching up to 6×10^6 cells mg $protein^{-1}$ and 2 x 10^6 cells g $tissue^{-1}$ respectively. Large quantities of these symbiotic *Symbiodinium* cells are released by hosts continually. For example, the scleractinian coral *Pocillopora damicornis* released up to 1000 cells cm^{-2} hr^{-1} during ambient conditions (Stimson and Kinzie 1991), and up to 36.66×10^5 cells cm^{-2} hr^{-1} during conditions of thermal stress (Stimson et al. 2002). Although expelled cells are competent and photosynthetically active (Ralph et al. 2001; Ralph et al. 2005; Hill and Ralph 2007), the definitive survival of these cells *in situ* is unknown. *Symbiodinium* cells are arrested in a non-motile vegetative cyst state while *in symbio* (although the basal bodies of flagella have been observed in *Symbiodinium* cells within endodermal cells, see Schoenberg and Trench (1980)), and it is likely that these cells act as passive particles upon release. During calm conditions (e.g. low wave or current energy), microalgae can sink to the bottom and settle, and given the right physical environment, become incorporated into the microphytobenthos (MacIntyre et al. 1996). It is thus possible that the non-motile *Symbiodinium* cells released by corals are continuously supplementing benthic populations of free-living *Symbiodinium* cells on coral reefs.

The purpose of this study was to investigate the possibility that adult scleractinian coral hosts contribute to the standing stock of free-living *Symbiodinium* cells in the calcium carbonate sediments of a coral reef. We aimed to detect changes in the abundance of *Symbiodinium* cells in the sediment in relation

to the proximity of coral colonies and the presence or absence of coral colonies. Our hypotheses were: A) the greatest abundance of benthic *Symbiodinium* cells would be found at the immediate basal area of coral colonies; and B) after the removal of coral colonies the abundance of *Symbiodinium* cells would decrease. An alternative hypothesis to the seeding of free-living *Symbiodinium* cells is that, C) a positive chemotaxis of free-living *Symbiodinium* cells to coral colonies may occur, and this will be addressed through the experimental design and method development. This work may provide evidence that adult corals are an important source of free-living *Symbiodinium* cells.

2.3 Materials and methods

2.3.1 Study area and permanent site selection

Heron Reef is located approximately 70 km offshore from Gladstone (Queensland, Australia), situated in the Mackay/Capricorn management area of the Great Barrier Reef Marine Park. The elevated reef has a maximum length and width of approximately 9.6 km and 4.5 km respectively, with an acute western end containing a vegetated coral cay (Maxwell et al. 1964). Three permanent sites on the reef-flat of Heron Reef were identified based upon two criteria: A) from the centre of each site there was uninterrupted bare sand (containing no scleractinian coral or coral rubble/skeleton) spanned at least 10 m in every direction, and B) the sites were within 100 m of each other.

An extensive 'sandy corridor' on the reef-flat approximately 300 m SE of the vegetated cay was selected. The GPS coordinates were 23.26'49.1" S - 151.55'00.7" E, 23.26'50.1" S - 151.55'02.1" E and 23.26'51.2" S - 151.55'03.6" E for sites one (S1), two (S2) and three (S3) respectively.

2.3.2 Experimental design

Each site was investigated for the presence of '*Symbiodinium*-like' dinoflagellates associated with the benthos in three phases; 1) a 'baseline' phase of sampling, 2) a 'seeding' phase, following the translocation of a coral colony to the centre of each site and 3) an 'after' phase following the returning of the site to its baseline state of bare sand.

The baseline sediment samples (method of collection described below) were collected on the

1st, 2nd and 3rd of April, 2012, for S1, S2 and S3 respectively. Three *Acropora millepora* colonies (approximately 30 × 30 × 25 cm) were selected from the surrounding reef flat and translocated, one to the centre of each site, immediately following the collection of the baseline samples. *Acropora millepora* has a seasonal pattern of symbiont density (Moothien Pillay et al. 2005) and each site was therefore sampled three times during the seeding phase in order to detect any temporal variation in the seeding of benthic *Symbiodinium*. The seeding-phase samples were collected on the 25th-27th of June, 2012 (winter), the second on the 21st-23rd of November, 2012 (summer) and the third on the 23rd – 25th of June, 2013 (winter). *Acropora millepora* colonies were removed from each site immediately following the final seeding-phase sample collection on the 25th of June, 2013. A final set of samples was collected on the 4th – 6th of October, 2013 (spring) in the absence of *Acropora millepora* colonies.

2.3.3 Sediment collection and processing

Sediment samples were collected from each site and processed (on the same day as collection), using identical methodology at each time-point. A transect was marked from the centre of the site (or directly at the base of the coral colony), extending 200 cm. At 0 (base), 25, 50, 100 and 200 cm, sediment samples were collected *via* coring. Coring was carried out by inserting a PVC tube of known volume into the benthos, enclosing both the sediment and surface water (200 mL water plus 50 mL sediment). The corer was sealed at the top to create an upwards suction force while pulling the container and sample from the water. The end of the tube was rapidly capped to recover the sample and then transferred to a processing container (Littman et al. 2008). Two cores were taken at each distance, one from either side of the transect, and combined in the processing container to create a sample total of 400 mL water and 100 mL sediment. A sixth sample was taken at 500 cm from the site centre as a 'control' to compare within-site changes in the abundance of free-living *Symbiodinium* cells. This sampling design was replicated four times at each site, with each replicate transect rotating 90° in aspect from the previous to give N = 4 samples per distance increment and a total of 24 samples per site at each time point. All samples were collected at low tide and as close as possible to mid-day to avoid any confounding factors of MPB diel vertical migration.

The samples were immediately transported to Heron Island Research Station (The University of Queensland, Australia) for processing. The overall coarse composition of the carbonate sediment (Supplementary Fig.2.3) allowed for the use of a sieve tower to separate the sediment into a range of size fractions and the clearing of microphytobenthic algae from the interstitial spaces. Stainless

steel, woven-wire sieves (Endecott, London, UK) with mesh sizes of 2000, 1000, 500, 250, 125 and 63 μm were sequentially combined and the sediment sample added to the top. The interstitial water was allowed to drain through the sieve array and collect in a reservoir. The sediment retained by each mesh was thoroughly washed with 0.22 μm filtered seawater (FSW) in a step-wise manner until each mesh had been rinsed and the reservoir contained 1 L of diluted interstitial water (an addition of 600 mL of FSW). A sub-sample of 200 mL was taken from the reservoir and concentrated through centrifugation at 3950 rpm (3000 g) for 5 min to create a pellet of all the benthic algae removed from the sediment. The supernatant was decanted and the pellet re-suspended in 1 mL of FSW and each sample was preserved with 4% formalin for counting at a later date.

2.3.4 Quantification of Symbiodinium *cells*

A library of *Symbiodinium* cell images was generated and used as a reference identification guide. *Symbiodinium* spp. are known to vary in size (LaJeunesse et al. 2014) and therefore cell characteristics of symbionts from all three colonies were investigated to ensure that what was counted in the sediment was comparable to the potential source population. To do this, a single branch (5 cm in length) from each source colony was removed and the tissue blasted with pressurized FSW. The resulting tissue slurry was centrifuged (3950 rpm for 5 min) to separate symbiotic *Symbiodinium* cells from the host tissue. The concentrated pellet was resuspended in 2 mL of FSW and prepared for imaging with an Olympus BX51 microscope mounted with a calibrated Olympus DP71 camera. A total of 20 *Symbiodinium* cells from each colony were imaged for analysis of cell size. Length and width of each cell were measured using the image analysis software Image J (version 1.46r).

Symbiodinium cells may degrade (both visually and physiologically) once expelled from a coral host (Bhagooli and Hidaka 2004; Hill and Ralph 2007). To further supplement the reference library, changes in cell volume following isolation from *A. millepora* were analysed with non-linear regression. Cell volume was calculated on the basis of the dimensions of an ellipsoid, where volume = (4/3)πabc, (a, b, and c are equal to half the length, width, and height). It was assumed that cell height was identical to cell width, as in LaJeunesse et al. (2014). A four parameter sigmoidal model was used to interpolate at which point the cell volume declined by half (Magnusson et al. 2010): Y=Min + (Max-Min)/(1+10^((LogIC50-X)*Slope)), and it was assumed that *Symbiodinium* cells that fall below this volume would be unidentifiable in samples from the environment. *Symbiodinium* cells isolated from *A. millepora* were incubated in seawater for seven days. A branch of *A. millepora* (5 cm, taken from

the *A. millepora* colony at S1) was transferred to a 50 mL tube with sterile (autoclaved at 134°C for 20 min) FSW (SFSW) and *Symbiodinium* cells were removed from coral tissues through light abrasion. The resulting tissue slurry was homogenised for 2 min and then centrifuged (3950 rpm for 5 min) to separate *Symbiodinium* cells from the host tissue. The supernatant was decanted and the concentrated pellet was resuspended in 50 mL of SFSW. The above centrifugation process was repeated three times to remove as much coral tissue and mucus as possible. Finally, the pellet was again resuspended in SFSW and diluted to approximately 500 cells/mL. The diluted cell suspension was then divided into seven 50 mL, clear tubes sealed with parafilm. Each tube was transferred to an outdoor water bath with circulating water at ambient temperature and covered with 50% shade cloth. At 24 h increments, a single tube was removed and the cells concentrated (as above) into a pellet. After resuspension in 1 mL of SFSW, the cells were imaged (as above), and the volumes of 20 cells were calculated. Note that no changes in cell morphology indicative of a transition into a gymnodinoid motile-phase (as is commonly observed in *Symbiodinium* cultures) was observed during cell imaging.

To quantify the density of *Symbiodinium* cells in the interstitial water retrieved from sediment samples, the conventional plankton identification method of light microscopy (as described in Littman et al. (2008) for free-living *Symbiodinium* quantification) was used. The number of *Symbiodinium* cells in each sample was quantified using an Improved Neubauer haemocytometer (Boeco, Hamburg, Germany) for counting (N = 8 counts) under a high powered light microscope, using the reference guide and the major cell characteristics of *Symbiodinium,* including: a golden brown colour, cell shape, reticulated chloroplast and large accumulation body.

2.3.5 Statistical analyses

Cell size was analysed with two-way ANOVA with colony (three levels) and diameter (two levels) as the interacting factors. Two-way ANOVA with repeated measures was used to investigate the interactions between site (three levels) and distance from colony (six levels) in determining free-living *Symbiodinium* abundance at each time point, with Dunnett's multiple comparisons test to locate significant differences from the control distances and changes from the baseline abundances. The Shapiro-Wilk normality test, Levene's homogeneity of variance test, and Epsilons deviation from sphericity test were performed on all data to ensure that the assumptions of the analyses were met. Graphpad Prism (version 6.02) was used to perform all analyses and all results presented were tested against an alpha value of 0.05.

2.4 Results

2.4.1 Symbiodinium *cell characteristics*

There were no differences in average cell size between the three coral colonies (Fig. 2.1, Supplementary Fig. 2.1) for the freshly isolated *Symbiodinium* cells. *Symbiodinium* cells were not spherical (as has been previously assumed) and a range of physical proportions were recorded, the smallest being 8.40 × 7.751 µm, and the largest 11.275 × 9.707 µm (length × width) (Table 2.1, Supplementary Fig. 2.1). During the incubation experiment, cell volume was found to decline by 50% after 3.76 d (supplementary Fig. 2.1, four parameter sigmoidal model, $R^2 = 0.805$). After 3 days of incubation in seawater, the characteristic accumulation body was no longer visible (Fig. 2.2) and the cell contents underwent apoptotic-like compartmentalisation and were thus considered visually unidentifiable as *Symbiodinium* from this point onwards.

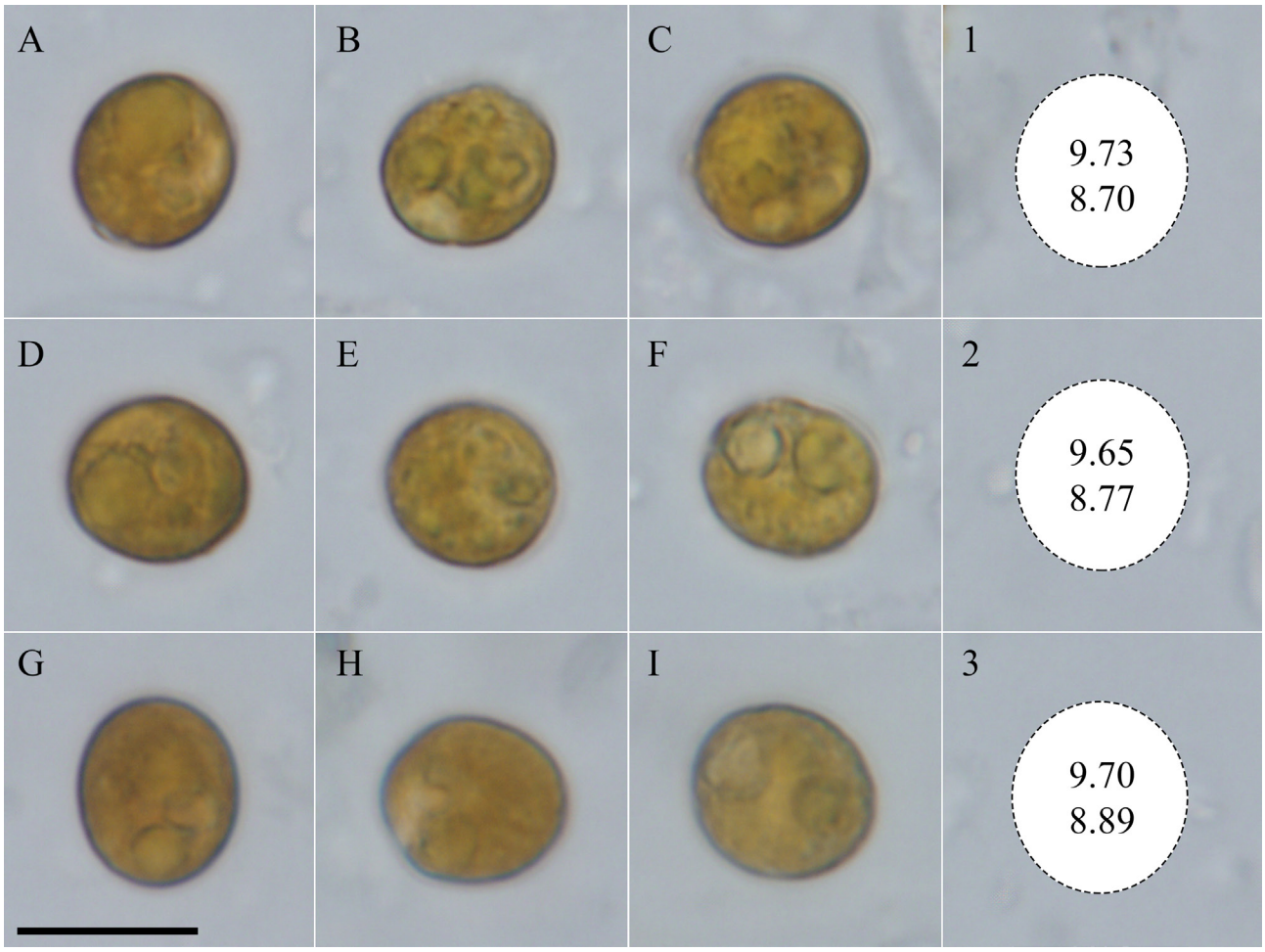

Figure 2.1 Light micrographs (Olympus BX51 microscope) of freshly isolated *Symbiodinium* cells from *Acropora millepora* colonies at Site 1 (A-C), Site 2 (D-F) and Site 3 (G-I). Values in panels 1-3 represent mean lengths and widths (N=20) of cells. Scale bar (black) = 10 µm.

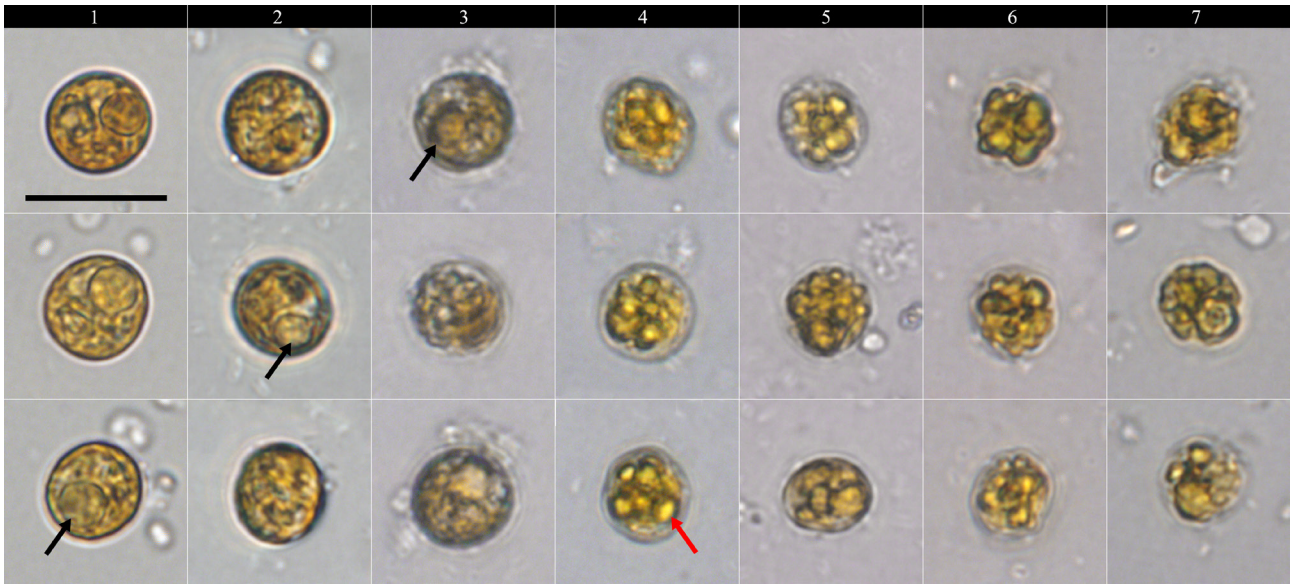

Figure 2.2 Light micrographs (Olympus BX51 microscope) of *Symbiodinium* cells from an *Acropora millepora* branch incubated in FSW. Each column contains three representative cells from the specified day. Black arrows indicate the characteristic accumulation body of coccoid *Symbiodinium* cells. Red arrows indicate membrane bound apoptotic-like bodies. Scale bar (black) = 10 µm.

Table 2.1 Statistical output of two-way ANOVA with aspect ratio (cell length and width) and site as factors. Test p-values presented that are below the alpha value of 0.05 are considered statistically significant. Note: The significant main effect of aspect ratio confirms that cell lengths and widths are significantly different.

Source of Variation	F (df)	P value
Aspect Ratio × Site	F (2, 114) = 0.1988	P = 0.8200
Aspect Ratio	F (1, 114) = 48.89	P < 0.0001
Site	F (2, 114) = 0.4928	P = 0.6122

2.4.2 Between-site effects

A significant interaction between time × site is apparent for between-site differences in *Symbiodinium* cells mL^{-1} (Table 2.2, Two-way ANOVA, $F_{(70, 252)} = 9.115$, $P < 0.0001$), however, the pattern of *Symbiodinium* seeding was similar across all three sites (Fig. 2.3). While the *post-hoc* tests for differences between sites do indeed show a number of significant comparisons, this is driven by the permutations of the test which have limited ecological value. For example, the comparisons of *Symbiodinium* cells mL^{-1} at S1 from a distance of 200 cm from the colony, to *Symbiodinium* cells mL^{-1} of S3 at 25 cm from the colony, there is a significant difference during the sampling in November 2012.

For the densities of *Symbiodinium* cells mL^{-1} between sites at the equidistant increments (assumed to be the relevant approach when investigating the coral colony as the source) there were no significant differences between sites (i.e. at the base of the coral colony, 0 cm, S1 = S2 = S3; at 25 cm, S1 = S2 = S3; etc). These similarities between sites are consistent through time and therefore the within-site changes in *Symbiodinium* cell density are of greater significance.

2.4.3 Within-site effects

A distinct change in *Symbiodinium* cell abundance was observed at each site (Fig 2.3), and a significant interaction between time × distance was apparent (Table 2.2, Two-way ANOVA, $F_{(70, 210)} = 10.35$, $P < 0.0001$).

Baseline period

During the baseline sampling period in April 2012, a background or 'ambient' population of *Symbiodinium* cells was visible in all sediment samples across all three sites (Fig. 2.3). During this period, the within-site abundances of cells varied widely, yet there were no significant or clear patterns in existing *Symbiodinium* distributions. Abundances ranged from 1.88×10^3 ($\pm 8.87 \times 10^2$) cells/mL to 7.03×10^3 ($\pm 1.17 \times 10^3$) cells/mL (values represent means ± SE) across all sites.

Seeding period

Approximately two months following the translocation of coral colonies to the centre of each site (June 2012), significant differences from the baseline period were observed. At the base of coral colonies (0 cm) 2.72×10^4 ($\pm 2.34 \times 10^3$), 2.91×10^4 ($\pm 1.59 \times 10^3$) and 2.44×10^4 ($\pm 8.84 \times 10^2$) cells/mL were enumerated from sediment samples for S1, S2 and S3 respectively. This represents increases of 483, 885, and 742% relative to the baseline period. Within each site, sediment at the base of the coral colony had significantly greater densities of *Symbiodinium* than at control distances of 500 cm (Dunnett's multiple comparisons test, p values < 0.001 for all comparisons) and these were unchanged relative to control distances during the baseline period. At 25 cm from the colony, only S1 and S2 recorded significant differences relative to the control distance, recording increases of 193% and 263% relative to baseline abundances.

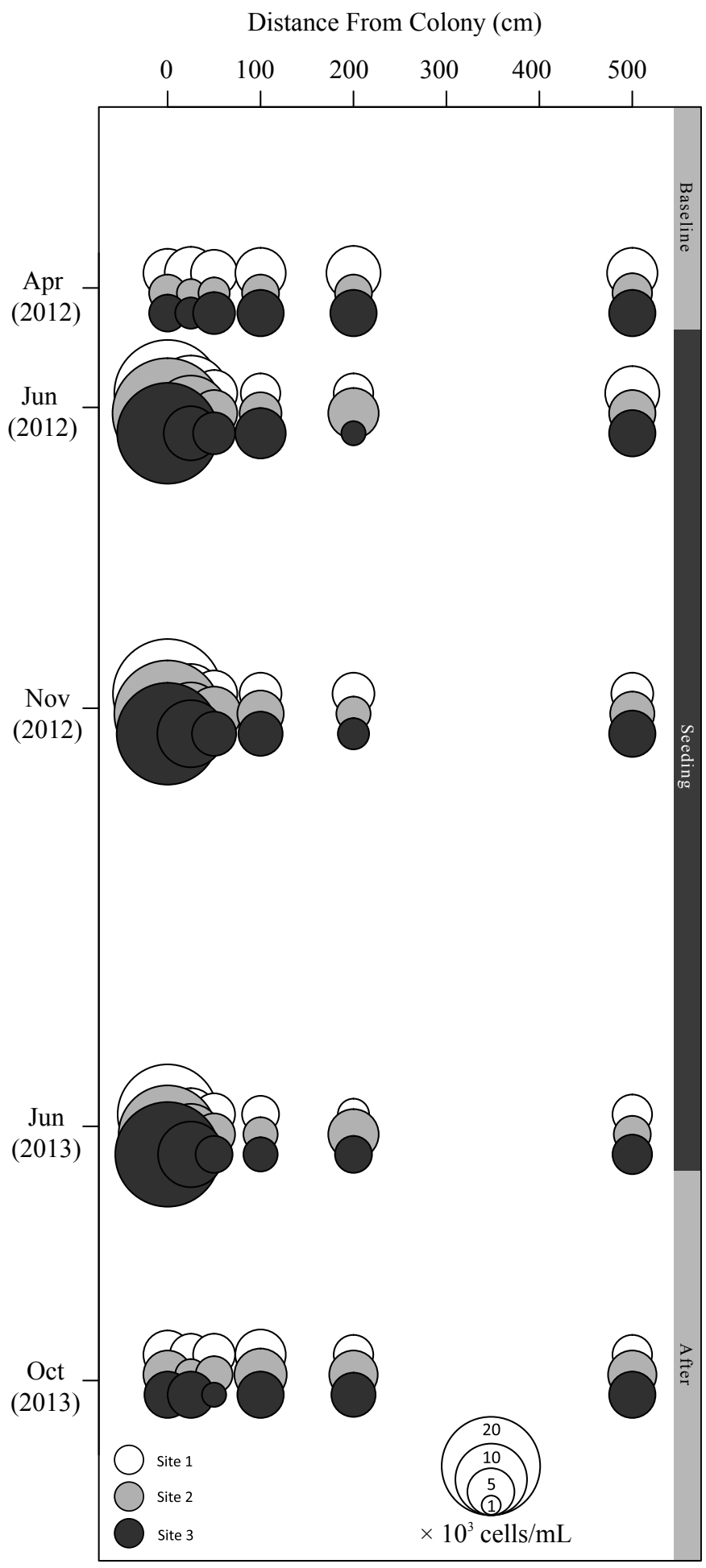

Figure 2.3 Abundances of *Symbiodinium* cells mL^{-1} at increasing distances (horizontal axis) from the base of the coral colony (0 cm) to the control distance (500 cm), visualised through time (vertical axis). *Symbiodinium* cells/mL are mean values represented as a function of circle area (πr^2). N = 4

After five months (November 2012, seven months post-translocation), sediment at the base of coral colonies continued to contain a high abundance of *Symbiodinium* cells in comparison with control distances. Cells were in abundances similar to the previous time point in June (2012), with 2.86×10^4 ($\pm 1.65 \times 10^3$), 2.72×10^4 ($\pm 1.56 \times 10^3$) and 2.48×10^4 ($\pm 2.82 \times 10^3$) cells/mL for S1, S2 and S3 respectively. At 25 cm from the colony, only S3 recorded a significantly greater abundance of *Symbiodinium* cells than the control distance, with 1.08×10^4 ($\pm 2.82 \times 10^3$) cells/mL.

After seven months (June 2013, 14 months post-translocation), sediment at the base of coral colonies continued to have a higher abundance of *Symbiodinium* cells than those at control distances. Densities did not change as before between November (2012) and June (2012), with 2.39×10^4 ($\pm 3.09 \times 10^3$), 2.30×10^4 ($\pm 2.47 \times 10^3$) and $2.63 \times 10^4 \pm (3.78 \times 10^3)$ *Symbiodinium* cells/mL for S1, S2 and S3 respectively. No sites recorded significantly greater abundances of cells at 25 cm from the colony, or at any of the other distances by comparison to the control distance of 500 cm.

After period

After approximately four months following removal of coral colonies, abundances of benthic *Symbiodinium* returned to baseline levels (Fig. 2.3). Cells were at 24%, 25% and 19% of the previous sampling point. All other densities recorded were equal to the densities at the control distance within each site and abundances ranged from 3.75×10^4 ($\pm 8.98 \times 10^3$) to 6.56×10^4 ($\pm 8.01 \times 10^3$) cells/mL.

Table 2.2 Statistical output of two-way ANOVA with repeated measures for between and within sites as subjects. Test p-values presented that are below the alpha value of 0.05 are considered statistically significant.

Subject	Source of Variation	F (df)	P value
Between Sites	Time × Site	F (70, 252) = 9.115	P < 0.0001
	Time	F (14, 252) = 14.82	P < 0.0001
	Site	F (5, 18) = 121.0	P < 0.0001
Within Sites	Time × Distance	F (70, 210) = 10.35	P < 0.0001
	Time	F (14, 42) = 9.282	P < 0.0001
	Distance	F (5, 15) = 135.2	P < 0.0001

2.5 Discussion

Many juvenile cnidarians initially draw their symbionts from the environment (Richmond and Hunter 1990; Schwarz et al. 2002; Barneah et al. 2004; Thornhill et al. 2006; Baird et al. 2009), and specifically, juvenile corals acquire *Symbiodinium* cells from the sediment (Adams et al. 2009; Cumbo et al. 2013). We address the need for a deeper understanding of the drivers of free-living *Symbiodinium* distribution and test the hypothesis that the abundance of benthic *Symbiodinium* cells would be directly related to the proximity of scleractinian coral colonies (*Acropora millepora*). Our data support these hypotheses in that an 8 fold increase in the abundance of benthic *Symbiodinium* cells at the immediate base of translocated *A. millepora* colonies relative to baseline abundances. This increase at the base of coral colonies was consistent between all three sites throughout the sampling period, with no detectable seasonal patterns in *Symbiodinium* abundance. Periodically, elevated abundances of *Symbiodinium* were detected up to 25 cm from the coral colonies. This work provides evidence in support of the hypothesis that scleractinian coral colonies act as significant sources of benthic *Symbiodinium*.

2.5.1 Symbiodinium *cell characteristics*

In our comparison of *Symbiodinium* cell size from branches of each *A. millepora* colony we found that there were no significant differences in the cell size between the three coral colonies. The phenotype of a vegetative *Symbiodinium* cell (*in hospite*) generally has a diameter of 5-15 μm, is brown and coccoid in shape (Stat et al. 2006); this non-descript morphology led early researchers to conclude the existence of a single species, *Symbiodinium microadriaticum* (Freudenthal 1962). Investigations of *Symbiodinium* at the molecular level have since revealed that there is widespread cryptic diversity within the genus. While three formally described species, *Symbiodinium boreum, Symbiodinium eurythalpos* and *Symbiodinium trenchii* were recently found to differ significantly in cell size and volume (LaJeunesse et al. 2014), species of *Symbiodinium* cannot be distinguished solely upon the basis of morphological data. As *Symbiodinium* cells isolated from *A. millepora* colonies in the current study were not identified to species/clade level, we can only speculate on their identity based on previous studies of *A. millepora* at Heron Reef. Fisher et al. (2012) reported that *A. millepora* colonies (N = 7) hosted *Symbiodinium* ITS2 type C3. These were also the findings of LaJeunesse et al. (2003) (N=1) and Stat et al. (2008) (N = 7) (although *A. millepora* colonies host other *Symbiodinium* types in other regions of the Great Barrier Reef (Tonk et al. 2013)). Given the apparent specificity of the symbiosis at Heron Reef it is unsurprising that there were no significant differences in the cell sizes of

Symbiodinium between the three colonies of *A. millepora* used in this study, however, the uniformity in morphology aided in the efficiency of *Symbiodinium* cell counting from sediment samples.

In this study, we incubated freshly isolated *Symbiodinium* cells in FSW for seven days to further supplement the reference library of *Symbiodinium* cells from *A. millepora.* After 3 d the *Symbiodinium* cells lost their characteristic size and structure, and would have been visually unidentifiable if sampled from the MPB community. Degraded *Symbiodinium* cells are irregular in shape, lack a cell wall and are less than half the size of healthy cells (Bhagooli and Hidaka 2004; Hill and Ralph 2007), and the incubated cells in this study followed this description. The incubated *Symbiodinium* cells also showed the characteristics of apoptosis as described in Strychar et al. (2004), with the condensing of cells and the compartmentalisation of cell contents into membrane bound apoptotic bodies. Although expelled *Symbiodinium* cells have been reported to be viable and photosynthetically active during ambient conditions and periods of physiological stress (Ralph et al. 2001; Ralph et al. 2005; Hill and Ralph 2007), it is possible that many types are only transiently free-living following expulsion. Hill and Ralph (2007) collected *Symbiodinium* cells expelled from the coral *Pocillopora damicornis* finding that, while initially photosynthetically active, the free-living *Symbiodinium* cells diminished rapidly in seawater. It was suggested that unless the expelled *Symbiodinium* cells encountered a more favourable habitat such as sediment, their ability to act as a source of symbionts for other hosts appears limited. The viability of *Symbiodinium* cells that become incorporated into the sediment following expulsion needs further investigation, but it is probable that the benthic cells observed in this study may only represent a transiently viable population that was constantly replenished by the coral colony.

2.5.2 Free-living Symbiodinium *cells*

The ecology of free-living *Symbiodinium* has received limited attention in comparison with other marine dinoflagellates. Littman et al. (2008) provide the only quantification of free-living *Symbiodinium* cells in coral reef sediment, enumerating between 1.0×10^3 to 4.0×10^3 cells/mL at Lizard Island, Great Barrier Reef, Australia. Venera-Ponton et al. (2010) also attempted to isolate free-living *Symbiodinium* cells from the sediments of Heron Reef with molecular techniques, but were unsuccessful. In the current study, the baseline or background abundance of *Symbiodinium*

cells was similar to that reported by Littman et al. (2008), with a range of 1.88×10^3 cells/mL to 7.03×10^3 cells/mL. The presence of a background population of *Symbiodinium* cells in the sediment suggests that *Symbiodinium* sp. may be widespread within reef ecosystems. Between the advection and diffusion of cells that are frequently expelled by hosts (Stimson and Kinzie 1991; Stimson et al. 2002) and the aided dispersal by corallivorous organisms (Castro-Sanguino and Sanchez 2012), there is the potential for free-living *Symbiodinium* cells to become ubiquitous in coral reef habitats, especially for those reefs with high coral cover. Indeed, Littman et al. (2008) found free-living *Symbiodinium* cells to be in highest abundance at sites with relatively high coral cover. Free-living *Symbiodinium* cells have also been found in macroalgae (Loeblich and Sherley 1979; Venera-Ponton et al. 2010) and the water column (Manning and Gates 2008), however, abundance data for these habitats are both limited. If *Symbiodinium* are indeed widespread in coral reef habitats, further studies might investigate whether horizontal transmission is density dependent, and whether the varying abundances of free-living *Symbiodinium* correlate with symbiont acquisition.

In the current study there were no significant seasonal differences in the abundances of benthic *Symbiodinium* cells during the seeding period. This is surprising, given that *in hospite Symbiodinium* densities are known to vary according to season (Stimson 1997; Fitt et al. 2000; Moothien Pillay et al. 2005) and also that large numbers of *Symbiodinium* are expelled by corals during elevated temperatures. However, the sea-surface temperatures at Heron Reef often reach their annual maximum in January (Bainbridge et al. 2010). It is possible that seasonal changes in free-living *Symbiodinium* abundance would have been detected if sampling had been conducted during the warmest months. There were, however, fine spatial differences in the distribution of free-living *Symbiodinium* cells within-sites. After the translocation of *A. millepora* colonies to the centre of each site, *Symbiodinium* cell abundance increased significantly in the sediment directly adjacent to the colony. The maximum density reached was 2.91×10^4 ($\pm 1.59 \times 10^3$) cells/mL which represents a greater than 7 fold difference when compared with the maximum abundance recorded by Littman et al. (2008). While this represents a high density relative to that reported by Littman et al. (2008), other genera of benthic and epiphytic dinoflagellates are known to reach high densities, including *Amphidinium britannicum* which reached more than 10^4 cells cm^2 (Azovsky et al. 2013) and *Ostreopsis lenticularis,* reaching densities of 23.58×10^4 cells g^{-1} *Dictyota* sp. (Ballantine et al. 1988). The increase of *Symbiodinium* cells directly at the base of colonies suggests that a portion of the cells expelled by the adult colony are retained in close proximity. This may be due to cells becoming trapped within the flow-reduced boundary layer that exists to varying extents around all corals (Nakamura and Van Woesik 2001; Jimenez et al. 2011), followed by the eventual settling of cells into the nearby sediment. The significant increase

of *Symbiodinium* cells up to 25 cm from the base of the colony (at 1.08×10^4 cells/mL) suggests that the diffusion of non-motile cells from the base of the colony through the interstitial spaces of the sediment is not detectable beyond this distance.

The increase of *Symbiodinium* cells at the base of the translocated coral colonies may also represent the aggregation of *Symbiodinium* cells near potential hosts, as suggested by Littman et al. (2008). Using the octocoral *Heteroxenia fuscescens* as a host species, Pasternak et al. (2006) demonstrated that *Symbiodinium* cells in flume chambers will swim towards cues from host polyps in still water and downstream flow treatments. Yamashita et al. (2014) also demonstrated that certain *Symbiodinium* types are attracted to *Acropora tenuis* larvae. This alternative hypothesis would be consistent with the fact that the removal of the coral colonies (and the potential host attractant) was followed by a decrease to baseline abundances of *Symbiodinium.* However, only coccoid *Symbiodinium* cells similar to those isolated from the coral colonies were counted, and therefore any gymnodinoid dinoflagellates that had structure of a motile *Symbiodinium* cell (commonly observed in culture but rarely seen *in situ,* but see Fujise et al. (2014) for a novel attempt) were not counted, and thus we consider this hypothesis less parsimonious than the coral seeding the sediment with *Symbiodinium* cells. To avoid all assumption and definitively differentiate between these hypotheses would require the molecular labelling of *Symbiodinium* cells *in hospite*, followed by their retrieval following expulsion.

2.5.3 The reinforcement of local Symbiodinium *strains*

Seeding the local environment with *Symbiodinium* cells has implications for the stability and specificity of coral-*Symbiodinium* symbioses. There is an opportunity to combine studies of free-living *Symbiodinium* cell populations (such as the present experiment) with observations of the successful propagation of *Symbiodinium* strains, genotypes, or species *in hospite*. For example, Baums et al. (2014) investigated the specificity of the *Acropora palmata* + *Symbiodinium fitti* symbioses across the entire range of *A. palmata* in the Caribbean, characterising the genotypic diversity of both host and symbiont. Baums et al. (2014) demonstrated that the effective gene flow of *S. fitti* was an order of magnitude lower than the host, and that unique genotypes of *S. fitti* dominated localised stands of coral hosts. When a *Symbiodinium* genotype is ubiquitous and well established within corals of a specific reef, it is reasonable to assume a quick infection of newly recruiting juveniles if the adult corals are

seeding the environment with free-living *Symbiodinium* cells (Baums et al. 2014). This 'symbiosis-loop' of intergenerational passing of symbionts may be all that is required for a *Symbiodinium* genotype or species to maintain a dominant or stable presence in the locality, however, further information of potential competitors in the reservoir of free-living *Symbiodinium* cells is required to predict how the symbioses may change with varying selection pressures (i.e. changes in climate). It is at this point is where the two fields of study can merge to provide a more complete picture of *Symbiodinium* + coral interactions, and this will be especially useful in predicting the outcomes of adaptation through novel symbioses. An example of combining the study of free-living *Symbiodinium* and *in hospite Symbiodinium* is the work conducted with aposymbiotic *Cassiopea xamachana* scyphistomae by Thornhill et al. (2006) in Florida Bay (USA). Symbiont-free *C. xamachana* were cultured and placed directly adjacent to a stand of *Montastrea faveolata* corals that are dominated by *Symbiodinium* sub-type B1. After 5 days of exposure to this new environment, *C. xamachana* acquire *Symbiodinium* cells belonging to clades A, B, C and D, signifying that the natural reservoir of *Symbiodinium* extends far beyond the immediate diversity within hosts. Prior to this study, the assumption that aposymbiotic recruiting corals would immediately be infected with the locally dominant symbiont, *Symbiodinium* B1, are indeed false, owing to the potential differences in prevalence and host-locating ability of the free-living *Symbiodinium* populations.

2.5.4 Conclusions

Benthic dinoflagellates are a major constituent of the MPB community on the reef-flat of Heron Reef, and reports of chlorophyll content there are among the highest recorded in the literature for marine sediments (Heil et al. 2004). Here, we show that the dinoflagellate *Symbiodinium* was in relatively high abundance in the sediment adjacent to translocated scleractinian corals (exceeding the single highest reports of *Symbiodinium* cell density in Littman et al.(2008)). Given the large concentration of cells at the base of adult coral colonies, recruits that draw their symbionts from the environment may potentially benefit from settling near a conspecific and the exposure to a larger pool of prospective symbionts, especially for those with type specificity (Weis et al. 2001). It therefore follows that aposymbiotic juveniles recruiting to recovering or damaged reefs lacking specific *Symbiodinium* types (and their sources), may receive relatively limited exposure to potential symbionts. Future studies may also investigate how abiotic factors such as sediment characteristics (see Supplementary Fig. 2.3 for the sediment characterisation of each site), nutrient availability (Taguchi and Kinzie Iii

2001) and varying photosynthetically active radiation (Robison and Warner 2006) affect the survival and distribution of free-living *Symbiodinium*.

2.6 Acknowledgements

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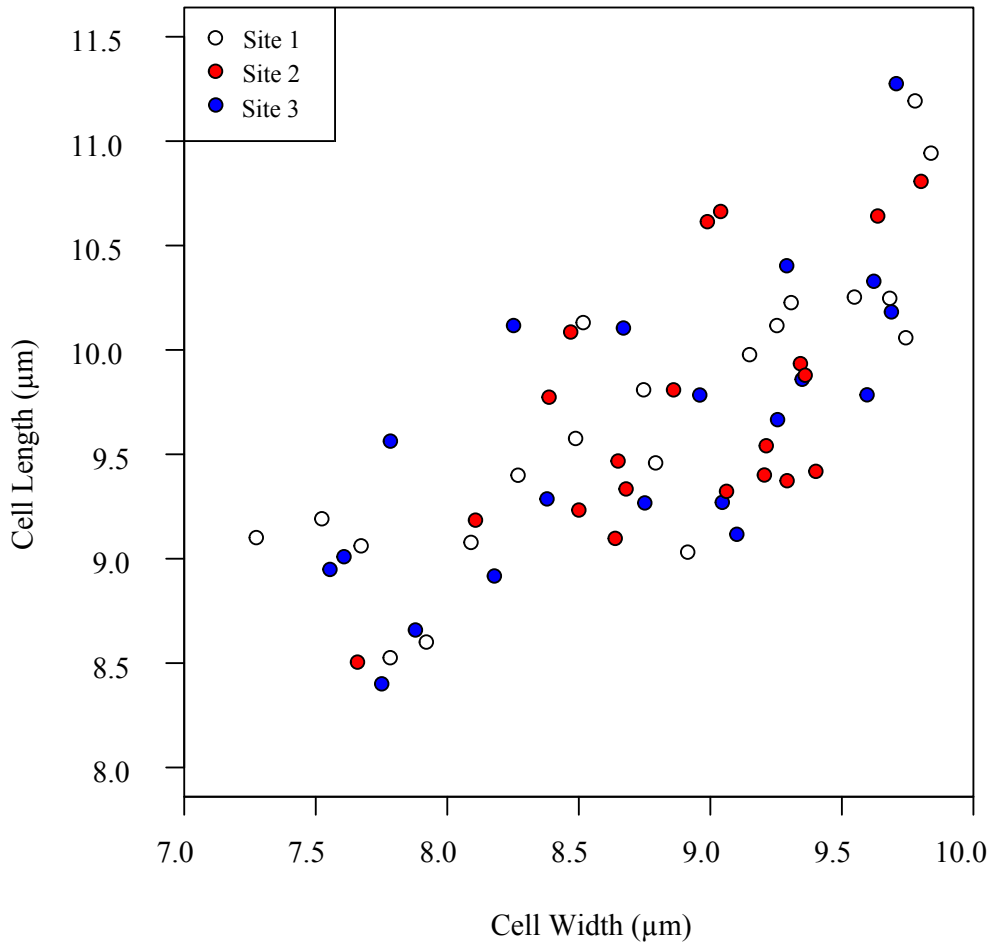

Figure S2.1 *Symbiodinium* cell lengths × widths (N = 20) from an *A. millepora* branch collected from each colony. White, red and blue circles represent cells from sites 1, 2 and 3 respectively. Cell sizes were not different between each site (Two-way ANOVA, $P > 0.05$ for all Tukey's multiple comparisons tests).

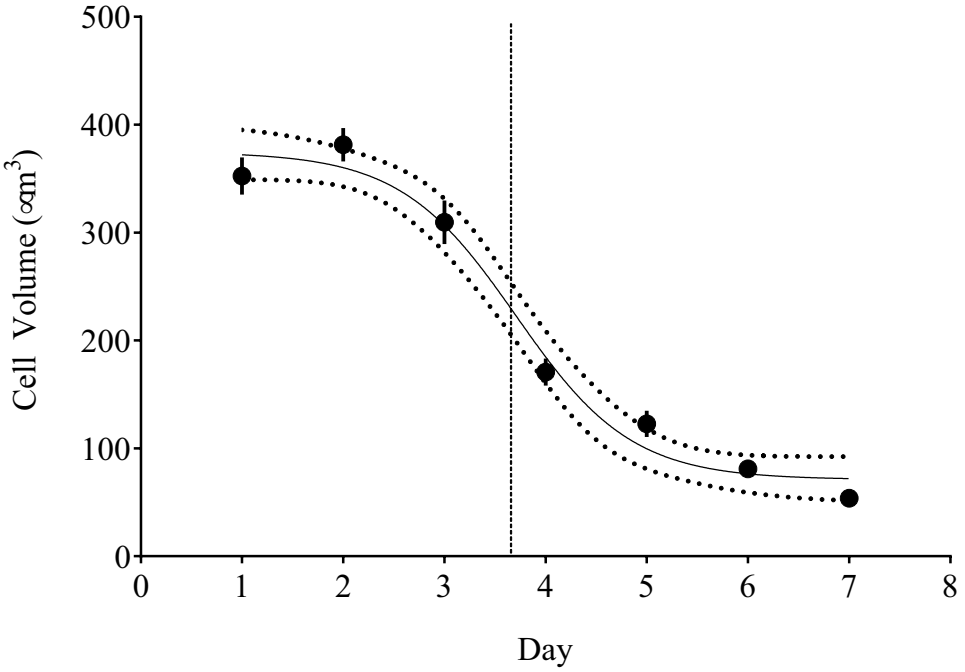

Figure S2.2 *Symbiodinium* cell volumes (N = 20) from an *Acropora millepora* branch, incubated in FSW for 7 days. Values (points) are means in µm³, assuming the *Symbiodinium* cell is an ellipsoid, ± SE. The solid line represents the four parameter sigmoidal model and its 95% confidence interval (dotted line). The vertical hashed line represents the interpolation for when cells halve in volume (relative to the entire range of volumes observed).

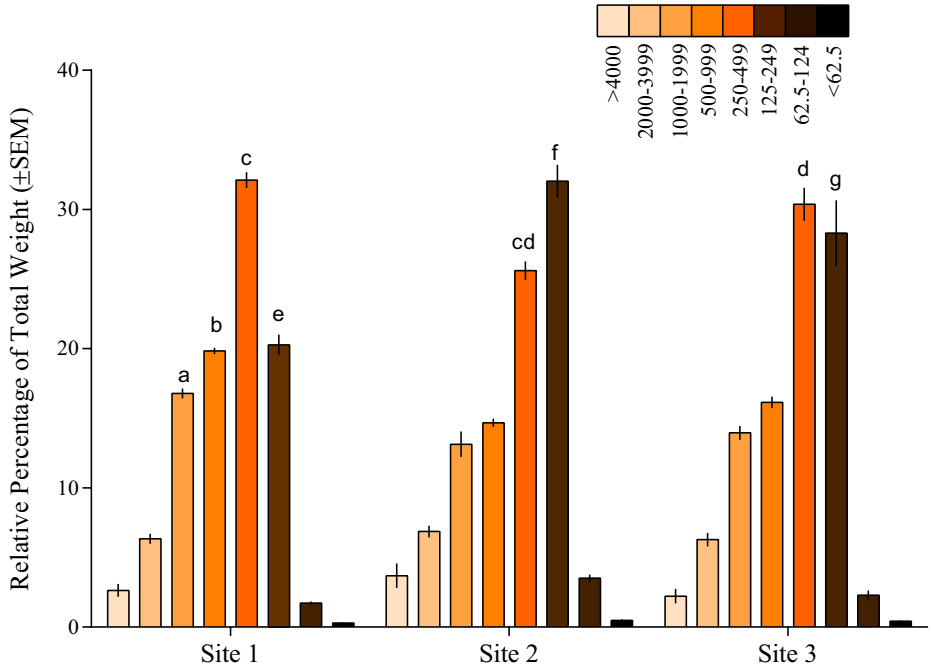

Figure S2.3 Complete sediment cores (collected as described above) were taken at each site to investigate the sediment size class composition. Samples were dried to constant weight at 60°C and then processed through an array of stainless steel, woven-wire sieves (Endecott, London, UK) with mesh sizes of 2000, 1000, 500, 250, 125 and 63 µm with an automatic shaker for 10 min. Each size fraction was then weighed and converted to a relative percentage of the total weight. Values represent means (N = 4) ± SE. Letters denote significant groups according to Tukey's multiple comparisons tests ($P < 0.05$).

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Chapter 3: *Pocillopora damicornis* fragments seed the sediment with a pulse of free-living *Symbiodinium* cells

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3.1 Abstract

Taxa within the genus *Symbiodinium* exist in two states: as intracellular symbionts (*in hospite*) and free-living. *Symbiodinium* cells have recently been identified in reef sediment, however at present it is unclear whether these cells represent a persistent population of *Symbiodinium* or transiently free-living cells displaced from adult hosts. Here, we test the hypothesis that scleractinian corals can release viable *Symbiodinium* cells that persist in the benthos. Fragments of the coral *Pocillopora damicornis* were collected from Kaneohe Bay (Hawaii) and either buried under a layer of carbonate sediment, or placed on the bottom of aquaria (control). Maximum quantum yield of Photosystem II (F_v/F_m) and the density of both *in hospite* and free-living *Symbiodinium* cells were recorded over a period of 12 days. A bleaching response was initiated within 2 days for branches buried in the sediment, and F_v/F_m declined by 50% relative to control fragments, for which this parameter remained unchanged. After 7 days we observed significant tissue loss, accompanied by a decline of the *in hospite Symbiodinium* to 1.66% (relative to controls) and F_v/F_m fell to zero. Branches had lost all tissue after 10 days. The concentration of free-living *Symbiodinium* cells in the sediment peaked at 4 d with 5.3 x 10^5 cells/mL, with an F_v/F_m of 0.270. Following this peak, *Symbiodinium* cell numbers declined and by 12 days > 50% of cells remaining in the sediment appeared degraded. These findings show that expelled *Symbiodinium* cells, while transiently free-living, can persist for a period of at least 7 days in reef sediment. For the many recruiting cnidarian hosts that initially draw their symbionts from the environment, this time-frame may be sufficient to make contact with their symbionts.

3.2 Introduction

The genus *Symbiodinium* comprises a group of marine dinoflagellates that occur in two states: symbiotic and free-living (Stat et al. 2006). During symbiosis, *Symbiodinium* cells can potentially meet most of the energetic demands of their hosts through the exchange of the products of photosynthesis (Falkowski et al. 1984; Muscatine 1990). Free-living *Symbiodinium* cells are crucial for hosts that acquire their symbionts from the environment, a process known as horizontal transmission (as opposed to vertical or maternal transmission). Between 80-85% of reef building coral species acquire their intracellular symbionts using this mechanism (Richmond and Hunter 1990; Baird et al. 2009), as do many jellyfish (Thornhill et al. 2006), octocorals (Barneah et al. 2004) and sea anemones (Schwarz et al. 2002).

The distinct taxa within *Symbiodinium* are distinguished as clades (A-I), and multiple sub-clades or 'types' are recognised within each clade (Pochon and Gates 2010). A number of distinct species have also been delineated (LaJeunesse et al. 2014). Of the nine clades, seven (A, B, C, D, E, G and H) have been identified in samples from the environment in a 'free-living' state (Gou et al. 2003; Santos 2004; Manning and Gates 2008; Porto et al. 2008; Reimer et al. 2010; Castro-Sanguino and Sanchez 2012; Takabayashi et al. 2012). It is unclear however, whether the free-living isolates from each clade represent self-sustaining populations of *Symbiodinium* or transiently free-living cells that have been recently displaced from hosts.

Symbiodinium cells are frequently removed or expelled from adult scleractinian corals through thermal bleaching (Hill and Ralph 2007), release of symbiotic gametes and juveniles (Harii et al. 2010), expulsion of excess symbionts (Stimson and Kinzie 1991; Jones and Yellowlees 1997) and through tissue removal caused by physical abrasion (Castro-Sanguino and Sanchez 2012). Hill and Ralph (2007) showed that some released *Symbiodinium* cells are photosynthetically active and persist for several days. Although, the ultimate fate of these expelled cells may vary according to the stimulus of release and innate physiological differences of each *Symbiodinium* clade, it appears that large numbers of *Symbiodinium* cells are entering (at least transiently) a free-living state.

In a comparison of free-living *Symbiodinium* cells in the water column and sediment, Littman et al. (2008) estimated that the sediment contained up to 15-fold more cells than corresponding water column samples analysed from Lizard Island (Great Barrier Reef, Australia). Indeed, a number of studies have now identified free-living *Symbiodinium* cells in reef sand/sediment (Carlos et al. 1999; Hirose et al. 2008; Littman et al. 2008; Reimer et al. 2010; Takabayashi et al. 2012; Yamashita and Koike 2013). Moreover, juvenile corals are able to acquire a consortium of symbionts through

exposure to sediment (Adams et al. 2009; Cumbo et al. 2013). Benthic reservoirs of *Symbiodinium* may therefore be significant to species that acquire their symbionts *via* horizontal transmission.

When corals come into direct contact with sediment (e.g. through whole colony or branch fragmentation), commonly observed responses include bleaching (a visible paling of coral tissue from loss or degradation of symbiotic algal cells), tissue damage and whole branch mortality (Erftemeijer et al. 2012). Given that *Symbiodinium* cells reach high densities within coral tissues, often exceeding 2.0 x 10^6 cm^{-2} (Fitt et al. 2000), contact with sediment in some circumstances may result in the release of a large pulse of *Symbiodinium* cells, contributing to the free-living population. The purpose of this study was to quantify the vitality of *Symbiodinium* cells released into calcium carbonate reef sediment during coral tissue degradation. Our hypotheses were that: A) corals in contact with sediment would release viable *Symbiodinium* cells; and B) released *Symbiodinium* cells would persist in the sediment in a free-living state. This research may provide evidence that adult corals act as significant sources of free-living *Symbiodinium* cells.

3.3 Materials and methods

3.3.1 Sediment and coral collection

Carbonate sediment was collected from a patch reef in Kaneohe Bay (21°27'11.5"N 157°48'06.0"W – Oahu, Hawaii). The sediment composition was standardized by washing with 0.22 µm filtered sea water (FSW) through sieves with mesh sizes of 1000 µm and 125 µm, and then all grains inside this size range were retained for use in treatments. The sieved sediment was sterilised in an autoclave (heated to 134°C for 20 min). After this process the sediment was rinsed a second time with Milli-Q water, and dried to constant weight in sterile conditions.

A single colony of the coral *Pocillopora damicornis* was collected from the adjacent reef and transferred to an indoor aquarium on a 12 h light : 12 h dark (0700 : 1900) cycle. A total of 36 fragments (4 × 2.5 cm) of the colony were removed and transferred to an acclimation tank supplied with flowing seawater from Kaneohe Bay. The coral fragments were allowed to recover for seven days. During this time they were acclimated to an experimental irradiance of 309.3 µmol photons m^{-2} s^{-1} (± 4.78 SD) that was provided by a collection of eight Dual Daylight 65W fluorescent tubes (Current-USA, Vista, California, USA).

3.3.2 Experimental design

A sediment × time experiment was run in an ambient temperature water bath (23.78 °C ± 0.36 SD)

over a 12 day period (see Supplementary Fig. S3.1 for diel temperature profile). Coral fragments (N = 21) were rinsed in FSW and placed in individual 300 mL glass beakers before 1.5 cm (depth) of sediment was placed on top. Control fragments (N = 21) were similarly rinsed, but this time they were placed uncovered in the water bath. Physiological measurements were conducted, in triplicate (N = 3), at 7 time-points: days 0, 1, 2, 4, 7, 10 and 12, for both the controls and the experimental treatments. Thus the experiment required a total of 42 individual glass beakers. Each beaker formed a closed system with water circulation through aeration (Whisper 100 Air Pumps, Tetra, Blacksburg, Virginia, USA). After every 8 h, 50 mL were removed from each replicate and replaced with fresh FSW to prevent anoxia and evaporation.

3.3.3 In hospite Symbiodinium

Maximum dark-adapted quantum yield of photosystem II (F_v/F_m) was measured 20 min after the beginning of the dark cycle, using a Diving-PAM chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany; settings: saturating intensity = 8, saturating width = 0.8 s, actinic intensity = 3, actinic width = 300 s, absorption factor = 1, gain = 4, damping = 2). The fluorometer was auto-zeroed in FSW before each sampling point to ensure the settings were calibrated correctly. The measurement locations were also standardized between fragments, with all readings taken perpendicular to the fragment at 1 cm distance mid-way along the fragment after the fluorescence signal (F) had stabilized.

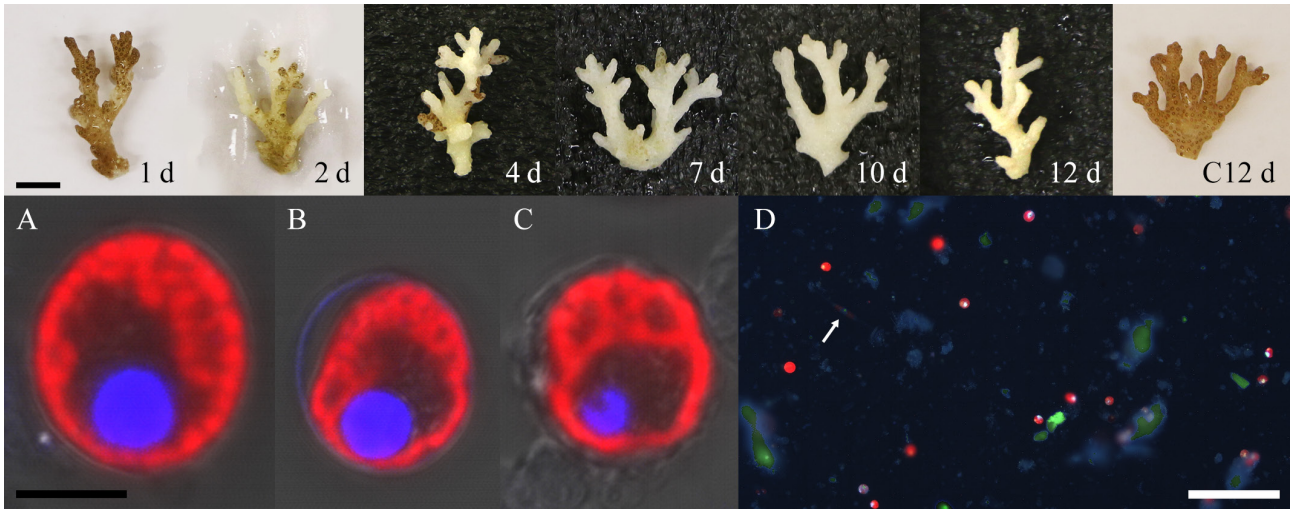

Figure 3.1 Progression of tissue loss in buried *Pocillopora damicornis* fragments and the free-living *Symbiodinium* cells extracted from the sediment. Upper panels: *P. damicornis* fragments after 1, 2, 4, 7, 10 and 12 d of burial and a control branch at 12 d (C12). Scale bar = 2 cm. Lower panels: *Symbiodinium* cells with regular morphology (A), and abnormal morphologies (B, C) viewed under a Zeiss LSM 710 Live Imaging confocal microscope with chlorophyll fluorescence (red) and DAPI stained (NucBlue® Fixed Cell ReadyProbes®, Life Technologies) DNA (blue). Scale bar = 5 µm. *Symbiodinium* cells extracted from sediment at 7 d (D) - note that the sediment crystals fluoresce green. The white arrow indicates a pennate diatom. Scale bar = 50 µm

Buried coral fragments were exposed for sampling by lightly transferring the top layer of sediment to the side of the aquarium using a Pasteur pipette. Once the fragments had been sampled, they were removed and frozen at -20°C for subsequent analysis of *Symbiodinium* density.

Each coral fragment was defrosted prior to processing. The coral tissue was removed by blasting the skeleton with pressurised FSW. The homogenate was decanted and centrifuged at 4000 × g for 5 min (22°C) to separate the *Symbiodinium* cells and the host tissue. After discarding the supernatant, the algal pellet was resuspended in 10 mL FSW, or 3 mL if the fragment appeared severely bleached. Individual *Symbiodinium* cells were counted from a 1 mL aliquot of each sample using an Improved Neubauer haemocytometer (Boeco, Hamburg, Germany, N = 8 replicated counts). The coral skeletons were then placed in 10% bleach for 24 h to remove any remaining tissue or cellular material. The surface area of each fragment was calculated using calibration cylinders and the double-dip, paraffin wax method as described in Holmes (2008). These values were subsequently used to normalise symbiont density.

3.3.4 Free-living Symbiodinium

After the coral fragment had been removed, free-living *Symbiodinium* cells were extracted from the interstitial spaces of the sediment. A 125 µm mesh was used to separate sediment from the seawater. The slurry was centrifuged at 4000 × g for 5 min (22°C). The supernatant was discarded and the algal pellet was resuspended in 1.5 mL FSW. The quantity of *Symbiodinium* cells expelled by the control (i.e. sediment-free) fragments was extremely low, and so the following analyses could only be performed on cells extracted from the treatment sediment.

The resuspended *Symbiodinium* cells were transferred to a 1.5 mL tube for measurement of F_v/F_m (using the settings described above). The Diving PAM was auto-zeroed to FSW in an identical 1.5 mL tube before the F_v/F_m of each sample was measured. *Symbiodinium* cells were dark adapted for 20 min and then aspirated into suspension. The fiber optic cable of the Diving PAM was inserted into the top of the tube until the tip was submerged. After F had stabilized, a single measure of F_v/F_m was acquired.

The density of free-living *Symbiodinium* cells was quantified from a 500 µL aliquot using the method described above. During the counts, 100 cells were examined and identified as either healthy – (approximately 10 µm in size, brown, circular and with an intact cell wall) or degraded (reduced size, irregular in shape and color) (Bhagooli and Hidaka 2004; Hill and Ralph 2007). The proportion of dividing cells was simultaneously recorded to generate a mitotic index. Finally, the presence and quantity of any other benthic microalgae that colonized the sediment (i.e. microphytobenthos; MPB) during the experiment was also noted during cell counts.

3.3.5 Statistical analyses

Two-way analysis of variance (ANOVA) was used to investigate the interaction between sediment (two levels – with and without sediment) and time (seven levels) for $F_v/F_{m.}$ and the density of *in hospite Symbiodinium*. When appropriate, Tukey *post hoc* and Sidak's multiple comparisons tests were used to locate significant differences. Free-living *Symbiodinium* cells were analysed using a one-way ANOVA to investigate the effect of time on $F_v/F_{m,}$ cell density, cell abnormality and mitotic index. Tukey *post hoc* tests were again used to identify significant differences. Kolmogorov-Smirnov normality tests and Brown-Forsythe homogeneity of variance tests were performed on all data to ensure that the assumptions of the analyses were met. Graphpad Prism (version 6.04) was used to perform all analyses and all results presented were tested against an alpha value of 0.05.

3.4 Results and Discussion

3.4.1 In hospite Symbiodinium

The photophysiology of *Pocillopora damicornis* reacted strongly to sediment exposure and a significant interaction between treatment and time was detected for F_v/F_m (Table 3.1). The F_v/F_m of control fragments was unchanged through time, ranging from 0.627 ± 0.01 (mean ± SE) to 0.663 ± 0.01 by day 12. This is consistent with previous values of dark adapted F_v/F_m for *P.damicornis* in ambient conditions (Ulstrup et al. 2005; Hill and Ralph 2007). The F_v/F_m of branches buried in the sediment, however, dropped rapidly. After two days of burial F_v/F_m had declined to 0.332 ± 0.02 and was significantly lower than for control branches (Sidak's multiple comparisons test, $p < 0.0001$). By day 7 branches were visibly devoid of tissue (or nearly so) and $F_v/F_m = 0$.

A linear relationship between sediment exposure and a decrease in F_v/F_m has previously been observed in the reef-building corals *Montipora peltiformis* by Philipp and Fabricius (2003), and also in *Montipora capitata* and *Porites lobata* by Piniak (2007), however the causal mechanism for this response has not yet been identified. In our experiment, corals fragments were maintained in almost constant darkness (through burial) for 12 days. Sediment smothering reduces the amount of light available to the symbiont for photosynthesis, which in turn may lead to hypoxia in the surrounding seawater. This renders the coral host and its symbiont almost entirely dependent on oxygen diffusion from the surrounding seawater to fuel their respiratory requirements (Kuhl et al. 1995), which may also be blocked by sediment smothering.

Symbiont density in control fragments averaged $1.38 \times 10^6 \pm 7.16 \times 10^4$ cells cm^{-2} which is within the

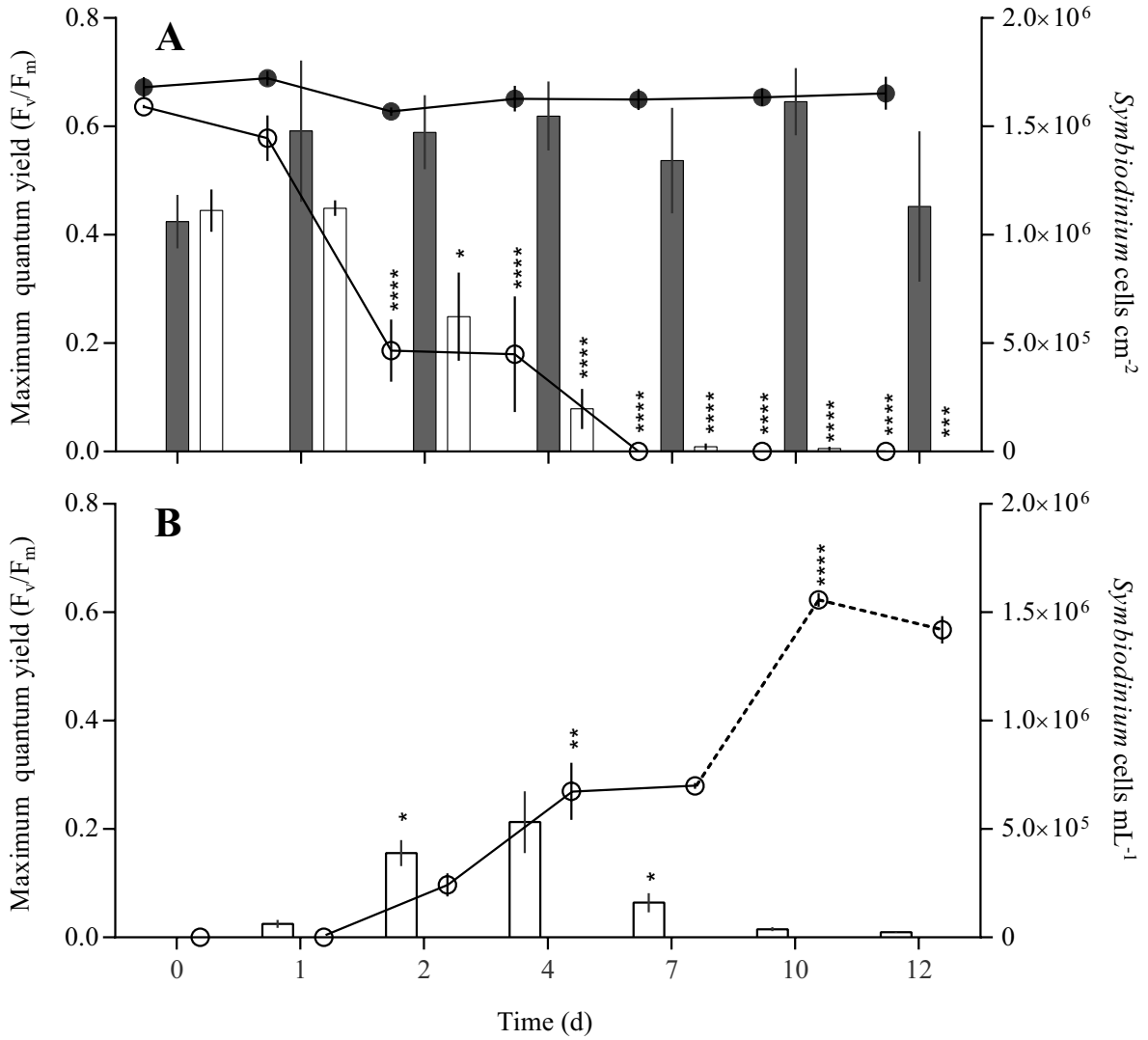

Figure 3.2 Maximum quantum yield of Photosystem II (lines) and *Symbiodinium* densities (bars) in hospite (A) and free-living (B). Controls are represented by dark bars/symbols and sediment treated as white bars/symbols. In 3.2.A the significance is indicated by Sidak's multiple comparison test that locate differences relative to controls, and in 3.2.B significance is indicated by Tukey's post hoc test that locates differences through time (e.g. 1 < 2 = 4 > 7 = 10 = 12). Values are means ± 1 SE (N = 3). * ≤ 0.05, ** ≤ 0.001, *** ≤ 0.001, **** ≤ 0.0001. Hashed lines indicate the onset of the diatom bloom.

range of measurements made in other studies from the same geographic region (Stimson 1997). In contrast, fragments buried within the sediment bleached rapidly by day 2 (Fig. 3.1), hosting only half as many symbionts as the control fragments (Sidak's multiple comparisons test, p = 0.0146). After day 7, 98.4% of symbionts had been lost relative to controls and branches appeared to be completely lacking tissues on days 10 and 12.

Some coral species, for example *Pleuractis granulosa, Lobactis scutaria* and *Herpolitha limax* can actively escape permanent burial through pulsed tissue inflation (Schuhmacher 1977; Bongaerts et

al. 2012), but this ability is rare and many corals are adversely affected when exposed to sediment. Notably, the severity of this response is species specific. *Porites lobata, Porites lutea* and *Montipora aequituberculata* for example, only exhibited partial bleaching after exposure to sediment for 6 d (Stafford-Smith 1993), whereas the burial of *Acropora* sp. branches for 20 h resulted in 100% mortality (Wesseling et al. 1999). Necrosis is another common response to sediment smothering, and it is suggested that microbial activity in the sediment exacerbates tissue damage (Hodgson 1990). In fact, host necrosis may be a mechanism by which symbionts are released (Gates et al. 1992; Weis 2008). Irrespective of the physiological mechanism of release, little is known about the viability of the symbiont cell following emergence or loss from the coral.

3.4.2 Free-living Symbiodinium

Pocillopra damicornis colonies expel photosynthetically active *Symbiodinium* cells (Ralph et al. 2005; Hill and Ralph 2007), however rapid declines in F_v/F_m have been observed following ejection, suggesting that free-living *Symbiodinium* cells have limited survival outside their hosts (Hill and Ralph 2007). In this study *Symbiodinium* cells were present in the sediment on day 1 (Fig. 3.2.B) but, due to their low concentration, the minimum fluorescence signal required for reading F_v/F_m was not reached. Yields of F_v/F_m on day 2 were the lowest values recorded in the experiment, at 0.097 ± 0.21. Following this, F_v/F_m increased significantly by day 4 to 0.270 ± 0.05 and remained at this level until day 7. On days 10 and 12, a significant increase in F_v/F_m coincided with the proliferation of other microphytobenthic algae (discussed below) and so cannot be interpreted as a recovery of the

Table 3.1 Statistical output of two-way ANOVA (for symbiotic corals) and one-way ANOVA (for free-living). Test p-values < 0.05 are considered statistically significant.

		Maximum quantum yield (F_v/F_m)	
State	**Source of Variation**	**F Statistic (df)**	**p value**
Symbiotic	Time × treatment	F (6, 28) = 5.603	P = 0.0006
	Time	F (6, 28) = 4.062	P = 0.0047
	Treatment	F (1, 28) = 97.49	P < 0.0001
Free-living	Time	F (6, 14) = 111.6	P < 0.0001

		***Symbiodinium* Density**	
		F Statistic (df)	**p value**
Symbiotic	Time × treatment	F (6, 28) = 5.603	P = 0.0006
	Time	F (6, 28) = 4.062	P = 0.0047
	Treatment	F (1, 28) = 97.49	P < 0.0001
Free-living	Time	F (6, 14) = 11.48	P = 0.0001

free-living *Symbiodinium* photosystem. However, the initial reductions of *in hospite Symbiodinium* densities are consistent with significant pulse of free-living cells into the sediment, which peaked on day 4 at 5.3 ± 1.4 x 10^5 cells/mL (Fig. 3.2.B). After this point, the concentration of cells decreased significantly to 1.6 x 10^5 ± 4.4 x 10^4 cells/mL by day 7 (Tukey's *post hoc* test, p = 0.0103) and reduced by a further 76% and 85% on days 10 and 12, respectively. The large pulse of cells released on day 4 had a higher F_v/F_m than those cells measured on day 2 and this may suggest that the most physiologically compromised cells were preferentially released, followed by a mass release of cells. Indeed, the initial release of cells on days 1 and 2 involved a visual paling of the coral tissues which is characteristic of bleaching (the loss of symbionts from host tissue), followed by rapid, necrotic-like tissue loss (Fig. 3.1). The potential biphasic cellular mechanisms of symbiont loss may contribute to the period of time that *Symbiodinium* cells persist in a free-living state.

The greatest mitotic index of 2.7% (± 0.33) occurred when the concentration of free-living cells peaked at day 4 (Tukey's *post hoc* test, p = 0.0031). This *Symbiodinium* division rate is close to those recorded in other studies (Wilkerson et al. 1988; Starzak et al. 2014), but were not enough to offset the degradation or loss of *Symbiodinium* cells over the duration of the experiment. The proportion of abnormal to healthy *Symbiodinium* cells was initially low, with only 10.7% (± 3.18) of free-living cells appearing degraded after day 7 (Supplementary Information, Table S3.1). A significant increase in abnormality was seen on days 10 and 12, with degraded cells comprising 33% (± 2.08) and 52.67% (± 2.40) of cells, respectively (Tukey's *post hoc* test, $p < 0.0001$ and p = 0.0002 respectively). No cells were observed with the gymnodinoid morphology of motile cells commonly observed in culture.

In a previous study, *Symbiodinium* cells expelled from *Stylophora pistillata* after 36 h of bleaching were approximately 50% dead or degraded (Franklin et al. 2004). Hill and Ralph (2007) examined cells up to 96 h after expulsion from *P.damicornis* during bleaching, finding between 60 and 80% of cells to be visually degraded. The stressors that initiated these symbiont expulsions differed from those in the present study and may explain the contrast in the speed with which the cells degraded. It is also possible that the staggered expulsion of the first 7 days would supplement the population of free-living cells, which would therefore consist of both recently expelled, viable cells and older, more degraded cells. However, ascertaining the photophysiological condition of *Symbiodinium* cells past day 7 in the present study, would have required cells to have been individually inspected with a microscope PAM (Ralph et al. 2005).

The presence of other microphytobenthic algae became apparent on day 7 (Fig. 3.1, Table S3.1). Initially concentrations were low, at just 4% of the total (i.e. *Symbiodinium* cells comprised the other 96%). However, by days 10 and 12, pennate diatoms had bloomed substantially, and significantly

outnumbered the declining population of *Symbiodinium* cells. The replicates were maintained in closed systems and the sediments were sterilized, so it is likely that the diatoms were present on the coral fragments themselves, an observation supported by other studies (Johnston and Rohwer 2007; Sudek et al. 2012).

3.4.3 Do corals seed substrates with free-living Symbiodinium*?*

Corals are regularly broken into fragments and smaller pieces have a very low chance of survival when in contact with the benthos (Smith and Hughes 1999). Here, we show that small fragments of the coral *P.damicornis* release a significant pulse of symbionts four days after being covered by sediment. While this ultimately resulted in the mortality of the coral, photosynthetically active free-living *Symbiodinium* cells were present in the sediment for at least 7 days, and some lasted as long as 12 days. In the days following the pulse, the viability of cells diminished rapidly, suggesting that these symbionts are only transiently free-living and that populations outside hosts require constant replenishment. Given the large concentration of cells lost and the viability for approximately seven days, this pulse may contribute importantly to the stock of free-living cells from which recruits draw their symbionts. In consideration of this, future experiments should aim to determine whether significant environmental stress, for example elevated temperature, shortens the window of this transiently free-living state, and if this influences the onset of symbiosis.

3.5 Acknowledgements

This fieldwork was supported by a Graduate School International Travel Award to MRN from The University of Queensland. We acknowledge Hollie Putnam, Danielle Claar, Grace Foulds and Chris Wall for their help in collection of the samples and assistance in setting up the experiment.

Table S3.1 Free-living cell characteristics. Percentages of abnormal and mitotic *Symbiodinium* cells and the ratio of other microphytobenthic algae (MPB) to *Symbiodinium*. Significance is indicated by Tukey's post hoc test that locates differences through time (e.g. 1 = 2 = 4 = 7 < 10 < 12). Values are means ± 1 SE (N = 3). * ≤ 0.05, ** ≤ 0.001, *** ≤ 0.001, **** ≤ 0.0001.

	Time (Days)					
Free-living cell characteristics	**1**	**2**	**4**	**7**	**10**	**12**
Abnormality (% ± SE)	1.262 (0.369)	9 (2.081)	4.333 (0.667)	10.667 (3.180)	33 (2.082) ****	52.667 (2.404) ***
Mitotic Index (± SE)	0.928 (0.038)	0.665 (0.168)	2.667 (0.333) **	2 (0.577)	0 **	0
Ratio of MPB:*Symbiodinium* (± SE)	0	0	0	0.093 (0.008)	5.465 (0.841) *	28.667 (2.028) ****

Table S3.2 Statistical output of one-way ANOVA for cell abnormality, mitotic index and the ratio of microphytobenthic (MPB) algae to *Symbiodinium*. Test p-values presented that are below the alpha value of 0.05 are considered statistically significant.

	Source of Variation	**F Statistic (df)**	**p value**
Abnormality	Time	F (5, 12) = 96.77	P < 0.0001
Mitotic Index	Time	F (5, 12) = 14.90	P < 0.0001
MPB:*Symbiodinium*	Time	F (5, 12) = 163.6	P < 0.0001

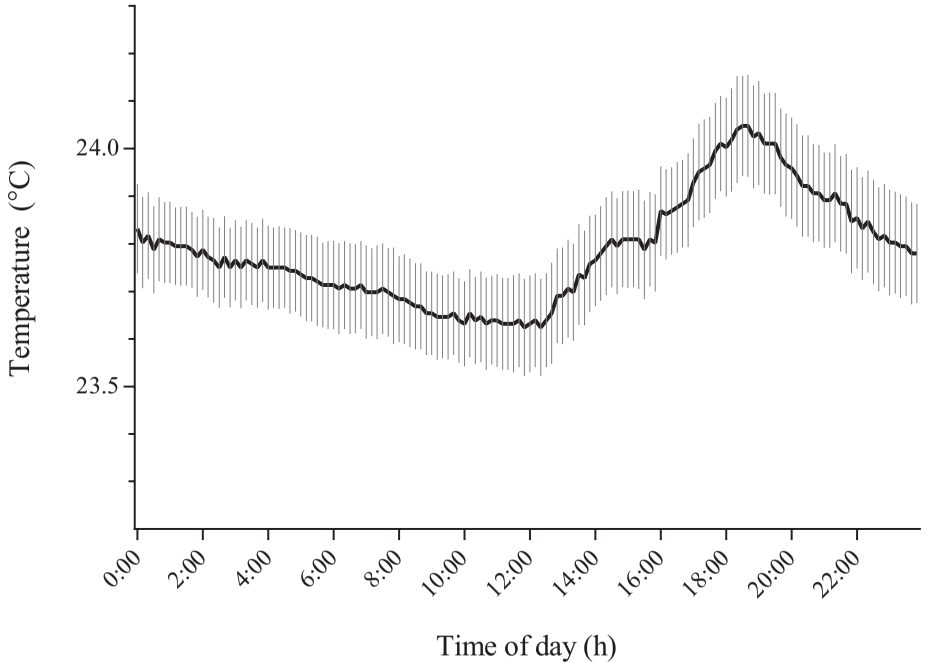

Figure S3.1 Diel temperature profile of the ambient water bath used for experimentation, recorded by HOBO data loggers (Onset Corporation, Bourne, MA, USA). Values are means ± 1 SE (N = 12).

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Chapter 4: The effect of elevated temperature and substrate on free-living *Symbiodinium* cultures

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4.1 Abstract

Elevated temperatures can produce a range of serious, deleterious effects on marine invertebrate - *Symbiodinium* symbioses. The responses of free-living *Symbiodinium* to elevated temperature, however, have been little studied, especially in the context of their natural habitat. In this study, we investigated physiological responses of two *Symbiodinium* cultures to elevated temperature, an exclusively free-living ITS2 clade A (strain HI-0509) and the symbiosis-forming ITS2 type A1 (strain CCMP2467). Free-living *Symbiodinium* strains have recently been isolated from benthic sediments, and both cultures were therefore grown with or without a microhabitat of carbonate sediment at 25°C, 28°C or 31°C. Maximum quantum yield of photosystem II (F_v/F_m) and specific growth rate were measured as response variables. In culture, *Symbiodinium* cells exhibit motility in a helical swimming pattern and therefore revolutions per minute (RPM) were also measured with video microscopy. The exclusively free-living clade A was physiologically superior to *Symbiodinium* A1 across all measured variables and treatment combinations. F_v/F_m remained relatively stable through time (at approximately 0.55) and was not substantially affected by temperature or presence or absence of sediment. Populations of the exclusively free-living *Symbiodinium* A reproduced faster with sediment than without and exhibited high levels of motility across all treatments (surpassing 300 RPM). In contrast, the F_v/F_m of A1 dropped to 0.42 in sediment (relative to cultures without sediment) and exhibited dramatic declines in cell concentration, most severely at 31°C. A >50% reduction in motility was also observed at 31°C. Even in the absence of sediment, elevated temperature was observed to reduce population growth and cell motility of type A1. We suggest that vital behaviours linked to motility (such as vertical migration and the locating of potential hosts) may become impaired during future thermal anomalies, and that populations of *Symbiodinium* A1 may only live transiently in sediment or outside coral hosts. Such differences in physiology between distinct *Symbiodinium* types may represent adaptations that are either conserved or lost depending on the differing selection pressures that come with living in symbiosis or free in the environment.

4.2 Introduction

The dinoflagellate genus *Symbiodinium* comprises a geographically widespread group of protists known for their symbiotic association with marine invertebrates, including corals, jellyfish and sea anemones. There are many ecologically and genetically distinct entities within the genus and these are currently delineated into different clades (A-I), and multiple 'types' are recognised within each clade (Pochon and Gates 2010). A mutualistic endosymbiosis with *Symbiodinium* can provision the associated host and meet its energy demands through the translocation of photosynthetically-fixed carbon (Falkowski et al. 1984; Muscatine 1990), and in the tropics are of paramount importance to the formation of coral reefs.

Many host species, including jellyfish of the *Cassiopea* genus (Mellas et al. 2014) and 80-85% of all scleractinian coral species (Richmond and Hunter 1990; Baird et al. 2009) initially lack symbionts (i.e. they are aposymbiotic). These hosts must therefore acquire symbionts from an environmental reservoir of *Symbiodinium,* yet little is known about the free-living *Symbiodinium* to which invertebrates have access (Littman et al. 2008; Adams et al. 2009). This mode of symbiont acquisition is referred to as horizontal transmission.

Symbiodinium occurs in two states: symbiotic and free-living. 'Free-living' refers to either: A) living outside a host while retaining the facility of forming symbioses (Hirose et al. 2008), including 'transiently free-living' cells recently expelled from their hosts that may not continuously persist in a free-living state (Yamashita and Koike 2013), or B) novel *Symbiodinium* types not currently known to engage in symbiosis that are permanently free-living (Takabayashi et al. 2012). These types are referred to as 'exclusively free-living' hereafter. While these exclusively free-living *Symbiodinium* types are not well understood, the ecology of free-living types that are ubiquitous in forming symbioses is of great interest due to their contributions to the nutrition of their potential hosts and ultimately the formation of diverse ecosystems such as coral reefs.

The molecular tools used to identify *Symbiodinium* types from within hosts are now also used to identify free-living types. For example, Littman et al. (2008) used a combination of genomic DNA analysis and cell counting, finding *Symbiodinium* in the benthos (Great Barrier Reef, Australia) at

densities approximately 15-fold greater in sediment than in water column samples, suggesting that a great proportion of free-living cells may be demersal. Carlos et al. (1999) identified a single clade A isolate in the interstitial water of reef sediment at Coconut Island, Hawaii. Pochon et al. (2010) and Takabayashi et al. (2012) also investigated the diversity of free-living *Symbiodinium* in sediment of Hawaiian coral reef environments, finding only limited overlap between endosymbiotic and free-living types. For example, Pochon et al. (2010) did not find the locally dominant scleractinian endosymbionts (C1, C1d, C3, C15, and C31) in sediment or water samples. Takabayashi et al. (2012) also found a low genetic diversity for identifiable free-living members of clade C, which is otherwise known to be highly diverse, containing many endosymbiotic types (LaJeunesse et al. 2003; Correa and Baker 2009). In contrast, free-living isolates belonging to clade A from the same area were highly diverse in comparison to the endosymbiotic diversity known for this clade and therefore may potentially represent exclusively free-living types (Correa and Baker 2009; Takabayashi et al. 2012). Hirose et al. (2008) and Reimer et al. (2010) also reported novel clade A types within sediment samples from Japan that may be exclusively free-living. These observations, combined with recent findings that reservoirs of *Symbiodinium* within reef sediment are an important source of symbionts for juvenile corals (Adams et al. 2009; Cumbo et al. 2013), call for an investigation of the physiology of free-living *Symbiodinium* cells in their microhabitat as the innate physiological differences between types will affect survivorship outside the host.

To understand the free-living reservoir of *Symbiodinium*, it is important to identify types that can utilize sediment as a microhabitat and explore how assemblages could change with varying environmental conditions. Other genera of dinoflagellates are well known for their ability to persist for extended periods of time within sediment (Lewis et al. 1999) and it is possible that *Symbiodinium* may similarly persist in this habitat. Benthic-pelagic coupling in dinoflagellates has been observed in cells vertically migrating into sediment (Sinclair and Kamykowski 2008), and also through the encystment and long term burial of cells that are viable up to after 100 years of dormancy (Lundholm et al. 2011). While most photosynthetic organisms experience periods of darkness on a daily basis, microalgae that associate with sediment may experience dark periods of days to weeks, or even longer (McMinn and Martin 2013). The energy dependent aspects of *Symbiodinium* physiology, such as motility (Fitt et al. 1981), are therefore worth considering if free-living reservoirs in sediment are to successfully make contact with potential hosts.

Investigations into physiological changes that occur after the breakdown of symbioses between

dinoflagellates and marine invertebrates, especially corals, are increasingly focused on what we may expect in the physical environment of the future. Prolonged exposure to elevated temperatures is known to cause a breakdown in the photosynthetic apparatus of the symbiont both in the field and in culture (Lesser and Farrell 2004; Fisher et al. 2012). When temperature reaches a stressful level, excess light energy not utilized in photochemical pathways ultimately increases the concentration of reactive and destructive compounds which impair the photosynthetic apparatus of *Symbiodinium* cells (Lesser and Farrell 2004; McGinty et al. 2012). For free-living dinoflagellate genera other than *Symbiodinium*, elevated temperatures (especially between 30 and 35°C) also cause sharp declines in cell swimming speed (Kamykowski and McCollum 1986). It is therefore important to understand how demersal reservoirs of *Symbiodinium* will respond, both physiologically and behaviourally, to future changes in temperature to gain insight into how this may affect symbiont acquisition.

The purpose of this study was to quantify the physiological performance of *Symbiodinium* types grown in calcium carbonate reef sediment across a range of temperatures. Our hypotheses were that; A) exclusively free-living *Symbiodinium* would be physiologically better suited to living within sediment than those that form endosymbiosis and B) that elevated temperatures would reduce photosynthetic yield of *Symbiodinium* in control (no-sediment) conditions more than cells that may find refuge in the light reduced interstitial spaces of the sediment. This work may provide evidence for sediment acting as a refuge from light-induced damage during high temperatures.

4.3 Materials and methods

4.3.1 Sediment collection

Carbonate sediment was collected from the reef flat of Heron Reef (23° 26’ 38.81”, 151° 54’ 46.73” - Great Barrier Reef) and processed at Heron Island Research Station. Free-living *Symbiodinium* cells have previously been isolated from sediment samples of 1-2 cm depth at other sites (Takabayashi et al. 2012) and the sediment for this experiment were collected accordingly. A 1-L bottle was used to scoop sediment and the sample was capped while submerged to retain the smaller grain-size fractions. A sieve shaker (Endecott Ltd, London) was used to separate sediment and for standardization the grain-size range of 500-1000 μm was retained as it was the dominant fraction by volume. Each batch of sediment used for experimentation was washed thoroughly through the sieve mesh with UV-treated Milli-Q water, autoclaved at 134°C for 20 min, washed a second time with UV-treated Milli-Q water, and dried in sterile conditions.

4.3.2 Symbiodinium *cultures*

The exclusively free-living *Symbiodinium* culture HI-0509 was acquired by Krueger and Gates (2012) during the cultivation of *Symbiodinium* cells associated with *Porites compressa* (Kaneohe Bay, Hawaii). The culture was found to belong to clade A (see Krueger and Gates (2012) for phylogenetic ITS2 placement) and to be closely related to other previously identified, potentially exclusively free-living types isolated from sediment - HA3-5 from Hawaii (Carlos et al. 1999) and Oku05, Oku09, Oku11, Oku12 and Oku17 from Okinawa, Japan (Hirose et al. 2008). *P.compressa* is not known to host *Symbiodinium* types belonging to clade A (Franklin et al. 2012), and the *Symbiodinium* cell that began the culture HI-0509 may have been within the mucus layer or stuck to the coral tissue (T. Krueger, pers. comm.). HI-0509 will be referred to as A-FLI (A – free-living isolate) hereafter as no ITS2 sub-clade was nominated (Krueger and Gates 2012).

Symbiodinium culture CCMP2467 (ITS2 type A1, *Symbiodinium microadriaticum*), described as a type having "thermal tolerance" (Ragni et al. 2010; Hawkins and Davy 2012; Kramer et al. 2012), was originally isolated from the scleractinian coral *Stylophora pistillata* by LaJeunesse (2001) in the Gulf of Aqaba (Red Sea). While *S. pistillata* transmits symbionts vertically from parent to offspring (Byler et al. 2013), A1 has also been isolated from corals that acquire their symbionts horizontally, such as *Acropora cytherea, A. longicyathus* and *A.muricata* (Stat and Gates 2008; LaJeunesse et al. 2009; Yang et al. 2012), however this may indeed represent a distinct lineage of *Symbiodinium* A1. A1 has also been isolated from the ambient environment, free-living and associated with the substrate while retaining the ability to form symbioses with octocorals (Coffroth et al. 2006).

Stocks of *Symbiodinium* A-FLI and A1 were grown at 25°C in silica-free sterile f/2-medium (pH 8.1) (Guillard and Ryther 1962) on a 12 h : 12 h (0700 : 1900) light/dark cycle. Stock cultures were acclimatised to experimental light levels of 75-80 µmol photons m^{-2} s^{-1} under Phillips TLD 36W/840 cool white tubes for 1 week. To ensure an even light field, an air-calibrated LI-COR LI250A was used to ensure minimal variation in intensity. *Symbiodinium* types were confirmed through the sequencing of the ITS2 region of nuclear ribosomal DNA (LaJeunesse 2002).

4.3.3 Experimental setup

A sediment × temperature experiment was run separately in two independent trials over 4 days, once for each *Symbiodinium* type. Stock cultures were diluted 5 h prior to experimentation to 100,000 cells mL^{-1} (8 replicate cell counts using a haemocytometer) with fresh, sterile f/2 medium. An 80 mL aliquot of culture was added to nine control flasks (no sediment) and nine flasks containing sediment. Flasks were conical (150 mL) and sediment treatments each contained 55 g (± 0.04 g) of sediment; this sediment displaced 20 mL of liquid medium and attained a depth of 1.5 cm. The flask sides were covered with multiple layers of black electrical tape below the surface line of the sediment layer to prevent the transmission of light; no-sediment flasks were left free of tape. These 18 flasks were then sealed with a cap made of tinfoil and placed into a temperature bath at ambient temperature (25°C ± 0.47) where they were allowed to acclimatise for 12 h. All flasks were then distributed at random between three temperature treatments, each containing three sediment flasks and three no-sediment flasks. *Symbiodinium* cultures were exposed to an ambient temperature of 25.22°C (± 0.47), a medium temperature of 27.80°C (± 0.21) or a high temperature of 30.63°C (± 0.27). Light conditions were kept constant between treatments at 75-80 μmol photons m^{-2} s^{-1} (Phillips TLD 36W/840 cool white tubes). Desired temperatures were reached by manually increasing the temperature in 1°C increments every two hours (i.e. a duration of 6 h to reach 28°C and 12 h to reach 31°C from ambient), using Weipro MX-1019 temperature controllers and 500 W water heaters. Temperature treatments were monitored every 10 min using HOBO Pendant® Temperature Data Loggers (Onset Computer Corporation, USA).

4.3.4 Maximum quantum yield of photosystem II

Dark-adapted maximum quantum yield of photosystem II (F_v/F_m) was measured throughout the experiment at 12 h intervals with a Water-PAM chlorophyll fluorometer (Heinz Walz GmbH, Germany). A baseline measure of F_v/F_m was also taken before increasing the temperature (0 h). Measurements were taken 3 h after the beginning of the light period and 3 h after dark. Measurements of *Symbiodinium* F_v/F_m taken during the day were dark-adapted for a minimum of 20 min. *Symbiodinium* cells within the flask were stirred into suspension before the period of dark adaptation and a 1 mL aliquot was sampled from each replicate flask. The method of stirring in both control and sediment flasks was identical. A sterile glass rod was inserted into each flask until it touched the bottom and a single clockwise revolution was made lasting 3-4 s following the edge of the flask opening. This gentle stirring minimized the risk of damage to the cells, while ensuring that the cells both within the interstitial spaces of the sediment and on the bottom of the flasks in the no-sediment treatments were re-suspended. The aliquot was taken from either 1 cm above the sediment-medium interface

for sediment treatments or 1 cm above the bottom of flasks without sediment. Each aliquot was transferred to a quartz glass cuvette (15 mm diameter) for analysis. The Water-PAM chlorophyll fluorometer applied a saturating pulse of light to the cuvette after the fluorescence signal became steady (gain = 5 or 6; saturating intensity = 10; saturating width = 0.8; measuring light intensity = 8; actinic intensity = 5 and actinic width = 300 s). The aliquot was discarded after the measurement of F_v/F_m.

4.3.5 Specific growth rate

Culture growth was monitored by taking a 1 mL aliquot for counting, after resuspension and harvesting as described above, followed by fixation with formaldehyde (4%). Aliquots were taken at mid-day at 24 h intervals. A haemocytometer was used to quantify cells mL^{-1}, with eight replicate counts per sample. The cell specific growth rate (μ day^{-1}) was calculated and averaged for each treatment (Guillard 1973).

4.3.6 Motility

Symbiodinium cells are known to exhibit a diel rhythm of motility that begins with the initiation of the light period and lasts for 8-9 h, peaking after 2.5-4 h (Yacobovitch et al. 2004; Pasternak et al. 2006), so measurements of motility were made between 1000 h and 1200 h during the 0700 h : 1900 h, light : dark regime. A motile, or mastigote *Symbiodinium* cell has both longitudinal and transverse flagella (Freudenthal 1962) that move the cell in helical and tight-cyclic patterns (Pasternak et al. 2006). The motility of individual *Symbiodinium* cells was quantified using video analysis prior to temperature increase (baseline) and at the termination of the experiment. Cells were stirred into suspension (as previously described) and a 1 mL aliquot from each flask (in the same location as previously mentioned) was immediately transferred to a Sedgewick Rafter cell counting slide. A Canon EOS 550D digital SLR camera was used to record motile cells under 10× magnification (compound light microscope) at high definition (1920 × 1080 pixels) and 25 frames per second. The open source program Avidemux (version 2.6) and Tracker (version 4.82 - Open Source Physics) were used to analyze video recordings frame-by-frame. A single frame represents the passing of 40 ms. Individual *Symbiodinium* cells were tracked and observed rotating in the field of view (see Fig. 4.1). For each replicate, the mean number of revolutions per minute (RPM) for 10 mastigote cells was recorded.

4.3.7 Statistical analyses

Two-way ANOVA with repeated measures was used to investigate the interactions between temperature (three levels) and sediment (two levels) on F_v/F_m at each time point, with Tukey *post hoc* comparisons to locate significant differences. Changes in motility were similarly analysed with repeated measures two-way ANOVA, comparing baseline values of RPM to the next time point at the termination of the experiment. Two-way ANOVA was also used to investigate differences between growth rates (μ). The Shapiro-Wilk normality test, Levene's homogeneity of variance test, and Epsilons deviation from sphericity were performed on all data to ensure that the assumptions of the analyses were met. Graphpad Prism (version 6.02) was used to perform all analyses and all results presented were tested against an alpha value of 0.05.

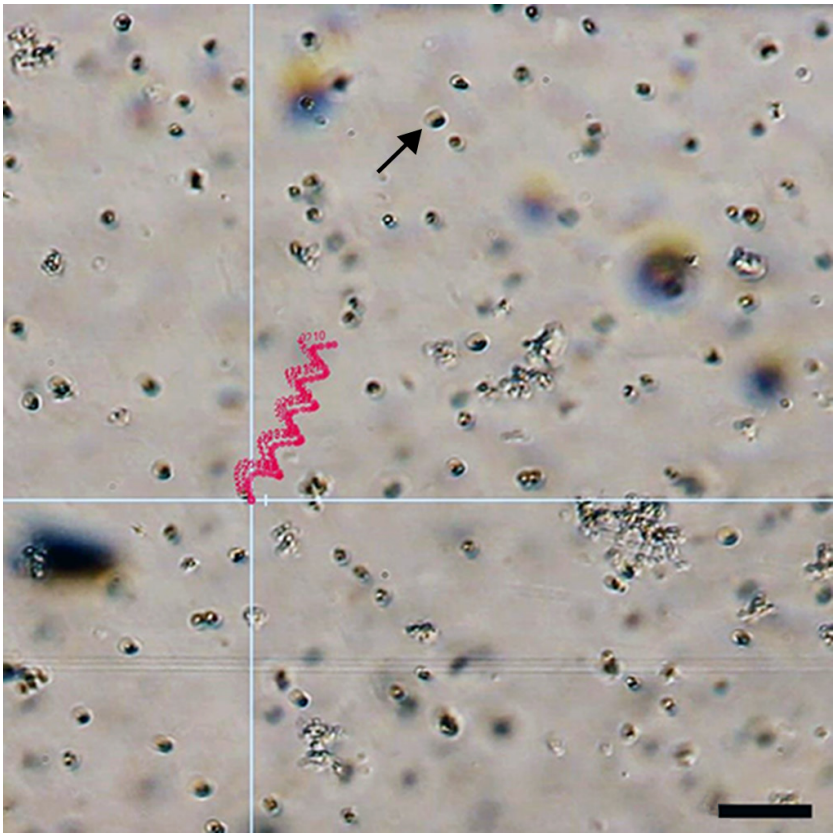

Figure 4.1 An example of path-tracking of *Symbiodinium* type A1 cells using the open source program Tracker (version 4.82 - Open Source Physics). The red path represents the movement of a single cell in a high temperature × no-sediment treatment over a duration of 2.28 seconds (57 individual video frames) and four complete revolutions (104 RPM). The intersecting white axes track with individually selected cells. Scale bar (black) represents 50 μm and arrow (black) indicates a single *Symbiodinium* cell (note that some cells are out of focus as they swim beyond the focal plane).

4.4 Results

4.4.1 Maximum quantum yield of photosystem II

The maximum quantum yield of *Symbiodinium* A-FLI was resilient to changes in temperature and the presence of sediment. Statistically, there was a significant interaction between treatment and time on F_v/F_m (Table 4.1). F_v/F_m in the ambient × no-sediment treatment remained unchanged from time 0 through to 86 h (Fig. 4.2.A, Tukey *post-hoc* test, $p > 0.05$ for all comparisons). A small, but significant increase in F_v/F_m was observed by the end of the experiment in all elevated temperature treatments (Fig. 4.2.A, Tukey *post-hoc* test, $p < 0.05$ for all comparisons). Cells in the high temperature × sediment treatment had a significantly lower F_v/F_m at 36 h than at 0 h, but this temporary decline did not continue through to the later stages of the experiment.

For symbiotic *Symbiodinium* A1, a different trend was observed as maximum quantum yield was reduced by both elevated temperature and the presence of sediment. Overall, there was a significant interaction between treatment and time on F_v/F_m (Table 4.1). All treatments exhibited a significant decline in F_v/F_m by the end of the experiment relative to initial levels (Fig. 4.2.B, Tukey *post hoc* test, $p < 0.05$ for all comparisons). Both medium and high temperature × sediment treatments exhibited significant decreases in F_v/F_m immediately after the completion of the incremental temperature increase, and then continued to decline. F_v/F_m reached its lowest value in the presence of sediment, with F_v/F_m in the high temperature × sediment treatment declining to 0.428 (± 0.009 SE) by 36 h. At 86 h, cells in the ambient temperature × no-sediment treatment had a significantly greater F_v/F_m (0.532 ± 0.003 SE) than those in the high temperature x no-sediment (0.466 ± 0.001 SE) and high temperature × sediment (0.469 ± 0.025 SE) treatments ($p = 0.0203$ and 0.0295 respectively, Tukey *post hoc* test).

4.4.2 Specific growth rate

For *Symbiodinium* A-FLI, temperature had no effect on reproduction. The main effect of presence or absence of sediment was, however, significant (Fig. 4.3.A, Table 4.1). Cells in the medium and high temperature × sediment treatments had significantly greater μ-values than in the ambient × no-sediment treatment ($\mu = 0.32$ day^{-1} ± 0.01 and $\mu = 0.32$ day^{-1} ± 0.01, respectively, Tukey *post hoc* test, $p < 0.05$ for all comparisons). This is equivalent to a doubling time of approximately 2.61 days.

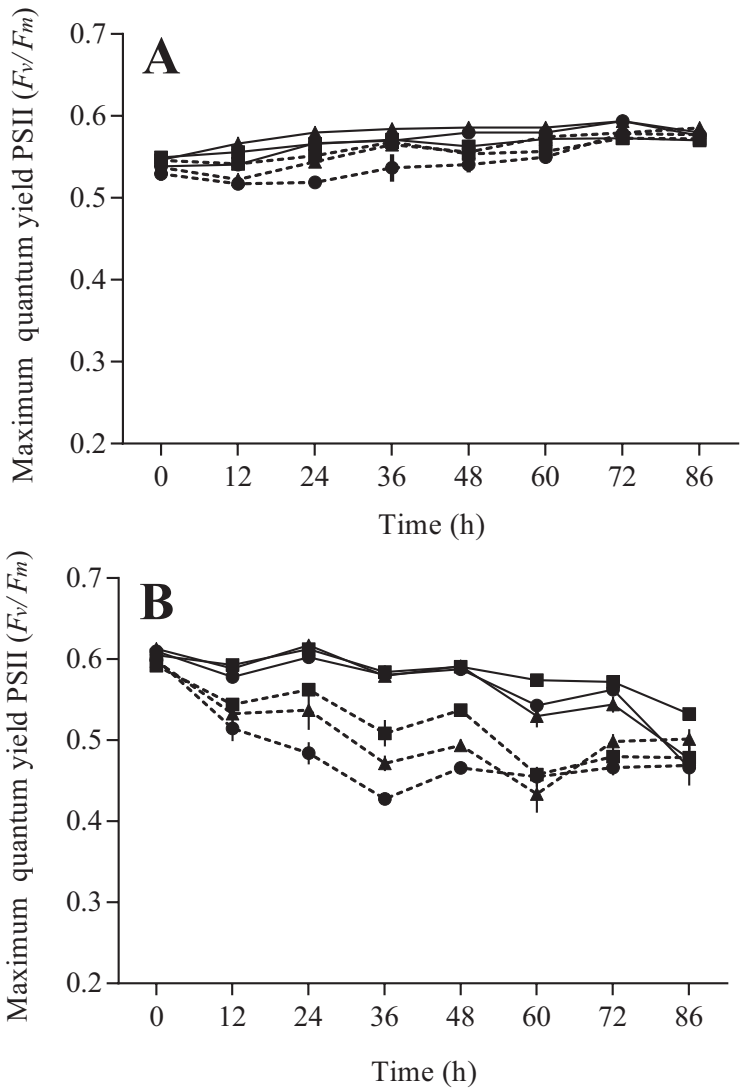

Figure 4.2 Maximum quantum yield of photosystem II (PSII) versus time of *Symbiodinium* types A-FLI (A) and A1 (B), under a range of temperatures and sediment loads (no sediment, sediment). Solid lines represent 'no sediment' control treatments and dashed lines represent 'sediment treatments'. Square, triangle and circle points represent 25, 28 and 31°C respectively. Temperatures were increased from ambient to treatment levels during the first 12 hours. Values are means ± 1 SE (N = 3).

Symbiodinium A1 responded differently to A-FLI and a range of growth rates was observed across treatment combinations. There was a significant interaction between treatments for cell specific growth rate (Table 4.1). Specific growth rate differed between all treatments except the ambient × no-sediment and medium × no-sediment treatments which were statistically identical (Fig 4.3.B, Tukey *post hoc* test, $p < 0.05$ for all comparisons). For the high temperature × no-sediment treatment, the specific growth rate was $\mu = 0.04$ day^{-1} (± 0.037), representing an approximate doubling time of 17.33 days. All temperature × sediment treatments produced negative values of μ. The high temperature ×

sediment treatment resulted in the greatest decline, with $\mu = -0.58$ day^{-1} ($\pm$ 0.067 SE), approximately equivalent to a halving of the population every 1.26 days.

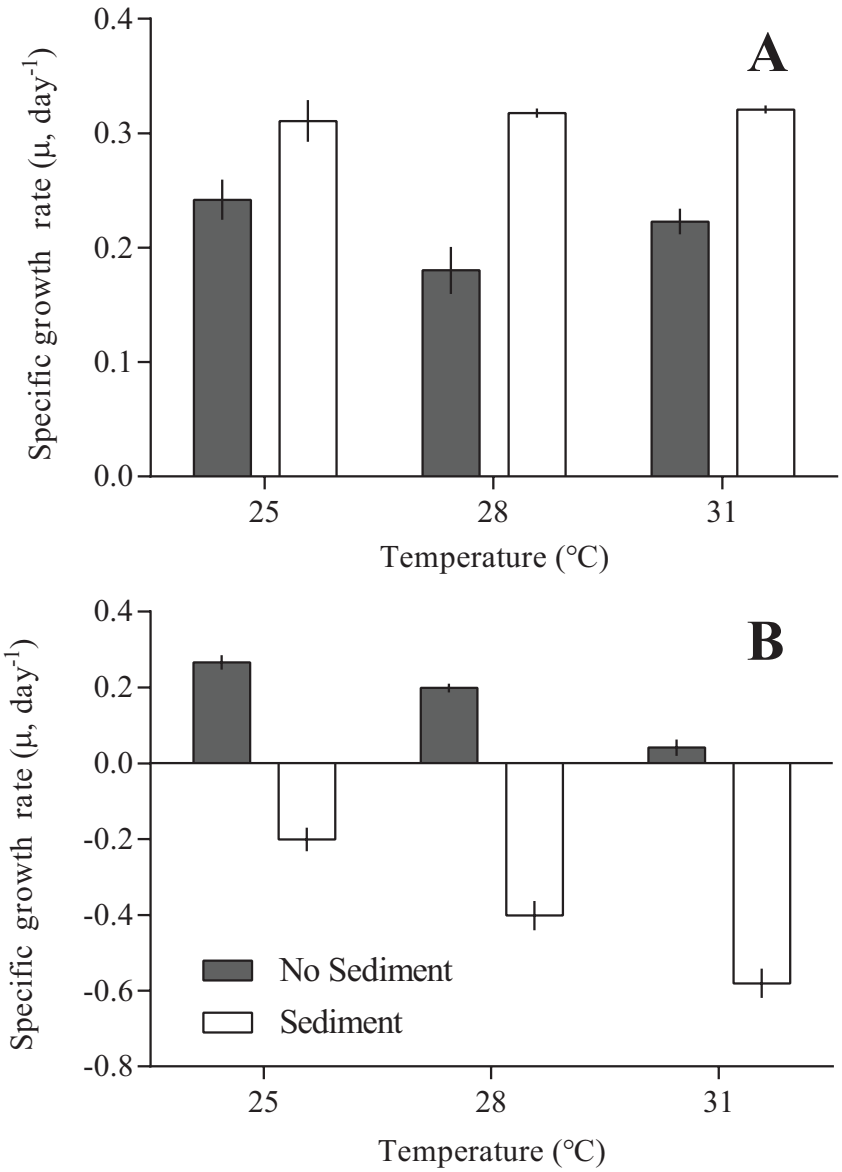

Figure 4.3 Specific growth rates of *Symbiodinium* types A-FLI (A) and A1 (B) under a range of temperatures (25, 28 and 31°C) and sediment loads (no sediment, sediment). Note that $\mu = 0.69$ day-1 is equivalent to a culture doubling time of 1 day. Values are means $\pm$ 1 SE (N = 3)

4.4.3 Motility

The exclusively free-living *Symbiodinium* A-FLI displayed high levels of activity (in comparison with A1), reaching a peak motility of 305.76 RPM ($\pm$ 4.17 SE) in the ambient $\times$ sediment treatment There were no significant interactions between time and treatment, only a significant main effect of

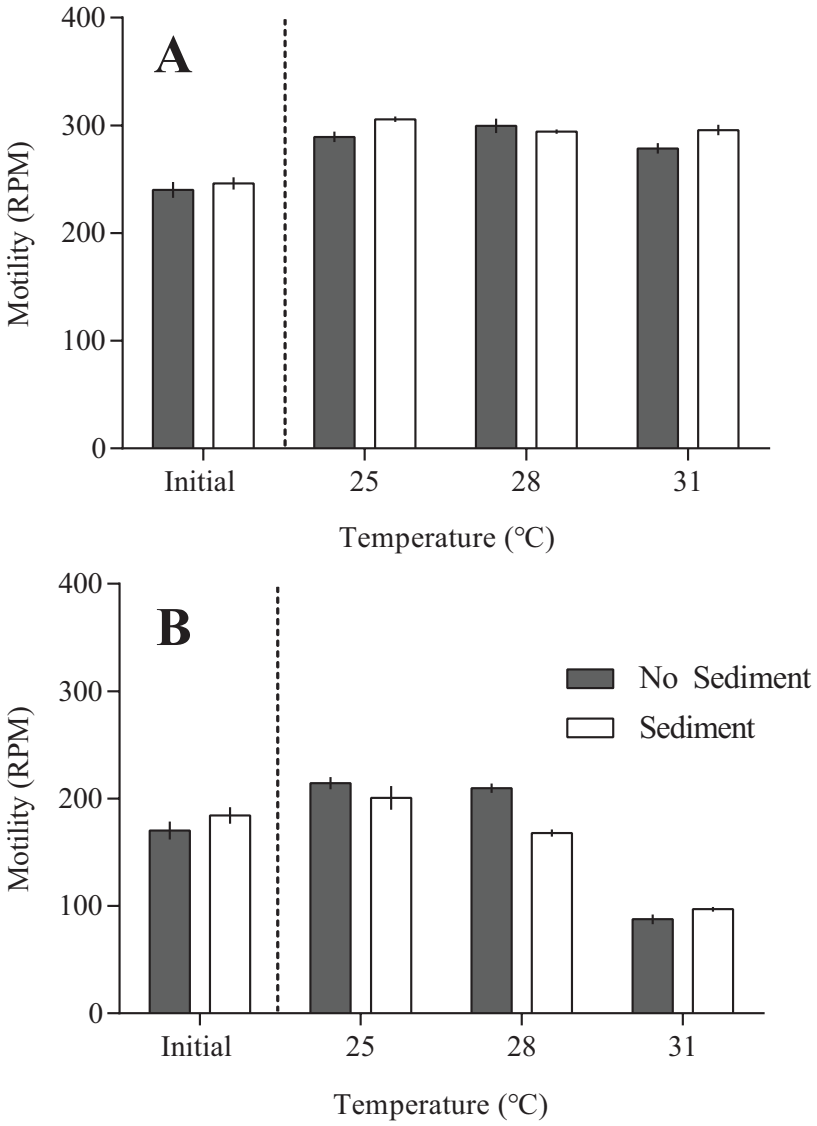

Figure 4.4 Motility of *Symbiodinium* types A-FLI (A) and A1 (B) under a range of temperatures (25, 28 and 31°C) and sediment loads (no sediment, sediment). Initial refers to the motility levels of cells prior to the beginning of the temperature increase. Values represent mean cell revolutions per minute (RPM) ± 1 SE (N = 3).

time (Table 4.1). Motility was greater in all treatment combinations at the end of the experiment than at the start (Fig. 4.4.A, Tukey *post hoc* test, $p < 0.05$ for all comparisons). There was also a difference in motility between the ambient temperature × sediment and high temperature × no-sediment control treatments, with 305.76 RPM (± 2.41 SE) and 278.61 RPM (± 4.78 SE) respectively (Tukey *post hoc* test, $p < 0.0001$).

For *Symbiodinium* A1, a peak of 214.41 RPM (± 9.94 SE) was observed in the ambient × no-sediment treatment, approximately 91 fewer RPM than the peak recorded for A-FLI. A significant

Table 4.1 Statistical output of Two-Way ANOVA with repeated measures (for maximum quantum yield and motility) and ordinary Two-Way ANOVA (for specific growth rate). Test p-values presented that are below the alpha value of 0.05 are considered statistically significant.

		Maximum Quantum Yield		Motility			Specific Growth Rate	
Type	**Source of Variation**	**F (df)**	**P value**	**F (df)**	**P value**	**Source of Variation**	**F (df)**	**P value**
						Sediment ×		
AFLI	Time × Treatment	F (35, 96) = 3.112	P < 0.0001	F (5, 24) = 0.9885	P = 0.4453	Temperature	F (2, 12) = 2.931	P = 0.0919
	Time	F (7, 96) = 46.96	P < 0.0001	F (1, 24) = 240.8	P < 0.0001	Temperature	F (2, 12) = 2.128	P = 0.1618
	Treatment	F (5, 96) = 35.70	P < 0.0001	F (5, 24) = 2.000	P = 0.1151	Sediment	F (1, 12) = 76.19	P < 0.0001
						Sediment ×		
A1	Time × Treatment	F (35, 95) = 6.824	P < 0.0001	F (5, 12) = 37.82	P < 0.0001	Temperature	F (2, 12) = 4.322	P = 0.0386
	Time	F (7, 95) = 85.54	P < 0.0001	F (1, 12) = 13.78	P = 0.0030	Temperature	F (2, 12) = 56.12	P < 0.0001
	Treatment	F (5, 95) = 119.5	P < 0.0001	F (5, 12) = 30.14	P < 0.0001	Sediment	F (1, 12) = 583.1	P < 0.0001

interaction between time × treatment was found for the motility of A1 (Table 4.1). Cells in both the ambient and medium temperature × no-sediment treatments were significantly more motile than at the start (Fig. 4.4.B, 214.41 RPM (± 9.94 SE) and 209.64 RPM (± 7.43 SE) respectively, Tukey *post hoc* test, $p < 0.05$). Cells in both the high temperature × no-sediment and high temperature × sediment treatments were significantly less motile than they were prior to the treatment and in all other treatment combinations. Cells in the high temperature × no-sediment and high temperature × sediment treatments were 51.42% and 52.62% less motile than prior to the treatment, respectively.

4.5 Discussion

Given the increasing number of studies that have demonstrated the presence of *Symbiodinium* free-living in reef sediment (Hirose et al. 2008; Littman et al. 2008; Takabayashi et al. 2012; Yamashita and Koike 2013), we address an important knowledge gap in the physiology of *Symbiodinium* cells. Until this study, the effects of temperature stress on the functioning of free-living *Symbiodinium* had not been examined in the context of their microhabitat. We found that an exclusively free-living *Symbiodinium* culture reproduced faster in sediment than cultures without sediment and that elevated temperature × sediment did not cause notable changes in either maximum quantum yield of photosystem II or cell motility. In contrast, the symbiosis-forming *Symbiodinium* A1 exhibited severely depressed growth rates in sediment (also in comparison with cultures grown without), most severely at 31°C. The combination of elevated temperature × sediment caused a significant decline in maximum quantum yield for A1 and temperatures elevated to 31°C also caused large reductions in cell motility in comparison with ambient (25°C) and medium (28°C) temperatures. Thus, a comparison of the physiological responses of the two types supports the hypothesis that the exclusively free-living type is better suited to survival in sediment than the symbiosis-forming A1. However, these data are inconsistent with our hypothesis that physiologically stressed cells may find refuge from temperature stress if within the light-reduced interstitial spaces of sediment. Our findings may reflect different competencies for survival in a natural habitat, differences that could potentially play a role in shaping the free-living assemblages of *Symbiodinium*.

The relationship between the impairment of photosynthesis and high temperature has been well explored for *Symbiodinium in hospite* (Hennige et al. 2011; Hill et al. 2011; Fisher et al. 2012) and in culture (Robison and Warner 2006; Ragni et al. 2010). We report that the exclusively free-living type proved to be tolerant of changes in temperature and exposure to sediment, exhibiting no significant decline in F_v/F_m over four days. The average summer/autumn water temperature for Kaneohe Bay

(from where A-FLI was originally cultured) is 27°C (Rodrigues et al. 2008) and yearly maxima are yet to reach 30°C (Massaro et al. 2012), therefore the F_v/F_m data presented for 31°C in this study suggest that exclusively free-living A-FLI, in its natural habitat, may be living well within its thermal limits (although this extrapolation would need to be tested *in situ*). While it is thought that this is an exclusively free-living type, it may be useful to investigate its potential to establish novel symbioses by challenging aposymbiotic hosts with this culture, given it clearly has the ability to maintain efficient photochemical pathways across a range of temperatures and exposures to a dynamic microhabitat such as sediment.

The F_v/F_m of the symbiosis-forming *Symbiodinium* A1 followed a rhythmic pattern, with higher values recorded during the day than at night, consistent with previous studies (Brown et al. 1999; Hoegh-Guldberg and Jones 1999; Sorek and Levy 2012). *Symbiodinium* A1 has been described as 'thermally tolerant' (Hawkins and Davy 2012) and the original culture was acquired from the Gulf of Aqaba, where temperatures near 30°C during thermal abnormalities from summer averages (Fine et al. 2013). The high temperature × sediment treatment, however, exhibited consistent declines in F_v/F_m over the first 36 h, suggesting that the ability to withstand thermal abnormalities are initially compromised while buried in sediment.

An examination of the photosynthetically active radiation (PAR) light field available to cells within the interstitial spaces of sediment may explain declines in the maximum quantum yield of *Symbiodinium* A1. PAR penetration into sediment is limited by the size distribution of grains and generally decreases with decreasing grain size (Kuhl et al. 1994). This emphasizes a need to investigate a range of sediment size classes to recreate the varying light environments experienced *in situ*. For a light intensity of 1000 μmol photons m^{-2} s^{-1} (approximately 12.5× the intensity used in this study) and a sediment size class comparable to that used in this study (500-1000 μm), Ichimi et al. (2008) calculated the depth by which irradiance reaches 1% of the incident light is only 2.2 mm. As light is expected to attenuate exponentially below 1 mm sediment depth (Kuhl et al. 1994), it is reasonable to assume that any *Symbiodinium* cells buried in sediment in this experiment were in a physical environment of near darkness. However, *Symbiodinium* is certainly capable of existing across a great range of light fields while *in hospite,* including those associated with mesophotic reefs as deep as 70 m (Bongaerts et al. 2011). If *Symbiodinium* cells migrate from within sediment to the sediment / medium interface, intense light scattering by sediment grains can potentially increase the irradiance intensity by up to 280% (Kuhl et al. 1994; McMinn and Martin 2013). If A1 migrated vertically out of the sediment

and into the sediment / medium interface, it is possible that excess light caused photoinhibition and the observed decline in F_v/F_m. Robison and Warner (2006) found that A1 is tolerant of changes in temperature, however, declines in dark adapted F_v/F_m were observed when this type was grown at higher light levels. Measuring the vertical position of *Symbiodinium* would be required to distinguish how much time cells were spending buried beneath the sediment compared with the sediment / medium interface.

While the growth rate of type *Symbiodinium* A1 in culture has been reported previously, there is an absence of information on the growth of exclusively free-living *Symbiodinium* in culture. Temperature had no significant effect on the growth rate of *Symbiodinium* A-FLI, however the presence or absence of sediment affected it significantly. Previous studies have demonstrated positive correlations between irradiance and growth rate (Robison and Warner 2006; Hennige et al. 2009; Kramer et al. 2012), however the *Symbiodinium* cultures grown in sediment during this study would have been periodically exposed to extreme low light conditions while buried. While *Symbiodinium* cells *in hospite* are fixed in the non-motile coccoid stage and confined to endodermal tissues (Fitt and Trench 1983), free-living *Symbiodinium* cells are flagellate and thus motile. However, *Symbiodinium* cells cease their motility in darkness when exposed to a regular light : dark regime (Yacobovitch et al. 2004), and it is assumed in this study that these non-motile cells would sink onto or into the sediment and potentially become buried. Following the activation of motility at the start of the light period cells have the potential to orient themselves favourably through vertical migration. It is conceivable that *Symbiodinium* A-FLI is able to use such behaviours to escape burial and maintain a favourable position for photosynthesis and reproduction. Alternatively, *Symbiodinium* cells have been shown to be mixotrophs (Jeong et al. 2012), and although the cultures started under sterile conditions, it is possible that *Symbiodinium* A-FLI fed on bacteria that may have colonized the sediment, contributing to an increase in population growth. It is also possible that there are adaptive differences between exclusively free-living types and endosymbiotic types in their ability to phagocytose food particles and act as mixotrophs.

The specific growth rate ($\mu = 0.27$ day^{-1}) of *Symbiodinium* A1 at ambient temperature is comparable to values reported previously for cultured *Symbiodinium*; for example, $\mu = 0.31$ day^{-1} was reported by Kramer et al. (2012) and $\mu = 0.17$ day^{-1} by Robison and Warner (2006), when grown under 145 and 100 μmol photons m^{-2} s^{-1} at 25 and 26°C, respectively. A1 responded negatively to high temperature in the absence of sediment, with a growth rate that has effectively ceased at $\mu = 0.04$ day^{-1}. Kramer et al. (2012) noted a decline in the specific growth rate for A1 at elevated temperatures, but not to

such an extent as reported here (declining to $\mu = 0.19$ day^{-1} at 32°C from $\mu = 0.31$ day^{-1} at 25°C). The differences in the experimental irradiances of these studies could potentially have driven these observed differences in growth rate. At high temperatures in the current study, there was an apparent uncoupling of photosynthesis and growth, with F_v/F_m at the end of the experiment declining to a relatively functional 0.47 in the no-sediment treatment, yet reproduction had effectively ceased. This result is similar to that of Robison and Warner (2006), who suggested that the cellular energy requirements of repairing photosystem protein contributed to a decline in reproductive capacity. Cultures in sediment showed a net decline in cell concentration through time. The combined effect of high temperature and sediment led to an 82% decline in the cell concentration by the end of the experiment; at this rate of decline ($\mu = -0.58$ day^{-1}), this culture would have been extinguished within a further 1.5 days. It is possible that physiologically impaired cells may have been unable to withstand the mechanical disturbance during the experiment, and that collisions between sediment grains (and between sediment grains and the flask wall) physically contributed to cellular disintegration. Indeed, sediment stability and disturbances associated with particle collision are thought to play a strong role in the distribution of microphytobenthic organisms, along with the ability to adhere to sediment grains and resist abrasion (Paterson and Hagerthey 2001). These physical and biological processes may influence the fine-scale distribution of free-living *Symbiodinium*, however they remain unexplored.

The motility of *Symbiodinium* cells has received limited attention. The few investigations of *Symbiodinium* motility, such as those of Fitt (1984) and Pasternak et al. (2006), have focused on how *Symbiodinium* cells responded to a potential 'host cue'. Motile *Symbiodinium* cells also migrate into wavelengths of green light (Hollingsworth et al. 2005) It is unknown how, or if at all, cellular stress is reflected in cell swimming behaviour. In the present study there was little difference in motility between the treatments for *Symbiodinium* A-FLI. It is reasonable to assume that maintaining motility is essential for the successful navigation of the interstitial spaces of sediment, allowing cells to avoid permanent or extended periods of darkness. It is possible that a resilience in motility across a range of temperatures allowed a great proportion of *Symbiodinium* A-FLI cells to navigate to the sediment surface and gain access to PAR. Future studies could investigate fine-scale positioning of cells and their interactions with the benthos, including other potential benthic microhabitats such as macro and turf algae (Venera-Ponton et al. 2010). Under ambient conditions *Symbiodinium* A-FLI completed up to 91 more RPM on average than did A1, consistent with the putatively free-living nature of this type and its apparent capacity to tolerate living in sediment.

Symbiodinium A1 completed only half as many revolutions at high temperature (31°C) than at the ambient temperatures. Kamykowski and McCollum (1986) investigated the thermal optima of swimming speeds for a range of dinoflagellate species and observed abrupt decreases in motility between 30°C and 35°C, suggesting a hard upper limit and a narrow margin between the fastest swimming rate and cell immobility. The observed decrease in motility seen here highlights the importance of investigating non-lethal aspects of stress other than commonly measured parameters such as chlorophyll content and photosynthetic function. In particular, while A1 is considered to be thermally tolerant based on its photosynthetic performance, and only sub-lethal declines in F_v/F_m were detected in this study, decreases in motility may significantly impair the ability for the survival of wild populations, as swimming is thought to allow dinoflagellates to take advantage of varying light, nutrient and temperature conditions (McKay et al. 2006).

It is interesting to consider the potential of an exclusively free-living *Symbiodinium* type with heat tolerance and the ability to persist within microhabitats. Starzak et al. (2014) demonstrated that a free-living *Symbiodinium* strain (originally isolated from a single cell in Wellington Harbour, New Zealand) could partner with *Aiptasia pulchella* (sea anemone from the Indo-Pacific) over an experimental period of 12 weeks. The symbiosis, however, did not reach full autotrophy (with respect to carbon) and may be suggestive of an interaction that leans towards parasitism. If exclusively free-living *Symbiodinium* types (such as the heat-tolerant A-FLI used here in this study) are making contact and forming symbioses with hosts during the process of initial symbiont acquisition (even transiently), the potential for adaptation to environmental stress through novel partnerships may extend beyond the types already known to form stable symbioses. This idea requires further investigation of the genetic and physiological diversity of free-living *Symbiodinium* types.

Clear differences were found between the two *Symbiodinium* types and we suggest that free-living populations of A1 may only exist transiently in reef sediment and especially briefly during times of elevated temperatures. However, corals are known to continually shed their symbionts, possibly as a mechanism that maintains and regulates numbers of *Symbiodinium in hospite* (Baghdasarian and Muscatine 2000). Therefore, hosts presumably replenish free-living populations of *Symbiodinium* constantly. Future experiments must consequently be conducted on freshly-isolated or recently expelled cells. For example, Hill and Ralph (2007) found reduced *Symbiodinium* viability after expulsion from coral hosts that were exposed to elevated temperatures. They also report that, the longer after the thermal stress the symbionts are expelled, the less their duration of viability, suggesting that the

condition of the symbiosis may play a role in the successful replenishment of the environment with free-living cells. In the current study, the observed differences in physiology between two types of a single *Symbiodinium* clade may represent adaptations that are either conserved or lost depending on the differing selection pressures that come with living in symbiosis or free in the environment. *Symbiodinium* A1 was significantly less motile at high temperatures, suggesting that vital behaviours such as vertical migration and location of hosts (Pasternak et al. 2006) might become increasingly impaired in future climate conditions. Future studies will be needed to investigate the requirements of *ex hospite* survival for other important *Symbiodinium* types, and crucially, more of those that form symbioses.

4.6 Acknowledgements

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Chapter 5: Benthic sediment aids the horizontal transmission of *Symbiodinium* cells between adult and juvenile corals

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5.1 Abstract

Of all reef-building coral species, 80-85% initially draw their intracellular symbionts (dinoflagellates of the genus *Symbiodinium*) from the environment. Although *Symbiodinium* cells are crucial for the growth of corals and the formation of corals reefs, little is known regarding how corals first encounter free-living *Symbiodinium* cells. We report how the supply of free-living *Symbiodinium* cells to the benthos by adult corals can increase the rate of horizontal symbiont acquisition for conspecific recruits. Following three consecutive reproduction events (Heron Island, Great Barrier Reef, Australia), newly-settled, aposymbiotic (i.e. symbiont-free) corals were maintained in open systems containing: sterlised sediment + adult coral fragments; adult coral fragments; sterilised sediment or seawater. In all instances, the combination of an adult coral and sediment resulted in the highest acquisition rates by juvenile corals (up to 5-fold greater than seawater alone). Juvenile corals exposed to individual treatments of adult coral or sediment produced an intermediate acquisition response (< 52% of recruits), and symbiont acquisition from unfiltered seawater was comparatively low (< 20% of recruits). Additionally, the abundance of benthic free-living *Symbiodinium* cells in each treatment was quantified, reaching the highest densities in the adult coral + sediment treatment (up to 1.2×10^4 cells mL^{-1}). Our results suggest that corals seed microhabitats with free-living *Symbiodinium* cells suitable for acquisition by conspecifics during the process of coral recruitment. The quantity of these symbiosis-ready *Symbiodinium* cells may prove to be a new aspect of coral reef health worthy of monitoring.

5.2 Introduction

Reef building (scleractinian) corals are declining in many areas of the world due to concurrent stressors, the foremost including: thermal bleaching; ocean acidification; disease; coastal development; overfishing; and crown-of-thorns sea star predation (Bellwood et al. 2004). Many millions of people around the globe rely on coral reefs for the intrinsic ecosystem services and sustenance they provide (Hoegh-Guldberg 1999), and the preservation of these ecosystems is of paramount importance.

Photosynthetic endosymbionts (genus *Symbiodinium*) can potentially meet the energy demands of cnidarian hosts through the translocation of photosynthetically-fixed carbon (Falkowski et al. 1984; Muscatine 1990), and for reef-building corals that inhabit nutrient-poor waters, this energy subsidy is obligatory for growth. Moreover, the resilience of individual coral colonies to disturbances such as thermal bleaching has, in part, been attributed to the identity of the *Symbiodinium* clade they harbor (Sampayo et al. 2008). There are nine 'clades' (A-I) of *Symbiodinium*, and numerous 'sub-clades' or 'types' within each clade (Pochon and Gates 2010), and the formal description of *Symbiodinium* species is progressing (LaJeunesse et al. 2014).

Intra-colony populations of *Symbiodinium* cells usually comprise a dominant type (by proportion) and often a cryptic type (or types) in low abundance (Mieog et al. 2007; Silverstein et al. 2012). Certain *Symbiodinium* types (e.g. some members of clade D) may afford a tolerance to temperature extremes (Baker et al. 2004; Fabricius et al. 2004; Rowan 2004; Sampayo et al. 2008) and coral communities exposed to marginal or more extreme conditions (e.g. as a result of a thermal anomaly) may host a greater proportion of these tolerant symbiont types (Baker et al. 2004). It is suggested that acquiring a favorable complement of symbionts requires the "shuffling" of the proportions of the *Symbiodinium* types already existing within the coral colony (Berkelmans and Van Oppen 2006). There is no evidence to suggest that adult corals acquire exogenous free-living *Symbiodinium* cells (although see (Coffroth et al. 2010) for a transient acquisition of *Symbiodinium* by bleached *Porites divaricata* colonies) and therefore the primary acquisition of a consortium of *Symbiodinium* types during recruitment is a crucial stage in the life-history of corals.

Reef building corals (and many other cnidarians) utilize two pathways of *Symbiodinium* cell acquisition.

The first is a parent-to-offspring pathway known as vertical transmission (Baird et al. 2009). The second pathway, by which coral recruits acquire free-living *Symbiodinium* cells from the surrounding environment, is referred to as horizontal transmission (Abrego et al. 2009b). The adopted pathway of transmission is largely related to reproductive mode (Richmond and Hunter 1990). Thus, the majority of coral species broadcast spawn their gametes that, once fertilized, develop into aposymbiotic (i.e symbiont-free) larvae (Richmond and Hunter 1990; Baird et al. 2009). Such larvae must eventually acquire free-living *Symbiodinium* cells during recruitment. Notwithstanding this basic dichotomy, evidence is mounting that the vertical transmission of *Symbiodinium* cells may not be a 'closed' system, and that recruits with maternally inherited symbionts (transfer of *Symbiodinium* cells to oocytes) may also acquire additional *Symbiodinium* cells horizontally from the environmental (Byler et al. 2013). These cnidarian-*Symbiodinium* symbioses are relatively well studied, however little is known regarding the process by which juvenile corals initially encounter free-living *Symbiodinium* cells.

It has been suggested that free-living *Symbiodinium* cells are mainly demersal (Littman et al. 2008). Indeed, Adams et al. (2009) demonstrated that *Acropora monticulosa* larvae exposed to reef sediments acquired *Symbiodinium* cells earlier and in greater densities than those exposed only to seawater. Furthermore, Cumbo et al. (2013) detected the larval uptake (for *Acropora millepora* and *Acropora tenuis*) of multiple *Symbiodinium* clades during exposure to sediments from a number of locations on the Great Barrier Reef (GBR), Australia. These observations are further supported through the molecular-based identification of benthic free-living *Symbiodinium* cells (Carlos et al. 1999; Hirose et al. 2008; Littman et al. 2008; Reimer et al. 2010; Takabayashi et al. 2012; Yamashita and Koike 2013).

At present, little is known about the composition of benthic free-living *Symbiodinium* populations. Pochon et al. (2010) investigated the diversity of free-living *Symbiodinium* cells in coral reef sediment in Hawaii, noting that the dominant endosymbionts of nearby corals were not present in sediment or seawater samples. Takabayashi et al. (2012) isolated a highly diverse assemblage of free-living clade A *Symbiodinium* (distinct from clade A types known to form symbioses), suggesting a potential guild of exclusively free-living specialists. Hirose et al. (2008) and Reimer et al. (2010) also reported the isolation of novel clade A types from the sediment (Japan) that may be exclusively free-living. The assemblages of benthic free-living *Symbiodinium* cells are therefore comprised of a mixture of *Symbiodinium* types; those that have the potential to form symbioses, and those that do not (Coffroth

et al. 2006). The distribution of free-living *Symbiodinium* cells that do form symbioses, however, will likely be of critical importance to juvenile corals.

Scleractinian corals expel viable symbionts (Ralph et al. 2001; Hill and Ralph 2007) constantly, and in large quantities (Stimson and Kinzie 1991; Stimson et al. 2002). Some of these may temporarily maintain their viability in reef sediment (Nitschke et al. 2015), and we aim to test whether these cells are a significant source of symbionts for recruiting corals. Here, we test the hypothesis that corals may seed the benthos with free-living *Symbiodinium* cells and that juvenile corals may acquire their symbionts at an accelerated rate when exposed to this symbiont-seeded sediment. First, we demonstrated that a small proportion of juvenile corals acquired symbionts from the seawater, suggesting that there are free-living *Symbiodinium* cells in suspension and that these may therefore be widespread in the study system. Secondly, *Symbiodinium*-like cells found in coral-exposed sediment were at least twice as abundant as in all other treatments, suggesting that reef-building corals play an important role in the localised abundance of benthic free-living *Symbiodinium* cells. Thirdly, we observed an increased rate of symbiont acquisition by recruits when incubated simultaneously with sediment and adult corals, and we suggest that benthic sediment aids the horizontal transmission of *Symbiodinium* cells.

5.3 Materials and methods

5.3.1 Study site

Heron Reef is located approximately 70 km from Gladstone (Queensland, Australia) in the Capricorn/Bunker management area of the GBR Marine Park (23.26 °S, 151.54 °E). The elevated reef is 9.6 km × 4.5 km with a vegetated coral cay at the western end (Maxwell et al. 1964). The following experiments were conducted at Heron Island Research Station (HIRS, The University of Queensland, Australia).

5.3.2 Study species and reproduction

Many scleractinian corals on the Great Barrier Reef (GBR) have an annual gametogenic cycle that culminates in a synchronized, mass spawning event (Harrison et al. 1984; Babcock et al. 1986). *Acropora millepora* and *A. selago* are broadcast spawning corals (Babcock et al. 1986) with horizontal

transmission of *Symbiodinium* (i.e. their gametes are aposymbiotic) and were selected as study species. Gravid colonies of *A. millepora* (N = 5) were identified on the southern reef flat of Heron Island following the full moon on the 12th November, 2011. Each colony (approximately 30 cm × 30 cm) was collected and maintained in an individual aquarium at HIRS, where it could be monitored for spawning behavior (retracted tentacles at night and setting of gametes in oral opening of polyp). Five nights following the full moon, synchronous spawning was observed at approximately 2130 h.

In the following year, reproductive colonies of *A. selago* were identified at Coral Canyons (N = 5) and Libby's Lair (N = 5) following the full moon on the 29th October, 2012. Each colony (approximately 30 cm × 30 cm) was brought into one of two large holding aquaria at HIRS where it could be monitored for spawning behavior as above. *A. selago* colonies spawned in synchrony 11 nights following the full moon at approximately 2300 h.

The coral *Isopora palifera* is a brooding species that releases aposymbiotic planula larvae (Harii et al. 2009), which is a rare combination of reproductive mode and symbiont acquisition (Baird et al. 2009). Adult colonies (N = 5) were collected from the reef flat of Heron Reef and were maintained in individual aquaria. The larvae released from each coral were collected on the 25th and 26th October (2013), and were then pooled for use in the following experiment.

5.3.3 Rearing and settlement of juvenile corals

The following rearing procedures were used for *Acropora* spp. The gametes from each coral were outcrossed among aquaria and transferred to closed system rearing tanks (200 L) for larval development. Whether juvenile corals initially acquire their symbionts pre-settlement (as larvae) (Adams et al. 2009; Cumbo et al. 2013) or post-settlement (Harrison and Wallace 1990) (as recruits) is currently unknown in the definitive sense, however, the timing *in situ* is likely to be dependent on differences in the regional abundances of free-living *Symbiodinium* cells (Littman et al. 2008). In the present study, post-settlement and firmly attached juveniles were used due to the high water-flow (described below) of the open system. After four days of larval development, pre-conditioned terracotta tiles (5 cm × 5 cm) were collected from the reef-flat of Heron Reef and cleaned of filamentous algae before introduction to rearing tanks as settlement substrate. The settlement tiles were suspended approximately 1 cm from the rearing tank bottom to allow larvae to settle on both sides of the tile.

Larvae were given four days to settle and metamorphose. A census count of juveniles on each tile was conducted and those tiles with sufficient numbers of recruits were maintained for the experiment. From selected tiles, *A. millepora* settled at a density of 0.84 recruits cm^{-2} (± 0.11) and *A. selago* at 0.83 recruits cm^{-2} (± 0.18) (values are means ± SE). The above process was repeated for *I. palifera* larvae, except that tiles were provided immediately following collection.

5.3.4 Experimental design

The following treatments were initiated immediately following the spawning event. Seawater was filtered through a zelbrite-sand filter (16 µm, Crystal Clear 21, Davey, Victoria, AUS) to remove particulates and sediment before distribution to the treatment aquaria (20 × 1 L), at a high flow rate of 0.4 L min^{-1}. The selected settlement tiles were randomized among four treatments (Fig. 5.1): seawater (SW); seawater + adult coral (SWC); sediment (SED); and sediment + adult coral (SEDC) (N = 5 aquaria per treatment). Sediment used in SED and SEDC treatments was collected from the reef flat and passed through a stainless steel, woven-wire sieve (Endecott, London, UK) with a mesh size of 2000 µm, to standardize grain size. Sediments were further standardized *via* sterilization (134ºC for 20 min) and rinsing with 0.22 µm filtered seawater (FSW) to remove the microphytobenthic community and any potential confounding free-living *Symbiodinium* cells. Approximately 150 mL of autoclaved sediment (1.5 cm depth) was distributed to SED and SEDC treatments. Adult coral branches (5 cm × 1 cm) were collected from the recently spawned colonies for SWC and SEDC treatments, and three branches placed in each aquarium. The mean symbiont density for *A. millepora* and *A. selago* branches was 1.89 × 10^6 cells cm^{-2} (± 0.08 × 10^6) and 1.91 × 10^6 cells cm^{-2} (± 0.04 × 10^6), respectively.

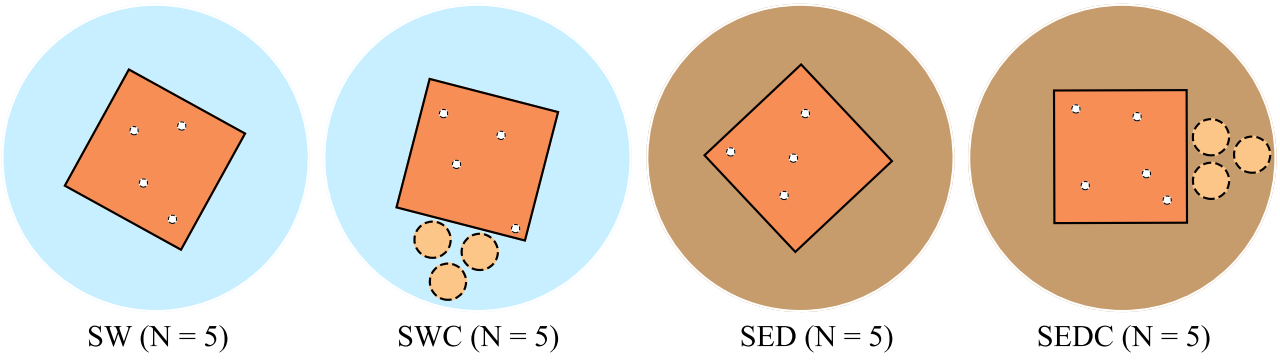

Figure 5.1 The experimental design used for *Acropora millepora*, *Acropora selago* and *Isopora palifera*. Settlement tiles (squares) with recruits were randomized among 1 L treatment aquaria (closed circles): seawater (SW); seawater + adult coral (SWC); sediment (SED), sediment + adult coral (SEDC). The adult coral branches are represented as hashed circles. Note: The experiment with *Isopora palifera* contained single adult branches.

The above process were repeated for *I. palifera* larvae, however, due to the large diameter of the branches, only a single adult fragment was introduced to the SWC and SEDC treatments. The mean symbiont density for *I. palifera* branches was 1.46×10^6 cells cm^{-2} ($\pm 0.04 \times 10^6$).

5.3.5 Symbiont acquisition and mortality

Each recruit was investigated for the acquisition of *Symbiodinium* cells through visual counting at six time points: *A. millepora* after 4, 6, 7, 9, 11 and 12 days of exposure to treatment conditions, and *A. selago* after 3, 5, 7, 9, 11 and 12 days. *I. palifera* recruits were investigated for the acquisition of *Symbiodinium* cells each day for 8 days. Settlement tiles were transferred to a microscope dish and a 2 min acclimation period was allowed for the recruits to assume regular morphology (i.e. full extension of tentacles). Light for microscopy was supplied in two directions (above and horizontal) to provide contrast and aid in the detection of *Symbiodinium* cells within the coral tissues. Aposymbiotic recruits were translucent and devoid of pigmentation, with the developing skeleton clearly visible under the tissue. A recruit was recorded as 'symbiotic' if *Symbiodinium* cells were clearly visible in the developing polyp (Fig 5.2). Recruits that did not follow typical morphological development were not included in counts and were considered as dead. The number of recruits at the end of the experiment was subtracted from the initial number of coral recruits on each tile and converted to a relative percentage for comparisons of mortality among treatments.

5.3.6 Free-living Symbiodinium *cell counts*

Following the final score for symbiont acquisition, each aquarium was investigated for the presence of free-living *Symbiodinium*-like cells. The sediment and interstitial water from the SED and SEDC treatments were transferred to a stainless steel, woven-wire sieve array (Endecott, London, UK) with mesh sizes of 1000, 500, 250, 125 and 63 µm. The sediment was spread evenly across the sieves and the interstitial water was allowed to drain and collect in a reservoir. A sub-sample of 200 mL was taken from the reservoir and concentrated through centrifugation at 3000 × g for 5 min to create a pellet of all the benthic micro-organisms removed from the sediment. The supernatant was decanted and the pellet re-suspended in 1 mL FSW. Each sample was preserved with 4% formalin for counting at a later date. The above process was repeated with 1 L of water from the SW and SWC treatments without the sieve processing.

To quantify the density of *Symbiodinium* cells retrieved from each sample, the conventional plankton identification method of brightfield microscopy was used (as in (Littman et al. 2008)). The number of *Symbiodinium* cells in each sample was quantified using an Improved Neubauer haemocytometer (Boeco, Hamburg, Germany) for counting (N = 8 counts per sample), using *Symbiodinium* cells from the adult branches as a reference and the general cell characteristics of *Symbiodinium,* including: a golden brown colour; spherical shape; reticulated chloroplast; and a large accumulation body and/or pyrenoid.

5.3.7 Statistical analyses

Each species was analyzed in independent tests. Nonlinear regressions were fit to the symbiont acquisition data of each treatment and an extra-sum-of-squares test confirms whether data sets differ from one another. The exponential model, $Y = START \times e^{(KX)}$, was fit to the data of each treatment, where Y is symbiont acquisition and X is time. At X=0, Y equals START and Y increases geometrically with a doubling time equal to 0.6932/K. Differences in mortality and free-living *Symbiodinium* counts were analyzed with one-way ANOVA, with Tukey *post hoc* comparisons to locate significant differences. The Shapiro-Wilk normality test and Levene's homogeneity of variance test were performed on all data to ensure that the assumptions of the analyses were met. Due to the low number of *I. palifera* larvae, post-settlement recruits could be followed individually throughout the duration of the experiment. Kaplan-Meier survival curves for each treatment were generated from symbiont acquisition and mortality data. The Mantel-Cox test was performed for all permutations of between-treatment curve comparisons to locate significant differences. Graphpad Prism (version 6.02) was used to perform all analyses and all results presented were tested against an alpha value of 0.05.

5.4 Results

5.4.1 Symbiont acquisition by Acropora *spp*

Similar patterns of symbiont acquisition were observed for the two broadcast spawning coral species (*Acropora millepora* and *A. selago*) and the distributions were described with non-linear regression (Table 5.1). The first appearance of symbionts in *A. millepora* was observed after 7 days in the SWC treatment only. Following this, symbiont acquisition occurred rapidly and symbiotic recruits were

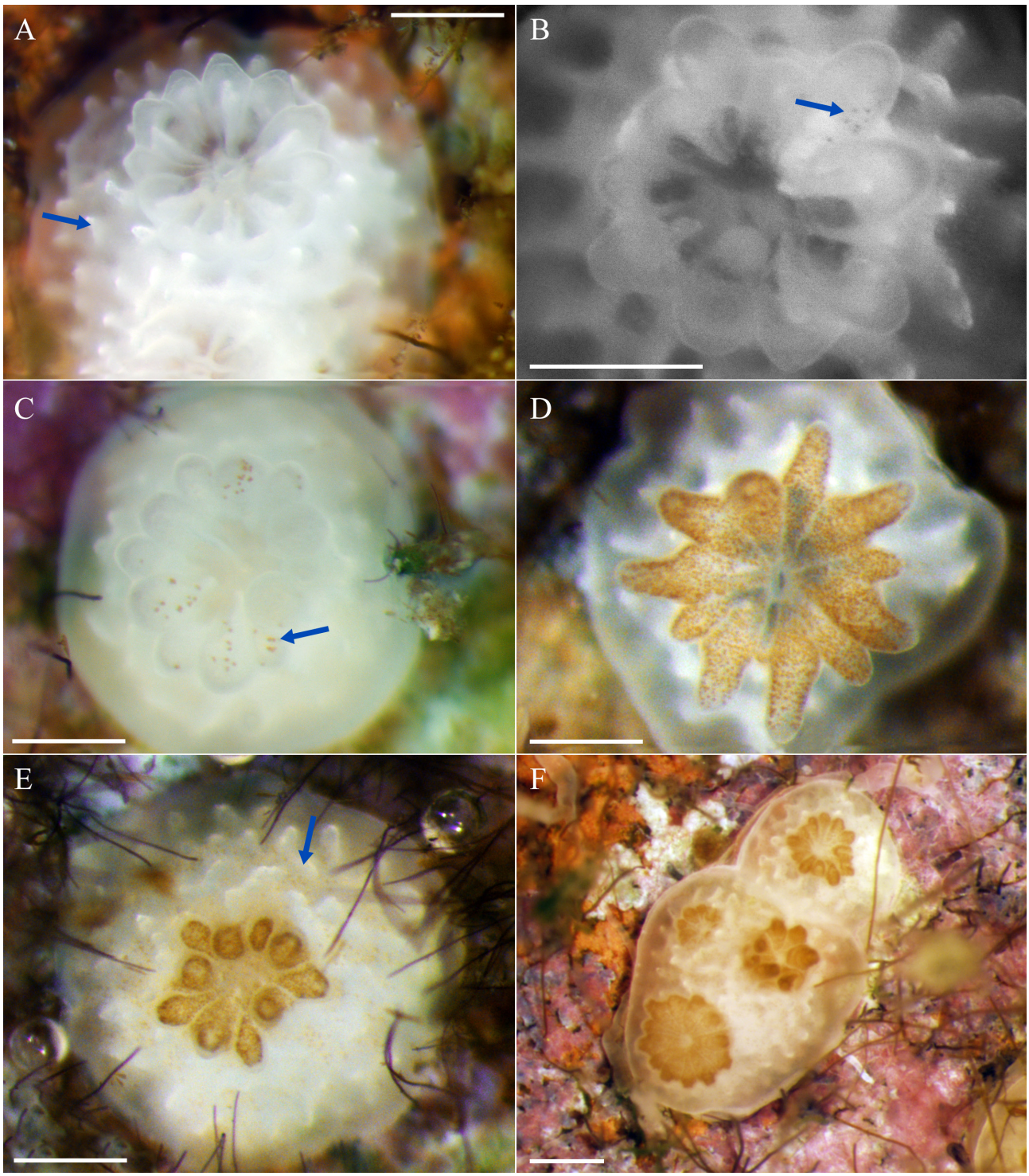

Figure 5.2 *Acropora millepora* recruits and the process of symbiont acquisition: A) a translucent aposymbiotic recruit with clear development of underlying skeleton (arrow); B) black and white, high contrast microscopy detailing the first observation of *Symbiodinium* cells (arrow) in a single tentacle; C) the distribution of *Symbiodinium* cells among multiple tentacles and apparently proliferating *Symbiodinium* cells (arrow); D) *Symbiodinium* cells densely populating tentacles; E) distribution of *Symbiodinium* cells across the recruit body (arrow) and F) highly pigmented corals with budding polyps. Scale bars = 200 µm.

observed in all other treatments. After 11 days, up to 48% (± 7.9) of juvenile corals had acquired *Symbiodinium* cells in the SEDC treatment (Fig. 5.3.A). After 12 days of exposure, the SW treatment had the lowest percentage of symbiotic *A. millepora* juveniles of the four treatments at 19.06% (± 5.75). The SWC and sediment-alone SED treatments produced an intermediate acquisition response at 46.63% (± 3.54) and 51.77 (± 3.71) respectively. The SEDC treatment produced 72.62% (±3.61) acquisition by the end of the experiment. The SEDC and SED treatments exhibited strong goodness-of-fit to an exponential curve, with $R^2 = 0.92$ and $R^2 = 0.94$, respectively (Fig. 5.3.A, Table 5.1). A comparison-of-fit test indicated that the data have significantly different data distributions ($F_{(6,112)} = 37.93$, $P < 0.0001$). *Acropora selago* juveniles followed the same pattern of symbiont acquisition as *A. millepora*, however the first symbiotic recruit was observed two days earlier in the SWC treatment than for *A. millepora*. After 12 days of exposure, the SW treatment had the lowest percentage of symbiotic juveniles at 14.67% (± 2.78). In comparison, the SWC and SED treatments produced an intermediate acquisition response at 38.49% (± 10.22) and 42.36 (± 3.84) respectively, while the SEDC treatment produced 70.4% (± 2.43) acquisition, greater than all other treatments. The goodness-of-fit for the exponential curves of the SEDC and SED treatments was strong with $R^2 = 0.94$ and $R^2 = 0.82$, respectively (Fig. 5.3.B, Table 5.1). A comparison-of-fit test indicated that the data have significantly different data distributions ($F_{(6,112)} = 49.16$, $P < 0.0001$).

5.4.2 Mortality of Acropora *spp*

Different patterns of mortality were observed for *Acropora* spp. Mortality in each treatment was highly variable between treatments for *Acropora millepora* (one-way ANOVA, $F_{(3, 16)} = 1.969$, $P = 0.1593$). For example, mortality on individual tiles in the SW treatment ranged from 13.11% to 78.57%. There was no significant difference across all treatments; 41.53% (± 12.57), 21.8% (± 6.82), 26.76% (± 8.67) and 43.14% (± 7.96) of juveniles died over the course of the experiment in the SW, SWC, SED and SEDC treatments, respectively (Fig. 5.3.C). In contrast with *A. millepora,* there were differences in the mortality of *A. selago* juveniles between treatments (one-way ANOVA, $F_{(3, 16)} = 5.727$, $P = 0.0074$). The mortality of recruits in the SWC treatment was lower than in the SED and SEDC treatments (Tukey *post hoc* test, $p = 0.0061$ and 0.0331, respectively). The mortality after 12 days was higher overall than for *A. millepora,* with 69.03% (± 3.44), 45.39% (± 5.04), 79.87% (± 6.60) and 72.46% (± 9.22) mortality over the course of the experiment for the SW, SWC, SED and SEDC treatments, respectively (Fig. 5.3.D).

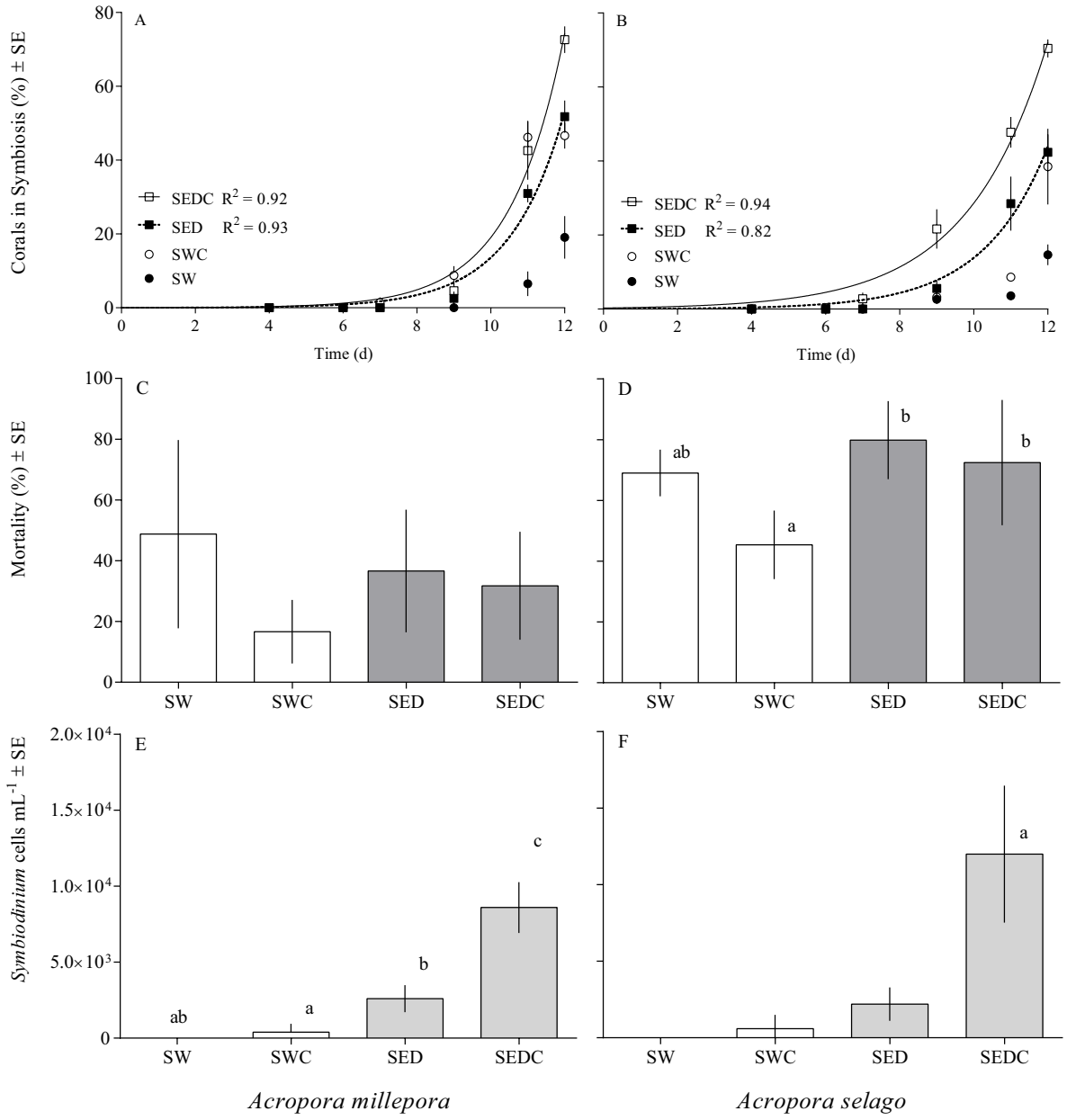

Figure 5.3: Symbiont acquisition, free-living *Symbiodinium* cell density and recruit mortality for *Acropora millepora* and *Acropora selago*. Figures on the left (A, C, and E) are data for *A. millepora* and figures on the right (B, D, and F) for A. selago. Mean percentages of symbiotic recruits (A, B) in: seawater (SW, closed circle); seawater + adult coral (SWC, open circles); sediment alone (SED, closed squares); and sediment + adult coral (SEDC, open squares) treatments. Nonlinear regressions (lines) of SED (hashed lines) and SEDC (complete lines) represent exponential growth models (note: not all regressions are presented). Recruit mortality (C, D) and free-living *Symbiodinium* densities (E, F) are represented as bars. All values are means ± 1 SE (N = 5). Significant differences are indicated by Tukey's *post hoc* test groups and are denoted by lower case letters, a-c.

Table 5.1 Model parameter estimates of nonlinear regressions for symbiont acquisition in: seawater (SW); seawater + adult coral (SWC); sediment alone (SED); and sediment + adult coral (SEDC) treatments. When time (X) = 0, symbiont acquisition (Y) equals START and Y increases geometrically with a doubling time equal to 0.6932/K.

		Acropora millepora		
Treatment	**SW**	**SWC**	**SED**	**SEDC**
Start	0.0000	0.2477	0.0209	0.0148
K	1.111	0.4465	0.6813	0.6823
Doubling time	0.6237	1.552	1.017	1.016
Start 95% CI	-0.0002698 to 0.0003315	-0.1444 to 0.6397	-0.02046 to 0.06234	-0.01120 to 0.04078
K 95% CI	0.2930 to 1.930	0.3096 to 0.5834	0.5132 to 0.8493	0.5330 to 0.8317
Doubling time 95% CI	0.3592 to 2.366	1.188 to 2.239	0.8161 to 1.351	0.8334 to 1.301
Degrees of freedom	28	28	28	28
R^2	0.6251	0.8508	0.9238	0.9393

		Acropora selago		
Treatment	**SW**	**SWC**	**SED**	**SEDC**
Start	0.0000	0.0000	0.2819	0.0431
K	1.262	1.442	0.462	0.5773
Doubling Time	0.5494	0.4806	1.5	1.201
Start 95% CI	-2.624×10^{-5} to 3.399×10^{-5}	-1.144×10^{-5} to 1.377×10^{-5}	-0.0001346 to 0.5640	-0.06709 to 0.1532
K 95% CI	0.6099 to 1.913	0.5400 to 2.345	0.3756 to 0.5483	0.3586 to 0.7959
Doubling Time 95% CI	0.3623 to 1.136	0.2956 to 1.284	1.264 to 1.846	0.8709 to 1.933
Degrees of freedom	28	28	28	28
R^2	0.7678	0.7213	0.9381	0.821

5.4.3 Free-living Symbiodinium *cell counts*

The abundance of *Symbiodinium* cells varied significantly between treatments for the *Acropora millepora* (one-way ANOVA, $F_{(3, 16)} = 80.75$, $P < 0.0001$) and *A. selago* (one-way ANOVA, $F_{(3, 16)} = 28.62$, $P < 0.0001$) experiments. In the experiment with *Acropora millepora*, although no free-living *Symbiodinium* cells were observed in the SW treatment, cell counts in the SWC, SED and SEDC treatments were significantly different from one another (Fig. 5.3.E, Tukey *post hoc* test, $p < 0.05$ for all comparisons). The SWC treatment had the lowest *Symbiodinium* density at 2.0×10^2 ($\pm 2.44 \times 10^2$) cells mL^{-1}, while the SED and SEDC treatments contained 2.6×10^3 ($\pm 4.0 \times 10^2$) and 8.6×10^3 ($\pm 7.48 \times 10^2$) cells mL^{-1}, respectively. For *A. selago*, differences in the density of *Symbiodinium* cells were only apparent in the SEDC treatment (Fig 5.3.F, Tukey *post hoc* test, $p < 0.001$ for all comparisons). Again, no free-living *Symbiodinium* cells were observed in the SW treatment. In contrast, the SWC treatment contained a relatively low density of *Symbiodinium* cells at 6.0×10^2 ($\pm 4.10 \times 10^2$) cells mL^{-1}, while the SED and SEDC treatments contained 2.2×10^3 ($\pm 4.9 \times 10^2$) and 1.2×10^4 ($\pm 2.0 \times 10^3$) cells mL^{-1}, respectively.

5.4.4 Isopora palifera

Symbiont acquisition and mortality were investigated for the brooding coral *Isopora palifera* using survival analysis. There was a significant difference in the Kaplan-Meier curve for the SEDC treatment when individually compared with each treatment (Table 5.2, Fig 5.4.A, Mantel-cox test, $P < 0.05$ for all comparisons). The first symbiotic juveniles were observed after four days in the SED and SEDC treatments. The rate of acquisition was significantly greater in the SEDC treatment, and after nine days, 61.53% of the initial recruits were symbiotic, compared with 18%, 18% and 27.27% in the SW, SWC and SED treatments, respectively. There were no significant differences in the Kaplan-Meier mortality curves between any treatments and only two or three individuals had died in each treatment after more than 9 days (Fig 5.4.B).

Table 5.2 The significant comparisons of the Kaplan-Meier curves for the symbiont acquisition of *Isopora palifera* in: seawater (SW); seawater + adult coral (SWC); sediment alone (SED); and sediment + adult coral (SEDC) treatments. P values are Mantel-cox test results and non-significant comparisons are not presented.

	Significant comparisons		
	SW - SEDC	**SWC - SEDC**	**SED - SEDC**
X^2	4.951	7.064	4.498
Degrees of freedom	1	1	1
P value	0.0261	0.0079	0.0339

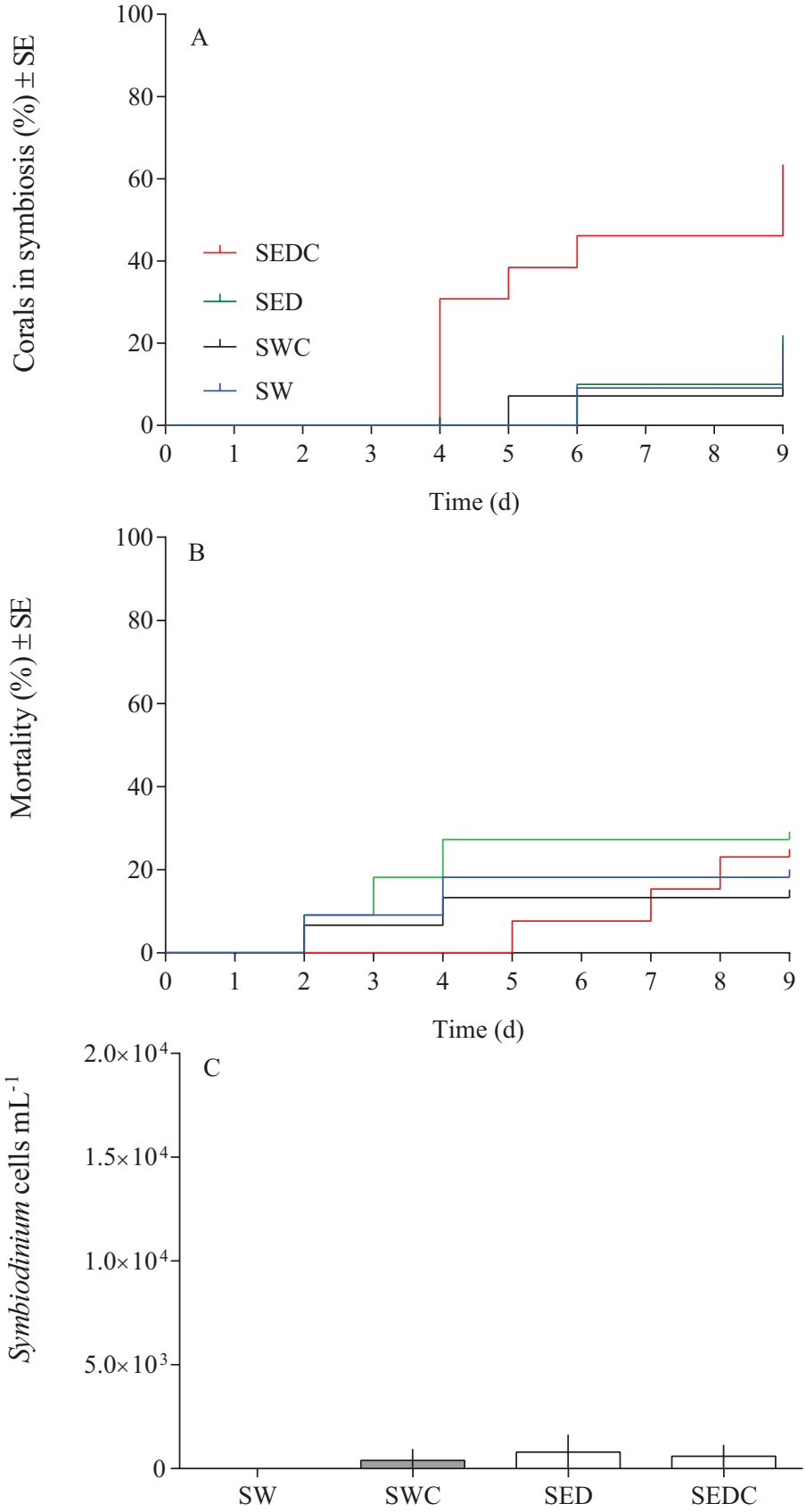

Figure 5.4 Kaplan-Meier curves for symbiont acquisition (A) and mortality (B) of *Isopora palifera* recruits in: seawater (SW, blue); seawater + adult coral (SWC, black); sediment alone (SED, green); and sediment + adult coral (SEDC, red) treatments. Free-living *Symbiodinium* densities (C) are represented as bars (note: for comparitive purposes, the y-axis is identical to Fig. 5.3E and F). All values are means ± 1 SE (N = 5).

For *I. palifera,* the density of free-living *Symbiodinium* cells in each treatment was low in comparison with the previous experiments for the *Acropora* spp, and no significant differences were observed between treatments (one-way ANOVA, $F_{(3, 16)} = 1.795$, $P = 0.1887$). No *Symbiodinium* cells were seen in the SW treatment, however low densities of *Symbiodinium* cells were seen in the SWC, SED and SEDC treatments, with an average of 6.0×10^2 ($\pm 2.8 \times 10^2$) cells mL^{-1} (Fig. 5.4.C).

5.5 Discussion

In our study, reef-building coral recruits were exposed to a conspecific adult (one of three species), combined with a known microhabitat of free-living *Symbiodinium* cells (sediment). In this treatment, up to a five-fold increase in the number of symbiotic juveniles was detected in the presence of adult corals and sediment, compared to when juveniles were incubated in unfiltered seawater only. This result was consistent across three coral species, representing two reproductive strategies. Although previous studies have shown that juvenile corals can draw their symbionts from the benthos (Adams et al. 2009; Cumbo et al. 2013), this is the first study to link adult corals with an increased abundance of benthic free-living *Symbiodinium* cells and enhanced symbiont acquisition by recruits. The findings of this study provide three novel insights. First, we identified that a small proportion of juvenile corals acquired symbionts from seawater alone, suggesting that there are free-living *Symbiodinium* cells in suspension at Heron Reef and that these may therefore be widespread in this ecosystem. Secondly, *Symbiodinium*-like cells found in coral-exposed sediment were at least twice as abundant as in all other treatments. This suggests that reef-building corals play an important role in the localized abundances of benthic free-living *Symbiodinium* cells. Thirdly, the increased rate of symbiont acquisition by recruits when incubated with sediment and adult corals suggests that benthic sediment aids the horizontal transmission of *Symbiodinium* cells between adult and juvenile corals.

Our results suggest that seawater alone may be a source of *Symbiodinium* cells for recruiting corals. However, in the seawater treatment, only 19%, 14% and 18% of *Acropora millepora, Acropora selago* and *Isopora palifera* recruits, respectively, acquired *Symbiodinium* cells by the end of the experiment. This is consistent with a previous report of *Acropora monticulosa* larvae that acquired *Symbiodinium* cells from seawater after 12 days (Adams et al. 2009). Free-living *Symbiodinium* cells have been isolated from the water column (Koike 2007; Littman et al. 2008; Manning and Gates 2008), and Littman et al. (2008) estimated there to be as few as 14 cells mL^{-1} in suspension (Lizard Island, GBR, Australia). *Symbiodinium* cells entering through the aquarium system in our study were below visual levels and this low abundance likely explains why so few recruits acquired

Symbiodinium in the SW treatment. Successful infection experiments have been conducted with various densities of *Symbiodinium* cells in the water (*in vitro*). Up to millions of cells mL^{-1} (Harii et al. 2009) and as few as one *Symbiodinium* cell per larva (Yamashita et al. 2014) have been used to infect coral larvae, yet the density dependence of *Symbiodinium* acquisition from the water column requires further investigation *in situ*. At present, it is unclear whether free-living *Symbiodinium* cells are only temporarily in suspension or are perennial members of the phytoplankton. Conceivably, the *Symbiodinium* cells acquired by the recruits in the seawater-only treatment may indicate the presence of a background population of free-living *Symbiodinium* cells associated with the settlement tile microhabitats (Coffroth et al. 2001). Venera-Ponton et al. (2010) reported free-living *Symbiodinium* cells from algal turfs at Heron Reef, and although all settlement tiles in the present study had algal turfs removed, some epiphytic *Symbiodinium* cells may have remained. Controlling for this potential source of *Symbiodinium* cells is difficult, as the successful removal of the microalgal/microbial community from settlement substrates might disrupt the natural metamorphosis of the larvae (Webster et al. 2004).

A supply of homologous symbionts from a nearby conspecific adult may assist juveniles with reaching a stable symbiosis. Over a period of 12 days, an intermediate acquisition response was observed, where approximately 40% and 50% of *Acropora* spp recruits established symbioses when incubated in seawater + coral and sediment only, respectively. In the case of seawater + coral, it is possible that *Symbiodinium* cells released from the coral branches in the aquaria made contact directly with the recruit or with the settlement tile (and subsequently with the recruit). Indeed, a low concentration of '*Symbiodinium*-like' cells was visible in the water column in this treatment. It should be noted, however, that the identity of the *Symbiodinium* cells acquired by juvenile cnidarians does not always match those present in adult colonies (Gomez-Cabrera et al. 2008; Abrego et al. 2009b). For example, some *Symbiodinium* types have been described as opportunistic and highly infectious (Gomez-Cabrera et al. 2008; Abrego et al. 2009b; Stat and Gates 2010), and corals which host these more 'selfish' types may grow up to 38% slower than those with the typical dominant type (Jones and Berkelmans 2010). It can take up to three years for *Symbiodnium* populations of juveniles to reach equilibrium and match what is observed in adults of the same region (Abrego et al. 2009a). Whether this period is reduced by the proximity to a conspecific adult would be a valuable future study.

Free-living *Symbiodinium* cells are constituents of the microphytobenthos (Hirose et al. 2008; Littman et al. 2008; Reimer et al. 2010; Takabayashi et al. 2012; Yamashita and Koike 2013), and there may be

type-specific differences in the capacity to persist within sediments (Nitschke et al. 2015). Sterilized sediments were used in the present study to reduce the potential confounding effect of an ambient population of *Symbiodinium* cells (Littman et al. 2008). It is assumed therefore, that the '*Symbiodinium*-like' cells harvested from the interstitial waters of the sediment-alone treatment moved from the settlement tiles or entered through the flow-system of the aquaria. Adams et al. (2009) reported that 80% of *Acropora monticulosa* larvae acquire *Symbiodinium* cells from natural sediment after only six days of exposure. A similar result was reported for *Acropora tenuis* and *Acropora millepora* larvae after six days of exposure (Cumbo et al. 2013), with natural sediment from three different locations on the GBR. In the sediment treatment in the present study, acquisition reached 51% and 42% for *A. millepora* and *A. selago*, respectively, after 12 days. The slower acquisition rate in the present study than in previous studies may be due to the different state of the sediments used in treatments (and also that previous studies used larvae rather than juvenile recruits). As the present study used sterilized sediment, this suggests that *Symbiodinium* cells can quickly colonize or accumulate in the sediment, which may naturally filter free-living *Symbiodinium* cells from the water column.

The symbioses of adult and juvenile corals may be linked by the microhabitats of free-living *Symbiodinium* cells. When coral recruits were simultaneously incubated with sediment and adult coral, symbiont acquisition reached its highest percentage (>70% of recruits) after 12 days for *Acropora* spp, and after 9 days for *Isopora palifera* (>60% of recruits). We suggest that *Symbiodinium* cells frequently expelled from the adult corals accumulate in the sediment where they may persist, either transiently or indefinitely (Nitschke et al. 2015). *Symbiodinium*-like cells were present in the sediment at a density of approximately 8.6×10^3 and 12.0×10^3 cells mL^{-1} in the *A. millepora* and *A. selago* experiments, respectively. In comparison, relatively few *Symbiodinium* cells were detected in the presence of *I. palifera* (6.0×10^2 cells mL^{-1}), though, a higher rate of symbiont acquisition was still observed in this treatment. The comparatively lower abundance of *Symbiodinium* cells in the *I. palifera* incubation may well reflect the lower number of coral branches used (one *versus* three). We suggest that free-living *Symbiodinium* cells become trapped in the sediment and are either disturbed from the seabed or eventually become motile (Yacobovitch et al. 2004). The predominant mechanism of transmission is unknown, but a parsimonious suggestion is some combination of motile *Symbiodinium* cells actively infecting corals, and the chance ingestion of coccoid cells recently expelled from hosts.

Early post-settlement mortality is typically high for coral recruits (Wilson and Harrison 2005; Penin et al. 2010) and given the widely demonstrated importance of the *Symbiodinium* symbiosis, we were

surprised that there were no prominent differences in mortality between the treatments. These data suggest that the failure to acquire symbionts in the early stages of settlement (< 2 weeks) does not have a significant effect on survivorship in this experimental situation. Graham et al. (2008) reported that aposymbiotic coral larvae can survive in the water column for over 200 days *in vitro*, and therefore recruits may survive for extended periods without *Symbiodinium* cells. It would be valuable to consider whether recruits acquire different types of *Symbiodinium* from different microhabitats, and whether there are long-term growth or mortality implications as a result of this.

Our research has demonstrated a clear pattern in the acquisition of *Symbiodinium* cells for both broadcast spawning and brooding coral species. We suggest that the brooded larvae of other species probably encounter and acquire (and least temporarily) free-living *Symbiodinium* cells through similar processes (Byler et al. 2013). It appears that adult conspecifics supply *Symbiodinium* cells to substrata from which juvenile corals can draw their symbionts. It therefore follows that juveniles recruiting to recovering or damaged reefs devoid of specific *Symbiodinium* cells and their potential sources may be limited in exposure to their crucial symbionts. The presence or absence of symbiosis-ready, free-living *Symbiodinium* types could prove to be a new ecological aspect of coral reef health worthy of monitoring. The ecology of free-living *Symbiodinium* types and their potential to form symbioses with juvenile corals is an emerging field that warrants further investigation, especially in light of the many concerns for coral reef ecosystems.

5.6 Acknowledgments

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Chapter 6: General discussion

Coral recruitment and symbiont acquisition are inextricably bound processes that function in the maintenance and recovery of coral reef ecosystems (Bellwood et al. 2004). As *Symbiodinium* spp. can contribute to coral resilience during physical stress (Rowan et al. 1997; Berkelmans and Van Oppen 2006; Sampayo et al. 2008), the ecology and physiology of free-living (FL) *Symbiodinium* cells are indirect but important parameters of coral reef health. It is thought that adult corals do not acquire FL *Symbiodinium* cells (Coffroth et al. 2010) and are, therefore, limited to the complement of *Symbiodinium* types acquired during the early stages of coral ontogeny. This is concerning in that coral colonies reach significant age and are likely to experience a rapidly changing climate (Hughes et al. 2003; Hoegh-Guldberg et al. 2007).

Little is known regarding the capacity of *Symbiodinium* spp. to persist as free-living cells. There is also some confusion relating to whether some *Symbiodinium* types are exclusively FL or transiently FL. To reach a comprehensive understanding of cnidarian/*Symbiodinium* symbioses requires: data for the distribution and microhabitats of FL *Symbiodinium* cells; data for the life cycle of FL *Symbiodinium* spp. and the importance of cell morphologies (i.e. coccoid or mastigote); data for distinguishing between putatively FL and transiently FL cells; and a characterisation of how FL *Symbiodinium* populations first contact hosts. The aim of this thesis was to address these issues and experimentally manipulate populations of free-living *Symbiodinium* cells to characterise the connections between known microhabitats of FL *Symbiodinium* cells and scleractinian coral hosts.

The major findings of each chapter are summarised in a conceptual diagram (Fig. 6.1) on the following page.

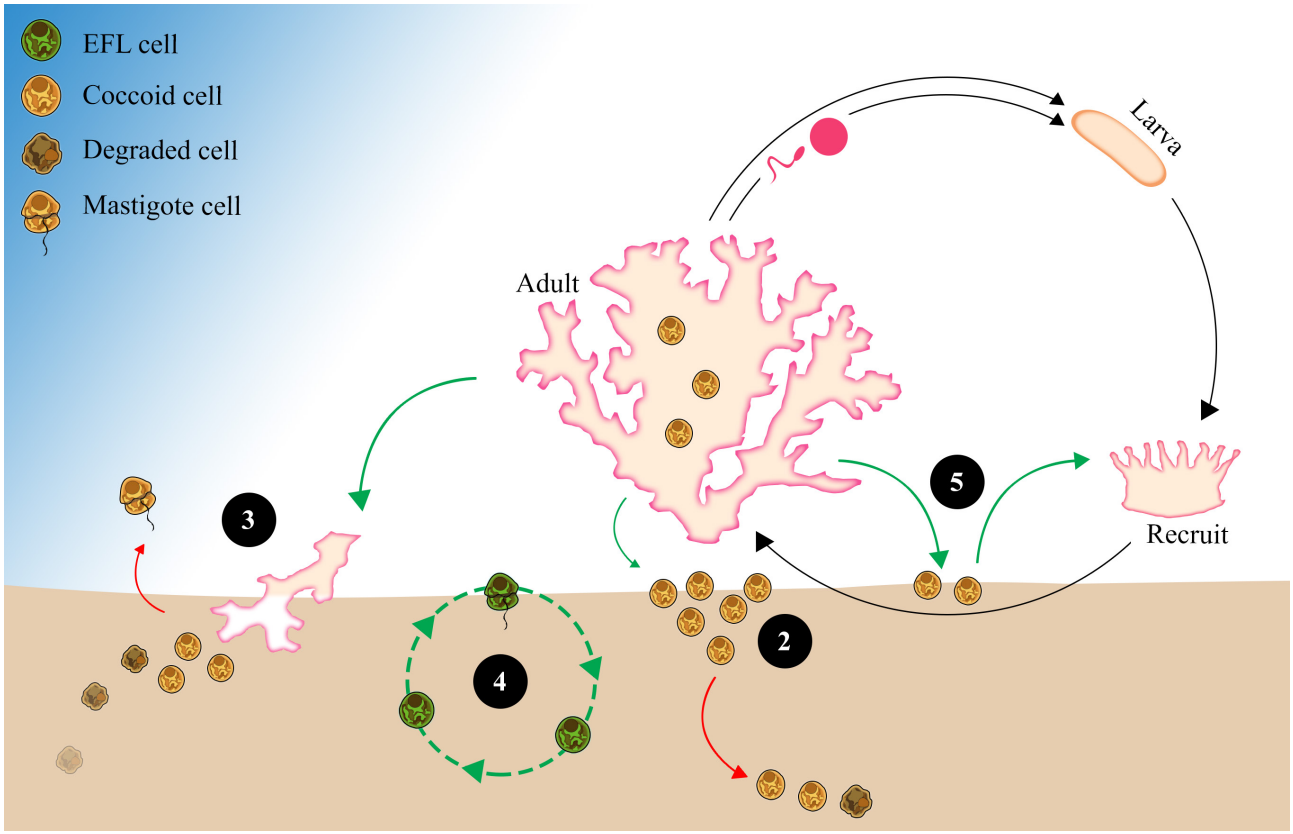

Figure 6.1 Some described and conceptual links between adult corals, sediment, free-living *Symbiodinium* cells and recruiting corals. Black arrows represent the coral life history. Green arrows represent processes observed in this thesis research (black circles refer to chapters). Red arrows represent theorised, but not yet observed processes. Chapter 2: The seeding of sediment by coral colonies and the diffusion or loss of cells. Chapter 3: The bleaching and degradation of coral tissues when in contact with sediment and transiently free-living *Symbiodinium* cells. A small portion of these cells may become motile. Chapter 4: Exclusively free-living *Symbiodinium* cells that can persist in sediment, interchanging between coccoid and motile cells. Chapter 5: Horizontal transmission of free-living *Symbiodinium* cells aided by sediment.

Chapter 2 *Free-living* Symbiodinium *cells at the base of translocated scleractinian coral colonies*

I explored the potential for a link between adult scleractinian corals and FL *Symbiodinium* cells. I aimed to determine if *Acropora millepora* colonies contribute to the standing stock of FL *Symbiodinium* cells and hypothesized that cell abundances in reef sediment would be directly related to the presence and proximity of colonies. I found evidence for a previously undescribed link between a source population of *Symbiodinium* cells (the coral host) and sediment associated FL *Symbiodinium* cells in support of these hypotheses. An eight-fold increase in the abundance of FL *Symbiodinium* cells at the immediate base of translocated *A. millepora* colonies was evident (relative to baseline abundances). This was consistent between all three sites on Heron Reef (Great Barrier Reef) and was sustained throughout the sampling period. No seasonal patterns in *Symbiodinium* cell abundance were detected, but elevated abundances of *Symbiodinium* cells were periodically detected up to 25 cm from the coral colonies.

Acropora millepora was an ideal species to use in the present study in that: the species has been extensively studied; there is an abundance of mature colonies at Heron Reef; and it was also used for coral spawning work (**chapter 5**). However, the patterns produced in this study using the coral *Acropora millepora* as a source of *Symbiodinium* cells may not reflect what occurs across all coral species. For example, the rate of *Symbiodinium* cell expulsion varies significantly between coral species (Stimson et al. 2002). If *Symbiodinium* cells are only released at low rates (or colonies are situated in areas of high water flow), they may be removed from the surrounding area through diffusion and advection before accumulations can be observed. Therefore, large aggregations of FL *Symbiodinium* cells may be common in stable lagoonal or reef-flat areas of coral reefs, as in the present study, and by Littman et al. (2008).

The filtering of sediment and manual counting of *Symbiodinium* cells requires extensive processing time (Littman et al. 2008), and in the present study this prevented a multi-coral species approach. The development of molecular techniques from qualitative, 'presence or absence' methods (such as denaturing gradient gel electrophoresis – DGGE), towards quantitative, next-generation methods (such as qPCR and gene barcoding) will eventually provide a protocol for the robust determination of FL *Symbiodinium* cell abundance. Previous studies that have attempted to extract and amplify *Symbiodinium* cell DNA from sediment samples have had their reagents inhibited by unidentified substrates, suggesting that sample purification methods will also need to be further developed (Erdner et al. 2010).

One implication from this study is that coral recruits that draw their symbionts from the environment may benefit from settling near a conspecific individual through exposure to a pool of prospective

symbionts. Thus, aposymbiotic juveniles recruiting to recovering or damaged reefs lacking specific *Symbiodinium* types (and their sources), may receive little exposure to potential symbionts. Coral colonies at the margins of their range distributions will provide natural laboratories for assessing how the populations of FL *Symbiodinium* cells and hosts are correlated. For example, at the high latitude coral reefs of Lord Howe Island, *Symbiodinium* endemicity has been described in corals that acquire symbionts both horizontally or vertically (Wicks et al. 2010). Deploying aposymbiotic organisms as FL *Symbiodinium* sampling arrays (Coffroth et al. 2006) in such locations may provide an interesting snapshot of the symbionts that are available to hosts, and how they compare with the local endosymbiotic populations. It is conceivable that populations of *Symbiodinium* cells will contain greater proportions of "free-living specialists" at higher latitudes or locations where only a few host species occur.

Chapter 3 Pocillopora damicornis *fragments seed the sediment with a pulse of free-living* Symbiodinium *cells*

I investigated whether *Symbiodinium* cells survive beyond coral tissue death. Small fragments of the coral *Pocillopora damicornis* were buried under a layer of carbonate sediment. Over a period of 12 days, F_v/F_m and the density of *in hospite* and free-living *Symbiodinium* cells were recorded as the buried coral became increasingly stressed. A bleaching response was initiated within two days of burial, and after seven days, significant loss of coral tissues had occurred. At this stage, the *in hospite Symbiodinium* population had declined dramatically (< 2%, relative to controls) and F_v/F_m fell to zero. A pulse of free-living *Symbiodinium* cells was observed in the sediment after four days, followed by a steep decline in FL *Symbiodinium* abundance over the following eight days, at which > 50% of cells in the sediment appeared degraded. These findings show that expelled *Symbiodinium* cells, although transiently free-living, can persist for a substantial period (at least seven days) in reef sediment.

The fragmentation of corals is a common occurrence (especially at the margins of colonies with branching morphologies), however, smaller pieces have a low chance of survival (Smith and Hughes 1999). Contact between sediment and corals has been reviewed extensively (Erftemeijer et al. 2012) and is generally described as negative for coral physiology. The major finding of this study is that *Symbiodinium* cells survive the mortality of sediment-covered *P. damicornis* branches and are ejected as a 'pulse' of cells. A pulse-release of *Symbiodinium* cells may ensure that types which are only transiently FL are periodically represented in the reservoir of FL *Symbiodinium* cells at high densities. For the many recruiting cnidarian species that initially draw their symbionts from the environment, the time-frame of viability observed in this study may be sufficient to make contact with their symbionts. However, this duration of viability also suggests that in **chapter 2**, the timeframe of

sampling following the removal of *Acropora millepora* colonies needed to have been within seven days of colony removal to detect the natural decline in *Symbiodinium* cell abundance.

Sediment microcosms have been widely used to replicate the habitats of microalgae and infaunal invertebrates (Caffrey et al. 1993; Enoksson 1993; Jönsson et al. 1993; Conley and Johnstone 1995; Tuominen et al. 1999), however confident extrapolation to the natural environment can be limited by the constraints of working *in vitro.* A well-designed microcosm will eliminate the technical problems inherent in field experiments and preserve most of the complexity of the *in situ* environment (Wagner-Döbler et al. 1992). In the present study (and in **chapter 4** below), the experimental sediment was sterilised and rinsed to remove any photosynthetic organisms that would confound the measurements of F_v/F_m. This was especially necessary for this experiment, as free-living *Symbiodinium* cells have been isolated from the benthos at the study location (Carlos et al. 1999; Pochon et al. 2010; Takabayashi et al. 2012). The process of sterilisation would have removed organic and inorganic nutrients that are present in natural sediment to an unknown degree, and it is possible that this would have altered the longevity of the recently expelled *Symbiodinium* cells. Moreover, free-living *Symbiodinium* cells are both predators (Jeong et al. 2012) and prey (Jeong et al. 2014), and the removal of infaunal microorganisms would also alter the longevity of recently expelled cells. It would be useful to manipulate these variables in future microcosm studies, and ultimately, such experiments are needed to predict which *Symbiodinium* spp. persist continuously outside of hosts.

Characterising the free-living phenotype of *Symbiodinium* spp. can aid the placing of symbioses on the parasitism-mutualism continuum. Moreover, investigating *Symbiodinium* cell viability following expulsion for correlation with traits such as: host specificity; cultivability; and mode of transmission, can test the evolutionary theories of parasitism and mutualism. By default, horizontal acquisition is the ancestral mode of transmission of *Symbiodinium* cells. Pressures which select for the minimisation of conflict between corals and *Symbiodinium* cells will favour certain traits, such as: vertical transmission of symbionts to conserve types with highly-integrated metabolisms; genetic uniformity of symbionts, which reduces intra-colony competition of *Symbiodinium* types; and little or no requirement of a free-living state (Lesser et al. 2013). A number of *Symbiodinium* types have been reported to be particularly ephemeral following expulsion or isolation from hosts. For example, when removed from the tissues of *Montipora* spp, *Symbiodinium* ITS2 type C15 cells degrade rapidly in seawater (Wang et al. 2011) and it is suggested that C15 has coevolved with corals to the point that it does not survive outside them (Krueger and Gates 2012). *Symbiodinium* type C15 appears to be transmitted vertically exclusively, and has only been observed as a free-living isolate by Venera-Ponton et al. (2010). For species that are on the various trajectories of adaptation from parasitism to mutualism, latency in the degradation of free-living *Symbiodinium* cells ensures that there is a window of opportunity for

acquisition. Future experiments should aim to determine whether significant environmental stress (for example elevated temperature, as apparent in **chapter 2**) shortens the window of this transiently free-living state, and if this in turn, influences the reservoir of FL *Symbiodinium* cells and the onset of symbiosis.

Free-living *Symbiodinium* cells may further extend their viability following ejection through mechanisms common to other dinoflagellates that are yet to be observed in *Symbiodinium* spp. For example, following sexual reproduction, dinoflagellates may enter a 'dormant cyst' stage where cells rapidly sink to the benthos (Pfiester 1975; Heiskanen 1993). These cysts are highly resistant to degradation and remain viable during extended periods of darkness and anoxic conditions (Dale 1983). While gametes of *Symbiodinium* cells have never been observed, sexual reproduction is suggested to occur at some stage of the *Symbiodinium* life cycle, based upon the high allelic diversity that has been described in the genus (Baillie et al. 2000; LaJeunesse 2005; Stat et al. 2006). In view of the fact that little is known about the life cycle of FL *Symbiodinium* cells *in situ*, it is conceivable that there are specialised morphologies of *Symbiodinium* spp common to other dinoflagellates (such as resting cysts and peduncles for heterotrophic feeding (Jeong et al. 2012)) which may allow for extended persistence in a free-living state.

Chapter 4 *The effect of elevated temperature and substrate on free-living* Symbiodinium *cultures*

I used thermal stress to challenge the cellular physiology of *Symbiodinium* cells in sediment microcosms. The physiology of an exclusively free-living (EFL) *Symbiodinium* type (belonging to clade A) was compared with a symbiosis-forming type (A1) under a range of temperatures (25, 28, 31 ºC) and culturing conditions (sediment, no sediment). The EFL *Symbiodinium* clade A reproduced faster in sediment than in cultures without sediment, and elevated temperature did not cause notable changes in the photobiology or motility of cells. In contrast, the symbiosis-forming *Symbiodinium* A1 exhibited depressed reproduction in sediment (relative to cultures without sediment), most severely at 31°C. The combination of elevated temperature and sediment caused a significant decline in the photosynthetic efficiency of cells and major reductions in cell motility. Thus, a comparison of the physiological responses of the two types supported the original hypothesis of the study, that the exclusively free-living type is better suited to survival in sediment than the symbiosis-forming A1. My findings indicate that there are type-specific competencies for survival in a natural habitat that may define the free-living assemblages of *Symbiodinium* cells.

Symbiodinium cell motility could prove to be a new aspect of cellular 'health' or function worthy

of measuring. Although it is unknown how cellular stress is reflected in cell motility or swimming behaviour, it is conceivable that the maintenance of cell motility will allow *Symbiodinium* cells to: locate hosts (Pasternak et al. 2006); navigate microhabitats; avoid permanent burial in substrates; and gain access to PAR. In the present study, only a moderate reduction (28%) in the dark-adapted yield of PSII (F_v/F_m) was detected for *Symbiodinium* type A1, at elevated temperatures. Contrastingly, the motility of cells was reduced by >50% during elevated temperatures and is, therefore, more sensitive to thermal stress. If similar reactions to elevated temperature are observed for other symbiosis-forming clades, vital behaviours for persistence in a FL state (such as vertical migration), and the location of potential hosts (Pasternak et al. 2006), may become increasingly impaired in the climate predicted for the future. The requirements of *ex hospite* survival for other important *Symbiodinium* types, and crucially, more of those that form symbioses, need to be explored.

I proposed that FL *Symbiodinium* cells may find refuge in light-reduced interstitial spaces of the sediment during thermal stress as the second hypothesis of this study. The data reported are inconsistent with this hypothesis, however further information is required to determine how *Symbiodinium* cells experience the micro-light environments of sediment. It would be useful to investigate how *Symbiodinium* cells position themselves throughout the light period, and measure the capacity of cells to navigate the interstitial spaces of benthic sediment. I have conducted preliminary work in this area, finding type-specific differences in the propensities of cells to migrate through a range of sediment size fractions. For example, *Symbiodinium* type B1 (a common endosymbiont of corals in the Caribbean (Franklin et al. 2012)) showed a stronger tendency to migrate vertically out of sediments than the EFL *Symbiodinium* clade A (the strain used in the present study). Contrastingly, EFL *Symbiodinium* clade A showed a strong tendency to migrate downwards into the sediment, opposite to the behaviour of *Symbiodinium* type B1. There have been few studies of *Symbiodinium* cell motility, and of these few, most have focused on rhythms associated with the cell life-cycle (Fitt and Trench 1983; Yacobovitch et al. 2004; Fujise et al. 2014). To better understand how free-living *Symbiodinium* cells persist in the environment, additional studies are needed where the cell motility of *Symbiodinium* types is challenged with various physical (i.e. temperature, salinity, and pH), mechanical (i.e. complex microenvironments) and chemical (i.e. host cues) conditions. The observed differences in motility between types of a single *Symbiodinium* clade may represent adaptations that are either conserved or lost depending on the differing selection pressures that come with living in symbiosis or free in the environment.

Chapter 5 *Benthic sediment aids the horizontal transmission of* Symbiodinium *cells between adult and juvenile corals*

I aimed to determine if juvenile corals acquire symbionts from microhabitats seeded with free-living *Symbiodinium* cells. *Acropora millepora*, *Acropora selago*, and *Isopora palifera* recruits were exposed to a conspecific adult, combined with a known microhabitat of free-living *Symbiodinium* cells (sediment). From this treatment, up to a five-fold increase in the number of symbiotic juveniles was detected, relative to the treatment of unfiltered seawater. This pattern was consistent across two reproductive strategies, broadcast spawning and brooding. Although previous studies have shown that juvenile cnidarians can draw their symbionts from the benthos (Coffroth et al. 2006; Adams et al. 2009; Cumbo et al. 2013), this is the first study to link adult corals with an increased abundance of benthic free-living *Symbiodinium* cells, followed by an enhanced symbiont acquisition in recruits.

In response to rising seawater temperatures it is expected that some marine organisms will expand their range margins (Precht and Aronson 2004; Yamano et al. 2011). It has been suggested that this may be especially true of coral species situated on reefs with pole-ward currents (i.e. the eastern coasts of Australia, South America, Africa and the USA) (Yamano et al. 2011). Although coral larvae have the potential to disperse large distances (Graham et al. 2008), the absence of free-living *Symbiodinium* cells may preclude the recruitment of corals, especially during range shifts beyond conspecifics. For example, if a juvenile coral with horizontal transmission of symbionts has a high specificity for a transiently free-living *Symbiodinium* type, it is conceivable that the presence of a host may be required to seed the environment with symbionts prior to recruitment. Before range expansion can occur, the seeding of new environments with FL *Symbiodinium* cells may require the dispersal of symbiotic recruits that carry generalist symbionts, or through other vectors, such as corallivorous fish (Castro-Sanguino and Sanchez 2012). Ultimately, to accurately model and predict how corals may shift their range to new locations as a result of changes in sea surface temperature will require (in combination with the other crucial physical parameters, e.g. aragonite saturation) an improved understanding of how adult corals, FL *Symbiodinium* cells and juvenile corals are linked.

The morphology of the *Symbiodinium* cell during primary acquisition is an area that requires further study. The predominant mechanism of horizontal transmission is unknown, but options include: the chance encounter and ingestion of a TFL coccoid *Symbiodinium* cell recently expelled by hosts; the chance encounter and ingestion of a FL *Symbiodinium* coccoid cell; and motile *Symbiodinium* cells actively seeking hosts. Kinzie (1974) noted that the uptake of *Symbiodinium* cells by aposymbiotic recruits of the gorgonian *Pseudopterogorgia bipinnata* only occurred when motile cells were used during inoculation. In **chapter 4** I observed different capacities for motility, and certain types of

Symbiodinium have been previously described as 'infectious' and may have strong host-location abilities (Gomez-Cabrera et al. 2008; Abrego et al. 2009b; Stat and Gates 2010). Future experiments might explore which FL *Symbiodinium* types interchange between motile and coccoid cells *in situ*, and if this occurs in the diel rhythms commonly observed in culture (Yacobovitch et al. 2004). For example, observing the division of *Symbiodinium* cells recently-expelled by hosts may reveal whether motile cells arise after becoming FL in the environment. These studies would also be useful to conduct on the *Symbiodinium* spp that are difficult to culture (Santos et al. 2001) and may not have the ability to generate flagella (Krueger and Gates 2012). If some *Symbiodinium* types have indeed lost the motile form, then the horizontal acquisition of *Symbiodinium* cells by hosts is likely to be density dependent and affected by hydrodynamics.

Summary of major findings and areas future research

The fine-scale abundance of benthic FL *Symbiodinium* cells is related to the proximity of scleractinian corals, and high-abundances of cells are found at the base of coral colonies (**thesis aim 1)**.

Some *Symbiodinium* cells initially survive the degradation of coral tissues during contact with sediment, yet persist only transiently with an approximate window of viability of seven days (**thesis aim 2**).

There are type-specific capacities for survival in sediment during thermal stress, including differences in the maintenance of: cell division; photophysiology; and cell motility (**thesis aims 3 and 4**).

The supply of free-living *Symbiodinium* cells to sediment by adult corals can increase the rate of horizontal symbiont acquisition for conspecific recruits (**thesis aim 5**).

Areas identified as valuable future experiments include:

The development of sample purification and quantitative molecular protocols to identify the populations of FL *Symbiodinium* cells from a range of habitats, including: sediment, macroalgae and turf algae, CCA surfaces and the water column.

The identification of model organisms which can be deployed to habitats in an aposymbiotic form as 'FL *Symbiodinium* cell sampling arrays'.

An investigation of whether *Symbiodinium* cells undergoing constant periods of darkness or permanent burial in sediment enter a 'dormant or resting cyst' phase, as is observed in many other dinoflagellates

species.

The development of culturing microcosms which better reflect the natural complexity in the environments of FL *Symbiodinium* cells, with the introduction of: microhabitats, dissolved and particulate organic matter; prey species; and predators.

An investigation of the interchange between coccoid and motile cell morphologies following isolation from hosts, and whether the capacity for motility is lost in any highly derived *Symbiodinium* types.

If the climate-related changes expected to occur in the next 50-100 years will impact the distribution of FL *Symbiodinium* cells, and alter the duration of viability for TFL *Symbiodinium* cells.

The examination of whether or not the range of FL *Symbiodinium* cells that form symbioses extends beyond the range of the scleractinian corals that host them.

Concluding remarks

In conclusion, this thesis has described links between adult corals, free-living *Symbiodinium* cells, microhabitats, and juvenile corals. I have documented free-living *Symbiodinium* cell abundance and viability, in relation to source populations (adult corals) and the physical environment (temperature). Patterns were identified in the process of symbiont acquisition, and I show that adult corals seed the environment with symbiosis-ready *Symbiodinium* cells. As we enter an age of climate modification, exceeding all rates of change previously experienced by coral reefs (Hughes et al. 2003; Hoegh-Guldberg et al. 2007), I propose that the abundance, distribution, and physiology of these symbiosis-ready, free-living *Symbiodinium* cells could prove worthy of monitoring as a parameter of coral reef health. We must attempt to understand the synergy of physiological stressors imposed upon the coral/*Symbiodinium* symbiosis in concert with free-living *Symbiodinium* cells, and elicit how any potential degradation of this *Symbiodinium* cell reservoir will preclude future generations of recruiting corals.

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Thank you.

The end.

A: Appendices

A.1 ITS2 nucleotide sequences for cultured *Symbiodinium* strains

CCMP2467 (ITS2 type A1)

CGTGCATTGCGCTCTTGGGATATGCCTGAGAGCATGTCTGCTTCAGTGCTTC
TACTTTCATTTTCT GCTGCTCTTGTTATCAGGAGCAGTGTTGCTGCATGCTTCTGCAAGT
GGCACTGGCATGCTAAATATCAAGTTTTGCTTGCTGTTGTGACTGATCAACATCTCAT
GTCGTTTCAGTTGGCGAAACAAAAGCT CATGTGTGTTCTTAACACTTCCTAGCAT
GAAGTCAGACAA

HI-0509 (undesignated, exclusively free-living member of clade A, referred to here as A-FLI)

GTGAATTGCAGAACTCCGTGAACCAATGGCCTCTTGAACGTGCATTGCGCTCTTG
GGATATGCCTGAGAGCATGTCTGCTTCAGTGCTTCTACTTTTCCTATTCCTGCT
GCTCCTTTCAAGGGGTGGTGCTGCTGTGTGCTACTGCATACTTTGCATTGGCATGCT
CAGTATTAAGCATTGCCCACTGTGTTGACTGATCAACTTTTCATGTCTTTTTCAGTCGGG
CAAATCAACACCTGGTGTCTTGAACACTTCCTAGCATGAAGTCAGACAAGTGAACCCG
CCGAATTTAAGCATATA

Ap1 (ITS2 type B1)

CGTGAACCGATGGCCTCCTGAACGCGCATTGCGCTCTCGGGATTTCCTGAGAG
CAGGTCTGCTTCAGTGCTTAGCATTATCTACCTGTGCTTGCAAGCAGCATGTAT
GTCTGCATTGCTGCTTCGCTTTCCAACAAGTCATCGATCGCTTTTGTGTTCGTA
AATGGCTTGTTTGCTGCCTGGCCCATGCGCCAAGCTTGAGCGTACTGTTGTT
CCAAGCTTTGCTTGCATCGTGCAGCTCAAGCGCGCAGCTGTCGGGATGCTGATGCATGC
CCTTAGCATGAAGTCAGACAAGAGAACCCGCTGAATTTAAGCATAT

A.2 Preliminary work

The vertical migration of *Symbiodinium* cells through interstitial spaces

Chapter 3 initially consisted of two experiments: A) The effect of elevated temperature and substrate on free-living *Symbiodinium* cultures (these data are described in chapter 3), and, B) The vertical migration of *Symbiodinium* cells through interstitial spaces. Preliminary research was conducted for Part B of this chapter, however, additional research is required to complete the study.

Part B aimed to investigate the capacity of *Symbiodinium* cells to navigate between the spaces of sediment grains in upwards and downwards directions.

It was hypothesized that: 1) The exclusively free-living *Symbiodinium* type would should a stronger capacity for vertical migration through sediment interstices than those that form symbioses, and 2) that successful migration through the sediment will be positively correlated with sediment grain size.

A.2.1 Methods and protocol

Carbonate sediment was collected from the reef flat of Heron Reef (as in chapter 3) and a sieve shaker (Endecott Ltd, London) was used to separate the sediment into the following size classes: very coarse (2000-1001 μm); coarse (1000-501 μm); medium (500-251 μm); fine (250-126 μm); and very fine (125-63 μm). Each batch of sediment used for experimentation was washed thoroughly through the sieve mesh with UV-treated Milli-Q water, autoclaved at 134°C for 20 min, washed a second time with UV-treated Milli-Q water, and dried in sterile conditions.

Stocks of *Symbiodinium* ITS2 type B1 (strain Ap1) and A1 were grown (as in chapter 3) at 25°C in silica-free sterile f/2-medium (pH 8.1) (Guillard and Ryther 1962) on a 12 h : 12 h (0700 : 1900) light/dark cycle. Stock cultures were acclimatised to experimental light levels of 75-80 μmol photons m–2 s–1 under Phillips TLD 36W/840 cool white tubes for 1 week. To ensure an even light field, an air-calibrated LI-COR LI250A was used to ensure minimal variation in intensity.

Upwards migration (Fig A.2.1)

Symbiodinium cells may produce flagella and are commonly seen to become motile during the light phase, when in culture (Yacobovitch et al, 2004). The following experiment was begun at 2400 h (dark) and completed at 1200 h (light).

1) At 2400 h, 10 mL of culture was added to a 15 mL tube from stock cultures. This was repeated

for 5 replicate tubes. At this time, 1 mL of stock culture was also preserved in a separate tube (4% formalin).

2) The bottom 2 mL of the tube was then covered with black electrical-tape to prevent light transmission through the side of the tubes.

3) Each tube was centrifuged at 400 × g for 2 min. This speed concentrated *Symbiodinium* cells without forming a dense pellet.

4) Sediment grains from a single size class were gently transferred to the tube, to a depth of 1 mL.

5) Each tube was then transferred to a holding rack under the same culturing conditions (water temperature and light environment) as described above.

6) After 12 h (6 h of dark and 6 h of light), the top 8 mL of each tube was pipetted out and transferred to a second tube, where the cells were then concentrated through centrifugation (4000 × g), and preserved for counting (4% formalin)

7) A haemocytometer was used to quantify cells mL^{-1}, with eight replicate counts per sample. The concentration of the cells that had successfully migrated through the sediment was compared with the starting concentration of cells.

8) The above process was repeated for all sediment size classes, and a control (no-sediment).

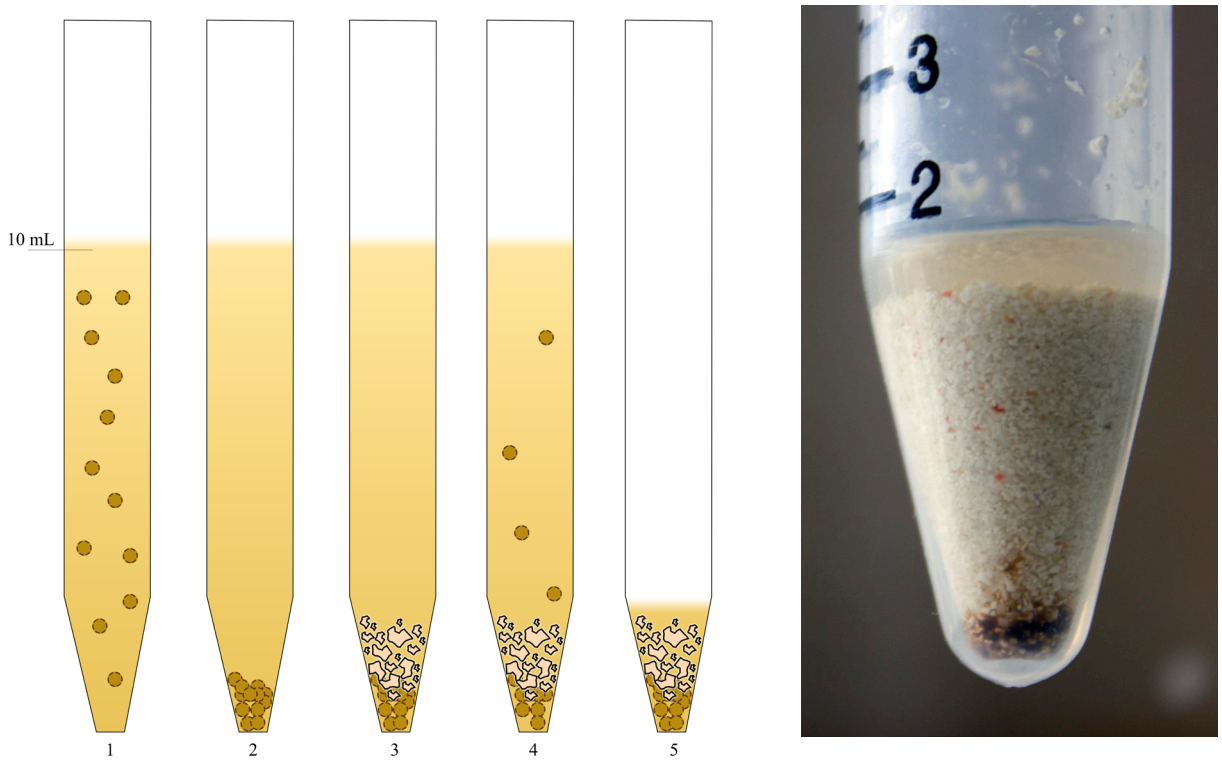

Figure A.2.1 Left: Experimental protocol for examining upwards-migration of *Symbiodinium* cells. 1) The addition of 10 mL of stock cell culture. 2) The concentration of *Symbiodinium* cells into a loose pellet. 3) The addition of sediment grains ontop of the pellet. 4) The successful migration of motile cells through the sediment layer. 5) The removal of the top 8 mL of the tube for enumeration of cells in suspension. Right: *Symbiodinium* cell pellet buried in sediment.

Downwards migration (Fig. A.2.2)

1) At 1200 h (an inverted design to the upwards-migration experiment) 10 mL of 0.22 µm FSW was added to the bottom of modified 50 mL syringes (N = 5).

2) A mesh layer (65 µm pore size) was pressed between two rubber o-rings at the surface of the FSW. Care was taken to ensure there were no bubbles trapped below the mesh.

3) Sediment (prepared as above) was evenly layered onto the mesh until a depth of 1 cm was reached.

4) 10 mL of culture was added to the syringe, flooding the sediment layer. At this time, 1 mL of stock culture was also preserved in a separate tube (4% formalin).

5) Each syringe was then transferred to a holding rack under the same culturing conditions (water temperature and light environment) as described above.

6) After 12 h (6 h of light and 6 h of dark), the lower half of each tube was drained out through

a tap and transferred to a second tube, where the cells were then concentrated through centrifugation (4000 × g), and preserved for counting (4% formalin)

7) A haemocytometer was used to quantify cells mL^{-1}, with eight replicate counts per sample. The concentration of the cells that had successfully migrated through the sediment was compared with the starting concentration of cells.

8) The above process was repeated for all sediment size classes, and a control (modified syringe without sediment).

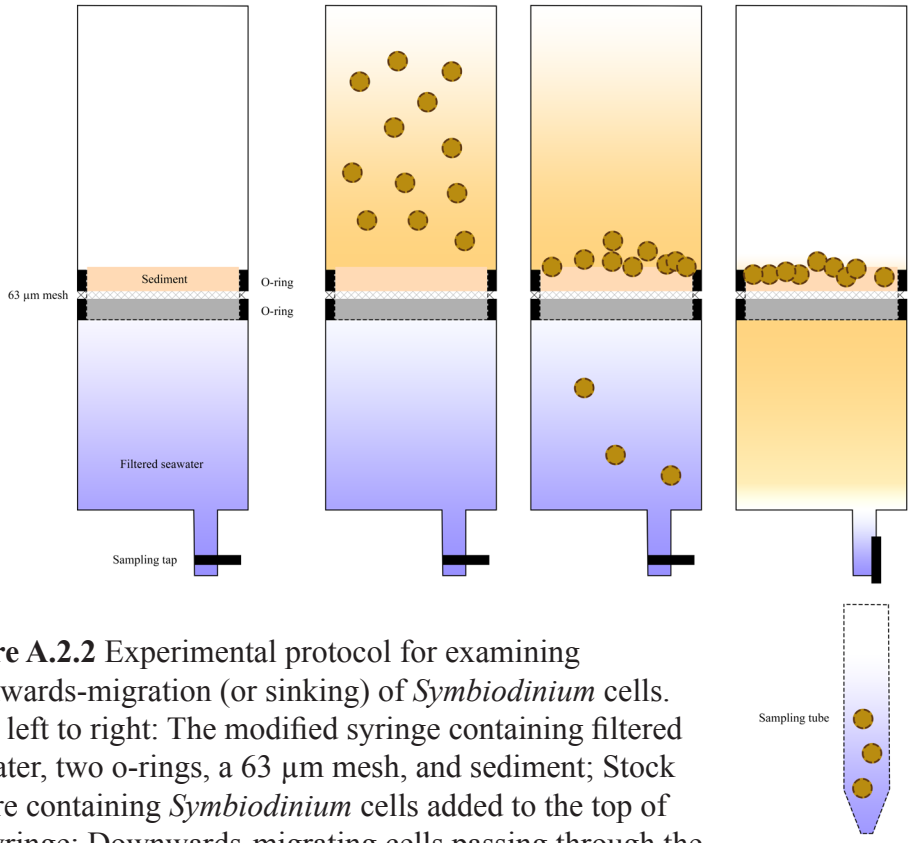

Figure A.2.2 Experimental protocol for examining downwards-migration (or sinking) of *Symbiodinium* cells. From left to right: The modified syringe containing filtered seawater, two o-rings, a 63 µm mesh, and sediment; Stock culture containing *Symbiodinium* cells added to the top of the syringe; Downwards-migrating cells passing through the sediment layer; Draining the lower half of the syringe and *Symbiodinium* cells into a sample tube.

A.2.3 Results and discussion

Two-way ANOVA was used to analyze the interaction between sediment size class (6 levels) and *Symbiodinium* type (2 levels), for both upwards and downwards-migration experiments. A significant interaction was observed for both upwards and downwards directions (Two-way ANOVA, $F_{(5, 48)} = 7.872$, $P < 0.0001$ and $F_{(5, 48)} = 11.06$, $P < 0.0001$, respectively).

Sediment of all size-classes significantly reduced the proportion of successful upwards, for both B1 and A-FLI relative to the control (Fig A.2.3, Tukey *post-hoc* tests, $P > 0.05$ for all comparisons). For B1, 31.65% (± 1.64 SE) of the cells introduced to the tube were motile (or in suspension) at the time of sampling (half-way through the light phase, 1200 h). This was significantly greater than A-FLI, which had only 19.89% (± 0.81) of cells in suspension (Tukey *post-hoc* tests, $P < 0.0001$). This difference was consistent across all sediment size classes, excepting the coarse sediment. Evidence was not found to support the hypothesis that fine sediments would reduce the number of successful migrations. Rather, the opposite was found, and the least number of successful migrations was observed in the coarse sediment.

For downwards migration (or sinking) of *Symbiodinium* cells, a different trend was observed. *Symbiodinium* A-FLI was found below the sediment layer (or mesh layer for controls) in significantly greater proportions than *Symbiodinium* type B1, across all treatments (Fig A.2.3, Tukey *post-hoc* tests, $P > 0.05$ for all comparisons). This potentially represent type-specific differences in specific gravity and cell sinking rates, rather than active 'downwards-migration' into sediment interstices. The 'very fine' sediment treatment produced the lowest proportion of downward cell migrations for both *Symbiodinium* types (Tukey post-hoc tests, $P > 0.05$ for all comparisons). This is in partial support of our hypothesis, that the migration through the sediment is correlated with sediment grain size.

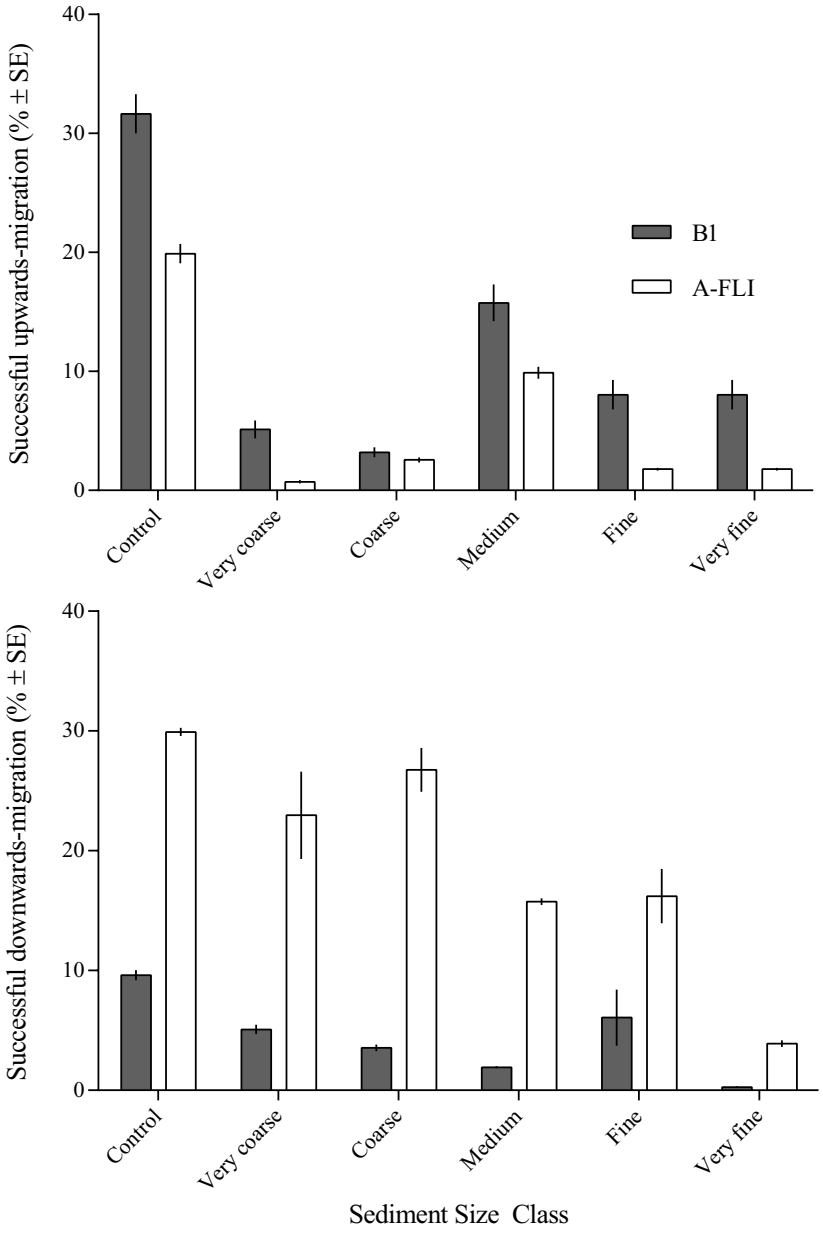

Figure A.2.3 Vertical migration of *Symbiodinium* types B1 (dark bars) and A-FLI (light bars) through a range of sediment size-classes. Values are means (± SE) expressed as the percentage of cells that have moved upwards (upper graph) or downwards (lower graph) through the sediment. The unsuccessful migrations or non-motile cells (remaining in the sediment) were not counted.