THE ECOLOGY OF BACTERIALLY MEDIATED BLEACHING IN A CHEMICALLY DEFENDED SEAWEED

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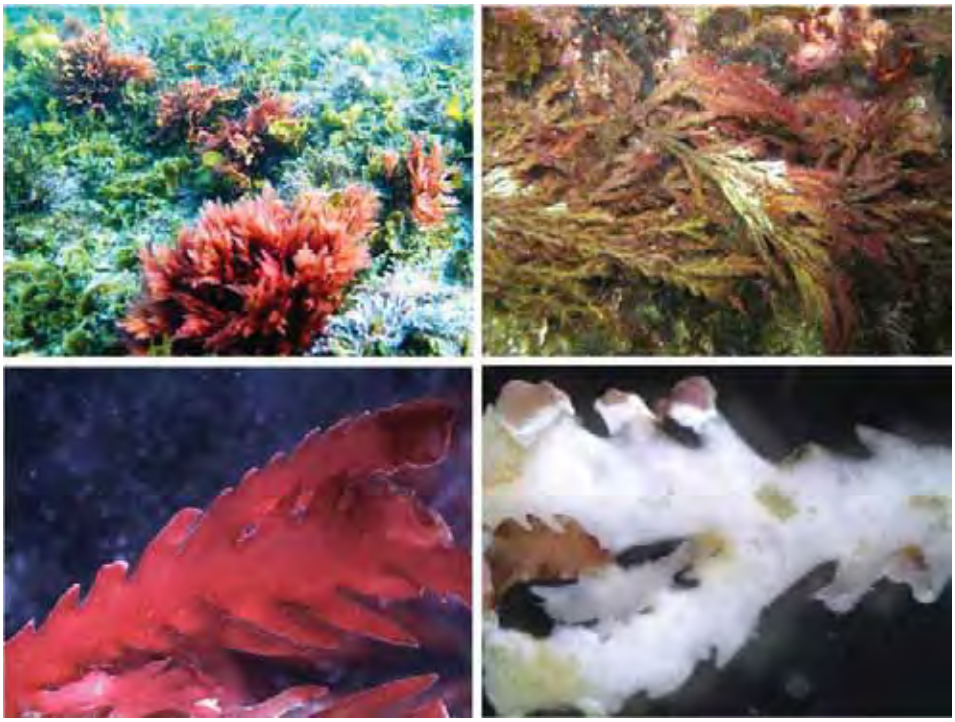

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As global climates warm and habitats are modified, disease is emerging as a major threat to biodiversity. Understanding the causes and consequences of disease and the influence of environmental change on both host organisms and pathogens is critical for the conservation of important ecosystems. Current rates of environmental change are unprecedentedly rapid, due largely to anthropogenic disturbances, further necessitating the study of disease dynamics. In this thesis, I assessed the causes and consequences of a bleaching phenomenon afflicting a chemically-defended macroalga, *Delisea pulchra*. Broadly, the aims of this study were to characterise patterns of bleaching in *D. pulchra* and to assess whether they related (directly or indirectly) to environmental factors, either via influences on algal chemical defences and/or bacterial pathogens. Additionally, I aimed to understand the consequences of disease for *D. pulchra* and its trophic interactions. Bleaching was more common in summer when water temperatures were elevated and was also more prevalent in shallow water habitats than at depth. Bleached algae supported significantly different microbial communities on their surfaces and also had depleted chemical defences (furanones) compared to co-occurring, healthy conspecifics. Bleaching was more likely at high temperatures when algal chemical defences were low or absent, and microorganisms were abundant and this was evident both in natural populations and in experimental manipulations. Inoculation with a candidate pathogen, *Ruegeria* sp. R11 caused bleaching in novel field inoculation experiments, but infection and bleaching were moderated by algal defences. Exposure to excessive solar radiation indirectly increased algal susceptibility to bleaching by causing a precursor condition ('fading'), which had depleted furanones. Association with shade-providing kelps prevented fading and bleaching in *D. pulchra* individuals. Bleaching had significant, sub-lethal performance consequences for affected algae and also altered trophic interactions between this seaweed and locally abundant macroherbivores. This work provides strong evidence that bleaching in *D. pulchra* is the result of an environmentally-mediated bacterial infection(s) that occurs when algal defensive chemistry is low. Understanding how environmental change and disease affect habitat-forming organisms like macroalgae is crucial for the study and management of important natural ecosystems under increasing stress.

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Abstract

As global climates warm and habitats are modified, disease is emerging as a major threat to biodiversity. Understanding the causes and consequences of disease and the influence of environmental change on both host organisms and pathogens is critical for the conservation of important ecosystems. Current rates of environmental change are unprecedentedly rapid, due largely to anthropogenic disturbances, further necessitating the study of disease dynamics. In this thesis, I assessed the causes and consequences of a bleaching phenomenon afflicting a chemically-defended macroalga, *Delisea pulchra*. Broadly, the aims of this study were to characterise patterns of bleaching in *D. pulchra* and to assess whether they related (directly or indirectly) to environmental factors, either via influences on algal chemical defences and/or bacterial pathogens. Additionally, I aimed to understand the consequences of disease for *D. pulchra* and its trophic interactions. Bleaching was more common in summer when water temperatures were elevated and was also more prevalent in shallow water habitats than at depth. Bleached algae supported significantly different microbial communities on their surfaces and also had depleted chemical defences (furanones) compared to co-occurring, healthy conspecifics. Bleaching was more likely at high temperatures when algal chemical defences were low or absent, and microorganisms were abundant and this was evident both in natural populations and in experimental manipulations. Inoculation with a candidate pathogen, *Ruegeria* sp. R11 caused bleaching in novel field inoculation experiments, but infection and bleaching were moderated by algal defences. Exposure to excessive solar radiation indirectly increased algal susceptibility to bleaching by causing a precursor condition ('fading'), which had depleted furanones. Association with shade-providing kelps prevented fading and bleaching in *D. pulchra* individuals. Bleaching had significant, sub-lethal performance consequences for affected algae and also altered trophic interactions between this seaweed and locally abundant macroherbivores. This work provides strong evidence that bleaching in *D. pulchra* is the result of an environmentally-mediated bacterial infection(s) that occurs when algal defensive chemistry is low. Understanding how environmental change and disease affect habitat-forming organisms like macroalgae is crucial for the study and management of important natural ecosystems under increasing stress.

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CHAPTER 1

General Introduction

Human impacts are now so ubiquitous in natural ecosystems that prominent scientists have suggested naming the current geological epoch the 'anthropocene' (Crutzen 2002, Crutzen 2006). Global climate change and other anthropogenic pressures have caused extensive changes to much of the planet (Vitousek et al. 1997). Consequences of these changes have had severe impacts upon biodiversity (e.g. Sih et al. 2000, Airoldi and Beck 2007), which are likely to persist for many thousands of years (Berger and Loutre 1996). Increasingly, human activities and impacts are concentrated in coastal regions, placing growing pressure on these important and sensitive ecosystems (Airoldi and Beck 2007), such that truly 'pristine' marine habitats are becoming progressively more rare (Jackson 2001, Jackson et al. 2001, Stachowitz 2003).

Organisms can respond to environmental change via adaption, shifts in distributions, or if unable to do either, may face local extinction (Holt 1990). Many organisms are shifting their ranges poleward and to areas of higher elevation at average estimated speeds of 6.1 km per decade, in pursuit of their receding ecological niche (Parmesan and Yohe 2003). If unable to move quickly enough to remain within their niche, an organism must adapt to its new niche or risk extinction. However, the rapidity of extant anthropogenic

environmental change is beyond the capacity for adaptation in many organisms, leading to extremely high extinction rates (Mainka 2002, IUCN 2007) and a so-called biodiversity crisis (Novacek and Cleland 2001, Olson et al. 2002).

Environmental change, microbes and disease

Recently, there have been suggestions that the interactions between macro-and micro-organisms (including bacteria, archaea, viruses and some fungi and protists) are shifting in response to environmental change. In coastal ecosystems for example, the influence of microbes is argued to be increasing, possibly at the expense of 'higher' organisms (Jackson 2001, Rosenberg and Ben-Haim 2002, Harvell et al. 2007). Microorganisms are ubiquitous in all environments and are critically involved, often in obligate symbiotic relationships, with many 'macro'-organisms. For example, bacterial endosymbionts provide nutrition to 10-15% of the world's insects (reviewed by Baumann et al. 2006). Microbes contribute significantly to important processes including global biogeochemical cycles (Morel and Price 2003) digestion in animals (including humans; Mackie 2002) and growth of at least 90% of plants (Smith and Read 1997). Microorganisms have also strongly influenced the evolution and ecology of macroorganisms. The existence and persistence of sex and dioecy, for example, have been credited to microbial pathogens (reviewed by Clay and Kover 1996, Lively 2009).

Considering that such fundamental relationships exist between micro- and macro- biota, environmentally-mediated changes to these interactions could have important consequences for individuals, communities and ecosystems. The emergence and re-emergence of diseases in natural ecosystems is one manifestation of this environmentally-mediated shift and as a result, the incidence and severity of diseases

appear to be increasing (Harvell et al. 2002, Lafferty and Holt 2003, Harvell 2004, Lafferty 2009). In addition to an increase in the abundance and virulence of microbial pathogens, environmental change and local stressors including warming, can also affect a host organism's susceptibility to disease (Lafferty and Holt 2003). For example, amphibian immune systems are suppressed at high temperatures (Raffel et al. 2006), Red Sea corals are more susceptible to bacterial bleaching in warmer waters (Banin et al. 2000) and extreme salinity conditions increase seagrass susceptibility to pathogens that cause wasting disease (McKone and Tanner 2009). In the past few decades, diseases have caused mass mortalities of diverse organisms including sea urchins (Lessios et al. 1984b), oak trees (Davidson et al. 2003), amphibians (Pounds et al. 2006), seals (Kennedy et al. 2000) and corals (e.g. Harvell et al. 2001, Kim and Harvell 2004).

Avoiding the impacts of warming and disease

Behavioural and phenological responses to climate change have now been observed, indicating that adaptation or its (phenotypic) precursor is occurring. For example, in order to reduce thermal stress some animals are spending less time foraging during the hottest parts of the day than previously (e.g. moose; Dussault et al. 2004). Additionally, flowering in many angiosperms, emergence in insects and migration of birds is occurring earlier as summers lengthen (reviewed by Walther 2002, Parmesan 2006, Cleland et al. 2007), and body sizes are decreasing as conditions warm (Daufresne et al. 2009). Evolutionary adaptation to these unprecedented, swift environmental shifts may however, be restricted to organisms with relatively short life spans. Shorter generation times should confer an evolutionary advantage when conditions change rapidly. For example, Bell and Gonzales (2009) demonstrated that 'evolutionary rescue' from a previously lethal environment was possible in yeast cultures within 25 generations which, for yeast

equated to about 35 hours. In this context, microorganisms may thus be better equipped to deal with rapid, anthropogenic environmental change than most macroorganisms.

Most 'higher' organisms have mechanisms with which they can defend themselves against pathogens and other natural enemies. Many marine organisms produce chemical defences against consumers and pathogens (reviewed by Engel et al. 2002, Steinberg et al. 2002). Defences can be costly to produce and some models predict that these will be less concentrated when conditions are sub-optimal (e.g. Cronin 2001, Agrawal et al. 2002, Marak et al. 2003, Dworjanyn et al. 2006b). In stressful environments, organisms may thus become less well defended (e.g. Koricheva et al. 1998, Van Alstyne and Pelletreau 2000) and therefore more susceptible to pathogen attack.

Diseases affecting habitat-forming organisms

The impacts of disease are of particular concern when they affect habitat-forming organisms, as impacts can cascade through entire communities. For example, epidemic infections by an invasive fungal pathogen led to mass mortalities within native eucalypt forests in Australia in the 1900s. Mortality of trees had impacts on mid- and under-storey plants and eventually led to significant regional shifts in vegetation structure and a decrease in biodiversity on an ecosystem-scale (reviewed by Burgess and Wingfield 2002). Plant diseases have also had severe impacts in marine ecosystems. Seagrass die-backs have led to losses of commercially important invertebrates and phytoplankton blooms (reviewed by Fourqurean and Robblee 1999). Marine ecosystems are arguably at a higher risk of diseases than terrestrial habitats, given the high density of potentially pathogenic organisms in seawater (Reinheimer 1992), difficulties associated with applying traditional disease control methods like quarantine and immunisation and the ideal dispersal

medium that water provides. Therefore, it is perhaps not surprising that diseases affecting marine organisms appear to have increased (Harvell et al. 1999, Lafferty et al. 2004).

One high-profile example of disease affecting a marine habitat-forming organism is coral bleaching, several cases of which have been attributed to environmentally-mediated pathogen infections (e.g. Porter et al. 2001, Kim and Harvell 2004, Williams and Miller 2005, Baker et al. 2008). Infection of the coral *Oculina patagonica* by the bacterium *Vibrio shiloi* has been particularly well-studied (reviewed by Rosenberg and Falkovitz 2004). This bacterium becomes virulent when water temperatures are elevated (Kushmaro et al. 1998, Banin et al. 2000) and attacks the coral's symbiotic zooxanthellae (Ben-Haim et al. 1999), leading to widespread bleaching. Coral bleaching and disease have a myriad of potential ecological and socioeconomic implications, including reef degradation and associated increased risks for coastal habitats, infrastructure and communities, habitat loss for reef fishes and other organisms and significant transitions in reef community composition (reviewed by Baker et al. 2008).

Analogous to coral reefs in the tropics, macroalgae are the dominant habitat-forming organisms in hard-substrate, temperate marine ecosystems. Macroalgae are responsible for a significant proportion of global primary production (Charpy-Roubaud and Sourina 1990) and support temperate marine biodiversity from the bottom-up by providing complex, three-dimensional habitat and food for other organisms (Steneck et al. 2002). In tropical ecosystems, encrusting coralline algae stabilise coral reefs (Littler and Littler 1995). Loss or degradation of macroalgal populations can have ecosystem-level consequences (Eckman et al. 1989, Kennelly 1989, O'Connor and Anderson 2010).

Recently, there have been increasing reports of declines of vast stands of these important organisms (e.g. Cole and Babcock 1996, Carballo et al. 2002, Edwards and Estes 2006, Airoldi and Beck 2007, Coleman et al. 2008, Connell et al. 2008). Such declines are often related to environmental anomalies like El Niño and diseases are often implicated in mass mortalities, but actual relationships between environmental stressors, microbial pathogens and macroalgae are rarely evaluated experimentally.

Disease was confirmed as the causative agent in the formation of galls on the Californian red seaweed *Prionitis lanceolata*, but the pathogenic bacterium could not be isolated or identified beyond its clade (roseobacter; Apt and Gibor 1989, Ashen and Goff 1996, Ashen and Goff 1998). Similarly, a bacterial pathogen was confirmed as the infective agent causing coralline lethal orange disease (CLOD; Hale and Mitchell 1995), which bleached large tracts of encrusting coralline algae, leaving behind bare skeleton that was quickly covered by fast-growing foliose algae (Littler and Littler 1995). In neither of these examples were the authors able to test the importance of environmental factors on the incidence or prevalence of disease.

Correa and colleagues described diseases affecting several rhodophytes (e.g. Correa and McLachlan 1991, Correa et al. 1993, Correa et al. 1994, Correa et al. 1997). Although seasonal variation in disease incidence was observed, the role of the environment in mediating disease was not confirmed experimentally. The implications of significant macroalgal disease and declines to temperate marine ecosystems and coastal communities are no less important than coral reef degradation in the tropics. Understanding the mechanisms behind such declines (e.g. whether diseases are involved)

and the influence of environmental change, is crucial for the conservation and management of these important organisms and the ecosystems they support.

Thesis aims and organisation

Broadly, the aim of the project outlined in this thesis was to examine interactions between the environment, host defences and bacterial pathogens in a habitat-forming organism: the red seaweed *Delisea pulchra* (Greville) Montagne (Bonnemaisonales: Rhodophyta). "Bleaching" of a chemically-defended macroalga is characterised by a localised region of affected thallus whitening. The phenomenon is not restricted to a specific or uniform area of the alga, but rather, appears anywhere on the alga and spreads (Case et al. 2011). Anecdotal reports have suggested that bleaching occurs more commonly in summer when water temperatures are elevated and levels of algal chemical defences are typically low (Wright et al. 2000). Bleaching can be induced by bacterial inoculations under certain laboratory conditions (Case et al. 2011) but it is not clear whether bleaching in natural populations of this habitat-forming seaweed are bacterially-mediated.

Throughout this project, I aimed to understand whether bleaching in *D. pulchra* populations is the result of bacterial infection and whether infection is moderated by concentrations of algal defensive chemistry and/or environmental stressors including water temperature and solar radiation. I conducted a monitoring program in which patterns of bleaching and their relationships with environmental variables, microbial communities and algal chemical defences were characterised. I also used field and laboratory-based manipulative experiments to test the importance of both specific bacterial strains and ambient seawater microorganisms on algal bleaching. In some of

these experiments I also manipulated water temperature and/or algal chemical defences to assess whether these factors influenced bacterial bleaching in *D. pulchra*. In another series of experiments aiming to test the importance of solar radiation on algal condition, chemical defences and susceptibility to bacterial bleaching, I manipulated light levels *in situ* and monitored algae and associated microbial communities. Finally, I conducted an algal survivorship study, ran herbivore surveys and feeding preference experiments to assess whether bleaching had any biological or ecological consequences for *D. pulchra* or its associates. More specific details on the hypotheses tested within each chapter follow below.

In chapter 2, patterns of bleaching in *D. pulchra* observed during the monitoring program are characterised and any relationships between the prevalence of bleaching and water temperature, solar radiation and salinity levels are assessed. In order to understand whether algal bleaching is associated with changes in chemical defences, concentrations of halogenated furanones are compared among bleached and healthy individuals and within bleached individuals. Microbial communities associated with the surfaces of algae are also compared between bleached and healthy thalli and within bleached algae, in order to understand whether bleaching is associated with microbial community shifts. In this chapter I also test whether halogenated furanones can prevent or reduce the severity of bleaching in *D. pulchra*, by manipulating algal production of these chemical defences and exposing algae to natural field conditions. Finally, to test whether the presence or abundance of microorganisms affects bleaching in *D. pulchra* and whether higher water temperature or depleted concentrations of halogenated furanones facilitate bacterially-mediated bleaching, these factors were manipulated in a three-factor laboratory experiment.

Chapter 3 involves an assessment of a candidate bacterial pathogen *Ruegeria* sp R11 (hereafter 'R11') and its role in the bleaching of *D. pulchra*. Here, I test whether this bacterium can infect and bleach this seaweed by conducting inoculation experiments, in which water temperature and the production of algal chemical defences in cultured sporelings are also manipulated, in order to assess whether these factors affect R11's ability to cause bleaching. Additionally, to investigate whether R11 can cause bleaching in established, chemically defended *D. pulchra* individuals and whether damage by scraping (thereby mimicking herbivory wounds and creating an infection site) facilitates infection and bleaching, novel field inoculation and damage experiments are presented.

In Chapter 4, I assess the how exposure to high levels of solar radiation affect established *D. pulchra* individuals' susceptibility to bleaching, their chemical defences and surface-associated microbial communities, by manipulating light levels *in situ* during shading and transplant experiments. A putative 'precursor' condition to bleaching ("fading") is also characterised in this chapter, and photosynthetic pigments from bleached, faded and healthy individuals are quantified. Finally, to see whether bleached individuals were closer to or further from shade than healthy conspecifics the role of canopy-forming macroalgae as shade-providers is also investigated in a 'nearest neighbour' study.

In Chapter 5, I conduct an assessment of the biological (i.e. survival) and ecological (i.e. interactions with consumers) consequences of bleaching for *D. pulchra*. A tagging study, in which the effect of bleaching on algal survival and condition were monitored over six months, is described. Additionally, in order to assess whether bleaching affects *D. pulchra*'s role as habitat, surveys of arborescent herbivores on bleached and healthy individuals are presented. Additionally, feeding preference experiments, involving locally

abundant herbivores being offered bleached and healthy algal tissues are also presented, in order to test whether bleached individuals are more or less likely to be consumed.

Chapter 6 provides a general discussion for this thesis.

Chapters 2 to 5 of this thesis comprise four stand-alone papers that have been prepared for publication in peer-reviewed journals. This format involves some inevitable repetition of material throughout the thesis, particularly in the introduction of each Chapter, however I have reduced this, where possible through cross-referencing.

CHAPTER 2

Patterns of bleaching in a seaweed: links to environmental stress, chemical defences and microbial pathogens

INTRODUCTION

Worldwide, environments are undergoing change at unprecedented rates due to global warming (IPCC 2007) and other anthropogenic stressors (Jackson 2001, Halpern et al. 2008). Current rates of environmental change exceed many organisms' capacities for adaptation or tolerance, leading to physiological stress (Chapin et al. 1993). Environmentally-mediated stress, defined as negative impacts of the environment on the normal physiology of an organism, has recently been observed in a diversity of organisms (e.g. Larsson et al. 1986, Dethier et al. 2005, Hoegh-Guldberg et al. 2007, Obermuller et al. 2007) with negative effects on survival (Toohey and Kendrick 2007) and/or performance (Karsten et al. 2001). Environmental stress associated with rapidly changing climates can have severe consequences, including species range shifts (Parmesan and Yohe 2003, Root et al. 2003, Ling et al. 2009) and extinctions (Pounds et al. 1999, Pounds et al. 2006).

In habitats that are affected by anthropogenic disturbances, stressors rarely operate in isolation (Halpern et al. 2008). Instead, multiple biotic and/or abiotic stressors can act

simultaneously and cumulatively, potentially exacerbating each other's effects (Crain et al. 2008). For example, exposure to heavy metal pollution decreases drought resilience in mountain birch trees (Eranen et al. 2009). Similarly, acidic conditions lower sea urchin thermal tolerance (O'Donnell et al. 2009). Stressed organisms are also typically at greater risk of disease. Stress due to climate change and other anthropogenic influences has been linked with diseases in diverse organisms including corals (Bruno et al. 2003, Garrett et al. 2006, Bally and Garrabou 2007), amphibians (Raffel et al. 2006), seagrasses (Vergeer et al. 1995), trees (Marcais and Breda 2006) and abalone (Travers et al. 2009).

Environmental change can alter disease dynamics by affecting both hosts and pathogens (Lafferty and Holt 2003). Some organisms defend themselves from natural enemies, including pathogens by producing chemical defences (reviewed by Steinberg et al. 2001, Engel et al. 2002). Such chemical defences can be metabolically costly to produce and maintain (e.g. Cronin 2001, Agrawal et al. 2002, Marak et al. 2003, Dworjanyn et al. 2006b) and may be depleted when organisms are stressed (e.g. Koricheva et al. 1998, Van Alstyne and Pelletreau 2000), thereby increasing susceptibility to attack by pathogens.

Defensive metabolites can moderate a host organism's interactions with microbes on its surface (Steinberg et al. 2002). In fact the living surface of any organism represents a biological niche in which specific microbial communities thrive (Donlan 2002). Any physiological or chemical changes that occur within a host organism due to stress will likely also alter the composition of the microbial community associated with its surface (Thompson et al. 1993). This has been observed in microbial communities associated with animal gastrointestinal tracts: 'gut flora' assemblages change significantly with changes in

pH (Savage 1977). Similarly, soil acidification leads to a shift in soil-associated microbial communities (Steenwerth et al. 2002).

Environmental change can also act upon pathogens directly by influencing their virulence, reproduction or dispersal (Lafferty et al. 2004). Many pathogens have temperature-regulated virulence (Konkel and Tilly 2000, Klinkert and Narberhaus 2009) and there are accounts of previously innocuous microorganisms becoming pathogenic under certain abiotic conditions (e.g. Vergeer and den Hartog 1994). Even when stressed, microorganisms arguably have a higher potential for adaptation to rapid environmental change than most 'higher' organisms (Bell and Gonzalez 2009). Their relatively short generation times, their capacity for horizontal gene transfer (Garcia-Vallve et al. 2000, Stephens and Murray 2001), plasmid acquisition (Selifonova et al. 2001), and the presence of ' contingency' genes in bacterial genomes (Moxon et al. 1994) facilitates more rapid adaptation, giving microbes an evolutionary edge in a rapidly changing world.

Considering the potential for higher abundances of more virulent pathogens in more stressful environments, recent increases in disease frequency and severity in natural ecosystems (Harvell et al. 2002, Lafferty 2009) are perhaps unsurprising. Diseases are particularly problematic in the world's oceans (Harvell et al. 1999, Lafferty et al. 2004), where organisms are exposed to a persistently high abundance of potentially pathogenic microbes (Reinheimer 1992). Furthermore, water-borne diseases tend to be more severe than others (Ewald 1994) and traditional disease-control methods such as quarantine and immunisation are particularly challenging underwater. One high profile example of marine diseases comes from tropical reefs around the world, where diverse microbial diseases are contributing to widespread coral bleaching and the decline of these important habitat-

forming organisms (Aronson and Precht 2001, Harvell et al. 2001, Hughes et al. 2003, Williams and Miller 2005, Bruckner and Bruckner 2006, Ainsworth et al. 2007, Ellner et al. 2007).

In temperate, hard substrate marine ecosystems, the dominant habitat-forming organisms are macroalgae. Negative impacts on macroalgal stands due to environmental stress and/or disease could also have severe consequences, analogous to those resulting from declines of coral reefs in tropical ecosystems (Edwards and Estes 2006, Ling 2008). Declines (Edwards and Estes 2006, Coleman et al. 2008, Connell et al. 2008) and diseases (Correa et al. 1994, Littler and Littler 1995, Largo et al. 1998) of macroalgal stands have been reported recently. Gaining an understanding of how environmental stressors interact with disease and affect these crucial organisms is necessary for the management and study of important communities as global climates continue to change.

Delisea pulchra is a chemically-defended marine macroalga, abundant in subtidal habitats around temperate Australia. A bleaching phenomenon affecting populations of this alga near Sydney, Australia has been observed and anecdotal reports indicate that this bleaching occurs more commonly in summer. *D. pulchra* has been well-studied, particularly with respect to its defensive chemistry. It produces secondary metabolites called 'halogenated furanones' (De Nys et al. 1993), which deter herbivores (Wright et al. 2000, Williamson et al. 2004, Wright and Davis 2006), inhibit surface biofilm formation (Maximilien et al. 1998), the settlement of fouling organisms (Dworjanyn et al. 2006a) and virulence of pathogens (Manefield et al. 2001). Production of furanones by this seaweed is costly and results in reduced growth (Dworjanyn et al. 2006b). *D. pulchra*'s defensive metabolites can be readily quantified and experimentally manipulated. Considering this background and recent

observations of bleaching (Case et al. 2011), this alga was used to investigate how environmental stressors and microbial communities interact with defensive chemistry and bleaching in a habitat-forming organism.

In this study, I characterised temporal and spatial patterns of algal bleaching in populations of *D. pulchra* over a three-year period and assessed whether these patterns fluctuated seasonally and/or were related to variation in water temperature, solar radiation and salinity. To investigate whether patterns of bleaching were related to patterns of algal chemical defences in both individuals and populations of *D. pulchra*, concentrations of halogenated furanones were compared among healthy and bleached thalli collected throughout the monitoring period from replicate sites. If environmental stress affects algal defensive chemistry and/or pathogen abundance or virulence and these changes result in algal bleaching, then a shift in the composition of microbial communities associated *D. pulchra* thalli would be expected on bleached algae relative to healthy conspecifics. Previous studies, investigating the potential roles of pathogens in 'disease-like' syndromes, assessed whether biofilm communities associated with the host changed as symptoms appeared (e.g. Frias-Lopez et al. 2002, Pantos et al. 2003). Thus, I also compared algal surface-associated microbial communities (SAMCs) from bleached and healthy thalli collected throughout the monitoring period.

Based on the patterns observed in these descriptive data, I conducted several manipulative experiments to more closely assess the importance of algal chemical defences, microbial pathogens and water temperature in *D. pulchra*'s bleaching. More specifically, to test whether algal chemical defences could prevent or reduce the severity of bleaching, I cultured chemically 'defended' and 'undefended' sporelings and then exposed them to

natural field conditions in which bleaching was commonly observed in established algal populations. Additionally, to test whether higher temperatures led to more frequent or severe bleaching, I also exposed defended and undefended sporelings to 'sterile' and 'natural' biofilm and seawater conditions, at 'high' and 'low' temperatures in the laboratory. These experiments each tested parts of the hypothesis that bleaching in natural populations of this seaweed is the result of temperature-mediated microbial infections.

MATERIALS & METHODS

Study sites and organisms

Delisea pulchra (Greville) Montagne (Bonnemaisonales: Rhodophyta) is a red macroalga that is common in subtidal habitats fringing the southern, temperate reefs of Australia, the sub-Antarctic islands and the Antarctic continent (Womersley 1996). It occurs frequently between one and fifteen meters depth in coastal habitats around Sydney, Australia. *D. pulchra*'s defensive metabolites (furanones) prevent the colonisation of many bacteria onto the surface of the alga (Maximilien et al. 1998), in part by acting as chemical antagonists to bacterial cell-cell signalling systems or 'quorum sensing' (Manefield et al. 1999, Manefield et al. 2002).

Surveys and collections were conducted at two locations: Bare Island in Botany Bay (151°13'50'' E, 33°59'32'' S) and Long Bay in Malabar (151°14'42'' E, 33°58'19'' S). Both locations are near Sydney, Australia and include shallow (1-4 m) and deep (7-9 m) rocky reefs which are dominated by macroalgae, most commonly the foliose species *Ecklonia radiata* (C. Agardh) J Agardh (Laminariales: Heterokontophyta), *Sargassum linearifolium* (Turner) C. Agardh (Fucales: Heterokontophyta), *S. vestitum* (R. Brown ex. Turner) C. Agardh (Fucales: Heterokontophyta), *Dilophus*, spp. (Dictyotales: Heterokontophyta), *D. pulchra* and the turfing corallines *Corallina officinalis* (Linneaus) (Corallinales: Rhodophyta) and *Amphiroa anceps* (Lamark) Decaisne (Corallinales: Rhodophyta). *D. pulchra* was common, during the period of this study, in both shallow and deep water rocky reefs at both locations.

Patterns of algal bleaching and its relationships with environmental variables

To quantify seasonal patterns in the prevalence of *D. pulchra*'s bleaching, algal surveys were conducted approximately monthly. Bleaching was defined as whitening of any part of the algal thallus (Figure 2.1). It was not restricted to any specific or uniform section of thalli, but appeared variously within thalli. Individuals with any visible whitening were counted as 'bleached' and those without visible bleaching as 'healthy'. Algal populations growing in 'deep' (7-10 m) and 'shallow' (1-4 m) water at Bare Island and Long Bay were assessed from August 2006 until April 2009 so that three summers and two winters were included during the survey period. On each survey date, six replicate 1 m^2 quadrats were haphazardly thrown within populations of *D. pulchra* at each depth and location. Individuals within each quadrat were inspected for visible signs of bleaching and counted as either 'bleached' or 'healthy'.

Each sampling year was defined as beginning in the austral winter and ending the following winter rather than as a calendar year, so that each sampling year contained a complete summer. Water temperatures were warmer during months that occurred in summer and autumn and so those months were grouped in this matter for analyses and are hereafter referred to as 'warm' sampling times. Cooler water temperatures occurred during the months in winter and spring and these were similarly grouped in analyses and are hereafter referred to as 'cool' sampling times. At the time of each survey, replicate algal samples (healthy and bleached algae, depending on occurrence) were also collected for characterisation of the surface-associated microbial communities and quantification of furanones.

Algae were placed inside individual clip-seal plastic bags *in situ* and transported to the laboratory, where they were rinsed in filtered seawater three times to remove any epibionts that were not attached or part of the biofilm. Tissue samples from each alga were aseptically transferred into sterile 1.5 ml Eppendorf tubes and frozen at -80 ^{o}C, then freeze-dried and stored at -20^{o}C until processing. Three classifications of algal tissue were sampled: (1) 'healthy' tissue from completely healthy algae that showed no signs of bleaching, (2) 'bleached' tissue from bleached algae and (3) 'adjacent' unbleached tissue from bleached algae.

Data on several environmental variables were also collected simultaneously with algal condition surveys. Water temperature, depth and salinity were measured using a 'YeoKal' water quality instrument (model 611). Solar radiation (photosynthetically active radiation (PAR) and ultra-violet radiation a and b (UVAR and UVBR)) were measured using a Skye light meter ('Spectrosense 2' Display Meter SKL 904) with a single channel light sensor for PAR and two high output light sensors for UVAR and UVBR. Three to six replicate measurements of each variable were taken at each depth, survey date and location.

Variation in chemical defences

To assess whether levels of chemical defences were different in bleached and co-occurring healthy algae, furanones were extracted and quantified from 'healthy', 'bleached' and 'adjacent' algal tissues collected during the monitoring program from 'deep' and 'shallow' reefs at both Bare Island and Long Bay. Because concentrations of halogenated furanones in healthy populations of *D. pulchra* are quite variable spatially and temporally (Wright et al. 2000), I also assessed whether population-level seasonal variation in chemical defences may affect susceptibility to algal bleaching. To do this, furanone concentrations in healthy algae

from deep and shallow reefs at both locations in 'cool' winter and 'warm' summer months were compared. As furanone concentrations within *D. pulchra* thalli vary from the base to the tips (Dworjanyn et al. 1999) care was taken to consistently sample tissue from the mid-tip region of thalli throughout all studies. To extract furanones, I used methods modified from (Dworjanyn et al. 1999). Freeze-dried algal tissue (50-150 mg) was homogenised by grinding with a mortar and pestle, weighed and transferred into 1.5 ml Eppendorf tubes. Analytical grade dichloro-methane (DCM; 500 µl) was added and then the samples were sonicated for 30 minutes. The tubes were then centrifuged at 10,000 x g for 10 minutes and approximately 400 µl of supernatant was transferred into another 1.5 ml Eppendorf tube.

This extraction process was repeated twice and the final aliquots of supernatant (approximately 1200 µl total from all three extractions) combined and evaporated overnight in a fume cupboard. The dried extract was resuspended in 1 ml HPLC grade ethyl-acetate (EtoAc) and stored in the dark at -20 ^{o}C until quantification by gas chromatography-mass spectrometry (GC-MS). 50 µl of each sample was diluted 1:20 into a GC-MS vial with 950 µl EtoAc containing an internal standard of 5 µl/ml synthetic furanone 8 (F8). F8 was chosen as an internal standard as it does not occur in nature but is structurally similar to naturally-occurring furanones 1-4 and hence has similar ionisation properties for mass spectrometric detection.

Concentrations of furanones 1-4 were analysed using methods modified from (Dworjanyn et al. 1999), with coupled chemical ionisation (CI) gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890N gas chromatograph (GC) and a polyimide-coated fused silica capillary column (ZB1-MS, 30m length, 0.25 mm ID). All injections (1 µl) were performed in the splitless mode with an inlet pressure of 11.4 psi. The injection port was held at 250˚C

and the GC-MS interface at 300˚C. The GC was held at 100˚C for 3 min, ramped at 10˚C/min to 200˚C and then ramped at 20˚C/min to 280˚C, with a final run time of 17 minutes. The carrier gas was helium. Mass spectrometry was performed on an Agilent Technologies 5973 inert mass spectrometer. Ions characteristic of the internal standard and the analytes were monitored in the selected ion monitoring (SIM) mode. The GC-MS system was controlled by Agilent Chemstation software (D.01.00 Build 75 2003). The ratio of peak areas (analyte/internal standard) in the sample was calculated and converted to concentration by reference to a standard curve. Concentrations of the four main furanone compounds (furanones1-4) in each sample were calculated as µg/mg freeze-dried material.

Patterns of microbial communities associated with bleached and healthy algae

To determine whether surface-associated microbial communities (SAMCs) differ between co-occurring bleached and healthy *D. pulchra* individuals, I extracted bacterial DNA and characterised communities associated with algae collected over the survey period using the polymerase chain reaction (PCR) based DNA fingerprinting technique, terminal restriction fragment length polymorphisms (TRFLP, Liu et al. 1997). Such methods are widely used to characterise and compare the composition and diversity of microbial communities, theoretically with a species resolution, but usually to the widely accepted 'operational taxomonic unit' (OTU; Nocker et al. 2007).

To carry out a t-RFLP analysis, phylogenetic marker genes within the sample DNA are amplified using the polymerase chain reaction (PCR) with a fluorescent dye attached to the 5' end or the forward primer. PCR products are then digested using restriction enzymes, which results in DNA fragments of variable length. These fragments are then physically separated in sequencing capillaries and the labelled terminal fragments are detected using a

laser, producing an electropherogram. A size standard labelled with a different fluorophore is also analysed, allowing the fragment lengths to be estimated with a resolution of one base pair (Liu et al. 1997, Nocker et al. 2007). Each OTU is represented by a different fragment length and thus the composition and diversity of the community can be estimated based on the polymorphism of the terminal restriction fragment lengths from a sample of community DNA (Walker et al. 2004). Like most DNA fingerprinting techniques that use PCR, t-RFLP is biased towards the most abundant members of the microbial community (Crosby and Criddle 2003).

To compare SAMCs from replicate healthy, bleached and adjacent algal tissue collected throughout the monitoring period from both depths and locations, DNA was extracted from selected freeze-dried algal samples (50-100 mg) using a ZR Soil Microbe DNA extraction kit (Zymo). A fragment of the 16S rDNA gene was amplified using the polymerase chain reaction (PCR) with the forward primer labelled with a fluorescent marker (6-FAM). PCR conditions and volumes were modified from those presented by Walker et al. (2004) for t-RFLP. Briefly, PCR volumes were 25 µl, including 12.5 µl Econo Taq 2 X mastermix (Lucigen), 0.25 µl of 10 µmol forward and reverse primers (27F, labelled with 6-FAM fluorophore :5'-AGAGTTTGATC(AC)TGGCTCAG-3' and 519R: 5'-GWATTACCGCGGCKGCTG-3'), template DNA and molecular water. 'Touchdown' (variable annealing temperatures) PCR was conducted in a thermocycler, where the samples were initially denatured at 94 °C for 3 minutes, followed by 27 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 55 °C for 45 seconds, followed by enzyme extension for 2 minutes at 72 °C, another 30 seconds at 94 °C, 45 seconds at 54 °C, another 2 minutes at 72°C, 30 seconds at 94°C, 45 seconds at 53 °C,

2 minutes at 72 °C, 30 seconds at 94 °C, 45 seconds at 52 °C and another 2 minutes at 72 °C. This was followed by a final extension at 72 °C for 5 minutes.

PCR products were checked on 1% agarose gels stained with gel red (BIOTIN), purified using a Zymo DNA Clean and Concentrator Kit 5 (Zymo Research) and quantified using a nano-drop ND1000 (Thermo Scientific). Approximately100 ng of purified PCR product was digested in 25 µl reactions containing 5 units of the restriction enzyme RsaI (NE Biolabs) with 14 µl 1 X NE buffer. Digestion reactions were incubated at 37 ^{0}C for four hours. Digestion products were de-salted and re-purified using the Zymo DNA Clean and Concentrator Kit 5 (Zymo Research), as high salt concentrations can interfere with electrokinetic injection into capillaries during TRFLP. Finally, 2 µl of each desalted, purified, digestion product (with an approximate DNA concentration of 10-20 ng/µl) was submitted to the Ramaciotti Centre at UNSW for fragment analysis using an AB3730 capillary sequencer (Applied Biosystems) in genetyping mode. Electropherograms were visualised and data were extracted using 'Peak Scanner v 1.0' (Applied Biosystems).

Transplanting defended and undefended **D. pulchra** *sporelings into the field*

One potential explanation for observations of low concentrations of halogenated furanones in populations with high frequencies of bleaching, as well as in bleached individuals relative to healthy conspecifics, is that these chemical defences protect *D. pulchra* from bleaching. To test this idea (specifically, whether halogenated furanones prevent or reduce the severity of bleaching in *D. pulchra*), I manipulated the production of chemical defences in cultured *D. pulchra* sporelings and transplanted them into natural field conditions that were associated with high bleaching frequencies during the monitoring study (i.e. shallow waters with 'high' summer temperatures). Furanones produced by *D. pulchra* are brominated, so their

production can be manipulated *in vivo* by removing bromine from growth media as per Dworjanyn et al., (1999). To culture *D. pulchra* sporelings, tetraspores were harvested from fertile adult tetrasporophytes collected from Long Bay. Spores were cultured initially in filtered seawater (FSW) enriched with 5 ml/L 5x Provasoli's Enrichment Solution (PES) for one week. Then, to manipulate the production of halogenated furanones, half were transferred into artificial seawater (ASW) containing bromine at natural concentrations and the other half into ASW lacking bromine (as per Dworjanyn et al. 1999, Dworjanyn et al. 2006b). Sporelings were cultured in ASW (Br +/-; water changed weekly) at 19-20 ^{0}C under a 15:9 hour light: dark cycle on an orbital mixer, until they reached 2-4 mm length (generally 4-6 weeks).

Algae cultured in media containing bromine produce halogenated furanones ('furanone (+)' algae) and those grown in media lacking bromine do not ('furanone (-)' algae). In furanone (-) algae, the gland cells that normally contain these compounds are atrophied, but the sporelings are otherwise morphologically similar to furanone (+) sporelings (Dworjanyn et al. 1999). Dworjanyn *et al.*, (2006b) demonstrated that furanone (-) sporelings grow at the same rate as those maintained in FSW and that another red alga, *Bostrychia moritziana*, which does not produce brominated compounds, is unaffected by manipulation of bromine.

To further test whether manipulating bromine affects *D. pulchra* other than in its ability to produce halogenated furanones, we increased the concentration of bromine in growth media above normal levels and monitored algal growth. Six replicate, two-week old sporelings were maintained in ASW containing natural concentrations of bromine (0.083 mg/L) and six in ASW containing twice that concentration (0.166 mg/L). We compared growth (change in length) over a two-week period by analysing images of algae at initial and

final stages of the experiment using the image analysis software 'Image J'. There was no difference in growth between bromine treatments (mean change in length of natural bromine levels: 3.82 mm ±0.35 mm; mean change in double-natural bromine levels: 4.16 mm ± 0.21 mm; $F_{1,9}$ =0.746, p =0.410), providing further evidence that manipulating bromine levels in growth media does not affect algae except in its ability to produce brominated compounds.

To assess whether halogenated furanones could prevent or reduce the severity of bleaching in *D. pulchra* sporelings from bleaching under natural field conditions, five replicate furanone (+) and furanone (-) sporelings were sealed within 50 ml 'flow-through chambers', which had mesh sides so that water (and water-borne microbes) could pass through easily but algae were kept within (Figure 2.2). These chambers were deployed on the benthos by attachment to masonry nails via cable-ties in approximately 3 m of water at Long Bay, Malabar for 24 hours. Because furanone (-) algae begin producing halogenated furanones and regain typical levels of chemical defences after 48 hours of exposure to brominated media such as seawater (R. De Nys *pers. comm.*), this experiment was conducted for only 24 h. After collection, sporelings were photographed under a dissecting microscope and the proportion of each thallus that bleached was quantified using the image analysis software 'Image J'. This experiment was conducted during the summer of 2008-2009 and again the following summer (2009-2010).

Manipulating algal chemical defences, water temperature and microbes

Patterns of variation in chemical defences, temperature and surface microbes suggest that they interact to affect bleaching in *D. pulchra*. To test this idea, I exposed furanone (+) and furanone (-) sporelings to sterile and non-sterile conditions. Water temperature was also

manipulated to assess whether warmer temperatures influence bleaching in the presence/absence of pathogens and chemical defences. To reduce bacterial biofilms from algal surfaces, furanone (-) and furanone (+) sporelings were soaked in an ASW antibiotic solution containing 20 mg/L streptomycin, 10 mg/L kanamycin and 10 mg/L penicillin for 24 hours and were then rinsed three times in ASW. Control algae were treated the same way but soaked in ASW lacking antibiotics. Treatment with antibiotics does not damage *D. pulchra* sporelings (Longford 2008) or other red algae (Paul et al. 2006). It is not possible to completely remove bacteria from the surface of *D. pulchra* (Longford 2008) or other marine algae (Huggett et al. 2006, Paul et al. 2006), so this treatment represents a significant disturbance to biofilm communities, rather than a complete removal of bacteria. Sporelings with disturbed or intact biofilms were then transferred into 3 cm diameter plastic Petri dishes. Antibiotic-treated sporelings were placed into dishes containing sterile Br+/- ASW and sporelings that were not treated with antibiotics were placed into dishes containing unfiltered, non-sterile seawater that had been collected from Long Bay several hours previously.

Sporelings were then maintained at high (25-26 ^{o}C) or low (20-21 ^{o}C) temperatures inside incubators for four days. These temperatures were chosen based on measurements collected throughout parts of *D. pulchra*'s geographical range as part of a broader study not detailed in this thesis. The water temperature component of that study, in which temperatures were measured along a latitudinal gradient within the north-eastern part of *D. pulchra*'s Australian range (from the subtropics and through several degrees of latitude within the temperate zone, as classified by Stern et al. (2000)) is included in this chapter. Two incubators were set at each temperature such that 'incubator' was nested within

'temperature' in the experimental design, with three replicates in each incubator (and thus a total of six replicates in each temperature treatment). The dishes were gently agitated on orbital shakers at 60 rpm and maintained under a 15:9 light:dark cycle. After four days, each sporeling was photographed under a dissecting microscope and the proportion of each experimental thallus that bleached was quantified using image analysis software 'Image J'.

Statistical analyses

The proportion of bleaching that occurred in populations of *D. pulchra* was analysed using a five-factor Analysis of Variance (ANOVA), with p-values generated via permutation, where the factors were sampling year (1, 2, 3), 'season' (warm, cool), sampling date (nested within season), location (Bare Island, Long Bay) and depth (deep, shallow). Throughout this study, 'location' was treated as a fixed factor because the study sites were chosen based on (i) the presence of rocky reefs supporting macroalgal assemblages in shallow and deep waters and (ii) a high abundance of *D. pulchra* occurring at both depths. Due to the preponderance of zero values in this dataset (bleaching was rare during winter, for example), these data failed to conform to ANOVAs assumptions of normality and homogeneity of variances and although transformations were attempted they did not improve the dataset with respect to these assumptions. Therefore, raw data were used in analyses and marginal results should be interpreted with caution, although ANOVA assumptions are less important in datasets with so many replicates (Underwood 1997). However, to reduce the likelihood of Type I error in interpreting analyses of this dataset, 'α' was lowered to 0.01. Relationships between the prevalence of bleaching and environmental variables (temperature, solar radiation and salinity) were analysed using linear regressions, although data were not distributed normally and thus, relationships should be interpreted with caution.

Concentrations of total furanones in bleached and healthy *D. pulchra* were compared using a three-fixed-factor ANOVA, where the factors were location (Bare Island, Long Bay), depth (deep, shallow) and condition (healthy, bleached). Furanone concentrations in bleached and adjacent unbleached tissues from bleached individuals were compared using a paired T-test. Furanone concentrations in unbleached 'adjacent' tissues were compared to those in completely healthy algae using an unpaired T-test. To compare seasonal and spatial variation in furanone concentrations in healthy algae, a three-fixed factor ANOVA with the factors season (warm, cool), location (Bare Island, Long Bay) and depth (deep, shallow) was conducted.

After visualisation and data extraction in Peak Scanner (Applied Biosystems), t-RFLP electropherrograms were analysed using the statistical platform 'R'. Peak areas were standardised by total peak areas, background noise was filtered, fragments were aligned via binning to 1 base pair (bp) and the presence/absence of each fragment length in each sample was calculated. These data were then used to compare SAMCs from bleached, healthy and adjacent algal tissues ('condition') from different sampling years (1,2,3), locations (Bare Island, Long Bay) and depths (deep, shallow), using Bray-Curtis similarity coefficients and permutational multivariate analyses of variances (PERMANOVAs, Anderson 2001, Anderson and Ter Braak 2003).

Assumptions of normality and homogeneity of variances were checked by inspecting scatterplots of residuals. Transformations were conducted where appropriate and are reported alongside tables. All ANOVAs and linear regressions were conducted using Systat v 13.00.05. PERMANOVAs were conducted using PRIMER 6 v 6.1.11 & PERMANOVA + v 1.0.1.

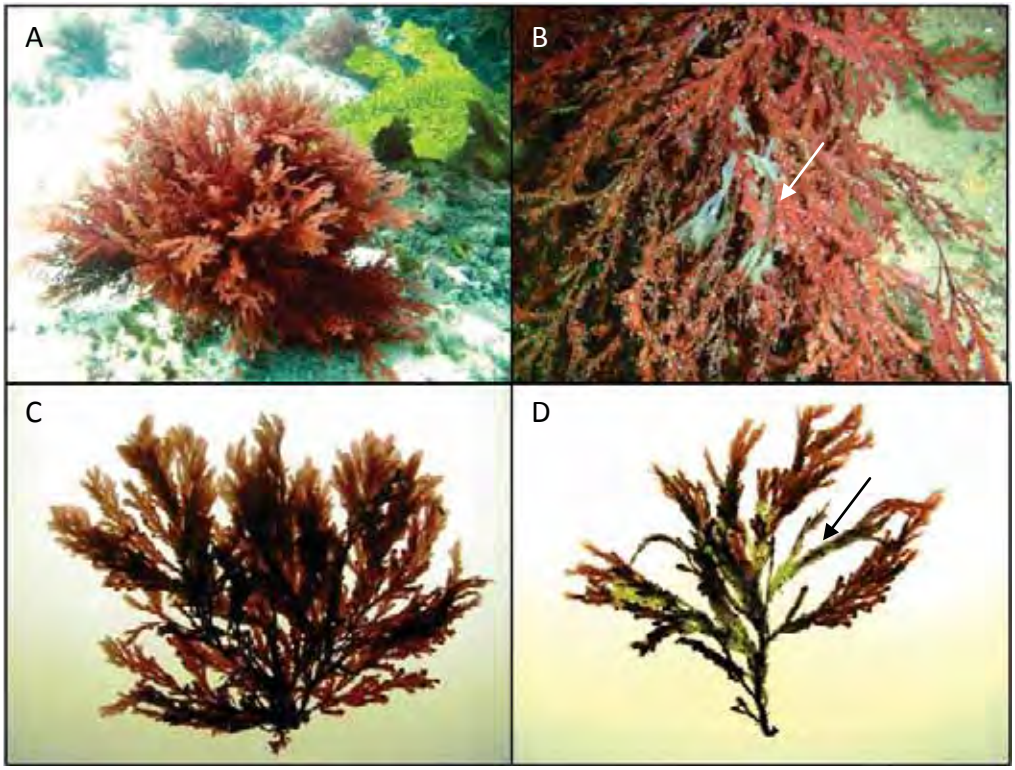

Figure 2.1. Habit photographs of **(A)** healthy and **(B)** bleached *Delisea pulchra*, both taken at Bare Island near Sydney, Australia and photographs of sections of **(C)** healthy and **(D)** bleached thalli. Note that bleaching is not restricted to the tips of thalli but may appear anywhere, and that adjacent to bleached portions of algal thalli tissue remains unbleached in appearance. Arrows indicate bleached sections of *D. pulchra* thalli.

Figure 2.2. Flow-through chamber containing a *D. pulchra* sporeling (furanone +/-) deployed on the benthos at Long Bay during one of the outplanting experiments.

RESULTS

Patterns of algal bleaching and relationships with environmental variables

Bleaching was more prevalent during 'warm' summer than 'cool' winter months and this was observed across all three sampling years at both locations (Figure 2.3, Table 2.1). Bleaching occurred in both shallow (1-4 m; Figure 2.3A) and deep (7-10 m; Figure 2.3B) water populations of *D. pulchra* but during 'warm' months was more common in the shallows. Additionally, the seasonal pattern of bleaching was more obvious in the shallows than at depth and more bleaching occurred in deep populations in 'warm' summer months than in shallow populations in 'cool' winter months, and during 'cool' months, bleaching prevalence was similar among depths (although these depth-specific seasonal differences were slightly non-significant with α=0.01; Figure 2.3, Table 2.1). Bleaching was more common in the first and second years of sampling than the third (slightly non-significant difference with α=0.01). There was also significant temporal variation in the prevalence of bleaching between sampling dates (which were nested within 'seasons'), particularly at some depths and locations, throughout the study (Table 2.1).

Patterns of seawater temperature were similar to patterns of bleaching, with peak bleaching prevalence coinciding with peak temperatures during mid-late 'warm' months across all survey years (Figure 2.3). Overall, bleaching prevalence was weakly, positively related to water temperature (R^2 =0.063, *df* =565, p <0.001; Figure 2.4). However, further analyses done for each depth separately, revealed that this relationship was stronger among shallow algae (R^2 =0.238, *df* =277, p <0.001; Figure 2.4A), whereas no relationship existed between bleaching and temperature in deep populations (R^2 =0.006, *df* =289, p =0.192; Figure 2.4B). These relationships were similar at both locations. Water temperature was

typically 1-2 ^{o}C higher in the shallows than at depth. This difference was significant ($F_{1,554}$ =7.52, p =0.006) and consistent across sampling years, seasons and locations.

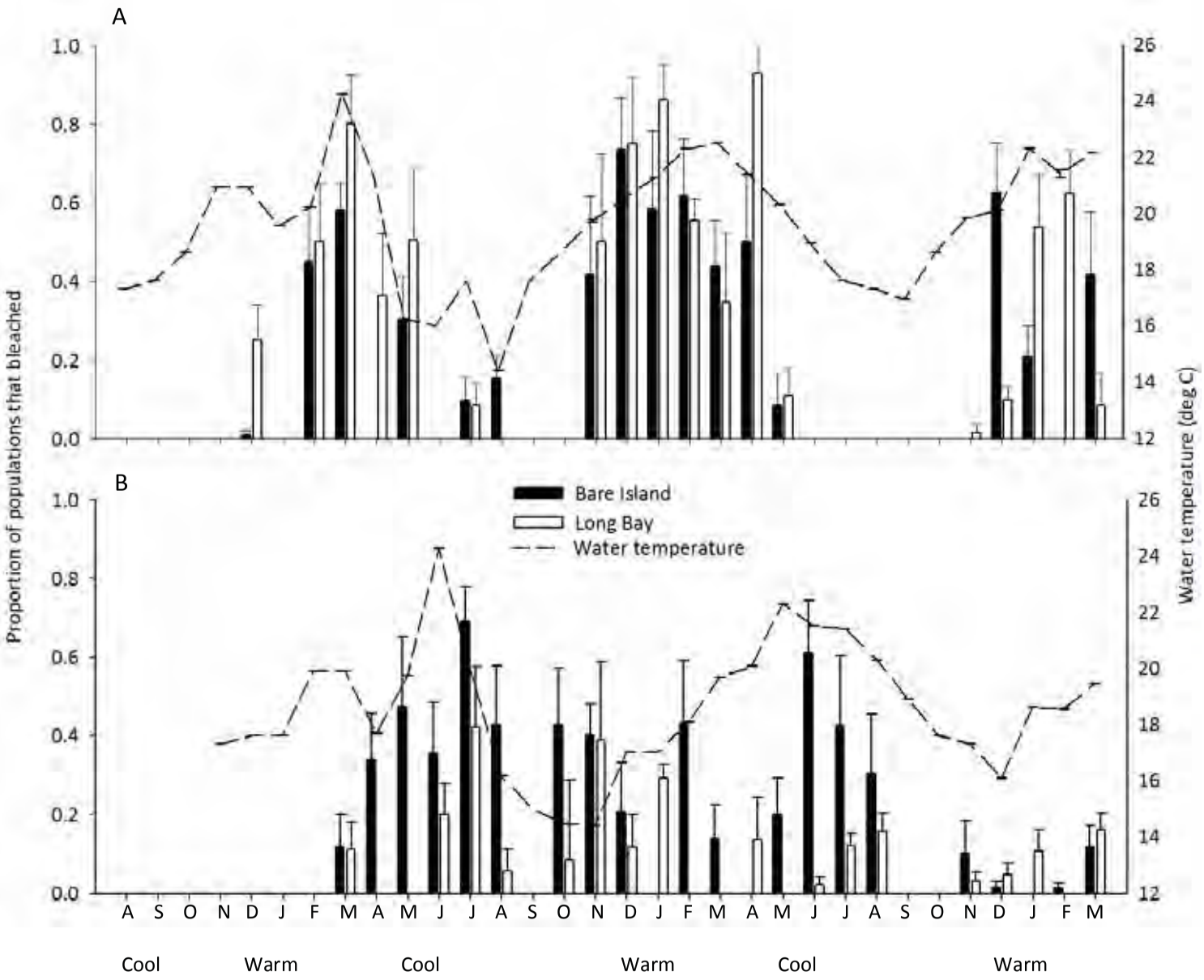
A
B
Bare Island
Long Bay
Water temperature
Proportion of populations that bleached
Water temperature (deg C)
1.0
0.8
0.6
0.4
0.2
0.0
26
24
22
20
18
16
14
12
A S O N D J F M A M J J A S O N D J F M A M J J A S O N D J F M
Cool
Warm
Cool
Warm
Cool
Warm

Figure 2.3. (On previous page) Proportions of *D. pulchra* (means +SE) growing in **(A)** shallow (1-4 m) and **(B)** deep (7-10 m) water that bleached during surveys (corresponding with left y-axis) at Bare Island (black bars) and Long Bay (white bars) and water temperature (dashed line; corresponding with right y-axis, in ^{o}C) with n = 6.

Table 2.1. Five-factor ANOVA, with p values generated via permutation, comparing the prevalence of bleaching in *D. pulchra* populations from Bare Island and Long Bay across three sampling years in deep and shallow water. '**' represents significant effect of factor on the prevalence of bleaching, with α= 0.01 and '*' represents significant effect of factor with α=0.05 (the latter should be interpreted with caution due to non-normal data).

Source of variation	df	MS	F	P(perm)
Sampling year "Y"	2	1.188	6.3526	0.014*
Season "S"	1	2.212	24.312	0.005**
Sampling time(Season) "T(S)"	1	0.127	2.089	0.036*
Location "L"	1	0.008	0.007	0.798
Depth "D"	8	0.006	0.522	0.507
Y x S	2	0.108	0.580	0.585
Y x L	2	1.466	1.129	0.324
Y x D	2	0.133	1.095	0.351
S x L	1	0.003	0.248	0.653
S x D	1	1.016	8.413	0.024*
L x D	1	0.227	1.255	0.287
Y x T(S)	12	0.241	3.957	0.001**
T(S) x L	8	0.174	2.864	0.006**
T(S) x D	8	0.192	3.166	0.001**
Y x S x L	2	0.003	0.368	0.694
Y x S x D	2	0.367	3.023	0.099
Y x L x D	2	0.005	0.230	0.803
S x L x D	1	0.439	2.428	0.178
Y x T(S) X L	9	0.009	1.574	0.112
Y x T(S) X D	12	0.147	2.415	0.004**
T(S) x L x D	8	0.334	5.500	0.001**
Y x S x L x D	2	0.104	0.500	0.639
Y x T(S) x L x D	9	0.243	3.997	0.001**
Error	471	0.006		

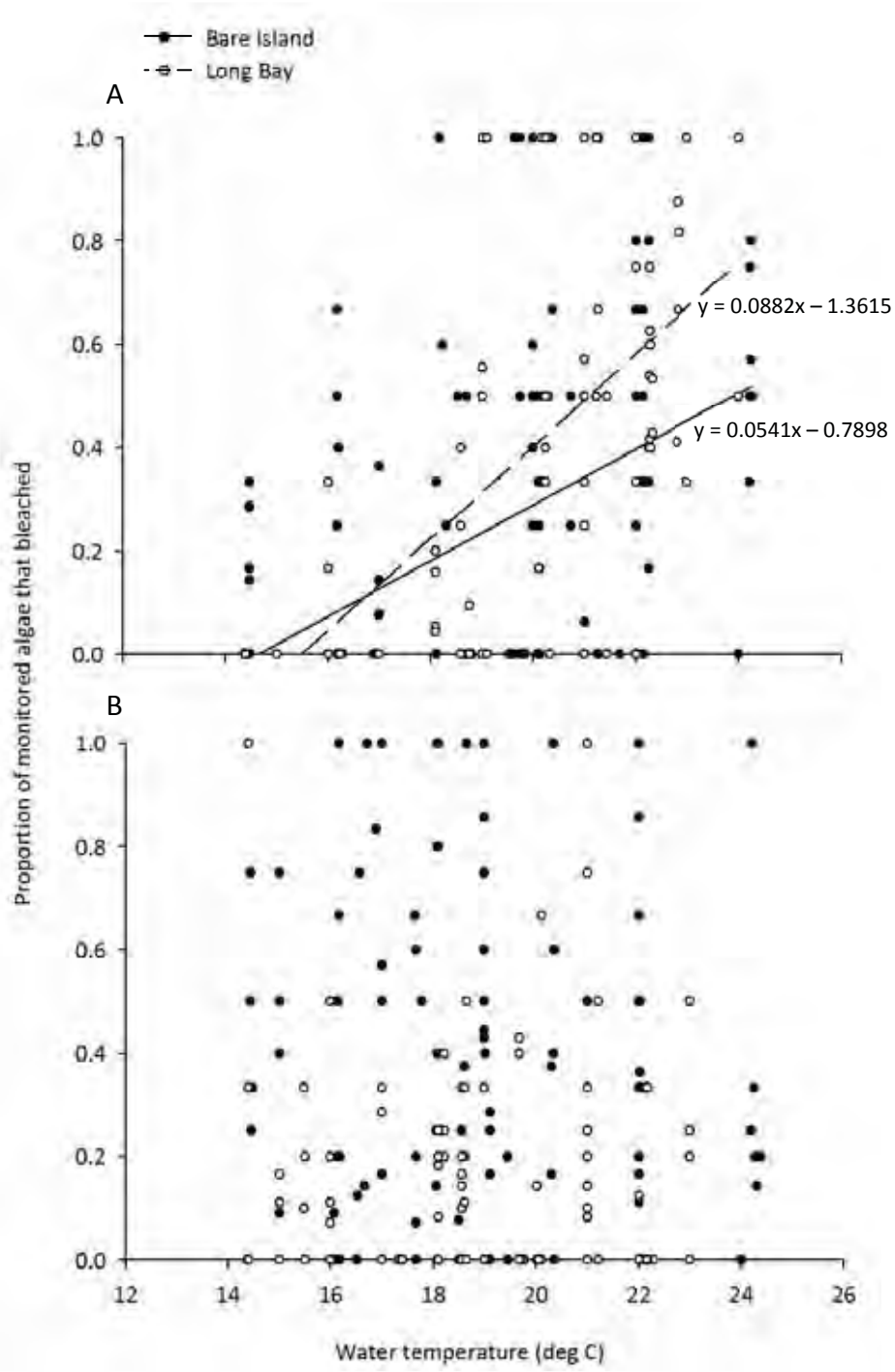

Figure 2.4. The proportion of bleaching at Bare Island (black circles, solid line) and Long Bay (white circles, dashed line) plotted as a function of water temperature in **(A)** shallow and **(B)** deep water populations of *D. pulchra* with *n* = 278. Significant, linear relationships are indicated by regression lines and are described by formulae included on graph.

When sites were pooled and analysed together, the overall incidence of bleaching was weakly (not significantly, with $\alpha = 0.01$) and negatively related with photosynthetically active radiation (PAR; R^2 =0.010, *df* =329, p =0.058; Figure 2.5A&D). Similarly weak (and marginally non-significant) but positive relationships between bleaching incidence and UVAR (R^2 =0.015, *df* =329, p =0.026; Figure 2.5B&E) and UVBR (R^2 =0.021, *df* =329, p =0.058; Figure 2.5c&f) were also detected. The relationships between bleaching and solar radiation were also assessed for deep and shallow algal populations separately as solar radiation was higher in the shallows than at depth (Figure 2.5). Additionally, due to site-specific differences in these relationships, sites were also compared separately but in all cases, relationships between solar radiation and algal bleaching were inconsistent and weak (Table 2.2).

The prevalence of bleaching was negatively related to salinity at Bare Island in shallow water populations, with more bleaching occurring when salinity was low, although this relationship was very weak and not observed at other locations or depths (Figure 2.6, Table 2.3). Thus overall, the physical variable that was most strongly related to patterns of bleaching in *D. pulchra* was water temperature, particularly in shallow water populations of the alga, and this was consistent at both study locations and across all three sampling years.

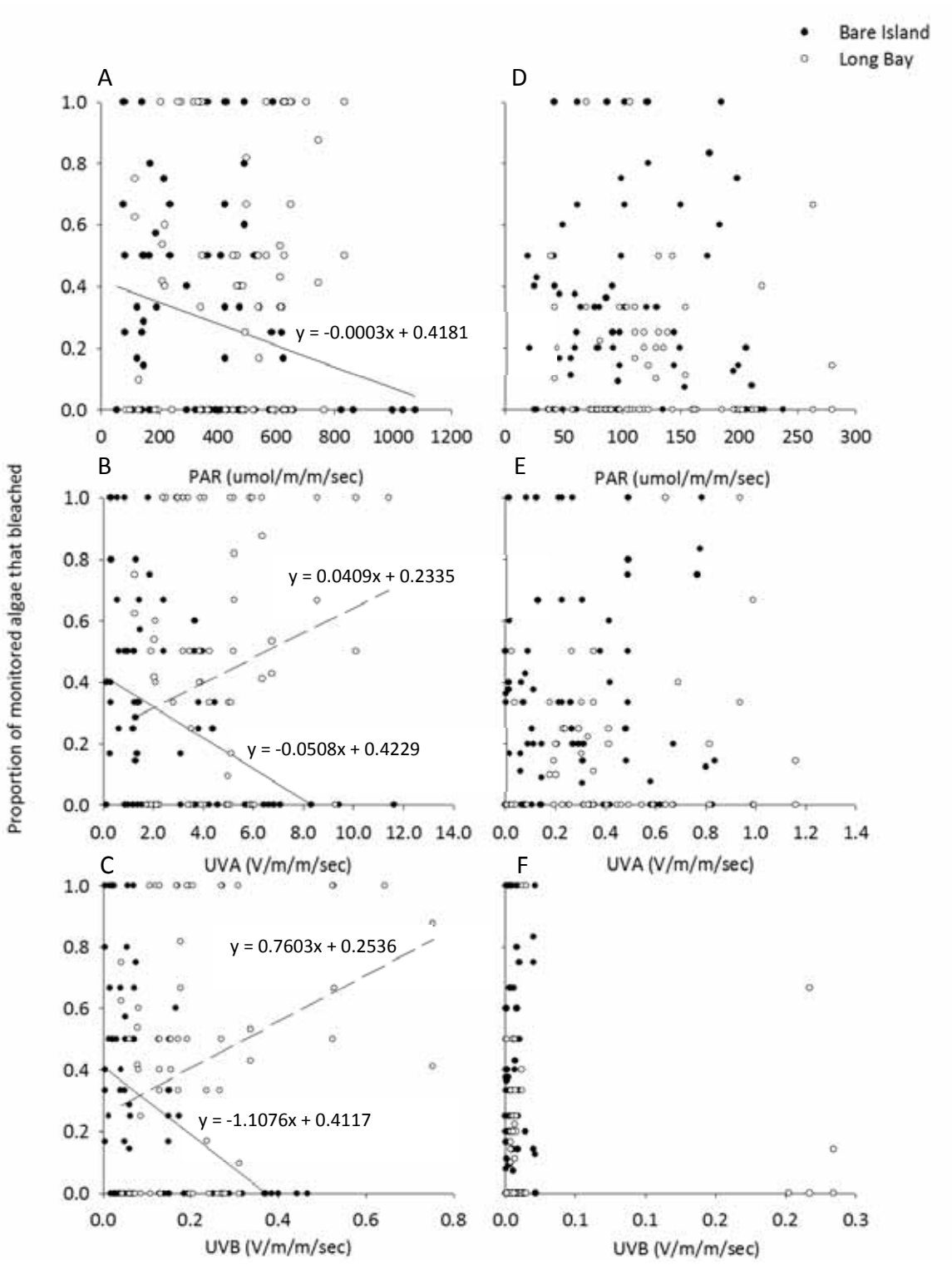

Figure 2.5. The prevalence of bleaching in *D. pulchra* from Bare Island (black circles, solid lines) and Long Bay (white circles, dashed lines) plotted as a function of different wavelengths of solar radiation in shallow **(A-C)** and deep **(D-F)** populations with *n* =78-90. Significant, linear relationships are indicated with regression lines described by formula.

Table 2.2. Linear regression analyses of relationships between the prevalence of bleaching in shallow populations of *D. pulchra* at two study locations and solar radiation wavelengths photosynthetically active radiation (PAR) and ultra-violet radiation A (UVAR) and B (UVBR) in shallow and deep water populations. '**' Indicates significant linear relationships with α= 0.01 and '*' represents a significant relationship with α=0.05.

	Bare Island			Long Bay		
shallow	R^2	df	p	R^2	df	p
PAR	0.053	84	0.035*	0.042	77	0.072
UVAR	0.115	84	0.002**	0.059	77	0.032*
UVBR	0.123	84	0.001**	0.094	77	0.006**
deep	R^2	df	p	R^2	df	p
PAR	0.012	89	0.301	0.001	77	0.767
UVAR	0.000	89	0.970	0.040	77	0.078
UVBR	0.005	89	0.524	0.000	77	0.902

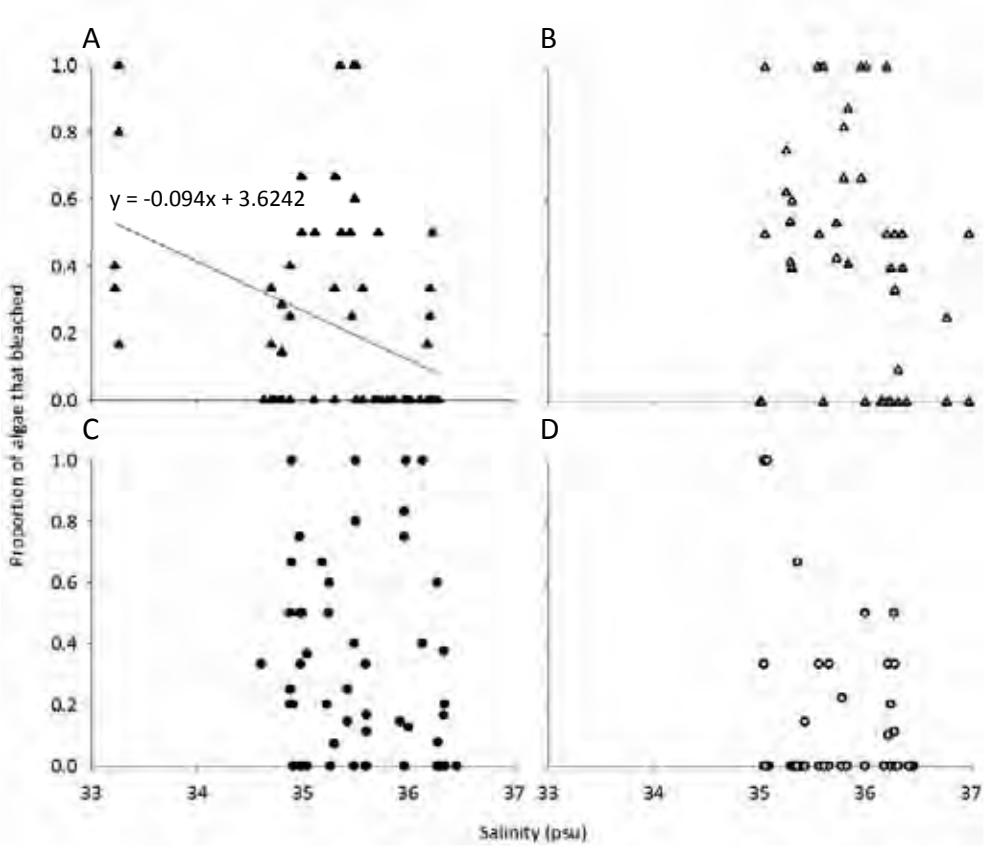

Figure 2.6. The prevalence of bleaching in **(A, B)** shallow (triangles) and **(C, D)** deep (circles) water populations of *D. pulchra* at Bare Island (black symbols) and Long Bay (white symbols) plotted as a function of salinity, with n = 60-70. The significant, linear relationship is indicated by the regression line, which is described by the formula.

Table 2.3. Linear regression analyses of relationships between prevalence of bleaching and salinity in deep and shallow water at the two study locations. '**' indicates a significant relationship with α= 0.01.

	Bare Island			Long Bay		
	R2	df	p	R2	df	p
shallow	0.141	71	0.001**	0.047	59	0.098
deep	0.054	59	0.073	0.012	59	0.396

Variation in chemical defences

To assess whether concentrations of chemical defences were different among bleached and healthy algae, I compared furanone concentrations between healthy and bleached tissue. Because replicate bleached, healthy and adjacent tissue samples from each year, location and depth combination were not available, I grouped algae from multiple 'warm' sampling periods (when bleaching was most frequent) and compared furanone concentrations in bleached vs. healthy individuals from each location and depth combination. Bleached individuals had significantly depleted concentrations of total furanones compared to co-occurring healthy algae and this was observed at both locations but was more pronounced in deep than shallow waters. Often, furanone concentrations in bleached algae were so low they were below the sensitivity of the GC-MS detector and were thus quantified as zero. There were no overall differences in chemical defences among the two study locations or between depths (Figure 2.7, Table 2.4).

When total furanone concentrations in 'bleached' and 'adjacent' unbleached tissues from bleached individuals were compared, I found no significant (marginally) difference (paired T = 1.89, p =0.084), with furanone concentrations comparably low throughout bleached thalli (Figure 2.8). Tissue from completely healthy algae had much higher furanone concentrations than 'adjacent' tissue from bleached algae (unpaired T =3.58, p =0.001; Figure 2.8). When seasonal and spatial comparisons were made between furanone concentrations in healthy algae (to determine whether some populations may be more or less defended at particular times), there was a strong, depth-specific seasonal influence on chemical defences. Shallow algae had much higher furanone concentrations during the 'cool' winter months than the 'warm' summer months, whereas there was no seasonal influence in deeper waters and this was observed consistently at both locations (Figure 2.9, Table 2.5).

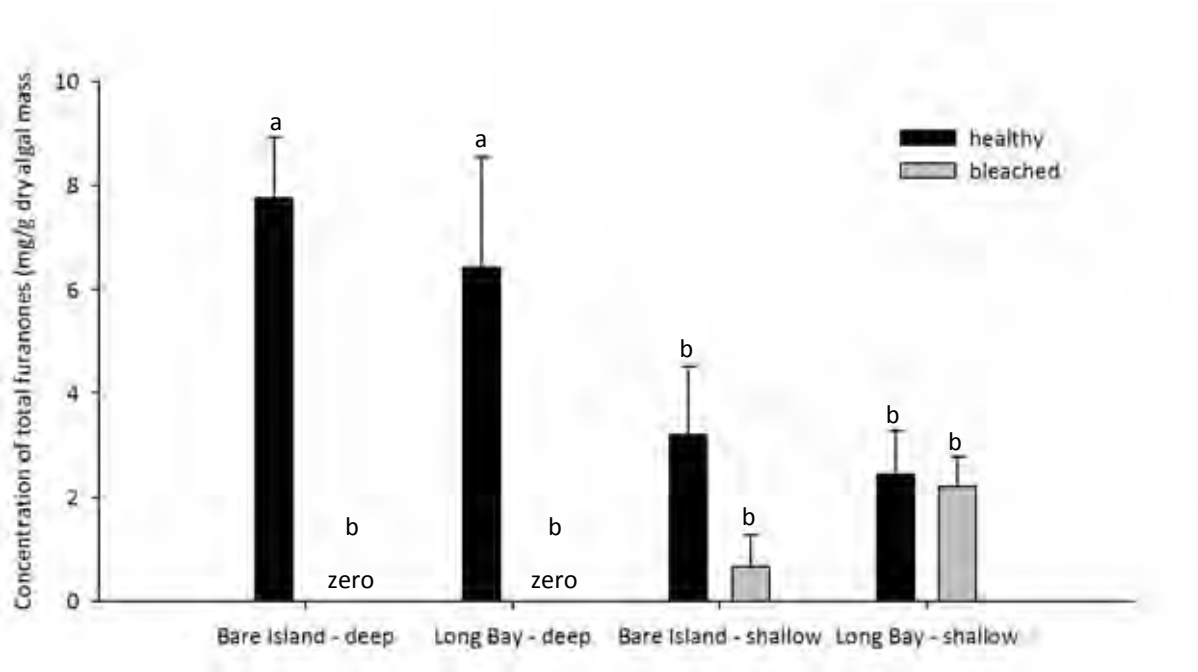

Figure 2.7. Concentrations of total furanones (means +SE) in bleached (grey bars) and healthy (black bars) *D. pulchra* from shallow and deep water habitats at Bare Island and Long Bay. Shared letters indicate statistical similarity according to Tukey's multiple pairwise comparisons tests.

Table 2.4. Three-factor ANOVA comparing total furanone concentrations from bleached and healthy algae collected from deep and shallow waters at Bare Island and Long Bay. '*' indicates significant effect of the relevant factor on total furanone concentrations with α=0.05.

Source of variation	df	MS	F-ratio	p
Location "L"	1	0.032	0.00	0.950
Depth "D"	1	10.764	1.33	0.255
Condition "C"	1	120.113	14.89	0.000*
L x D	1	0.755	0.09	0.761
L x C	1	3.662	0.45	0.504
D x C	1	51.516	6.38	0.016*
L x D x C	1	1.501	0.19	0.669
Error	10	8.069		

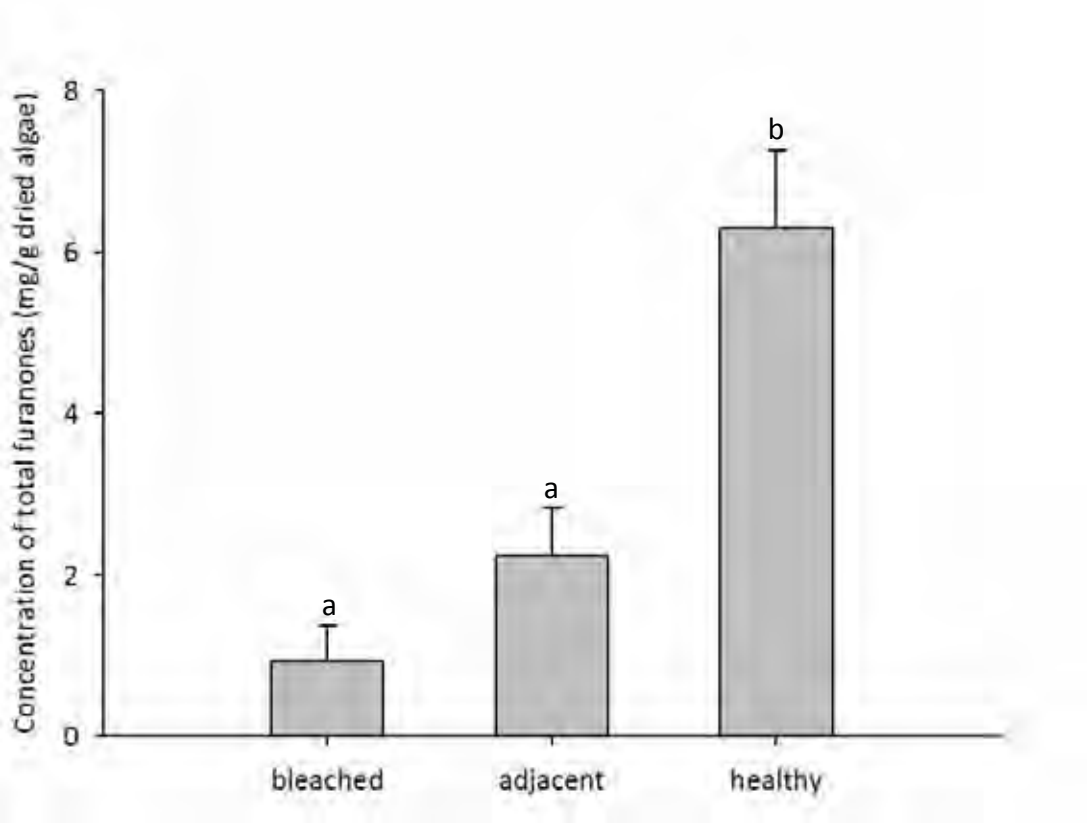

Figure 2.8. Concentrations of total furanones (means +SE) in *n*=15 randomly selected bleached (bleached and adjacent unbleached tissues) and healthy *D. pulchra* individuals. Shared letters represent statistical similarity according to T-tests (paired: bleached vs. adjacent; unpaired: healthy vs. adjacent).

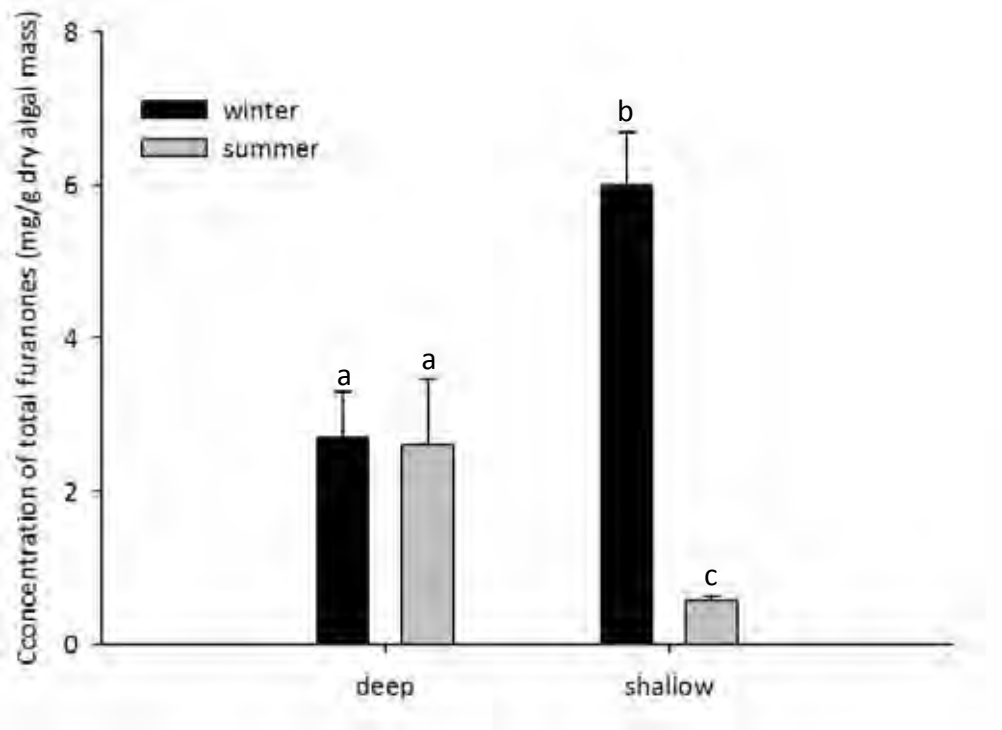

Figure 2.9. Concentrations of total furanones (means +SE) from healthy algae collected during summer and winter at deep and shallow populations (data from locations pooled as there was no effect of location) with *n*=5-8. Shared letters indicate statistical similarity according to Tukey's multiple pairwise comparisons tests.

Table 2.5. Three-factor ANOVA comparing total furanone concentrations from healthy algae collected from deep and shallow populations at the two study locations in summer and winter. Data were square-root transformed to conform to the assumptions of ANOVA. '*' indicates a significant effect of the relevant factor on furanone concentrations with α=0.05.

Source of variation	df	MS	F-ratio	p
Season "S"	1	7.928	20.78	0.000*
Location "L"	1	0.501	1.31	0.258
Depth "D"	1	0.152	0.40	0.531
S x L	1	2.864	7.51	0.009*
S x D	1	9.142	23.96	0.000*
L x D	1	0.693	1.82	0.185
S x L x D	1	0.024	0.06	0.805
Error	45	0.382		

Patterns of microbial communities associated with bleached and healthy algae

Surface-associated microbial communities (SAMCs) from healthy *D. pulchra* individuals were significantly different to those associated with co-occurring bleached algae (Figure 2.10, Table 2.6). This difference in SAMCs due to algal condition was consistent across all sampling years, locations and depths. On bleached thalli, SAMCs associated with unbleached parts of thalli, adjacent to visibly bleached areas were not significantly different from bleached areas and were also not significantly different from communities from co-occurring healthy *D. pulchra*. This pattern was observed consistently across all study years, at both locations and depths. SAMCs were similar among locations, but varied temporally in a location-specific way. SAMCs also varied between depths and this variation was not consistent from year to year, or between locations (Table 2.6).

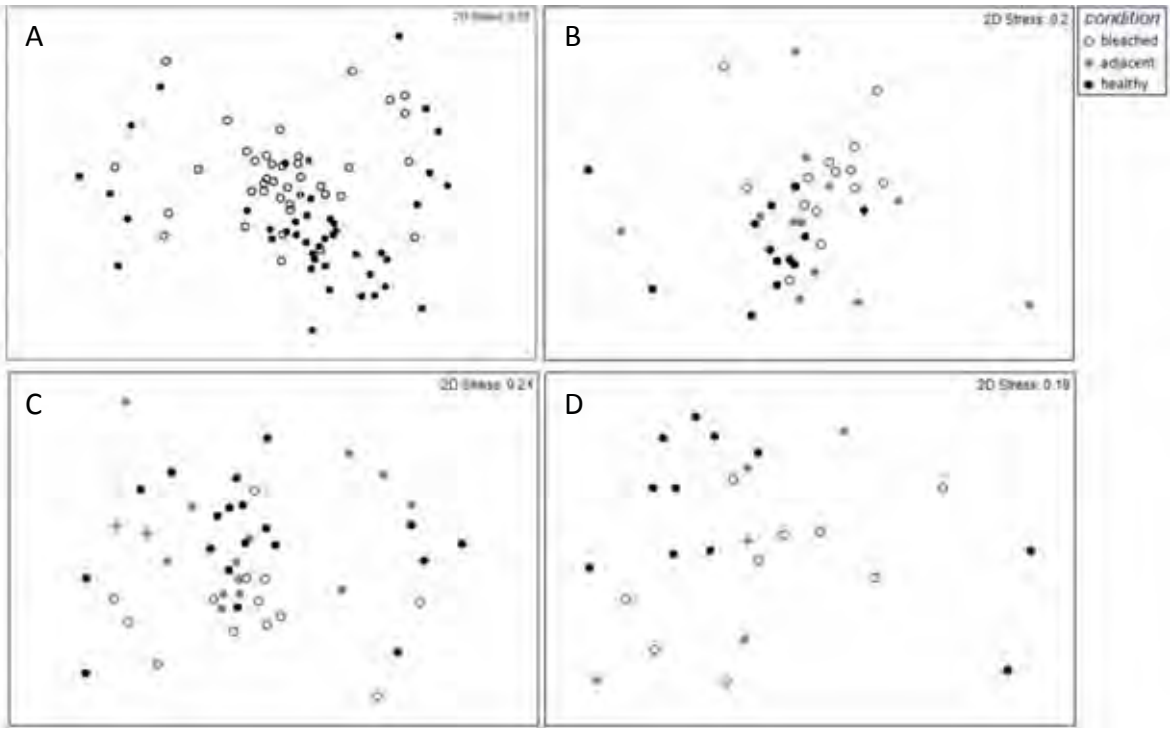

Figure 2.10. Non-parametric multi-dimensional scaling (nMDS) plots showing similarities between surface-associated microbial communities (SAMCs) associated with bleached individuals (bleached tissue (open circles) and adjacent unbleached tissue (grey circles)) and healthy individuals (black circles) **(A)** overall and in the **(B)** first, **(C)** second and **(D)** third sampling years. Data from both locations and depths are pooled in all plots and in plot **(A)**, community data from 'adjacent' algal tissues have been omitted for clarity.

Table 2.6. PERMANOVA comparisons of SAMCs from healthy *D. pulchra* individuals and bleached and adjacent tissue from co-occurring bleached individuals collected across three sampling years, at Bare Island and Long Bay from deep and shallow waters. '*' indicates significant effect of the relevant factor on SAMCs, with α=0.05.

Source of variation	df	MS	Pseudo-F	p(perm)
Sampling year "Y"	2	6210.9	2.8009	0.001*
Location "L"	1	3136.1	1.4142	0.138
Depth "D"	1	5004.6	2.2569	0.012*
Condition "C"	2	5583.0	2.5177	0.001*
Y x L	2	4132.2	1.8635	0.004*
Y x D	2	3683.6	1.6611	0.023*
Y x C	4	1905.6	0.8593	0.733
L x D	1	3968.0	1.7894	0.032*
L x C	2	2230.6	1.0059	0.437
D x C	2	2768.3	1.2484	0.163
Y x L x D	2	3079.6	1.3888	0.091
Y x L x C	4	2234.1	1.0075	0.448
Y x D x C	4	1964.1	0.8407	0.799
L x D x C	2	2315.5	1.0442	0.401
Y x L x D x C	2	2373.3	1.0702	0.352
Residual	80	2217.5		

Transplanting defended and undefended **D. pulchra** *sporelings into the field*

Furanone (-) algae bleached significantly more than furanone (+) algae in both outplanting experiments ($F_{1,13}$ =17.44, p =0001; Figure 2.11). The effect of furanones on the extent of bleaching was consistent among experiments.

Manipulating algal chemical defences, water temperature and microbes

Temperature varied predictably along the latitudinal gradient, with higher temperatures (25-26 ^{o}C) in the northern, sub-tropical waters than in the temperate zone (20-21 ^{o}C; Figure 2.12). In the antibiotic experiments, bleaching was consistently more common and extensive in warmer treatments. Sporelings exposed to non-sterile seawater bleached more extensively than those maintained in sterile conditions. Bleaching was less frequent and severe in furanone (+) sporelings than furanone (-) algae, except when algae were exposed to microbes (Figure 2.13; Table 2.7). However, furanone (+) sporelings treated with antibiotics bleached significantly less than furanone (-) sporelings treated with antibiotics and this was the case at both temperatures. Additionally, furanone (+) sporelings treated with antibiotics bleached significantly less than untreated, non-sterile furanone (+) sporelings. Furanone (-) sporelings treated with antibiotics still bleached at both temperatures, but to a significantly lesser degree than 'non-sterile' furanone (-) sporelings in unfiltered seawater (Figure 2.13, Table 2.7).

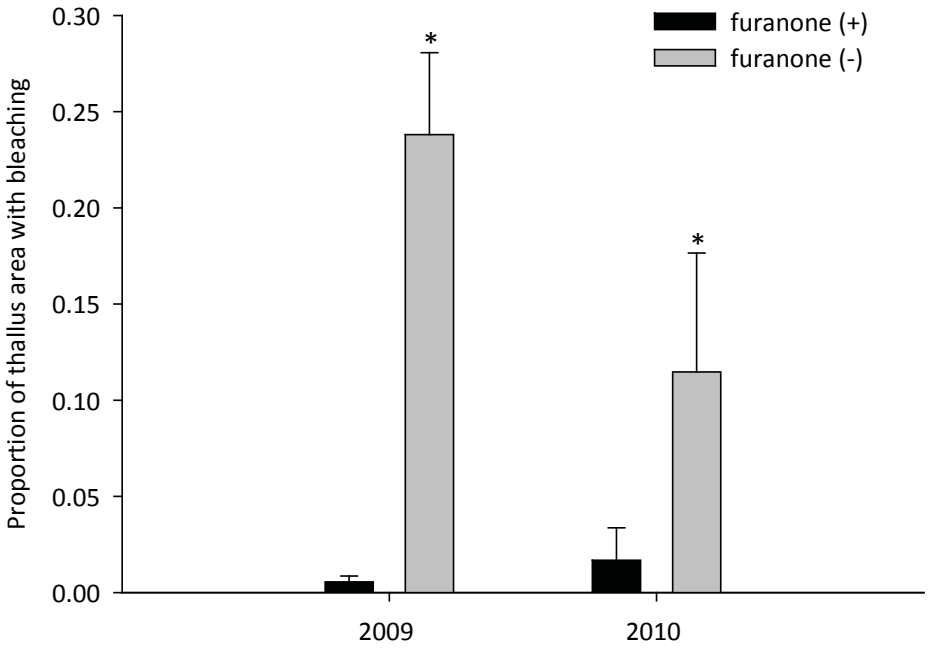

Figure 2.11. Proportion of thallus bleaching (means +SE) in furanone (-) (grey bars) and furanone (+) (black bars) *D. pulchra* sporelings deployed at Long Bay during the outplanting experiments, *n*=4-6. '*' indicates a significant difference in the extent of thallus bleaching between furanone (+) and (-) algae.

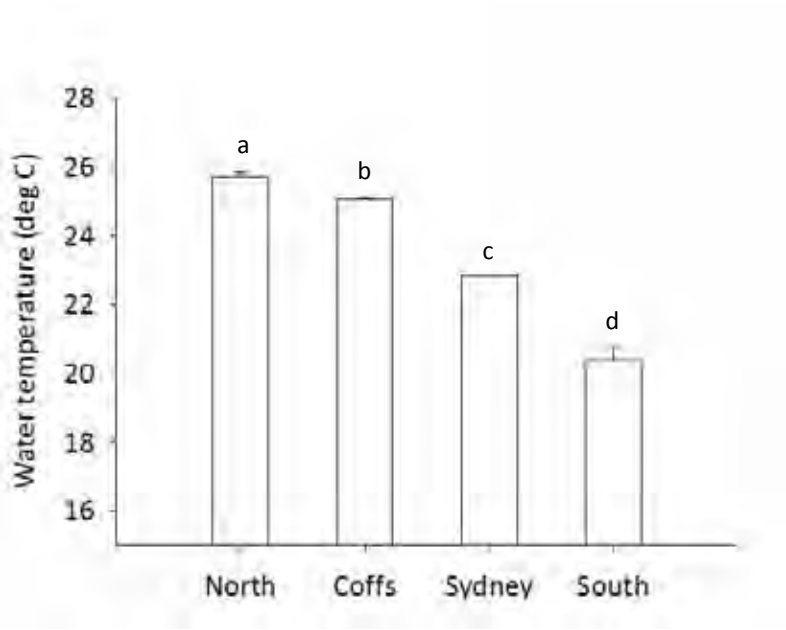

Figure 2.12. Summer water temperatures (means +SE) along a latitudinal gradient encompassing *D. pulchra*'s north-eastern Australian range, from its northern limits ('North'), southwards through the warm extreme of the temperate zone ('Coffs'), Sydney and into the cool temperate zone ('South') with *n*=6. Shared letters represent statistical similarity according to Tukey's multiple pairwise comparisons tests. These observations were used to decide upon the 'high' and 'low' temperatures used in the antibiotic experiments.

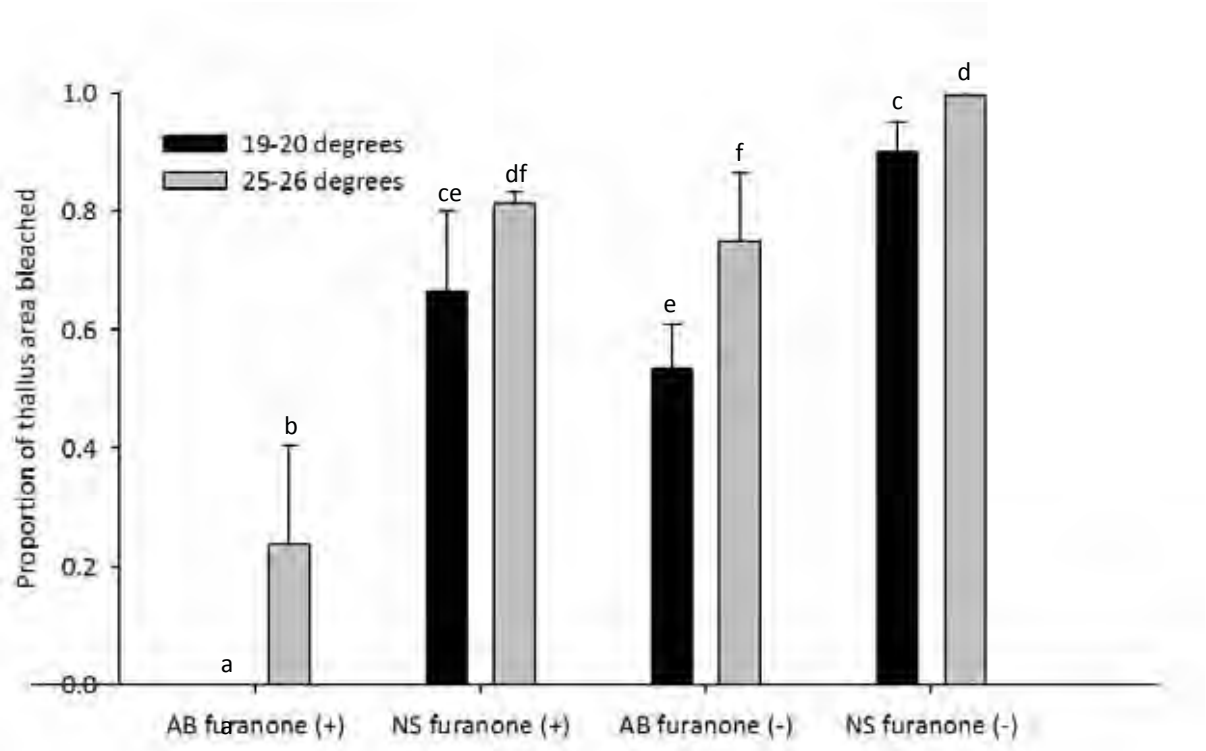

Figure 2.13. Proportion of thalli areas (means +SE) that bleached in antibiotic-treated (AB) and non-sterile (NS) furanone (+) and (-) *D. pulchra* sporelings exposed to high (grey bars) and low (black bars) water temperatures with replicates from individual incubators pooled into temperatures, giving *n*=6. Shared letters represent statistical similarity according to Tukey's multiple pairwise comparisons tests.

Table 2.7. ANOVA comparing the proportional areas of furanone (+/-) thallus bleaching exposed to high and low temperatures and the presence or absence of microbes. As there was no effect of individual incubators (which were nested within each temperature) the data from each incubator within each temperature were pooled for simplicity. '*' indicates significant effect of factor on the extent of thallus bleaching with α =0.05.

Source of variation	df	MS	F-ratio	p
Temperature "T"	1	0.336	6.70	0.014*
Furanones "F"	1	1.497	29.80	0.000*
Antibiotics "A"	1	2.391	47.60	0.000*
T x F	1	0.004	0.08	0.776
T x A	1	0.030	0.60	0.443
F x A	1	0.274	5.46	0.025*
T x F x A	1	0.000	0.01	0.915
Error	35	0.050		

DISCUSSION

Understanding the complex ways in which global change might affect important natural ecosystems (e.g. via disease) is paramount for biodiversity conservation and the management of natural resources. I observed effects of increasing temperatures on patterns of bleaching in an abundant, chemically-defended seaweed. Higher temperatures were correlated with higher incidences of bleaching and lower levels of algal chemical defences (halogenated furanones). Chemical defences were also significantly lower in bleached compared to healthy individuals and this reduction was apparent across entire thalli of affected algae. Furthermore, in the first experiments to demonstrate the importance of chemical defences in seaweeds *in situ*, I found that the presence of halogenated furanones in *D. pulchra* significantly reduced the severity of algal bleaching. Incidences of bleaching were associated with shifts in surface bacterial assemblages and these shifts were observed consistently through time and among locations. Bleaching could be induced in *D. pulchra* sporelings in laboratory experiments through manipulations of temperature and algal chemical defences, with exposure to ambient seawater microbes. These findings are consistent with the hypothesis that bleaching in natural populations of this seaweed is the result of a temperature-mediated bacterial infection.

Patterns of algal bleaching and environmental variables

Higher water temperatures were consistently associated with a higher frequency of bleaching in shallow populations of *D. pulchra*, and also increased the severity of bleaching in manipulative laboratory experiments. This relationship could potentially be explained by thermal stress: bleaching may have occurred directly in response to suboptimal temperature conditions. Anomalously high water temperatures have been linked to coral

stress and subsequent bleaching (reviewed by Hoegh-Guldberg 1999) and can affect physiological processes in marine macrophytes (Short and Neckles 1999, Staehr and Wernberg 2009). However, several observations in this study suggest that bleaching in *D. pulchra* is not a direct result of temperature. Despite the consistent relationship, water temperature explained less than one quarter of the variation in bleaching in shallow algae and less than 10% overall. A high degree of spatial and temporal variation in patterns of bleaching was not explained by temperature. For example, not all algae within an area exposed to the same water temperatures, bleached. Furthermore, when bleaching did occur, patterns of bleaching within affected individuals were non-uniform and not limited to any particular section of thalli, as would be likely if it were a direct response to environmental stress. Rather, bleaching occurred anywhere from the base to the tips of algae, further suggesting that it is not simply caused by high temperatures.

My observations suggest that the relationship between bleaching and temperature is complex and probably an indirect effect. In general, the environment strongly influences defensive chemistry in plants and algae (Coley et al. 1985, Caldwell et al. 1998, Bidart-Bouzat and Imeh-Nathaniel 2008) and I suggest that high water temperatures may affect the production of halogenated furanones in *D. pulchra*. If high temperatures lead to a decrease in defensive chemistry, this would have consequences for algal susceptibility to natural enemies, including pathogens.

Although infrequent, the negative relationship between salinity and the prevalence of bleaching in shallow algae at Bare Island is worth noting. During the monitoring study, this population of *D. pulchra* was influenced by a flooding river, resulting in anomalously low salinity for several weeks. Low salinity can compromise host defences (e.g. Green and

Barnes 2010) and associated run-off from urban cities may also introduce contaminants and pollutants into coastal environments via stormwater outlets (Makepeace et al. 1995, Birch 2000) potentially impacting subtidal macroalgae (e.g. Roberts et al. 2006) and microbial pathogens (e.g. Bruno et al. 2003). Salinity extremes have been linked to increased disease frequency and severity in oysters (Green and Barnes 2010), seagrass (Vergeer et al. 1995, McKone and Tanner 2009) and other red algae (Largo et al. 1995b) and as such, this could be an interesting avenue to pursue in further studies into *D. pulchra*'s bleaching. Importantly, solar radiation had no strong or consistent effects on patterns of algal bleaching in populations of *D. pulchra*, demonstrating that this phenomenon is unlikely to be 'photo-bleaching', which has been observed in other marine algae (e.g. Valentine and Johnson 2004).

Algal bleaching and chemical defences

At individual and population levels, throughout this study, bleaching was negatively related to concentrations of halogenated furanones. Although these observations are purely correlative, (i.e. furanones and patterns of bleaching may fluctuate independently), the importance of furanones in the bleaching phenomenon was confirmed experimentally, with well defended algae bleaching less frequently and severely than those lacking halogenated furanones. For example, when chemically defended and undefended *D. pulchra* thalli were transplanted into natural seawater *in situ*, the presence of halogenated furanones appeared to protect sporelings from bleaching. In the field, furanone concentrations are highly variable through time, within populations (Wright et al. 2000) and individuals (Dworjanyn et al. 1999). The ability to manipulate the production of these metabolites thus provides a

powerful tool with which the importance of chemical defences can be tested experimentally.

On a population level, concentrations of halogenated furanones in *D. pulchra* were also at their lowest when water temperatures were high. This relationship suggests that the production of furanones may be reduced at higher temperatures. Halogenated furanones are costly for *D. pulchra* to produce (Dworjanyn et al. 2006b) and thus, under 'stressful' conditions, resources may be reallocated away from defence and towards maintenance, resource acquisition, growth, etc (Cronin 2001). Wright et al., (2000) observed that along a latitudinal gradient, concentrations of halogenated furanones in shallow *D. pulchra* were generally lower in summer than winter and my observations of depth-specific seasonal variation of chemical defences support this. It has been established previously, that within healthy thalli, concentrations of halogenated furanones vary from the base of the alga to the tips (Dworjanyn et al. 1999). I have demonstrated that when *D. pulchra* bleaches, furanone concentrations are significantly depleted (often below the detection limits of GC-MS) across entire thalli. This last observation suggests that a reduction of chemical defences occurs thallus-wide, prior to visible bleaching. Such a reduction would affect *D. pulchra*'s susceptibility to natural enemies, including microbial pathogens, particularly when water temperatures are elevated.

Algal bleaching and the role of pathogens

Disease progressions are often associated with microbial shifts, such as those I observed between bleached and healthy *D. pulchra*. For example, communities associated with Caribbean corals shift significantly when they are affected by black band (Frias-Lopez et al. 2002) and white plague (Pantos et al. 2003) diseases. Although in general microbial shifts

associated with disease may not necessarily be involved causally, but rather may shift independently or in response to a change in their niche, changes in SAMCs associated with *D. pulchra* were detected prior to visible bleaching. Unbleached tissues adjacent to bleached parts of thalli supported 'transition communities', which were statistically similar both to communities from fully bleached and completely healthy tissues (the latter two being statistically distinct). Normally, the healthy, chemically-defended surface of *D. pulchra* supports relatively specific microbial communities (Longford et al. 2007, Longford 2008). The depletion of furanones, which help regulate surface microbial community composition and the expression of bacterial behavioural phenotypes via inhibition of bacterial quorum sensing (Maximilien et al. 1998, Manefield et al. 2001, Manefield et al. 2002), could thus facilitate the settlement of a different microbial community onto *D pulchra*'s surface.

Case et al., (2011) demonstrated that, under laboratory conditions when temperatures are high and chemical defences inhibited, a specific bacterium (*Ruegeria* sp. R11) can infect and bleach *D. pulchra* sporelings. Here, I have provided further evidence for a role of bacteria in bleaching of *D. pulchra* and also raised the possibility that a broad range of bacteria may have the capacity to infect and bleach marine algae. Sporelings that were exposed to natural seawater bleached much more frequently and extensively than conspecifics that were treated with antibiotics and maintained in 'sterile' media, especially when water temperatures were high and algal defences low. This suggests that ambient seawater microbial communities (and/or established biofilm communities) include opportunistic organisms that can exploit poorly defended hosts, particularly when temperatures are high. In further support of this general model of bacterial bleaching of algae, Case et al., (2011) found a second bacterium, isolated from a geographically distinct location in the U.S.A. that

infected and bleached *D. pulchra* sporelings cultured in Sydney, Australia. Thus, diverse bacteria may become virulent to susceptible hosts when temperatures rise.

Although the main effects of water temperature, chemical defences and microbes on algal bleaching appear clear, the antibiotic experiment revealed complex interactions between these factors in our system. I observed consistent bleaching in undefended algae from 'sterile' treatments, particularly when temperatures were high. This could indicate that some 'background bleaching' was unrelated to experimental manipulations, or may be the result of our inability to completely remove bacteria from algal surfaces (Huggett et al. 2006, Paul et al. 2006, Longford 2008). Any remnant, opportunistic, pathogenic microorganisms that might have persisted after treatment with antibiotics, could have exploited the lack of competition, proliferated and caused bleaching. Although this reveals a limitation of these experiments (where the inclusion of 'true controls' completely lacking bacteria is not possible), this manipulation of multiple, interacting factors (i.e. chemical defences, physical variables and microorganisms) is a strong approach to investigating complex ecological problems arising from global change.

Conclusions

Clear and consistent effects of high temperatures on the prevalence of bleaching and concurrent observations of furanone depletion and SAMC shifts in bleached *D. pulchra*, support the hypothesis that bleaching is a temperature-mediated bacterial infection. Analogous to many instances of coral bleaching in tropical marine ecosystems, I suggest that *D. pulchra*'s bleaching is a bacterially-mediated disease and that ocean warming (due to both seasonal variation and climate change) indirectly influences bleaching by causing a reduction in algal chemical defences and/or inducing or increasing pathogen virulence.

Many pathogens have temperature-regulated virulence (Konkel and Tilly 2000, Klinkert and Narberhaus 2009) and most 'higher' organisms become stressed when temperatures increase beyond their optimal range. Warming may simultaneously increase host stress and pathogen virulence, compelling a number of prominent ecologists to consider disease as one of the forces currently causing major transitions in many ecosystems around the world (Jackson 2001, Harvell et al. 2002, Bruno et al. 2007). Understanding the complex ways in which environmental change may affect habitat-forming seaweeds is crucial for their conservation and management of the ecosystems they support.

CHAPTER 3

Effects of temperature and host defences on the bleaching of *Delisea pulchra* by the bacterium *Ruegeria* sp. R11

INTRODUCTION

As climates change, disease is emerging as a major threat to natural ecosystems worldwide (reviewed by Harvell et al. 2002, Lafferty and Holt 2003, Lafferty et al. 2004). Disease outbreaks are often associated with temperature anomalies (Harvell et al. 2002, Pounds et al. 2006, Bruno et al. 2007) and have caused mass mortalities of diverse organisms, including seagrasses (Robblee et al. 1991, Vergeer et al. 1995), frogs (Pounds et al. 2006) and corals (Harvell et al. 2007). Despite these extreme and often catastrophic impacts, the pathogen(s) causing disease outbreaks are often difficult to identify and isolate (e.g. Apt and Gibor 1989, Cole and Babcock 1996, Richardson 1998, Cooney et al. 2002). Moreover, despite often compelling correlations (e.g. Correa et al. 1997, Altizer et al. 2004), the role of seasonality and environmental change on disease outbreaks and progression is often not established experimentally. Disease in marine environments, where microorganisms are ubiquitous (Reinheimer 1992) and pathogens tend to be particularly virulent (Ewald 1994), appear to be increasing in both frequency and severity (Harvell et al. 1999). South-eastern

Australia is projected to be a warming 'hot-spot' that has already experienced above-average ocean warming in the last couple of decades (Cai et al. 2007, Poloczanska et al. 2007), due largely to the intensification of the warm East Australian Current (Ridgeway 2007). Marine environments and organisms in this region may thus be particularly vulnerable to the potential impacts of climate change, including disease. Considering predicted global warming over the next century (IPCC 2007), understanding whether and how temperature increases and other stressors affect host-pathogen interactions is crucial.

Environmental change can have complex and variable effects on both hosts and pathogens (Lafferty and Holt 2003). Host organisms can become stressed if temperatures increase beyond their optimal thermal niche, potentially leading to depleted host immunological, chemical and other defences (e.g. Banin et al. 2000, Levitus et al. 2000, Raffel et al. 2006, Bidart-Bouzat and Imeh-Nathaniel 2008, Baird et al. 2009). Simultaneously, many pathogens have temperature-regulated virulence, becoming more virulent at higher temperatures (Konkel and Tilly 2000, Klinkert and Narberhaus 2009). Thus, in a warming world, more susceptible hosts may be exposed for longer periods to more virulent pathogens. Given both host and pathogen responses to temperature, the effects of environmental change on disease dynamics are likely to be complex but are poorly understood for most marine and terrestrial wildlife diseases.

Many marine organisms (including macroalgae) are chemically defended to protect themselves from water-borne microbes and other natural enemies (reviewed by Engel et al. 2002) but the production of these chemical defences can be metabolically costly. Although little empirical evidence is available for resource allocation to defence in marine algae (importantly excepting Dworjanyn et al. 2006b), theoretically at least, during times of stress

(e.g. anomalously high water temperatures) chemical defences may be depleted (Cronin 2001) and thus, host susceptibility to pathogens may be increased. Additionally, many marine organisms have physically protective structures and layers in place to protect themselves from ubiquitous seawater microbes and other natural enemies. For example, many algae are calcified (Nelson 2009) and some also have spines, thick outer layers and other structures that can protect them (Hanley et al. 2007). Any damage to these layers (e.g. via herbivory, acidification or other physical injury) could increase susceptibility to water-borne pathogens or other endosymbionts by creating infection sites (Klein and Perkins 1987, Borer et al. 2009, Longtin and Scrosati 2009). Indeed, many plants and algae have specific chemical defences to manage such wounds (Ross et al. 2006, Ruuhola and Yang 2006). Understanding how environmental change and other stressors may affect host defences and thus, influence pathogen infection and disease is important to understand host-pathogen interactions in increasingly stressful environments.

When habitat-formers are affected by disease, understanding environmental influences on host defences is particularly pertinent, as these organisms support communities from the bottom-up. In temperate marine ecosystems, macrophytes are the dominant habitat-forming organisms and play an important role in coastal ecosystems, analogous to that of coral reefs in the tropics, where disease is contributing to widespread coral bleaching (Aronson and Precht 2001, Rosenberg and Ben-Haim 2002, Williams and Miller 2005, Bruckner and Bruckner 2006, Harvell et al. 2007). Disease has also impacted global seagrass populations (Short et al. 1987, Robblee et al. 1991, Orth et al. 2006), with significant, ecosystem-scale consequences (Costanza et al. 1997). Similarly, recent reports of diseases affecting seaweeds are appearing in the literature (Correa et al. 1993, Correa et al. 1994,

Littler and Littler 1995, Correa et al. 1997, Largo et al. 1998), which has alarming, potential implications for temperate rocky reef ecosystems.

In Chapter 2, I described a bleaching phenomenon affecting the chemically-defended, foliose macroalga, *Delisea pulchra*. This habitat-forming seaweed is common within the southeast Australian climate-warming 'hot-spot' (Poloczanska et al. 2007). It is chemically defended, producing compounds called halogenated furanones (for more information about halogenated furanones, see Chapter 2), which protect it from natural enemies including epiphytic and pathogenic bacteria (De Nys et al. 1993, Dworjanyn et al. 1999, Manefield et al. 2001). Several locally abundant herbivores, including the urchin *Holopneustes purpurascens* (Williamson et al. 2004), the snail *Phasianotrochus eximius* (Wright et al. 2004) and the sea hare *Aplysia parvula* (Rogers et al. 2003) use this alga as habitat and food and thus, may cause damage to *D. pulchra* thalli via grazing or attachment. Shallow water (1-4 m) populations of this alga bleached more commonly during the summer and when algal chemical defences were low and I demonstrated experimentally that the presence of halogenated furanones reduced the frequency and severity of seaweed bleaching (Chapter 2). Also linked to bleaching was a shift in the microbial communities associated with algal surfaces and I established that, when algae were exposed to microorganisms, they were more likely to bleach (and to a greater extent) than algae exposed to 'sterile' conditions, especially when water temperatures were high (Chapter 2).

These findings, along with observations of non-uniform patterns of algal bleaching (both within populations and individuals) suggest that this bleaching phenomenon may be the result of an environmentally-mediated bacterial infection(s). Case et al. (2011) isolated a bacterium from *D. pulchra* (*Ruegeria* sp. R11, hereafter 'R11') and using laboratory

experiments, demonstrated that inoculation with this bacterial strain induced bleaching in algal sporelings when water temperatures were elevated and algal chemical defences experimentally inhibited. In these experiments, Koch's postulates were satisfied and Case et al. (2011) proposed R11 as a novel bacterial bleaching pathogen of *D. pulchra*. I used this host-pathogen system to further assess R11's role as a pathogen of *D. pulchra* in a series of manipulative laboratory and novel field inoculation experiments. Specifically, manipulating temperatures slightly above those used by Case et al. (2011), but within the temperature range predicted for the south-eastern Australian global warming 'hot-spot' over the next century (Poloczanska et al. 2007), I tested whether inoculation with R11 cultures led to algal bleaching, and whether this was influenced by water temperature or algal chemical defences. Secondly, to assess whether physical damage (similar to that inflicted by herbivores) could facilitate bacterial bleaching in *D. pulchra* by creating infection sites, I inflicted established algae with artificial wounds and exposed them to R11. These experiments tested the hypotheses that bleaching in *D. pulchra* is the result of a temperature-mediated infection by the bacterium R11 and that algal stress (either physical damage or the experimental inhibition of chemical defences) is required to facilitate infection.

MATERIALS & METHODS

Study sites and organisms

Delisea pulchra Greville) Montagne (Bonnemaisonales: Rhodophyta) is an abundant macroalga that occurs subtidally in temperate Australia (for more information about *D. pulchra*, see Chapter 2). Experiments and sampling of fertile adult tetrasporophytes were conducted at Long Bay, Malabar, near Sydney, Australia (Chapter 2). At this location *D. pulchra* is abundant on rocky reefs between 2-10 m depth *Nautella* sp. R11 belongs to the roseobacter clade within the Alphaproteobacteria phylum and was first isolated from *D. pulchra* collected from nearby Bare Island. This species can be cultured in the laboratory in marine broth (Difco) and has recently been observed from microbial communities associated with bleached *D. pulchra* individuals using 16S RNA gene sequencing techniques (Fernandes et al. in prep).

Manipulating temperature, defensive chemistry and pathogens in the laboratory

To assess whether the candidate pathogen R11 can cause bleaching in *D. pulchra* and whether its ability to do so is affected by algal chemical defences and water temperature, I exposed defended ('furanone (+)') and undefended ('furanone (-)') sporelings to bacterial cultures at high and low temperatures. Tetraspores were harvested from fertile adult tetrasporophytes collected from Long Bay, Malabar and cultured as detailed in Chapter 2. The removal of bromine from growth media affects the ability of *D. pulchra* to produce furanones (which are brominated compounds) but does not otherwise affect the algae (Chapter 2, Dworjanyn et al. 1999, Dworjanyn et al. 2006b). Prior to experimentation, the sporelings (approximately 2mm in length) were soaked for 24 hours in ASW solution containing 20 mg/L streptomycin, 10 mg/L kanamycin and 10 mg/L penicillin to reduce any

bacterial contaminants and biofilms, then rinsed three times (for at least 3 minutes per rinse) in sterile ASW to remove residual antibiotics. Furanone (+) or furanone (-) algae were added to individual, 3.5 cm diameter Petri dishes containing 5 ml sterile Br+/- ASW. Half of these were inoculated with R11 cultures, the other half were not inoculated and treated as controls.

R11 was cultured overnight to stationary phase (10^9 cells/ml; approx. 17 hours; Case et al. 2011) in 5 ml marine broth (Difco) medium within a 50 ml falcon tube at 30 ^{o}C on an orbital mixer set at 150 rpm. Cells were pelleted via centrifugation at 4500 rpm and the spent supernatant was discarded. Cells were then resuspended and rinsed in 5 ml either Br+ or Br- sterile ASW and pelleted again. The ASW was discarded and the wash step repeated. Finally, 'washed' cells were resuspended in clean ASW (Br+/-) and diluted to a concentration of 10^8 cells/ml at which time they were ready for use in inoculation experiments. 50 µl 10^8 cells/ml R11 cultures were added to appropriate 'inoculation' treatment dishes containing 5 ml ASW (and *D. pulchra* sporelings) to obtain a final cell concentration of 10^6 cells/ml. Case et al. (2011) previously determined R11's growth curves and time until stationary phase and also established that 10^6 cells/ml is an effective cell density for inoculation assays. This density and higher is often used in inoculation experiments testing the efficacy of pathogens against their host (e.g. Cervino et al. 2008).

Three replicate dishes were placed in one of four incubators. Two incubators were set at low (20-21 ^{o}C) and the other two at high (25-26 ^{o}C) temperatures, such that 'Incubator' was a nested treatment within the treatment 'temperature'. These experimental temperatures were chosen based on surveys of water temperature within part of *D. pulchra*'s Australian range (Chapter 2). They represent temperatures that populations of *D. pulchra* experience

currently and are likely to experience more frequently as oceans warm. The dishes were agitated at 30 rpm on an orbital mixer throughout the experiment and were placed under a 15:9 light:dark cycle for three days. After this time, each sporeling was photographed under a dissecting microscope and any bleaching was quantified as the proportion of thallus area bleached using the program 'Image J'. Three replicate algae in each treatment were placed within each incubator, giving n=6 for each treatment combination. This experiment was conducted a total of three times.

Inoculation experiments in the field

To assess whether R11 could cause bleaching in established, chemically-defended *D. pulchra* individuals and whether artificially inflicted damage facilitated infection, I exposed damaged and undamaged algae to R11 *in situ* and monitored them for signs of bleaching for 48 hours. All experimental algae were inspected prior to experimentation and classified as healthy, (i.e. they had no visible signs of bleaching and no obvious fouling) and were all approximately 10-20 cm length. The bacterium was grown in 1 L of marine broth, using the methods described above except that for these experiments, the cultures were kept at a concentration of 10^9 cells/ml. This higher inoculum density was used in the field inoculation experiments to account for dilution within bags (see below), which were 3500 ml in volume, giving a final density R11 of approximately 10^7 cells/ml.

Eight replicate algae were enclosed inside plastic bags *in situ*, with a 50 ml falcon tube containing R11 culture (Figure 3.1). The tubes were opened and then the algae were left enclosed within the bags, exposed to the bacteria for two hours, after which time the bags were removed. Another eight replicate algae were damaged via gentle scraping with a razor blade (with the intention of removing the top couple of cell layers, thus mimicking grazing

damage inflicted by a gastropod) and also inoculated with R11 as described above. Another set of replicate algae were damaged but not inoculated with R11. In two procedural control treatments, eight replicate algae were bagged but not inoculated or damaged and another eight were handled but not bagged, damaged or inoculated. Finally, eight algae were left as unmanipulated controls. Thus, there were six experimental treatments each with eight replicates: (1) inoculation only, (2) damage + inoculation, (3) damage only, (4) bag control, (5) handling control and (6) unmanipulated control.

After 48 hours, each alga was revisited and the presence of any bleaching was recorded. Because bleaching occurred more frequently in summer than winter (Chapter 2), this experiment was conducted during summer 2008-2009, when the water temperature was 21-22 ^{o}C, again in winter 2009 when the water temperature was approximately 17-18 ^{o}C and a third time in summer 2009- 2010 when water temperatures were approximately 21-22^{o}C to assess whether R11's ability to cause bleaching in *D. pulchra* varied seasonally. These experiments were conducted in deep water habitats due to the rarity of completely healthy algae in the shallows during summer.

Statistical analyses

The proportional areas of furanone (+/-) sporeling thalli that bleached in the laboratory inoculation experiments were initially compared in a five-factor ANOVA, with the factors 'experiment time' (*n*=3), temperature (high (26 ^{o}C), low (20 ^{o}C)), incubator (nested within temperature with *n* =3 in experiment 1 and *n* =2 in experiments 2 and 3), furanones (+/-) and inoculation (+/-). As there was consistently no significant effect of incubator, data from within each temperature were pooled for ease of analyses and interpretations. Additionally, there was no difference between experiments 1 and 2 so they were pooled, whereas

experiment 3 was analysed separately and the latter ANOVAs (with 'incubators' and 'experiments' 1 and 3 pooled) are presented. Differences in bleaching response (+/-; binary response variable) between treatments in the field inoculation experiments were compared using a two-factor PERMANOVA where the effects of the factors experiment number (summer 1, winter, summer 2), and treatment (inoculated only, damaged and inoculated, damaged only, bag control, handling control and unmanipulated control).

Figure 3.1. The *in situ* inoculation of an established *D. pulchra* individual with the bacterium R11 during the field inoculation experiments carried out at Long Bay in summer 2008-2009, winter 2009 and again in summer 2009-2010.

RESULTS

Effects of temperature, halogenated furanones and pathogens in the laboratory

In all three experiments, bleaching occurred much more frequently when water temperatures were elevated (Figure 3.2). Data from experiment 3 were significantly different from those in experiments 1 and 2 so they were analysed separately. In experiments 1 and 2, the effect of temperature on bleaching varied depending on the presence of furanones and inoculation with R11 (Table 3.1A, Figure 3.2A), with the most extensive bleaching occurring in inoculated furanone (-) algae at high temperatures. In experiments 1 and 2, bleaching was rare in furanone (+) algae, regardless of temperature or inoculation (Figure 3.2A).

Different results were obtained from experiment 3. Here, inoculation with R11 did not influence the extent of bleaching at any temperature, regardless of whether algae were chemically defended or not. In experiment 3, inoculated furanone (+) bleached to a similar degree as inoculated furanone (-) algae when temperatures were high. Uninoculated furanone (+) algae bleached to a lesser extent than furanone (-) algae at both temperatures. When the water was cool, furanone (-) algae bleached significantly more often and severely thank furanone (+) sporelings, regardless of whether or not they had been inoculated with R11 (Table 3.1B, Figure 3.2B).

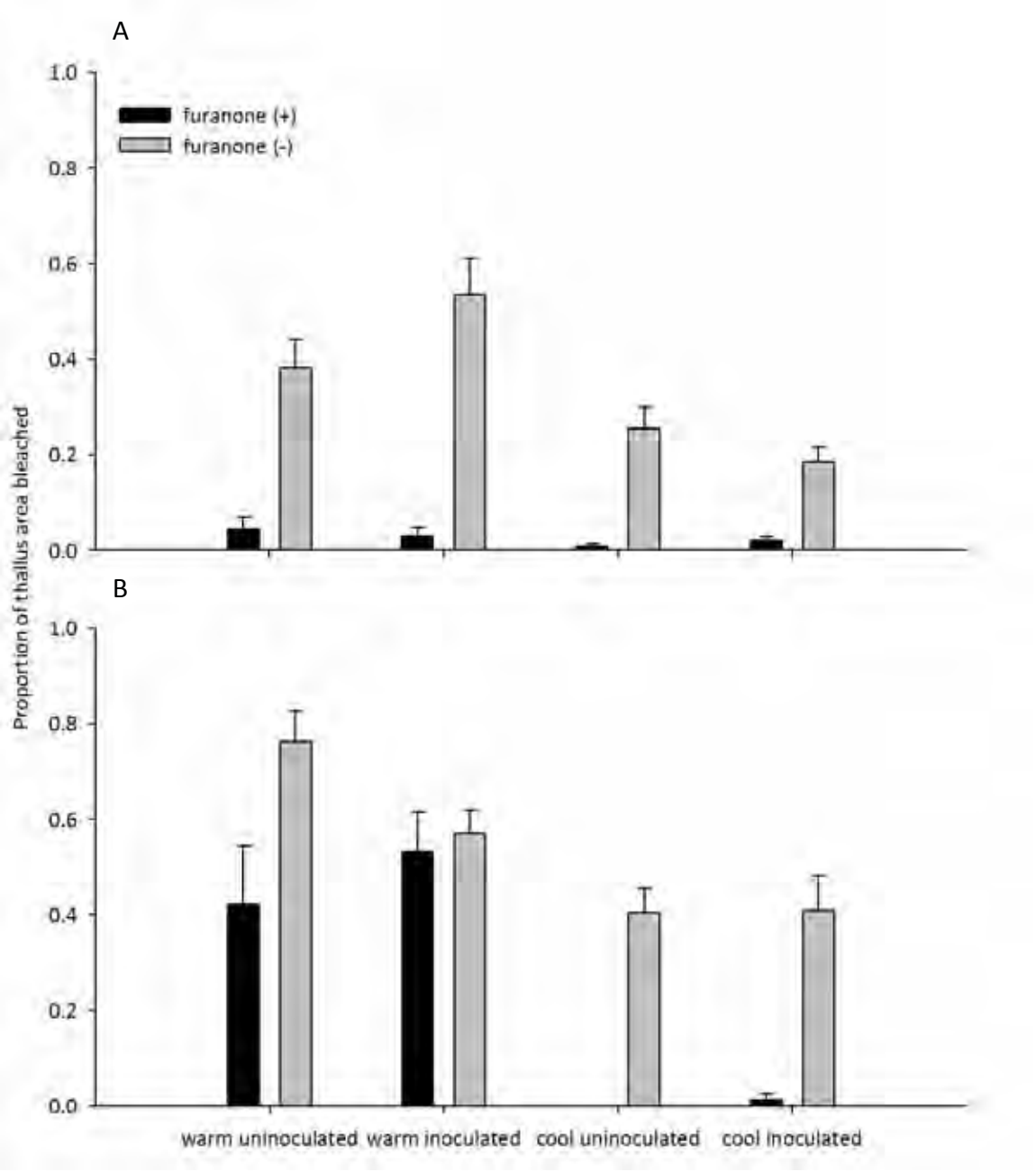

Figure 3.2. Proportional areas of experimental sporeling thalli that bleached (means +SE) in experiments **(A)** 1 and 2 and **(B)** 3 in which water temperature, furanones and the presence of R11 was manipulated, with *n*=6-9.

Table 3.1. ANOVA comparing the extent of bleaching among furanone (+/-) sporelings exposed to cultures of the bacterium R11 under high and low water temperatures in **(A)** experiments 1 and 2 and **(B)** experiment 3. '*' indicates significant effect of relevant factor on bleaching with $\alpha = 0.05$.

A

Source of variation	df	MS	F-ratio	p
Temperature "T"	1	0.507	20.45	0.000*
Furanones "F"	1	2.891	116.65	0.000*
Inoculation "I"	1	0.012	0.49	0.487
T x F	1	0.348	14.04	0.000*
T x I	1	0.072	2.92	0.090
F x I	1	0.012	0.49	0.486
T x F x I	1	0.111	4.47	0.037*
Error	110	0.025		

B

Source of variation	df	MS	F-ratio	p
Temperature "T"	1	1.361	64.37	0.000*
Furanones "F"	1	0.888	42.01	0.000*
Inoculation "I"	1	0.003	0.13	0.719
T x F	1	0.114	5.39	0.026*
T x I	1	0.006	0.29	0.594
F x I	1	0.061	2.89	0.098
T x F x I	1	0.055	2.60	0.116
Error	34	0.021		

Effects of damage and inoculation in the field

Algae that were both inoculated and damaged were significantly more likely to bleach than algae in any other treatment, in all three experiments (Figure 3.3, Table 3.2). There was no effect of time on the results, with 'stress + inoculation' algae consistently bleaching more than other treatments regardless of the time of year the experiment was conducted. Pairwise comparisons of treatments showed that algae in the 'damage + inoculation' treatment bleached significantly more than 'damage only' ($p=0.003$) and 'inoculation only' ($p=0.011$) treatments and more than all the control treatments, in which no bleaching was observed. Although there was some bleaching observed in 'damage only' and 'inoculation only' treatments, this was infrequent and not significantly different from frequencies of bleaching in control treatments (which was zero). Neither simply scraping, nor just inoculating algae led to bleaching (with the exception of one replicate in each treatment). Bleaching observed throughout these experiments appeared to be restricted to the experimentally damaged regions of thalli (Figure 3.4).

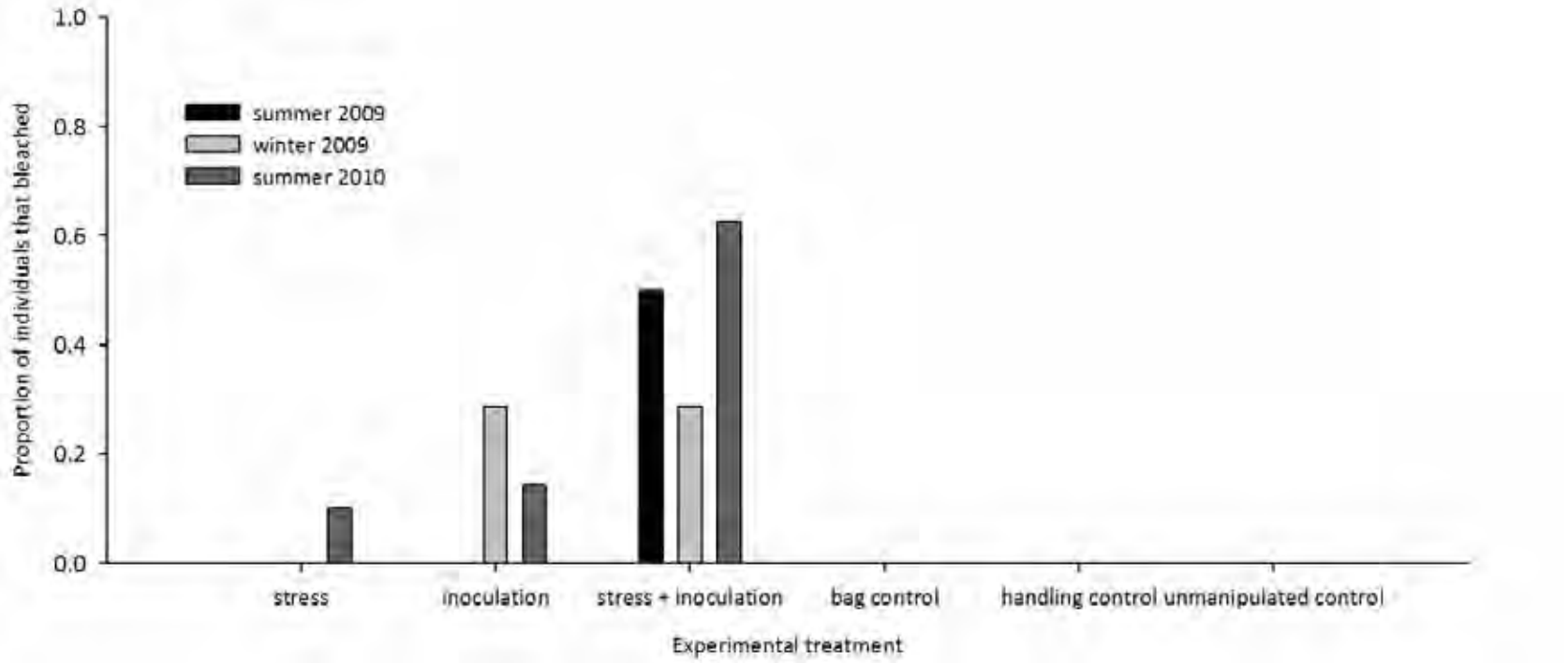

Figure 3.3. Proportion of algae within treatments that bleached during the field inoculation experiments conducted in summer 2009 (black bars), winter 2009 (light grey bars) and summer 2010 (dark grey bars) in deep waters at Long Bay.

Table 3.2. Permutational ANOVA comparing the incidence of bleaching in *D. pulchra* manipulated during the field inoculation experiments. '*' indicates significant effect of factor on bleaching incidence

Source of variation	df	MS	F	P
Experiment "E"	2	0.005	0.638	0.558
Treatment "T"	2	0.757	10.072	0.001*
ExT	10	0.007	0.9974	0.442
Residual	113	0.008		

Figure 3.4. The extent of bleaching observed in three replicate algae in the 'damage + inoculation' treatment, from one of the field inoculation experiments. Bleaching was restricted to areas that had been experimentally damaged (indicated by arrows).

DISCUSSION

In this chapter, I have demonstrated that, under certain laboratory and field conditions, the putative pathogen, *Ruegeria* sp. R11 can cause bleaching in the seaweed *Delisea pulchra*. Its ability to do so appears to be moderated by water temperature and algal chemical defences (in laboratory experiments), as well as algal physical defences (in field experiments). In two out of three laboratory inoculation experiments, inoculated, undefended *D. pulchra* sporelings maintained at high temperatures bleached more severely than defended algae (inoculated or no) in cooler waters. This result was not obtained in the third repeat of the experiment however, where undefended algae at higher temperatures bleached more severely than defended algae at low temperatures, regardless of whether or not they had been inoculated with R11. In a novel field inoculation experiment involving marine macroalgae, R11 was clearly able to cause localised bleaching on *D. pulchra* thalli that had also been experimentally damaged. In these field experiments, algae that had been both damaged and inoculated were significantly more likely to bleach than algae in other treatments, regardless of the water temperature at the time of experimentation. These findings confirm the potential role of R11 as a putative pathogen of *D. pulchra* but also highlight complexity in this particular host-pathogen system and the influence of water temperature.

R11 as an algal pathogen

In all field and the majority of laboratory inoculation experiments, the exposure of chemically undefended sporelings or experimentally damaged adults, to cultures of R11 led to bleaching (although in the laboratory experiments, this was only the case when water temperatures were elevated). In the final laboratory experiment, the high water

temperature sporelings (defended and undefended) all bleached substantially and the presence of R11 had no influence on this. At lower temperatures, however, undefended sporelings bleached more often and severely than defended conspecifics, regardless of whether they had been inoculated with R11. The lack of effect of inoculation with R11 in this final experiment could be due to contamination or another experimental issue, in which all algae were accidentally exposed to another stressor (not related to my experimental manipulations) that led to bleaching, making the addition of R11 inconsequential.

Case et al. (2011) consistently found that, as per my first two laboratory inoculation experiments, when undefended sporelings were exposed to R11 at high temperatures, the bacterial cells predictably formed biofilms and invaded the algal cells, leading to bleaching. The high temperatures used in this study were purposefully 1-2 ^{o}C higher than those used by Case et al. (2011) and this temperature may be approaching *D. pulchra*'s thermal tolerance limits. Senescence due to heat stress would likely have also involved pigment loss as cells died and released their contents. However, had stressfully high water temperatures had directly negative effects on the sporelings, leading to the observed bleaching, then it should have influenced defended and undefended algae similarly in all experiments, but this was not the case. In light of these observations and the discussion of residual bacteria in Chapter 2, the most likely explanation for the bleaching observed in laboratory experiment 3 is that residual bacteria, not removed by antibiotic treatments, were able to infect and bleach *D. pulchra* sporelings at those high temperatures.

This suggestion raises two interesting possibilities. Firstly, these high temperatures, which are within the predicted range for south-eastern Australia over the next century (Poloczanska et al. 2007), may have stressed defended sporelings and led to reduced

concentrations of halogenated furanones and thus, increased susceptibility to opportunistic pathogens. This scenario is alarming for *D. pulchra* populations occurring within the south-eastern Australian global warming 'hot-spot', if indeed the production of halogenated furanones is reduced when waters warm. Little is known about how warming may affect the production of chemical defences in seaweeds and what is known about terrestrial plants demonstrate a high degree of species and compound specificity (reviewed by Bidart-Bouzat and Imeh-Nathaniel 2008). My findings in Chapter 2 and previous work by Wright et al. (2004) suggest that *D. pulchra*'s chemical defences may be lower when water temperatures are warmer.

The second possibility arising from the explanation that bleaching in uninoculated sporelings in the third laboratory inoculation experiment was the result of residual bacteria, is that other microorganisms in algal biofilms may become virulent when temperatures increase, then infect and bleach *D. pulchra*. Certainly, many bacteria have temperature-regulated virulence (Konkel and Tilly 2000, Klinkert and Narberhaus 2009). The idea that microbial consortia, rather than specific pathogens, are involved in disease is becoming more widely accepted, for example, in coral (Cooney et al. 2002) and dental diseases (Kleinberg 2002). In Chapter 2 I demonstrated that exposure to ambient seawater microbes (not specific cultures) could induce bleaching in undefended sporelings, particularly at high temperatures. Additionally, Case et al. (2011) found an additional, geographically distinct bacterium that could also infect and bleach *D. pulchra* under particular laboratory conditions. Thus, it appears possible that pathogens in addition to R11 may be involved in *D. pulchra*'s bleaching.

Damage and infection

The ability of R11 to cause bleaching in established algae was consistent during the field inoculation experiments, but dependent on experimentally inflicted damage. This damage treatment was intended to mimic wounds inflicted by grazing gastropods, which are commonly associated with *D. pulchra* (Wright et al. 2000, Wright et al. 2004), or other physical disturbances, and provide an infection site for bacterial pathogens. Although experimentally-inflicted damage does not always elicit the same plant-response that actual herbivory does (e.g. Pavia and Toth 2000), in this case artificial damage to *D. pulchra* thalli appears to have facilitated infection by R11 and led to subsequent, localised bleaching. These results are particularly interesting with respect to herbivores, which may cause similar damage to *D. pulchra* when feeding or using thalli as habitat. If herbivore-mediated damage facilitates infection by R11 or other bacterial pathogens, then populations of *D. pulchra* with higher abundances of herbivores may be at greater risk of bleaching.

As a first line of defence against attack from natural enemies and/or physical damage, many seaweeds produce an 'oxidative burst', which is a sudden and short-lived release of reactive oxygen species to impose oxidative stress on the affected cells thereby preventing the growth and spread of pathogens and resisting further infection (Bouarab et al. 2001, Dring 2006). An oxidative burst can occur in response to the detection of degrading compounds produced by pathogens (e.g. Potin et al. 2002) or mechanical damage (e.g. Collén et al. 1994). It is therefore possible that the bleaching I observed in the field inoculation experiments was in fact a response to physical damage to prevent further infection. The localised nature of observed bleaching in this experiment supports this idea, but if the observed bleaching was simply an algal defensive response to damage rather than a

bacterial infection *per se*, then it should have been as commonly observed in the algae that were damaged only, as it was in those that were damaged and inoculated, but this was not the case.

It is therefore likely that damage facilitated infection of *D. pulchra* cells by R11 cells and that this (rather than experimental damage alone) caused algal bleaching. This suggests that, unlike the laboratory inoculation experiments, these field experiments provided less evidence for ubiquitous and opportunistic microbial pathogens: damage and exposure to ambient seawater microorganisms resulted in only infrequent bleaching (although it did occur). This could be due to normal levels of chemical defences in these established, healthy, adult algae and their protective role against bacterial pathogens (Manefield et al. 2001).

An unexpected observation from these experiments was that the prevailing water temperature appeared to have little influence on whether or not R11 caused bleaching in damaged *D. pulchra* individuals. There was a non-significant trend for more bleaching in inoculated and damaged algae in the two summer experiments than the winter experiment and it is possible that with greater replication a temperature effect would be observed. The water temperature differences between when the 'summer' (21-22 °C) and 'winter' (17-18 °C) experiments were conducted, were much less extreme than the temperature differences used in the laboratory experiments presented here, in Chapter 2 and by Case et al. (2011). Overall, these experiments provide support for the hypothesis that bleaching in *D. pulchra* is bacterially mediated and that R11 can cause bleaching in this seaweed under certain conditions.

Conclusions

The bacterium R11 caused bleaching in *D. pulchra*, but its ability to do so was largely moderated by algal chemical defences, algal physical defences and water temperature. In most cases, the presence of halogenated furanones prevented (or reduced the severity of) bleaching in *D. pulchra* sporelings. In established adult algae, R11 was only able to infect thalli and cause bleaching if algae were also experimentally damaged to create and infection site, similar to the damage inflicted by grazing herbivores. Results from this study demonstrate complexity in this host-pathogen system and raise interesting questions about (i) the role of herbivory wounds, (ii) the generality of bleaching and virulence in marine microorganisms, especially at higher temperatures and (iii) the temperature extremes at which defended *D. pulchra* are unable to produce or maintain 'normal' concentrations of halogenated furanones. This work supports the hypothesis that bleaching in *D. pulchra* is bacterially-mediated, and that R11 can, under some circumstances, infect and cause bleaching in this habitat-forming seaweed.

CHAPTER 4

Too much of a good thing? Excessive solar radiation indirectly increases algal susceptibility to bacterial bleaching

INTRODUCTION

Many organisms need light, but too much can be damaging (Franklin and Forster 1997, Caldwell et al. 1998, Bancroft et al. 2007). Excessive solar radiation, especially in the ultra-violet range can disrupt photosystems (Franklin and Forster 1997, Bischof et al. 2002) and damage proteins, nucleic acids and lipids inside living cells, with a myriad of potential consequences at the organismal level (Bancroft et al. 2007). Exposure to high levels of solar radiation often leads to reduced performance or survivorship (Caldwell et al. 1998, Hader et al. 1998, Bancroft et al. 2007) and may also reduce growth, fecundity and offspring survival (Coelho et al. 2000, Bancroft et al. 2007, Nahon et al. 2010). In recent decades, solar radiation levels (in particular, harmful UVR) have increased, due largely to atmospheric ozone depletion (Solomon 1990). Additionally, habitat destruction and modification have also altered levels of solar radiation reaching many ecosystems. For example, the removal of canopy-forming organisms such as trees from forests drastically increases light levels reaching understory communities (e.g. Powell and Bork 2007).

Although many organisms have mechanisms to avoid, tolerate or moderate exposure to solar radiation (e.g. the production of 'sunscreen' (light-absorbing) pigments, such as melanins (Riley 1997) and carotenoids (Goodwin 1986), behavioural adaptations including avoidance, phenological adaptations such as moderating the timing of photosynthesis, and morphological traits including thickening of outer body surfaces (Franklin and Forster 1997)), current levels are often beyond those tolerance limits. Hence, prevailing light conditions are can be sub-optimal and have the potential to negatively impact the normal physiological functioning of many organisms, leading to stress (Chapin et al. 1993) and photo-damage (Franklin and Forster 1997, Bischof et al. 2002, Bancroft et al. 2007). In addition to these direct impacts, increased solar radiation can also affect organisms indirectly. Generally, exposure to one abiotic stressor can reduce an organisms' resilience to another (Crain et al. 2008). For example, exposure to high levels of solar radiation reduces the thermal tolerance range of seaweeds (Hoffman et al. 2003) and intertidal molluscs (Przeslawski et al. 2005).

High light environments can also interfere with trophic interactions, many of which are chemically mediated (see reviews by Levin 1976, Hay and Fenical 1988, Hay 2009). Light can alter the production of defensive chemistry (Coley et al. 1985, Caldwell et al. 1998, Bidart-Bouzat and Imeh-Nathaniel 2008) and thereby also alter an organisms' susceptibility to natural enemies, including consumers and pathogens. In some terrestrial plants, exposure to UVR can lead to an increase in polyphenolic compounds and have positive, negative or no effects on other compounds (including flavinoids, terpenoids and lignin ; reviewed by Roberts and Paul 2006, Bidart-Bouzat and Imeh-Nathaniel 2008). In brown seaweeds, exposure to UVR can lead to an increase (Pavia et al. 1997), decrease (Cronin and Hay 1996)

or have no effect on concentration of phlorotannins (Henry and Van Alstyne 2004, Fairhead et al. 2006). Beyond this, little else is known about the effects of solar radiation on the production of other defensive metabolites produced by other chemically-defended organisms.

As well as influencing trophic interactions by affecting the defensive chemistry of a host, solar radiation may also affect the consumer and/or pathogen. The effects of solar radiation on host-pathogen interactions are likely to be particularly complex, since light can have severe effects on pathogens (Lafferty and Holt 2003). Microorganisms are generally less tolerant to changes in solar radiation than eukaryotes (Jeffrey et al. 1996) and the composition and density of microbial communities shift strongly in response to different light regimes (Dobretsov et al. 2010). In general, when UVR is high, bacteria are less active (Herndl et al. 1993) and less virulent (Suttle and Chen 1992, Fine et al. 2002), however some evidence suggests that increased light can enhance pathogen infection (Schaffelke et al. 1996). Overall however, few studies have directly assessed the effects of exposure to high levels of solar radiation on disease dynamics.

If exposure to solar radiation stress increases a hosts' susceptibility to disease, then growing near a shade-providing neighbour could be beneficial for organisms living in high light environments (Callaway 1995) and this may be considered as associational resistance to disease. Associational resistance is an example of a positive interaction between plants or algae. To date, it has predominantly been investigated in the context of avoiding herbivory through association with chemically-defended or otherwise protective neighbours (e.g. Pfister and Hay 1988, Wahl and Hay 1995, Hamback et al. 2000). The potential for such facilitative interactions among organisms is high in productive habitats such as rainforests,

coral reefs, and seaweed communities, where light microhabitats are diverse on relatively small spatial scales (Endler 1993, Chazdon et al. 1996, Anthony and Hoegh-Guldberg 2003).

Temperate, hard-substrate marine ecosystems are dominated by macroalgae, which are important habitat formers and also create shaded microhabitats beneath their canopies in both intertidal (Benedetti-Cecchi et al. 2001, Bischof et al. 2002) and subtidal habitats (Wood 1987, Toohey et al. 2004). Despite light attenuation in water providing some protection against solar radiation in subtidal habitats, light (including harmful ultra-violet B radiation (UVBR)) still penetrates to an extent that benthic organisms in shallow water environments are exposed to excessive and potentially damaging light conditions (Bancroft et al. 2007). Photo-bleaching in subtidal algae is usually associated with exposure to damaging light levels, often due to a loss or reduction of canopy cover (e.g. Scrosati and DeWreede 1998, Valentine and Johnson 2004).

In Chapters 2 and 3, I assessed patterns of bleaching in a chemically-defended red seaweed, *Delisea pulchra*, and investigated the influence of abiotic factors, halogenated furanones (defensive metabolites produced by the alga) and bacterial pathogens on this phenomenon. There were no direct relationships between solar radiation (PAR or UVR) and bleaching in *D. pulchra.* However, bleaching was more common in shallow (1-4 m) water habitats than at depth (7-10 m) and light levels also varied significantly between depths (Chapter 2). In the course of investigating the relationship between bleaching and solar radiation, I observed another phenomenon, whereby the colour of *D. pulchra* in shallow waters changed from deep red to yellow, and this appeared to occur seasonally. I called this algal 'fading' and in this chapter, I characterised patterns of fading and assessed relationships between the

prevalence of this phenomenon and water temperature, solar radiation (PAR, UVAR and UVBR) and salinity.

I hypothesised that exposure to ambient solar radiation in shallow water habitats indirectly increases *D. pulchra*'s susceptibility to bacterial bleaching by lowering furanone concentrations and causing algal fading. To test this idea, I manipulated solar radiation *in situ* (by shading and transplanting algae), monitored algal condition (i.e. fading and bleaching) and after several weeks, measured concentrations of halogenated furanones and characterised microbial communities on algal surfaces. Finally, to investigate whether co-occurring kelps provided shade for *D. pulchra* and thus, prevented fading (and potentially also bleaching), I conducted a 'nearest-neighbour' study to see whether bleached and faded algae were closer to or further from large kelps, compared to healthy, co-occurring *D. pulchra*. Thus, the broad hypothesis tested within this chapter is that algal fading may be a precursor condition which increases algal susceptibility to bleaching by lowering chemical defences.

METHODS

Study sites and organisms

Delisea pulchra (Greville) Montagne (Bonnemaisonales: Rhodophyta) occurs from southern Australia to Antarctica (Chapter 2). Two populations of *D. pulchra* were studied: Bare Island in Botany Bay and Long Bay in Malabar, which are both near Sydney, Australia (Chapter 2). Both locations have similar subtidal habitats, importantly including areas dominated by turf-forming algae beds (including *D. pulchra*) but lacking large (>30 cm) canopy-forming kelps, as well as immediately adjacent areas with patchy stands of the kelp *Ecklonia radiata* (C. Agardh) J. Agardh (Laminariales: Heterokontophyta). *E. radiata* is a relatively large macroalga or kelp that grows to approximately 1 m and is among the most significant kelp forests in subtidal habitats (2-15 m depth) in coastal habitats within the Sydney region.

Patterns of fading and their relationships with environmental variables

I predicted that the prevalence of algal fading in *D. pulchra* populations was related to high levels of solar radiation. Fading was defined qualitatively as a yellowing of the upper part of the thallus (Figure 4.1D-F). Faded individuals were readily discernable from healthy algae *in situ*, which were deep red in colour (Figure 4.1A-C). Algal fading was distinct from bleaching in that it caused a uniform part of affected thalli (tips) to change from deep red to yellow, whereas algal bleaching, which did not affect any uniform part of the thalli. Rather, it caused whitening of localised patches of thalli and was not restricted to the tips (Figure 4.1G-I).

To quantify fading and my qualitative observations of associated change in algal pigmentation, I conducted crude pigments extractions in acetone and compared absorbance spectra for chlorophylls and phycobiliproteins between healthy, faded and bleached individuals. To do this, six replicate bleached, faded and healthy freeze-dried algal samples

(50-100 mg) were finely ground using a mortar and pestle. Acetone (10 ml) was then added to the ground algal tissue and pigments were extracted in the dark at 4 ^{o}C for 60 minutes inside 15 ml falcon tubes. The tubes were then spun in a centrifuge at 4 ^{o}C and 2500 x g for 10 minutes and then the supernatant was transferred to a clean falcon tube. Absorbance at 450-500 nm is likely due to chlorophylls and absorbance at 600 and 650 nm is likely due to the phycobiliproteins, phycocyanin and phycoerythrin, respectively (French and Young 1952) and to estimate the relative concentrations of those pigments in healthy, faded and bleached *D. pulchra* algae, absorbance at the above wavelengths were compared.

To assess whether fading affected *D. pulchra* when solar radiation was high, I monitored algal populations and assessed the proportion of sampled individuals that faded over a period from August 2006 until April 2009. Two populations, at Bare Island and Long Bay in deep (7-10 m) and shallow (1-4 m) water habitats were surveyed approximately monthly. On each sampling date, location and depth, six replicate 1 m^2 quadrats were thrown haphazardly within *D. pulchra* habitat and individuals falling within quadrats were counted as either healthy or faded. Samples of healthy and faded algal tissue were also collected throughout the study for furanone quantification. These were enclosed within individual clip-seal bags and suspended in a bucket containing seawater collected onsite in order to maintain ambient temperature *en route* back to the laboratory. There they were processed as described in chapter 2 and stored once freeze-dried, at -20 ^{o}C until processing.

To assess whether the incidence of fading was related to the environment, several (environmental) variables were also measured on survey dates. Water temperature and salinity were recorded using a 'YeoKal' water quality instrument (model 611). Solar radiation (photosynthetically active radiation (PAR) and ultra-violet radiation (UVAR and UVBR) were

measured using a Skye light meter ('Spectrosense 2' Display Meter SKL 904) with a single channel light sensor for PAR and two high output light sensors for UVAR and UVBR. Three to six replicate measurements of each variable were taken at each date, location and depth.

Fading and variation in chemical defences

Furanones were extracted and quantified in faded and healthy algae to assess whether algal fading affected *D. pulchra*'s chemical defences and thus its susceptibility to bacterial bleaching. As there were very few completely healthy algae in the shallows during summer that had not, at least partially faded, and there were no faded algae in deeper waters, the possible comparisons of furanone concentrations between faded and healthy co-occurring *D. pulchra* individuals were limited. Thus, these data are unavoidably confounded and need to be interpreted with caution. Total furanone concentrations in faded algae collected in the shallows during summer, were compared those from healthy algae collected at the same time from deeper waters. Additionally, furanone concentrations from shallow faded algae collected during the summer, were compared to those from shallow healthy algae collected the following winter. Extraction and quantification methods were as detailed in Chapter 2 and total furanone concentrations were calculated as mg/g of dry algal mass.

Assessing the role of shade-providing neighbours

To assess whether shade from co-occurring, canopy-forming kelps (*Ecklonia radiata*) affected the likelihood of fading and/or bleaching in *D. pulchra*, I assessed the condition (healthy, faded, bleached) and biomass of *D. pulchra* individuals at the end of summer 2008-2009 and compared this to their distance from large, canopy-forming (> 30 cm) *E. radiata* individuals. To estimate biomass of *D. pulchra in situ*, I developed a biomass index number (BIN) scale, which was validated prior to this study being conducted and involved the

classification of each alga into a size classes between 1 and 10. To check the effectiveness of the scale for *in situ* biomass estimations, I classified size-classes of twenty-nine replicate algae using the BIN scale, destructively sampled each alga and returned them to the laboratory. There the algae were padded dry, weighed to determine wet mass, then freeze-dried and re-weighed to determine dry mass. The BIN scale size classes were compared to actual algal wet and dry masses using linear regression analyses.

This study was conducted at Long Bay where there was a region of co-existence and interspersion of *D. pulchra* and *E. radiata*. There, I randomly selected twenty 'healthy' and twenty 'faded and bleached' *D. pulchra* individuals, classified them into a BIN size class and measured their distances from the nearest *E. radiata* individual greater than 30 cm high.

Manipulating solar radiation

Shading experiment: To determine whether solar radiation causes fading in shallow (1-4 m) water *D. pulchra*, and whether protection from solar radiation affects algal susceptibility to bleaching disease, a shading field experiment was conducted at Long Bay. In this experiment, I manipulated solar radiation exposure for established, healthy *D. pulchra* individuals. For each light treatment, eight replicate patches of algae (consisting of two to five healthy, fully-pigmented *D. pulchra* individuals) were covered with either: (i) full shades made from opaque Perspex, which significantly reduced total solar radiation (PAR and UVR (ultra-violet A radiation (hereafter UVAR) and UVBR)), (ii) UVR shades made from polycarbonate, which significantly reduced UVR (UVAR and UVBR) but not PAR, (iii) shade controls, which did not significantly alter light levels but mimicked any changes in conditions due to the frames articulating the shade materials, thereby serving as a procedural control, or (iv) unmanipulated controls that were not covered.

Shades were 50 cm x 30 cm in area and were elevated approximately 20 cm above the substratum by wire frames, high enough so that algae underneath were not physically disturbed by the shades, but low enough so as to ensure effective manipulations of light. Shades were cleaned twice every week using pot scourers to remove fouling organisms and maintain the experimental treatments. Shade frames, which were made of wire grid (approximately 3 cm^2; Figure 4.2), were also cleaned regularly to ensure that water flow and light levels remained unaltered throughout the experiment.

Large holes cut into the sides of each frame ensured that macroherbivores and other organisms could access the areas underneath the shades. 'Dynabolts' drilled into the benthos fastened the corners of each shade to the substratum. Unmanipulated control patches had flagged bolts similarly attached to each corner to mark their location. Replicate light measurements (PAR, UVAR and UVBR) were taken under each shade at the experiments completion to ensure that the experimental manipulations of solar radiation remained effective throughout the experiment. Once the shades had been established, the condition of each alga was recorded as either: healthy, faded or bleached. Because growth of algae in this experiment could not be estimated by measuring changes in length (as algae were beneath shades), I used the BIN scale as described above, to estimate biomass *in situ*.

Each alga was revisited and its condition (healthy, faded, bleached) and biomass (BIN scale: 1-10) assessed approximately fortnightly from late spring until early summer in 2008. After eight weeks (two weeks prior to the completion of the experiment), an individual alga was randomly chosen from each replicate patch, destructively sampled and returned to the laboratory. There it was freeze-dried and stored at -20 ^{o}C until processing. Surface-associated microbial communities (SAMCs) on algae from all treatments were characterised

and compared using t-RFLPs as detailed in Chapter 2. Furanone concentrations of experimental algae were also extracted and quantified using GC-MS as described in Chapter 2 and compared among light treatments.

Transplant Experiment: To further assess whether environmental differences (including solar radiation) explain observed differences in bleaching incidence between deep (7-10 m) and shallow (1-4 m) populations of *D. pulchra* (chapter 2), I conducted a reciprocal transplant experiment. Ten healthy (fully pigmented) replicate individuals were either: (i) transplanted from deep (7-9 m) water to shallow (1-4 m) water, (ii) transplanted from shallow to deep water, (iii) detached from the substratum then reattached in their original environment/depth as procedural controls, or (iv) left as unmanipulated controls. Transplantations were conducted in early spring 2008 and algae were carefully reattached to the substratum via masonry nails and small cable-ties. Care was taken to firmly secure the algae without damaging the thalli. Algal condition (healthy, faded, bleached) and biomass (BIN scale: 1-10) were monitored weekly for approximately three weeks.

Statistical analyses

Absorbance spectra corresponding to the wavelengths 450, 600 and 650 nm (approximating chlorophyll a and phycobiliproteins, respectively) were compared among healthy, faded and bleached individuals using two single-factor analyses of variances (ANOVAs), where the response variable was absorbance and the factor was algal 'condition'. Seasonal patterns of fading were compared among seasons (summer, autumn, winter and spring), locations (Bare Island, Long Bay) and depths (deep, shallow) using a three-fixed-factor ANOVA. Due to the preponderance of zero values in this dataset (fading was rare during winter, for example), these data failed to conform to the ANOVA assumptions of normality and

homogeneity of variances and although transformations were attempted they did not improve the dataset with respect to these assumptions. Therefore, raw data were used in analyses and marginal results should be interpreted with caution.

Relationships between the prevalence of fading and physical variables (temperature, solar radiation (PAR, UVAR, UVBR) and salinity) were analysed using linear regressions and principal components analysis (PCA). Again, due to the preponderance of zero's in this dataset these data did not conform to the assumptions of linear regression analyses and these were not improved with transformations so marginal results should be interpreted with caution. To reduce the risk of Type I error, α values were lowered to 0.01 for these analyses. Concentrations of total furanones were compared among faded and healthy *D. pulchra* individuals using single-fixed-factor ANOVAs, where the factor was condition (faded, healthy).

Relationships between the *in situ* BIN scale and actual wet and dry mass of algae were checked using linear regression analyses. To assess whether the condition of *D. pulchra* individuals was related to their distance from *E. radiata* individuals > 30 cm height, distances were analysed using a single-fixed-factor ANOVA where the factor was 'algal condition' (healthy, faded & bleached) and the dependant variable was distance. The relationship between distance from *E. radiata* individuals and biomass in *D. pulchra* was analysed using a linear regression analysis. The effects of shade treatments on light levels (PAR, UVAR, UVBR) reaching the benthos beneath were analysed using a series of single-factor ANOVAs, where the factor was 'treatment' (full shade, URV only shade, procedural control, unmanipulated control). The effects of light treatments on algal condition were analysed using a series of Pearson's chi-square analyses. Effects of light treatments on algal biomass and total

furanones were compared using single-factor ANOVAs where the factor was 'treatment' (full shade, UVR only shade, procedural control, unmanipulated control). The relative abundance of fragment sizes from t-RFLP analyses were compared among shading treatments using a single-factor Permutational multivariate analyses of variances (PERMANOVAs) based on similarity matrices. The effects of origin and destination on the condition of transplanted and control algae were analysed using Pearson's chi-square analysis and effects on biomass were analysed using a single fixed-factor ANOVA comparing 'habitats' (origin, destination).

Assumptions of normality and homogeneity of variances were checked by inspecting scatterplots of residuals and conducting K-S and Levene's tests respectively. Transformations were conducted where appropriate and reported alongside tables. All ANOVAs, linear regressions, Pearson's chi-squared analyses and PCAs were conducted using Systat v 13.00.05. Electropherrograms were downloaded and checked using the program 'Peak Scanner' and then the statistical platform 'R' was used to define and filter 'true peaks' from noise and bin fragment sizes between samples. 'Strawberry Perl' was used to generate programming script for R. PERMANOVAs were conducted using PRIMER 6 v 6.1.11 & PERMANOVA + v 1.0.1.

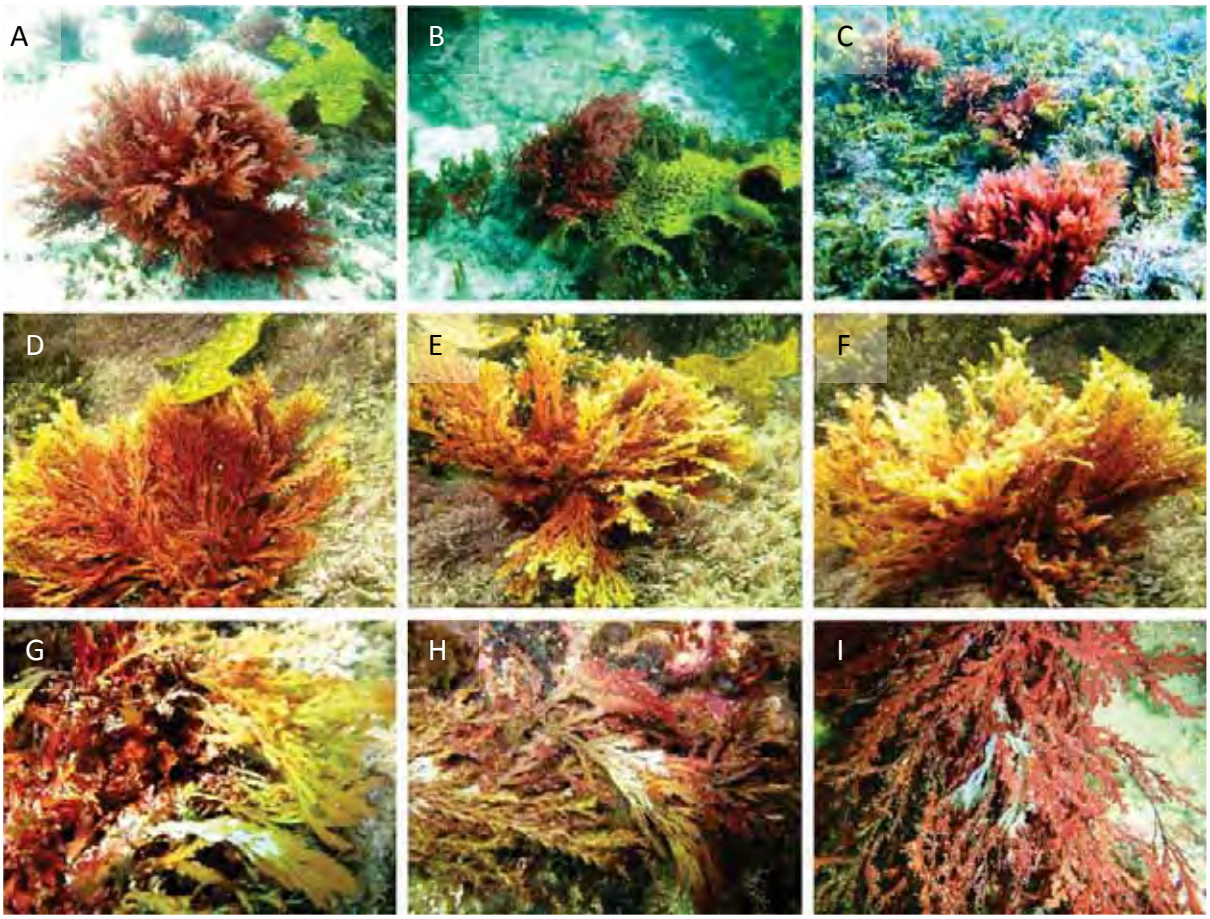

Figure 4.1. Healthy **(A-C)**, faded **(D-F)** and bleached **(G-I)** *D. pulchra* individuals *in situ*. Note that fading causes a yellowing of the thallus and tends to affect a uniform part of the thallus (usually the tips), whereas bleaching causes thallus non-uniform whitening and is not restricted to any specific area but rather appears anywhere. The individual in photograph (G) is both faded at the tips and bleached mid-thallus.

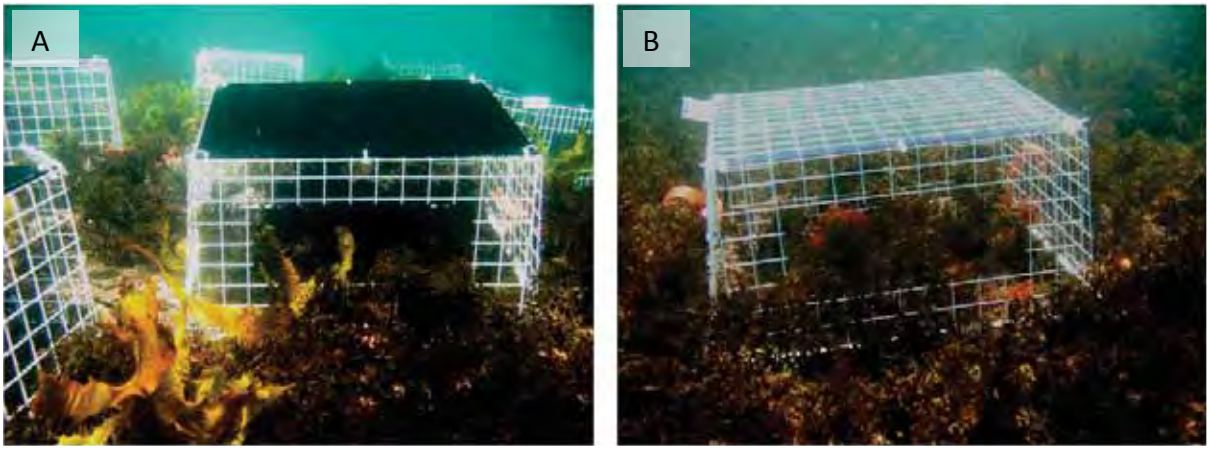

Figure 4.2. Shading experiment treatments including **(A)** full opaque Perspex shades, which reduced levels of total solar radiation (PAR, UVAR and UVBR) and **(B)** UVR only polycarbonate shades, which significantly reduced UVAR and UVBR but not PAR. Not pictured are procedural controls, which included the white metal frame (but no shade) and unmanipulated controls.

RESULTS

Patterns of fading and their relationships with environmental variables

There was no significant difference in absorbance spectra (standardised for dry mass of algae pigments were extracted from) at wavelengths corresponding to chlorophyll (500 nm) between healthy (3.48 ±2.53), bleached (1.62 ±0.66) and faded (0.72 ±0.10) algae ($F_{2,15}$ =0.207, p = 0.161). However, absorbance spectra at wavelengths corresponding to the accessory pigments phycocyanin and phycoerythrin were higher in healthy (7.19 ±5.56 and 1.26 ±1.06, respectively) algae than both faded (1.66 ±0.35 and 0.23 ±0.04, respectively) and bleached (4.98 ±2.03 and 0.10 ±0.04, respectively) algae, which had comparably low levels of absorbance (phycocyanin: $F_{2,15}$ =3.60, p =0.053; phycoerythrin: $F_{2,15}$ =3.64, p =0.051), although statistically, these differences were marginally non-significant, probably due to the high variance within 'conditions'.

To determine whether fading was related to solar radiation, water temperature and salinity, the prevalence of fading in populations of *D. pulchra* was monitored. There was no effect of sampling year on the patterns of fading ($F_{2,731}$ =0.47, p =0.626), so seasonal data from all years were pooled for further analysis of the effects of season, location and depth on the prevalence of fading. Fading was not observed in deep water habitats at any time. There was a strong seasonal effect on the prevalence of fading in shallow water populations, with more fading occurring during the summer, spring and autumn and less during winter (during winters, the prevalence of fading was negligible; Figure 4.3, Table 4.1). Patterns of fading were similar at the two locations (Table 4.1).

At both locations, the prevalence of fading was significantly positively related to total solar radiation and to all wavelengths individually (Figure 4.4, Table 4.2). A very weak (and

marginally non-significant, with α= 0.01) positive relationship was detected between the prevalence of fading and water temperature (R^2_{569} =0.011, p =0.012). There was also a very weak but non-significant (with α= 0.01) relationship between fading and salinity (R^2_{270}=0.014, p =0.053).

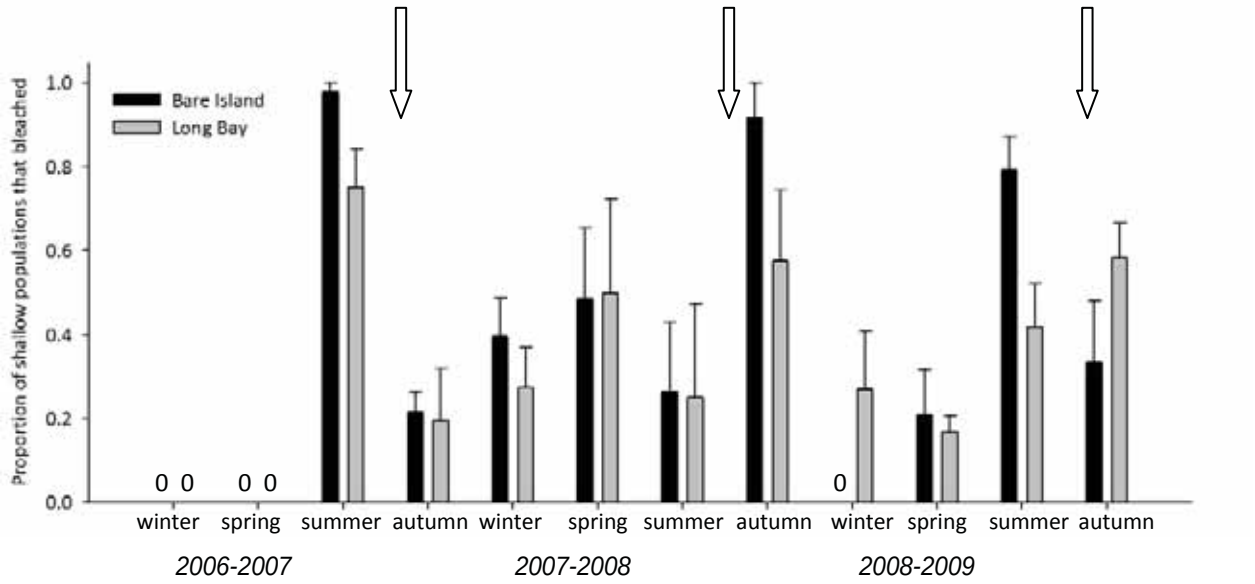

Figure 4.3. Proportions of shallow *D. pulchra* populations (means +SE) at Bare Island (black bars) and Long Bay (grey bars) that faded during the monitoring period. There was no fading observed in deep water populations. Arrows indicate peak periods of bleaching in shallow water populations, as detailed in Chapter 2.

Table 4.1. Four-factor ANOVA of the factors influencing the patterns of fading in *D. pulchra* populations. '**' indicates a significant effect of factor on the prevalence of fading in populations of *D. pulchra,* with α= 0.01.

Source of variation	df	MS	F-ratio	p
Season "S"	3	0.473	7.714	0.000**
Location "L"	1	0.016	0.256	0.613
Depth "D"	1	9.332	152.061	0.000**
S x L	3	0.063	1.031	0.379
S x D	3	0.473	7.714	0.000**
L x D	1	0.016	0.256	0.613
S x L x D	3	0.063	1.031	0.379
Error	241	0.061		

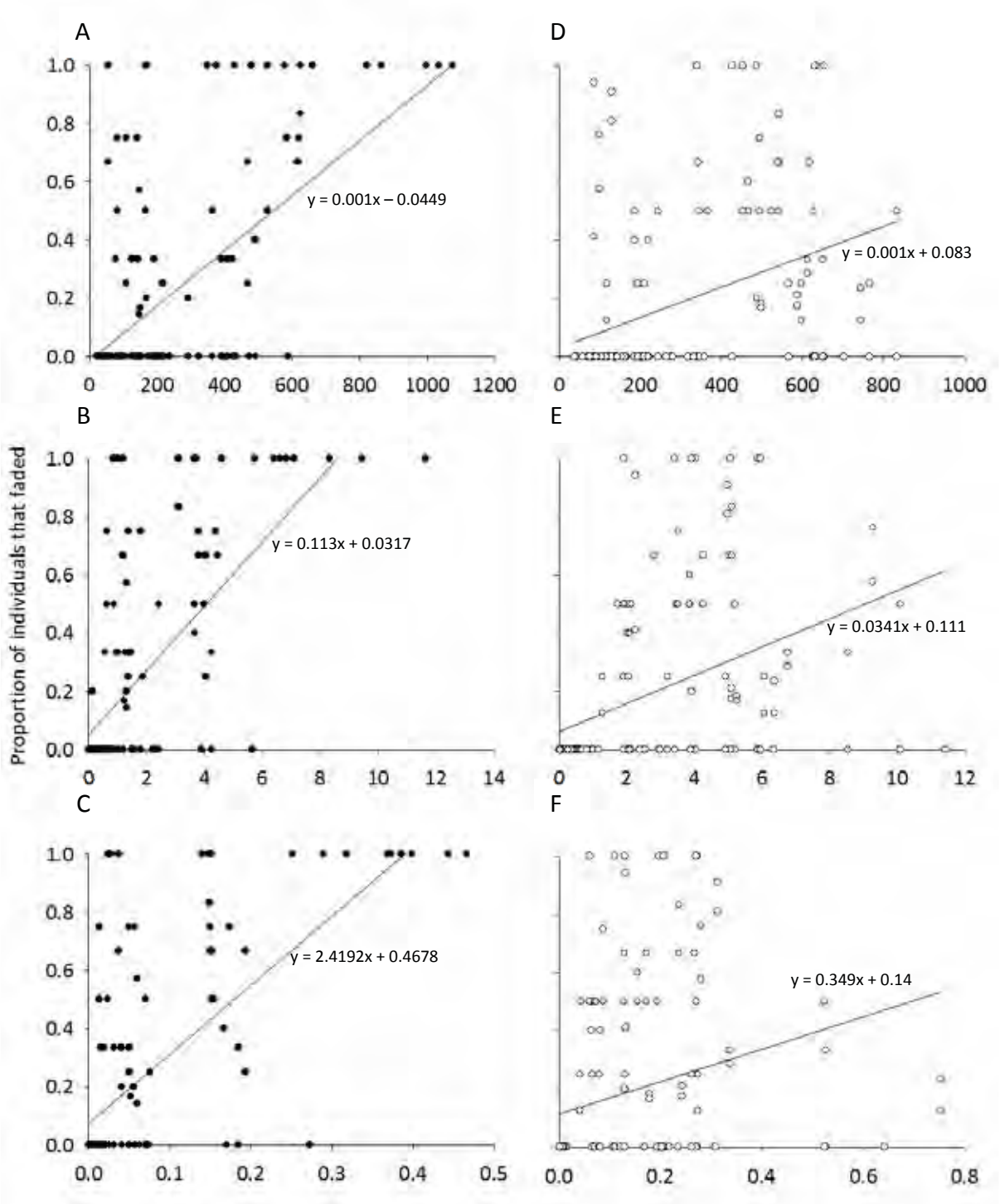

Figure 4.4. The prevalence of fading in populations of *D. pulchra* (over the survey period) plotted as a function of solar radiation wavelengths **(A, D)** PAR, **(B, E)** UVAR and **(C, F)** UVBR at **(A-C)** Bare Island (black circles) and **(D-F)** Long Bay (white circles). Significant, linear relationships are indicated by regression lines and described by formulae.

Table 4.2. Linear regression analyses of relationships between the prevalence of bleaching and solar radiation in deep and shallow water at the two study locations. '**' indicates a significant relationship, with $\alpha = 0.01$.

	Bare Island			*Long Bay*		
	R^2	*df*	p	R^2	*df*	p
PAR	0.366	173	0.000**	0.152	156	0.000**
UVAR	0.455	173	0.000**	0.188	156	0.000**
UVBR	0.460	173	0.000**	0.085	156	0.000**

Fading and variation in chemical defences

During the summer, faded shallow algae had significantly lower furanone concentrations than healthy algae in deeper waters ($F_{1,15}$ =6.06, p = 0.027) and similarly, shallow faded algae collected in the summer had significantly lower furanone concentrations than shallow healthy algae collected in the winter ($F_{1,16}$ =27.07, p < 0.001; Figure 4.5).

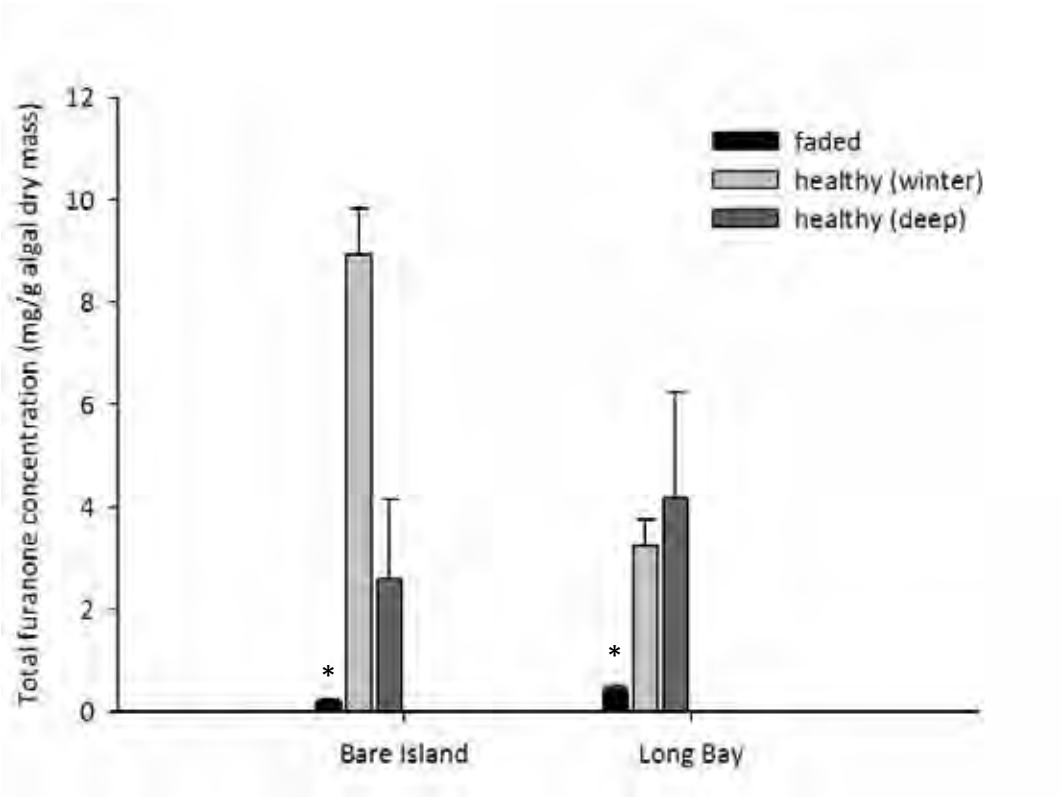

Figure 4.5. Concentrations of total furanones (means +SE) in faded (black bars) *D. pulchra* collected from during summer 2008-2009, healthy algae collected at the same time from deeper water (dark grey bars) and healthy *D. pulchra* collected from the shallows during winter (light grey bars). '*' represent statistical difference in furanone concentrations among conditions with α= 0.05.

Assessing the role of shade-providing neighbours

Healthy *D. pulchra* individuals growing close to large (>30 cm tall) *Ecklonia radiata* individuals were more likely to be 'healthy' than conspecifics growing further away, with distance from kelps having a significant influence on algal condition ($F_{1, 38}$ =47.407, p =0.000; Figure 4.6A). Similarly, there was a significant, negative linear relationship between algal biomass and distance from canopy-forming neighbours, with larger *D. pulchra* individuals growing close to *E. radiata* canopies (R^2_{39} =0.236, p =0.001; Figure 4.6B).

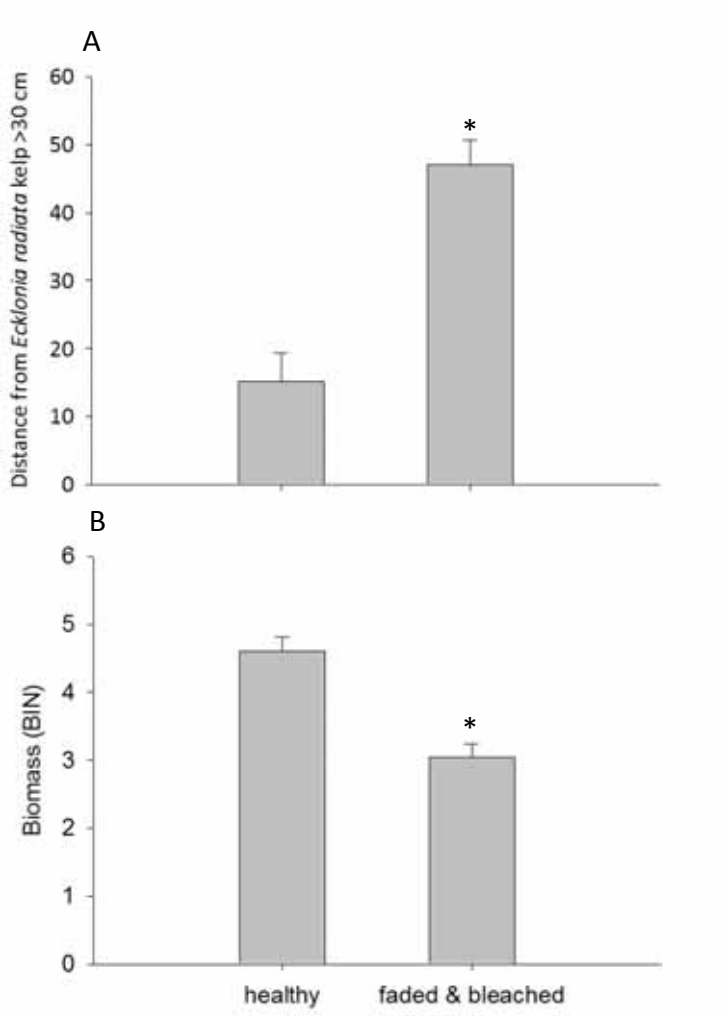

Figure 4.6. (A) Distance (means + SE) to large *E. radiata* individuals and **(B)** Biomass Index number (BIN; means + SE) of 'healthy' and 'faded & bleached' *D. pulchra* individuals in the nearest neighbour study with *n* = 20.'*' indicates significant difference between algal conditions with α= 0.05.

Manipulating solar radiation

Shading experiment: Full shades significantly reduced PAR, UVAR and UVBR reaching the benthos beneath, whereas UVR-only shades significantly reduced UVR (A and B) but did not significantly alter PAR. Procedural control shades did not affect solar radiation relative to unmanipulated control light measurements (Figure 4.7). *D. pulchra* shaded from all light (full shades) faded significantly less frequently than control algae and remained healthy for much longer than algae from other treatments (Figure 4.8). The effect of full shades on algal fading was evident after only two weeks (χ^2_3 =59.426, p <0.005) and persisted until the experiments completion (χ^2_3 =54.065, p <0.005) such that by the end, the only healthy algae (unfaded and unbleached) remaining in the experimental area were beneath full shades. Fading was only observed in algae from this treatment towards the end of the experiment (after nine to ten weeks) and even then, less than 10% algae beneath full shades faded. Algae that were shaded from UVR (but not PAR) took several weeks longer to fade than unshaded control algae and after two weeks the amount of fading in the UVR only shade treatment was still similar to the full shade treatment (χ^2_1 =2.650, p =0.104).

After six weeks, significantly more algae under UVR only shades were faded than the full shade treatment (χ^2_1 =9.664, p =0.002) and this persisted for the remainder of the experiment (Figure 4.8). At this time, however, significantly less UVR only shaded algae faded than unshaded controls (40% compared to 100% fading respectively; χ^2_4 =27.180, p <0.005). By the following week (seven weeks into the experiment) the prevalence of fading beneath UVR only shades was similar to that among unshaded control algae (100% fading; χ^2_2 =3.722, p =0.156), and this similarity remained for the rest of the experiment.

There was no significant effect of shading on total furanone concentrations ($F_{3, 15}$ =1.35, p =0.297) but there was a non-significant trend for higher furanone concentrations in shaded algae (Figure 4.9). Shading treatments did, however, significantly alter microbial communities associated with algal surfaces ($F_{3,17}$ =1.785, p =0.032; Figure 4.10). Communities associated with fully-shaded D*. pulchra* were significantly different to those associated with unshaded control algae (procedural (p =0.028) and unmanipulated (p = 0.007) controls), but were no different from communities on algae shaded from UVR only. Communities associated with algae shaded from UVR only were not significantly different from controls (Figure 4.10).

Algae protected from total solar radiation (full shades) were also significantly less likely to bleach than unshaded control algae throughout the experiment (e.g. after eight weeks: χ^2_2 =10.351, p =0.006; Figure 4.11). Of the algae under complete shades, only 7-8% bleached throughout the entire experiment, compared to >20% UVR only shade treatment and >60% unshaded controls (Figure 4.11). Surprisingly, I was unable to detect a significant difference in the prevalence of bleaching between UVR shaded algae and either unshaded controls or fully shaded controls, though this was probably due to the small sample size within this treatment by the end of the experiment (discussed below). The apparent reduction in the proportion of algae that bleached in the 'UVR only shade' treatment was due to the loss of bleached replicates, rather than algal recovery from bleaching. There was no effect of shading on algal biomass in any weeks of the experiment (e.g. final week: $F_{3,62}$ =2.046, p =0.117).

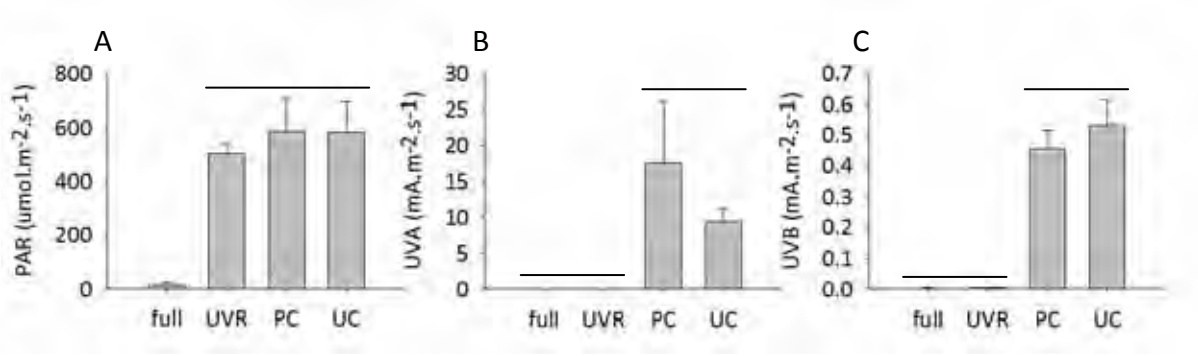

Figure 4.7. Levels of **(A)** PAR**, (B)** UVAR and**(C)** UVBR measured beneath experimental shades at the experiment's completion. Light treatments are indicated along the x-axis and abbreviations are as follows: 'full': full shades; 'UVR': UVR only shades; 'PC': procedural control; 'UC': unmanipulated control with *n*=8. Lines above bars represent statistical similarity according to Tukey's HSD pairwise comparisons.

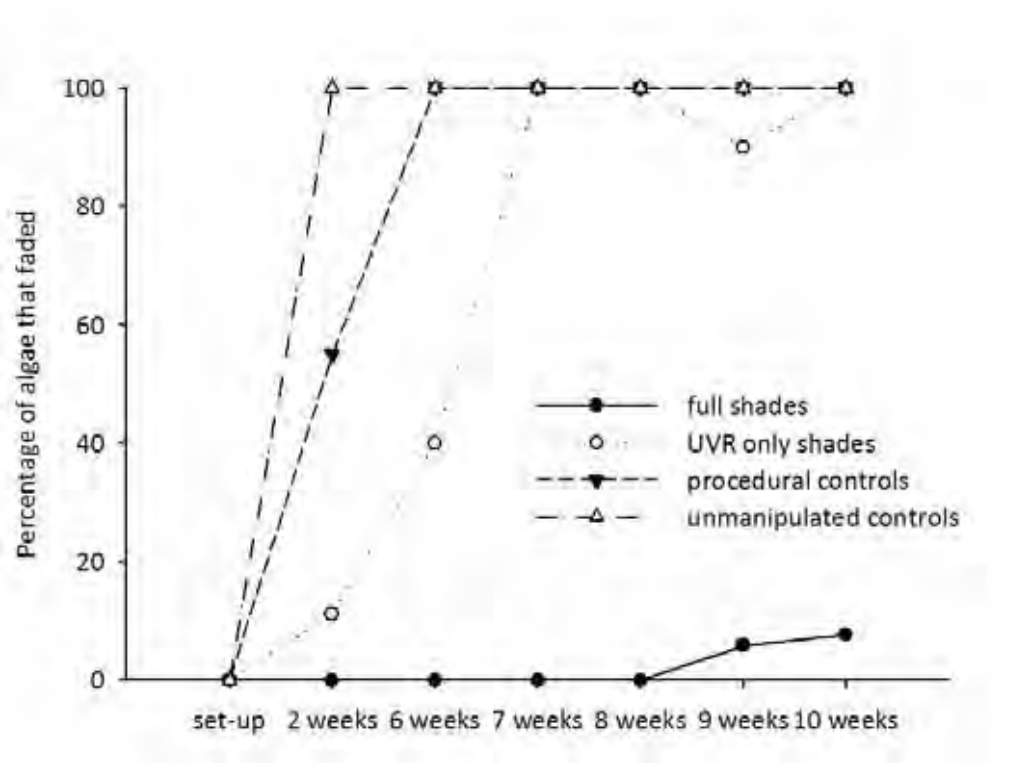

Figure 4.8. The percentage of experimental *D. pulchra* individuals that faded during the shading experiments, in either the full shades (black circles, solid line), UVR only shades (white circles, dotted line), procedural controls with shade frames but no alterations to irradiance (black, inverted triangles, dashed line) or unmanipulated controls (white triangles, long-dashed line) treatment, with *n*=8 replicate algal patches.

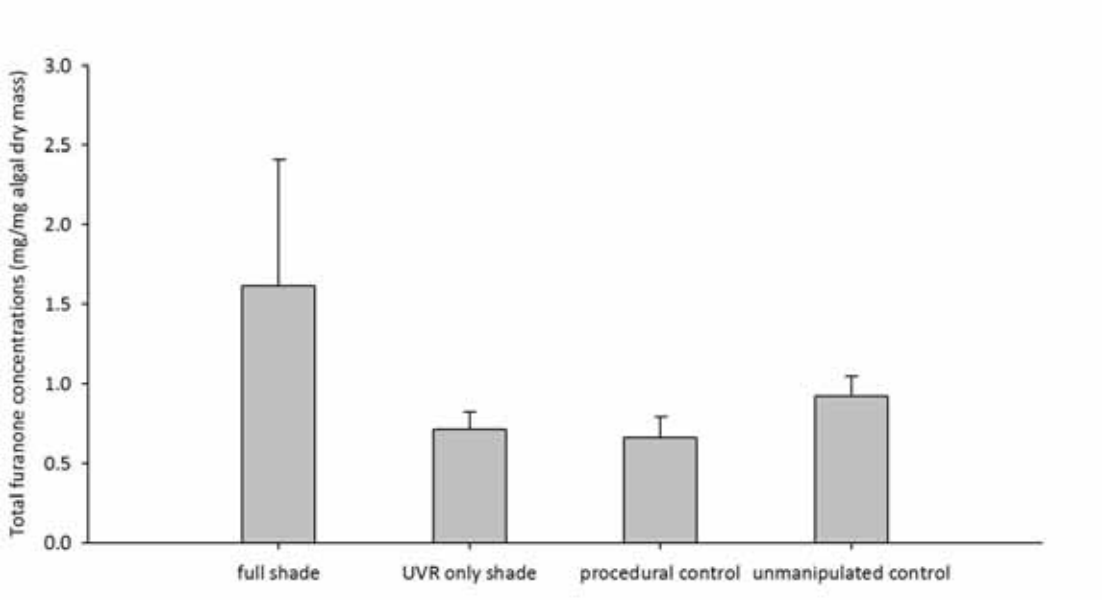

Figure 4.9. Total furanone concentrations (means +SE) in *D. pulchra* individuals collected after eight weeks beneath experimental shades, with *n*=5 algae from each treatment.

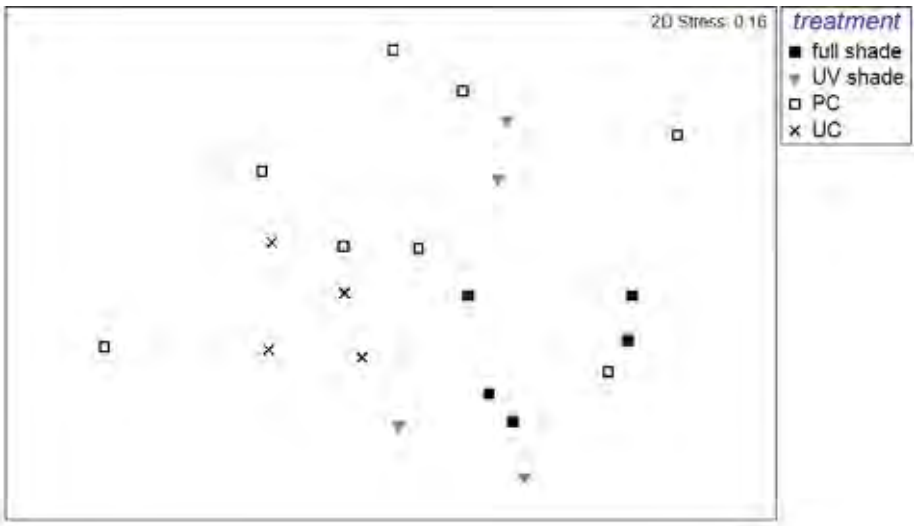

Figure 4.10. Non-parametric multi-dimensional scaling plot comparing surface-associated microbial communities from experimental algae after 8 weeks exposure to either: fully shaded (black squares), shaded from UVR only (grey, inverted triangles), or unshaded in procedural ('PC'; white squares) or unmanipulated controls ('UC'; crosses) treatments, with *n*=5.

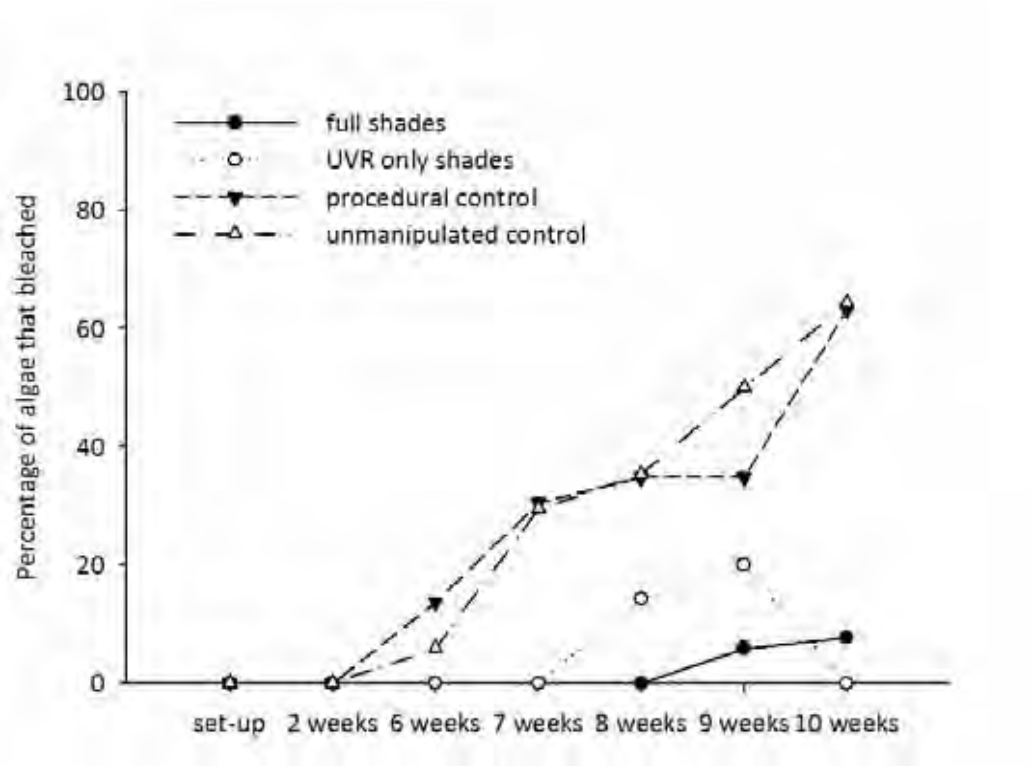

Figure 4.11. The percentage of experimental *D. pulchra* individuals that bleached during the shading experiments, in either the full shades (black circles, solid line), UVR only shades (white circles, dotted line), procedural controls with shade frames but no alterations to irradiance (black, inverted triangles, dashed line) or unmanipulated controls (white triangles, long-dashed line) treatment, with *n*=8 replicate algal patches.

Transplant experiment: Individuals that were transplanted to, or remained in the shallows were much more likely to fade than those that were transplanted to, or remained in deeper waters. In fact, algae whose 'destination' was the deep water environment did not fade at all. This effect of 'destination' environment on algal condition was observed after only one week (χ^2_2 =8.738, p =0.013) and throughout the second (χ^2_2 =27.231, p <0.005) and third (χ^2_2 =8.082, p =0.018) weeks of the experiment (Figure 4.12A). There was no statistically detectable effect of experimental procedures on the prevalence of bleaching in *D. pulchra* possibly due to loss of replicates and resulting low power, however, two out of three treatments with shallow 'destination' habitats ended-up with (non-significantly) higher proportions of bleaching than other treatments (Figure 4.12B).

The biomass of algae was significantly affected by transplantation treatment: the 'destination' environment significantly affected algal biomass ($F_{2,97}$=25.380, p <0.005), the environment of origin did not ($F_{2,97}$=0.015, p =0.902; Figure 4.13). Essentially, shallow algae (transplants and controls) ended the experiment smaller than deep water (transplants and controls) conspecifics. This effect was observed after only one week ($F_{1, 52}$ =7.855, p =0.007) and again after two weeks ($F_{1, 29}$ =15.217, p = 0.001) but was no longer significant by the final week ($F_{1, 16}$ =3.747, p = 0.071), presumably due to loss of replicates (discussed below).

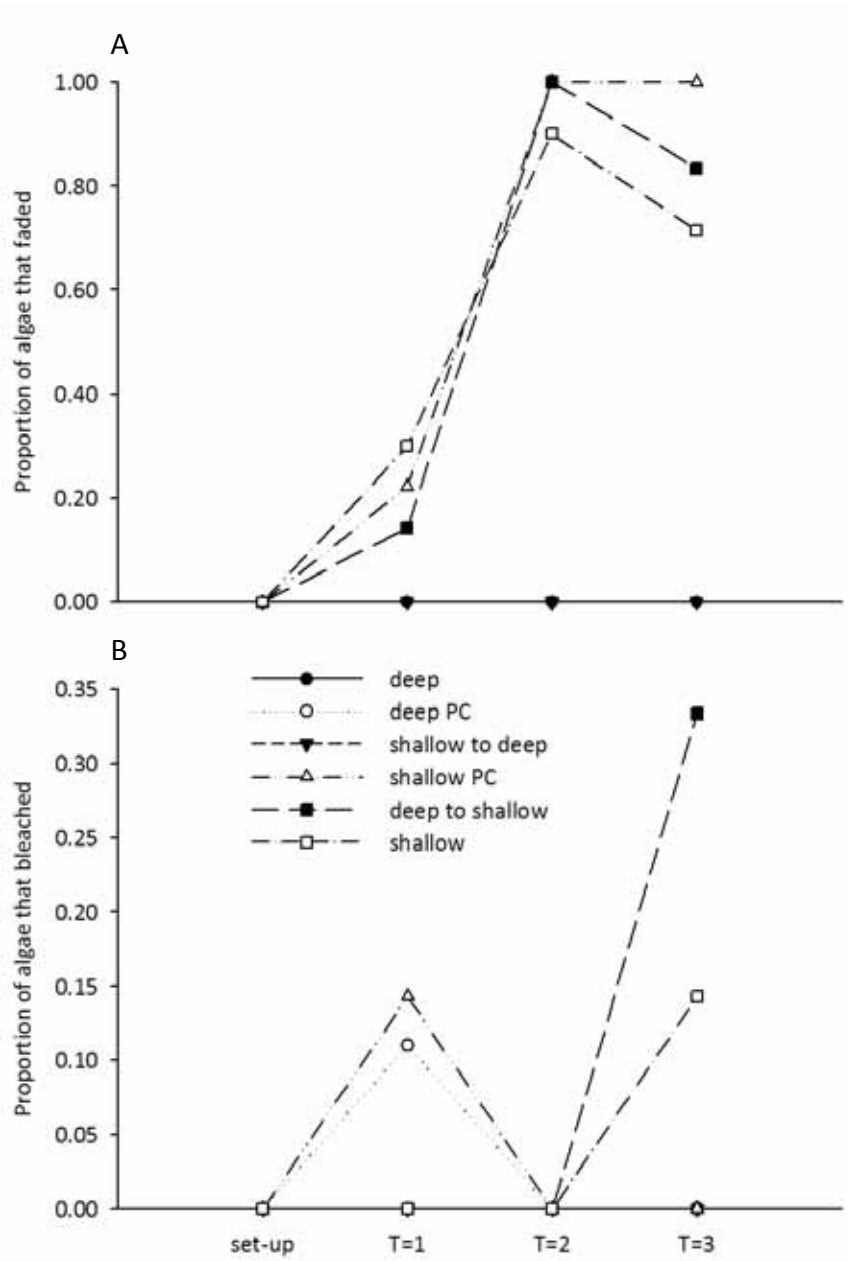

Figure 4.12. The proportion of *D. pulchra* individuals that **(A)** faded and **(B)** bleached during the transplant experiment in which algae were either left unmanipulated at depth ('deep'), transplanted from deep to deep environments, as a procedural control ('deep PC'), transplanted from shallow to deep habitats ('shallow to deep'), transplanted from shallow to shallow waters as another procedural control ('shallow PC'), transplanted from deep to shallow waters ('deep to shallow') or left unmanipulated in the shallows ('shallow'), with initial *n*=10. Note different scale of y-axes.

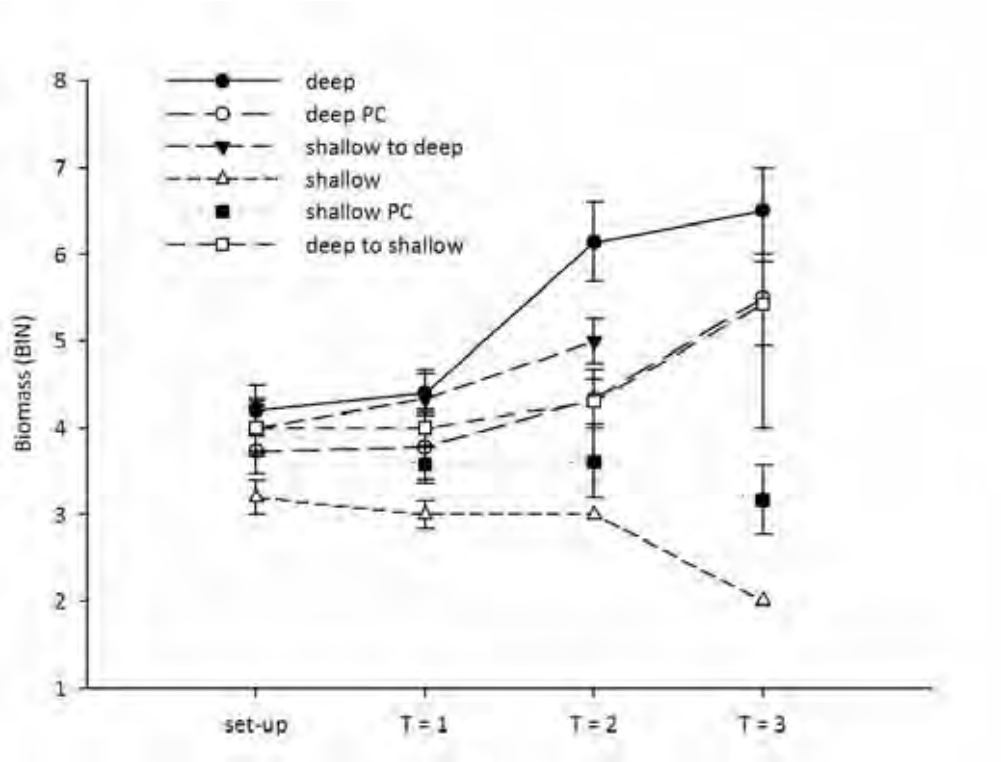

Figure 4.13. Biomass Index Number (BIN; mean ±SE) assigned to of *D. pulchra* that were either transplanted between deep and shallow habitats ('deep to shallow' or 'shallow to deep'), transplanted within their own habitats as procedural controls ('deep PC' or 'shallow PC') or left unmanipulated in their environment of origin ('deep' or 'shallow') during the transplant experiment with initial n = 10.

DISCUSSION

Fading in *Delisea pulchra* was strongly, positively correlated with solar radiation and was widespread in shallow-water populations of this alga during spring, summer and autumn but was less frequent during winter and absent in deeper waters. This increase in the prevalence of fading occurred immediately prior to peaks in the incidence of bleaching (the latter reported in Chapter 2). Faded algae had reduced absorbance spectra corresponding to photosynthetic accessory pigments relative to healthy individuals. Faded *D. pulchra* also had depleted levels of furanones relative to healthy individuals, although comparisons between faded and healthy individuals were unavoidably confounded either spatially (deep vs. shallow) or temporally (winter vs. summer). *D. pulchra* individuals growing adjacent to large (>50 cm), canopy-forming *Ecklonia radiata* kelps were less likely to bleach and were also larger than conspecifics growing far from *E. radiata*. Shading *D. pulchra* individuals from total solar radiation (PAR, UVAR and UVBR) prevented fading and also decreased algal susceptibility to bleaching. Surprisingly, we did not detect a significant effect of shading on concentrations of halogenated furanones, however this was likely due to low replication as a consistent (but non-significant) trend was observed. Transplanting algae to deeper environments with lower light levels prevented fading and in most cases, also appeared to decrease the likelihood of bleaching. These results suggest that high levels of solar radiation may be stressful for *D. pulchra* and cause fading. Faded algae have reduced concentrations of halogenated furanones than healthy individuals, potentially increasing algal susceptibility to pathogens that cause bleaching.

Solar radiation, algal fading and chemical defences

Exposure to full-strength solar radiation led to fading in *D. pulchra.* Evidence of a correlation between high light levels and the prevalence of fading in *D. pulchra* populations was observed in the surveys and causality was confirmed via the manipulative shading and transplant experiments. *D. pulchra* individuals that grew at depth or during winters, were transplanted into deeper waters, or were shaded by canopy-forming neighbours or experimental manipulations were much less likely to fade, than others. This suggests that levels of solar radiation reaching shallow (1-4 m) habitats at these two locations are sufficient to be a stressor for shallow *D. pulchra* populations, potentially altering its normal physiological functioning. Franklin & Forster (1997) suggested that levels of light reaching benthic habitats in shallow (<10 m) water is far in excess of what is required for photosynthesis. Ambient PAR and UVR can damage photosystems, leading to performance consequences for macrophytes (Franklin and Forster 1997). I have provided evidence of this for *D. pulchra:* the loss of photosynthetic pigments in faded algae (implied by the reduced absorbance at relevant wavelengths) suggests that exposure to solar radiation in shallow waters caused damage to photosystems in this seaweed.

As well as evidence for damage to photosystems, exposure to high light levels appeared to deplete furanone concentrations. This was observed in surveys, where faded algae had significantly reduced furanone concentrations compared to healthy individuals. However, these comparisons were either spatially or temporally confounded, because completely healthy algae were scarce in the shallows during summers (when the samples were collected and fading was most prevalent) and must therefore be interpreted with caution. Re-sampling these populations when both healthy and faded specimens can be obtained

from the same habitat is required. Furthermore, in order to properly assess whether exposure to high light levels affects algal chemical defences, solar radiation needs to be manipulated in replicated experiments (e.g. my shading experiment). Although in this experiment I did not observe a statistically significant effect of shading on *D. pulchra*'s halogenated furanones, there was a consistent, non-significant trend for higher concentrations of chemical defences in fully shaded algae, relative to unshaded controls and algae shaded from UVR only. Because quantifying halogenated furanones requires destructive sampling, only *n*=5 individuals from each treatment were collected in order to allow the field experiment to continue for as long as possible and as such, statistical power in this analysis was quite low (whereas variance within the shaded treatment was high). Previous work by Steinberg et al. (unpubl. data), in which shallow *D. pulchra* individuals were shaded from PAR, demonstrated clearly that protection from solar radiation increased concentrations of halogenated furanones.

If, as Steinberg et al.'s previous work and the non-significant trend reported here suggest, high levels of solar radiation do lead to a reduction in *D. pulchra*'s halogenated furanones, this could be explained by resource allocation away from defence in a stressful environment (Cronin 2002). Furanones can be costly to produce (Dworjanyn et al. 2006b), so it is possible that when conditions are sub-optimal, they are produced less. Alternatively, the normal rate of production of halogenated furanones may be maintained under high irradiance regimes, but they may be destroyed or otherwise altered by high levels of solar radiation after production. Whatever the mechanism, faded algae had lower concentrations of halogenated furanones and photosynthetic pigments than healthy algae, providing

compelling evidence that high levels of solar radiation in shallow water habitats cause fading and alter the normal physiological functioning of *D. pulchra.*

In this study, total solar radiation (PAR, UVAR and UVBR) rather than UVR alone had the most significant and prolonged impact on *D. pulchra*'s condition. This combined effect of PAR and UVR was also observed by (Bischof et al. 2002) who suggested that these two wavelengths may interact synergistically, particularly when one or both are elevated. Similar results have been obtained from experiments involving cyanobacteria, which suffered the greatest photoinhibition and took longest to recover when exposed to high levels of PAR and UVR (Gao et al. 2007). PAR and UVR can both damage algal photosystems, and do so in different ways (Franklin and Forster 1997) and the combination of these wavelengths appears to have the greatest impact of *D. pulchra*'s condition and potentially also its performance. The present study also supports the suggestion by Bischof et al. (2002) and others, that studies assessing the effects of UVR alone may be underestimating the potential impacts of total solar radiation on organisms.

An interesting observation from the surveys was that the relationships between solar radiation and the prevalence of fading were much stronger at Bare Island than Long Bay. The cause for this is unknown, however over the course of the monitoring study, I observed that water quality (and thus light penetration) usually appeared lower at Bare Island than Long Bay. Potentially, algae growing in shallow water environments at Bare Island had adapted to lower light environments than those at Long Bay and were thus more sensitive to increases in solar radiation. This has been observed in terrestrial plants, with those acclimated to high light levels having higher levels of energy-dissipating molecules than

shade leaves, thus experiencing less stress and photo-damage from excessive solar radiation than shaded conspecifics (Demmig-Adams and Adams 1996).

Early life history stages of most macrophytes are typically shade-adapted to allow them to grow within the low-light conditions of an understory environment and are smaller with high surface-area: volume ratios (Franklin and Forster 1997). For these reasons, algal recruits may be more susceptible to solar radiation stress than adult conspecifics (reviewed by Coelho et al. 2000). Many marine rhodophytes have reproductive strategies that involve temporally separated life history stages that are thought to maximise offspring success in the most favourable environments (e.g. *Asparagopsis armata*; Verges et al. 2008). Although *D. pulchra* reproduces year-round (Wright and Steinberg 2001) and the effects of solar radiation stress on different life history stages of *D. pulchra* were not assessed here, these data suggest that the light characteristics of a settlement micro-habitat may influence adult *D. pulchra* condition and performance. For example, propagules that settle where solar radiation is low (e.g. during winter or in shaded micro-habitats or deeper water) will potentially yield larger, more healthy and more chemically-defended *D. pulchra* individuals relative to those that settle in high light environments (e.g. during summer, in unshaded micro-habitats or shallow water).

Solar radiation and susceptibility to bleaching

Algae that were not experimentally or naturally shaded from ambient solar radiation were more likely to bleach. In the shading experiment, individuals that were shaded from total solar radiation bleached significantly less than algae from other light treatments, suggesting that exposure to solar radiation increases the risk of bleaching in *D. pulchra*. A study into the effects of UVR on the distribution of another marine macroalga, the kelp *Laminaria*

saccharina, demonstrated that increased UVR exposure led to higher infection rates by an endophytic alga *Sterblonema acceidioides* (Schaffelke et al. 1996). Results from the present study support these findings, in that exposure to levels of solar radiation ambient in shallow subtidal marine environments can increase disease susceptibility to a putative disease in a habitat-forming seaweed. The exact mechanisms of this remain somewhat unclear. Although I detected only a non-significant trend of higher levels of chemical defences in shaded algae, light treatments did have a clear influence on microbial communities associated with algal surfaces. This suggests that microbial communities shifted either in direct response to a change in the light regime, or indirectly, due to changes in their host caused by different light environments.

If, as previous work and the trend observed here suggest, that exposure to high levels of solar radiation does lead to a decrease in halogenated furanones in *D. pulchra*, this could have several important ecological consequences. Halogenated furanones mediate a range of important ecological interactions for *D. pulchra*, including the prevention of settlement by pathogenic (Manefield et al. 2001) microorganisms onto its surface, so a reduction in these compounds would likely increase risk of pathogen attack, fouling (Dworjanyn et al. 2006a) and herbivory (Wright et al. 2004). Individuals with depleted chemical defences are also more susceptible to bleaching pathogens (Chapters 2 and 3). The microbial community shifts observed here could be explained by a depletion of halogenated furanones in unshaded individuals. Such shifts in surface-associated microbial community (SAMC) have been linked to diseases in other marine organisms (e.g. Frias-Lopez et al. 2002, Pantos et al. 2003).

Although there is little existing knowledge of the importance of solar radiation to disease susceptibility of seaweeds or other marine organisms, data on the influence of light on plant-herbivore interactions in terrestrial environments are relatively abundant. These are useful to review in this context, as herbivores and pathogens exert similar selective pressures on plants and algae (Fritz and Simms 1992), so understanding how abiotic stress influence plant-herbivore interactions may aid in predictions about environmental influences on host-pathogen interactions. For example, shading terrestrial herbaceous plants from UVBR led to increased rates of herbivory in an Argentinean national park (Rousseaux et al. 1998). Conversely, marine gastropods grazed preferentially on algae that had been exposed to UVR (Heaven and Scrosati 2004). High levels of UVR exposure increased oviposition by an invertebrate herbivore onto the terrestrial plant *Brassica oleracea* (Foggo et al. 2007). Interestingly, in the latter study, herbivore larvae that fed on plants exposed to UVR were then more likely to be parasitised than conspecifics that fed on UVR depleted host plants (Foggo et al. 2007), demonstrating the potentially complex consequences of exposure to solar radiation on affected individuals and their trophic interactions.

Observations presented here, in the context of those from Chapter 2, suggest that solar radiation levels in shallow subtidal marine environments result in algal fading and the depletion of halogenated furanones, thus indirectly increasing *D. pulchra*'s susceptibility to pathogens and its likelihood of bleaching. Furthermore, I suggest that algal fading may be a precursor condition to bleaching. Although not all algae that bleached had also faded, fading appears to increase an alga's probability of bleaching, at least on a population level. Observations of low levels of bleaching in fully-shaded algae (and those in deep water

habitats, Chapter 2) are interesting and demonstrate that solar radiation is certainly not the only stressor that can influence *D. pulchra*'s susceptibility to bleaching. Indeed, in previous chapters I documented a consistent influence of temperature and an occasional effect of salinity on the prevalence of bleaching and there are likely also other biotic and abiotic processes that can influence an alga's susceptibility to bleaching.

An observation from the transplant experiment illustrates this point: I observed some instances of bleaching in algae that were transplanted into shallow environments (either from depth or from the shallows, as procedural controls), but not shallow unmanipulated control algae. This suggests that the transplantation method used may have damaged or stressed the algae thereby increasing their susceptibility to bleaching pathogens by accidentally providing and infection site. This effect was not observed in deep algae (transplants or controls), suggesting that this combination of mechanical stress and the shallow water environment (high light and warmer temperatures; Chapter 2) led to bleaching in these treatments. Furthermore, these observations support those from Chapter 3, where a bacterial pathogen was able to infect and bleach *D. pulchra* thalli that had also been experimentally damaged. Another apparent result from this experiment that warrants mentioning is that the prevalence of bleaching appeared to fluctuate within treatments over time (i.e. bleached algae appeared to 'un-bleach'). This was not the case; instead replicates were occasionally lost which affected the proportional data that were plotted in graphs.

Associational resistance to stress and disease

D. pulchra individuals growing close to *E. radiata* individuals faded and also bleached less frequently than conspecifics growing far from canopy-formers. They were also larger. The shade provided by these neighbouring kelps could have prevented algal fading, appeared to

enhance algal performance (estimated by biomass) and also decreased algal susceptibility to bacterial bleaching. This result provides an example of a novel, positive interaction in which one organism can avoid abiotic stress and reduce the risk of pathogen attack by associating with a shade-providing neighbour. It also suggests that shaded habitats, such as underneath canopy-formers or deeper waters, may become more important as environments become more stressful. Deep water refugia have been suggested as sources for biodiversity of corals (Bongaerts et al. 2010) and kelps (Graham et al. 2007), as shallow water habitats become further impacted by climate change and other anthropogenic disturbances.

The availability of such naturally shaded microhabitats in shallow waters may, however, be decreasing. Evidence from many parts of the world indicates that stands of macroalgal kelps that provide shade and create understory microhabitats are in decline (e.g. Thibaut et al. 2005, Edwards and Estes 2006). In Australia and New Zealand, significant losses of important habitat-forming macroalgae have been reported for several species including *E. radiata* (Cole and Babcock 1996, Connell et al. 2008), *Phyllospora comosa* (Valentine and Johnson 2004, Coleman and Kelaher 2009) and *Macrocystis pyrifera* (Ling 2008). The loss of these habitat-formers typically has many consequences for other trophic levels, but also has specific impacts on understorey associates with respect to exposure to solar radiation. For example, the manipulation of *E. radiata* canopies in Western Australia leads to a decrease in the diversity of understorey communities (Toohey et al. 2004) and lowered survival of recruits (Toohey and Kendrick, 2007). The work presented here demonstrates that the loss or decline of *E. radiata* (and possibly other species, e.g. *Phyllospora comosa*; Coleman et al. 2008) can affect another algal species' susceptibility to pathogen attack and bleaching. Loss

of these canopy-formers may indirectly increase stress in coastal marine habitats, placing further pressure on already impacted organisms and communities.

Conclusions

Ambient levels of solar radiation in shallow subtidal marine environments were sufficient to cause fading (which I suggest is a precursor condition to bleaching) and concentrations of halogenated furanones were lower in faded algae than healthy conspecifics (collected at other times or depths). Exposure to solar radiation led to a shift in microbial communities associated with algal surfaces and increased risk of bleaching. Algae that were experimentally shaded from total solar radiation were less likely to fade, performed better and had a lower risk of bleaching compared to unshaded conspecifics and algae shaded from UVR only. Canopy-forming kelps provided shaded refugia for *D. pulchra*, which grew larger and bleached less when adjacent to a large *E. radiata* than in unshaded microhabitats. As stands of canopy-forming macroalgae are in decline worldwide, the shaded refuge they provide to understorey communities may become limited, potentially leading to increased stress and disease. This study highlights the complexity of interactions between environmental stress, chemical defences, pathogens and anthropogenic disturbances to natural habitats.

CHAPTER 5

Ecological consequences of bacterially mediated bleaching for a habitat former and its trophic interactions

INTRODUCTION

Diseases affecting natural ecosystems are occurring more frequently worldwide, due largely to climate change and other anthropogenic disturbances (Harvell et al. 2002, Lafferty 2009). The consequences of such diseases can be obvious and catastrophic, involving mass mortalities of host organisms over large spatial or temporal scales, or may be more subtle, involving sub-lethal impacts. Some examples of disease epidemics that have caused widespread deaths within affected populations include canine distemper virus, which infected Caspian seals (Kennedy et al. 2000), *Batrachochytrium dendrobatidis* or 'chytrid fungus', which has had severe impacts on global amphibian populations (Pounds et al. 2006), fungal infections of gorgonians (Martin et al. 2002), diverse microbial diseases of corals (Aronson and Precht 2001, Rosenberg and Ben-Haim 2002, Kim and Harvell 2004), sudden oak disease and fungal infections affecting woody trees (Rizzo and Garbelotto 2003) and seagrass wasting disease (Muehlstein et al. 1988, Robblee et al. 1991). In these and

similar cases, the direct consequences of disease for infected individuals and populations are clearly negative and severe.

Not all organisms that become infected by pathogens die. However disease avoidance, tolerance or recovery typically incur substantial fitness and/or performance costs (Gemmill and Read 1998, Boots 2008). For example, viral infections in the Indian meal moth don't usually result in mortality, but infected moths do have significantly reduced reproductive capacities relative to uninfected conspecifics (Sait et al. 1994). Similarly, fecundity decreases in tent caterpillars that survive pathogen infection (Myers and Kuken 1995) and protistan parasites may not kill all the economically important molluscs they infect, but survival of the molluscs incurs fecundity, growth and performance costs (Villalba et al. 2004). So disease resistance often leads to significant sub-lethal impacts, which can also have negative impacts on host populations.

Sub-lethal disease impacts may also have broader community-level consequences by altering trophic interactions, involving affected host organisms. So-called 'tripartite' or 'tri-trophic' interactions between a plant host, a pathogen and herbivore(s) have been studied in terrestrial plant and agricultural science fields, primarily with respect to how plant palatability and herbivore preferences change when plants are infected by diseases (reviewed by Stout et al. 2005). This body of work has demonstrated that it is difficult to predict exactly how a particular disease may affect trophic interactions of a particular host. For example, infection by disease may increase, decrease or have no effect on host susceptibility to consumption and this effect may vary among different consumers (e.g. Kluth et al. 2001). Given the closely-coupled, co-evolutionary relationships that exist

between plants and their herbivores (Agrawal 2007), changes to these interactions due to disease could have significant evolutionary consequences for both groups of organisms.

Considering the potential for disease to have such profound impacts on hosts and the organisms they interact with, it is surprising that the consequences of disease outbreaks are often poorly understood. Indeed, unless catastrophic over large spatial or temporal scales, diseases in natural ecosystems may even go unnoticed. Understanding how disease may impact host organisms and populations, as well as their interactions is crucial, especially as new diseases continue to emerge in nature (Harvell 2004). There is a particular need to increase our understanding of how diseases are likely to affect important habitat-forming organisms (e.g. corals, trees and seagrasses). Habitat-formers perform vital functions within ecosystems including the provision of three-dimensional structures as habitat, primary production, and an energy source for a myriad of consumers. Thus, any changes within these populations due to disease have the potential to cascade through the entire community (e.g. Aronson and Precht 2001).

In marine environments, organisms are arguably at greater risk of disease than those on land (Correa and Sanchez 1996, McCallum et al. 2004), due to the high abundance and dispersal potential of microorganisms (Reinheimer 1992), the tendency of water-borne diseases to be more severe than others (Ewald 1994) and difficulties associated with disease control underwater. In temperate marine ecosystems, the dominant habitat-formers are macrophytes. Diseases affecting marine macrophytes have been reported (Short et al. 1987, Apt and Gibor 1989, Correa et al. 1993, Correa et al. 1994, Largo et al. 1995a, Littler and Littler 1995, Peters and Schaffelke 1996, Correa et al. 1997, Faugeron et al. 2000, Bouarab et

al. 2001) but the consequences of disease for the host population or the communities they support are rarely described, except when obvious, large-scale losses occur.

In previous chapters, I have described a putative bleaching disease affecting a chemically defended red macroalga, *Delisea pulchra*. This seaweed suffers from tissue bleaching which is associated with bacterial infection, reduced chemical defences and increased water temperature (Chapters 2 and 3). Additionally, populations of *D. pulchra* occurring in shallow (1-4 m) waters frequently undergo 'fading' in response to increased levels of solar radiation (Chapter 4). During peak periods, these phenomena affect up to 80% and 100% of *D. pulchra* individuals respectively. Both conditions result in depleted concentrations of halogenated furanones (Chapters 2 and 4), chemical defences that moderate *D. pulchra*'s interactions with many microorganisms (Manefield et al. 2001, Jones et al. 2005) and consumers (Williamson et al. 2000, Wright et al. 2000, Wright and Steinberg 2001, Rogers et al. 2002).

Marine algae such as *D. pulchra* play critical roles in global primary production (Charpy-Roubaud and Sourina 1990) and also provide essential habitat and/or food for a large proportion of coastal marine biodiversity (Steneck et al. 2002). Herbivores exert selective pressure on *D. pulchra* and its production halogenated furanones (Wright et al. 2004) so environmentally-mediated changes to these interactions could have ecological and evolutionary consequences for this seaweed. Despite the high incidence of bacterially-mediated bleaching affecting this habitat-former, the biological and ecological consequences for individuals and the broader community are not currently known. In this chapter, I assessed whether bleaching had direct effects on *D. pulchra*'s survivorship or performance, by monitoring tagged individuals over a six month period. I also investigated

whether bleaching or fading influenced this seaweed's role as a habitat-former or food source for locally abundant herbivores that are commonly associated with the alga.

MATERIALS & METHODS

Study sites and organisms

Delisea pulchra (Greville) Montagne (Bonnemaisonales: Rhodophyta) is a common red alga in coastal, sub-tidal habitats around Sydney and southern Australia (Chapter 2). Two populations of *D. pulchra* were studied: at Bare Island in Botany Bay (33°59'32'' S, 151°13'50'' E) and Long Bay, Malabar (33°58'19'' S, 151°14'42'' E). Both locations are near Sydney, Australia and are similarly dominated subtidally by turf-forming algae beds, urchin barrens and sandstone boulders. These locations were selected because bleached, faded and healthy *D. pulchra* individuals co-occur there during the late summer (Chapter 2).

Effects of bleaching on survival and performance of **D. pulchra**

To determine whether bleaching in *D. pulchra* affected algal survival or performance, fifty replicate, haphazardly selected algae were tagged in the shallows (1-4 m) and in deep water (7-10 m) at Bare Island and Long Bay, giving a total of 200 algae tagged in September 2007. Uniquely numbered, flagged plastic tags were attached to the substratum directly adjacent to *D. pulchra* individuals so that the algae were not in direct contact with the tags but each tag corresponded obviously with only one individual. Due to storms and sand movement, a significant number of tags were lost within the first two months of the study, particularly from shallow areas, so a second tagging effort was carried-out in December 2007. At set-up, the tag number (unique three-digit code), location (Bare Island or Long Bay), depth (shallow or deep) and date were all recorded, and each alga was classified as either healthy, bleached, or faded (defined in Chapters 2 and 4).

I tracked changes in algal size as a proxy for algal performance and fecundity throughout the study period. Biomass was estimated *in situ* by measuring algal length. I first assessed

whether there was a relationship between algal height and biomass prior to set-up of the tagging study. To do this, I measured the length of twenty-nine replicate algae of varying sizes *in situ* and then destructively sampled and returned them to the lab. There the algae were padded dry and weighed to obtain wet-mass, then freeze-dried and re-weighed to obtain dry-mass. To assess whether algal biomass was related to fecundity, I destructively sampled twenty-eight replicate *D. pulchra* individuals of varying sizes (1 - 150 g) from both Bare Island and Long Bay. From each alga I randomly selected five replicate branches of similar sizes (approximately 3 cm in length) and counted the numbers of fertile tetrasporangia on each branch.

After set-up and once initial data had been collected for each alga, tagged algae were revisited monthly to assess condition (healthy, bleached or faded) and estimate their biomass (by measuring length). These surveys were conducted until April 2008 at which time many of the algae and tags had been lost, so data were collected for algae for up to six months, which approximates the mean life span for this species (Wright and Steinberg 2001). Algae were considered to have survived until they were no longer attached to the substratum adjacent to their unique tags.

At the end of the study, algae that had bleached at any time during the study were classified as 'bleached' (whitening of localised, non-uniform parts of the thallus; Chapter 2) and those that had not were classified as 'healthy'. By the second month of sampling, most shallow algae had faded (yellowing of the tips of algal thalli; Chapter 4). Many of these also went on to bleach but few tagged shallow algae remained neither bleached nor faded by the end of the study. For this reason, comparisons of survival and biomass change were made between (i) algae that bleached (mostly 'faded and bleached' algae in the shallows, 'bleached' only at

depth) and (ii) those that did not (mostly 'faded' only in the shallows and 'healthy' at depth). Survivorship (whether algae died during the study and biomass change corrected for differences in initial lengths (final length-initial length)/initial length) were compared among 'bleached' and 'unbleached' algae from both locations and depths.

Effects of algal condition on herbivore preferences

Several macroherbivores (> 5 mm) commonly associated with *D. pulchra* were surveyed and used in feeding assays: the urchin *Holopneustes purpurascens* (Figure 5.1A), the gastropod *Phasianotrochus eximius* (Figure 5.1B), and the sea hare *Aplysia parvula* (Figure 5.1C). *P. eximius* consumes *D. pulchra* despite the presence of furanones (Wright et al. 2004), as does *A. parvula*, although *D. pulchra* is not considered its preferred diet, as it preferentially consumes other red algal species when available (Rogers et al. 2002). Herbivory by *H. purpurascens*, which is often found living on *D. pulchra*, is largely inhibited by furanones (Williamson et al. 2004, Wright et al. 2004) and when this urchin is fed *D. pulchra* exclusively, it performs poorly and experiences lower survivorship than individuals fed other algal diets (Williamson et al. 2004).

Herbivore surveys: To determine whether herbivores were more or less likely to associate with bleached, faded or healthy algae, populations of *D. pulchra* growing subtidally at 1-4 m depth were surveyed and the abundances of *P. eximius*, *H. purpurascens* and *A. parvula* were assessed. From each location, thirty replicate algae in each condition (healthy, bleached or faded) were destructively sampled and fully enclosed *in situ* within sealed bags. In the laboratory the algae were rinsed thoroughly in seawater in an examination tray and the numbers of the three herbivores (*P. eximius*, *H. purpurascens* and *A. parvula* >5 mm) counted. These taxa were consistently the most numerous macroherbivores observed on *D.*

pulchra during the survey. Algae were padded dry, weighed and the density of herbivores (numbers per gram (wet-mass) of algae) on each alga was calculated.

Because bleached *D. pulchra* tended to be smaller than healthy conspecifics, there was a risk that any differences I observed in herbivore density between bleached and unbleached algae may be confounded by condition-specific differences in biomass. To investigate whether algal biomass influenced herbivore density, I sampled thirty algae of varying sizes (1-150 g) that were all in the same condition (healthy) and assessed whether there was a relationship between herbivore density and algal biomass. Algae were destructively sampled from both Long Bay and Bare Island and processed as described above. Finally, to assess whether herbivore densities varied between deep and shallow habitats, I also destructively sampled thirty-two replicate healthy algae of similar sizes from deep and shallow habitats at Long Bay and compared herbivore densities among depths.

Feeding assays: To assess whether herbivores preferred to consume healthy or bleached algal tissues, I conducted three separate paired choice feeding experiments, in which herbivores were offered a choice between two portions of algal tissue. All experiments were run separately for each herbivore species (*P. eximius*, *H. purpurascens* and *A. parvula*). In the first experiment (Assay 1) eighteen replicate herbivores were offered a choice between (i) bleached tissue and (ii) adjacent healthy looking tissue from the same algae. Each replicate herbivore was offered bleached or adjacent unbleached tissue from the same individual alga such that eighteen replicate algae were also used in this assay. In the second experiment (Assay 2) eighteen replicate herbivores were offered a choice between (i) faded tissue and (ii) adjacent 'unfaded' tissue from a faded individual such that, as in Assay 1, each herbivore was offered two tissue samples from the same replicate alga. In the third

experiment (Assay 3) eighteen replicate herbivores were offered a choice between (i) healthy tissue from a completely healthy alga and (ii) visibly unbleached tissue from a bleached individual, such that each replicate herbivore was offered tissue from two different algal individuals. For each assay, an equal number of replicates were set-up with no herbivores to control for autogenic changes in biomass.

In all three assays, the initial (wet) mass of algal tissue samples was measured. Experimental algae and herbivores (or controls which lacked herbivores) were gently aerated in compartmentalised aquaria for 48 hours at a constant temperature of 19-20 ^{o}C with a 16: 8 hour light: dark cycle. After 48 hours, changes in algal mass in the presence of herbivores were compared to changes in mass in control algae from which herbivores were absent. Because within-alga furanone concentrations vary from the base to the tips (Dworjanyn et al. 1999), care was taken to ensure that herbivores were consistently offered algal tissue from the same area of each plant in each experiment.

Statistical analyses

Throughout this study, I treated 'location' as a fixed factor in analyses, as these locations were selected specifically because of the presence of high density populations of *D. pulchra* in both deep and shallow water and the coexistence of bleached, faded and healthy algae in shallow water. Survivorship of tagged *D. pulchra* was compared using non-parametric permutational analyses of variances (PERMANOVAs) where survival in tagged *D. pulchra* was compared among locations (Bare Island, Long Bay), depths (deep, shallow) and condition (bleached, unbleached). Relationships between (i) algal length and biomass, (ii) biomass and fecundity and (iii) herbivore densities and algal biomass were analysed using linear regressions. Proportional changes in biomass (final length-initial length)/initial length) of

tagged *D. pulchra* individuals were compared using a three-fixed-factor ANOVA comparing change of algal length among locations, depths and condition.

Densities of each herbivore species (*P. eximius*, *H. purpurascens* and *A. parvula*) and 'total' herbivores were compared between bleached, faded and healthy algae at the two study locations using two-fixed-factor ANOVAs, with algal biomass as the covariate. Herbivore densities on deep and shallow *D. pulchra* individuals were compared using a permutational analysis of variance (PERMANOVA) as these data did not conform to assumptions of ANOVA. Finally, herbivore feeding preferences were compared for each species and assay using single-factor ANOVAs with the factor as 'condition (healthy vs. bleached in Assay 1, faded vs. unfaded in Assay 2, and healthy vs. 'adjacent' unbleached in Assay 3).

Assumptions of normality and equal variances of all data were checked by inspecting scatterplots of residuals and by Levene's and K-S tests respectively. When appropriate, data were transformed to conform to assumptions (transformations reported alongside tables). ANOVAs and regressions (linear and logistic) were performed using the program SYSTAT 13 Version 13.00.05 and T-tests were conducted in Microsoft Office Excel 2007. PERMANOVAs were performed using PRIMER 6 v 6.1.11 & PERMANOVA + v 1.0.1.

Figure 5.1. Herbivore taxa included in surveys and used in feeding assays: **(A)** the urchin *Holopneustes purpurascens*, **(B)** the sea hare *Aplysia parvula* and **(C)** the gastropod *Phasianotrochus eximius*. The herbivores shown here are being offered choices between fragments of *D. pulchra* thalli in various conditions as food.

RESULTS

Effects of bleaching on survival and performance of **D. pulchra**

Bleaching did not affect survivorship in deep populations of *D. pulchra*, but in shallow populations, survivorship of bleached *Delisea pulchra* was higher than for healthy conspecifics at both locations (Figure 5.2, Table 5.1). At depth, bleached algae survived at similar rates as they did in the shallows. Survivorship rates of healthy algae were much higher at depth than in the shallows. Bleached algae experienced significantly less increase in length (proxy for growth) than co-occurring healthy algae and this difference was more pronounced at Long Bay than Bare Island at both depths (Figure 5.3, Table 5.2). In many cases, bleached algae (and some healthy algae at Long Bay) lost biomass during the study period. This negative effect of bleaching on change in algal length was consistent among locations and depths (Table 5.2). There was also a significant effect of depth on changes in algal length (Figure 5.3) with shallow algae gaining significantly less length throughout the study than deep conspecifics, regardless of condition (Figure 5.3).

Algal length was a reliable predictor of algal biomass (Figure 5.4) both with respect to dry (R^2 =0.519, *df* =28, p <0.001) and wet (R^2 =0.481, *df* =28, p <0.001) mass. Biomass was positively related to fecundity (Figure 5.5; R^2 =0.570, *df* = 57 p <0.001), and this relationship was similar at both Bare Island (R^2 =0.272, *df* =28, p =0.003) and Long Bay (R^2 =0.244, df =27, p =0.006): at both locations, larger *D. pulchra* individuals had more fertile tips per branch than smaller conspecifics.

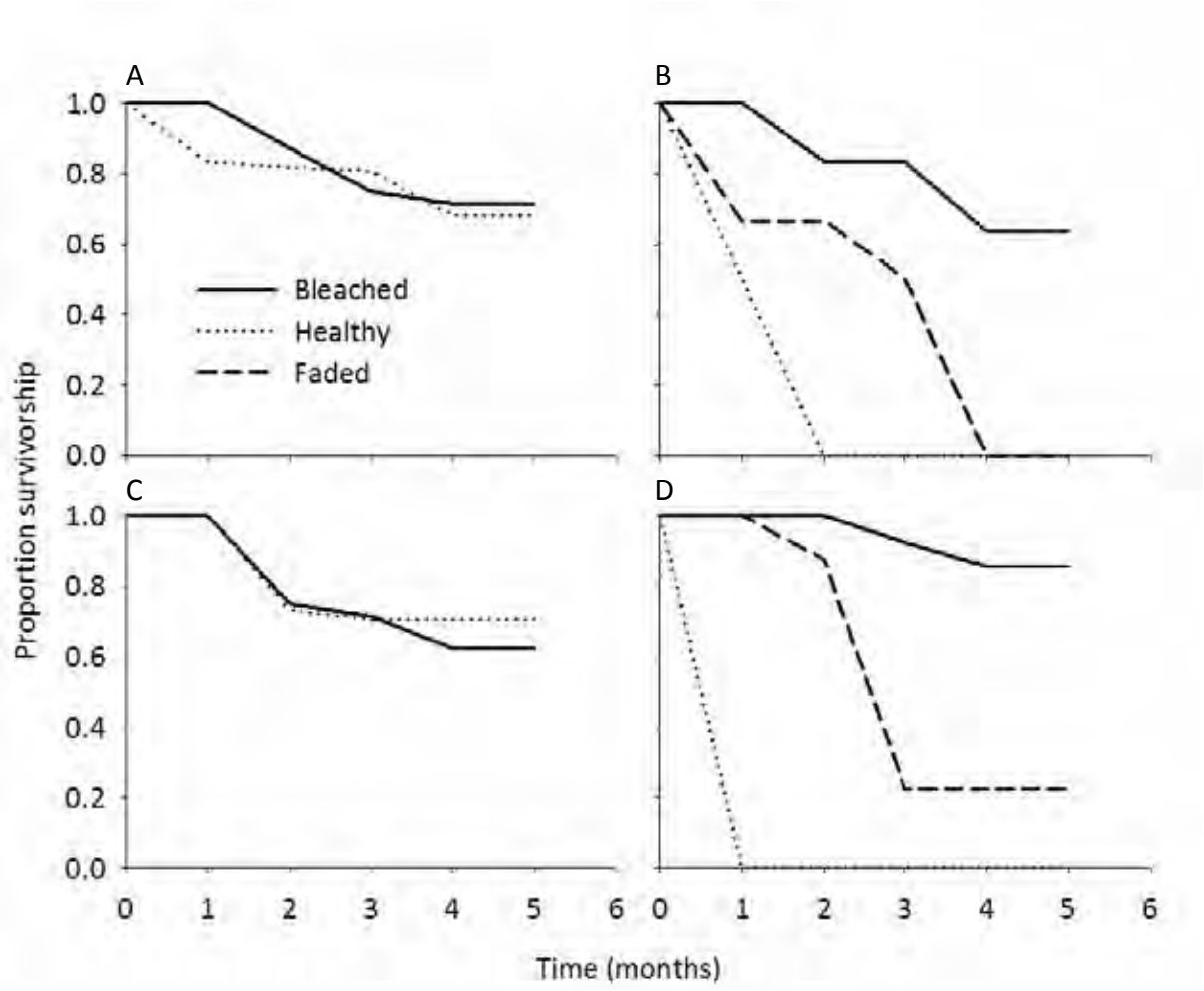

Figure 5.2. Survivorship curves showing the cumulative proportion of tagged *D. pulchra* that survived during the study including bleached (solid line), healthy (dotted line) and faded (dashed line) algae from **(A)** deep and **(B)** shallow water sites at Bare Island and **(C)** deep and **(D)** shallow water sites at Long Bay. No fading occurred within deep populations with initial n = 50.

Table 5.1. PERMANOVA comparing survivorship among tagged *D. pulchra* individuals from deep and shallow waters at Bare Island and Long Bay that either bleached or remained healthy survived throughout the study. '*' indicates significant effect of factor on survivorship with α= 0.05

Source of variation	*df*	*MS*	*Pseudo-F*	*P(perm)*
Location (L)	1	0.141	0.782	0.394
Depth (D)	1	1.233	6.863	0.005*
Condition (C)	1	1.071	5.963	0.020*
L x D	1	0.397	2.044	0.158
L x C	1	0.004	0.203	0.688
D x C	1	1.675	9.325	0.001*
L x D x C	1	0.000	0.003	0.852
Residual	80	0.180		

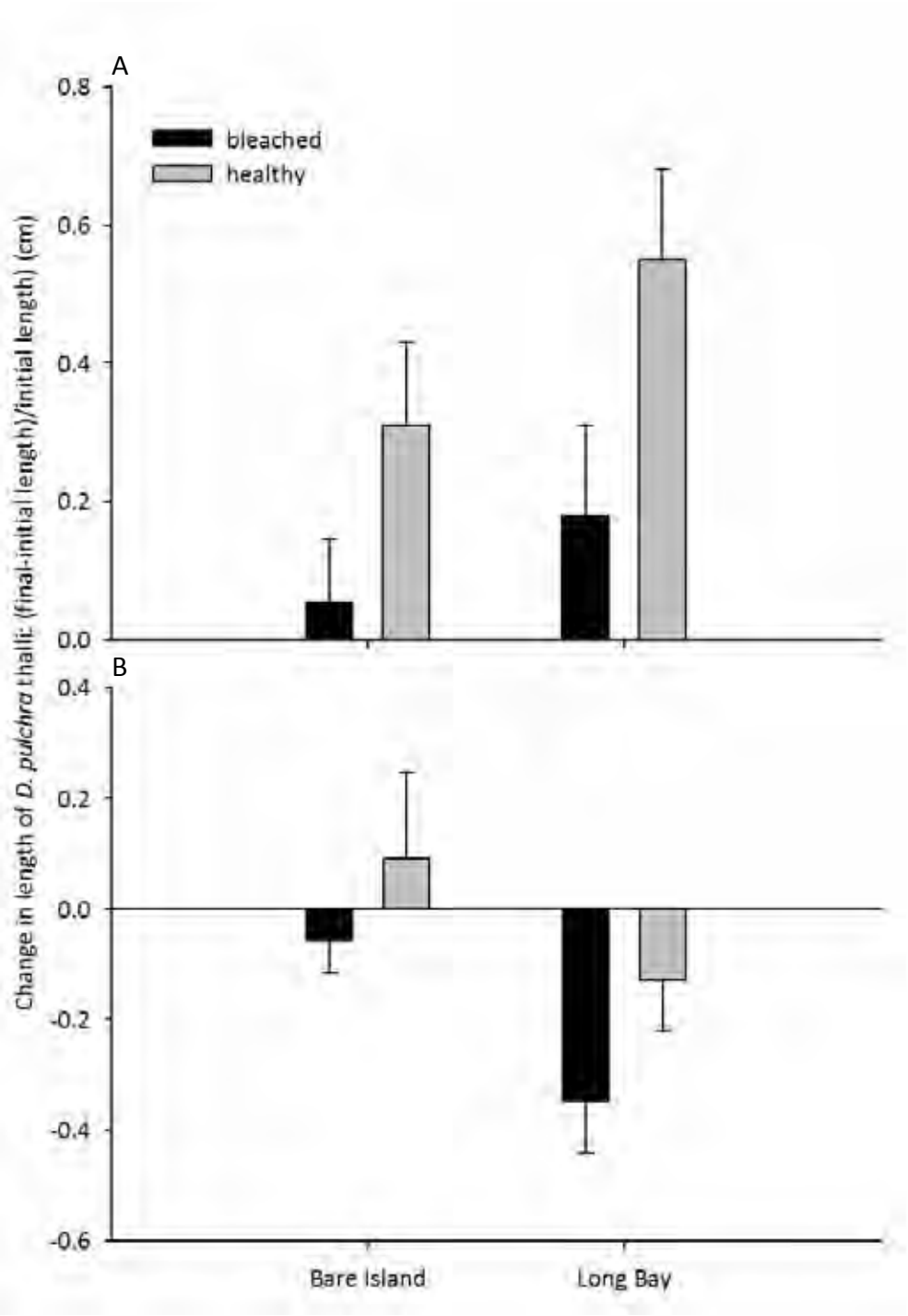

Figure 5.3. Change in algal length (means +/- SE; (final-initial length)/initial length)) of **(A)** deep and **(B)** shallow tagged *D. pulchra* thalli that bleached (black bars) and did not bleach (grey bars) throughout the study, with initial *n* = 50.

Table 5.2. ANOVA comparing changes in length ((final-initial)/initial height) of tagged *D. pulchra* that either bleached or did not bleach during the study, from deep and shallow waters at Bare Island and Long Bay. '*' indicates a significant effect of the relevant factor on change in length, with α= 0.05 Data were 'log+1' transformed to conform to the assumptions of ANOVA.

Source	*df*	*MS*	*F*	*p*
Location (L)	1	0.102	2.34	0.128
Depth (D)	1	0.907	20.74	0.000*
Condition (C)	1	0.420	9.59	0.002*
L x D	1	0.444	10.14	0.002*
L x C	1	0.029	0.65	0.420
D x C	1	0.000	0.00	0.964
L x D X C	1	0.009	0.20	0.653
Error	171	0.044		

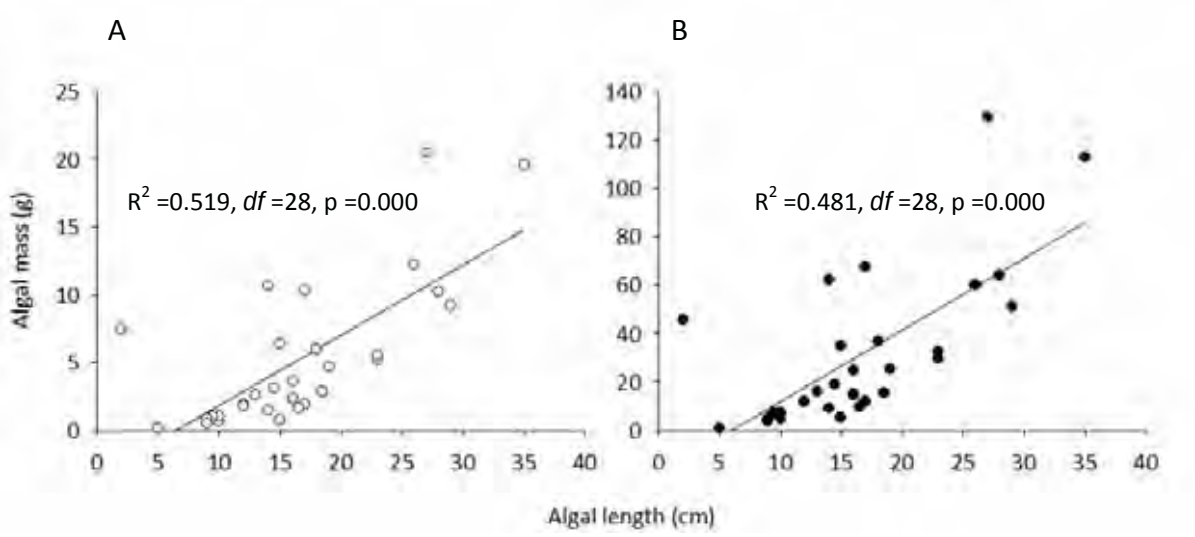

Figure 5.4. Algal **(A)** dry and **(B)** wet mass plotted as a function of algal length measured in situ with *n*=30. Significant, linear relationships are indicated with regression lines and regression analyses are shown in graph areas.

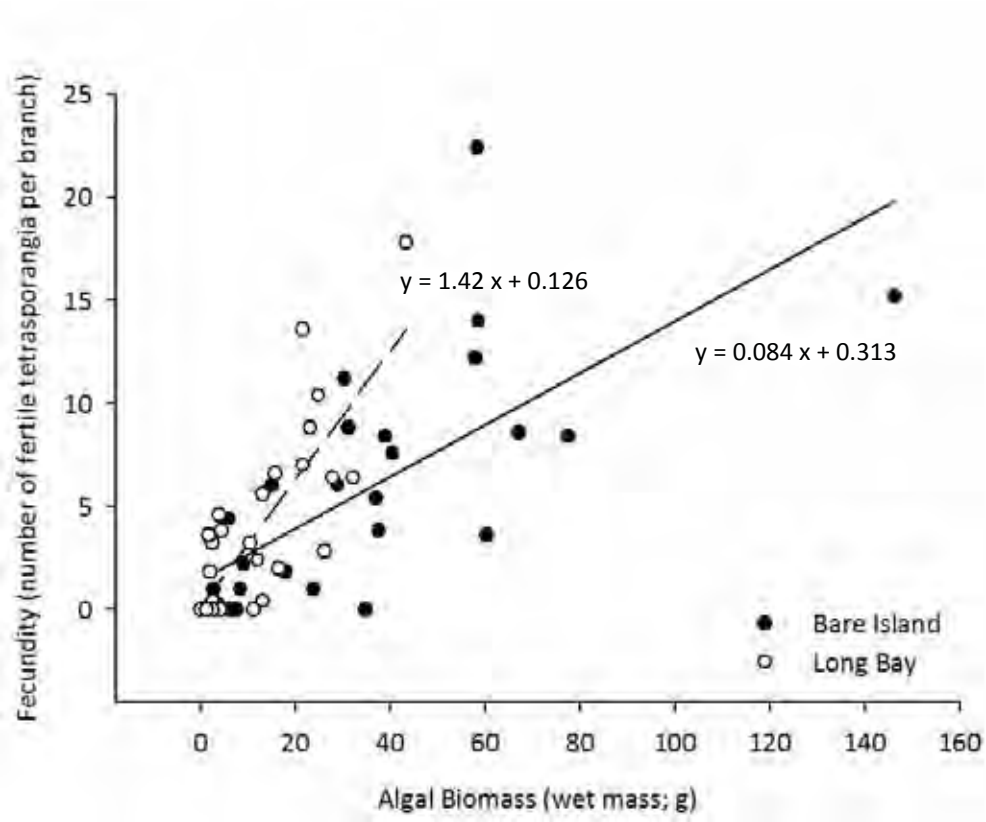

Figure 5.5. Fecundity, measured as the number of fertile tetrasporangial sori from five randomly selected branch from *n*=29 replicate algae from Bare Island (closed circles, solid line) and *n*=28 replicate algae form Long Bay (open circles, dashed line) plotted as a function of algal biomass. Significant, linear relationships are indicated by regression lines and described by formulae.

Effects of algal condition on herbivore preferences

Herbivore surveys: The total density of herbivores (*Phasianotrochus eximius*, *Holopneustes purpurascens* and *Aplysia parvula* combined) was significantly higher on bleached *D. pulchra* algae than on co-occurring healthy and faded algae (Figure 5.6A, Table 5.3). Total herbivore densities were very similar on healthy and faded algae. These patterns were observed at both Long Bay and Bare Island and neither location nor algal mass influenced total herbivore density (Table 5.3), although there was some species-specific variation. *P. eximius* densities were higher on bleached *D. pulchra* than on co-occurring healthy and faded algae (the latter two supporting similar densities) at Long Bay, however, there was no difference in densities of the gastropod found on bleached, faded or healthy algae at Bare Island (but there was a trend for higher densities on bleached algae; Figure 5.6B). Urchins (*H. purpurascens*) were more abundant on bleached than healthy and faded algae at both locations (although this difference was more pronounced at Bare Island than Long Bay) and were significantly more abundant on all algae at Bare Island than Long Bay (Figure 5.6C). *H. purpurascens* density was influenced by algal biomass, with proportionally more urchins found on smaller algae than large individuals (Table 5.3).

A. parvula densities were higher on bleached compared to healthy algae at Bare Island, but there was no difference in densities among bleached and faded algae and no difference between faded and healthy algae (Figure 5.6D). Densities of *A. parvula* were very low at Long Bay and despite a trend for more sea hares on bleached individuals, there was no significant effect of algal condition on density of this herbivore (Figure 5.6D). All herbivores were significantly more abundant on *D. pulchra* individuals growing in shallow water habitats ($F_{1,31}$ = 19.015, p = 0.001) than on deep algae, where no herbivores were found at

all. This pattern was consistent at both locations (Figure 5.7). There were no relationships between healthy algal biomass and herbivore density for total herbivores (R^2 =0.009, *df* =58, p =0.473), or any individual species (*P. eximius*: R^2 =0.017, *df* =58, p =0.331; *H. purpurascens*: R^2 =0.002, *df* =58, p =0.728; *A. parvula*: R^2 =0.033, *df* =58, p =0.168).

Feeding assays: In Assay 1, where herbivores were offered bleached tissue and adjacent healthy looking tissue, *P. eximius* and *H. purpurascens* strongly preferred bleached tissue over adjacent unbleached tissue from the same alga (Figure 5.8A; Table 5.4A). In contrast, *A. parvula* did not show a preference for bleached tissue, although the power of this analysis decreased due to the death of many replicate sea hares during this assay (discussed below) and a non-significant trend for higher consumption of bleached tissue was observed (Figure 5.8A; Table 5.4A). In Assay 2, no herbivores showed any preferences for either faded tissue or adjacent, unfaded tissue (Figure 5.8B; Table 5.4B). Similarly in Assay 3, where herbivores were offered a choice between visibly healthy tissue from a bleached alga and tissue from a completely healthy alga, no herbivores showed any preference for either tissue type (Figure 5.8C; Table 5.4C).

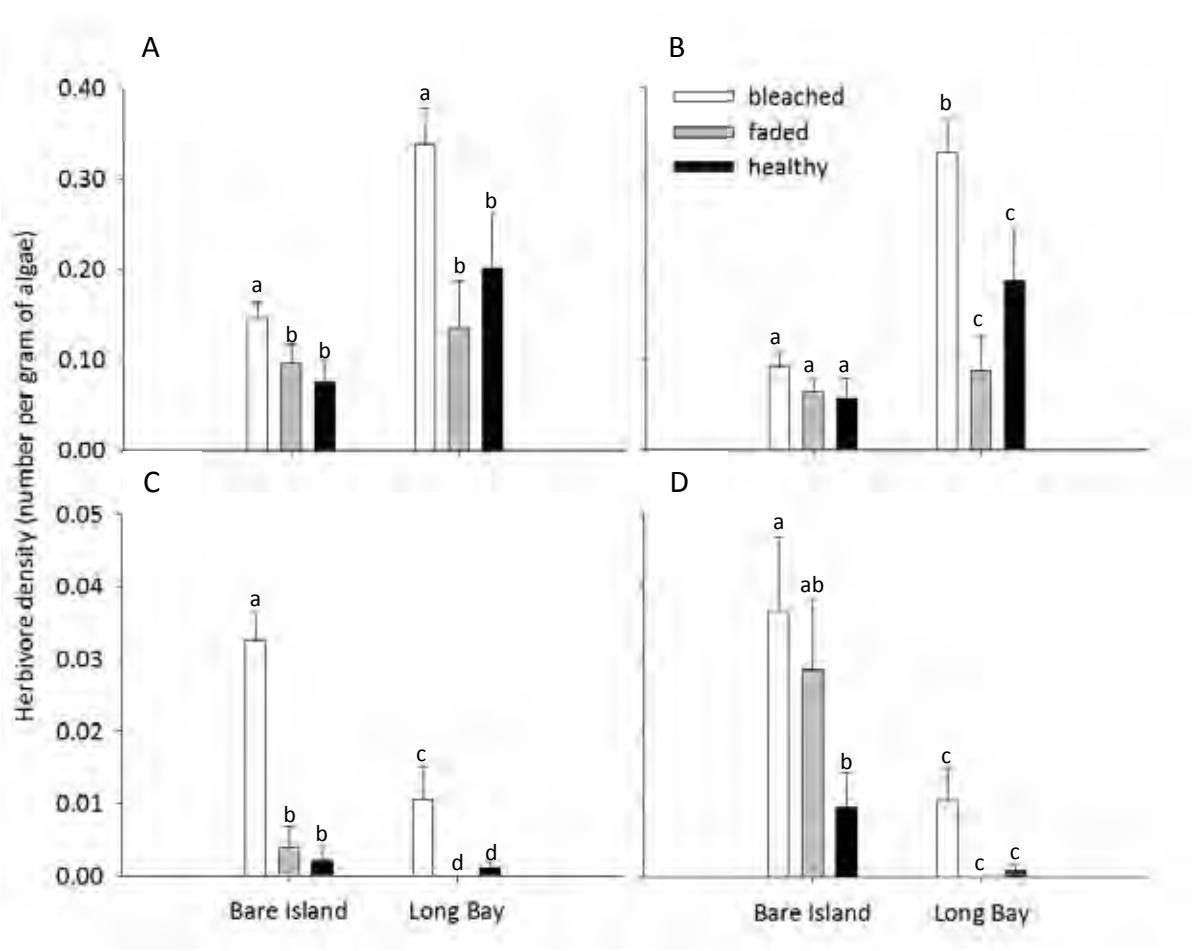

Figure 5.6. Densities (means + SE) of **(A)** 'total' herbivores, **(B)** the gastropod *P. eximius*, **(C)** the urchin *H. purpurascens* and **(D)** the sea hare *A. parvula* on bleached (white bars), faded (light grey bars) and healthy (black bars) *D. pulchra* algae at Bare Island and Long Bay with *n*=30. Shared letters represent statistical similarity according to Tukey's multiple pairwise comparisons tests.

Table 5.3. Analyses of covariance (ANCOVAs) of densities of total herbivores, the urchin *H. purpurascens*, the gastropod *P. eximius* and the sea hare *A. parvula* on bleached, healthy and faded algae from Bare Island and Long Bay with algal mass as the covariate and n = 30. '*' indicates significant effect of relevant factor on herbivore density with α= 0.05.

	total herbivores†				*H. purpurascens*			
Source of variation	*df*	*MS*	*F*	*p*	*df*	*MS*	*F*	*p*
Location (L)	2	0.043	0.831	0.363	1	0.002	10.763	0.001*
Condition (C)	1	0.252	4.905	0.009*	2	0.002	8.438	0.000*
L x C	2	0.029	0.566	0.561	2	0.001	4.079	0.019*
Algal mass	1	0.065	1.272	0.261	1	0.001	6.877	0.010*
Error	164	0.051			164	0.000		

	P. eximius‡				*A. parvula*			
Source of variation	*df*	*MS*	*F*	*p*	*df*	*MS*	*F*	*p*
Location (L)	2	0.215	5.272	0.023*	1	0.044	23.881	0.000*
Condition (C)	1	0.186	4.556	0.012*	2	0.006	3.222	0.042*
L x C	2	0.110	2.692	0.071	2	0.005	2.813	0.063
Algal mass	1	0.017	0.407	0.524	1	0.001	0.512	0.475
Error	164	0.041			164	0.002		

† Data were Arcsine transformed to meet the assumptions of ANOVA

‡ Data were reciprocally transformed to meet the assumptions of ANOVA

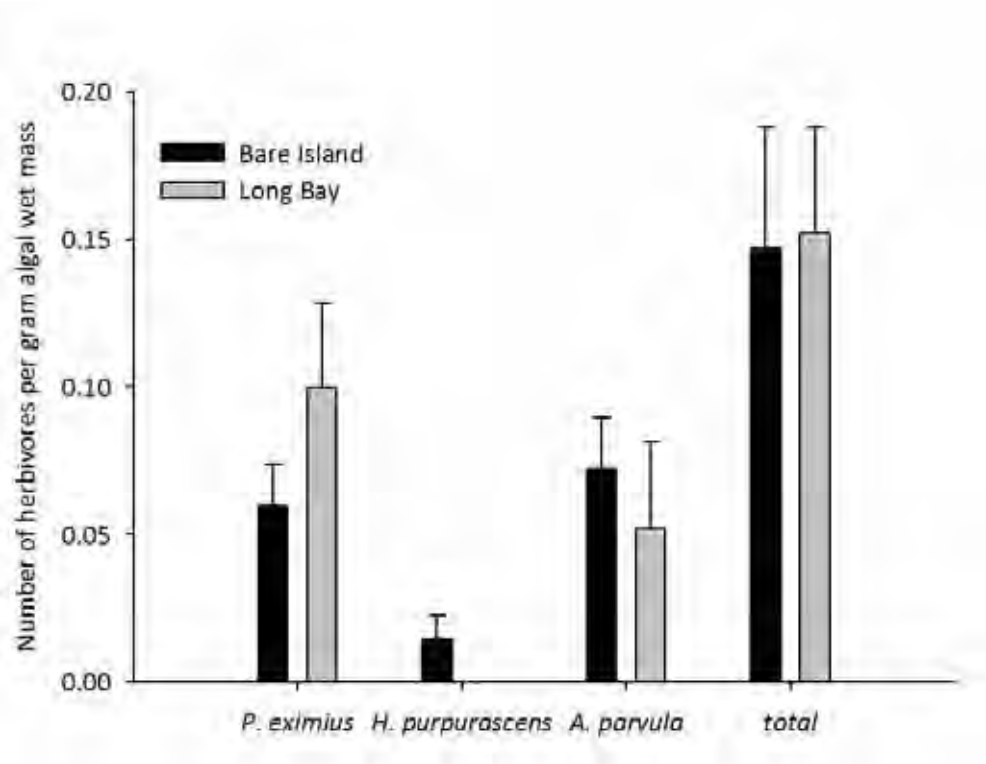

Figure 5.7. Densities of *P. eximius, H. purpurascens, A. parvula* (means + SE) and total herbivores on healthy *D. pulchra* individuals growing in shallow Bare Island (black bars) and Long Bay (grey bars) with *n*=10. No herbivores were found on *n*=10 *D. pulchra* individuals from deep water habitats at either location and so these data were omitted from the graph.

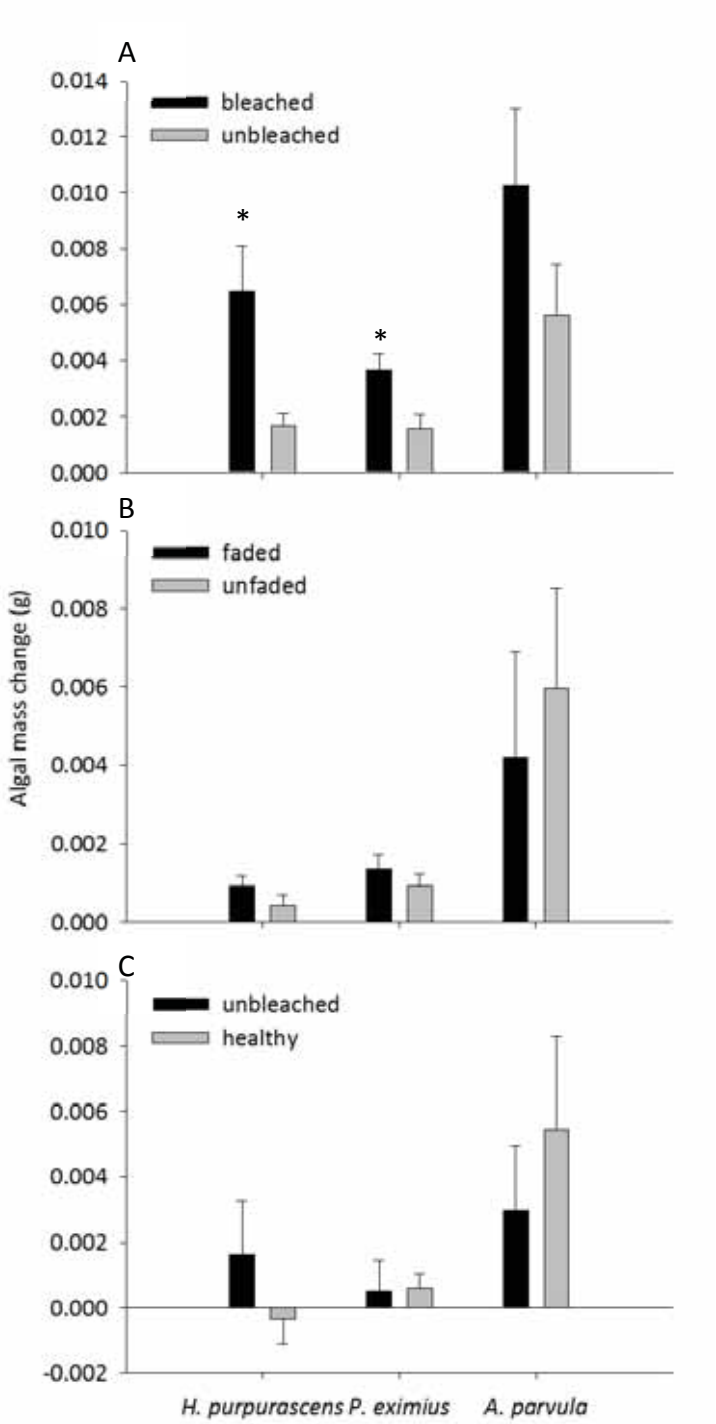

Figure 5.8. Mass changes (means +/- SE) of experimental *D. pulchra* offered to herbivores *H. purpurascens*, *P. eximius* and *A. parvula* in **(A)** Assay 1 (bleached vs. unbleached tissue), **(B)** Assay 2 (faded vs. unfaded tissue) and **(C)** Assay 3 (healthy tissue vs. unbleached tissue from bleached alga) with *n* = 18 for *H. purpurascens* and *P. eximius* and *n*=8 for *A. parvula*. '*' indicates a significant difference in consumption by herbivores between algal tissue types.

Table 5.4. ANOVAs comparing algal mass change during feeding choice experiments **(A)** Assay 1, **(B)** Assay 2 and **(C)** Assay 3, where each experiment was conducted separately using *P. eximius*, *H.s purpurascens* and *A. parvula* and compared to control treatments with no herbivores.'*' indicates a significant effect of algal condition on consumption by herbivores, with α= 0.05.

A

	H. purpurascens				*P. eximius†*				*A. parvula*			
Source of variation	*df*	*MS*	*F*	*p*	*df*	*MS*	*F*	*p*	*df*	*MS*	*F*	*p*
Algal condition	1	$0.205e^{-3}$	8.056	0.007*	1	$0.399e^{-4}$	7.815	0.008*	1	$0.846e^{-4}$	1.960	0.183
Error	33	$0.245e^{-4}$			33	$0.432e^{-4}$			13	$0.432e^{-4}$		

B

	H. purpurascens				*P. eximius†*				*A. parvula*			
Source of variation	*df*	*MS*	*F*	*p*	*df*	*MS*	*F*	*p*	*df*	*MS*	*F*	*p*
Algal condition	1	$0.011e^{-4}$	2.280	0.142	1	0.000	0.830	0.369	1	$0.754e^{-4}$	0.040	0.850
Error	29	$0.009e^{-4}$			31	$0.020e^{-4}$			15	$0.616e^{-4}$		

C

	H. purpurascens				*P. eximius†*				*A. parvula*			
Source of variation	*df*	*MS*	*F*	*p*	*df*	*MS*	*F*	*p*	*df*	*MS*	*F*	*p*
Algal condition	1	$0.001e^{-4}$	0.000	0.291	1	$0.212e^{-4}$	0.010	0.929	1	$1.904e^{-4}$	0.600	0.451
Error	33	$0.304e^{-4}$			33	$0.099e^{-4}$			15	$0.458e^{-4}$		

† Data were square-root transformed to conform to assumptions of ANOVA

DISCUSSION

Bleaching had direct, negative impacts on algal performance, with reduced biomass (estimated by change in algal length) in bleached algae relative to healthy conspecifics. As biomass was strongly related to fecundity in *Delisea pulchra*, this reduction in growth and biomass loss associated with bacterially-mediated bleaching, likely also had negative impacts on algal fecundity, reproduction and fitness. Bleaching had no effect on survivorship of algae from deep water populations, but had an unexpectedly positive effect on survivorship in shallow algae, which I argue may be due to beneficial 'disease pruning'. There were also indirect consequences of algal bleaching: host use by mesoherbivores was higher in bleached individuals relative to healthy conspecifics, and bleached tissue was also preferentially consumed by several locally abundant herbivores. Thus, bacterially-mediated bleaching had significant sub-lethal consequences for *D. pulchra* and also altered its trophic interactions. Given that up to 80% of individuals are affected by bleaching at peak periods, these consequences could have implications at the population scale.

Direct effects of bleaching on survival and performance

Algal biomass (as estimated through changes in algal length, which was strongly correlated with biomass) was significantly lower in bleached *D. pulchra* relative to healthy conspecifics. Reduced biomass could be explained by two mechanisms: lower growth rates in bleached algae and/or thallus breakage. Observations of biomass loss in bleached individuals during this study suggest that breakage of bleached thalli did occur. Either way, smaller size is likely to result in reduced fecundity, because biomass was strongly related to fecundity and breakage would inevitably cause the loss of distal tips, where reproductive structures are

located. Considering this last point and my measure of relative (rather than total) fecundity, it is likely that the possible effects of bleaching on fecundity have been underestimated here. Disease has been linked to reduced fecundity in terrestrial invertebrates (Sait et al. 1994, Myers and Kuken 1995) and plants (Burdon 1987) and although less is understood about how disease affects fecundity in seaweeds, biomass loss has been shown to negatively impact fecundity in another red alga, *Gelidium sesquipedale* (Santos and Duarte 1996) and Giant Kelp in California (Graham 2002).

In addition to negative effects on fecundity, reduced growth or biomass also generally represents lower metabolic rates, with smaller algae performing poorly relative to larger conspecifics within a cohort (Gillooly et al. 2001, Raven and Kubler 2002). Additionally, seaweeds are less differentiated than terrestrial plants and are mostly photosynthetic (Hay 1991). Therefore, biomass loss (and reduction of photosynthetic pigments associated with bleaching; Chapter 4), could lead to decreased photosynthetic activity and/or potential. Considering that photosynthesis by benthic algae accounts for up to 10% of total marine primary production (Charpy-Roubaud and Sourina 1990), and that up to 80% of individuals in some populations of *D. pulchra* can be affected by bleaching during peak periods (Chapter 2), bleaching certainly has the potential to reduce primary production in this seaweed.

The enhanced survivorship observed in bleached individuals in shallow but not deep habitats, was not expected and is unusual in the context host-pathogen interactions. The depth-specificity of this survivorship effect (it was only observed in shallow populations) suggests that there may be characteristics associated with the shallow-water habitat that are absent or reduced at depth, that may interact with algal condition to influence survivorship of bleached individuals. One key difference between deep and shallow habitats

is wave energy, which is much higher in the shallows (Koehl 1986). Therefore, shallow algae may be at greater risk of detachment than conspecifics in deeper water, which has direct survivorship effects because detachment usually means death for macroalgae (Koehl and Wainwright 1977). Since bleached algae were consistently smaller than healthy conspecifics, this reduction in size may have conferred some benefit with respect to detachment risk in shallow water habitats exposed to relatively high energy environments. Indeed, smaller algae are less likely to detach due to waves and storms than larger conspecifics (Carrington 1990, Gaylord et al. 1994, Santos 1994, Blanchette 1997) and beneficial 'pruning' of marine macrophytes by herbivores has been demonstrated to reduce detachment risk by reducing algal size in other species (e.g. Black 1976). Analogous 'disease pruning' may be occurring in this system and influencing survivorship of bleached thalli by lowering detachment risk.

This apparently positive effect of bleaching on survivorship was only observed in shallow populations: there was no effect of bleaching on survivorship of algae in deeper waters. Certainly, I did not detect any negative impacts of bleaching on the survival of *D. pulchra* individuals in this study, suggesting that this seaweed can tolerate some bleaching. Host organisms can display multiple mechanisms to resist, recover from or tolerate diseases (Medel 2001, Kover and Schaal 2002, Boots 2008). *D. pulchra* resists bacterial bleaching by producing furanones (Chapter 2). The production of furanones is heritable and potentially selected for by herbivores (Wright et al. 2004) and possibly also by pathogens, but they are metabolically costly to produce (Dworjanyn et al. 2006b). As such, the lack of negative effects of bleaching on survivorship in *D. pulchra* suggests that this macroalga may have multiple mechanisms in place, initially to resist bleaching pathogens, and later, to tolerate

bleaching when it does occur, but suffers significant performance (and possibly also fitness) costs as a consequence.

Indirect effects of bleaching and fading on algal-herbivore interactions

Herbivory generally has negative outcomes for the plants and algae being consumed (although it can sometimes benefit a plant; e.g. Black 1976, Brawley and Adey 1981, Ruesink 2000). To avoid being eaten or too heavily colonised, many plants and algae have chemical, physical and/or phenological adaptations to avoid, tolerate or recover from herbivory (Hay and Fenical 1988, Simms 1992, Agrawal 2007). *D. pulchra*'s furanones deter many herbivores (Williamson et al. 2004, Wright et al. 2004) so depletion of these compounds in bleached and faded algae (Chapters 2 and 3) may increase the susceptibility of these individuals to consumption or alter their attractiveness as hosts. My observations of increased host-use of bleached algae and enhanced consumption of bleached tissue support this model of chemically-mediated interactions between *D. pulchra* and associated herbivores. They also provide a novel example of a marine tripartite or tri-trophic interaction, where infection by pathogens can alter a habitat-forming seaweed's interactions with herbivores that live upon and consume it.

There were some interesting species-specific responses to bleached individuals and tissues by the herbivores in this study. Colonisation by *Phasianotrochus eximius* and *Aplysia parvula* only appeared affected by algal condition at one of two locations (although the other location typically showed a non-significant trend for increased host use on bleached individuals), whereas the effect of algal condition on the colonisation of *Holopneustes purpurascens* was consistent among locations. Interestingly, at one location, *A. parvula* was more abundant on bleached and faded algae than healthy conspecifics, potentially

suggesting that this herbivore, relatively tolerant to *D. pulchra*'s halogenated furanones (Rogers et al. 2002), was attracted to less chemically-defended hosts (Chapters 2 and 3).

Consumption of *D. pulchra* by *H. purpurascens* is limited by furanones (Williamson et al. 2004), so its preference for less chemically defended, bleached tissue during the feeding assays is perhaps not surprising. Conversely, the preference of *P. eximius* for bleached tissue is more surprising, since it is one of the most widespread herbivores associated with *D. pulchra* (Wright et al. 2000) and is generally tolerant of furanones, even feeding preferentially on the tips of the thallus (Wright et al. 2004) where furanone concentrations are highest (Dworjanyn et al. 1999). Potentially, like *A. parvula* (Rogers et al. 2002), *P. eximius* tolerates furanones but performs better when it avoids them. Alternatively, other chemical or physiological changes associated with bleaching may influence the gastropod's feeding preferences. Although I did not detect a preference for any tissue types in *A. parvula*, there was a non-significant trend for higher consumption of bleached relative to healthy tissue, consistent with the other herbivores. Unfortunately, a high proportion of replicate sea hares died during the feeding trials, presumably due to sub-optimal conditions in aquaria, resulting in low statistical power and a limited ability to draw conclusions about this herbivore and whether its consumption of *D. pulchra* is affected by bleaching. This experiment should be repeated in a flowing seawater system where survival should be higher.

When considered collectively, increased herbivory and colonisation of bleached *D. pulchra* support the 'plant-stress hypothesis' (White 1969, 2009) which predicts that stressed plants will be preferentially occupied and consumed over healthy conspecifics. Haggitt and Babcock (2003) observed a similar phenomenon when a die-back affected large stands of

the kelp *Ecklonia radiata* in New Zealand. Herbivorous amphipods grazed preferentially on "bleached" kelp tissue, which had relatively low levels of phlorotannins. In this case, although disease was suggested as a potential cause of the die-back (Cole and Babcock 1996), it was not confirmed. Little else is known about how diseases of marine macrophytes may impact herbivory, and further investigations are needed as oceans continue to warm and disease incidence increases (Harvell et al. 2002).

In all surveys, herbivores were only found in shallow waters (none were collected on deep plants), an observation that is consistent with previous studies showing that grazing pressure often decreases with depth (e.g. Hay et al. 1983, Korpinen et al. 2007, Brokovich et al. 2010). This is interesting with respect to spatial variation in patterns of bleaching in *D. pulchra* (Chapter 2), as herbivores appear to be more abundant where bleaching is most prevalent. As well as suggesting that herbivores were attracted to less chemically-defended food sources, this observation also raises the possibility that these animals may be involved in the infection process. Consumers can act as transmission vectors or pathogen reservoirs for infectious diseases (e.g. Sussman et al. 2003) and damage inflicted by herbivores can facilitate disease by providing infection sites (Borer et al. 2009). Indeed, an entire suite of plant defences have evolved to manage herbivory wounds (Ross et al. 2006, Ruuhola and Yang 2006) and this would be an interesting avenue for future research into the bacterial bleaching of *D. pulchra*.

Algal fading, which is caused by exposure to excessive solar radiation and results in depleted furanones and photosynthetic pigmentation (Chapter 4), did not appear to alter herbivore host use or feeding preferences in *D. pulchra*, except for *A. parvula* at Long Bay, although there was a consistent (non-significant) trend for higher densities of herbivores on faded

algae, relative to healthy individuals. Similarly, herbivores showed no preference between adjacent unbleached tissue from bleached individuals and healthy tissue from healthy individuals. This is interesting with respect to the 'whole thallus' effect of bleaching on furanone concentrations observed in Chapter 2 and suggests that herbivore preferences for bleached tissue may be related specifically to chemical or mechanical changes that occur within infected parts of the algae as visible bleaching occurs. Increased herbivory appears to be limited to the visibly bleached portion of affected thalli.

Conclusions

In this study, I provided experimental evidence that bleaching has consistent, negative sub-lethal consequences for *D. pulchra*, by reducing biomass and fecundity with potential performance and fitness consequences. Nevertheless, bleaching had inconsistent but positive effects on survival in shallow algae potentially suggesting a trade-off between survival and fecundity. This provides evidence that *D. pulchra* may tolerate bleaching, but with performance and potentially also fitness consequences. Bleaching also had important indirect effects on *D. pulchra*, by altering its trophic interactions with locally abundant herbivores: bleached algae were more likely to be used as hosts and bleached tissue was more likely to be consumed. As the incidence and severity of diseases in natural systems continues to increase, understanding how they are likely to affect habitat-forming organisms like macroalgae and their important interactions with other trophic levels in ecosystems is crucial for the study and managements of natural systems under stress.

CHAPTER 6

General Discussion

In this thesis, I studied a bleaching phenomenon that affects a chemically defended, habitat-forming seaweed, *Delisea pulchra*. Specifically, I assessed the relative importance of environmental stressors, algal chemistry and microbial pathogens in *D. pulchra*'s bleaching. I demonstrated a strong relationship between the prevalence of bleaching, high water temperatures and depleted levels of defensive metabolites (furanones). Often, bleaching in algae is attributed to direct abiotic stress (e.g. solar radiation). In this case, however, I presented experimental evidence that bacterial pathogens are involved in bleaching of *D. pulchra*. I tested this specifically for one bacterium (*Ruegeria* sp. R11), in novel *in situ* inoculation experiments, and demonstrated that this pathogen could cause bleaching in established *D. pulchra* that had also been experimentally damaged. Exposure to excessive solar radiation had an indirect influence of bleaching in *D. pulchra*, by stressing algae and causing a precursor condition ('fading') in which furanones and photosynthetic pigments were reduced. Shade from experimental manipulations and co-occurring kelps protected *D. pulchra* from this stressor and decreased its susceptibility to pathogens. Bleaching had significant, sub-lethal performance consequences for *D. pulchra* and also altered its

functional role within the ecosystem as a habitat-former, and increased its susceptibility to consumption by locally abundant herbivores.

Is bleaching in D. pulchra an environmentally-mediated disease?

Bleaching in *D. pulchra* was induced via specific experimental manipulations of bacterial pathogens and in natural populations of the seaweed (Chapters 2 and 3), was associated with clear and consistent shifts in microbial biofilm communities (Chapter 2). Surface-associated microbial communities (SAMCs) can shift in response to changes that occur within their niche (i.e. the surface on which they live; Thompson et al. 1993). In general, disease has been associated with shifts in the composition of host surface-biofilm communities. For example, dental plaque biofilms adjacent to disease sites are different from those in healthy areas (e.g. Marsh 2003). Similarly, corals in the Caribbean experience significant SAMC shifts during and after infection by black band (Frias-Lopez et al. 2002) and white plague-like (Pantos et al. 2003) diseases. In the latter example, SAMC shifts were observed 'colony-wide', before visible bleaching had affected all coral tissues.

In *D. pulchra* the visibly unbleached 'adjacent' sections of bleached thalli supported a 'transition' microbial community, which were not significantly different from SAMCs on bleached tissue or on healthy, co-occurring individuals (but communities from bleached and healthy tissues were distinct from each other), suggesting that the microbial community on *D. pulchra*, shifted in response to some physiological change(s) within the algal host. Bleached algae also had lower furanone concentrations across whole thalli, relative to healthy conspecifics, so the microbial communities may have responded to this reduction in chemical defences. Furanones interfere with bacterial quorum sensing (Manefield et al. 2002), so a depletion of these compounds would likely affect which bacteria could colonise

D. pulchra's surface. Certainly, experimental manipulation of furanones had consistently strong implications for *D. pulchra*'s bleaching, especially when undefended algae were exposed to bacteria at high temperatures (Chapters 2 and 3). Concentrations of halogenated furanones were consistently and negatively correlated with water temperature, and exposure to solar radiation caused algal fading, a possible precursor condition to bleaching, in which chemical defences were depleted. The clear protective role of furanones in this bleaching phenomenon provides further evidence that bleaching is the result of bacterial infection(s).

Physical defences against disease also appear to be important. In Chapter 3, I demonstrated that R11 required physical damage to algae for infection and bleaching to occur. This was also observed, to a lesser degree in Chapter 4, when transplanted algae in shallow waters also bleached adjacent to the parts of thalli that were attached to nails via cable-ties. This was likely due to unintended damage inflicted to the algae during attachment (and was not observed in unmanipulated individuals exposed to the same environmental conditions). Furthermore, herbivores were more abundant and fed preferentially on bleached algae (Chapter 5). Together, these observations suggest that physical damage (caused by herbivores or other disturbances) could facilitate infection by pathogens and increase the risk of bleaching in *D. pulchra*. Herbivory wounds have been implicated in disease (e.g. Borer et al. 2009) and damage inflicted to trees by other disturbances (e.g. acid rain and frost) increases their susceptibility to infection by fungal pathogens (Klein and Perkins 1987). In terms of consumer effects, the marine fireworm, *Hermodice carunculata*, was demonstrated to transmit bacterial pathogens between coral colonies and provide a winter refuge habitat

for the bleaching pathogens, which were not observed on corals during those times (Sussman et al. 2003).

The importance of physical and chemical defences in the prevention of bleaching in *D. pulchra* emerged clearly in this study. When algae were chemically undefended, the removal of bacteria from growth media or the disturbance of algal surface biofilm communities with antibiotics led to a reduction in the severity of bleaching in *D. pulchra* sporelings (Chapters 2 and 3). This provides further evidence that bleaching in *D. pulchra* is bacterially-mediated. Whilst it is not possible to completely remove bacteria from algal surfaces (Huggett et al. 2006, Paul et al. 2006, Longford 2008), treating sporelings with antibiotics reduced the prevalence of bleaching in *D. pulchra*. This highlights that, in these experiments, true control treatments (with no bacteria) are not possible, and that residual bacterial can also cause bleaching in *D. pulchra*.

The pathogenic bacterium R11 caused bleaching in established algae, but only when the algae had also been experimentally damaged (Chapter 3). The role of R11 as a pathogen for *D. pulchra* was less clear in laboratory experiments, in which residual bacteria were not removed with antibiotics appeared to cause bleaching to a similar extent as R11. This result was not expected and did not conform to previous work on this algal pathosystem (Case et al. 2011). Therefore, this study adds complexity to the picture of R11 as a pathogen of *D. pulchra*. Additionally, these results suggest that the ability of bacteria to infect and bleach *D. pulchra* may be quite a general phenomenon, rather than a specific host-pathogen system, and this concept is becoming more widespread in disciplines concerned with pathology.

This point is well illustrated by recent research into dental caries, which are a widespread and costly disease in much of the developed world. Until relatively recently, most cases of

this disease were attributed to the bacterial pathogens *Lactobacillus acidophilus* and *Streptococcus mutans*. However, it's becoming more accepted within this field that dental caries are caused by pathogenic microbial consortia in dental plaque, rather than those specific pathogens (Kleinberg 2002, Marsh 2003).

Similarly, pathogenic microbial consortia, rather than specific pathogens are often implicated in coral bleaching diseases (e.g. Cooney et al. 2002). In the coral disease literature, researchers have suggested that, in response to rapidly changing environmental conditions, corals can adapt by altering the composition of their microbial symbionts to play a protective role against pathogenic organisms (Reshef et al. 2006). Corals have very specific and diverse microbial flora associated with their tissues and mucous layer and these can change seasonally (e.g. Oculina patagonica in the Red Sea; Koren and Rosenberg 2006). By proposing the 'coral probiotic hypothesis', Reshef et al. (2006) suggest that corals can alter their mucous-associated microbial communities to adapt to environmental change and develop resistance to pathogens.

The generality of algal bleaching suggested by observations in Chapters 2 and 3, and the lack of a consistent influence of R11 on algal bleaching (Chapter 3), suggests that bleaching in *D. pulchra* may also be the result of opportunistic, pathogenic, microbial communities, rather than a specific pathogen. Based on the evidence presented in this thesis, I conclude that bleaching in *D. pulchra* is the result of bacterial infections, which are facilitated by high water temperatures and depleted algal chemistry. I suggest that abiotic factors cause a reduction in *D. pulchra*'s furanones, thereby increasing algal susceptibility to multiple, non-specific, opportunistic pathogens which may also become virulent under the prevailing environmental conditions.

Climate change and disease

If bleaching in *D. pulchra* is the result of non-specific bacterial infections that are more common at higher temperatures when algal chemical defences are depleted, this suggests that temperature-regulated virulence is a common trait in bacteria (Konkel and Tilly 2000, Klinkert and Narberhaus 2009). This is an alarming thought, as ocean temperatures have already experienced warming over the last century of 0.1 ^{o}C globally (IPCC 2007) and of approximately 2 ^{o}C in south-eastern Australia, where these studies were carried out (Cai et al. 2007, Poloczanska et al. 2007). Bleaching was more common and extensive in *D. pulchra* when water temperatures were high, both in natural populations and experimental manipulations (Chapters 2 and 3). Evidence presented in this thesis suggests that *D. pulchra*'s production of furanones is depleted when water temperatures are high (Chapters 2 and 3).

Throughout this thesis, I presented data which demonstrated that the shallow water environment at these locations was more stressful for *D. pulchra* than deeper water habitats, with respect to water temperatures (Chapter 2), solar radiation (Chapter 4), wave energy and herbivore densities (Chapter 5). Bleaching in this habitat was also more prevalent than at depth and these multiple biotic and abiotic stressors may have combined to affect *D. pulchra's* susceptibility to bleaching pathogens, potentially by reducing concentrations of halogenated furanones in algal tissues. These factors may have also influenced the density, transmission and virulence of pathogens as well. The environment can strongly influence microorganisms to the extent that sometimes, seemingly innocuous microorganisms can become pathogenic under certain conditions (e.g. Vergeer and den Hartog 1994). As climates continue to warm and change, gaining a better understanding of

how the production of chemical defences and the important processes they mediate might change in response, is crucial.

Implications for individuals, populations and ecosystems in a rapidly changing world

Bleaching had significant, sub-lethal performance consequences for affected *D. pulchra*, which were smaller and likely less fecund than healthy conspecifics, but there were no consistent or negative impacts of bleaching on algal survival (Chapter 5). This suggests that *D. pulchra* can tolerate bleaching, but does so with serious performance (and probably also fitness) costs. *D. pulchra*'s roles as a habitat-former and food for locally abundant macroherbivores were also altered by bleaching. Macroalgae are important habitat-formers that provide a myriad of bottom-up ecosystem services (Steneck et al. 2002). It is thus important to consider the implications of negative impacts on macroalgae in the context of the communities they support. Worldwide, stands of larger macroalgae and kelps are in decline (Cole and Babcock 1996, Thibaut et al. 2005, Edwards and Estes 2006, Coleman et al. 2008, Connell et al. 2008). These declines are usually linked to climate anomalies (Cole and Babcock 1996) and/or the destructive effects of urbanisation (Thibaut et al. 2005, Airoldi and Beck 2007). Often, disease is suggested as a possible cause of declines (Cole and Babcock 1996, Coleman et al. 2008) but this is rarely confirmed experimentally. This body of work thus provides a novel example of climate-mediated seaweed-bacteria interactions that have significant consequences for the host, an important habitat-forming organism.

Hosts and pathogens both face fitness trade-offs during disease progression, and these are widely varied and debated in the field of evolutionary epidemiology (e.g. May and Anderson 1983, Galvani 2003). Regardless of how virulent a particular pathogen may be to a particular host, as a group, pathogens exert enormous selective pressures on host organisms, and are

widely accepted to influence the morphology, life history, reproductive strategy and genetic diversity of host populations (Clay and van der Putten 1999, Kareiva 1999). Considering the importance of these fundamental relationships, any alterations via anthropogenic climate change or other disturbances could have profound and far-reaching consequences for organisms and communities (discussed by Kareiva 1999). Understanding the mechanisms underlying complex interactions between environmental change, host defences and pathogen virulence, is essential for the study and conservation of important organisms and ecosystems in an increasingly stressful world.

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