THE CHEMICAL ECOLOGY OF THE SOFT CORAL - ZOOXANTHELLAE ASSOCIATION AND ITS SIGNIFICANCE TO THE BLEACHING PROCESS

Thesis submitted by
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THESIS DEDICATION

This thesis is dedicated to the loving memory of my great-grandmother Josefina and to my husband Andreas - the most wonderful man alive!

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ABSTRACT

Biochemical changes in soft corals (*Lobophytum compactum* and *Sinularia flexibilis*) affected by bleaching were investigated, with the ultimate aim of understanding how molecular changes are linked to biological responses observed in the wake of bleaching events.

Annual variation in photo-protective mycosporine-like amino acids (MAAs) in soft coral populations provides the first evidence that MAA tissue concentrations are positively correlated with seasonal cycles in both solar irradiance and sea-surface temperatures. The timing of peak concentrations of MAAs in summer when exposure to solar irradiance and sea temperatures are greatest, and in female colonies prior to spawning, further corroborate their role as photo-protectants. In manipulative bleaching experiments elevated UVR and temperature were found to act synergistically in the bleaching process. However, chemical degradation of MAAs during exposure to elevated temperature is not, as previously assumed, the key to understanding the synergistic interaction. In fact, MAA levels increased in response to simultaneous exposure to high temperature and UVR, in both experimentally and naturally bleached colonies, suggesting increased resource allocation towards photo-protection in corals already experiencing thermal stress.

Soft coral responded to bleaching by increasing the production of terpenoid secondary metabolites, which aid in the prevention of microbial and algal fouling. While changes in secondary metabolites were short-lived, a substantial increase of an anti-microbial agent in *S. flexibilis* may have contributed to the prevention of fouling by opportunistic bacteria. This suggests that soft corals may alter their secondary metabolite chemistry to prevent fouling by opportunistic bacteria following bleaching. These experimental results were validated through analysis of soft corals affected by the 1998 mass bleaching event, where bleached colonies with high levels of algaecides remained free of fouling, while conspecifics with substantially lower levels were found to be overgrown. This suggests that soft corals are capable of surviving short-term bleaching events and detrimental algal overgrowth that is often associated with bleaching, by regulating their secondary metabolite chemistry to counteract fouling.

Understanding the role of symbiotic zooxanthellae in the production of terpenoid secondary metabolites is integral to evaluating the full impact of bleaching disturbances on the soft coral host. Zooxanthella cross-infection experiments with freshly metamorphosed polyps of *Lobophytum compactum* demonstrated that control over the production of secondary metabolite lies with the animal host. Moreover, the equivalence of secondary metabolite chemistry in apo-

and symbiotic polyps clearly shows that the algal partner is not essential for biosynthesis. Despite no direct algal involvement in terpene production, a strong correlation between polyp growth and investment into terpenes suggests that, via their contribution to coral nutrition through primary metabolism, zooxanthellae have the capacity to indirectly influence secondary metabolism. The implications for bleached soft corals are that while the host, with or without energetic contributions from the algal symbiont controls the production of ecologically important terpenes, energy reserves may be insufficient to maintain the production of ecologically significant concentrations.

Severe experimental bleaching was found to have long-term sub-lethal impacts on soft corals, reducing overall reproductive output of *Lobophytum compactum* for at least two spawning seasons. Polyp fecundity and mean egg diameters were inversely correlated with the degree of bleaching, with complete failure of fertilisation in heavily bleached colonies in the first year and significantly reduced fecundity in the second year after the bleaching event. Although bleached corals recovered their zooxanthellae within 4 months, protein, lipid, MAA and carotenoid concentrations were reduced for at least eight months in adult tissues. The reductions were amplified when they were passed on to gametes, with the greatest reductions occurring in lipid and protein concentrations. Although reductions in MAAs were relatively smaller when passed on to gametes, even minor proportional reductions have significant implications for larval survival, given that MAA levels are approximately three times higher in eggs than in maternal tissues. By the second spawning season (20 months after experimental bleaching) the biochemical compositions of both adult tissues and their gametes were indistinguishable from those of control (unbleached) corals.

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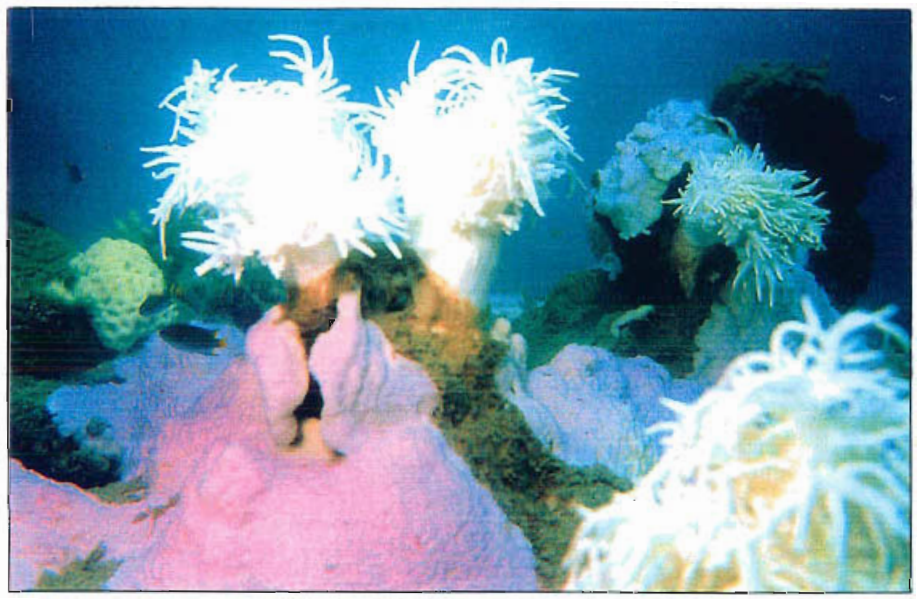
TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

General Introduction



Sinularia flexibilis during a natural bleaching event at Orpheus Island, March 1998

1. GENERAL INTRODUCTION

1.1. Background and significance of coral bleaching

The focal point of this thesis is the disruption of the symbiotic relationship between soft coral hosts and their dinoflagellate algal partner due to stress, a phenomenon known as coral bleaching. The animal–plant relationship, which evolved in scleractinian corals during the Triassic, is believed to have greatly contributed to the radiation and evolutionary success of today's tropical corals (Stanley, 1981; Stanley and Swart, 1995). Their symbiotic success is primarily based on provision of photosynthetically-derived carbon to the animal host (Cook, 1983) and efficient uptake and recycling of nitrogen and phosphorus in the association (Miller and Yellowlees, 1989; Sutton and Hoegh-Guldberg, 1990; Wang and Douglas, 1998), particularly in highly oligotrophic systems. Bleaching, which can occur via loss of zooxanthellae from the association and/or the degradation of photosynthetic pigments within the algal partner (Hoegh-Guldberg and Smith, 1989a), therefore has the potential to compromise the fitness and survival of corals.

Coral bleaching has been described for decades and is a well-established disturbance on reefs (Yonge and Nicholls, 1931; Goreau et al., 1964). Before the 1980s, however, bleaching episodes were generally confined to small areas that had been subjected to identifiable local stresses, such as heavy rainfalls or river discharges which lowered salinity (Yonge and Nicholls, 1931; Goreau et al. 1964). Sudden temperature drops due to upwelling phenomena (Glynn and Steward, 1973), sedimentation, aerial exposure due to low tides and resulting long exposure to high irradiances or contact with pollutants are also known to lead to coral bleaching (reviewed in Williams and Bunkley-Williams, 1990; Brown, 1997a; Hoegh-Guldberg 1999). During the past two decades, however, acute and chronic bleaching of tropical corals has occurred on both dramatically increased scale and intensity and these have predominantly been correlated with high sea surface temperature anomalies (Brown and Ogden, 1993; Gleason, 1993; Hoegh-Guldberg and Salvat, 1995; Brown et al., 1996; Jones et al., 1997; Berkelmans and Oliver, 1999). Among the recognised multiple biological effects of bleaching are reduced coral growth, calcification, and possibly reduced reproductive output and increased mortality (Clausen and Roth, 1975; Szmant and Gassman, 1990; Leder et al., 1991; Goreau and Macfarlane, 1990). Ecological consequences of bleaching include shifts in community structure and decreases in both species and habitat diversity (Fisk and Done, 1985; Gleason, 1996; Glynn, 1996; Marshall and Baird, 1999). Until 1998, the most severe and extensive (10 000 km^2) bleaching event recorded occurred in the Eastern Pacific during 1982-83, where 51-97% of coral cover between Panama and the Galapagos Islands was lost (Glynn 1988) and two

species almost became locally extinct (Glynn and de Weerdt, 1991; Glynn and Feingold, 1992). In 1998, however, mass coral bleaching was recorded on an even greater scale throughout the Pacific region, the Indian Ocean, the Red Sea, the Persian Gulf, the Mediterranean and the Caribbean region (reviewed in Hoegh-Guldberg, 1999). Along the 1500 km Great Barrier Reef (GBR), 87 % of inshore reefs were affected by this disturbance (Berkelmans and Oliver, 1999). This increase in scale and severity of bleaching events is accompanied by evidence for increased frequency of such events (Jones et al., 1997) and has thus led to suggestions that coral bleaching could be a manifestation of global warming (Williams and Bunkley-Williams, 1990; Hoegh-Guldberg, 1999).

1.2. Causal factors and interactions of elevated solar irradiance and temperature in the bleaching process

The physiological basis for speculations that corals are possible indicators of global climate change is that they live precariously close to their upper thermal limits (Coles and Jokiel, 1977; Jokiel and Coles, 1990; Berkelmans and Willis, 1999). Consequently, increases of 1 - 2° C in the tropical belt, as predicted by a number of ocean-atmospheric models (Manabe et al., 1991; Bijlsma et al., 1995) could exceed the thermal tolerance limits of some species. More importantly, under natural conditions an organism is exposed to a whole framework of physico-chemical (stress) factors and it is their combined impact rather than the effects of a single factor, which define the limits for survival. Given that coral reef environments are typically characterised as having oligotrophic, clear waters in which high ambient solar radiation can penetrate to considerable depth, high irradiance has been identified as an important selective pressure in the development of symbiotic associations (Jerlov, 1950; Jokiel and York, 1982; Fleischman, 1989; reviewed in Shick et al., 1996). There is a great deal of experimental evidence supporting the notion that high visible and ultraviolet light are additional triggers (Jokiel and York, 1982; Gleason and Wellington, 1993; Jones et al., 1998), which greatly intensify the bleaching response when occurring simultaneously with elevated temperatures (Lesser et al., 1990; Salih et al., 1998; Jones et al., 1998). Explanations for the nature and the molecular level at which an interaction of the two factors occurs, however, are only just emerging. A model providing a fundamental basis for understanding the interaction of high temperature and light in the photosynthetically active region has recently been provided by Jones et al. (1998). The authors demonstrated that increases in temperature in *Stylophora pistillata* result in a decreased capacity to process the excitation energy coming from the dark reactions of photosynthesis as a primary effect. Subsequently, zooxanthellae become more sensitive to light, resulting in an over-reduction of biochemical components of the light

reactions, which eventually leads to destruction of chloroplasts and photoinhibition (Jones et al., 1998). Subsequent discarding of thermally damaged and dysfunctional zooxanthellae from the coral association (Suharsono et al., 1993) therefore presents an explanation for bleaching. An alternative hypothesis suggests oxidative stress caused by the algal partner to be the proximal cause of bleaching (Kühl et al., 1995; Lesser 1997). Loss of zooxanthellae in this case has consequently been interpreted as a "damage limiting" response, attained by decreasing concentrations of the algal partner and consequently reactive oxygen species in the host tissue.

While the pioneering work by Jones and co-workers (1998) provides one basis for understanding the interactive effects of light and temperature, namely that photoinhibition is a function of thermal stress affecting the functioning of chloroplasts, interactions between these two factors on other molecular levels cannot be ruled out. The thermal degradation of mycosporine-like amino acids (MAAs), which constitute part of the coral's defence system against high irradiance and particularly UVR, may also be involved in such interactions (Lesser et al., 1990; Glynn et al., 1992).

1.3. MAAs and mechanisms of biochemical defence against high irradiance

Given their evolutionary exposure to high solar irradiance over evolutionary time scales, it is not surprising that reef-dwelling organisms have evolved a range of biochemical defence systems, such as MAAs, to counteract damage caused by exposure to high irradiance in the PAR (photosynthetically active region, 400-700 nm) and UV region (280-400 nm) (reviewed in Dunlap and Shick, 1998). Amongst the recognised multiple effects of UVR are damage to DNA and proteins, inactivation of enzymes and oxidation of lipids (reviewed in Shick et al. 1996). The underlying biochemical mechanism of damage caused by UV-B radiation (290-320 nm) is the absorption of the long wavelength frequencies by DNA, and subsequently UV-B is largely responsible for the direct damaging effects of sunlight. Many other cellular components, however, also absorb in the UV-A region (320-360 nm) and enter an excited state wherein the excitation energy can be transferred to molecular oxygen, causing *indirect* oxidative damage by the photodynamic production of reactive oxygen species, such as singlet oxygen ($^1O_2$) or the superoxide anion (O_2^-) (Booth et al., 1997). One of the strategies to reduce photodamage is the production of water-soluble (ascorbate gluthathione, urate) and lipid-soluble anti-oxidants (β-carotene, α-tocopherol), while another is the expression of anti-oxidant enzymes such as catalase and superoxide-dismutase (Lesser et al., 1990; Dunlap and Shick, 1998).

As another "line of defence" in response to extremely high UV exposure, many shallow-water marine organisms have evolved a group of unusual amino-acids, collectively called mycosporine-like amino acids (MAAs). These are a generic class of water-soluble cyclohexenone or cyclohexenimine chromophores conjugated with the nitrogen substituent of an amino acid or amino alcohol (Favre-Bovin et al., 1976), which have received considerable attention for their high absorption maxima ranging from 310 to 360 nm (reviewed in Dunlap and Shick, 1998). Depending on the amino acid that the chromophore is conjugated with, the MAA absorbs radiation at a slightly different wavelength, and thus the whole class of MAAs each with a slightly different absorption maximum, acts as a broadband UVR filter. In addition to UV absorbing properties, mycosporine-glycine, the simplest of MAAs, also features anti-oxidative properties, thus reducing oxidative stress by two modes, absorbance of radiation and scavenging of reactive oxygen species (Dunlap and Yamamoto, 1995). MAAs occur in phylogenetically diverse groups, including marine bacteria (Arai et al., 1993), cyanobacteria (Garcia-Pichel et al., 1993), phytoplankton (Carreto et al., 1990), macro-algae (Karentz et al., 1991; Karsten et al., 1998), molluscs (Ishikura et al., 1997; Carefoot et al., 1998), anthozoans (Dunlap et al., 1986; Stochaij et al., 1994; Teai et al., 1997) echinoderms (Adams and Shick 1996), and fish (Dunlap et al., 1989) and range geographically from tropical to Antarctic waters (Karentz et al., 1991; Shick et al., 1992).

The UV-protecting function of MAAs has been inferred from the fact that shallow water corals typically have higher MAA tissue concentrations than their deeper conspecifics, and because the compounds are light inducible (Jokiel and York, 1982; Dunlap et al., 1986; Gleason, 1993; Shick et al., 1995; Kuffner et al., 1995). The fact that a diverse range of marine benthic organisms provide their planktonic larvae with high concentrations of MAAs further corroborates their important role as photo-protectants (Chiocara, 1986; Adams and Shick, 1996; Carefoot et al., 1998). Direct evidence for their "sunscreen" role has been provided by Garcia-Pichel and co-workers (1993) who showed that the presence of MAAs in the cyanobacterium *Gleocaspa* prevented 23-30% of UV radiation at 320 nm from reaching cellular targets. MAAs are produced via the shikimic acid pathway in both terrestrial organisms (Favre-Bovin, 1976) and corals (Shick pers. comm.). Given that the shikimic acid pathway is thought to be found only in bacteria, fungi and algae (Bently, 1996), symbiotic zooxanthellae are probably the producers of MAAs in corals. Consequently, rates of MAA synthesis in bleached corals should be lower than in their non-bleached counterparts, potentially increasing damage due to high irradiance under conditions of thermal stress. To date there have been no systematic studies undertaken, however, in which MAA concentrations in bleached corals have been compared to those of their unbleached counterparts. Suggestions by Lesser et al. (1990) and Glynn et al. (1992) that MAAs may be thermolabile, raises the possibility that MAAs are the site at which

potential molecular interactions between high irradiance and temperature take place. Thermal degradation of MAAs under an increased temperature scenario would expose a coral to additional irradiance stress when it is already experiencing thermal stress and thus could serve as a model to explain the greatly accelerated bleaching response observed when the two stress factors come together. The implications of decreased protection against irradiance stress due to reduced MAA levels could be grave, particularly given the increased light-sensitivity of zooxanthellae in coral associations under thermal stress (Jones et al., 1998).

To date our understanding of natural variations in concentrations of MAAs in corals under non-stress conditions is very limited. Therefore any attempt to assess the effects of high temperature and irradiance on the functioning of MAAs requires investigation of natural variations as a prerequisite. So far only one study of mucus of *Fungia repanda* has documented a positive correlation between solar irradiance, based on changes in the absorption at 320 nm, however, individual MAAs were not identified (Drollet et al., 1997). Given the possibility that MAAs may play a major role in the understanding of possible synergistic interactions, research into the fate of MAAs during the bleaching process has been highlighted as an area warranting study in a recent review of MAAs (Dunlap and Shick 1998).

1.4. Potential roles of secondary metabolites in the bleaching response of soft corals

Research into bleaching has almost exclusively focused on scleractinian corals (Harriott, 1985; Gates, 1990; Hoeksema, 1991; Glynn, 1993; Glynn, 1996; reviewed in Hoegh-Guldberg, 1999), despite the frequent bleaching of soft corals (Oliver, 1985; Fabricius, 1999; pers. obs.). This omission is particularly striking, as alcyonacean corals are important contributors to both biomass and cover of Indo-Pacific reefs (Dinesen, 1983; Fabricius, 1996). Given that the symbiotic association between zooxanthellae and the host is very similar for both hard and soft corals (Kinzie and Chee, 1979; Benayahu et al., 1989; Trench, 1997), the underlying mechanisms in the bleaching process are likely to be the same for both hard and soft corals. Soft corals, however, may be affected differently by bleaching because of the strong dependence on certain so-called secondary metabolites which are virtually absent in hard corals (reviewed in Coll, 1992). Secondary metabolites are compounds which enhance the coral's fitness by increasing their defence against predation (La Barre et al., 1986a; Uchio et al., 1985; Wylie and Paul, 1989; Slattery et al. 1995a), algal and microbial fouling (Slattery et al. 1995b; Leone et al., 1995; Aceret et al., 1995; Koh, 1997; Michalek and Bowden, 1997), by increasing their competitive ability (Sammarco et al., 1985; La Barre et al., 1986b; de Nys et al., 1991; Maida et al., 1995a,b; Atrigenio and Aliño, 1996) or by acting as spawning - specific compounds to

ensure reproductive success (Coll et al., 1989; Pass et al., 1989; Coll and Clayton, 1990; Coll et al., 1995; Coll, 1992; Slattery et al., 1999). Because many of the highly specific functions of secondary metabolites are only just emerging, often decades after their isolation, the terms "fundamental" and "complementary" have recently been proposed to replace "primary" and "secondary" metabolites in recognition of our increasing understanding of their ecological significance (Sammarco and Coll, 1996).

While it is difficult to estimate the exact metabolic costs involved in producing terpenoid secondary metabolites in soft corals, a substantial part of the carbon necessary for their synthesis is probably of autotrophic origin. The underlying rationale for this notion is that the algal partner translocates up to 95% of its photosynthates to the coral host, thereby providing up to 143% of the daily energetic costs of the association (Muscatine et al., 1984; Davies, 1991). Hence, bleaching and subsequent reduced photosynthetic output will translate into nutritional constraints for the coral host. While the contribution of heterotrophy to the energy budgets of soft corals must not be underestimated, (Lewis, 1982; Fabricius, 1995a), heterotrophic feeding may be highly reduced under bleaching conditions (Fabricius, 1999). Given the integral role of zooxanthellae in uptake and recycling of phosphorus and nitrogen in the coral association (Miller and Yellowlees, 1989; Sutton and Hoegh-Guldberg, 1990; Wang and Douglas, 1998), bleaching can also have strong negative impact on the balance of the two nutrients. Consequently, energy allocation towards the production of significant concentrations of secondary metabolites may be compromised in bleached soft corals. If individuals lack adequate concentrations of key secondary metabolites, they will be more susceptible to factors such as increased predation and algal overgrowth (Coll et al., 1987), which could inhibit recovery of soft corals after bleaching. Moreover, besides their well-established role as primary producers, there is also (controversial) evidence that zooxanthellae may be involved directly in the production of specific secondary metabolites (Kokke et al., 1984; Anderson, 1985; Papastephanou and Anderson, 1982; Ciereszko, 1989). Despite a 30-year debate, the question as to which partner of the coral association controls the production of e.g. algaecides has never been fully resolved.

In summary, bleaching has the potential to compromise the production of secondary metabolites via two modes, indirectly through nutritional constraints, or directly if the compounds are indeed produced by the zooxanthellae. In either case, bleached soft corals could be more susceptible to bacterial or algal fouling and more vulnerable to predation. To date no systematic studies into the effects of bleaching on soft coral secondary metabolites have been carried out.

1.5. Longer-term effects of bleaching – implications for reproductive output

The duration and scale of an environmental disturbance dictate the severity of the bleaching response, and mortality generally only follows when stress is prolonged (Harriott, 1985; Glynn, 1996; Baird and Marshall, 1998). In many cases corals relieved of environmental stresses have been known to regain their zooxanthellae (Cook et al., 1990; Szmant and Gassman, 1990), but most studies of recovery of corals have only focused on short-term issues; i.e. the regaining of zooxanthellae densities (reviewed in Brown, 1997a,b). Given the nutritional constraints associated with bleaching and possible re-allocation of resources towards regeneration (Meesters and Bak, 1993), longer-term effects associated with the loss of zooxanthellae are likely to occur. To date there are indications that bleaching has negative impacts on a coral beyond recovery of zooxanthellae, particularly reduced calcification (Clausen and Roth, 1975; Leder et al., 1991) or growth (Szmant and Gassman, 1990; Goreau and Macfarlane, 1991). Pioneering work by Szmant and Gassman in 1990, has also provided the first, if very limited, evidence for impairment of gametogenesis in bleached colonies of *Montastrea annularis*. At present it is unknown whether bleaching can affect the "next generation" by reducing maternal energy reserves normally allocated for reproduction. The effects of bleaching could be passed on to the next generation via two modes: through reduced reproductive output and/or through biochemically less well-equipped offspring, with possibly reduced potential for fitness and survival. Given the integral role of sexual reproduction for the persistence and maintenance of reefs, it is important to establish the implications of bleaching for reproduction.

1.6. Specific aims

The research presented in this thesis investigates aspects of the chemical ecology of the soft coral – zooxanthellae interaction, to increase our understanding of how bleaching may affect the biochemistry and subsequently the physiology and ecology of soft corals. My specific aims were:

1. **To investigate the effects of temperature and UV radiation on concentrations and function of UV protecting agents.** The possibility that temperature and UV radiation may act synergistically to break down UV protecting agents and hence trigger bleaching has grave implications for the health and survival of reef organisms if predictions of global warming are accurate. This study will therefore provide a basis for the understanding of the role of MAAs during bleaching episodes, induced by elevated temperature and solar radiation.

2. **To measure seasonal variations in the concentrations of UV protecting agents in soft corals.** Knowledge of seasonality in concentrations of UV - blocking compounds in field populations of soft corals will aid in determining the role of these compounds in the biochemistry of reef organisms and is essential for interpreting manipulative bleaching experiments (as referred to in objective 1.).

3. **To determine if secondary metabolite production in soft corals changes after bleaching, and if so, to investigate the ecological implications of such changes.** If symbiotic zooxanthellae control or contribute to the production of secondary metabolites that act as anti-fouling agents or feeding deterrents, then bleaching may compromise the survival of soft corals through impacts in addition to those associated with nutritional constraints.

4. **To establish which partner of the coral alga association controls the production of species – specific secondary metabolites.** If zooxanthellae control the production of secondary metabolites this can have grave implications for bleached soft corals, since the maintenance of significant concentrations of these ecologically important compounds could not be sustained, even if resources were available.

5. **To examine if bleaching affects the reproductive success of selected soft corals, as a long-term sub - lethal impact of bleaching.** The loss of zooxanthellae may result in strong nutritional constraints or impaired production of ecologically important secondary metabolites, which could potentially decrease the reproductive success of bleached soft corals.

Chapter 2

Seasonal variation in MAA levels of soft corals in relation to annual solar irradiance and seawater temperature cycle

Lobophytum compactum recovering its zooxanthellae after a natural bleaching event at Orpheus Island, March 1998

2.1. ABSTRACT

This chapter focuses on the nature and extent of variation in MAAs in relation to annual cycles in solar radiation, seawater temperature and reproduction in reef-flat populations of soft corals. The results presented clearly show that MAA tissue concentrations in shallow water colonies of *Lobophytum compactum* and *Sinularia flexibilis* are significantly correlated to annual cycles in solar radiation and seawater temperature. Evidence of seasonal cycles in MAA levels in the tissues of shallow reef invertebrates positively correlated with annual cycles in solar radiation corroborates the importance of MAAs as photoprotectants. Furthermore, prior to spawning in both years, female colonies of both soft coral species featured substantially higher MAA levels than their male counterparts, presumably to provide a high level of protection against irradiance stress for progeny. In addition, this study demonstrates that levels of carotenoids, chlorophyll and proteins also vary seasonally, with maxima for the former group, and minima in the latter groups during the Australian summer, suggesting that carotenoids in particular may contribute to the photo-acclimatory responses of soft corals.

2.2. INTRODUCTION

The UV-protecting role of mycosporine-like amino acids (MAAs) in marine organisms has been inferred from numerous studies since Dunlap and Chalker (1986) first suggested they function as broad–band filters in corals (reviewed in Dunlap and Shick, 1998). MAAs appear to be ideal screening agents for symbiotic associations involving phototrophic organisms, because they are transparent to radiation in the PAR region (400-700 nm), but absorb strongly in the potentially damaging UV range between 310 – 360 nm. Functionally, MAAs are believed to absorb radiation of high energy in the UV region, which is then dissipated as vibrational energy, thereby minimising structural and physiological damage to tissues (M. Shick, pers. comm.).

Support for the assumption that the primary role MAAs is protection against high irradiance damage is mainly based on indirect evidence that MAAs are light inducible by radiation both in the PAR (Scelfo, 1985; Karsten et al., 1998) and UVR region (Lesser et al., 1990; Shick et al., 1991; Kinzie, 1993) and that shallow individuals typically have higher tissue concentrations than their deeper counterparts (Dunlap et al., 1986, Gleason, 1993; Shick et al., 1991; Kinzie, 1993). Their role as photoprotectants is further supported by the fact that a diverse range of marine benthic organisms provide their planktonic larvae with higher concentrations of MAAs than are found in adult tissues (Chiocarra, 1986; Adams and Shick, 1996; Carefoot et al., 1998, see also Chapter 7). The first direct evidence for their photo-protective role was provided by Garcia-Pichel and co-workers in 1993, who showed that the presence of MAAs in the cyanobacterium *Gleocaspa* prevented 23-30% of UV radiation at 320 nm from reaching cellular targets. Given that solar irradiance stress has been implicated as a causative factor in a number of bleaching events, both as a sole factor (Fisk and Done, 1985; Gleason and Wellington, 1993; Brown et al., 1994) and in combination with increased temperature (Harriott, 1985; Brown and Suharsono, 1990), MAAs have the potential to play an important role in the prevention of bleaching.

Although both direct and indirect evidence suggest that MAAs have a photo-protective function, experimental studies carried out by Scelfo (1985) and Stochaij and co-workers (1994) on a number of cnidarians failed to demonstrate MAA concentration increases in response to longer-term exposure to UVR. To date the only positive correlation between MAA concentrations and solar radiation cycles were established for the digestive tract of the sea urchin *Sterechinus neumayeri* (Karentz et al., 1997) and the mucus of the solitary coral *Fungia repanda* (Drollet et al., 1997). In fact, a variety of factors other than high solar irradiance have been proposed to affect MAA tissue concentrations in corals, including water velocity (Jokiel et al., 1995; Jokiel et al., 1997) and mechanical stress (Scelfo, 1985), suggesting that MAAs may

function other than as photoprotectants. Bandaranayake et al. (1997) proposed that MAAs may perform a role similar to reproductive hormones, after finding no correlation between MAA concentrations and annual solar irradiance, but a positive correlation with the reproductive cycle in the sponge *Dysidea herbacea.*

Evidence of natural cycles of MAA concentrations in the tissues of sessile organisms that correlate with annual cycles in solar radiation would corroborate the importance of MAAs as photoprotectants. The soft corals *Lobophytum compactum* and *Sinularia flexibilis* thrive on reef flats (and occur to around 20m) predominantly in the inner- and midshelf reefs of the GBR, which makes them ideal study organisms to examine the effects of high seasonal fluctuations in solar irradiance and temperature on MAA levels. If MAAs are an important component of the photo-protective system of soft corals, a seasonal trend in MAA concentrations should therefore be reflected in tissues of these reef flat corals, with maxima in summer when incident radiation is highest. Since both soft coral species are broadcast-spawners with positively buoyant eggs (Aliño and Coll, 1989), substantial investment into biochemical defence to counteract solar damage should also occur in the highly exposed eggs and early planktonic life stages. If maternal provision of offspring with MAAs is indeed an important life-strategy in corals, female colonies should have significantly higher concentrations prior to spawning than males, as has been observed in sea-urchins (Adams and Shick, 1996) and sponges (Bandaranayake et al., 1997).

The aim of this chapter is to determine the nature and extent of variation in MAAs in relation to annual cycles in solar radiation, seawater temperature and reproduction in reef-flat populations of the soft corals *Lobophytum compactum* and *Sinularia flexibilis*. Specifically, I will examine whether MAA levels are highest when protection against high incident radiation and, thus possibly bleaching, becomes most crucial. Knowledge of natural variations in MAA levels will also provide a basis for the interpretation of experimental MAA manipulations (Chapter 3). Since MAAs are most likely produced by the zooxanthellae partner in the coral association (Chapter 1), variation in MAA levels will be investigated in the framework of variations in zooxanthellae densities, chlorophyll and protein concentrations, which all contribute to quantifying natural variations in symbiont densities. Given that carotenoids are not only one of the light-harvesting photosynthetic pigments, but may also function as photoprotectants, I will also investigate annual cycles in carotenoid (peridinin) levels. Furthermore, sex-specific differences in the MAA pool will be investigated because of potential maternal provision of UV-absorbing compounds to progeny.

2.3. MATERIALS AND METHODS

2.3.1. Site description

This study was carried out between December 1995 and December 1997 in Pioneer Bay, Orpheus Island in the Central section of the Great Barrier Reef. Orpheus Island is part of the Palm Islands Group (18°46'S, 146°51'E), a group of continental islands surrounded by extensive fringing reefs (Figure 2.1). These reefs typically do not extend deeper than 15 m and are relatively sheltered on the western leeward side of islands. Turbidity is generally high, with underwater visibility at Pioneer Bay rarely exceeding 10 m (Baird and Marshall, 1999).

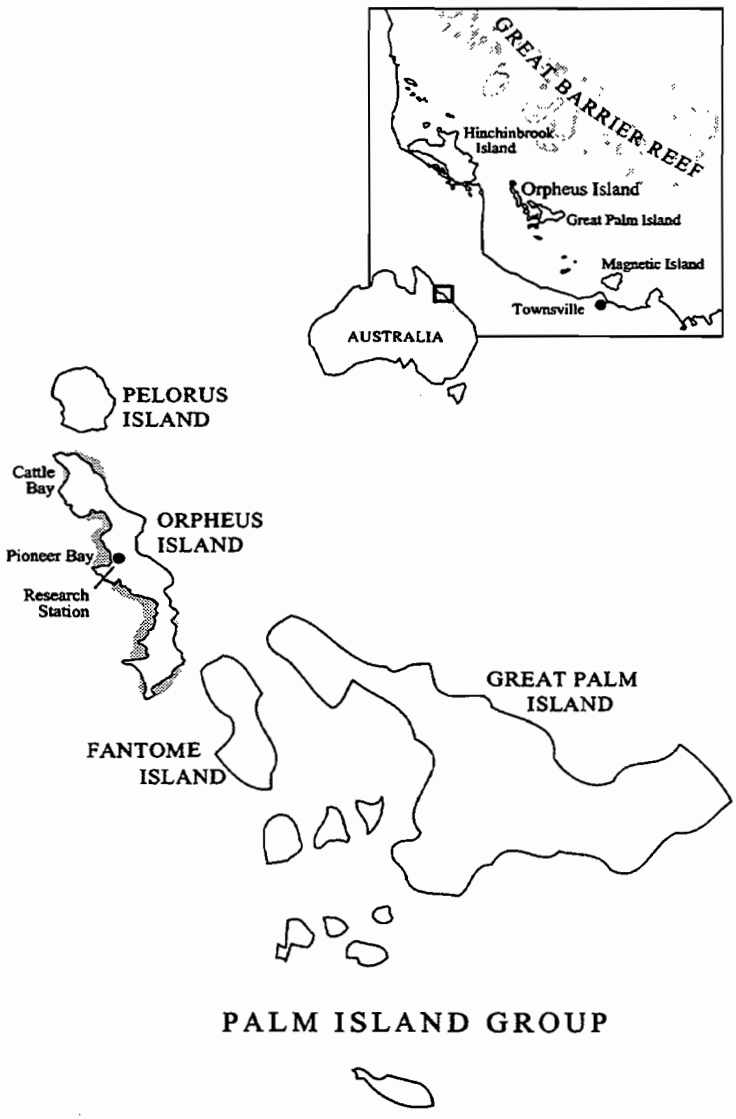


Figure 2.1. Location of study site, Pioneer Bay, Orpheus Island, in the Central Great Barrier Reef

2.3.2. Species description

The alcyonacean soft corals *Sinularia flexibilis* Quoy & Gaimard (Versefeld, 1980) and *Lobophytum compactum* Tixier-Durivault, 1956, (Tixier-Durivault, 1958) which are common reef flat corals on the inner and mid-shelf reefs of the Great Barrier Reef (Dinesen, 1983), were used for most experiments. Their contrasting growths forms, *Lobophytum compactum* featuring a solid leathery growths form, whereas *Sinularia flexibilis* has a comparatively delicate structure with fine branchlets), offers scope for comparisons between differential susceptibility towards thermal and solar stress between solid and more delicate species.

2.3.3. Sampling regime and preparation of experimental corals

To determine if there are annual cycles in MAA tissue concentrations, colonies located on the reef slope at a depth of 2-3 m were tagged *in situ* in November 1995 (Figure 2.2). I sampled colonies of the dioecious soft corals *Sinularia flexibilis* and *Lobophytum compactum* at monthly intervals over two subsequent years, from January 1996 through December 1997. Five large mature male and female colonies of similar colony size were used (stalk diameters of 10-13 cm for *Sinularia flexibilis* and colony diameters averaging 70-90 cm for *Lobophytum compactum*). I collected tissue samples of all 20 test corals at each sampling date. To determine the level of intra-colony variation in tissue concentrations of various biochemical parameters, four random tips (a-d; 1-2 g wet weight/tip) were collected from each colony. Samples were snapfrozen at -20° C and kept under dark conditions until analysed biochemically. All samples were subdivided and analysed for zooxanthellae densities and chlorophyll a + c_2, MAA, carotenoid and protein concentrations, so that there were 4 replicate determinations per colony for each biochemical parameter (Figure 2.2).

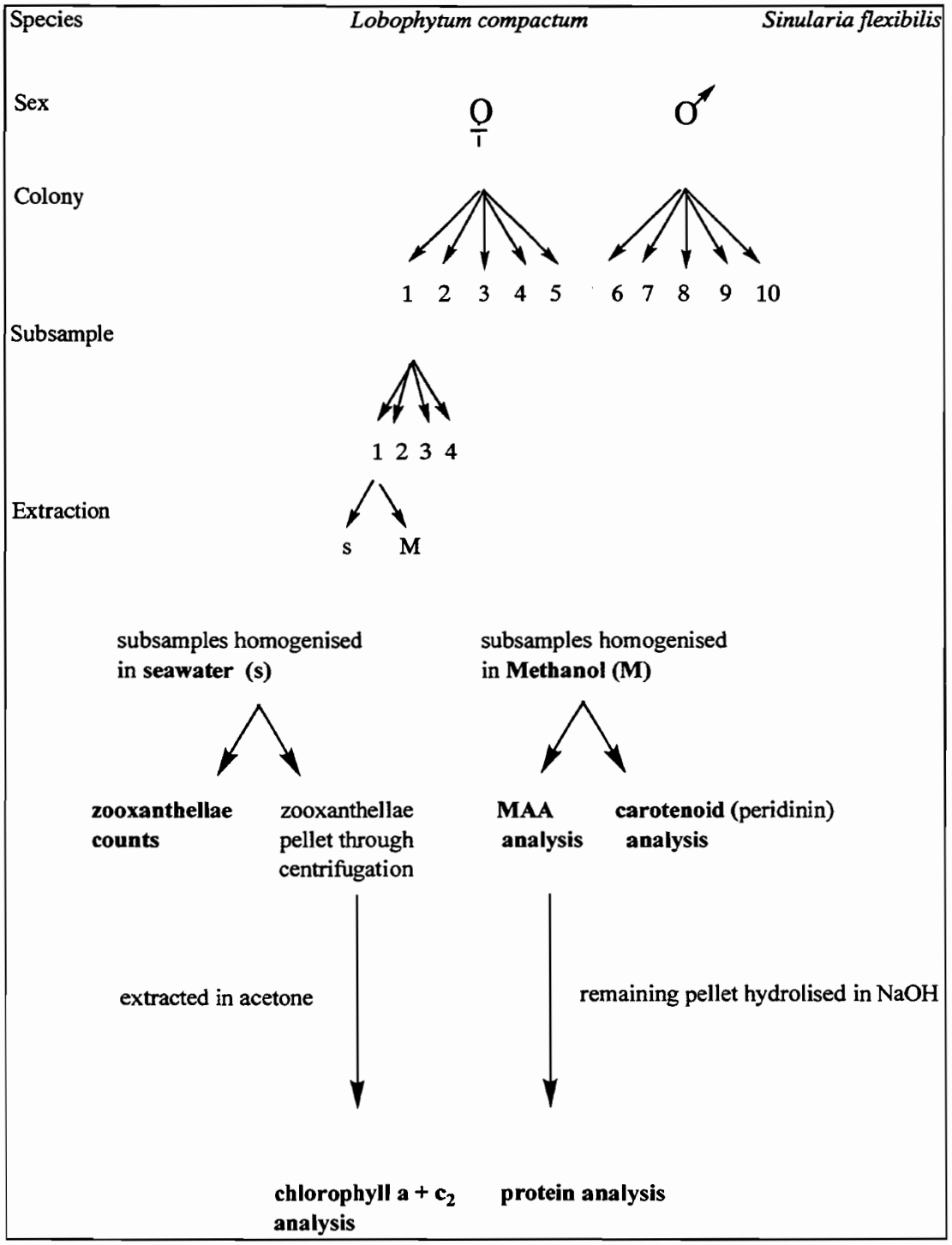


Figure 2.2. Overview of experimental design and sample preparation for the different biochemical analyses, N = 40 determinations (i.e. 10 colonies x 4 subsamples for each biochemical parameter).

2.3.4. Biochemical analysis of zooxanthellae densities, chlorophyll, carotenoid, MAA and protein concentrations

In order to monitor potential photo-acclimatory responses through time, zooxanthellae, chlorophyll and carotenoid concentrations were determined as follows.

1. Quantification of zooxanthellae densities. Zooxanthellae population densities were monitored as indicators of possible bleaching and recovery of host tissues. Tissue samples for the analysis were fully homogenised in 0.45 μm filtered and autoclaved seawater. An aliquot of each sample was fixed in formalin and stored at 4° C until analysis. Zooxanthellae densities were calculated from quadruplicate haemocytometer counts for each of four sub-samples per colony and normalised to coral wet weight. Coral wet weight was determined prior to freezing by weighing dry blotted samples.

2. Chlorophyll analysis. Homogenised tissue samples were centrifuged, the supernatant decanted and the zooxanthellae containing pellet extracted at 4° C for 24 hours in 100% acetone under dark conditions. After extraction, samples were centrifuged again and the supernatant used to quantify chlorophyll a and c_2 spectro-photometrically using equations in Jeffrey and Humphrey (1975).

3. Carotenoid (peridinin) analysis. Peridinin, the main carotenoid in dinoflagellelates (Kazlaukas et al. 1976) was quantified in order to examine its contribution as an accessory pigment and possible photoprotectant. Weighed and defrosted tissue samples were fully homogenised and sequentially extracted 3 times for 60 minutes in 3 ml of 100% methanol. To avoid photo-degradation of carotenoids (and also MAAs) samples were kept on ice and protected from light during extraction. The combined extract was clarified by centrifugation, and then peridinin immediately quantified spectro-photometrically using equations in Jeffrey and Haxo (1968).

4. Analysis of mycosporine-like amino acids (MAAs). Individual MAAs were separated and quantified by reverse-phase, isocratic high-performance liquid chromatography (hplc). Separation was accomplished using a Brownlee RP-8 column (spheri-5, 4.6. mm i.d. x 250 mm), protected with an RP-8 guard column (spheri-5, 4.6. mm i.d. x 30 mm). The mobile phase consisted of 25% methanol and 0.1% acetic acid (v: v) in water and was run at a flow rate of 0.8 mL/min. Peaks were detected by UV absorbance at 313 nm and 340 nm (dual wave detection). Prior to hplc analysis, MAA samples were processed in the same fashion as the

carotenoid samples and stored at – 20° C. Additional treatment involved filtration of samples through 0.45 μm millepore filters to ensure removal of particulate matter from the solution.

Identification of MAAs was attained by co-chromatography with verified standards of palythine and palythinol from the zoanthid *Palythoa tuberculosa*; porphyra-334 and shinorine from the red alga *Porphyra tenera*, and asterina-330 and palythene from the ocular lens tissue of the coral trout *Plectropomus leopardus*. Peak areas were integrated on a Hewlett-Packard integrator and quantification of individual MAAs was achieved by comparing the integrated hplc peak areas with pre-determined peak areas of chromatographically quantified standards (standards and calibration courtesy W. Dunlap). All measurements were corrected for the extraction efficiencies of individual MAAs of each species by the method described in Dunlap and Chalker (1986) and ranged from 95.5% - 99.6%.

5. Partitioning of MAAs between zooxanthellae and soft coral host. In order to determine whether MAAs are stored in the animal or algal part of the coral association, cells were separated and the MAA concentrations of each part determined. Entire lobes (*Lobophytum compactum*) or "branchlets" (*Sinularia flexibilis*) of test corals (3 females, 2 males) were collected and divided in half. One half was immediately frozen and later used to determine protein and MAA concentrations of the entire coral association. The second half was homogenised in artificial seawater to yield the zooxanthellae fraction of the association. This was achieved by repeated centrifugation, discarding of supernatant and washing until a pellet free of animal cells was achieved (confirmed by microscopy of extracts). Quantification of MAAs and protein present in the zooxanthellae fraction and in the entire association was then carried out as described below. Protein content and MAA concentration of the animal tissue were calculated as the difference between the whole fraction and the zooxanthellae fraction.

6. Protein determination. Protein concentrations were determined in order to normalise MAA concentrations to a representative unit of tissue. After methanol extraction, tissue residues were kept frozen until their protein content was determined. Hot 1N NaOH was used to digest tissue samples, which were then brought to room temperature and the mean protein content of duplicate aliquots measured using the method of Lowry et al. (1951). Bovine albumin was used as a standard.

7. Statistical analysis. I used repeated measures analyses to test for differences in biochemical parameters over time. Two factorial ANOVA was used to test for differences between sexes. Prior to analysis of variance, data were tested for normality (Shapiro-Wilks statistics) and homogeneity of variances (Bartlett and Cochran tests) and data transformed where appropriate.

After transformation, variances in protein levels in *Sinularia flexibilis* were still found to be heterogeneous, however, to a lesser degree. Given that ANOVA is considered robust to all but major deviations from homogeneity (Underwood, 1981), repeated measures analyses were carried out with the data. The significance of changes in the composition of MAAs through time was determined by MANOVA of arc sine transformed percentage compositions of MAAs (Sokal and Rohlf, 1995). Simple and multiple linear regression analyses were carried out in order to determine the relationship between levels of a number of biochemical parameters and solar irradiance or seawater temperature. I used STATVIEW, SUPERANOVA and SPSS software packages for the above tests.

2.3.5. Relationship between seawater temperature, solar radiation and biochemical parameters

In order to determine the relationship between variations in MAA concentrations and either annual solar radiation or seawater temperature variation, I acquired the appropriate data from two sources. Temperature data were obtained from the Great Barrier Reef Marine Park Authority (GBRMPA) temperature-monitoring program (courtesy R. Berkelmans). Temperatures were monitored at 2-3 m depth on the reef flat at Cattle Bay, which is within 3-4 km to my study site in Pioneer Bay on Orpheus Island (see Figure 2.1 for location). Means are based on approximately 1440 readings per month of permanently installed, calibrated temperature data loggers.

The environmental ambient radiation data were made available by Aurel Moise at the School of Mathematics, Computer Sciences and Physics at James Cook University and the Australian Radiation Laboratory (Yallambie, Victoria). All environmental radiation data were collected at Townsville, approximately 70 km from Orpheus Island.

Monthly means were used for regression analyses between a number of different combinations of biochemical parameters. Similarly, average monthly doses of total solar downward flux (300 – 2800 nm) and total UVR (285-400 nm) were used for regression analyses with the same biochemical parameters.

2.4. RESULTS

2.4.1. MAA analysis

1. MAA profiles in Lobophytum compactum and Sinularia flexibilis

The typical set of MAAs I identified in both species included shinorine, porphyra–334, palythine, asterina-330, palythinol and palythene (Figure 2.3).

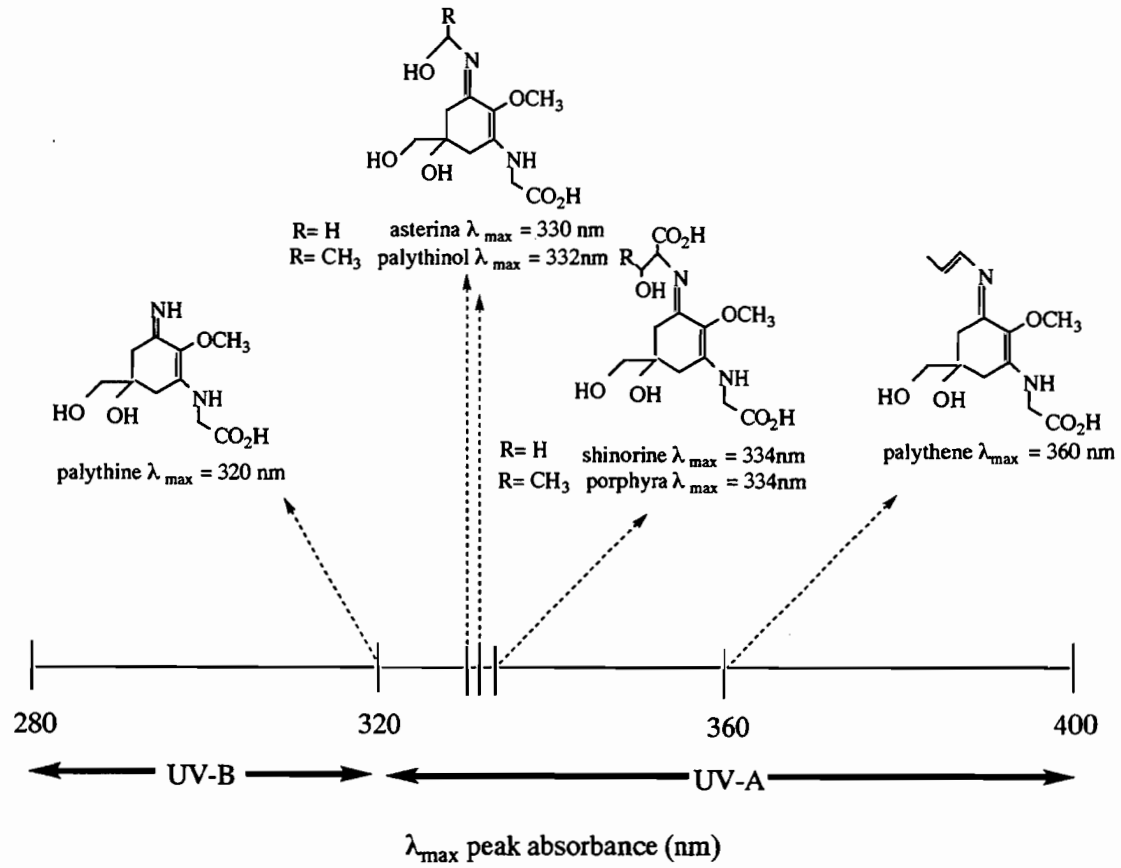


Figure 2.3. Wavelength and absorbance maxima (λ_{max}) of MAAs present in both the tissues of *Lobophytum compactum* and *Sinularia flexibilis*.

In both coral species palythine made by far the highest contribution to the MAA pool, and on average comprised 92.79 ± 0.49% and 93.26 ± 0.40% of the pool for *Lobophytum compactum* and *Sinularia flexibilis* respectively (Figure 2.4). The second strongest fraction differed between the two species, with shinorine contributing, on average, 3.25 ± 0.26% for *Lobophytum compactum*, and palythene contributing 4.32 ± 0.34% in *Sinularia flexibilis*. The concentrations of the remaining four MAA species were very low and collectively did not contribute more than 4.00 ± 0.20% and 2.43 ± 0.14% to the total pool in *L. compactum* and *S. flexibilis* respectively.

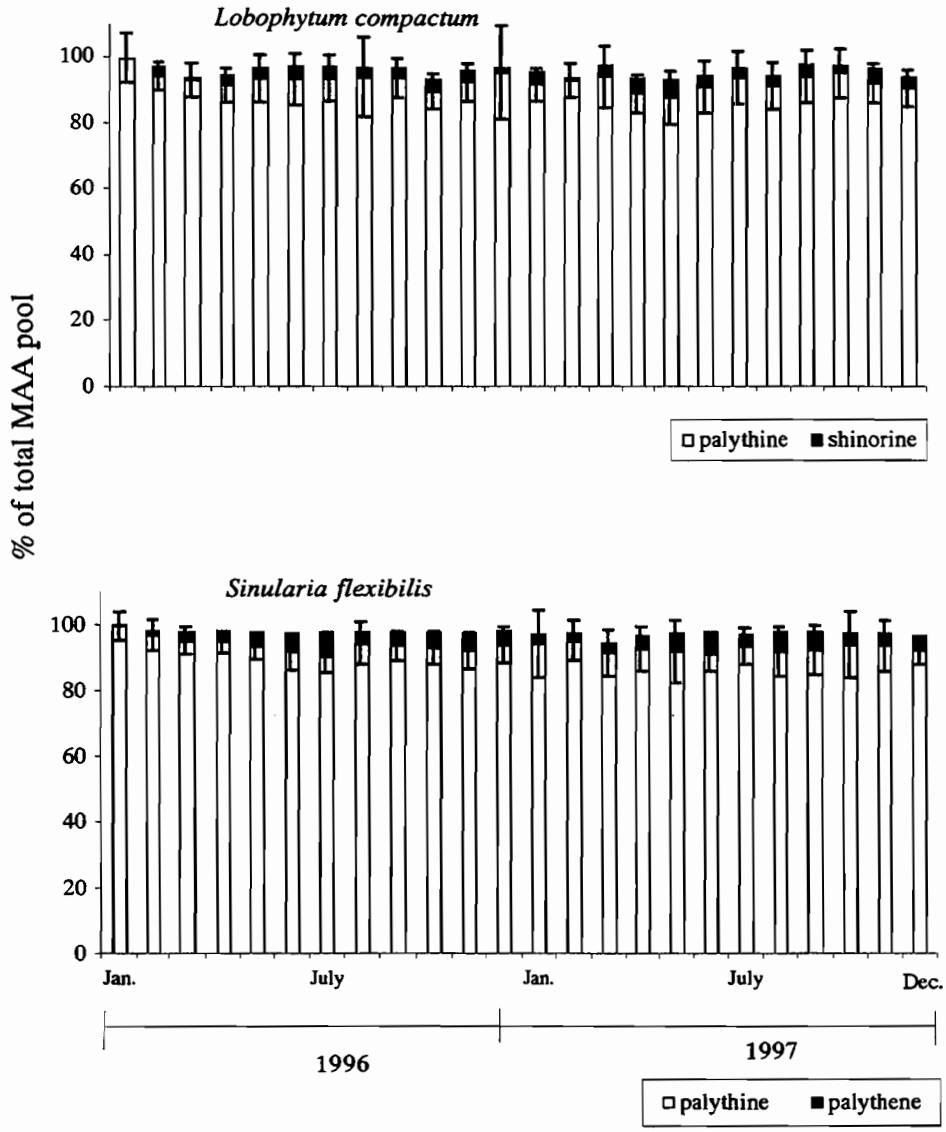


Figure 2.4. Percentage contribution of palythine, shinorine and palythene to the total MAA pool in *Lobophytum compactum* (top) and *Sinularia flexibilis* (bottom). Error bars indicate standard errors for palythine only, N = 40 determinations (i.e. 10 colonies x 4 subsamples per sampling date and species).

The relative proportions of each MAA with respect to the total pool did not change over time in either species (MANOVA, F = 0.03, df =23, p = 0.999 for *Lobophytum compactum* and F = 0.09, df = 23, p = 0.999 for *Sinularia flexibilis*).

2. Changes in MAA tissue concentration with time

In general, there was a trend for total MAA concentrations to vary with season in both *Lobophytum compactum* and *Sinularia flexibilis*. Mean MAA concentrations were highest in February and lowest in June in both species (Figure 2.5.).

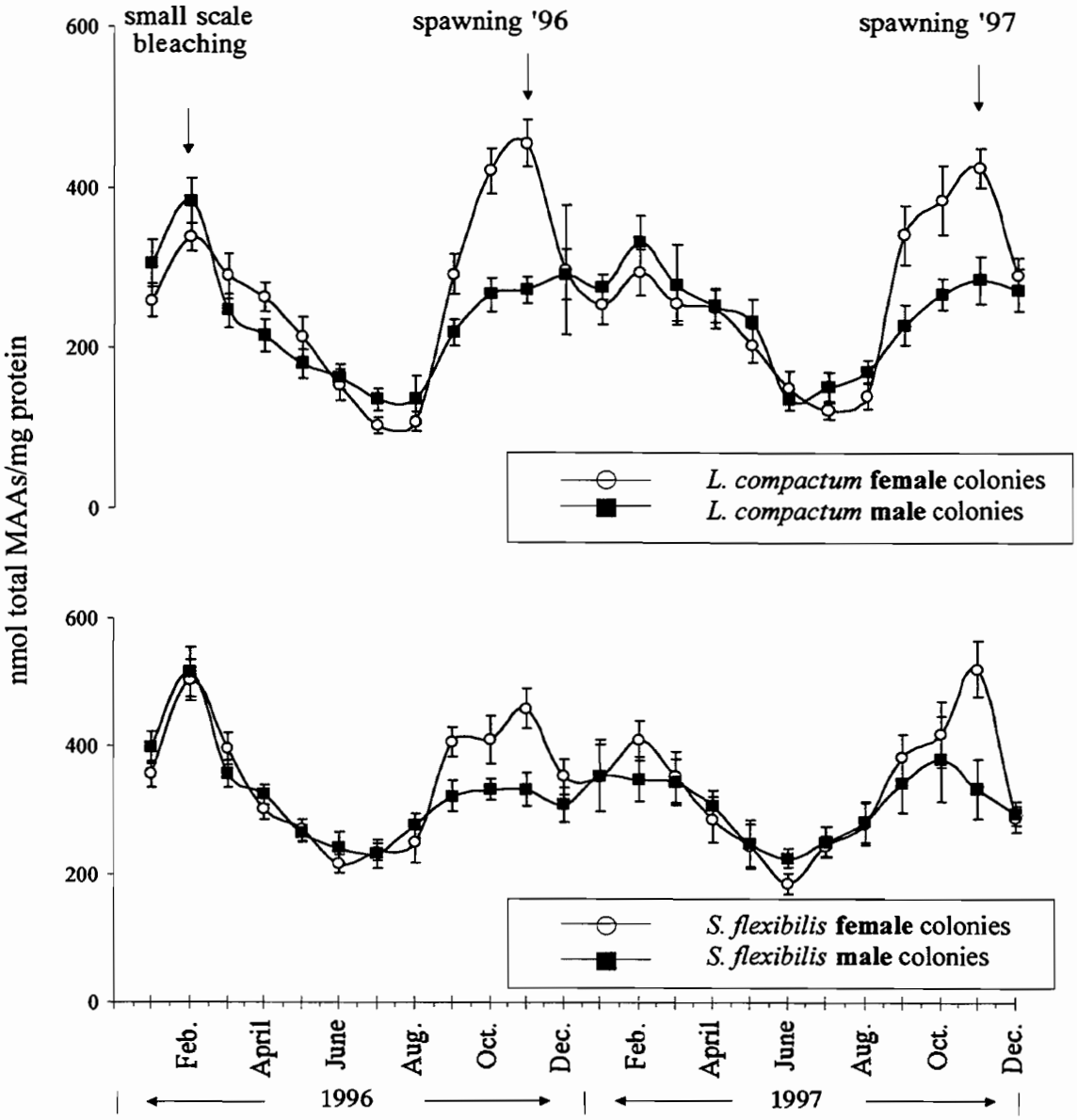


Figure 2.5. Changes in MAA levels through time in female and male colonies of *Lobophytum compactum* (top) and *Sinularia flexibilis* (bottom). Error bars indicate standard errors, N = 20 determinations (i.e. 5 males and 5 females per species x 4 subsamples, per month).

Two-factorial repeated measures ANOVA demonstrate that MAA concentrations vary significantly with time in both species ($p < 0.0001$; Table 2.1), but the significant first order interaction effect indicates that temporal patterns in MAA concentrations differ among colonies ($p < 0.0001$; Table 2.1).

Table 2.1. Summary of results for 2-factorial repeated measures ANOVA, testing for differences in MAA concentrations between colonies and through time. Asterisks denote significant differences ($\alpha = 0.05$).

MAA tissue concentrations over time			
Source of variation	df	F-ratio	p-value
Lobophytum compactum			
colony	9	1.935	0.0849
time	23	18.701	< 0.0001*
colony x time	207	1.803	< 0.0001*
Sinularia flexibilis			
colony	9	3.362	0.0058*
time	23	14.424	< 0.0001*
colony x time	207	2.456	< 0.0001*

The increase in MAA levels from January to February in both species was a function only of protein losses experienced during the small scale bleaching episode, since no significant changes occurred with respect to wet weight levels (Scheffé, $p = 0.70$ and $p = 0.33$ for *Lobophytum compactum* and *Sinularia flexibilis* respectively).

3. Sex-specific differences in MAA tissue concentrations

Differences in the MAA concentrations of male and female colonies were detected, with females featuring up to 67% and 56% higher concentrations immediately prior to spawning in *Lobophytum compactum* and *Sinularia flexibilis* (Figure 2.5). The magnitude of differences in MAA levels between males and females depended on the time of sampling resulting in a significant interaction term ($p < 0.0001$ for *L. compactum* and $p < 0.044$ for *Sinularia flexibilis*). Differences between the sexes were greatest in the three months prior to spawning in both species (Figure 2.5).

Table 2.2 Summary of results for 2-factorial ANOVA testing for differences in MAA levels between sexes and through time. Asterisks denote significant differences ($\alpha = 0.05$).

Sex-specific differences in MAA tissue concentrations			
Source of variation	df	F-ratio	p-value
Lobophytum compactum			
time	23	16.59	< 0.0001*
sex	1	10.78	0.001*
sex x time	23	3.26	< 0.0001*
Sinularia flexibilis			
time	23	10.82	< 0.0001*
sex	1	0.44	0.508
sex x time	23	1.57	0.044*

4. MAA partitioning between animal and plant tissue

Comparison of MAA concentrations between animal and plant tissue in *Lobophytum compactum* and *Sinularia flexibilis* revealed that concentrations in the animal tissue are approximately 9 fold higher then those in the zooxanthellae fraction. This corresponds to MAAs in host tissues comprising, on average, 92.36 ± 1.44% and 92.82 ± 1.07 % of the total pool for the symbiont – coral association in *Lobophytum compactum* and *Sinularia flexibilis* respectively.

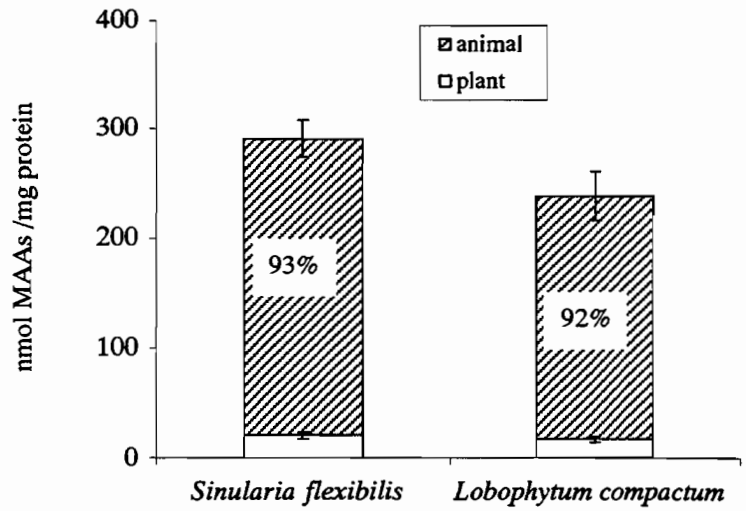


Figure 2.6. Partitioning of MAAs between animal and plant tissue in *Lobophytum compactum* and *Sinularia flexibilis* (n = 5). Figures within the column represent the percentage of MAAs of the total pool associated with the animal tissue.

2.4.2. Seasonal responses in biochemical parameters

1. Zooxanthellae analysis

With the exception of February 1996, when a short-term bleaching event occurred, zooxanthellae densities remained virtually constant throughout the 24-month monitoring period in both species (Figure 2.7a). Repeated measures analysis of all 23 monitoring months (excluding February 1996) confirmed that no significant changes occurred during that period ($F = 0.641$, $df = 22$, $p = 08955$ for *Lobophytum compactum;* $F= 1.302$, $df = 22$, $p = 0.1605$ for *Sinularia flexibilis*). The bleaching episode in February 1996 corresponded to significant zooxanthellae losses in the range of 22% for *Lobophytum compactum* and 10 % for *Sinularia flexibilis*. Both species, however, recovered to background levels within two months (Figure 2.7a). Zooxanthellae concentrations did not differ significantly among colonies independently of time (Table 2.3). Analysis of all 24 months (i.e. including February 1996; Table 2.3), revealed that zooxanthellae levels were dependent on the time of sampling in *Lobophytum compactum* ($p < 0.0001$; Table 2.3). Zooxanthellae densities did not differ among sampling times in *Sinularia flexibilis*, and the absence of significant first order interactions indicates that the overall temporal pattern in zooxanthellae levels did not differ among colonies ($p = 0.060$ for *Lobophytum compactum* and $p = 0.962$ for *Sinularia flexibilis*; Table 2.3).

2. Chlorophyll analysis

Levels of chlorophyll were consistently higher in winter than in summer in both *Lobophytum compactum* and *Sinularia flexibilis,* which largely explains the significant effect of time on chlorophyll concentrations (Figure 2.7b; Table 2.3). The significant interaction term (Table 2.3) suggests that the overall pattern of no variation in chlorophyll among colonies was not consistent at the level of individual months. Thus, the magnitude of differences in chlorophyll levels among colonies varied temporally, being significant in some months and inconsequential in others (Table 2.3).

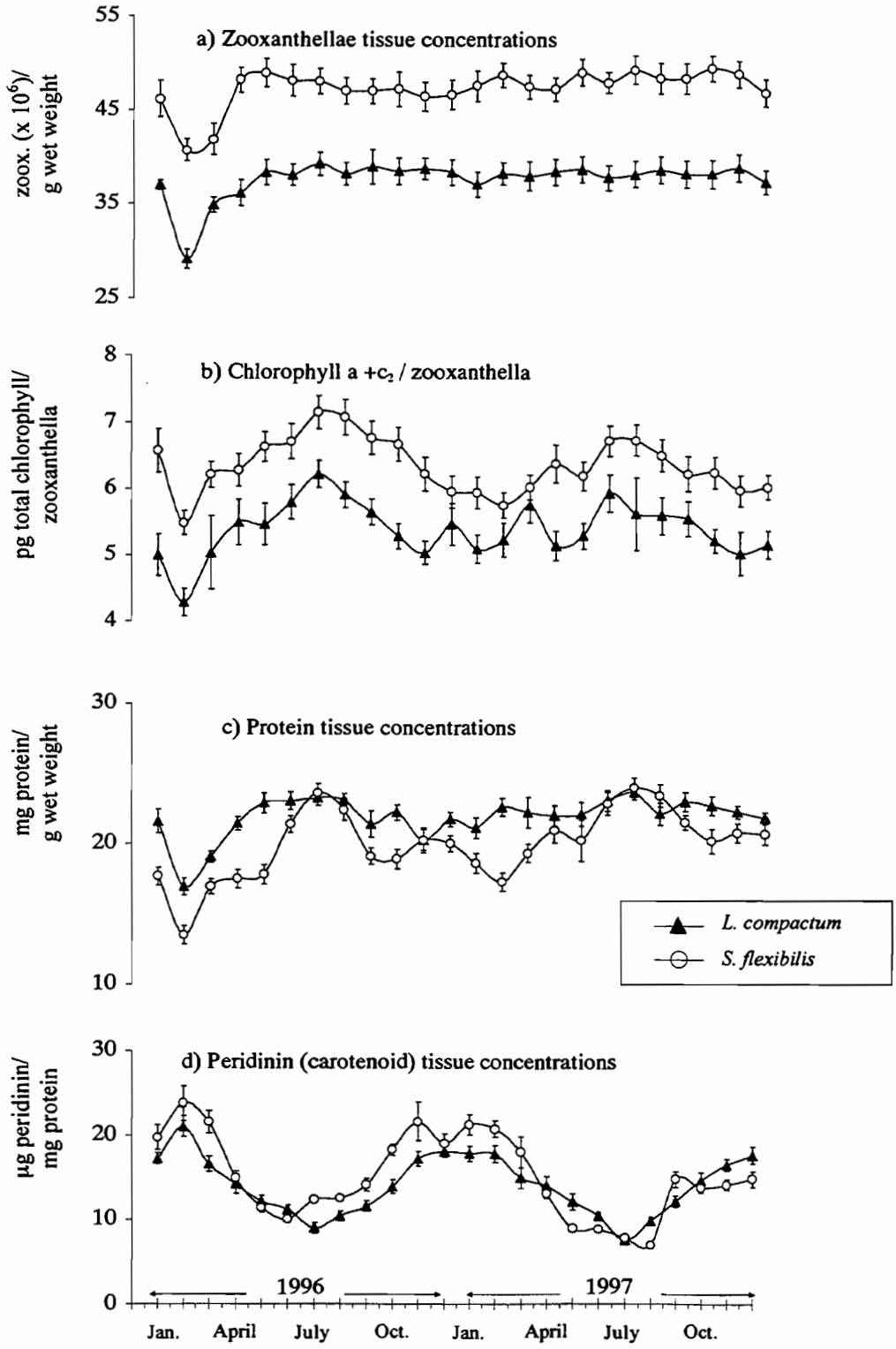


Figure 2.7. Mean concentrations of a range of biochemical parameters in the tissues of the soft coral *Lobophytum compactum* and *Sinularia flexibilis*: a) mean densities of zooxanthellae, b) mean chlorophyll a + c_2 concentrations, c) mean protein levels and d) mean peridinin concentrations during a two-year monitoring period. Errors indicate standard errors, N = 20 determinations (i.e. 5 male, 5 female colonies x 4 subsamples per colony, per month).

Table 2.3. Summary of results of 2-factorial repeated measures ANOVA testing for differences in zooxanthellae densities, total chlorophyll, carotenoid (peridinin) and protein concentrations between individuals through time. Asterisks denote significant differences ($\alpha = 0.05$).

Source of variation	*Zooxanthellae densities*			*total chlorophyll/ zooxanthellae*		*carotenoid (peridinin)*		*protein*	
	df	F-ratio	p-value	F-value	p-value	F-ratio	p-value	F-ratio	p-value
L. compactum									
colony	9	1.711	0.1333	0.58	0.802	4.222	0.0013*	3.578	0.004*
time	23	2.578	<0.0001*	2.2	0.001*	20.722	<0.0001*	12.76	<0.0001*
colony x time	207	1.186	0.0606	1.422	0.0006*	1.82	<0.0001*	1.572	<0.0001*
S. flexibilis									
colony	9	3.006	0.112	1.902	0.903	1.952	0.0821	1.035	0.4373
time	23	0.83	0.6951	3.69	<0.0001*	39.10	<0.0001*	5.318	<0.0001*
colony x time	207	0.814	0.9629	1.859	<0.0001*	4.622	<0.0001*	1.65	<0.0001*

3. Carotenoid analysis

Concentrations of peridinin (the main carotenoid) were consistently higher in summer than in winter. Averaging winter (July) values over two years, levels were only 47.25% and 49.95% of summer (January) levels for *Lobophytum compactum* and *Sinularia flexibilis* respectively (Figure 2.7d). The significant first order interaction term in both species indicates that the magnitude of differences temporal in peridinin levels differ among colonies varied throughout the sampling period ($p < 0.0001$; Table 2.3). In general, peridinin levels differed among colonies in some months but not in others, however, the overall temporal pattern was clear.

4. Protein analysis

Protein concentrations varied over time for both *Lobophytum compactum* and *Sinularia flexibilis* with highest levels in winter (July) and lowest in summer (January) for the latter species (Figure 2.7c). For *Lobophytum compactum* no clear seasonal pattern could be discerned. Protein concentrations varied significantly between colonies at any given month in *Lobophytum compactum*, whereas variation in protein levels between colonies of *Sinularia flexibilis* were not significant (Table 2.3). The presence of a significant interaction term again suggests that the magnitude of differences in protein concentrations among colonies varied temporally (Table 2.3).

2.4.3. Environmental parameters monitored during the sampling period

1. Solar radiation data for Townsville during the monitoring period

Total solar downflux and total UV radiation followed a pronounced seasonal trend, with peaks in summer and lows in winter (Figure 2.8).

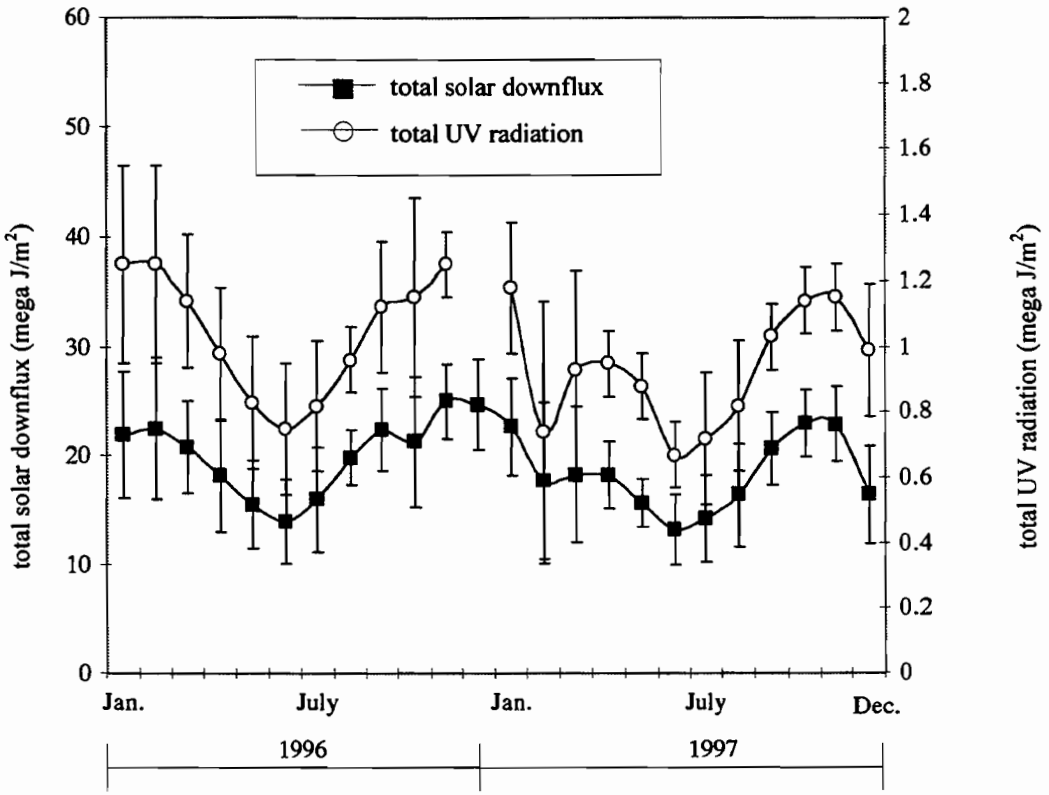


Figure 2.8. Mean total solar downflux (300-2800 nm) and UV radiation (285-400 nm) data during 1996 and 1997 (courtesy A. Moise, JCU). Error bars present the standard error (n = 144/ day). No data were available for UVR in January 1997.

Relationship between biochemical tissue concentrations and solar radiation data

Levels of MAAs in the tissues of both soft coral species were positively and highly significantly related to the total solar downflux (Table 2.4a) and also to the UV radiation fraction of the spectrum (Table 2.4b). Levels of zooxanthellae, chlorophyll, peridinin and protein in *Lobophytum compactum* were not significantly correlated with either the total solar downflux or UVR. For *Sinularia flexibilis*, however, zooxanthellae densities were significantly and negatively correlated with both solar downflux and UVR, whereas peridinin levels were positively correlated (Table 2.4). Total solar radiation explained between 1% and 44% of the variation of these parameters and UVR explained between 4% and 42%.

Table 2.4 Summary of regression analyses between coral tissue parameters (MAA, zooxanthellae, chlorophyll, protein and peridinin concentrations) and the monthly doses of total a) solar downflux and b) UV radiation in *Lobophytum compactum* and *Sinularia flexibilis*, (n = 23). Asterisks indicate a significant relationship ($\alpha = 0.05$).

	Lobophytum compactum			*Sinularia flexibilis*		
Parameter	p-value	nature of relationship	Coefficient of determination R^2	p-value	nature of relationship	Coefficient of determination R^2
a). Total solar downflux (280-3000 nm)						
MAAs	0.0006*	y = 64.76+0.466x	0.437	0.0005*	y = 62.62+0.45x	0.415
Zooxanthellae	0.6223	y = 39.92-0.0021x	0.011	0.0299*	y = 52.54-0.0091x	0.205
Chlorophyll a + c_2	0.2183	y = 6.99-0.0010x	0.077	0.1006	y = 6.16-0.0013x	0.081
Protein	0.2025	y = 23.68-0.0065x	0.032	0.0937	y = 24.79-0.005x	0.086
Peridinin (carotenoid)	0.0641	y = 6.71+0.0127x	0.153	0.0164*	y = 1.18+0.024x	0.244
b). UV radiation (285-400 nm)						
MAAs	0.0012*	y = 114.78+7.43x	0.400	0.0008*	y = 106.28+7.38x	0.424
Zooxanthellae	0.3368	y = 39.71-0.6995x	0.047	0.0275*	y = 51.81-0.154x	0.210
Chlorophyll a + c_2	0.3177	y = 6.80-0.014x	0.098	0.1176	y = 6.02-0.0216x	0.124
Protein	0.1317	y = 23.66-0.0065x	0.104	0.1027	y = 24.30-0.082x	0.121
Peridinin (carotenoid)	0.1030	y = 8.49+1.88x	0.121	0.0335*	y = 4.50+0.366x	0.197

2. *Sea surface temperatures*

Sea surface temperatures on the reef flat at Cattle Bay followed a clear annual cycle with minima around 20°C in winter and maxima around 32° C in summer during the two years (Figure 2.9). While average summer temperatures for the bay reached approximately 29° C, temperatures over 31° C were recorded in early 1996. This rise in water temperature coincided with the observed loss of zooxanthellae (Figure 2.7a) and a sharp increase in MAA levels (Figure 2.5) in both species.

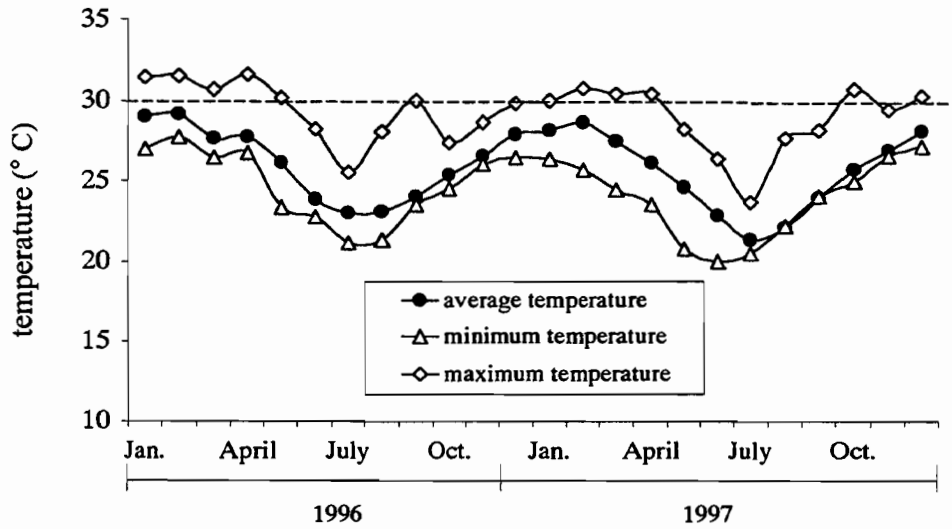


Figure 2.9. Sea temperatures at 2-3 m depth on the reef flat at Cattle Bay (Orpheus Island) during the monitoring period (courtesy R. Berkelmans, GBRMPA). Full circles represent the average monthly temperatures, while open triangles and diamonds present the minimum and maximum temperatures respectively.

Relationship between biochemical tissue concentrations and sea-surface temperatures

Seawater temperatures were positively and significantly related with MAA and peridinin concentrations in both *L. compactum* and *S. flexibilis* (Table 2.5). Conversely, temperature was negatively and significantly correlated with zooxanthellae, chlorophyll, and protein concentrations in both species (Table 2.5). Seawater temperature explained between 32% and 88% of the seasonal variation in these parameters. Multiple linear regression analyses revealed that 70.8% of variation in MAA levels in *L. compactum* and 71% in *S. flexibilis* could be explained by solar radiation and temperature combination (R^2 = 0.708, F = 24.25, df = 2, $p < 0.0001$ for *L. compactum* and $R^2 = 0.710$, F = 24.50, df = 2, $p < 0.0001$ for *S. flexibilis*).

Table 2.5. Summary of regression analyses between monthly average sea temperatures at the study site and MAA, zooxanthellae, chlorophyll, protein and peridinin tissue concentrations in *Lobophytum compactum* and *Sinularia flexibilis* (n = 24). Asterisks indicate that a relationship is significant ($\alpha = 0.05$).

	Temperature					
	Lobophytum compactum			***Sinularia flexibilis***		
Parameter	p-value	nature of relationship	correlation coefficient R^2	p-value	nature of relationship	correlation coefficient R^2
MAAs	0.0006*	y = -197.89+20.35x	0.417	0.0004*	y = -198.86+20.04x	0.412
zooxanthellae	0.011*	y = 49.33-0.45x	0.541	0.014*	y = 58.81-0.444x	0.585
chlorophyll a + c_2	<0.0001*	y = 8.55-0.122x	0.477	<0.0001*	y = 9.91-0.137x	0.537
protein	<0.0001*	y = 42.42-0.867x	0.654	0.0022*	y = 31.54-0.37x	0.321
peridinin (carotenoid)	<0.0001*	y = -21.77+1.38x	0.879	<0.0001*	y = -35.63+1.97x	0.757

2.5. DISCUSSION

The results presented here clearly show that MAA concentrations in shallow water colonies of *Lobophytum compactum* and *Sinularia flexibilis* are significantly correlated to annual cycles in seawater temperature and solar radiation. Furthermore, prior to spawning in both years, female colonies of both soft coral species featured substantially higher MAA levels than their male counterparts. Levels in female corals did not differ from those of males immediately after spawning, presumably because eggs were supplied with high levels of MAAs, providing corroborative evidence for the importance of MAAs for the survival of juvenile soft corals. In addition, this study demonstrates that annual variations in levels of carotenoids, chlorophyll and protein concentrations occur and follow seasonal (temperature) trends, with maxima for carotenoids, and minima for the other parameters during the Australian summer.

2.5.1. The relationship between solar radiation, temperature and MAA levels

Annual patterns in MAAs suggest they are responding to a seasonal cue, which could be driven by solar radiation and temperature alike. Multiple linear regression analyses revealed that 70.8% of variation in MAA concentrations in *Lobophytum compactum* and 71% in *Sinularia flexibilis* can be explained by solar radiation and temperature together. Patterns of high MAA tissue concentrations in summer when incident solar radiation is highest and minima in winter when radiation is lowest are consistent with MAA's functioning as photoprotectants (Jokiel and York, 1982; Shick et al., 1991, Gleason and Wellington, 1993, Shick et al. 1995). Comparable changes in MAA levels were found to be correlated to annual cycles of solar radiation in the mucus of *Fungia repanda* (Drollet et al., 1997).

Production of MAAs involves a substantial investment of nitrogen (Figure 2.3), which is considered to be highly limited in the nutrient-poor waters of coral reefs (Cook, 1983). The observed increase in biosynthesis of MAAs in summer (approximately twice the winter production) when environmental conditions are most extreme, is consistent with their induction constituting a strategy as is commonly reported in terrestrial (reviewed in Greshenzon, 1994) and marine systems (Paul and Fenical, 1986; Coll et al., 1987). Induction of energetically costly MAAs only in response to environmental stress minimises metabolic cost until the ecological benefit of their production can be realised (Hay, 1996). In absolute terms, average concentrations of 251.29 ± 17.32 nmol per mg protein in *Lobophytum compactum* and 328.78 ± 16.05 nmol per mg protein in *Sinularia flexibilis*, were in the range of those reported for

Acropora microphthalma (approximately 220 nmol/mg protein) at comparable depths (Shick et al., 1995), suggesting that soft corals do not invest in the production of MAAs to a higher degree than their hard coral counterparts.

The positive and significant relationship detected between MAA concentrations and seawater temperatures (Table 2.5) is intriguing in light of previous evidence that MAA levels are negatively correlated with temperature in the range of 30- 32° C (Lesser et al., 1990; Glynn et al., 1992). The experimental studies of Lesser and co-workers (1990) on the effects of temperature on MAA levels in *Palythoa sp.*, in fact suggested that these compounds are thermolabile. The positive correlation between MAAs and temperature discerned in this study, however, suggests that degradation of MAAs under typical summer seawater temperatures may not take place. Thermal degradation accompanied by compensatory biosynthesis, masking any temperature effects on MAA levels can not be excluded. To date a protective function of MAAs against thermal stress has not been proposed, yet multiple functions of such compounds cannot be excluded. In fact, observations that mechanical stress (Scelfo, 1985) and water velocity (Jokiel et al., 1997) can also trigger an increase in MAA levels, suggest that stimuli other then high irradiance are involved in the regulation of MAAs.

2.5.2. Composition of MAAs in *Lobophytum compactum* and *Sinularia flexibilis*

The presence of a combination of MAAs with slightly different absorption maxima within marine organisms has been interpreted as a "broad-band" filter against UVR damage (Dunlap et al., 1986). My findings of a MAA profile composed of six different MAAs in *Lobophytum compactum* and *Sinularia flexibilis* is therefore consistent with results from other studies (Shick et al., 1991; Gleason, 1993; Shick et al., 1995). Shinorine, porphyra-p 334, palythine, asterina, palythinol and playthene have regularly been described as typical MAA components in other corals (Shick et al., 1995; reviewed in Dunlap and Shick, 1998). While palythine has been described as a major component in other corals (Shick et al., 1995; Jokiel et al., 1997; Teai et al., 1997), a predominance of nearly 95% (Figure 2.4) as observed in both *Lobophytum compactum* and *Sinularia flexibilis*, has not been reported before. Palythine has its absorption peak at 320 nm, and thus of all the types of MAAs present in *Lobophytum compactum* and *Sinularia flexibilis*, it absorbs radiation most closely in the UV–B region, a region which has been shown to be the major biocidal component in the solar spectrum (Chalker-Scott et al., 1992; Berghahn et al., 1993). UV-B radiation is particularly detrimental because its wavelengths of 290 – 320 nm overlap with the upper absorption range of DNA and proteins. Therefore, lower-end absorption

of UV-B by MAAs such as palythine possibly provide some direct protection for DNA and proteins (Carroll and Shick, 1996). The total radiant flux in the UV-A region is typically at least an order of magnitude higher than that of UV-B (Peterson, 1995), therefore stress by UV-A must not be underestimated. In fact, Warner et al. (1996) implicated UV-A and blue light, and not UV-B as a depressant of photosynthesis in zooxanthellae isolated from *Montastrea annularis* under increased temperature. Hence the primary function of MAAs, such as shinorine, porphyra-334, asterina, palythinol and palythene which all absorb in the UV-A region, is most likely to interact with such wavelengths before they interact with other cellular components to form reactive oxygen species. Dissipation of energy through vibration is believed to be the mechanism by which oxidative stress is alleviated by MAAs (M. Shick, pers. comm.).

2.5.3. Fluctuations of MAAs in soft corals– selective bioaccumulation or active regulation?

One of the most important questions arising from my findings of strong seasonal variations in MAA levels in soft coral tissues, is whether the observed variations merely reflect changes in phytoplankton availability and accumulation or a true compensatory response to changing environmental conditions experienced by the individual corals. To date the metabolic costs for the production, accumulation, and maintenance of MAAs in coral tissues are not known, and the origin of their production in corals has not been conclusively established. Given the high diversity of non-autotrophic organisms that contain MAAs in their tissues, dietary origin of the compounds must be important in organisms such as sea-urchins and holothurians (reviewed in Dunlap and Shick, 1998). For these groups, dietary accumulation and even modification of MAAs through the microbial gut flora have been demonstrated (Shick et al., 1992; Caroll and Shick, 1996). In the symbiotic algal–cnidarian association, however, evidence available to date suggests that the algal partner is the actual producer of the MAAs (Banaszak and Trench, 1995a,b; M. Shick pers. comm.).

Assuming that MAAs produced in the coral association are of algal origin, the question as to how much of the coral's MAA pool is derived from dietary sources and how much through their zooxanthellae partner, remains difficult to establish. Both heterothrophy (Sebens, 1987; Fabricius, 1995a,b,c; Anthony 1999) and autotrophy (Muscatine et al., 1984) are well-established sources of nutrition in corals. If the observed variations were a mere reflection of a highly dynamic phytoplankton availability and selective bio-accumulation, I would expect to see corresponding changes in the MAA profile, as has been observed in sea-urchins (Karentz et al.,

1997, Shick pers. comm.). Feeding experiments with sea-urchins on different food sources produced typical "signatures" of certain MAAs, which remained for a considerable time after the diet was terminated (M. Shick pers. comm.). In this study, however, the percentage contribution of the different MAAs to the total pool remained virtually constant throughout the two year monitoring period in both soft coral species (Figure 2.5). This supports the notion that MAAs were derived from the same source continually and not (predominantly) from variable exogenous sources. In conclusion, while partial dietary acquisition of MAAs cannot be excluded, I suggest *de novo* production in the coral association as a response to environmental conditions is the most likely scenario for the corals tested in this experiment.

2.5.4. Photoacclimatory responses and MAAs

Zooxanthellae densities did not change significantly in either species throughout the 2-year monitoring period, with the exception of a small-scale bleaching event in February 1996 (Figure 2.7a). This stability of the coral-algal symbiosis under-non stress situations during which neither partner outgrows the other, has been described as one of the characteristic features of the symbiosis (Drew, 1972; Hoegh-Guldberg and Smith, 1989b; Jones, 1995). The results of my study are in contrast to findings of Stimson (1997) and Fagoonee and co-workers (1999), however, who proposed seasonally reduced numbers in summer and increased densities in winter in *Pocillopora damnicornis* and *Acropora formosa* respectively. However, both those studies suffer from high variability, unbalanced designs and low replication, placing some doubt on the significance of their findings.

The stability of zooxanthellae levels I found for both species of soft coral suggests considerable host control over the numbers of symbionts with 38 and 47 million algal cells/ per gram wet weight, representing ideal mean densities in *Lobophytum compactum* and *Sinularia flexibilis* respectively. Nutrient and space limitations on the algal partner (Jones and Yellowlees, 1997) and the mitotic index of zooxanthellae specifically have been suggested as levels at which control is exerted by the host (Muscatine and Pool, 1979; McCloskey et al., 1996). McCloskey and coworkers (1996) found expulsion of algal cells to increase directly in response to elevated irradiance, yet the overall algal density remained constant. Similarly, Jones (1995) found the mitotic index of *Acropora formosa* to be highest, when zooxanthellae density was lowest. Both studies suggest that compensatory cell divisions may maintain ideal densities of zooxanthellae, most of which would explain the stability I found in zooxanthellae populations over two years.

In contrast to the stability found for zooxanthellae densities, total chlorophyll levels per individual alga fluctuated strongly with season in both species and were inversely correlated to the thermal conditions (Table 2.5). In absolute terms, levels of total chlorophyll varied between 4 and 6 pg per zooxanthellae in *Lobophytum compactum* and 5.5 and 7 pg per zooxanthellae in *Sinularia flexibilis* throughout the 24 months. This is consistent with the range of 4 -7 pg chlorophyll a per zooxanthellae described for *Acropora formosa* (Jones, 1995). The seasonal fluctuations discerned in this study are in concordance with a number of studies which reported photoinhibition via decreases in chlorophyll concentrations in response to high irradiance levels (Hoegh-Guldberg and Smith, 1989a; Kleppel et al., 1989; Lesser, 1996; Ambarsani et al., 1997; Salih et al., 1998), increased temperature (Iglesias-Prieto et al., 1992; Warner et al., 1996) and a combination of these factors (Lesser et al., 1990; Salih, 1998) in a wide range of coral – algal associations. The significant inverse correlation I found between chlorophyll levels and temperature is particularly intriguing because it is consistent with recent findings that photoinhibition is primarily a function of temperature, which only secondarily leaves zooxanthellae more light sensitive (Jones et al., 1998).

Another component of the photo-acclimatory response system of corals is the carotenoid class of compounds, of which peridinin constitutes as much as 77-84% of the total carotenoid pool, accompanied by smaller quantities of diatoxanthin, diadinoxanthin and β-carotene (Jeffrey and Haxo, 1968; Ambarsani et al., 1997). Peridinin concentrations underwent annual variations similar to those found in the MAA levels, with significantly higher concentrations in summer than in winter in both species (Figure 2.7d). Thus, carotenoid levels in *Lobophytum compactum* and *Sinularia flexibilis* were positively correlated to thermal conditions and those of the latter species also with the solar radiation. (Tables 2.4 and 2.5).

Some of the peridinin within zooxanthellae is non-covalently bound and highly ordered in the so-called peridinin-chlorophyll-protein (PCP) complex (Prézelin, 1976; Iglesias-Prieto, et al. 1992; Iglesias-Prieto and Trench, 1997). It has been firmly established that peridinin serves as a light-harvesting pigment, however, it has also been proposed to act as a photoprotectant of the photosynthetic apparatus (Chang and Trench, 1984; Jeffrey et al., 1997). In evolutionary terms, it is believed that the light-harvesting function was the primary role of carotenoids, with the latter function assumed only secondarily (Jeffrey et al., 1997). My findings of highest peridinin levels when chlorophyll levels were lowest, is possibly a function of changes to compensate for the degradation of chlorophyll, and is consistent with peridinin taking on a light harvesting function. In analogy, an inverse relationship between chlorophylls and xanthophylls has recently been confirmed on a diurnal scale, with higher xanthophyll to chlorophyll ratios after midday when photoinhibiton in zooxanthellate corals is strongest (Brown et al., 1999). The authors concluded

that photoprotection through xanthophylls was the underlying function for this inverse relationship.

Carotenoids are produced *de novo* by the algal partner of the association and within the limits of genetic disposition, high irradiation has been shown to greatly induce their production in a large variety of algae (Harding and Shropshire, 1980; Paerl 1984; Ambarsani et al., 1997). While the actual photo-regulation process is largely unknown, the photo-receptors that modulate carotenoid biosynthesis have been positively identified as chlorophyll, chlorophyllide, cryptochrome and phytochrome (Harding and Shropshire, 1980). Thus a positive feedback regulation mechanism possibly controls chlorophyll and peridinin production, providing a strategy to counteract a loss of efficiency in the photosynthetic apparatus under unfavourable environmental conditions.

While the observation of seasonal maxima for peridinin occurring in summer can be explained by the potential of peridinin to compensate for reduced chlorophyll levels, my findings are also consistent with a photoprotectant role for this carotenoid. Higher protection against further photoinhibition and ultimately degradation of chlorophyll becomes essential during exposure to high solar irradiance and temperature, a scenario corroborated in this study by the significant and positive correlation of MAA and peridinin levels with solar radiation and sea temperatures (Tables 2.4 and 2.5). The function of carotenoids is not based on a UVR absorption capacity which means they do not function as sunscreens as is the case with most MAAs (Shick et al., 1996). Their photoprotective functions are connected to their capacity to quench activated photosensitisers and singlet oxygen (Shimidzu et al., 1996; Jeffrey et al., 1997) and to their potential to act as chain-breaking antioxidants (Kohen et al., 1995). While typically diatoxanthin and diadinoxanthin are described as the photo-protectants in the zooxanthellar system (Ambarsani et al., 1997; Brown et al., 1999), I believe that peridinin has the structural basis to possibly also act in this capacity (for detailed discussion of the structure-function relationship see Chapter 7). Indirect corroborative support for this hypothesis that peridinin has a function other than as a light-harvesting accessory pigment, can be derived from my findings that the eggs of *Lobophytum compactum* are devoid of zooxanthellae, yet feature high peridinin levels (Chapter 7). Thus, my results are consistent with conclusions that peridinin may play either a light-harvesting role, compensating for decrease in chlorophyll concentration, or possibly a photoprotective role in soft corals, counteracting protein tissue damage and degradation of chlorophyll.

2.5.5. The role of MAAs in the reproductive cycle of soft corals

The increases in MAA concentrations in female colonies of the two soft coral species did not occur linearly during the 24 months of their gametogenic cycle. During the last 3 months prior to spawning, concentrations increased rapidly by up to 67% over that of male colonies. While both species followed this strategy. this trend was statistically significant only for *Lobophytum compactum* (Scheffé, $p < 0.0001$). The male-female comparison through time revealed that the trend was (just) non-significant in *Sinularia flexibilis* (Scheffé, $p = 0.0508$). The basis of the observed increase in maternal tissues is the strong accumulation of MAAs in female tissues immediately before spawning, presumably to provide eggs with the UV-protectants, since MAA levels in females dropped dramatically just after spawning. In *Lobophytum compactum*, eggs contain a subset of MAAs identical to those of the corresponding "mothers", however, at approximately 3-times higher levels (Michalek-Wagner and Willis, 2000b, see Chapter 7). My findings of strong increases in MAA levels in females of both soft coral species are consistent with similar patterns found for the sponge *Dysidea herbacea* and the sea-urchin *Mastocarpus stellatus*, where gonads underwent a similar rapid increase in MAA tissue concentrations prior to spawning (Bandaranyake et al., 1997; Carroll and Shick, 1996).

While both *Lobophytum compactum* and *Sinularia flexibilis* have two year oogenic cycles (Aliño and Coll, 1989), the provision of eggs with MAAs appears to occur only at the very final stages of egg maturation (Figure 2.5). The reason for this late provision could be to avoid potential metabolic turnover of MAAs in eggs. Information on metabolism of MAAs is very scarce, and clearance or turnover rates are largely unknown. An approximately six-weekly turnover rate has been suggested for *Zoanthus caribaerum* (Chalker pers. comm. to Scelfo, in Scelfo, 1985). Furthermore, Carreto and co-workers (1990) suggested "a rapid metabolism" for MAAs in the red-tide dinoflagellate *Alexandrium excavatum*. Helbling et al. (1996) provide the only other information available and propose a MAA loss rate 4-5 times less than the rates of synthesis in the alga *Thalassiosira*. My findings of delayed supplementation of soft coral eggs with MAAs are consistent with turnover rates being rapid, so that the investment into MAAs is avoided before it is needed.

The high metabolic cost involved in providing eggs with MAA levels 3-fold more than those of adult tissues, appears to be well compensated for by the photoprotective properties that have been established for MAAs in eggs. Gleason and Wellington (1995) provided evidence that eggs with higher MAA levels experienced lower mortality when exposed to UV-B than their conspecifics with lower levels. Photoprotection has also been confirmed for the eggs of the sea-

urchin *Strongylocentrotus droebachiensis* by the inverse relationship between MAA levels and UVR–induced inhibition of early cell division (Adams and Shick, 1996).

While protection against high irradiances has been conclusively established as an important function of MAAs in early life stages of benthic invertebrates, MAAs have also been implicated as development control regulators in terrestrial fungi (Leach, 1965) and some marine invertebrates (Grant, et al., 1985; Bandaranyake et al., 1997). For example Bandaranyake and co-workers (1997) found MAA concentration changes were linked to the reproductive cycle rather than to the solar radiation cycle, they concluded that MAAs probably act as reproductive regulators. Grant et al. (1985) observed that concentrations of MAAs decreased in developing cysts of *Artemia sp.*, with motile cysts being devoid of MAAs. At the same time, however, they observed an increase in an MAA precursor, gadusol (a potential antioxidant), suggesting that a "retrobiossynthetic" conversion had taken place (Grant et al. 1995; Dunlap and Shick, 1998). This in turn suggests yet another function of (some) MAAs and their precursors, i.e. to act as an antioxidant as has been shown for mycosporine-glycine and a number of gadusol-derivatives (Dunlap and Yamamoto, 1995; Dunlap and Shick, 1998). Thus, while my study did not attempted to assess the role of MAAs as reproductive hormones, it is possible that MAAs fulfil multi-functional properties in corals, extending the adaptive value of MAAs beyond UV protection.

2.5.6. Regulation of MAA synthesis and translocation

Dietary origin and bio-accumulation of MAAs in gonads prior to spawning has clearly been established in heterotrophic invertebrates (Carroll and Shick, 1996; Carefoot et al., 1998). Assuming that MAAs are produced by the algal partner in response to prevailing environmental conditions in soft corals, the significant concentration increases in female gonads suggest mechanisms of up-regulation other then environmentally induced changes. A phenomenon termed "host release factor" is recognized as the mechanism by which the animal regulates translocation of photosynthetic products from their algal partner (Cook, 1983). While the underlying molecular mechanism by which the control is exerted has not been conclusively established, it has been demonstrated experimentally that host extract added to zooxanthellae significantly increases translocation rates, and that these are correlated to host protein concentrations of the extract (Sutton and Hoegh-Guldberg, 1990; McDermott and Blanquet, 1991). By analogy,

it is possible that MAA production and translocation from the alga to the animal tissue is closely controlled by the host, thus ensuring increase in the gonads at times when environmental conditions would not stimulate intensive production of MAAs.

An alternative, yet not mutually exclusive, explanation for the regulation of MAAs prior to spawning includes the storage of the zooxanthellae-produced MAA precursor at all times in the animal tissue. It is possible that the algal partner does not produce the final MAA product, but only its precursor, gadusol, which could be modified by the host after translocation to build the final MAA product. A similar shared production is believed to occur with certain sterols, such as gorgosterol, in soft corals (Anderson, 1985). While my study did not attempt to establish whether shared production of MAAs is carried out within the soft coral zooxanthellae association, the possibility of a shared production serves as a suitable model to explain the sharp MAA increase prior to spawning. When partitioning MAA concentrations in animal and plant tissue I typically found less then 8 % of the MAA pool resident in the algal tissue (Figure 2.6). This is consistent with findings of Shick et al. (1995) who detected only about 5% percent of MAAs in the algal fraction of *Acropora microphthalma* at a comparable depth. The much higher MAA levels in the animal tissue is in accordance with the theory that the coral host is the "first line of defence" against photodamage of its symbiotic algae (Dunlap and Shick, 1998). However, it also suggests that irrespective of origin and the mode of regulation, most of the MAA pool is under host control and thus may be transferred to the eggs at any desired time.

2.5.7. Summary and conclusions

In conclusion, MAA production so closely linked to environmental conditions suggests that they are an integral component of the soft corals' biochemical defence system against high irradiance and/or temperature stress and thus bleaching. This is further corroborated by the higher production of MAAs in females than males prior to spawning, presumably to protect the eggs and progeny from high solar radiation. As much as MAAs may contribute to photoprotection, they cannot provide full protection against bleaching, rather they constitute one line of a sophisticated biochemical defence system which includes other components such as anti-oxidants and oxygen detoxifying enzymes.
My data suggest that carotenoids possibly play an important role in the prevention of photoinhibition, given the highly inverse relationship between peridinin and chlorophyll throughout the seasons. Findings of extensive seasonal fluctuations

in the levels of carotenoids and chlorophyll, combined with virtually constant zooxanthellae levels, furthermore suggest a considerable degree of plasticity in photo-acclimatory strategies available to soft corals.

Chapter 3

The effects of high irradiance and temperature on tissue concentrations of UV-absorbing MAAs

Bleached *Sinularia flexibilis* on partially bleached *Porites sp.* during a natural bleaching event at Orpheus Island, March 1998

3.1. ABSTRACT

Mycosporine-like amino acids (MAAs) are believed to be an important component of a coral's biochemical defence system against high irradiance, and thus could play a role in their protection against irradiance stress during coral bleaching episodes. This study investigates the role of temperature and UV radiation (UVR) alone and in combination on levels of MAAs, using manipulative bleaching experiments and data collected during a natural bleaching episode. UVR and temperature were found to act synergistically in the bleaching process in the soft corals *Lobophytum compactum* and *Sinularia flexibilis*, i.e. the combined treatment leading to greater losses of zooxanthellae and chlorophyll than the sum of single treatments. The long standing notion that MAAs are thermo-labile, and thus chemically degraded during a thermal bleaching event, however, could not be confirmed. To the contrary, MAA levels increased in response to simultaneous exposure to elevated temperature (32° C) and UVR, in experimentally and naturally bleached colonies, suggesting increased resource allocation towards photo-protection in corals already experiencing thermal stress. Hence I found no evidence that thermal degradation of MAAs in corals is the mechanism responsible for the accelerated bleaching responses observed in corals exposed simultaneously to elevated temperature and irradiance. Subsequent studies with high and low MAA-acclimatised soft corals, which examined whether increased investment into MAAs results in reduced bleaching, revealed that there was no ecological advantage in high MAA acclimatised colonies when bleaching was solely triggered by increased temperature. When solar radiation alone, or in combination with temperature, act as stress factors, however, increased MAA levels do translate into higher protection against bleaching in soft corals.

3.2. INTRODUCTION

While most large-scale bleaching events recorded during the last two decades have been attributed to thermal stress, there is an increasing understanding that other environmental co-factors have the potential to greatly increase the bleaching response (reviewed in Brown, 1997a; Hoegh-Guldberg, 1999). To date, a number of studies have addressed the interactive effects between temperature and salinity (Coles and Jokiel, 1978; Hoegh-Guldberg and Smith, 1989a), temperature and nutrients (Maté, 1997), temperature and pathogens (Kushmaro et al., 1996; 1997) and temperature and high irradiance (Coles and Jokiel, 1978; Reaka-Kudla, 1993; Dennis and Wicklund, 1993; Lesser et al., 1990; Lesser, 1996; Drollet et al., 1994; Salih et al., 1998) and provided some evidence for greatly increased bleaching response when both factors occur together. In fact, the severity of the 1998 mass bleaching event on the GBR, during which 87 % of inshore reefs showed signs of bleaching, is believed to be based on a combination of high freshwater run-off caused by cyclone Sid, followed by elevated temperatures and increased irradiance penetration due to very calm conditions (Berkelmans and Oliver, 1999). Among the (co-) factors causing bleaching, possible synergistic interactions between temperature and light, both in the visible and UV region have received most attention, since the highest incidence of coral bleaching usually occurs in shallow water (Berkelmans and Oliver, 1999) and because bleaching preferentially starts on the upper, light-exposed surfaces of corals (Harriott, 1985; Hoegh-Guldberg, 1999).

Key observations relating bleaching to the general phenomenon of photoinhibition have been brought forward for a number of years, suggesting that the disruption of photosystem II reaction centres and the subsequent loss of the oxygen-evolving complex as the initial site of temperature damage (Renger et al., 1989; Aro et al., 1993; Warner et al., 1996). More recent evidence suggests that the initial impact of temperature is disruption of the electron flow to the dark reactions of photosynthesis, leaving zooxanthellae subsequently more sensitive to light, which means that damage to the PS II is only secondary (Jones et al., 1998). This model therefore provides an important basis for the understanding of the interaction of light and temperature, where photodamage is primarily a function of temperature (Jones et al., 1998). It is based on findings that elevated temperatures *in Stylophora pistillata* initially results in a decreased capacity to process the excitation energy coming from the dark reactions of photosynthesis. Subsequently, zooxanthellae become more sensitive to light, resulting in an over-reduction of biochemical components of the light reactions, which eventually leads to destruction of chloroplasts and photoinhibition (Jones et al., 1998). The subsequent discarding of thermally damaged and dysfunctional zooxanthellae from the coral association (Suharsono et al., 1993) provides therefore an explanation for bleaching.

If corals under thermal stress are indeed more sensitive to light, investment into protection against high solar irradiance through biochemical defence systems, such as MAAs, could represent a crucial strategy to avoid a potentially fatal combination of the two stress factors. Yet, thermal degradation of MAAs, and hence protection against solar damage, has been suggested to take place in coral exposed to elevated temperatures (Lesser et al., 1990; Glynn et al., 1992). If this interpretation is correct, a coral already under thermal stress would be exposed to additional irradiance, and particularly UV stress (Lesser et al., 1990). The effects of interaction between temperature and light on MAAs levels could therefore serve as a further model to explain the observed exacerbated bleaching response when both stress factors occur simultaneously (Lesser et al. 1990; Coles and Jokiel, 1978; Salih et al., 1998). To date, there have been no systematic comparisons of MAA levels in bleached corals and compared to those of their unbleached counterparts, in order to test the hypothesis that lack of bleaching may be mediated by elevated production of MAAs. The very limited observations reported so far were by Drollet and co-workers (1997) who found no "noticeable shift" in MAA concentrations during a bleaching episode in Tahiti (yet did not supply any data). Given the possibility that MAAs may play a role in the understanding of a possible synergistic interaction between temperature and light, research into the fate of MAAs during the bleaching process have been identified as an area that warrants study in a major review of MAAs by Dunlap and Shick (1998).

My general aim in this chapter is to determine the nature of the interaction between temperature and UVR leading to the bleaching response in *Lobophytum compactum* and *Sinularia flexibilis*. If the interaction is synergistic, then I will investigate whether thermal destruction of MAAs is the key to understanding the interaction using an experimentally simulated bleaching disturbance. In order to evaluate the role of MAAs in soft corals during a natural bleaching disturbance, I will compare MAA levels in bleached and unbleached conspecifics. Particular emphasis will be paid to determining whether thermal destruction or (possibly) active up-regulation of MAA levels occurs under bleaching conditions. Finally, I will investigate whether higher resource allocation towards MAAs in *Lobophytum compactum* also translates into increased protection against bleaching. This will be done by comparing the bleaching responses in colonies with experimentally manipulated high and low MAA levels, following exposure to experimental thermal and irradiance stress.

3.3. MATERIALS AND METHODS

3.3.1. Effects of elevated temperature and UV radiation alone and in combination on MAA levels and bleaching responses

1. Experimental design: In order to examine the impact of elevated temperature and UVR separately and in combination on bleaching responses and MAA levels, I subjected colonies of *Lobophytum compactum* and *Sinularia flexibilis* to experimentally manipulated temperature and UVR treatments. Tank facilities at the Orpheus Island Research Station (OIRS) were used during May and June 1996 for experimental manipulations. Experimental colonies (n = 64) were randomly collected from the reef flat in Pioneer Bay, tagged, dislodged with some basal substratum attached, and left *in situ* (firmly wedged into crevices in the reef) to recover for four months prior to the start of the experiment. All colonies were similar in size, that is stalk diameters ranged from 5-7 cm for *Sinularia flexibilis* and colony diameters averaged 13-15 cm for *Lobophytum compactum*. In May 1996 corals were transported to facilities at OIRS, where they were mounted in 10 cm high stands and placed in aerated flow-through tanks under a solar weave™ roof, to prevent rainwater dilution and rain damage to the UV lights.

Each factor, temperature and UV radiation, was examined at two treatment levels, ambient and elevated. Thus, there were four treatments in total: a control in which both factors were maintained at ambient levels, an elevated temperature (ambient UVR) treatment, an elevated UVR (ambient temperature) treatment, and a treatment in which both factors were elevated (combined treatment) (Figure 3.1). To account for possible induction of MAA levels due to mechanical stress (as suggested by Scelfo, 1985), colonies of each species were tagged, but left *in situ* as handling controls and sampled at the same intervals as experimental corals. Each of the four treatments was replicated in four tanks, each of which was supplied with four individuals of both soft coral species (Figure 3.1). In order to simulate a slight, moderate and strong UVR and temperature stress episode, samples were collected at time 0 (before) and after one, two and three weeks of exposure. Two tips of each colony were collected, frozen immediately at -20° C and kept under dark conditions until analysed biochemically for MAA, zooxanthellae, protein and chlorophyll tissue concentrations. Coral colonies were returned to their site of origin upon termination of the experiment.

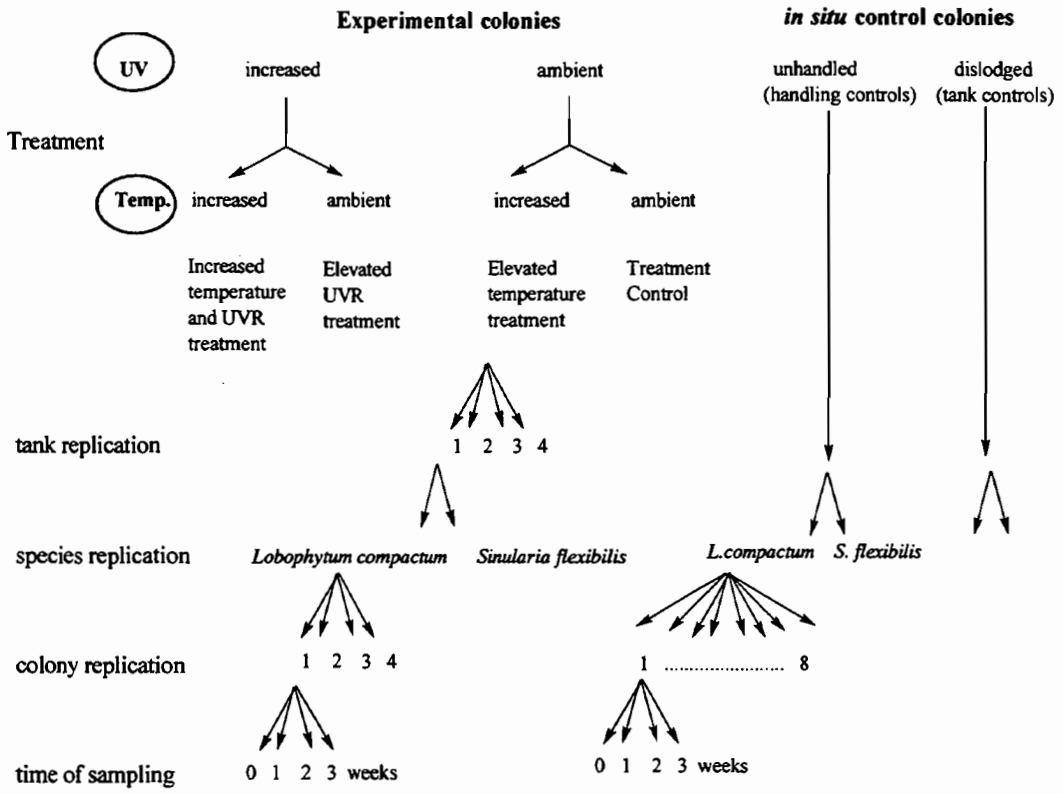


Figure 3.1. Experimental design and set-up to test the effects of UV radiation and temperature alone and in combination on biochemical parameters (MAA, zooxanthellae and chlorophyll tissue concentrations) in *L. compactum* and *S. flexibilis*. Colonies were sampled at time 0, 1, 2, and 3 weeks after exposure to the experimental treatments.

2. Technical details of experimental set-up: Elevated temperature treatments were heated to constant 32 ± 0.5° C and temperature monitored by a computer-controlled heating system developed by Ray Berkelmans and described in Berkelmans and Willis (1999). Seawater, of ambient (26 ± 2.0°C) and elevated temperature, was pumped through the 56 litre tanks at 1 litre/min, resulting in a complete turnover of resident water approximately every hour. In addition, submerged powerhead pumps were used to increase water circulation in the tanks. The elevated UVR treatment was achieved by using Philips TL'40 W/ 05 actinic lights, which have a radiation peak at 360 nm, fitted on top of the tanks (Figure 3.2). Spectral irradiance emitted in the UV-B region equates approximately to 1584 J/m^2/day and that of UV-A to 10132J/m^2/day (International light Inc. Radiometer/Photometer, model IL 1400A, courtesy Malcolm Shick). The UV-A to UV-B ratio was equivalent to natural conditions where UV-A is

typically one order of magnitude higher then UV-B (Peterson 1996). To put this into biological perspective, ambient fluxes were 21% and 17% higher for UV-B and UV-A respectively than fluxes typically experienced on an average cloudless summer day at Orpheus Island (calculations based on UVR data provided by W. Dunlap, unpublished). UV light was provided on a 10-hour-on 14-hour-off day/ night cycle (Figure 3.2).

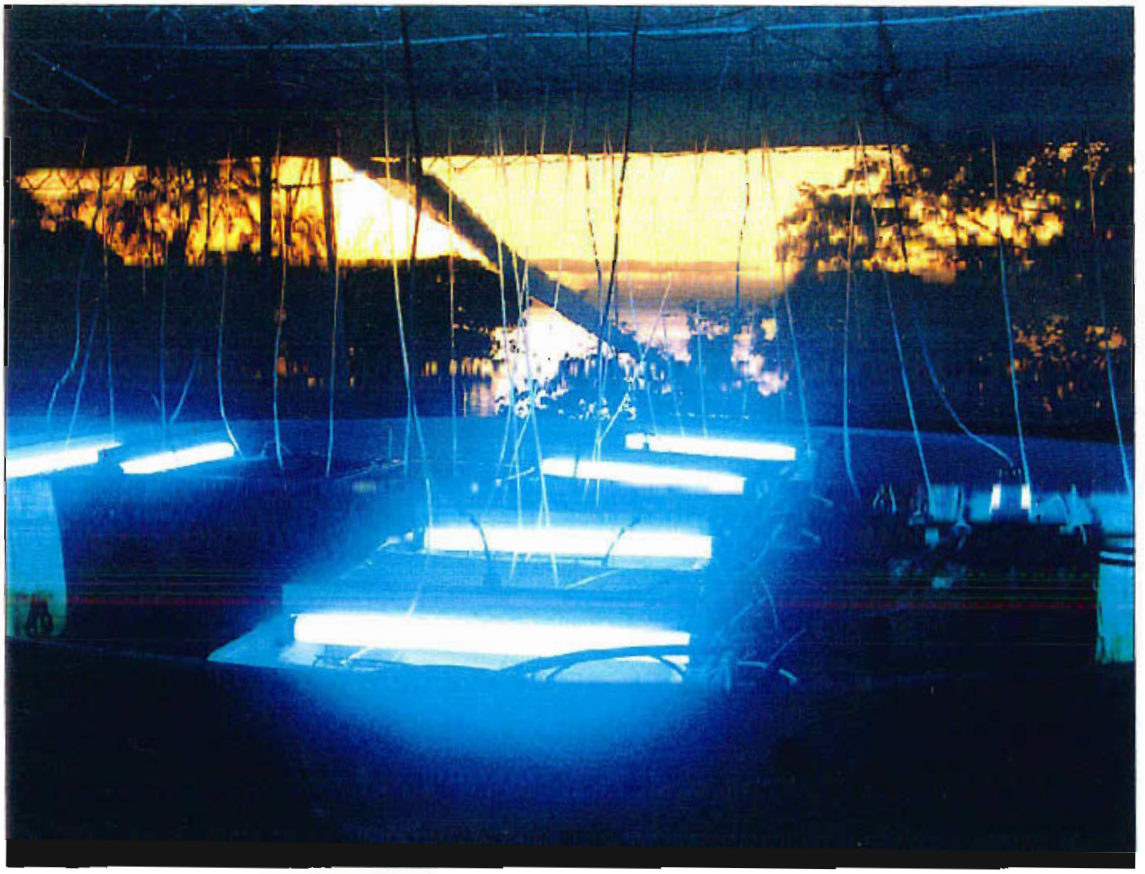


Figure 3.2. Experimental tank set-up under solar weave roof with UV lamps and aeration tubing at Orpheus Island.

The spectral quality of radiation experienced by corals under the solar weave roof was equivalent to PAR conditions at 4-5 m water depths in Pioneer Bay (calculations based on data acquired with a LiCor lightmeter, data courtesy of Ken Anthony). For corals in ambient light treatments, fluxes for UV-A were approximately 8% lower and those for UV-B were 14% lower than experienced by corals in their natural environment on a summer day, which was designated as ambient for the purposes of this experiment (calculations are based on UVR profiles carried out in Pioneer Bay, data courtesy Walt Dunlap, AIMS, unpublished). Given the high variability in incident solar radiation (Figure 2.8, Chapter 2), variations in the order of 8% and 14 % for UV-A and UV-B respectively are probably biologically insignificant. Yet, to

account for potential masking effects (i.e. falling MAA levels as a consequence of the experimentally slightly reduced UVR due to the solar weave roof), eight colonies of each species from the same depth were tagged and dislodged as described above for the experimental corals, but left *in situ* as tank controls and sampled at the same intervals.

3. Biochemical analysis: Zooxanthellae, chlorophyll, protein and MAA tissue concentrations were analysed as described in Chapter 2, section 2.4.

4. Coral survivorship: The survival of all test corals was assessed at the end of the 3-week treatment period and 3 months after their return to the reef of origin. Disintegration of colony tissue was used as a parameter to differentiate between dead and live corals.

5. Statistical analysis: Differences in zooxanthellae levels, chlorophyll, protein and MAA tissue concentrations were tested separately for each species, using 3-factorial nested ANOVA with time and experimental treatment as fixed and orthogonal factors with tank nested in the latter two. Considering the polyp to be the sampling unit, which was different for any given sampling time, "time" was used as a factor in a 3-factorial ANOVA. Post-hoc tests could not be done on the results of the 3-factor ANOVA to identify which experimental treatments and times were significantly different because the significant interaction terms indicated that patterns were not consistent through time. Therefore, to distinguish between the treatment effects at any given time one-factorial ANOVA, followed by multiple post hoc comparisons were carried out, using the Scheffé post-hoc test (Underwood, 1997). Prior to analysis of variance, data were tested for normality (Shapiro-Wilks statistic) and homogeneity of variances (Cochran and Bartlett's test) and where necessary data were square root transformed. All analyses were carried out using SPSS 7.5 and SUPERANOVA software.

3.3.2. Comparison of MAA levels in naturally bleached (mass bleaching event 1998) and unbleached soft corals

1. Sample collection: During a joint field trip and in co-operation with Katherina Fabricius, AIMS (on board MS "Harry Messel") in April 1998, bleached and unbleached colonies of the alcyonacean soft corals *Lobophytum compactum, Sinularia flexibilis, Sinularia capitalis* and *Sarcophyton sp.* (Figure 3.3) were collected at three sites at Fantome Island and one site at Great Palm Island (adjacent to Orpheus Island) (see Chapter 2, Figure 2.1). Collection from several sites in the Palm Islands was necessary to ensure sufficient replication of colonies from comparable depths.

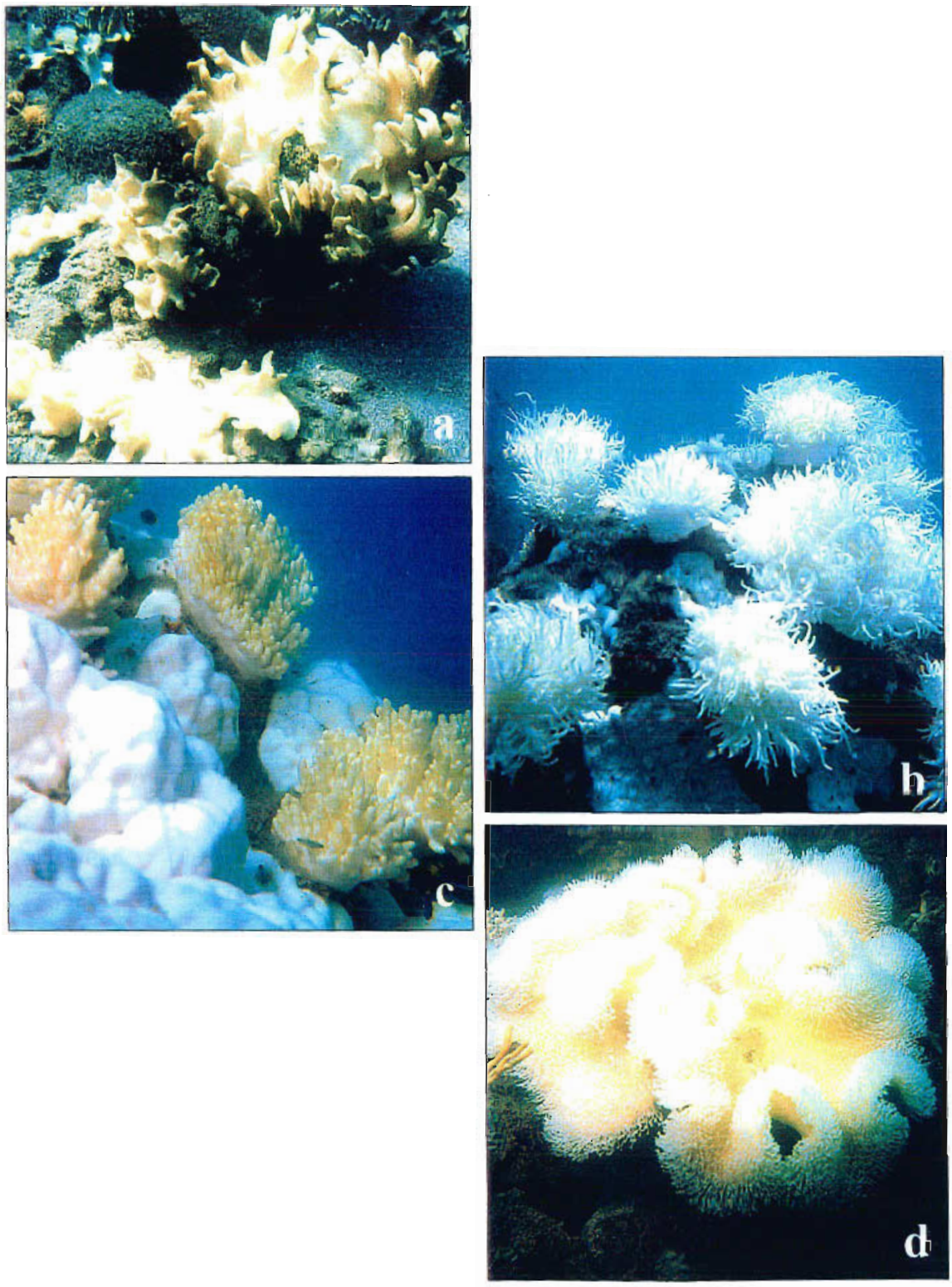


Figure 3.3. Bleached colonies of the four soft coral species: a) *Lobophytum compactum*, b) *Sinularia flexibilis*, c) *Sinularia capitalis* (on *Porites sp.)* and d) *Sarcophyton sp.* Sampled from the Palm Islands during the natural mass bleaching event in 1998.

*2. **Sampling regime and processing:*** Tissue samples of naturally bleached individuals were collected from 1-3 m, whereas unbleached counterparts were sampled at 5-6 m, since no unbleached colonies occurred above these depths. Determinations of zooxanthellae densities, plus chlorophyll, peridinin (carotenoid) and MAA concentrations were carried out as described in Chapter 2. To explore the relationship between depth and MAA concentrations in *L. compactum*, tissue samples of colonies collected prior to the bleaching event in January 1997 from 1 to 13 m depths were examined for their MAA levels (n = 5 colonies per depth). The compounds were extracted as described in Chapter 2 and MAA concentrations quantified spectro-photometrically as absorption at 320 nm.

*3. **Statistical analysis:*** Comparisons of MAA levels were carried out firstly between unbleached and bleached soft coral colonies affected by the 1998 mass bleaching event. Secondly, MAA levels were compared between colonies affected by the natural bleaching episode in 1998 and those of the same species at the same depths in non-bleaching years, which had been collected for 1996 and 1997 (presented in Chapter 2). The non-parametric Mann-Whitney test was used for both comparisons, since even after transformation data still failed to comply with the assumptions of ANOVA for homogeneity of variances. The data set collected during the natural bleaching event was much smaller than that collected in previous years, therefore three random equal number data sets were generated from the larger data set and tested using Mann-Whitney-tests. Regression analysis was carried out to establish whether the MAA - depth relationship in a non-bleaching year was statistically significant. In order to compare individuals from different depths in the bleaching year (1998), MAA concentrations for the deep samples were adjusted to values that would be predicted at the shallow depth for that sampling date (given no elevated exposure to UVR), using the relationship gained through regression analysis. The adjusted values were then compared using one-way ANOVA.

3.3.3. Do higher MAA levels protect against bleaching?

To test whether soft corals with higher MAA tissue levels experience less bleaching under elevated temperature or irradiance conditions, conspecifics with different MAA levels were submitted to experimental solar and temperature treatments, with a design similar to that described in section 3.3.1. (for details see below).

*1. **Translocation to achieve bathymetric MAA acclimation.*** To obtain colonies with different MAA levels, 32 individuals of *Lobophytum compactum* (disk diameter approximately 20 cm) were dislodged from the reef at 4 m in Cattle Bay, Orpheus Island in September 1996. Half of

them were left at the depth of origin and the remaining 20 were translocated from 4 m to 15 m within Pioneer Bay so that a bathymetric adaptation would result in colonies with low MAA levels individuals. Given that Pioneer Bay is a high turbidity environment, this translocation equated to fluxes in the PAR, UV-A and UV-B region of approximately 6.75%, 2.75% and 0.98% of those experienced at 3-4 m depths (calculations based on PAR and UV profiles carried out by Walt Dunlap in Pioneer Bay). Corals were maintained at the deep site for five months and in February 1997, after a decrease of MAA levels in the deep translocated corals had been confirmed, colonies were returned to their depth of origin (4m) in stages. Colonies were brought from 15m to 10m, then to 7m and finally to 4 m, remaining at each depth for 3 days to minimise light-related stress associated with a sudden increase in light intensity. This process almost certainly resulted in an increase in MAA levels (not analysed), yet analysis of samples immediately prior to the start of the experiment confirmed that levels were still approximately 50% lower than those of high-MAA acclimatised colonies.

2. Experimental solar irradiance and temperature treatment. After this step-by-step relocation of deep colonies to 4 m, "low-MAA" acclimatised colonies and those colonies that were maintained at 4m for the five months ("high-MAA acclimatised") were transferred to aerated flow-through tanks at OIRS. Colonies of both groups were then submitted to 4 experimental treatments, similar to those described in section 3.3.1. (control, high solar irradiance, increased temperature and a combination of the latter two factors) experimental treatment was only assessed at time 0 (before) and time 1 (after 10 days of exposure). The only difference in the experimental set-up was that increased irradiance was not supplied in the form of UV lights, but by placing tanks in the open sun, exposing test corals to increased PAR and UV radiation at the same time. All treatments were carried out in duplicate with four colonies in each tank. Zooxanthellae densities, chlorophyll, protein and MAA levels were analysed as described in Chapter 2, section 2.3.2. Two tissue subsamples per colony were collected before the experimental treatment and after 10 days (i.e. 4 treatments x 2 times x 8 colonies x 2 subsamples). Upon termination of the experiment, colonies were returned to their site of origin.

3. Statistical analysis: Three-factorial nested ANOVA, with MAA–levels and experimental treatment as fixed and orthogonal factors and tank being nested in treatment was used to test for significant differences in biochemical parameters. To distinguish between the treatment effects after 10 day exposure, post hoc comparisons were carried out using the Scheffé post-hoc test (Underwood, 1997). One-way ANOVA was used to compare MAA concentrations between low and high-light acclimatised colonies. As a conservative measure, MAA levels were re-normalised to mean protein concentrations of the unbleached colonies.

3.4. RESULTS

3.4.1. Effects of temperature and UV radiation alone and in combination on MAA levels and bleaching

Analysis of variance confirmed that in both *L. compactum* and *S. flexibilis*, experimental treatments and time of exposure had highly significant effects on zooxanthellae densities, as well as on chlorophyll, protein and MAA levels (Table 3.1). The presence of significant interaction terms between time and treatment for all parameters assessed (with the exception of protein concentration changes in *L. compactum*), however, suggests that the effect of treatment was highly dependent on the time of exposure. For all biochemical parameters assessed, tank had no significant effect, either as single factor, in first order interactions with time or treatment, or in second order interactions with treatment and time (Table 3.1). Thus irrespective of the treatment and the time of exposure to the treatment, placing the experimental colonies in tanks had no impact on levels of any of the biochemical parameters.

Table 3.1. Summary of results of 3-factorial ANOVA, testing the effects of time, experimental treatment and tank on levels of various biochemical parameters (MAAs, zooxanthellae, chlorophyll and protein) in two species of soft corals. Asterisks denote significant differences from controls for an alpha level of 0.05; (N = 32 determinations, i.e. 16 colonies per treatment x 2 subsamples per species).

Source of variation		Total MAAs		Zooxanthellae		Total chlorophyll		Protein	
L. compactum	df	F-ratio	p-value	F-ratio	p-value	F-ratio	p-value	F-ratio	p-value
time	3	16.94	<0.0001*	403.16	<0.0001*	86.81	<0.0001*	7.93	<0.0001*
treatment	3	10.41	<0.0001*	398.03	<0.0001*	90.98	<0.0001*	9.97	<0.0001*
tank	3	0.68	0.673	0.51	0.562	0.07	0.976	0.39	0.763
time x treatment	9	3.73	<0.0001*	138.91	<0.0001*	37.12	<0.0001*	0.96	0.471
time x tank	9	0.43	0.966	0.33	0.920	0.21	0.993	0.29	0.993
treatment x tank	9	0.72	0.95	0.44	0.694	0.09	0.999	0.21	0.977
time x treatment x tank	27	0.45	0.999	0.24	0.992	0.10	0.999	0.20	0.999
S. flexibilis									
time	3	19.42	<0.0001*	702.69	<0.0001*	138.32	<0.0001*	27.81	<0.0001*
treatment	3	13.34	<0.0001*	565.03	<0.0001*	131.04	<0.0001*	23.01	<0.0001*
tank	3	0.17	0.948	0.12	0.919	0.06	0.981	0.18	0.909
time x treatment	9	3.76	<0.0001*	177.18	<0.0001*	38.10	<0.0001*	5.34	<0.0001*
time x tank	9	0.29	0.997	0.16	0.977	0.61	0.789	0.25	0.986
treatment x tank	9	0.33	0.994	0.2	0.967	0.48	0.891	0.35	0.957
time x treatment x tank	27	0.49	0.999	0.25	0.987	0.53	0.976	0.64	0.919

1. Analysis of individual treatment effects on MAA, zooxanthellae and chlorophyll levels

A clear pattern of increase in MAA levels with ongoing treatment time was found in both *Lobophytum compactum* and *Sinularia flexibilis* in all experimentally manipulated colonies but not in the controls (Figure 3.4a, e). The reverse pattern of a decrease with increasing time of exposure was discerned for zooxanthellae densities, chlorophyll and protein concentrations in both species (Figure 3.4b-h). All colonies of both soft coral species submitted to experimental treatments and all field controls survived the course of the three-week experiment. The group means for all response variables are summarised in Table 3.2, while all ANOVA results are presented in Table 3.3.

Effects of elevated temperature and UV on MAA concentrations. Increases in MAA concentrations with time were detected in all but the control treatments in both soft coral species. (Figure 3.4a, e). Prior to the start of the experimental treatments, colonies randomly assigned to the four groups did not differ significantly in their MAA tissue levels which averaged 214.99 ± 21.21 SE nmol and 267.3 ± 30.9 SE nmol per mg protein for *Lobophytum compactum* and *Sinularia flexibilis* respectively (Table 3.3).

Lobophytum compactum: By the second week of exposure MAA levels had significantly increased by 41% in the combined treatment (Scheffé, p= 0.0019). MAA levels had also increased in the UVR and temperature treatments after two weeks of exposure by 12% and 20% respectively, but these increases were statistically not significant. After 3 weeks, marked and significant increases of 52% and 79% in comparison to controls were found in the UVR (Scheffé, $p = 0.040$) and the UVR and temperature combined treatments (Scheffé, $p < 0.0001$) (Figure 3.4a). Post hoc comparisons between MAA levels in the UVR and the combined UVR and temperature treatments after three weeks indicated that while MAA levels increased most in the combined UVR and temperature treatment, means in these two did not significantly differ from each other (Scheffé, $p = 0.371$). While the temperature treatment resulted in MAA levels increasing by 38 %, this was not significant according to Scheffé post hoc-tests (p= 0.054), which is the most conservative type of post hoc analysis. Analyses using three other post hoc tests (Fisher-PLSD, Student-Newman-Keuls, and Tukey-Kramer), however, all suggest that the increase in MAA concentrations is significant (all, $p < 0.05$). In order to test whether MAA increases were merely a reflection of the decrease in protein experienced at the same time, MAA tissue concentrations (at week 3) of all treatments were re-normalised to control (pre-bleaching) protein levels. A one-way ANOVA detected significant differences in MAA concentrations normalised to pre-bleaching protein levels among the 4 experimental and control treatments (one-way ANOVA, $F = 6.547$, $df = 1$, $p = 0.0004$). All treatments resulted in a significant increase in comparison to

controls (UVR: Scheffé, p = 0.0028; temperature: Scheffé, p = 0.00277; combination treatment: Scheffé, p < 0.0001). UVR and temperature were not different in their capacity to increase MAA concentrations (Scheffé, p = 0.4141). The combined treatment resulted in significantly higher MAA levels than the temperature treatment (Scheffé, p = 0.0403) but not the UVR treatment (Scheffé, p = 0.2126).

Sinularia flexibilis: Following one week of exposure no significant differences in MAA levels could be discerned among the four treatment groups (Table 3.3). Significant increases from 267.3 ± 30.9 SE nmol to 333 ± 31.18 SE nmol total MAAs per mg protein were detected in conspecifics exposed to the combined temperature and UVR treatment after two weeks (Scheffé, p = 0.0225), while the slight increases in temperature and UVR treated colonies were statistically non significant (Scheffé, p=0.2654 and p = 0.8902 respectively) (Figure 3.4e). After three weeks, as in *L. compactum* all treatments resulted in significant increases in MAA levels on a protein basis. Temperature treatment resulted in the mildest increase (32%, Scheffé, p < 0.0001), followed by UVR (43%, Scheffé, p < 0.0001) and the strongest impact was caused by the combined treatment (65%, Scheffé, p < 0.0001) (Figure 3.4e).

In order to analyse whether MAA increases were a reflection only of the decrease in protein experienced at the same time, MAA tissue concentrations (at week 3) of all treatments were re-normalised to "pre-bleaching" protein levels. The effects differed significantly among the four treatments (one-way ANOVA, F = 9.491, df = 1, p < 0.0001). Results of a post hoc, confirmed that significant increases occurred in the UVR, (Scheffé, p < 0.0001), temperature (Scheffé, p = 0.0175) and combined treatments (Scheffé, p < 0.0001). As in *L. compactum*, UVR and temperature did not differ in their capacity to increase MAA concentrations (Scheffé, p = 0.0603), the combined treatment, however, resulted significantly greater increases than temperature (Scheffé, p = 0.00182) but not the UVR treatment (Scheffé, p = 0.6199).

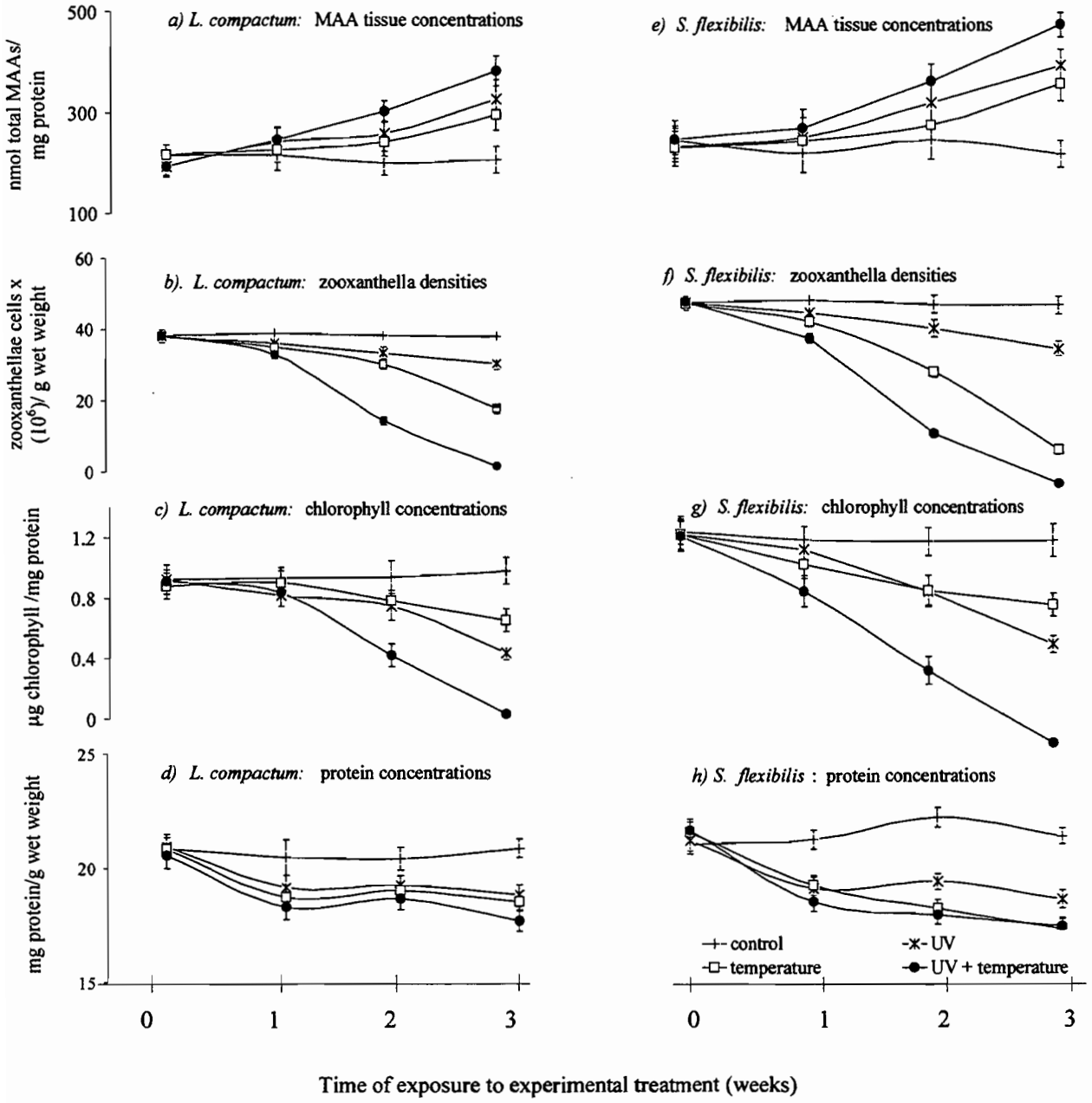


Figure 3.4. Changes in levels of biochemical parameters through time in tissues of *Lobophytum compactum* and *Sinularia flexibilis* submitted to experimentally elevated temperature and UVR treatments: a) + e) MAAs, b) + f) zooxanthellae, c) + g) chlorophyll and d) + h) protein. N = 32 determinations, (i.e. 16 colonies x 2 subsamples per species). Controls represent experimental treatment controls. Error bars denote standard errors.

Table 3.1. Summary of results of 3-factorial ANOVA, testing the effects of time, experimental treatment and tank on levels of various biochemical parameters (MAAs, zooxanthellae, chlorophyll and protein) in two species of soft corals. Asterisks denote significant differences from controls for an alpha level of 0.05; (N = 32 determinations, i.e. 16 colonies per treatment x 2 subsamples per species).

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time x treatment x tank	27	0.49	0.999	0.25	0.987	0.53	0.976	0.64	0.919

Table 3.2. Group means for response variables (MAAs, zooxanthellae, chlorophyll, protein) to four experimental treatments in *Lobophytum compactum* and *Sinularia flexibilis*; (con. = control; ↑UVR = increased UVR; ↑Temp. = increased temperature; ↑ Temp. + UVR = increased temperature and UVR); means are based on 32 determinations (i.e. 16 per treatment x 2 subsamples per species). Asterisks denote significant differences from the control.

Time of exposure to treatments	***Total MAAs*** *(nmol/mg protein)*				***Zooxanthellae*** *(cells x 106/ g wet weight)*				***Total chlorophyll*** *(µg /mg protein)*				***Protein*** *(mg/ g wet weight)*			
	Con.	↑ UVR	↑Temp	↑Temp. + UVR	Con.	↑ UVR	↑Temp	↑Temp. + UVR	Con.	↑ UVR	↑Temp	↑Temp. + UVR	Con.	↑ UVR	↑Temp	↑Temp. + UVR
L. compactum																
before	214.9	193.8	216.1	193.4	38.36	38.08	38.36	38.97	0.925	0.925	0.883	0.912	20.83	20.94	20.86	20.57
1 week	214.9	241.9	227.5	246.7	39.06	36.30	35.17*	32.92*	0.868	0.834	0.935	0.831	20.47	19.15	18.72	18.29
2 weeks	198.7	262.0	245.1	298.1*	38.27	33.46*	20.28*	14.30*	0.939	0.744*	0.782*	0.421*	20.41	19.27	19.03	18.66
3 weeks	208.4	334.6*	298.0*	381.6*	38.24	30.35*	17.86*	1.71*	0.980	0.437*	0.771*	0.032*	20.56	18.84*	18.52*	17.69*
S. flexibilis																
before	273.6	263.8	262.6	274.7	48.53	48.62	48.36	48.71	1.192	1.182	1.188	1.171	21.83	22.00	22.36	22.43
1 week	252.3	277.4	272.8	292.1	49.2	46.2	43.5*	39.3*	1.150	1.094	1.013	0.866*	22.04	19.94*	20.07*	19.35*
2 weeks	273.9	332.7	297.1	366.3*	48.23	42.02*	30.71*	14.65*	1.140	0.866*	0.872*	0.431*	23.00	20.23*	19.07*	18.79*
3 weeks	251.6	392.1*	362.8*	455.0*	47.85	35.59*	10.37*	1.49*	1.140	0.572*	0.789*	0.031*	22.18	19.46*	18.22*	18.30*

Effects of elevated temperature and UV on zooxanthellae densities. A decrease of zooxanthellae densities with increasing time of exposure was discerned for both soft coral hosts (Figure 3.4b, f). Prior to the start of the experiment, individual corals that had been randomly assigned to the 4 treatment groups did not differ significantly in their zooxanthellae densities in either soft coral species (average 38.36 ($x10^6$) ± 1.29 SE and 48.58 ($x10^6$) ± 1.32 SE algal cells per gram coral wet weight in *L. compactum* and *S. flexibilis* respectively) (Tables .3.2/ 3.3).

Lobophytum compactum: After one week of exposure, significant loss of algal cells was found in colonies submitted to the temperature treatment (Scheffé, $p < 0.0001$) and to an even greater degree in colonies exposed to the combined temperature and UVR treatment (Scheffé, $p < 0.0001$) (Figure 3.4b). The combined treatment resulted in significantly higher losses of zooxanthellae (Scheffé, $p = 0.0234$) than the sole elevated temperature treatment, while UVR alone did not result in any significant reduction of algal cells (Scheffé, $p = 0.053$) after one week. After two weeks of exposure, all but the control colonies showed significant losses in zooxanthella densities (Table 3.3.). UVR treatment alone had the mildest effect, sustaining a reduction in zooxanthellae densities of 13% (Scheffé, $p = 0.0084$), followed by a 21% reduction in the elevated temperature treatment (Scheffé, $p < 0.0001$) and a 62% reduction in the combined treatment (Scheffé, $p < 0.0001$). While elevated temperatures appear to have a greater effect than that of elevated UVR alone, zooxanthellae losses were not statistically significant between the two treatments (Scheffé, $p = 0.1519$). After 3 weeks, effects of all 3 experimental treatments were greater, however, the ranking of effects remained the same (Figure 3.4b). Corals submitted to the elevated UVR treatment alone showed 21% decreases in zooxanthellae numbers (Scheffé $p < 0.0001$), temperature-treated colonies sustained losses of 53% ($p < 0.0001$) and the combined UVR and temperature treatment resulted in a near total loss (96%) of the algal partner (Scheffé, $p < 0.0001$) (Tables .3.2/ 3.3). It is important to note that the losses in the combined treatment were significantly greater (one-way ANOVA, $F = 53.27$, $df = 1$, $p < 0.0001$) than the sum of losses in the separate elevated temperature and UVR treatments.

Sinularia flexibilis: A significant decrease in zooxanthellae densities was found in both the temperature (Scheffé, $p = 0.0006$) and temperature and UVR combined treatment after one week (Scheffé, $p < 0.0001$), while UVR alone did not affect zooxanthellae levels significantly (Scheffé, $p = 0.1327$) (Figure 3.4f, Table 3.3). The combined manipulation resulted in significantly greater losses (19 %) than the elevated temperature treatment (10 %) (Scheffé, $p = 0.0179$). After 2 weeks all experimental treatments resulted in significant losses of the algal partner from the association (Table 3.3.), losses averaging 13% in the UV treatment (Scheffé, $p = 0.0006$), 37% in the temperature treatment (Scheffé, $p < 0.0001$) and 70 % in the

combined treatment (Scheffé, $p < 0.0001$). As in *L. compactum,* after 3 weeks the UVR treatment resulted in a moderate (25%) but significant decrease in zooxanthella levels (Scheffé, $p < 0.0001$), while temperature and the combined UVR and temperature treatments resulted in strong and significant losses of 48% and 79 % respectively (Scheffé, both $p < 0.0001$). Unlike in *L. compactum* the effect of the combined manipulation (98 % loss) was not significantly different than the sum of the UVR and temperature treatments (one–way ANOVA, $F = 0.031$, $df = 1$; $p = 0.8608$).

Effects of elevated temperature and UV on chlorophyll concentrations. As with zooxanthellae densities, chlorophyll levels decreased with ongoing exposure in all but the control treatments in both *L. compactum* and *S. flexibilis*. Prior to the start of the experiment, chlorophyll tissue concentrations averaged 0.924 ± 0.056 SE and 1.19 ± 0.067 SE μg per mg protein in *L. compactum* and S. flexibilis respectively, and did not differ significantly between individuals assigned to the 4 groups (Figure 3.4c, g; (Tables .3.2/ 3.3).

Lobophytum compactum: After two weeks all colonies in experimental treatments sustained decreases in chlorophyll levels (Tables .3.2/ 3.3) although decreases were statistically significant for only the UVR treatment (Scheffé, $p = 0.0391$) and the combined UV and temperature treatment (Scheffé, $p < 0.0001$), with mean losses of 20% and 54% respectively (Figure 3.4c). After three weeks, however, all but the control corals experienced significant chlorophyll losses (Table 3.3) with the mildest loss (17%) caused by the temperature treatment (Scheffé, $p = 0.0040$), followed by a 53% loss in the UVR treatment (Scheffé, $p < 0.0001$) and a 97% loss in the combined treatment (Scheffé, $p < 0.0001$). Reductions in chlorophyll levels differed significantly among all treatments (Scheffé, $p < 0.0001$). After two and three weeks of exposure, the combined UVR and temperature treatment resulted in a greater decrease in chlorophyll concentration than the two single treatments summed together, the decrease due to the synergistic interaction being statistically significant at two weeks (one-way ANOVA, $F = 32.61$, $df = 1$, $p < 0.0001$), but not after in the three weeks (one-way ANOVA, $F = 1.092$, $df = 1$, $p = 0.300$).

Sinularia flexibilis: Photosynthetic pigment concentrations decreased in all treatments but the control after 1 week (Table 3.3.), yet, only the 27% loss in the combined UVR and temperature manipulation was significant (Scheffé, $p = 0.0059$) (Figure 3.4g). The losses caused by the temperature and UVR treatments reached 15% (Scheffé, $p = 0.3894$) and 8% (Scheffé, $p = 0.9175$) respectively, but were not significantly different from the controls or from each other (Scheffé, $p = 0.999$). A more pronounced reduction in chlorophyll levels was found after 2 weeks (Table 3.3.) with significant losses of 27%, 27% and 64% in comparisons to controls in

the UVR (Scheffé, p= 0.0021), the temperature (Scheffé, p = 0.0028) and the combined treatment (Scheffé, $p < 0.0001$) respectively. While the UVR and the temperature treatments had similar treatments effects, the combined treatment resulted in greater decreases in chlorophyll than either of the others (Scheffé, $p < 0.0001$). After 3 weeks, all three treatments led to strongly diminished chlorophyll levels (Table 3.3.), with the mildest reductions caused by the temperature treatment (38%, Scheffé, $p < 0.0001$), followed by that of UVR treatment (52%, Scheffé, $p < 0.0001$) and the strongest reductions experienced in the combined treatment (97%, Scheffé, $p < 0.0001$). While UVR effects appear stronger than temperature effects (Figure 3.4g), statistically, both treatments are not different (Scheffé, $p = 0.113$). The combined UVR and temperature treatment caused significantly higher loss of chlorophyll when compared to UVR (Scheffé, $p < 0.0001$) and temperature alone (Scheffé, $p < 0.0001$). Furthermore, as with chlorophyll decrease in *L. compactum* the combined treatment resulted in significantly greater losses after 2 weeks than the sum of the single factor treatments together (one-way ANOVA, $F = 22.36$, $df = 1$, $p < 0.0001$). After 3 weeks this over-additive effect was no longer statistically significant (one-way ANOVA, $F = 0.230$, $df = 1$, $p = 0.632$).

Effects of elevated temperature and UV on protein concentrations. At the start of the experiment, protein concentration in the 4 treatment groups averaged 20 .85 ± 0.787 SE and 21.83 ± 0.617 SE mg per gram coral wet weight in *L. compactum* and *S. flexibilis* respectively and did not differ significantly among the four treatment groups (Tables .3.2/ 3.3; Figure 3.4d, h).

L. compactum: After two weeks of exposure, significant changes were detected (Table 3.3.). in both the temperature (Scheffé, $p = 0.326$) and in the combined treatments (Scheffé, $p = 0.0068$) with losses of 9% and 11% respectively (Figure 3.4d). Elevated UVR resulted in protein losses of 8% but these were not significant (Scheffé, $p = 0.0766$). Three weeks of exposure led to significantly decreased protein levels in all three treatments (Table 3.3.), with 10% reductions in the UVR treatment (Scheffé, $p = 0.0138$), 11% reductions in the temperature treatment (Scheffé, $p = 0.0029$) and 15% reductions in the combined treatment (Scheffé, $p < 0.0001$) (Figure 3.4d).

S. flexibilis: In contrast to *L. compactum,* exposure of one week brought about significant decreases in the temperature and combined treatment in *S. flexibilis* (Scheffé, $p = 0.0172$, $p < 0.0001$ for the former and latter treatment respectively) (Figure 3.4h, Table 3.3.). The 8% decrease in the UVR treatment was not significant (Scheffé, $p = 0.090$). After two weeks, all experimental manipulations but the controls caused significant decreases of 7% (Scheffé,

p = 0.0094), 13% (Scheffé, p < 0.0001) and 14% (Scheffé, p < 0.0001) in the UVR, temperature and combined treatment respectively. After three weeks of exposure losses of 11%, 17% and 16% (all p < 0.0001) were sustained in the UVR, temperature and combined treatment respectively. Temperature and the combined treatment did not differ in their capacity to decrease protein in week 2 (Scheffé, p = 0.6011) or week 3 (Scheffé, p = 0.999). The effects of the combined treatments on protein concentrations were not found to be synergistic.

3.4.2. Controls of all parameters (experimental controls)

Lobophytum compactum and *Sinularia flexibilis* colonies held as experimental controls in ambient seawater underwent no significant release of zooxanthellae (one-way ANOVA, F = 0.0118, df = 3, p = 0.998 for *L.c.*; F = 0.2069, df = 3, p = 0.8914 for *S.f.*), nor changes in chlorophyll (F = 1.046, p = 0.374; for *L.c*; F = 0.8235, p = 0.302 for *S.f.*), protein (F = 0.541, p = 0.7204 for *L.c*; F= 0.910, p = 0.179 for *S.f.*) or MAA concentrations (F = 0.7958, p = 0.341 for *L.c*; F = 0.7197, p = 0.447 for *S.f.*) during the 3 week treatment period. Furthermore, experimental controls showed no significant differences at any time in comparison to handled (tank controls) and unhandled field controls in any of the parameters assessed (Table 3.4).

Table 3.4. Results of one-way ANOVA (df = 1) comparing experimental controls of each soft coral species with handled (tank controls) and unhandled field controls with respect to their zooxanthellae densities and chlorophyll, protein and MAA levels. For field controls, n = 16 determinations (i.e. 8 colonies x 2 subsamples), whereas for tanks controls n = 32 determinations (i.e. 16 colonies x 2 subsamples).

Parameter	**Unhandled field controls**		**Handled field controls**	
L. compactum	F-ratio	p-value	F-ratio	p-value
Zooxanthellae	0.1639	0.6875	0.0095	0.922
Chlorophyll	0.1863	0.6680	0.0342	0.854
Protein	0.4877	0.4885	0.0033	0.954
MAAs	0.0234	0.8791	0.0499	0.833
S. flexibilis				
Zooxanthellae	0.1639	0.6875	0.0095	0.922
Chlorophyll	0.1863	0.6680	0.0342	0.854
Protein	0.4877	0.4885	0.0033	0.954
MAAs	0.0234	08791	0.0499	0.833

3.4.3. Relationships between the biochemical parameters

Zooxanthellae levels in both species were found to be significantly and inversely correlated to MAA levels (Table 3.5). The nature of protein and MAA concentrations was similarly negative in both *L. compactum* and *S. flexibilis*, with increasing MAA concentrations with decreasing protein levels. With ongoing loss of zooxanthellae, protein levels decreased significantly in either species, thus resulting in a positive relationship between the two parameters.

Table 3.5. Regression analyses assessing the relationships between: 1) zooxanthella densities and MAA tissue concentrations, 2) protein concentrations and MAA tissue levels and 3) protein concentrations and zooxanthella levels. Asterisks denote significant relationships ($\propto$ = 0.05) while R^2 denotes the coefficient of determination, n = 512 determinations (i.e. 128 colonies x 4 sampling times).

Parameter				
Lobophytum compactum	F-ratio	p-value	relationship	R^2
1. zooxanthellae/MAAs	81.43	< 0.0001*	y = 364.13-3.7205x	0.138
2. protein/MAAs	11.15	0.0008*	y = 412.61-9.0766x	0.108
3. protein/ zooxanthellae	39.33	< 0.0001*	y = 11.239+0.2707x	0.370
Sinularia flexibilis				
1. zooxanthellae/MAAs	89.33	< 0.0001*	y = 414.49-2.8979x	0.149
2. protein/MAAs	17.78	< 0.0001*	y = 437.30-7.0029x	0.088
3. protein/ zooxanthellae	85.74	< 0.0001*	y = 11.358+0.11936x	0.391

3.4.4. Analysis of MAA levels in naturally bleached and unbleached soft corals

1. Comparison biochemical parameters between unbleached and bleached soft corals during the 1998 mass bleaching event

The bleaching episode in the summer of 1998 provided an ideal opportunity to test the relationship between bleaching as a result of a natural thermal disturbance and MAA levels in soft coral tissues. Analysis of tissue samples collected from naturally bleached soft corals following the thermal disturbance in the summer of 1998 shows that bleached colonies of *Lobophytum compactum, Sinularia flexibilis, Sinularia capitalis* and *Sarcophyton sp.* were characterised by increased MAA concentrations when compared to their unbleached counterparts. Conversely, chlorophyll, carotenoid and protein levels were lower in bleached than in unbleached colonies (Figure 3.5).

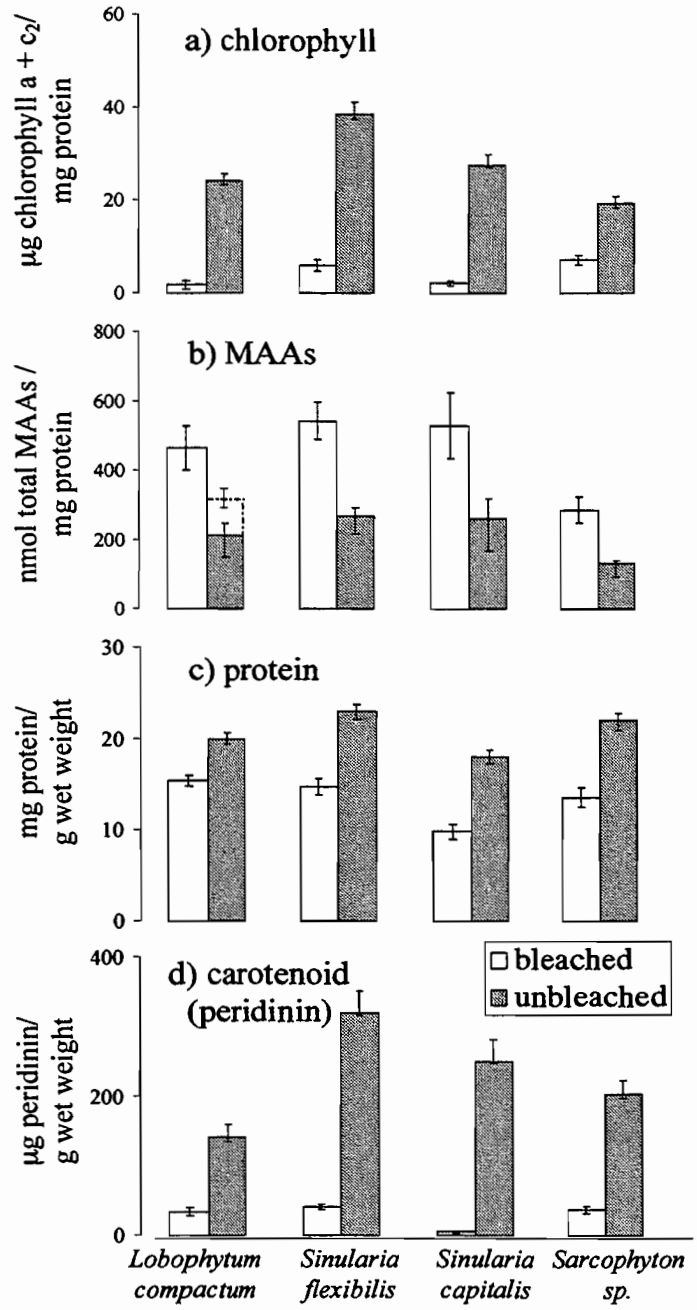


Figure 3.5. Comparison of biochemical parameters between bleached and unbleached colonies of four soft coral species collected during a natural thermal bleaching event: a) chlorophyll; b) MAAs; c) protein and d) carotenoid (peridinin) levels. Error bars denote standard errors (n = 14 determinations, i.e. 7 colonies x 2 subsamples). The depth-adjusted MAA level in unbleached *L. compactum* (b) is shown in the dashed column.

Statistical analyses confirmed that bleached and unbleached colonies of the four soft coral species sampled, differed significantly in their zooxanthellae, chlorophyll, protein and MAA tissue levels (Mann-Whitney tests) (Table 3.6)

Table 3.6. Results of Mann-Whitney tests, comparing biochemical parameters between bleached and unbleached conspecifics of 4 soft coral species sampled during the 1998 natural bleaching event. Asterisks denote significant differences, n = 14 determinations (i.e. 7 colonies x 2 subsamples per species).

Parameter	***Species***			
	L. compactum	*S. flexibilis*	*S. capitalis*	*Sarcophyton sp.*
chlorophyll	<0.0001*	<0.0001*	0.0001*	<0.0001*
MAAs	0.0011*	<0.0001*	0.0308*	0.0067*
protein	<0.0001*	<0.0001*	<0.0001*	<0.0001*
carotenoid	<0.0001*	<0.0001*	<0.0001*	0.0001*

While the results of the Mann-Whitney tests clearly provide support for significant differences between bleached and unbleached conspecifics of all four species for each of the parameters assessed, MAA comparisons are possibly confounded by the fact that they were collected at different depths and thus can have naturally different levels of MAAs. To correct for depth-related effects on MAA concentration, I calculated the relationship between depth and MAA concentrations (prior to the bleaching episode) and found a highly significant inverse relationship, for colonies of *Lobophytum compactum* in Pioneer Bay (regression analysis, $F= 117.40$, $p < 0.0001$) (Figure 3.6). The decline in MAAs with depth in unbleached colonies parallels reductions in light levels experienced at 6m depth, which equate to 42% less fluxes in the PAR region and 45% and 47% for the UV-A and UV-B regions respectively (calculations based on data provided by Walt Dunlap).

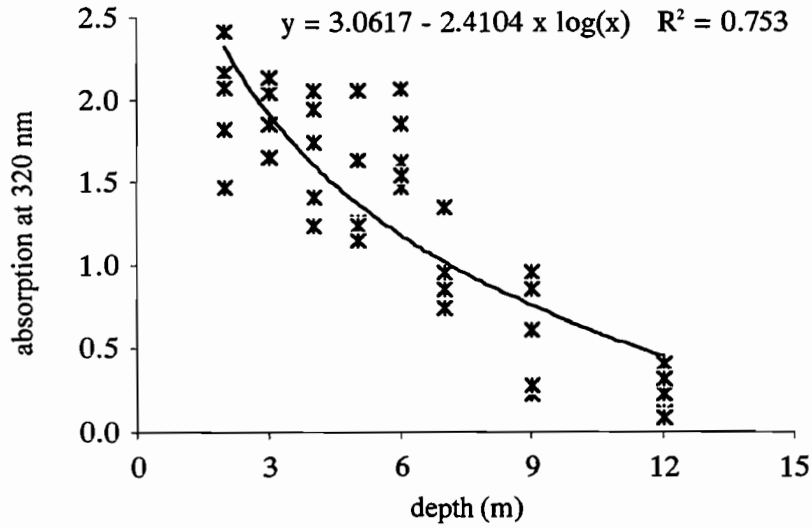


Figure 3.6. Relationship between depth and MAA concentrations in *Lobophytum compactum* at Pioneer Bay prior to the bleaching episode (January 1998; n = 5 per depth).

From the regression relationship between MAA level and depth, I calculated that individuals living at 6 m have MAA tissue concentrations approximately 48% below those of their conspecifics at 2 m at the study site. In order to compare the naturally bleached *Lobophytum compactum* colonies (all collected around 2 m) with the unbleached ones (collected around 6 m), the levels of the deeper colonies had to be adjusted by 48% prior to comparison. Also, in order to test whether increases in MAA concentrations *in L. compactum* represented true up-regulation, or only a reflection of reduced protein levels following bleaching, concentrations were then re-normalised to mean protein levels of the unbleached conspecifics. The analysis (Mann-Whitney tests) revealed that bleached *L. compactum* had significantly higher MAA tissue concentrations ($p = 0.0432$) when compared to the tissues of their unbleached counterparts corrected for both depth and protein loss. No comparisons using depth-adjusted MAA levels were carried out for the other three soft corals, since their relationships between depth and MAA levels are not known.

2. Comparison of MAA levels in 1998 with those of previous years

In another approach to establish whether increases in MAA concentrations are an active response to bleaching, I compared MAA concentrations in tissues collected during the April 1998 natural bleaching event, with concentrations in tissue samples collected at the same depth close to the study site at the same time of the year in the two previous years (see Chapter 2). MAA levels were approximately 79.5% and 63.1% higher in bleached samples of *Lobophytum compactum* and *Sinularia flexibilis* collected in April 1998, compared to levels in unbleached colonies collected in previous years (Figure 3.7).

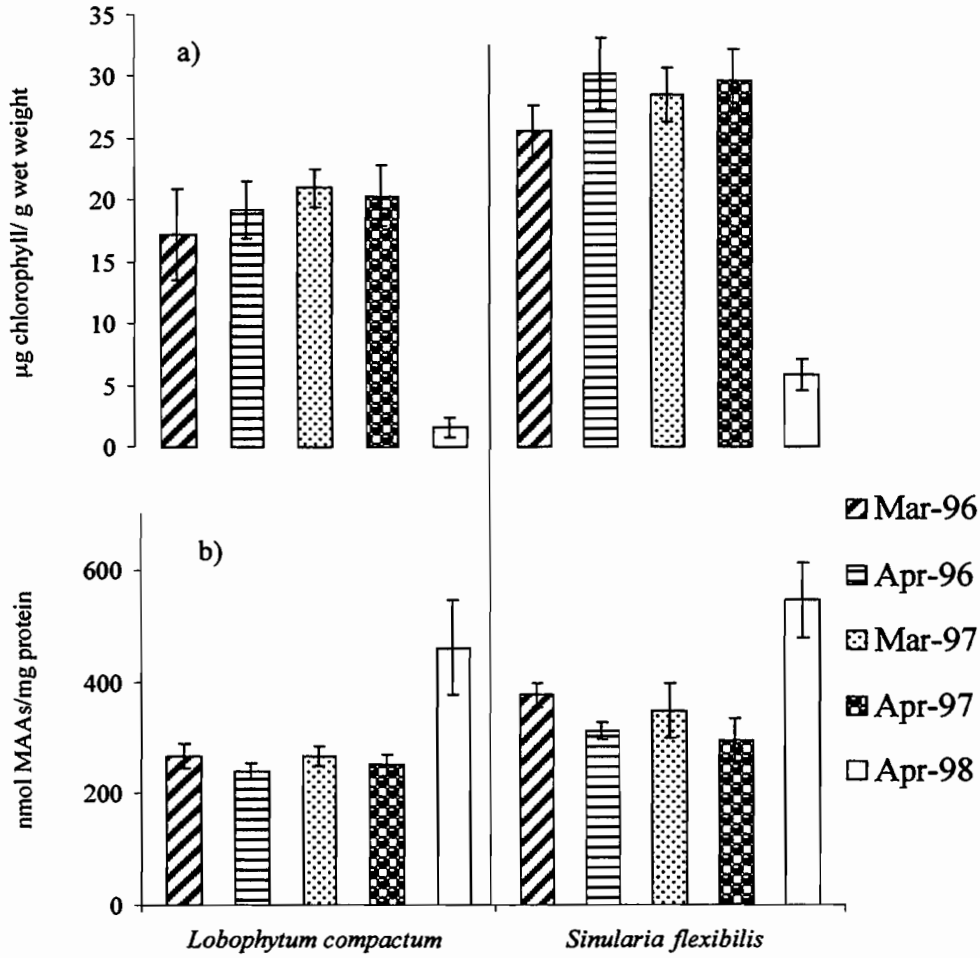


Figure 3.7. Comparison of **a**) chlorophyll and **b**) MAA levels in unbleached and bleached conspecifics of *L. compactum* and *S. flexibilis*. Data collected during March 1996 to April 1997 represent concentrations under non-bleaching conditions, whereas data collected in April 1998 represent bleached conditions. Each sampling period in 1996/1997 comprises n = 40 determinations (i.e. 10 colonies x 4 subsamples) whereas in April 1998, n = 14 determinations (i.e. 7 colonies x 2 subsamples).

In both *L. compactum* and *S. flexibilis* the higher concentrations of MAAs were statistically significant (Mann-Whitney test, $p = 0.0168$ and $p = 0.0021$ respectively), while at the same time chlorophyll levels were significantly decreased (Mann-Whitney test $p = 0.0001$ and $p = 0.0001$ respectively). Regression analyses confirmed for both species that the inverse relationship between MAA and chlorophyll concentrations is statistically significant ($F = 9.87$, $p = 0.0029$ and $F = 16.32$, $p = 0.0001$ for *L. compactum* and *S. flexibilis* respectively). MAA concentrations re-normalised to pre-bleaching protein levels were still significantly higher in the bleached than in unbleached colonies of *L. compactum* (Mann Whitney test, $p < 0.05$) and *S. flexibilis* (Mann Whitney test $p < 0.05$).

3.4.5. Do higher MAA levels protect against bleaching?

1. Effects of translocation on zooxanthella, MAA, chlorophyll and protein levels

Colonies of *L. compactum* responded to translocation from 4 to 15 m with a 43% decrease in MAA levels (from 303.93 ± 20.46 to 173.42 ± 11.25 nmol/ mg protein, one-way ANOVA, df = 1, F = 37.06, p < 0.0001), however, protein and zooxanthellae levels did not change significantly (one-way ANOVA, df = 1, F = 1.89, p = 0.174, and F = 0.11, p = 0.747 respectively). A significant acclimatory change was detected in chlorophyll concentrations which increased by approximately 63% from 0.99 ± 0.047 to 1.56 ±0.06 g/ mg protein (one-way ANOVA, df = 1, F = 52.27, p = 0.0001).

2. Effects of solar and thermal treatment on low and high MAA adjusted conspecifics

MAA level and experimental treatment both had highly significant impacts as single factors with respect to zooxanthellae and chlorophyll loss in *L. compactum* (3-factorial ANOVA, Table 3.7). More importantly, a first order interaction between the two factors occurred for the zooxanthella analysis, which means that the impact of the treatment (i.e. the degree of bleaching) is dependent on the MAA level. No significant tank effects were discerned, i.e. the use of tanks had no effects on the impact of any of the experimental treatments or the MAA level (Table 3.7).

Table 3.7. Summary of results of 3-factorial nested ANOVA, testing the effects of MAA-level and experimental treatment (fixed and orthogonal factors) and the nested factor tank, on zooxanthellae and chlorophyll levels. Asterisks denote significant differences from the control (α= 0.05); (n = 16 determinations, i.e. 8 colonies per treatment x 2 subsamples).

Source of variation		*Zooxanthellae*		*Total chlorophyll*	
	df	F-ratio	p-value	F-ratio	p-value
MAA-level	1	46.59	<0.0001*	7.83	0.007*
experimental treatment	3	117.04	<0.0001*	22.41	<0.0001*
tank	1	0.08	0.785	0.02	0.857
MAA-level x experimental treatment	3	10.52	<0.0001*	1.74	0.172

Effects of experimental treatments on zooxanthellae levels in low and high MAA acclimatised counterparts.

For comparison of the effects of each of the experimental treatments between the high and low-MAA acclimatised colonies, one-factorial ANOVA were used. In both high and low-MAA acclimatised corals, all but the control treatments did have a significantly decreasing effect on zooxanthellae densities (one–factorial ANOVA, df = 3, F = 35.79, $p < 0.0001$ and F = 89.02, $p < 0.0001$ for the former and latter group respectively; Figure 3.8).

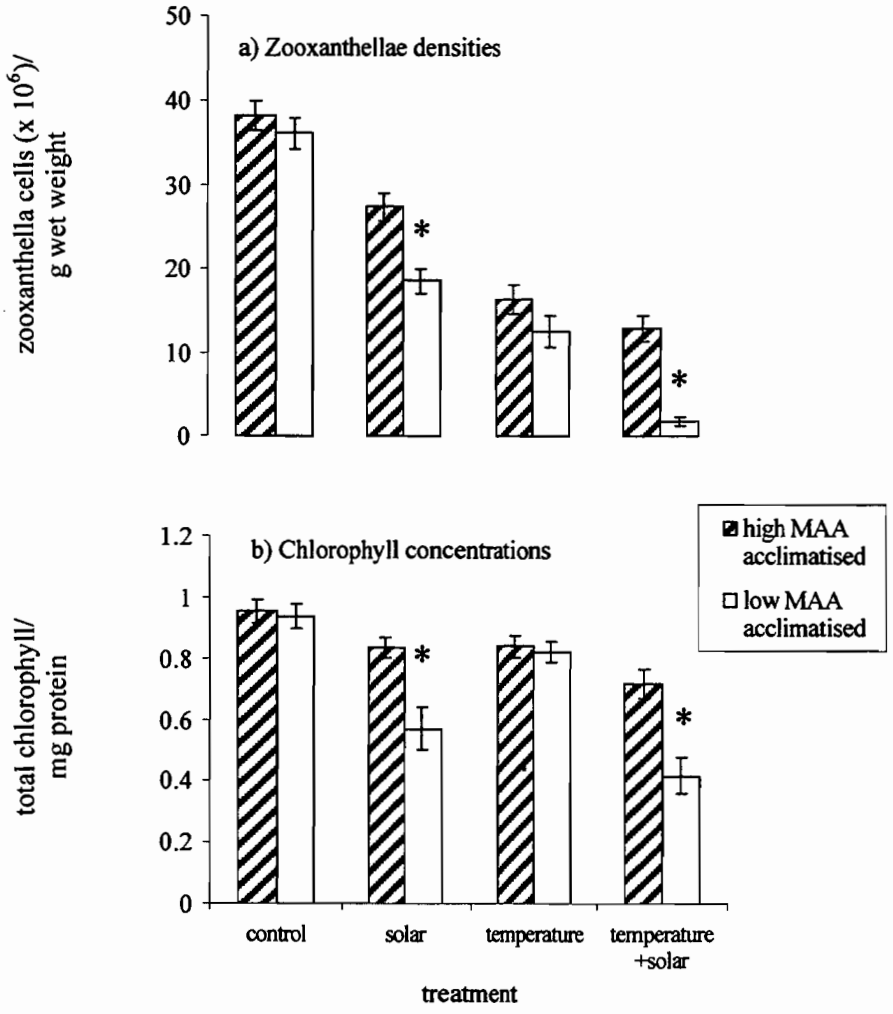


Figure 3.8. Bleaching response quantified as loss of **a**) zooxanthellae and **b**) chlorophyll levels of high and low-MAA acclimatised *L. compactum* conspecifics after 10-day exposures to increased temperature, solar radiation or a combination of both factors. Error bars denote standard error, n = 16 determinations, i.e. 8 colonies x 2 subsamples per treatment. Asterisks denote a significant difference in bleaching response between the two groups.

While low-MAA acclimatised control corals had undergone a limited (6%) loss of their algal partners by the end of the 10 day treatment, zooxanthellae densities of high-MAA and low-MAA acclimatised control corals did not differ significantly from each other (Scheffé, $p = 0.3494$). Low-MAA-acclimatised conspecifics, however, did undergo a significantly higher loss of zooxanthellae in the solar treatment (Scheffé, $p = 0.0391$) in comparison to their high-MAA adapted counterparts (Figure 3.8a). A similar but more pronounced trend was found in the combined UV solar and temperature treatments, where high-MAA adapted test corals showed a significantly lower bleaching response after 10 days than low-MAA adapted corals (Scheffé, $p = 0.0030$). Both high and low adapted conspecifics, exhibited statistically similar bleaching responses when submitted to the increased temperature treatment, with losses of 57.23 ± 4.48 % in the former and 65.16 ± 5.37 % in the latter group (Scheffé, $p = 0.5463$) (Figure 3.8a).

Effects of experimental treatments on chlorophyll concentrations in low and high MAA acclimatised counterparts. As found for zooxanthellae densities, chlorophyll levels did not differ significantly between corals maintained as low-MAA and high-MAA acclimatised control (Scheffé, $p = 0.999$) (Figure 3.8b).

Colonies with lower MAA levels, however, did undergo a significantly higher loss of chlorophyll in the solar treatment (Scheffé, $p = 0.0388$) than their high-MAA adapted counterparts (Figure 3.8b). As with zooxanthellae densities, chlorophyll levels in the combined UV solar and temperature treatments decreased in a more pronounced trend than in the solar treatment alone, where high-MAA adapted test corals showed a significantly lower bleaching response after 10 days than low-MAA adapted corals (Scheffé, $p = 0.0109$). Both high and low adapted colonies bleaching responses, statistically not different from each other when submitted to the increased temperature treatment (Scheffé, $p = 0.999$) (Figure 3.8b).

3.4.6. Summary of all results gained from experimentally and naturally bleached soft corals

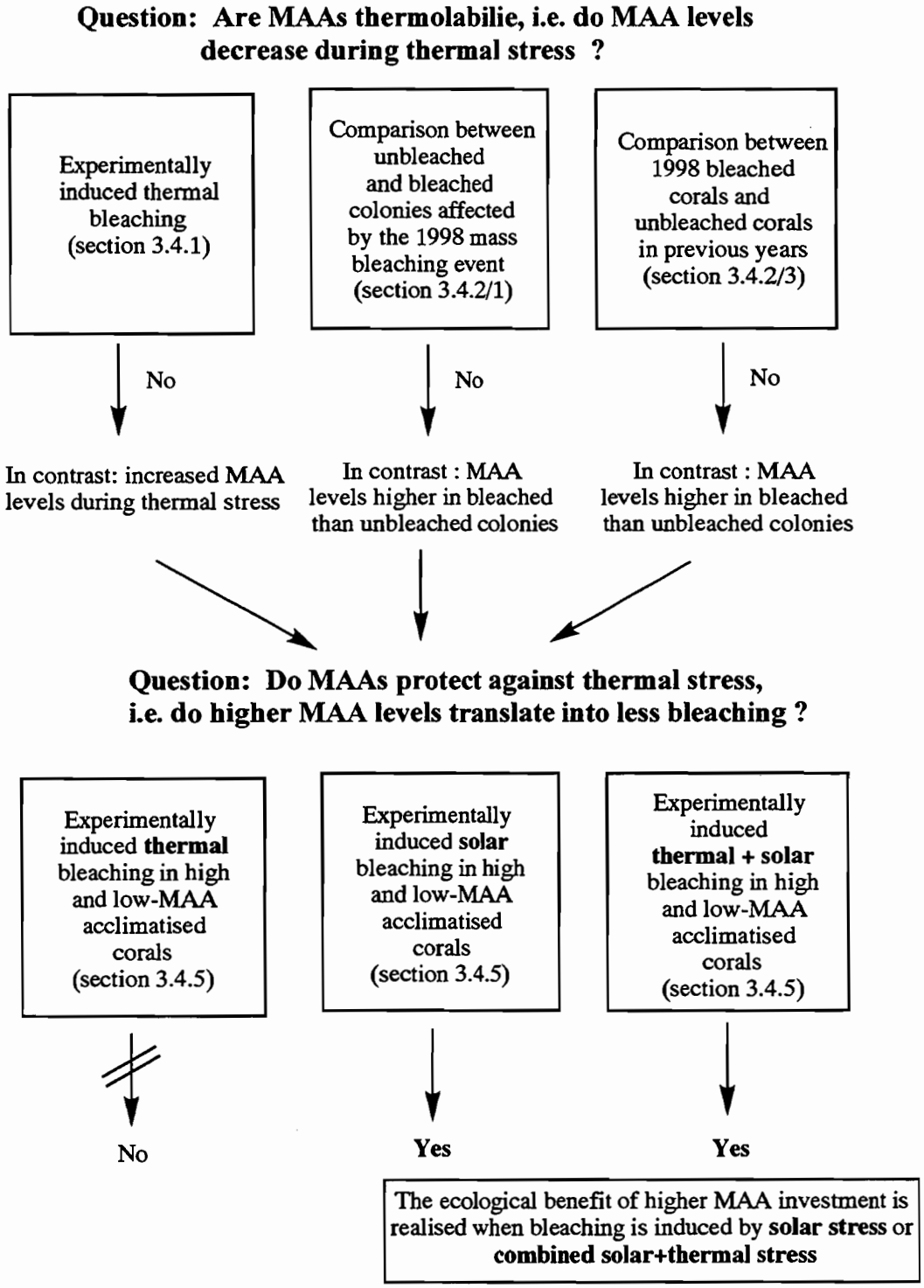


Figure 3.9. Overall summary of results of Chapter 3.

3.5. DISCUSSION

This study shows clearly that temperature and UVR have the capacity to act synergistically, resulting in a greatly increased bleaching response in *Lobophytum compactum* and *Sinularia flexibilis* when both factors occur simultaneously. The key to understanding this synergy, however, is not as previously assumed the degradation of MAAs (Lesser, 1990; Glynn, 1993), given that the compounds do not appear to be thermo-labile (within the range of temperature examined here). In fact, significant increases in MAA concentration were found in experimentally and naturally bleached soft corals that had been exposed to increased temperature. Moreover, the study with high and low-MAA acclimatised soft corals provides first evidence that metabolic investment into increased MAA production in soft corals translates into reduced bleaching, when a coral is exposed to high irradiance and combined high temperature and irradiance conditions.

3.5.1. The experimentally induced bleaching response in the soft corals *Lobophytum compactum* and *Sinularia flexibilis*

Exposure to elevated temperatures (32° C) between 1 and 2° C over summer maxima, (Chapter 2, Figure 2.9) clearly has the potential to cause severe bleaching via loss of zooxanthellae in both *Lobophytum compactum* and *Sinularia flexibilis*. Comparable temperature thresholds for inducing bleaching have been reported for a number of scleractinian corals (Jokiel and Coles, 1978; Glynn and D'Croz, 1990; Hoegh-Guldberg and Salvat, 1995; Berkelmans and Willis, 1999). In this study, the loss of zooxanthellae progressively increased in a time-dependent fashion in both species. Yet, *Sinularia flexibilis* is clearly more susceptible to increased temperature losing of 78% of its zooxanthellae population after 3 weeks, whereas *Lobophytum compactum* lost only 53% of its algal standing stock in this period. Greater thermal susceptibility of *Sinularia flexibilis* experimentally is consistent with field observations during the 1998 bleaching event, in which many colonies of *Sinularia flexibilis* were already "bone-white" when *Lobophytum compactum* at the same depths were still light brown in colour. Marked species-specific susceptibility to thermal stress has been observed frequently (Brown and Suharsono, 1990; Gleason, 1993; Hoegh-Guldberg and Salvat, 1995; Marshall and Baird, 1999), and has been suggested to be based on differential thermal susceptibility of the algal symbiont (Warner et al., 1996) or the host itself (Edmunds, 1994). The differences in bleaching susceptibility observed in my study, however, could also be based on the fact that *Lobophytum compactum* and *Sinularia flexibilis* feature very different tissue characteristics. While the former species has a thick leathery coenenchyme, into which the polyps can fully retract (which

provides shading for thermally stressed zooxanthellae), the latter species has a comparatively thinner coenenchyme, leaving zooxanthellae more exposed to light. Differences in tissue thickness and hence shading, have also been suggested to be the underlying reason for the observed differences in bleaching susceptibility between *Acropora spp.* and *Pocillopora spp.* (reviewed in Hoegh-Guldberg, 1999).

Increases of 21% and 17% over summer maxima in UV-B and UV-A also caused time-dependent decrease in symbionts, yet with losses of only 21% and 25 % of the algal standing stock in *Lobophytum compactum* and *Sinularia flexibilis* respectively, UVR treatment effects were much lower than temperature treatment effects. Thus, unlike in their thermal susceptibility, the two species were approximately equivalent in their susceptibility to UVR (Figure 3.4). If differences in tissue thickness were also to determine the hosts' susceptibility to solar stress, one would expect *Lobophytum compactum* also to be less susceptible to bleaching than *Sinularia flexibilis.* Other factors, such as the genetic variability of the host (Edmunds, 1994) or that the algal symbiont (Rowan et al., 1997), and thus potential differential susceptibility to UVR, are possibly responsible the observed differences in the (irradiance-induced) bleaching response of *Lobophytum compactum* and *Sinularia flexibilis.*

Elevated temperatures and UVR were equally potent in their capacity to reduce chlorophyll levels in zooxanthellae after 2 and 3 weeks of exposure in both *Lobophytum compactum* and *Sinularia flexibilis.* The degradation of chlorophyll after one week, (with overall losses rising to 36% and 35% in UVR treated colonies and 21 % and 47% in temperature treated colonies after 3 weeks in *L. compactum* and *S. flexibilis* respectively), is in agreement with a number of other studies. Degradation of algal chlorophyll under conditions of high irradiance (Hoegh-Guldberg and Smith, 1989a; Lesser, 1990; Kinzie, 1993; Ambarsani et al., 1997; Jones, 1997; Salih et al., 1998) and increased temperature (Iglesias-Prieto et al. 1992; Warner et al. 1996) has been widely documented for other dinoflagellate-cnidarian symbiotic associations.

3.5.2. Effects of synergistic interactions between high irradiance and temperature on zooxanthellae and chlorophyll level

The interactive effects of UVR and temperature have been recognised as early as 1978 (Coles and Jokiel), and several subsequent studies have shown a greatly enhanced capacity for bleaching when both factors occur together (Reaka-Kudla, 1993; Denis and Wicklund, 1993; Lesser et al., 1990; Drollet et al., 1997). While most studies have used the term synergy, none have compared the combined treatment response to the sum of individual treatment responses,

which should be significantly higher in a synergistic interaction. Thus, I believe the significantly greater loss in both zooxanthellae numbers and chlorophyll concentrations recorded in this study, when elevated temperature and UVR occurred in combination compared to the sum of losses when these two factors were elevated singly, represent the first records of truly synergistic with respect to bleaching. The synergistic response was observed for both *Lobophytum compactum* and *Sinularia* flexibilis, both on the level of zooxanthellae and chlorophyll loss. These findings can be readily explained by the light-temperature interaction model proposed by Jones et al. (1998), which suggests that thermally stressed zooxanthellae are more light-sensitive which subsequently leads to increased bleaching. Although synergistic interactions may not occur under all circumstances they may explain the intensity of some of the bleaching events for which temperature has been ruled out as a sole causative factor (Atwood, 1992). For example, the severity of the 1998 mass bleaching event in the GBR, in which bleaching was evident on 87% of inshore reefs along the coast has been suggested to be based on a combination of stress factors (hypo-salinity and elevated temperature and irradiance) (Berkelmans and Oliver, 1999).

3.5.3. Are MAAs the key to understanding the observed synergistic interaction?

Experimental exposure to elevated UVR significantly increased MAA concentrations in both *Lobophytum compactum* and *Sinularia flexibilis* in a time-dependent fashion as has been shown in a number of studies for hard corals (Jokiel and Coles, 1982; Kinzie, 1993; Gleason and Wellington, 1993). Most noteworthy, however, was that the highest increases occurred in the combined temperature and UVR treatment in both soft coral species (Figure 3.4.a, e). To date there is no evidence available to support that increased temperature can trigger the production of MAAs. In contrast, my initial assumption was that MAAs may be thermo-labile, thus are possibly chemically degraded during exposure to increased temperatures (Lesser et al., 1990; Glynn et al., 1992). Under this scenario, a coral experiencing thermal stress would be additionally exposed to UVR stress via degradation of its UV protection – a possible basis for the synergistic bleaching response. I found no evidence of degradation of MAAs in colonies exposed to the temperature treatment or the combined temperature and UVR treatment, however, suggesting that degradation of MAAs and subsequent increased exposure to solar stress is not the key to understanding the synergistic interaction between light and elevated temperature, at least for temperatures (32° C) used in experimental manipulations in this study. The model proposed by Jones et al. (1998), which suggests that increased light-sensitivity occurs in thermally stressed zooxanthellae thus provides, to date, the best understanding for the synergy between light and elevated temperature in the bleaching process.

3.5.4. Up-regulation of MAAs in response to thermal stress

Three independent investigations of MAA levels in response to thermal stress indicate that MAAs are not thermo-labile, but are actively up-regulated under thermal stress, and even more when thermal and irradiance stress occur simultaneously. Increases in MAA concentrations in soft corals exposed to elevated temperatures are intriguing, since to date MAAs have not been thought to provide protection against thermal stress. The possibility that the observed increases in MAA levels were the result of a dietary uptake during the experimental treatment, was dismissed on the grounds that no changes in MAA concentrations occurred in the field or tank controls during the 3 week treatment period (Table 3.4). Significant increases in MAA levels re-normalised to pre-treatment protein concentrations confirmed that increases were not merely a reflection of loss of protein levels, experienced in all bleached colonies, presumably due to host cell detachment (Gates et al., 1992). Therefore increases in MAAs in the experimentally elevated temperature treatment provided first evidence that active up-regulation of MAAs occurs in response to thermal stress. Secondly, this was further corroborated by my findings of increased MAA levels in bleached soft corals (in comparison to their unbleached conspecifics; Figure 3.7. and Table 3.6), which were sampled during the mainly temperature driven GBR bleaching event in summer of 1998 (Berkelmans and Oliver, 1999). Thirdly, more confirmatory support for the theory of MAA up-regulation under thermal stress was derived from comparisons between bleached colonies of both *L. compactum* and *S. flexibilis*, which had approximately 70% higher levels than their unbleached conspecifics collected in the appropriate season and depth in previous years (Figure 2.9.).

While my results, both from the experimentally induced and natural bleaching episodes demonstrated that bleached corals feature higher MAA levels than their unbleached counterparts, the question remained whether higher investment into MAA production also translated into increased protection against bleaching. Given the direct UV protecting function that has been shown for MAAs (Garcia-Pichel et al., 1993) and the increased light sensitivity of thermally stressed zooxanthellae (Jones et al., 1998), I expected some ecological advantage (i.e. less bleaching) to compensate for the investment into MAA production. This was confirmed by my findings that high MAA-acclimatised *Lobophytum compactum* experienced less bleaching when exposed to the elevated solar irradiance or the combined solar irradiance-temperature treatments than their low MAA-acclimatised counterparts. This strongly suggests that an ecological benefit is connected to investment into MAA production. The benefit provided by MAAs appears, however, to be only realised, when (elevated) irradiance is involved in the bleaching process, since both high and low MAA acclimatised corals responded equally strongly to thermally induced bleaching. Thus, while MAAs do not protect against

bleaching, when solely induced by thermal stress, in interaction with high solar irradiance they reduce loss of zooxanthellae significantly. This suggests some, yet to be established, interaction of MAAs with other cellular components at the level of light and temperature induced molecular damage.

In summary, in addition to the bulk of indirect evidence supporting the UV protecting role of MAAs (reviewed in Dunlap and Shick, 1998), this is first direct evidence that MAAs have the capacity to reduce the bleaching response in corals under high solar irradiance conditions. It is important to note, however, that irrespective of the experimental treatment, the presence of MAAs in the coral tissues did not completely prevent bleaching in any group, which is consistent with findings that MAAs prevent only about 20-30% of the UVR reaching cellular targets (Garcia-Pichel, 1993).

3.5.5. Possible regulation of MAA induction

Given that bleaching is typically associated with reduced photosynthetic rates (Glynn, 1993, Kinzie, 1993) and subsequent substantial loss of lipid reserves (Porter et al. 1989; see Chapter 4), it seems paradoxical to invest extra resources towards protection against a stress factor, other then the one experienced at that particular moment. Typically under thermal stress, energy is diverted to the expression of another part of the sub-cellular response system, the heat shock proteins (hsps) (Sharp et al., 1994; Black et al., 1995). Hsps have been implicated in various processes concerning avoidance and repair of protein damage in cnidarians, suggesting they play an important role in the thermo-tolerance of different species. Recently, however, the induction of heat-shock proteins has been found to be also activated by ultraviolet radiation in cell lines (Ohnishi et al, 1996). Given that incident solar fluxes and high sea surface temperatures typically occur simultaneously, selection has possibly not discriminated between the two environmental variables. This non-discrimination has also been suggested to be the underlying mechanism resulting in MAA induction by UVR, PAR and water flow (Jokiel et al., 1997), the authors speculating that MAA production could be increased via flow-modulated increase in photosynthesis and thus carbon availability for the synthesis of the compounds. Therefore a variety of regulators may be involved in the control MAA synthesis. Alternatively, resource allocation towards UV protection at a time when strong thermal stress is experienced, can be interpreted as a preventive strategy, designed to avoid a potentially lethal combination of stresses. The combination of sublethal UV-A and temperature stress, for example, has been shown to result in lethal consequences for cichlid fishes when administered simultaneously (Winckler and Fidhiany, 1996). Given that corals live so precariously close to their upper

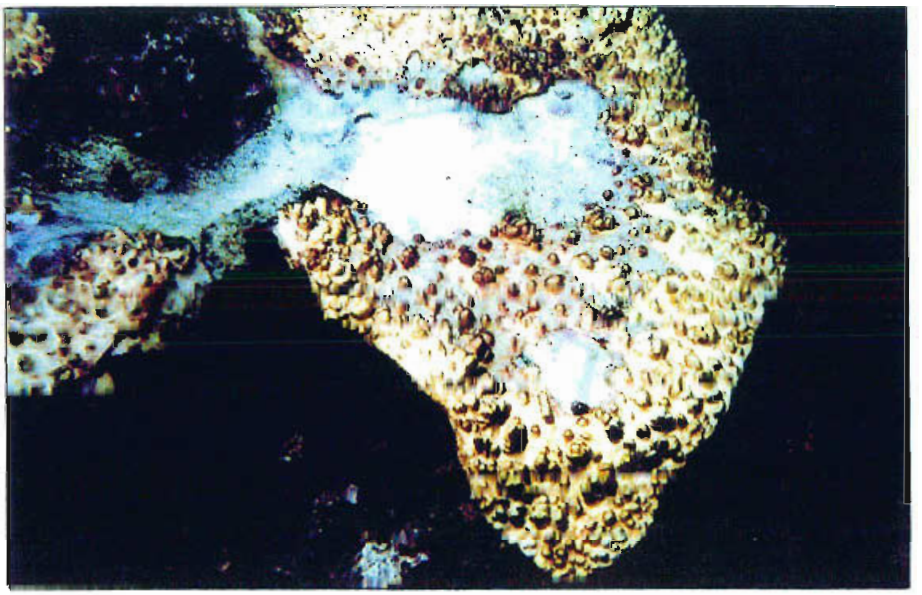
thermal limits (Coles and Jokiel, 1978; Jokiel and Coles, 1977; 1990; Berkelmans and Willis, 1999) a slight increase in solar radiation (as e.g. experienced under doldrum conditions) could prove similarly fatal, and can possibly be prevented by up-front investment into additional UVR protection. Finally, it can not be excluded that MAAs are non-specific, multi-functional compounds, which are produced in response to a variety of environmental stresses. Increases in MAA tissue concentration in response to mechanical stress and breakage have been reported in *Montipora verrucosa* (Scelfo, 1986) and *Zoanthus pacificus* (Scelfo, 1985). Scelfo (1985) interpreted immediate MAA amplification upon breakage as a feasible adaptation to possible storm damage, which could transport colony fragments to high-light environments. While all three hypotheses presented here (i.e. unknown regulators of MAA synthesis; thermally induced MAA synthesis to avoid combinations of stresses and up-front investment) are speculative, each is consistent with my findings of highest investment into production of MAAs in response to combined solar irradiance and temperature treatment.

3.5.6. Summary and conclusions

My results suggest that MAAs are not thermolabile, which means that the degradation of the compounds is not the key to understanding the synergistic relationship between irradiance and elevated temperature in the bleaching process. Converse to the notion of depressed MAA levels under thermal stress, both *Lobophytum compactum* and *Sinularia flexibilis* actively up-regulated their MAA levels in response to elevated temperatures and solar irradiance alike. While investment into MAAs does not confer an advantage when bleaching is induced solely by elevated temperature, when high irradiance and temperature act as stress factors simultaneously, increased MAA levels translate into higher protection against bleaching. Hence, the cost-to-benefit-ratio becomes positive for the coral not only under high irradiance situations, but also when exposed to increased temperature.

Chapter 4

The effects of bleaching on secondary metabolite chemistry of soft corals



Partial mortality on bleached and fouled *Sinularia sp.* during a natural bleaching event on the Solomon Islands, July 1996

4.1. ABSTRACT

The loss of zooxanthellae during a short term, experimentally simulated bleaching event resulted in significant changes to the secondary metabolite chemistry of the alcyonacean soft corals *Sinularia flexibilis* and *Lobophytum compactum*. The concentration of flexibilide, the most active cytotoxic secondary metabolite (with anti-microbial properties) in the tissues of *Sinularia flexibilis* increased by 126%, while that of its principal terpenoid algaecide, sinulariolide, simultaneously decreased to just 8% of that found in the controls. The changes were short-lived, however, with concentrations of both compounds returning to control levels one month after bleaching, although zooxanthellae levels of experimentally bleached colonies were still only approximately 20% of controls. Similarly, concentrations of isolobophytolide, the principal terpenoid secondary metabolite of *Lobophytum compactum,* underwent a significant reduction for one month following bleaching. Like their non-bleached counterparts, bleached individuals of both *Sinularia flexibilis* and *Lobophytum compactum* experienced neither significant algal overgrowth nor predation following bleaching. Full recovery of zooxanthellae to baseline levels in all bleached corals occurred within four months. These results suggest that some soft corals are capable of surviving short-term bleaching events and the detrimental algal overgrowth that is often associated with bleaching, possibly by regulating their secondary metabolite chemistry to counteract fouling. Support for the notion that resource allocation towards production of specific algaecides in bleached specimens translates into higher protection, was gained through analysis of naturally bleached soft corals. Bleached and overgrown individuals had significantly lower concentrations of species-specific algaecides than either their unbleached or bleached but unfouled conspecifics.

4.2. INTRODUCTION

Bacterial and algal fouling have been reported to pose some of the greatest threats to corals weakened by bleaching (Nair et al., 1987; Kushmaro et al., 1996, Fabricius, 1999). Algal fouling (Figure 4.1a - d) has the potential to reduce the coral's feeding capacity and compete with the coral for resources, and hence affect growth and ultimately survival rates (Coll et al., 1987; Leone et al., 1995).

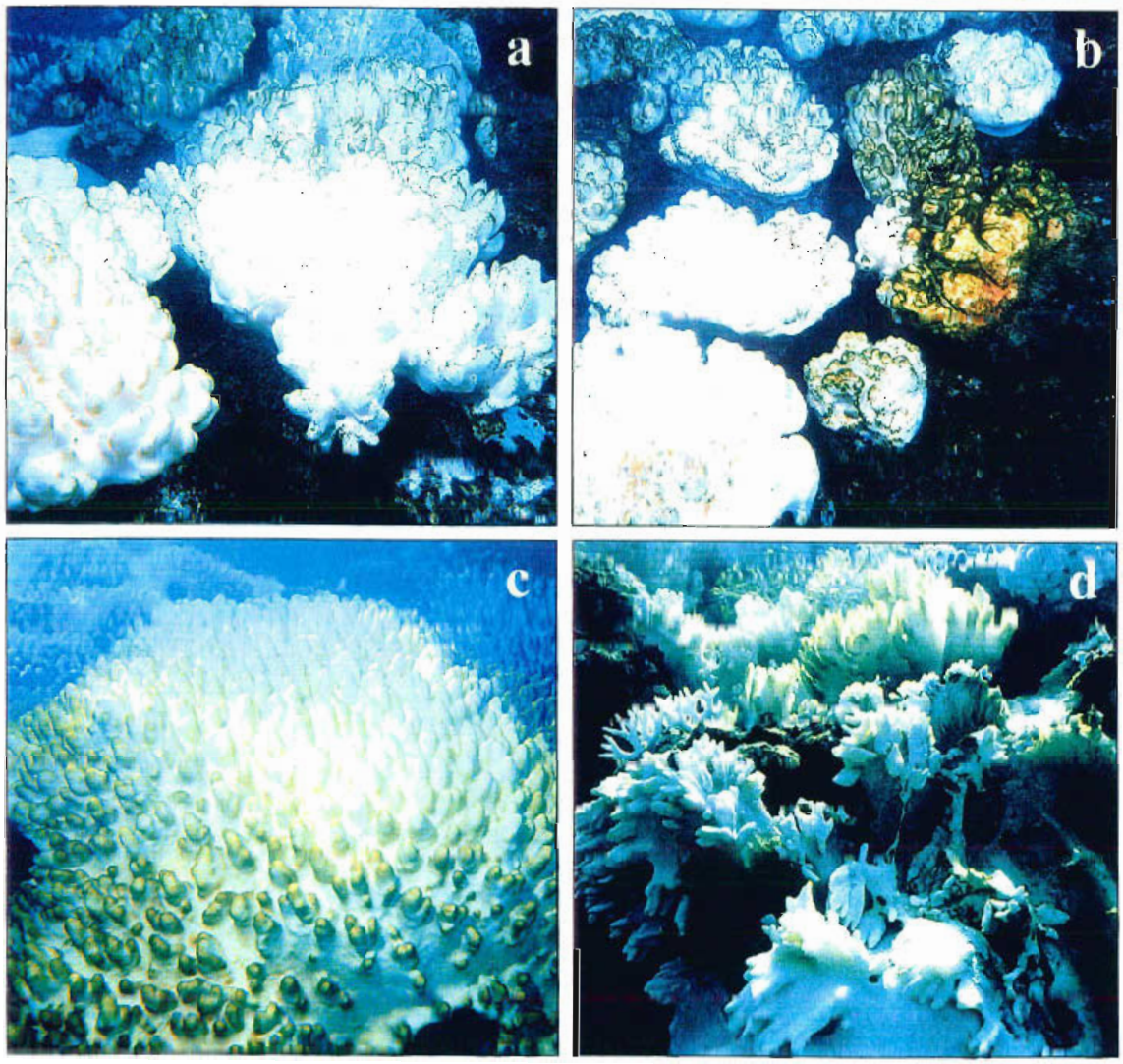


Figure 4.1. Progression of algal overgrowth on various bleached soft corals in the wake of the 1998 mass-bleaching event. **a**). Algae colonising the tips of bleached colonies of *Sinularia sp.;* **b**) Increased algal overgrowth on larger parts of colonies of bleached *Sinularia sp.;* **c**) Algae firmly engulf substantial parts of bleached *Sinularia sp.;* **d**) Tissue disintegration and death of bleached and overgrown *Sinularia sp.* (photo courtesy K. Fabricius).

Colonisation of corals by certain bacterial strains has been shown to be a normal part of reef ecology and is partially controlled by regular mucus sloughing (Ducklow, 1979; Rublee et al., 1980). However, infection of corals with bacterial strains not normally found on their surface has been reported, particularly in connection with thermally-induced bleaching events (Ritchie et al., 1993; Jindal et al., 1995). The connection between increased temperature and facilitated pathogen entry is believed to be due to changes in the bacterial adhesion function and virulence of certain associated bacterial strains (Kushmaro et al., 1996; 1997; Toren et al., 1998). Thus, bleaching obviously has the potential to alter the natural defence system of corals, thereby allowing opportunistic bacteria to capitalise on their weakened state.

Terpenoid secondary metabolites are an integral part of the natural biochemical defence system in soft corals and have been suggested to compensate for the absence of an immune system (Williams et al. 1989). Soft corals are amongst the most prolific producers of secondary metabolites which they use for functions such as anti-microbial, anti-fouling and anti-feeding agents (reviewed in Sammarco and Coll, 1990; Maida et al. 1995b; Aceret, 1995; Michalek and Bowden, 1997; Kelman et al., 1998). For example, flexibilide, a diterpene present in the tissues of the common alcyonacean soft coral *Sinularia flexibilis*, has general cytotoxic and antibacterial functions (Aceret, 1995). When released into the water column it acts as an allelopathic agent capable of disrupting respiration in neighbouring corals in concentrations as low as 5 – 10 ppm (Coll and Webb, 1984). Structurally very similar, but very different in function, are the diterpenes sinulariolide and sarcophytoxide, which act as specific algaecides in *Sinularia flexibilis* and *Sarcophyton sp.* respectively (Tursch, 1976; Coll et al., 1986; Michalek and Bowden, 1997). Feeding deterrents and ichthyotoxins were found in the aqueous extracts of 90% and 60% respectively of 160 soft coral species tested on the GBR (La Barre, 1986a). The ichthytoxin isolobophytolide is the principal terpenoid secondary metabolite in *Lobophytum compactum* and occurs in concentrations of up to 15% of the coral dry weight (Leone, 1995). Due to the presence of anti-feeding compounds in the tissues of soft corals, only a few specialised predators such as some chaetodontid fishes (Wylie and Paul, 1989) and the egg cowry *Ovula ovum* (Coll et al., 1983) are known to be able to overcome the coral's defence system. The latter predator enzymatically converts soft coral toxins i.e. sarcophytoxide to the less toxic compound 7, 8-deoxysarcophytoxide, allowing it to utilise this food source (Coll et al., 1983). Surveys on natural predation rates experienced by soft corals reveal that on average, only 1 % of individuals have bite marks (Griffith, 1994). This high level of protection against predation supports the premise that substantial metabolic investment into production of these compounds is compensated for by the important ecological role secondary metabolites play in the survival of soft corals.

While the exact metabolic costs for the production and turnover rates of terpenoid secondary metabolites are not known, it is believed that most of the carbon necessary for the synthesis is of autotrophic origin. The rationale for this premise is that the algal partner translocates up to 95% of its photosynthates to the coral host, thereby providing up to 143% of the daily energetic costs in the association (Muscatine et al., 1984; Davies, 1991). Furthermore, zooxanthellae are intrinsically involved in the uptake and efficient recycling of nitrogen and phosphorus of the association (Miller and Yellowlees, 1989; Sutton and Hoegh-Guldberg, 1990; Wang and Douglas, 1998) and play a key role in light- enhanced calcification of hard corals (Barnes and Chalker, 1990). Bleaching via loss of zooxanthellae or degradation of photosynthetic pigments, and subsequent reduced photosynthetic output, will therefore translate into nutritional constraints for the coral host. While heterotrophy cannot be discounted as an important factor contributing to nutrition of soft corals (Fabricius, 1995a; Lewis, 1982), heterotrophic feeding is highly reduced under bleaching conditions (Fabricius, 1999). Given that zooxanthellar recovery times in corals affected by strong bleaching events have been reported to last many months (Porter et al., 1989), extreme nutritional deprivation requiring metabolisation of lipids and proteins, can occur in bleached specimens. Energy allocation towards the production of significant concentrations of specific secondary metabolites may thus be compromised. Besides their well-established role as primary producers, there is also (controversial) evidence that zooxanthellae may be involved directly in the production of specific secondary metabolites (Papastephanou and Anderson, 1982). Bleaching could therefore indirectly impair the production of secondary metabolites through nutritional constraints, or directly interfere with metabolite production, if the metabolites are indeed produced by the zooxanthellae. In either case, bleached soft corals could be more susceptible to bacterial and algal fouling or more vulnerable to predation.

The general objective of this chapter is to determine if secondary metabolite production changes in soft corals as a result of bleaching and if so, to investigate ecological implications such as increased algal overgrowth and predation. I specifically focus on changes in concentrations of the algaecide, sinulariolide and the cytotoxic antimicrobial agent, flexibilide, which are species-specific to *Sinularia flexibilis*. For *Lobophytum compactum*, which has only one major terpenoid secondary metabolite, the ichthyotoxin isolobophytolide, I followed changes in the chemistry of bleached colonies and simultaneously looked for evidence of changes in rates of predation. The natural bleaching event that occurred in 1998 provided an opportunity to test my experimental findings. I compared the nature and extent of changes in the respective species-specific secondary metabolites associated with unbleached, bleached and bleached but not fouled conspecifics of *Lobophytum compactum, Sinularia flexibilis* and *Sarcophyton sp.*

4.3. MATERIAL AND METHODS

4.3.1. Site description

This experiment took place in Pioneer Bay and at the Orpheus Island Research Station (OIRS) during October 1996 and July 1997 (see Chapter 2, Figure 2.1 for more details).

4.3.2. Collection and preparation of experimental corals

Twenty random colonies of both *Sinularia flexibilis* (= ***S.f.***) Quoy & Gaimard and *Lobophytum compactum* (= ***L.c.***) Tixier-Durivault were collected from the reef flat of Pioneer Bay (3-4m depth). Entire colonies were chiselled off at their bases with some substratum attached and left to condition firmly wedged into reef crevices for five months prior to the start of the experiment. Stalk diameters ranged from 7-9 cm for the former species and colony diameters ranged from 20-25 cm for the latter species.

4.3.3. Experimental design

To test the impact of coral bleaching on the production of specific secondary metabolites, algal overgrowth and predation, 10 individuals of each coral species were artificially bleached while the remaining 10 colonies were left unbleached to serve as treatment controls (Figure 4.2). Five randomly chosen colonies of each species at 3-4 m depth in Pioneer Bay were left tagged *in situ* to serve as unhandled and unchiselled field controls. Colonies were sampled immediately before and after the bleaching treatment, and then at monthly intervals from March 1997 through July 1997.

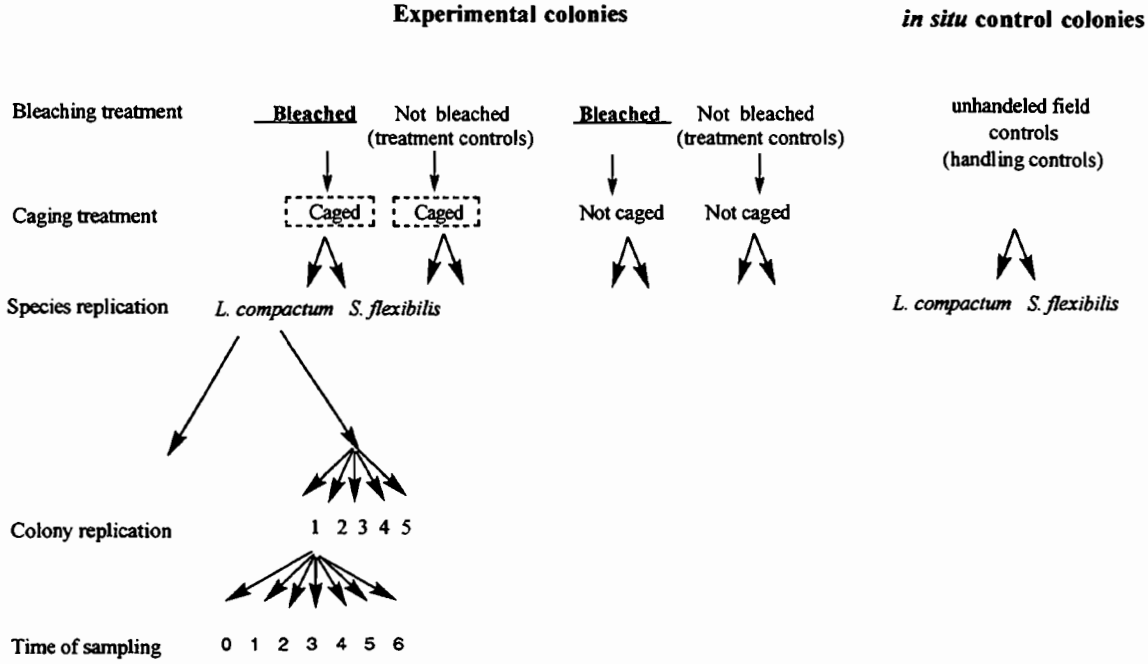


Figure 4.2. Experimental design and set-up to test the effects of bleaching on secondary metabolites, algal overgrowth and predation in *L. compactum* and *S. flexibilis* Colonies were sampled before the bleaching treatment (time 0), immediately after (1) and then in monthly intervals.

4.3.4. Bleaching treatment

The simulation of a strong bleaching event with zooxanthellae losses of more than 90% was achieved by treating the experimental corals with a combined increased temperature and solar irradiance treatment for 12 days. Temperature in the experimental flow-through tanks at Orpheus Island research station was increased from 29° C ambient to 31.5 ± 0.5° C using a computer controlled heating system (courtesy Ray Berkelmans, Great Barrier Reef Marine Park Authority, Townsville). Photosynthetically active radiation (PAR) and ultraviolet radiation (UVR) were increased through translocation of corals from 3-4 m to 50 cm (in the experimental tanks). This translocation equated to an approximately 50% increase in PAR and 33% increase in UVR respectively (calculations were based on UV/PAR depth profiles carried out at Orpheus Island in November 1996 by W. Dunlap, Australian Institute of Marine Science, Townsville, unpublished data). The timing of the experiment, March (1997), coincided with the most likely timing for a natural bleaching.

4.3.5. Quantification of zooxanthellae densities

Zooxanthellae population densities were quantified as described in Chapter 2, section 2.3.

4.3.6. Chemical analysis of secondary metabolites

Coral tissue was freeze-dried, weighed and the ground tissue was extracted 3 times with dichloromethane (DCM, 10 ml/ g dry weight of coral tissue) for 1, 8 and 24 hours respectively. Extraction efficiency for both corals was estimated by weighing the crude extract after each single extraction. Three consecutive extractions proved to be sufficient for both *Lobophytum compactum* and *Sinularia flexibilis* with mean extraction efficiencies of 97.5% for the former and 98.1% for the latter after 3 extractions. The 3 consecutive extracts were combined and the remaining solvent was removed *in vacuo*. A known amount of 2,4 - dinitrobenzene was added as an internal standard and deuterochloroform used as a solvent. Concentrations of selected secondary metabolites (flexibilide and sinulariolide for *Sinularia flexibilis;* isolobophytolide for *Lobophytum compactum*) were then determined by ^{1}H NMR, using a Bruker AM 300 NMR spectrometer.

A pulse delay of 4 seconds and a pulse angle of 60° were used to ensure complete relaxation of the internal standard and 16 scans of each sample were recorded. Spectra were Fourier transformed using zero line broadening and the appropriate signals integrated. Signals recorded for the internal standard 2,4 - dinitrobenzene resonated at δ7.81, δ8.57 and δ9.08 and signals used for flexibilide at δ6.46 and δ3.97 respectively. Sinulariolide was estimated on the basis of the methylene proton signal at δ6.21 and lipids were quantified by integration of their signal at δ1.25. Isolobophytolide was quantified by integrating the signal at δ5.94. Integration of each spectrum was carried out in triplicate, and mean values were used to estimate the absolute amount of each compound (in mg).

In order to determine whether secondary metabolites are stored in the animal or algal part of the coral association, cells were separated and concentrations of species-specific compounds of each partner of the association determined. Entire coral fingers (*Lobophytum compactum*) and "branchlets" (*Sinularia flexibilis*) of test corals were collected and divided in half (n = 5). One half was immediately deep frozen and later used to determine concentrations of the entire coral association. The second half was homogenised in artificial seawater to yield the zooxanthellae fraction of the association. This was achieved by repeated centrifugation, discarding of supernatant and washing steps until an animal-cell free pellet was achieved (confirmed by microscopy of extracts).

Quantification of isolobophytolide, sinulariolide and flexibilide present in the zooxanthellae fraction and in the entire association was carried out as described above. Concentrations of the secondary metabolites in the animal tissue were calculated by difference and normalised to protein concentrations.

4.3.7. Predation and algal fouling experiments

In order to test whether bleaching affected the susceptibility of soft corals to grazing, corals were returned to their site of collection immediately following the experimental bleaching. One half of the bleached and unbleached specimens were placed in cages (Figures 4.2/ 4.3). Cages were designed to prevent macro-grazers from grazing on algae growing on the corals, if indeed, algal overgrowth occurred during the monitoring period. Cages with a mesh size of 1 cm^2 were used, which excluded major grazers whilst not interfering with filter feeding of the corals. Cages were sufficient in size (2m x 1m) to avoid contact of colonies to cages or neighbouring test colonies (Figure 4.3). Fouling on the cages was reduced as much as possible by cleaning cages with wire brushes at each sampling date.

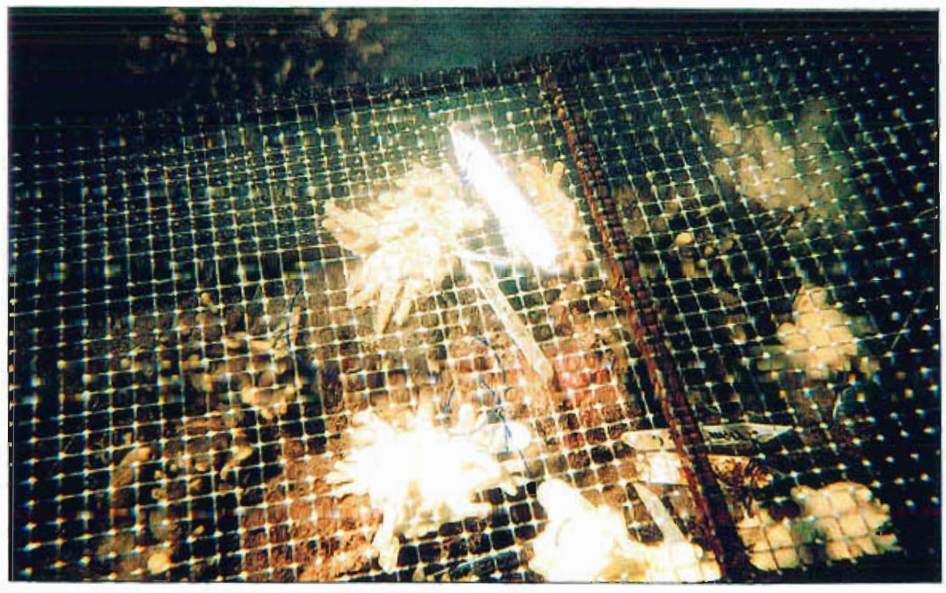


Figure 4.3. Caging set-up with bleached and unbleached specimen of *L. compactum*

1. Pilot experiment testing the effects of caging: Prior to the start of the experiment, the effects of caging (with 1 cm^2 mesh size) were tested in a nine-month pilot study for both *Lobophytum compactum* and *Sinularia flexibilis* (n = 5 caged, 5 uncaged colonies). The presence of extended polyps, algal smothering and tissue necrosis as well as the numbers of

zooxanthellae were assessed on a monthly basis from March 96 to November 96. Previous short term-tests with mesh sizes of 0.5 cm^2, which could have excluded some of the possible micro-grazers, showed algal overgrowth on the cages, leading to shading and smothering. Although cages with a mesh size of 1 cm^2, effectively prevent macro-grazing and macro-predation of coral tissue; micro-grazers such as amphipods, or smaller gastropods, however, could not be excluded with this method.

2. Assessment of algal overgrowth: Algal overgrowth was scored at monthly intervals as percentage area covered, and documented photographically with a Nikonos 5 underwater camera.

3. Assessment of predation: In order to examine whether bleaching affected the susceptibility of soft corals to predation, I monitored the five bleached and unbleached corals that remained uncaged at monthly intervals from March to July 1997. Bite marks inflicted by *Chaetodon auriga* and *Ovula ovum* (both specialised soft coral feeders) in tank experiments *with L. compactum and S. flexibilis* prior to the start of the experiment, confirmed that bite marks and grazing scars were visible for more than one month. While chaetodontid bite marks (Figure 4.4) were typically visible for 2-3 months, those of *Ovula ovum* were visible for over a year. Size and area of bite marks and scars on experimental colonies were scored monthly and documented photographically with a Nikonos 5 underwater camera. Each month, tissue samples (2 lobes of 1-2 g wet weight/ colony) were collected from the centre of each colony and snapfrozen at –20° C pending chemical analysis.

Figure 4.4. Bite marks inflicted on " fingers" of *Lobophytum compactum* by *Chaetodon auriga* during a pilot experiment, testing for persistence and nature of bite marks.

4. Statistical analysis: The effects of caging on zooxanthellae densities during the pilot study were estimated by 2-factorial ANOVA, with time, and caging as fixed factors. Variations in dry weight associated with bleaching were assessed by using one-factorial ANOVA. Differences in concentrations of secondary metabolites were assessed by 3-factorial ANOVA, with time, bleaching and caging as fixed and orthogonal factors, followed by Tukey's HSD post hoc tests for multiple comparisons. Square root and logarithmic transformation were carried out prior to analysis to comply with the assumptions of ANOVA for homogeneity of variances (Cochran's and Bartlett's tests). Considering the polyp to be the sampling unit, which was different for any given sampling time, "time" was used as a factor in a 3 factorial ANOVA. All analyses were carried out using SPSS 7.5 and SUPERANOVA software.

4.3.8. Analysis of *Lobophytum compactum*, *Sinularia flexibilis* and *Sarcophyton sp.* affected by a natural bleaching event in March/April 1998

In order to validate the findings of the experimentally simulated bleaching event, colonies of the alcyonacean soft corals *Lobophytum compactum*, *Sinularia flexibilis* and *Sarcophyton sp.* affected by the summer 1998 thermal bleaching episode were investigated for their bleaching status and their corresponding secondary metabolite levels.

1. Sampling regime and processing: Samples were collected during a joint field trip and in co-operation with Katherina Fabricius in April 1998 as described in Chapter 3. Tissue samples of bleached, semi-bleached unbleached, and bleached and overgrown conspecifics of the different species were collected, snapfrozen at – 20° C and later analysed for their chlorophyll, carotenoid, protein and MAA concentrations as described in Chapter 2. Analysis of terpenoid secondary metabolites was carried out as described above. Sarcophytoxide, the principal secondary metabolite in *Sarcophyton sp.*, which acts as an algaecide, was quantified by integrating the ^{1}H-n.m.r. signal at δ4.52.

***2. Statistical analysis*:** Non-parametric Kruskal-Wallis and Mann-Whitney tests were carried out to test for significance of differences in secondary metabolites between bleached (but unfouled), semi-bleached, unbleached, and bleached and overgrown conspecifics.

4.4. RESULTS

4.4.1. Results of caging pilot study

Caging had no effects at any given time throughout the nine-month pilot study on zooxanthellae densities of either *Lobophytum compactum* or *Sinularia flexibilis* (Table 4.1). No algal smothering on any of the colonies, nor tissue necrosis, which could potentially result from contact with the cage, was found in either species at any time.

Table 4.1. Summary of results of 2-factorial ANOVA testing the effects of caging and time on zooxanthellae densities *Sinularia flexibilis* and *Lobophytum compactum* during a nine-month pilot study. Asterisks denote a significant difference for an alpha level of 0.05.

		Sinularia flexibilis		*Lobophytum compactum*	
Source of variation	**df**	**F-ratio**	**p-value**	**F-ratio**	**p-value**
Caging	1	0.013	0.9092	0.820	0.6175
Time	8	1.573	0.1482	0.0.785	0.3683
Time x caging	8	0.737	0.6590	0.609	0.7674

4.4.2. Changes in terpenoid secondary metabolite chemistry in experimentally bleached *Sinularia flexibilis* and *Lobophytum compactum*

The factors bleaching status and time had highly significant effects on the concentrations of secondary metabolites in both coral species tested (3-factorial ANOVA, Table 4.2). More importantly, first order interactions between time and bleaching were discerned for all three secondary metabolites tested, which means that tissue concentrations in bleached *vs.* unbleached corals were highly dependent on the time at which the samples were collected. Caging had no significant effect on concentrations of sinulariolide, flexibilide and isolobophytolide, neither as a single factor, nor in first order or second order interactions with bleaching and time. Thus at all times, and irrespective of the bleaching status of a colony, caging had no effect on the levels of any of the three compounds investigated (Table 4.2).

Table 4.2. Summary of results of 3-factorial ANOVA testing the effects of time, bleaching and caging on tissue concentrations of the algaecide sinulariolide, the cytotoxin flexibilide of *Sinularia flexibilis* and the ichthyotoxin isolobophytolide of *Lobophytzum compactum*. Asterisks denote a significant difference ($\propto = 0.05$).

	sinulariolide			*flexibilide*		*isolobophytolide*	
Source of variation	**df**	**F-ratio**	**p-value**	**F-ratio**	**p-value**	**F-ratio**	**p-value**
Time	5	14.59	< 0.0001*	7.59	< 0.0001*	38.32	< 0.0001*
Bleaching	1	13.37	< 0.0001*	6.81	0.010*	33.47	< 0.0001*
Caging	1	1.10	0.297	1.00	0.319	0.06	0.809
Time x bleaching	5	15.05	< 0.0001*	7.43	< 0.0001*	7.39	< 0.0001*
Time x caging	5	0.46	0.803	0.45	0.811	0.82	0.541
Bleaching x caging	1	0.65	0.423	0.03	0.856	0.89	0.348
Time x bleaching x caging	5	0.32	0.900	0.33	0.895	0.37	0.867

1. Changes in Sinularia flexibilis: Immediately following the bleaching treatment, bleached specimens of *Sinularia flexibilis* underwent a significant change in the ratio of their specific secondary metabolites, flexibilide and sinulariolide (Figure 4.5). While concentrations of sinulariolide decreased by 92%, those for flexibilide increased by 126%. Metabolite levels in the tissues *of Sinularia flexibilis* were significantly different between February and July (Tukey's HSD test, $p < 0.001$) for both sinulariolide and flexibilide, although whereas levels of sinulariolide decreased over the period, levels of flexibilide increased. Multiple comparisons revealed that concentrations of sinulariolide in bleached corals were significantly different from their non-bleached counterparts and the field controls for a maximum of one month following the bleaching treatment (Tukey's HSD test, $p < 0.002$). Similarly, flexibilide levels were significantly different from controls only in March 1997 (Tukey's HSD test, $p < 0.0137$), and by April significant differences could no longer be discerned (Tukey's HSD test, $p = 0.2696$) (Figure 4.5).

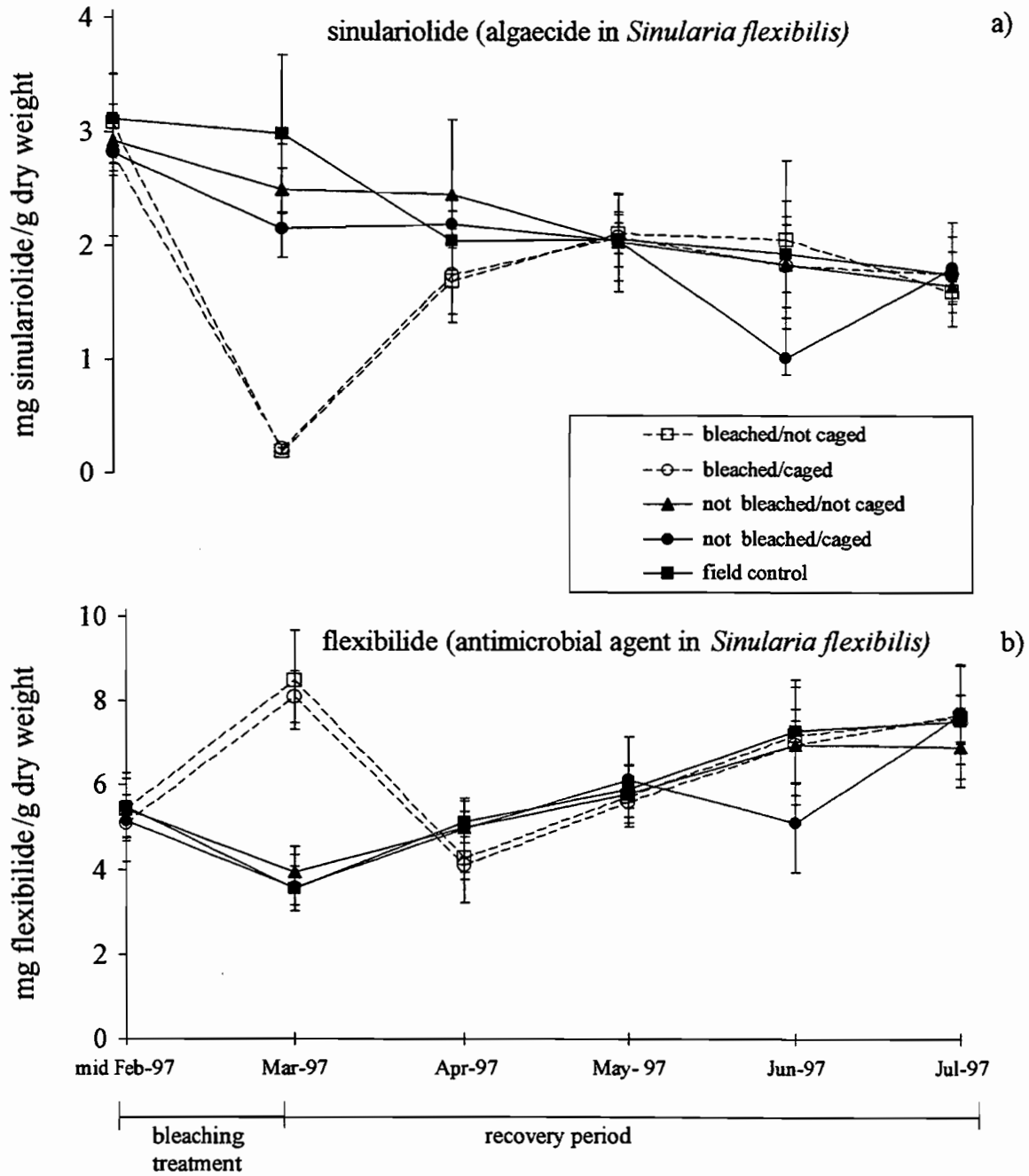


Figure 4.5. Changes in tissue concentrations of the algaecide sinulariolide and the cytotoxic antimicrobial agent flexibilide in *Sinularia flexibilis* through time. The bleaching treatment was carried out between mid February and the beginning of March 1997. Upon completion of the bleaching treatment corals were returned to the reef of origin, and sampled for 4 months during recovery. Error bars represent standard errors (n = 5 determinations per treatment/ per month).

2. Changes in Lobophytum compactum: Concentrations of isolobophytolide in *L. compactum* dropped significantly and stayed depressed for the three months following the bleaching (one-factorial ANOVA, $F = 55.43$, $df = 1$, $p < 0.0001$ for March, $F = 5.78$, $df = 1$, $p = 0.0246$ for April and $F = 12.68$, $df = 1$, $p = 0.0018$ for May) (Figure 4.6). In June levels were no longer different from controls (one-factorial ANOVA, $F = 1.039$, $df = 1$, $p = 0.319$). A significant natural increase in unbleached individuals (Tukey's HSD test, $p < 0.001$) from February to July was detected, similarly to the trend detected for flexibilide (Figure 4.6).

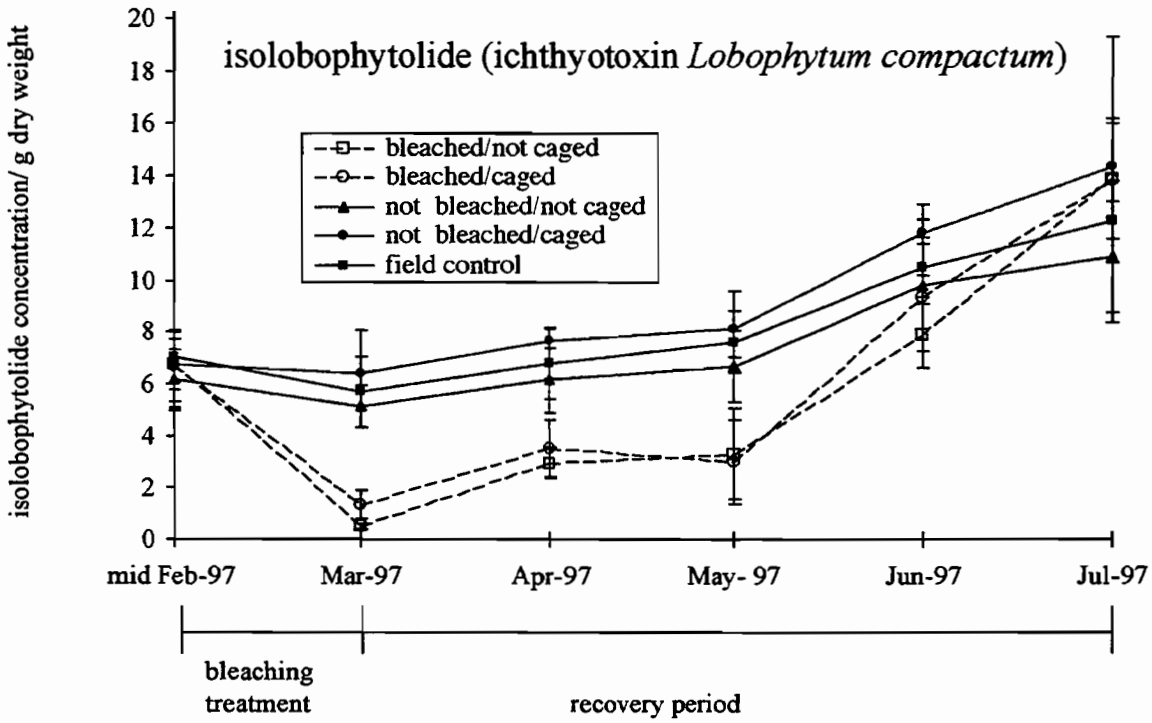


Figure 4.6. Changes in tissue concentrations of the ichthyotoxin isolobophytolide in *Lobophytum compactum* through time. The bleaching treatment was carried out between mid February and the beginning of March 1997. Upon completion of the bleaching treatment corals were returned to the reef of origin, and sampled for 4 months during recovery ($n = 5$ determinations per treatment). Error bars represent standard errors.

Analysis of ^{1}H.-n.m.r. spectra showed that bleaching in *Lobophytum compactum* did not result in the production of additional compounds, which might have been viewed as a stress-related induction of secondary metabolites.

4.4.3. Changes in relative lipid concentrations in experimentally bleached *Sinularia flexibilis* and *Lobophytum compactum*

Bleaching resulted in significant reductions in lipid levels for both species, however, these were dependent on time, since first order interactions between time and bleaching were found (3-factorial ANOVA, Table 4.3). Caging of colonies had no effect on lipid levels, not as a single factor, nor first or second order interactions with time and bleaching (Table 4.3). Thus, irrespective of the time and bleaching status of individuals, caging had no effect on the levels of lipids in either species.

Table 4.3. Summary of results of 3-factorial ANOVA testing the effects of time, bleaching and caging on lipid in *Sinularia flexibilis* and *Lobophytum compactum*. Asterisks denote a significant difference for an alpha level of 0.05.

		Sinularia flexibilis		*Lobophytum compactum*	
Source of variation	**df**	**F-ratio**	**p-value**	**F-ratio**	**p-value**
Time	5	5.62	< 0.0001*	25.67	< 0.0001*
Bleaching	1	60.55	< 0.0001*	58.64	< 0.0001*
Caging	1	0.05	0.831	2.60	0.110
Time x bleaching	5	6.47	< 0.0001*	10.57	< 0.0001*
Time x caging	5	0.36	0.872	0.16	0.976
Bleaching x caging	1	0.15	0.695	0.50	0.483
Time x bleaching x caging	5	0.14	0.982	0.05	0.998

One month after bleaching lipid levels were reduced on average by 82% in *Sinularia flexibilis* and 78% in *Lobophytum compactum* (Figure 4.7). Regression analysis of lipid recovery to control levels revealed that lipid concentrations were highly and significantly correlated to concentrations of symbiotic algae in *Sinularia flexibilis* and *Lobophytum compactum* respectively (R^2= 0.97/ 0.922; p = 0.005/ 0.02) (compare Figures 4.7 and 4.8).

The overall loss of lipids and zooxanthellae due to bleaching was also reflected in variations in the dry weight. In *L. compactum*, upon termination of the bleaching treatment, the dry weight decreased significantly from 26.59% ± 1.246 SE (in unbleached colonies) to 22.87% ± 1.199 SE (in bleached colonies) of the coral wet weight (one-way ANOVA, F = 5.049, df = 1, p = 0.0345). Similarly, in *S. flexibilis*, the dry weight in bleached colonies constituted, on average, only 22.42% ± 0.305 SE of the wet weight, whereas 24.85% ± 0.66 SE of the wet weight in unbleached colonies was made up by the dry weight (one-way ANOVA, F = 4.41, df = 1, p = 0.046) (data not shown).

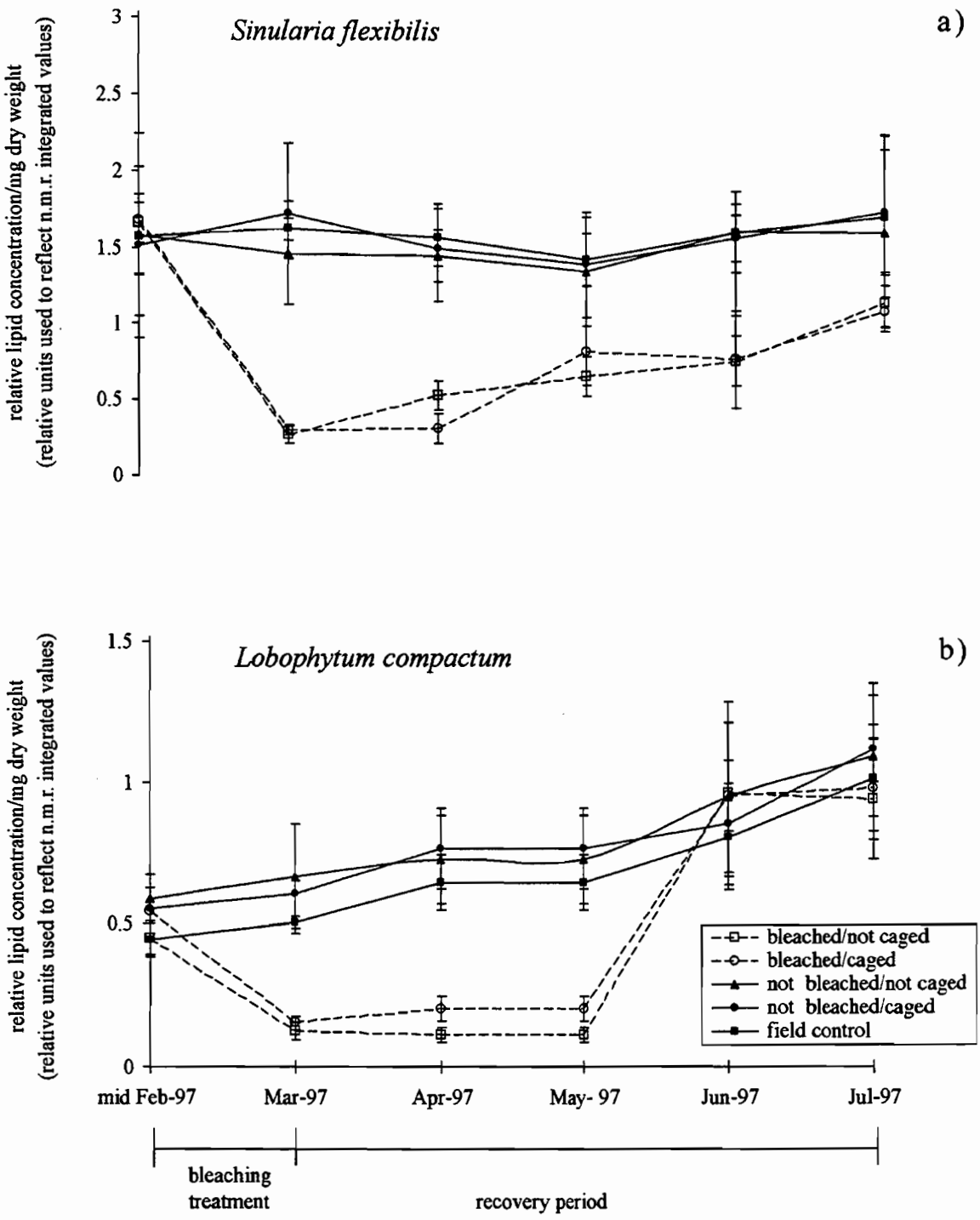


Figure 4.7. Changes in relative lipid concentrations in **a)** *Sinularia flexibilis* and **b)** *Lobophytum compactum* through time (n = 5 determinations per treatment). Error bars represent standard errors.

4.4.4. Partition of secondary metabolites between animal and algal tissue

On average 28.2 ± 3.45% of isolobophytolide, 21.4 ± 4.76% of flexibilide and 23.98 ± 4.36% of sinulariolide were found to be associated with the algal fraction (normalised to protein) of the coral-alga association.

4.4.5. Algal recovery in experimentally bleached *S. flexibilis* and *L. compactum*

Bleached individuals of both coral species regained their normal zooxanthellae population densities within four months of experimental bleaching (4.8). No total or partial mortality of test corals occurred during the monitoring period.

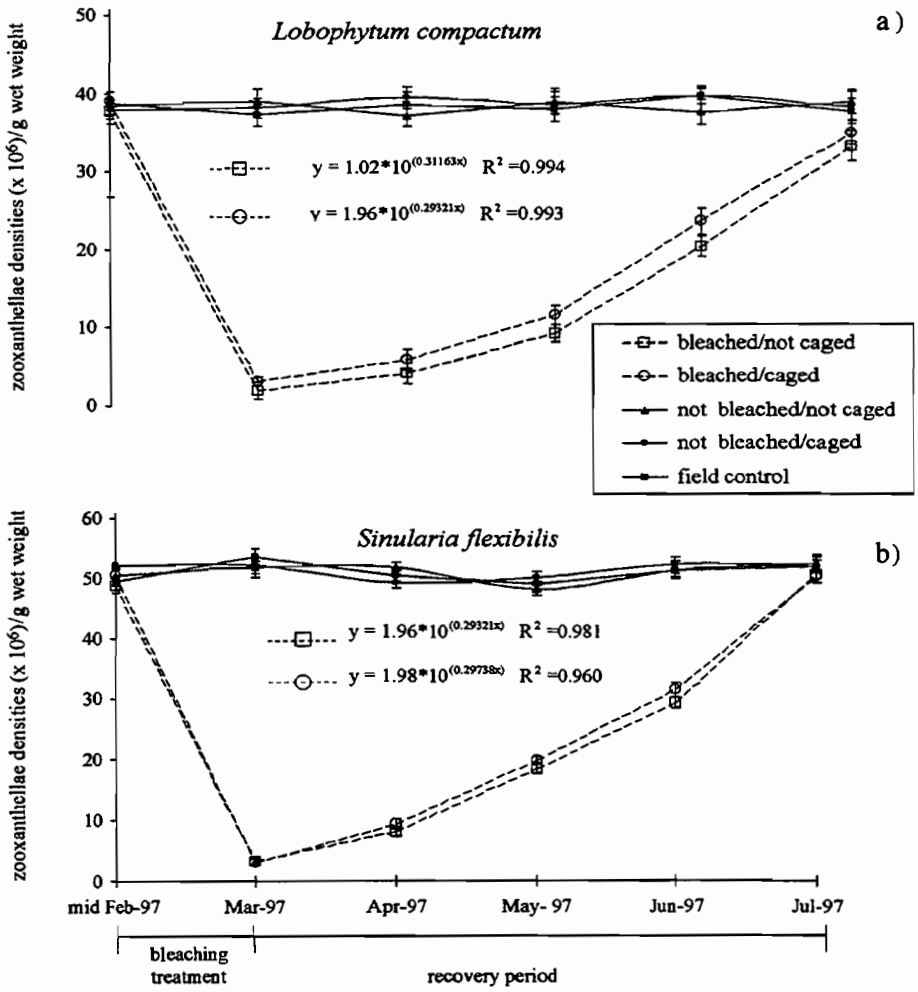


Figure 4.8. Changes in zooxanthella tissue densities in bleached and unbleached individuals of **a)** *Lobophytum compactum* and **b)** *Sinularia flexibilis* through time. Equations set inside the graph describe the exponential nature of recovery in bleached individuals (n = 5 determinations per treatment).

Recovery of algal symbionts followed a near exponential function for both species with correlation coefficients of R^2= 0.981/ 0.960 and R^2=0.993/ 0.994 for uncaged/caged individuals of *Sinularia flexibilis* and *Lobophytum compactum* respectively (Figure 4.8).

Density of zooxanthellae, i.e. recovery, was highly dependent on the time, however, density was not significantly affected by caging in either *Lobophytum compactum* or *Sinularia flexibilis* (Table 4.4). Furthermore, no first order interaction between caging and time was detected in either species (Table 4.4). Thus at no time during the monitoring period, and irrespective of the zooxanthella density of colonies, did caging affect the recovery of zooxanthellae.

Table 4.4. Summary of results of 2-factorial ANOVA, testing for the effects of time and caging on the recovery of zooxanthellae in experimentally bleached colonies of *Lobophytum compactum* and *Sinularia flexibilis*. Asterisks denote significant differences for an alpha level of 0.05.

Source of variation	*df*	*F-ratio*	*p-value*
Lobophytum compactum			
Time	5	485.61	< 0.0001*
Caging	1	3.06	0.082
Time x Caging	5	0.76	0.580
Sinularia flexibilis			
Time	5	485.61	< 0.0001*
Caging	1	3.06	0.119
Time x Caging	5	0.76	0.566

4.4.6. Predation experienced by bleached and unbleached conspecifics of *Sinularia flexibilis* and *Lobophytum compactum*

During the monitoring period from February to July 1997, bleached and recovering corals, like their unbleached counterparts, experienced very little predation (Table 4.5). The results equate to 5.3% and 6.7% of all colonies of *Sinularia flexibilis* and *Lobophytum compactum* respectively showing bite marks (bleached and unbleached treatments combined). No more than two bite marks were detected on any coral, and 75% of colonies were predation free. Tests for homogeneous distributions of the numbers of bite marks between bleached and unbleached colonies showed that these were normally distributed, both

in *Lobophytum compactum* (p = 0.9479) and *Sinularia flexibilis* (p = 0.4685) (Kolmogorov-Smirnov Goodness of Fit test). The diameter of bite marks that had occurred averaged 0.5 cm in both species and was thus not considered to be significant predation. However, it is noteworthy, that bite marks occurred in both uncaged and caged corals, suggesting that either cryptic predators were present within the substrate the corals were attached to, or predators small enough to fit through a 1 cm^2 mesh entered the cage (Table 4.5).

Table 4.5. Number of bite marks on test corals during the monitoring period. The figures in brackets denote the number of corals from which the bite marks were scored (n = 5 colonies per treatment). Shaded areas highlight bleached individuals.

Treatment	Feb. 97	Mar. 97	April 97	May 97	June 97	July 97
Sinularia flexibilis						
bleached/caged	2 (1)	0	1	0	0	0
bleached/not caged	0	1	0	0	0	0
not bleached/caged	0	0	2 (2)	0	1	0
not bleached/not caged	0	0	0	0	0	0
field controls	0	0	0	1	0	0
Lobophytum compactum						
bleached/caged	0	1	0	1	0	0
bleached/not caged	0	0	2 (1)	0	0	1
not bleached/caged	0	0	0	0	1	0
not bleached/not caged	1	0	0	1	0	1
field controls	1	0	0	1	0	0

4.4.7. Algal overgrowth experienced by bleached and unbleached conspecifics of *Sinularia flexibilis* and *Lobophytum compactum*

No algal-overgrowth was detected during the monitoring period on any of the test corals, irrespective of whether bleached or unbleached, caged or uncaged.

4.4.8. Analysis of *Lobophytum compactum*, *Sinularia flexibilis* and *Sarcophyton sp.* affected by the 1998 natural bleaching event in the Palm Island group

A comparison of bleached (but unfouled), unbleached, and bleached plus fouled colonies of *Sinularia flexibilis* and *Sarcophyton sp.* revealed highest tissue concentrations of their specific secondary metabolites in bleached (unfouled) colonies, followed by semi-bleached and unbleached ones. The lowest concentrations were found in either species in the bleached and fouled colonies (Figure 4.9). For *Lobophytum compactum,* only a comparison between bleached and unbleached counterparts could be carried out, since no semi-bleached and bleached and fouled colonies could be found. For quantitation of the bleaching status of colonies please see Figure 3.5 in Chapter 3.

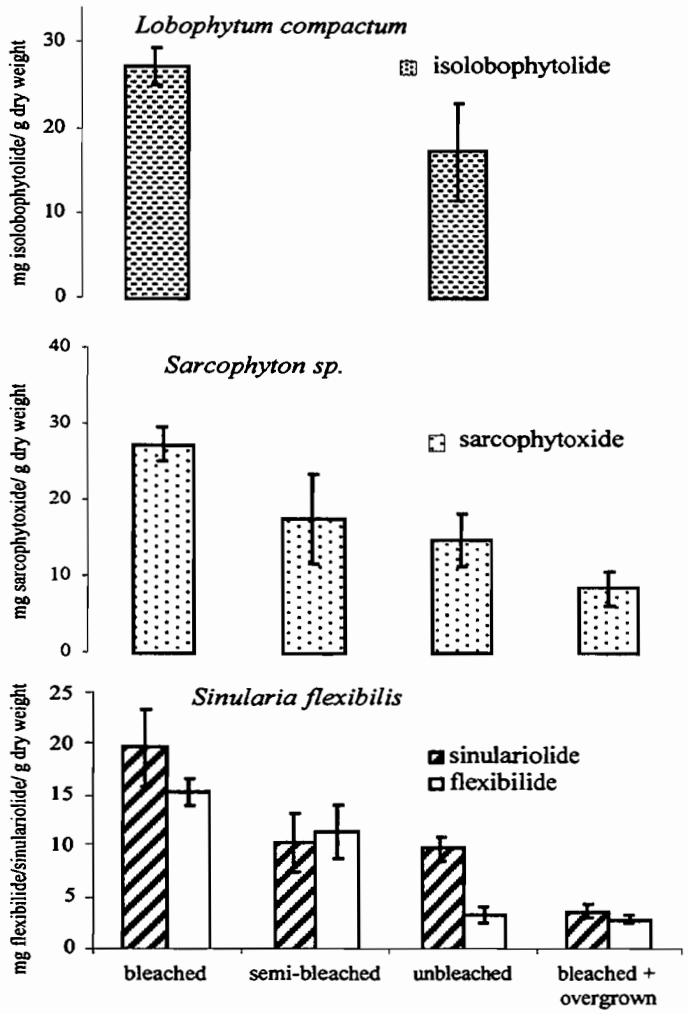


Figure 4.9. Tissue concentrations of species-specific secondary metabolites in bleached, semi-bleached, unbleached, and bleached and overgrown individuals of *Lobophytum compactum*, *Sarcophyton sp.* and *Sinularia flexibilis* (n = 14 determinations per category per species). No semi-bleached or bleached and overgrown individuals were available for *Lobophytum compactum*. Error bars represent the standard error.

For all three species, significant differences were found between bleached, semi-bleached, unbleached, and bleached and overgrown individuals with respect to their relative lipid levels and to their respective terpenoid secondary metabolites (Kruskal-Wallis tests, Table 4.6).

Table 4.6. Results of Kruskal-Wallis tests, testing for significant differences between bleached and unbleached conspecifics of soft coral species affected by the 1998 natural bleaching event. Isolobophytolide only occurs in *Lobophytum compactum*, sinulariolide and flexibilide are found in tissues of *Sinularia flexibilis*, while sarcophytoxide is specific to *Sarcophyton sp.* Asterisks denote significant differences (n = 7 determinations per species).

Parameter	***Species***		
	L. compactum	*S. flexibilis*	*Sarcophyton sp.*
Relative lipid concentration	0.0017*	0.0028*	0.0051*
Isolobophytolide	0.0017*	—	—
Sinulariolide	—	0.0025*	—
Flexibilide	—	0.0002*	—
Sarcophytoxide	—	—	0.0347*

Isolobophytolide (in *Lobophytum compactum*), sarcophytoxide (in *Sarcophyton sp.*), flexibilide and sinulariolide (both in *Sinularia flexibilis*) concentrations were significantly higher in bleached than in unbleached conspecifics (Mann-Whitney tests, Table 4.7) Similarly, for all compounds assessed, bleached colonies featured significantly higher concentrations than their bleached and fouled counterparts.

Table 4.7. Results of Mann-Whitney tests, comparing levels of secondary metabolites among bleached (unfouled), unbleached and bleached plus fouled conspecifics of soft coral species affected by the 1998 natural bleaching event. Asterisks denote significant differences (n = 7 determinations per species).

	L. compactum	**S. *flexibilis***		***Sarcophyton sp.***
	isolobophytolide	sinulariolide	flexibilide	sarcophytoxide
bleached *vs.* unbleached	0.0017*	0.0017*	0.0017*	0.0253*
bleached *vs.* bleached & fouled	—	0.0181*	0.0017*	0.0017*
unbleached *vs.* bleached & fouled	—	0.0040*	0.9491	0.1797

While bleached and overgrown *Sinularia flexibilis* had lower levels of flexibilide and sinulariolide when compared to unbleached colonies, this was statistically significant only for the latter compound. Likewise, mean levels of sarcophytoxide were lower in conspecifics that were bleached and overgrown than in unbleached individuals, however, this was not statistically significant.

4.5. DISCUSSION

The experimentally induced loss of zooxanthellae in the soft corals *Sinularia flexibilis* and *Lobophytum compactum* resulted in a significant – if short lived – changes in the composition of species-specific secondary metabolites. In bleached colonies of *Sinularia flexibilis*, sinulariolide concentrations fell significantly while flexibilide levels increased simultaneously. Similarly to decreases in sinulariolide, isolobophytolide levels dropped in bleached *Lobophytum compactum* and were accompanied by significant reductions in the lipid levels in both species.

Stress-induced increase in secondary metabolites is a well-established phenomenon in both terrestrial (reviewed in Greshenzon, 1994) and marine systems (Paul and Fenical, 1986; Coll, 1987; Paul and Van Alstyne, 1988). My findings of highly increased levels of flexibilide in experimentally bleached colonies of *S. flexibilis* and increased levels in all tested species–specific secondary metabolites in naturally bleached colonies, suggest that soft corals also follow this strategy. Sinulariolide and isolobophytolide, however, did not always follow this trend. Given that environmental stress, and increased temperatures in particular, are known to change the bacterial epifauna on corals, (Jindal et al., 1994 Nair et al., 1987, Ritchie et al., 1993), facilitate bacterial adhesion (Kushmaro et al., 1997) and increase virulence of some bacteria (Toren et al., 1998), increased investment into anti-microbial defence after bleaching may represent an important strategy.

4.5.1. Fouling of experimentally bleached soft corals

Flexibilide, which contains an α-methylene-δ-lactone moiety, is considered to be the most biologically active terpene present in *Sinularia flexibilis*. It acts as a cytotoxic agent (Aceret et al., 1995) and has strong allelopathic effects (including interference with photosynthesis, and eventual necrosis in neighbouring corals) (Webb and Coll, 1983). Its most important biological activities in relation to bleaching are its strong anti-bacterial properties (Aceret et al., 1995). The observed up-regulation of flexibilide may represent a direct strategy to prevent the adhesion and entry of bacterial pathogens into bleached specimens. Given that marine fouling is a multi-step successional process, where colonisation by bacteria precedes the establishment of micro-and macroalgal spores (Davis, 1989), the allocation of resources to an increased production of flexibilide in experimentally bleached *S. flexibilis* could be viewed as an attempt to interrupt the "fouling cascade" at an early stage.

Theoretically, the observed absence of algal fouling from all test corals could have been due to a general lack of algal fouling at the site during the course of the experiment, or perhaps to an insufficient monitoring timeframe. Both explanations, however, can be ruled out since the plastic tags I used to label the experimental colonies were so heavily overgrown by algae that they had to be renewed every month. Furthermore, the experimental timeframe of six months should have also been sufficient to observe algal overgrowth if it occurred, given that algal clearance experiments at One Tree Island have shown that within 30-60 days most reef sites recover their standing stock of epilithic algae (Hatcher and Larkum, 1983). Moreover, complete algal overgrowth colonies has been observed within four weeks for bleached hard corals (B. Schaffelcke, pers. comm., Marshall, pers. comm.), and five weeks for soft corals (pers. observ.). I therefore interpret the absence of algal fouling on experimentally bleached *Sinularia flexibilis* to be a function of co-occurring changes in their secondary metabolite chemistry. In particular the increase in the anti-bacterial agent, flexibilide, appears to have occurred at the expense of the algaecide, sinulariolide, possibly to avert the first successional stages of the fouling process.

The notion that regulation of secondary metabolites may aid in the prevention of algal fouling is further corroborated by data gained from the natural 1998 bleaching event. Bleached colonies of *Sinularia flexibilis* and *Sarcophyton sp* which remained free of fouling, also featured the highest concentrations of their respective algaecides, whereas those overgrown by algae had the lowest levels. Similarly, Coll and co-workers (1987) correlated the absence of certain secondary metabolites in *Lobophytum pauciflorum* to the occurrence of algal fouling on those conspecifics which lacked the compounds (Coll et al., 1987). Concentrations of secondary metabolites in the bleached and overgrown colonies were the lowest of all groups tested, suggesting that these colonies exhausted their energy reserves due to nutritional constraints. This hypothesis is consistent with reports that starvation of alcyoniid soft corals 2 - 3 months after the 1998 event, which led to volume reductions of 60 - 80% and were followed by algal overgrowth and death (Fabricius, 1999). It is likely that certain algaecidal thresholds have to be maintained in order to prevent fouling, and if production cannot be maintained due to nutritional constraints, fouling of weakened corals may occur. A second possibility includes the "shutdown" of secondary metabolite production, as a result of competitive interactions, which has been suggested to be the underlying mechanism for a failing isolobophytolide production in *Lobophytum compactum* where tissue necrosis of the coral is caused by contact with the red alga *Plocamium hamatum* (Leone et al., 1995).

4.5.2. Predation of experimentally bleached soft corals

Levels of isolobophytolide an ichthytoxin and principal terpenoid secondary metabolite in *Lobophytum compactum* dropped significantly in experimentally bleached colonies. Energetic constraints, as revealed by a 78% lipid level reduction following the bleaching, may again explain the decreased production of this compound. Unlike in *Sinularia flexibilis*, however, a change in resource allocation towards the production of another compound could not be detected by ^{1}H-n.m.r. analysis. The only function that has been experimentally demonstrated for isolobophytolide to date, is its function as an ichthyotoxin. The compound has the capacity to kill *Gambusia affinis* at concentrations of 10 ppm within 2 hours of exposure (Bowden, unpublished data). Despite levels of the compound decreasing by 83% following bleaching, neither significant predation (nor algal overgrowth) could be detected within the monitoring period. A number of reasons may explain this lack of observed predation. Firstly, isolobophytolide levels of 17% of pre-bleaching levels for a limited period could have been sufficient to avoid significant predation. In light of cost-efficiencies for defence this explanation is doubtful, given that under non-stress situations a much higher level of production is maintained.

Secondly, predation at the site may have been "unusually" low. This explanation, however, does not accord with the fact that bite marks were detected on average 5.3% of colonies of *Sinularia flexibilis* and 6.7% of colonies of *Lobophytum compactum*. These findings are slightly higher then results provided by Griffith (1994) who found predation on soft corals in Great Barrier Reef region to be generally very low (around 1%). Therefore some predation obviously took place at the site during the experiment. Given, that bite marks were found not only found on un-caged colonies, but also in the caged corals (mesh size 1 cm^2), predators other than chaetodontid fishes or the egg cowry *Ovula ovum,* known specialised soft coral predators, must have been responsible for the observed bite marks. Small species of cowries and nudibranchs, either hidden within the substrate to which the soft corals were attached (pers. observ.), or entering the cages through the mesh, could have been responsible for the observed bite marks on caged specimens.

A third explanation for the observed lack of major predation on bleached colonies includes the possibility of production of a compound or compounds that protect against predation, such as pukalide (Coll et al., 1989) or prostaglandins which have not been detected by ^{1}H-n.m.r.

A fourth alternative explanation is that potential predators have learned to avoid certain soft corals (*Chaetodon melannotus*, eg uses visual cues to select octocorals; Aliño, 1989), and this learned behaviour is maintained in spite of reduced tissue protection. This notion

is corroborated by the fact that bite marks were found to the same degree on bleached and unbleached corals, i.e. predators obviously did not distinguish between the two groups. In summary, no explicit protective role for isolobophytolide could be discerned, given the lack of major predation on experimentally bleached *L. compactum* despite reduced levels of this secondary metabolite. It cannot be ruled out, however, that the compound fulfils other, as yet unknown ecological functions in the coral and thus to be able to monitor the impacts of decreased levels adequately requires additional research.

4.5.3. Experimentally bleached versus naturally bleached soft coral conspecifics

Naturally bleached *Lobophytum compactum* featured significantly increased levels of isolobophytolide in comparison to their non-bleached counterparts. This is contrary to observations following experimentally induced bleaching and suggests some protective function for the ichthyotoxin isolobophytolide under extreme bleaching conditions. Likewise, I found that the levels of flexibilide in naturally bleached individuals were about twice as high as in the experimentally bleached ones, in addition to a simultaneous increase of sinulariolide in *Sinularia flexibilis*.

Part of the observed increases may be due to the fact that concentrations of species-specific secondary metabolites are normalised to the dry weight, which decreased slightly in bleached colonies due to losses in storage lipids, zooxanthellae cells and host protein. Based on the observed decrease in dry weight in bleached colonies of 14% and 10% in *L. compactum and S. flexibilis* respectively, only 10-14% of the increases can be explained with losses in dry weight. Given that e.g. flexibilide levels increased by 126% in experimentally bleached and by 361% in naturally bleached *S. flexibilis,* I interpret the increase as a true up-regulation response and not as a mere reflection of changes in the dry weight in bleached colonies.

Yet, the increased investment into production of secondary metabolites during the natural bleaching episode appears paradoxically because energy reserves would have almost certainly been more exhausted in the naturally than the experimentally bleached colonies, given that the magnitude of the latter bleaching was much higher. While the experimentally induced bleaching resulted in a loss of nearly 95% of the zooxanthellae standing stock, exposures to increased temperature and solar irradiance levels lasted for 12 days only. In contrast, the massive bleaching event experienced on the GBR in early 1998, is believed to have been caused by a number of stresses that occurred over several weeks. Preceding high water temperatures of 31–32° C (with occasional peaks of 33° C), freshwater run-off caused by cyclone Sid had already stressed corals via severely reduced

salinity (Fabricius, 1998; Berkelmans and Oliver, 1999). High irradiance penetration due to extremely calm conditions coincided with the increases in temperature and most likely greatly exacerbated the stress experienced by corals (Fabricius, 1998; Berkelmans and Oliver, 1999).

Secondary metabolites have been suggested to compensate for the immune system in lower invertebrates (Williams, 1989). Thus, extensive exposure to stress, such as the 1998 bleaching episode, may require an unusually high level of protection (i.e. high concentrations of algaecides and antimicrobial agents) to counteract fouling with opportunistic bacterial pathogens or algae. High investment into secondary metabolites under conditions of nutritional constraints, however, can only be maintained for a certain time. Thus, if time/ energy thresholds are exceeded, continued high investment into secondary metabolites may ultimately result in a fatal metabolic burden. While this interpretation is speculative, it is consistent with my findings of extremely low algaecide levels in bleached and fouled soft coral colonies, and reports that starvation in bleached alcyoniid soft corals resulted in colony death upon 2 –3 months following the 1998 GBR bleaching episode Fabricius (1999). In summary, I suggest that the differential responses in the experimentally and naturally bleached soft corals may be explained by the magnitude and the nature of the respective bleaching episodes and the metabolic investment connected to it.

Further research into the nature of regulation of secondary metabolites is required to ultimately determine whether the magnitude of a bleaching event and thus the energetic constraints connected to it do indeed determine the biochemical response. The rapid 92% decrease in levels of sinulariolide, the algaecide found in *Sinularia flexibilis* by the onset of experimental bleaching, for example, could be both the result of a resource re-allocation due to nutritional constraints or the outcome of a chemical conversion. On average only 24% of the compound was found to be associated with the algal partner; thus the loss of zooxanthellae can only explain up to one quarter of the decrease in sinulariolide concentrations. One explanation for the decrease in levels is that nutritional constraints associated with the loss of zooxanthellae, and mirrored by an 82% reduction in storage lipids, may have been indirectly responsible for a lower production of the algaecide. If nutritional constraints indeed required a redistribution of resources, flexibilide was clearly favoured over sinulariolide. Given that both compounds share a similar structural relationship, an alternative explanation could involve enzymatic inter-conversion of one compound into the other, as suggested by Maida and co-workers in 1993 (Figure 4.10).

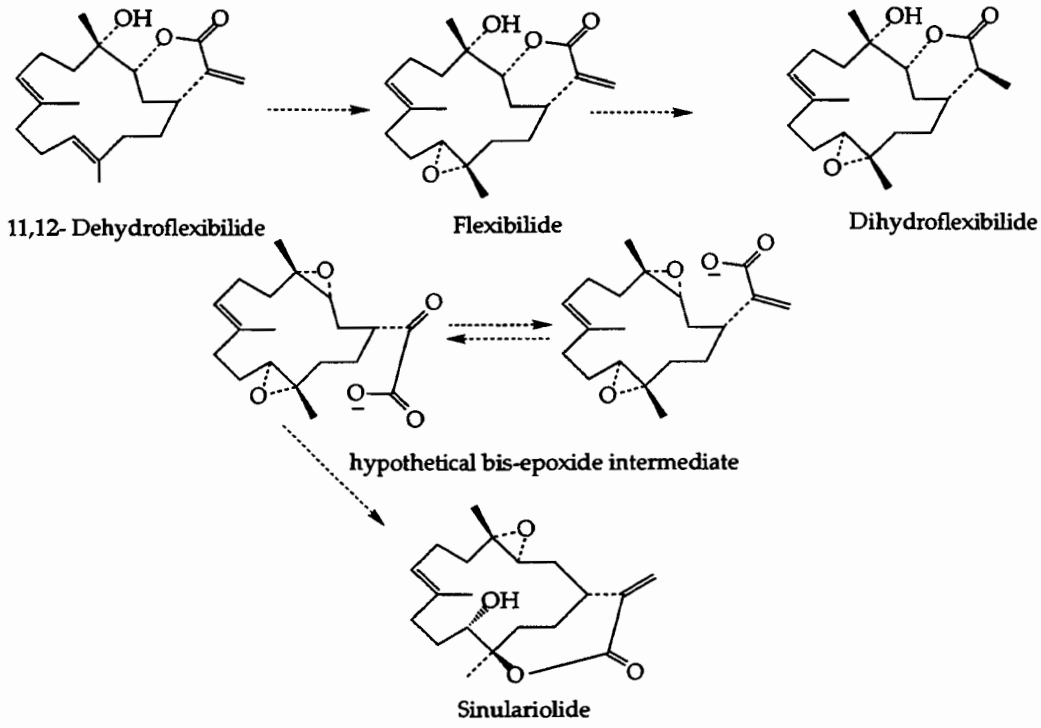


Figure 4.10. Cembranolide diterpene secondary metabolites derived from *Sinularia flexibilis* and possible biosynthetic pathways linking them, as suggested by Maida et al., 1993.

Seaweeds are known to immediately convert weak deterrents into strong deterrents upon mechanical damage of the alga (Paul and Van Alstyne, 1992). Thus, by analogy, the observed increase of flexibilide in experimentally bleached *Sinularia flexibilis* may not have been the result of a redirected resource allocation, but the outcome of a conversion of sinulariolide into flexibilide. If increased antibacterial properties of flexibilide were required quickly following bleaching and if normal levels were inaedequate, then biochemical conversion of sinulariolide, rather then *de novo* synthesis, may present an energetically preferable response to a short-term bleaching event. Rapid turnover of terpenoids has been confirmed for the eggs of *Lobophytum compactum*, in which terpenoid secondary metabolites can be fully metabolised within days (Heaton, 1988). Unfortunately, the collection of samples in this study was only carried out at monthly intervals, which precludes an analysis of the increase and decrease of flexibilide and sinulariolide respectively on a finer temporal scale. Yet, irrespective of the strategy involved in the increase in flexibilide, the absence of algal overgrowth in bleached colonies of *Sinularia flexibilis*, suggests that soft corals possibly do have some capacity to avoid bacterial and subsequent algal fouling after bleaching.

4.5.4. Summary and conclusions

This study has provided evidence that the soft corals *Sinularia flexibilis* and *Lobophytum compactum* are capable of surviving a severe short-term bleaching event, however, with the metabolic burden of strong reductions in the levels of lipids as a consequence. I suggest that their ability to control the production (or the conversion) of specific secondary metabolites to meet specific ecological requirements may play an important role in the survival of soft corals during short-term disturbances such as bleaching events. This notion is corroborated by the results from the intensive 1998 natural bleaching event, in which bleached but unfouled soft corals featured the highest concentrations of their respective algaecides/antimicrobial agents, whereas bleached and fouled colonies had the lowest tissue concentrations. Thus, investment into species-specific algaecides and antimicrobial agents, as found in naturally bleached soft corals obviously has the potential to prevent fouling in association with bleaching events.

Chapter 5

The effects of the zooxanthellae on the secondary metabolite chemistry and development of *Lobophytum compactum*

Detail of eight week old primary polyp of *Lobophytum compactum*. Brown spots represent clusters of zooxanthellae

5.1. ABSTRACT

The question of who controls the production of secondary metabolites in soft corals, the algal symbiont or the animal host has been debated for the last 30 years, but has not been resolved due to inherent problems with residual and/ or dietary terpenes in adult colonies. In a novel approach I have assessed the secondary metabolite chemistry of freshly metamorphosed coral polyps, with and without zooxanthellae using extremely sensitive electro-spray and fourier-transform mass spectrometry. In this chapter I report the effects different strains of zooxanthellae have on the terpenoid secondary metabolite chemistry and development of the alcyonacean soft coral *Lobophytum compactum.* Coral larvae of the same genetic background were reared, then inoculated with zooxanthellar strains of different taxonomic and geographic origin, and their terpenoid chemistry analysed.

Control over production of terpenoid secondary metabolites lies clearly with the animal host, since all algal-animal associations, irrespective of zooxanthellae origin, were found to produce isolobophytolide, the principal terpenoid in *Lobophytum compactum.* Moreover, even though the control group had no access to zooxanthellae they still expressed isolobophytolide confirming unequivocally that the algal partner is not essential for the production of terpenoids in the association. Importantly this implies that soft corals affected by bleaching can still maintain the production of these ecologically important compounds, if they have sufficient energy reserves to allocate towards secondary metabolite production.

5.2. INTRODUCTION

The evolutionary success of alcyonacean soft corals, a major component of Indo-Pacific reefs has been in part attributed to the quantity and diversity of terpenoid secondary metabolites in their tissues (Tursch, 1976; Sammarco and Coll, 1992). Whilst the production of secondary metabolites represents a significant metabolic cost, the energy expenditure for the production of these compounds is well compensated for by the multiple functions they fulfil within the organism, as discussed in Chapter 4. They enhance the coral's fitness in defending them against predation (LaBarre et al., 1986a; Wylie and Paul, 1989), increasing their competitive ability (Sammarco et al., 1983; Maida, 1995b) and enhancing mating opportunities (Coll, et al., 1989; Coll et al., 1995), as well as being active as anti-microbial (Aceret, 1995; Slattery et al., 1995b) and anti-fouling compounds (Kiefer and Rinehart, 1986; Michalek and Bowden, 1997).

While it has been established that zooxanthellae are the primary producers in the coral-alga association (Patton and Burris, 1983; Muscatine et al., 1984), the question of which organism carries out, and more importantly, controls the production of ecologically important secondary metabolites is still controversial. Historically, terpenes were viewed as plant products (Scheuer, 1973) and thus "by default" zooxanthellae were believed for a long time to be the sole producers of terpenoids in the symbiotic association. This premise was supported by results from one of the first studies on octocoral secondary metabolites carried out by Ciereszko in 1962. The author concluded that terpenes were of algal origin after he found high concentrations of the diterpene crassin acetate in zooxanthellae isolated from the gorgonian *Pseudoplexaura crassa*. While Papastephanou and Anderson (1982) confirmed through *in vitro* experiments the algal origin of the compound (using radioactively labelled precursors), the authors suggested that the gorgonian host controlled synthesis. Apparent evidence for possible host control of terpene production was provided by Ciereszko (1989) who found that several species of aposymbiotic soft corals harboured the same or similar types of terpenes in their tissues, but in much lower quantities than zooxanthellae bearing species. It remained ambiguous, however, whether or not the terpenes in aposymbiotic soft corals were acquired from dietary sources. Kokke et al. (1984) also concluded that terpenes were of animal origin, based on comparisons of $^{12}C/^{13}C$ ratios for products from different octocorals and their cultured zooxanthellae. In addition, Bandurraga and co-workers (1982) interpreted the isolation of terpenoids from the azooxanthellate gorgonian *Lophogorgia alba* as evidence that the animal itself was responsible for the production. As pointed out above, an inherent problem associated with all of these studies (with the exception of Papastephanou and Anderson, 1982) is the difficulty of excluding the possibility that terpenoids were of dietary origin. Thus, there is currently no conclusive evidence that the animal produces or controls the production of terpenes in symbiotic soft corals.

Quantitative and qualitative variations in secondary metabolite chemistry have been documented among soft coral species, both within and between species (Harvell and Fenical, 1989; Harvell et al., 1993; Bowden unpublished data). If indeed the algal partner is responsible for the production of secondary metabolites, then variation in terpenoid products between and within one species could be due to genetically different strains of zooxanthellae (Rowan and Powers, 1991; Loh et al., 1998) which may have different biochemical and physiological characteristics (Schoenberg and Trench, 1980 a-c). Evidence for substantial impact of the algal partner on cnidarian host physiology has been provided by Kinzie and Chee (1979) and Fitt (1985) who experimentally demonstrated significant differences in the host's growth rate, depending on the type of zooxanthella with which a cnidarian host became inoculated. While we are starting to understand the genetic, biochemical and physiological diversity of various zooxanthellar strains, their impact on soft coral metabolism remains virtually unresolved and warrants further study.

In this study I tested the effects of zooxanthellae of different geographic and taxonomic origin on the secondary metabolite chemistry of the alcyonacean coral *Lobophytum compactum.* To overcome confounding problems with residual terpenoids in adult colonies and possibly dietary origin of the compounds, I infected freshly metamorphosed polyps of the same genetic background with different algal symbionts, under controlled conditions that excluded dietary terpenoids. This soft coral species is particularly suitable for the study since it has only one major terpenoid secondary metabolite, the cembranoid diterpene, isolobophytolide, (Coll et al., 1986), an ichthyotoxin (Bowden, unpublished data). By infecting newly metamorphosed polyps with different strains of zooxanthellae, (while some were left uninfected) I addressed two questions – firstly, whether the zooxanthellar partner was necessary for the production of terpenoid secondary metabolites, and secondly, which partner controlled the production of certain metabolites.

5.3. MATERIALS AND METHODS

5.3.1. Gamete collection

Egg and sperm were collected in November 1996 during the annual mass coral spawning event in Pioneer Bay, adjacent to Orpheus Island Research Station (OIRS), in the Central section of the Great Barrier Reef. Eggs were collected *in situ* from one *Lobophytum compactum* female (to ensure genetic similarity of offspring) from the reef slope at 3-4 m depth by placing egg nets equipped with collecting jars on top of the colony. Egg nets were assembled approximately 1 hour prior to spawning (17:00) and retrieved 3 hours later (20:00). To collect sperm, three male colonies that had been dislodged from the reef substratum two months prior to spawning were arranged in well aerated flow-through aquaria at OIRS 4 days prior to spawning. Males were placed individually in highly aerated tanks only two hours prior to spawning. Water containing sperm from the male with the highest sperm count, was decanted immediately after spawning and diluted to optimal concentrations required for fertilisation success (10^6 sperm/ml, Willis et al., 1997).

5.3.2. Fertilisation

Spawned eggs were washed 3 times in millipore filtered seawater (0.45μm) and then incubated in ten 3L screw-top containers with sperm water at a concentration of 1.1 x 10^6 sperm/ml. Sperm from the most prolific male spawner was selected to fertlise all eggs. After 3 hours, sperm-water was discarded, the fertilised eggs washed in millipore filtered seawater, returned to the containers and left overnight in high flow-through aquaria at OIRS. Planulae were transported the next day to the James Cook University Aquarium facilities. To assess fertilisation rates, after exposure to sperm, an aliquot of each 3L container, containing the fertilised and washed embryos was fixed in Bouins solution for 24 hours and then stored in vials containing 70% ethanol. One hundred eggs per vial were examined and those that had undergone cleavage were scored as fertilised. Percent fertilisation was then calculated for 8 replicate vials per jar (n = 80 measures of percentage fertilisation).

5.3.3. Larval rearing

Sub-samples of 500 larvae were reared in separate 10 litre aquaria, supplied with filtered flow-through seawater and maintained under temperature controlled (25-29°C) conditions. Metal-halide lights were set on a 12 hour light: 12 hour dark cycle (light was approximately 800 $\mu Em^{-2}sec^{-1}$). Clay tiles which were autoclaved to ensure no zooxanthellae were present served as settlement substrata for the coral larvae. The tiles were left to pre-condition in 1µm filtered seawater for one month prior to the start of the experiment. A filter system which excluded all particles > 1 µm was used at all times to ensure dietary exclusion of terpenoids in phytoplankton or zooplankton. Furthermore, the aquarium system was kept free of adult soft corals to ensure no terpenoids could be taken up from the water column.

5.3.4. Experimental design and symbiont infection

The experimental set-up comprised 3 replicate tanks per treatment (= strain of zooxanthellae), each containing a sub-set of 500 larvae (n = 8 treatments x 3 tanks x 500 larvae) (Figure 1). After larvae had metamorphosed, one of seven mixtures of gymnodinoid swarmers and non-motile coccoid zooxanthellae were added to each of the treatment tanks on a daily basis for two weeks. The treatment strains included zooxanthellae derived from *Lobophytum compactum* (maternal strain), and from the soft coral species *Lobophytum microlobulatum, Sarcophyton trocheliophorum* and *Sinularia flexibilis,* which were growing in close vicinity to the "mother" coral in Pioneer Bay. Furthermore, zooxanthellae derived from the actinarian coral *Zoanthus sociatus* (*Symbiodinium pilosum*, isolated by B. Fitt, Jamaica), the jellyfish *Cassiopeia xamachana* (isolated by E. Dearne 1983, Florida, available as strain CS-153 from CSIRO micro-algal culture collection, Tasmania), and the clam *Tridacna squamosa* (isolated by R. Rowan, GBR central section) were offered for infection (Figure 5.1.). The terminology "strain" is used *senu* Kinzie and Chee (1979) and Fitt (1985) to denote that zooxanthellae are of different geographic and taxonomic origin, and vary in their morphology and swarming behaviour. No claim is made that these different types ("strains") of zooxanthellae necessarily represent genetically different clades. All algal strains were kept in monoclonal cultures at James Cook University (JCU). To check that no parental provision of zooxanthellae had occurred, some eggs and larvae were sacrificed for microscopical examination (light microscope and inverted microscope) prior to infection. Positive inoculation of polyps with zooxanthellae was confirmed *in situ* by examining polyps from all treatments at regular intervals, using stereo microscopy.

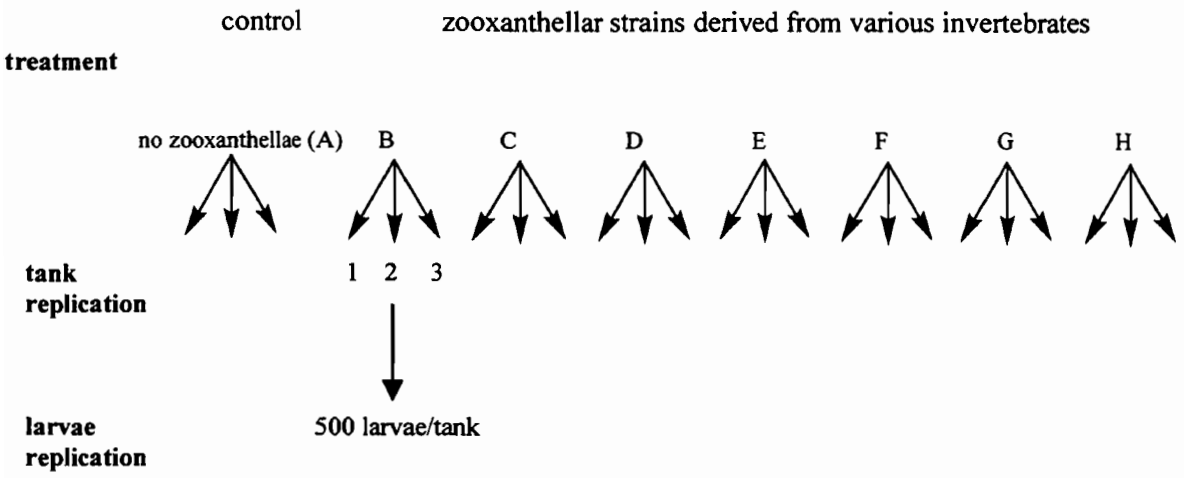


Figure 5.1. Experimental design for infection of coral polyps with zooxanthellae. While control polyps were not offered any zooxanthellae, all other treatments received zooxanthellae derived from various invertebrates of similar and different geographic origin: Treatment **A**: no zooxanthellae, **B**: *Lobophytum compactum* (maternal strain), **C**: *Lobophytum microlobulatum*, **D**: *Sarcophyton trocheliophorum*, **E**: *Sinularia flexibilis*, **F**: *Zoanthus sociatus.*, **G**: *Cassiopeia xamachana*, **H**: *Tridacna squamosa*. Each treatment was replicated in 3 tanks fitted with 500 planulae at the start of the experiment.

5.3.5. Offspring survivorship and polyp growth

The number of surviving polyps and their growth were assessed on a two-weekly basis for five months after fertilisation. Growth was recorded as the number of polyps per colony.

5.3.6. Preparation of polyps for terpenoid chemical analysis

After three months, eight single polyps from each treatment were harvested, freeze-dried and then washed three times with distilled water to remove excess salt. The whole animal was then extracted with 100% methanol (0.2 ml) under 10 min sonication. Samples were analysed on a Bruker 47e fourier transform mass spectrometer equipped with an atmospheric pressure Analytica electrospray ion source. Diluted extract was infused through an electrospray needle

at the rate of 100 µl per hour. Positive ion mode was used with a scan range from 80 to 3000 daltons and a cycle time of 1.3 sec. Resolution in the region of interest was approximately 10,000. Sixteen scans from each extract were summed and processed. The identity of compounds was established by accurate mass measurement of the sodium ion complex.

In addition to mass spectrometry, early larval chemistry, parental secondary metabolite chemistry and that of cultured zooxanthellae was confirmed by ^{1}H NMR, using a Bruker AM 300 NMR spectrometer. All samples were freeze dried, weighed and the ground tissue was extracted 3 times with dichloromethane (DCM, 10 ml/ g dry weight of coral tissue) for 1, 8 and 24 hours respectively. The 3 consecutive extracts were combined and the remaining solvent was removed *in vacuo*. Sixteen scans of each sample were recorded, spectra then Fourier transformed using zero line broadening and the appropriate signals integrated as described in Chapter 4, section 4.3.6.

5.3.7. Statistical analysis

The significance of differences in polyp growth rates was tested by two factorial-ANOVA with treatment as a fixed factor and tanks nested in treatment. Multiple comparisons (Tukey's HSD post hoc test) were then carried out to test differences between treatments. The SPSS 7.5 software package was used for the analysis.

5.4. RESULTS

5.4.1. Fertilisation

Fertilisation rates were high with sperm and eggs from the selected male and female colony under the given experimental conditions averaging 92.8% ± 1.8 standard error (SE).

5.4.2. Polyp settlement, metamorphosis and zooxanthellae infection

Polyps started to settle on the clay tiles 4 days after fertilisation and approximately 80% had settled after one week. Metamorphosis and development of the primary polyp followed 3 - 4 days after settlement. Post-settlement development began with the opening of the "mouth" (actinopharynx) and subsequent development of the tentacles and pinnules. Uptake (feeding) of all zooxanthellae strains offered followed immediately after metamorphosis. The establishment of a symbiosis (Figure 5.2), however, occurred only for 5 of the 7 strains offered.

Zooxanthellae derived from *Zoanthus sociatus* and *Lobophytum microlobulatum* did not result in the establishment of a symbiosis at any stage, and polyps died after 3 months. Thus no chemical analysis and growth rate data are provided for these two experimental groups. Control polyps, which were not offered any zooxanthellae, never became inoculated, thus confirming that no uncontrolled infection occurred during the experiment. These polyps started to degenerate after 2 months and were dead 3 months after spawning. It was established by ^{1}H - n.m.r. that none of the cultured strains of zooxanthellae contained isolobophytolide in their tissue prior to infection.

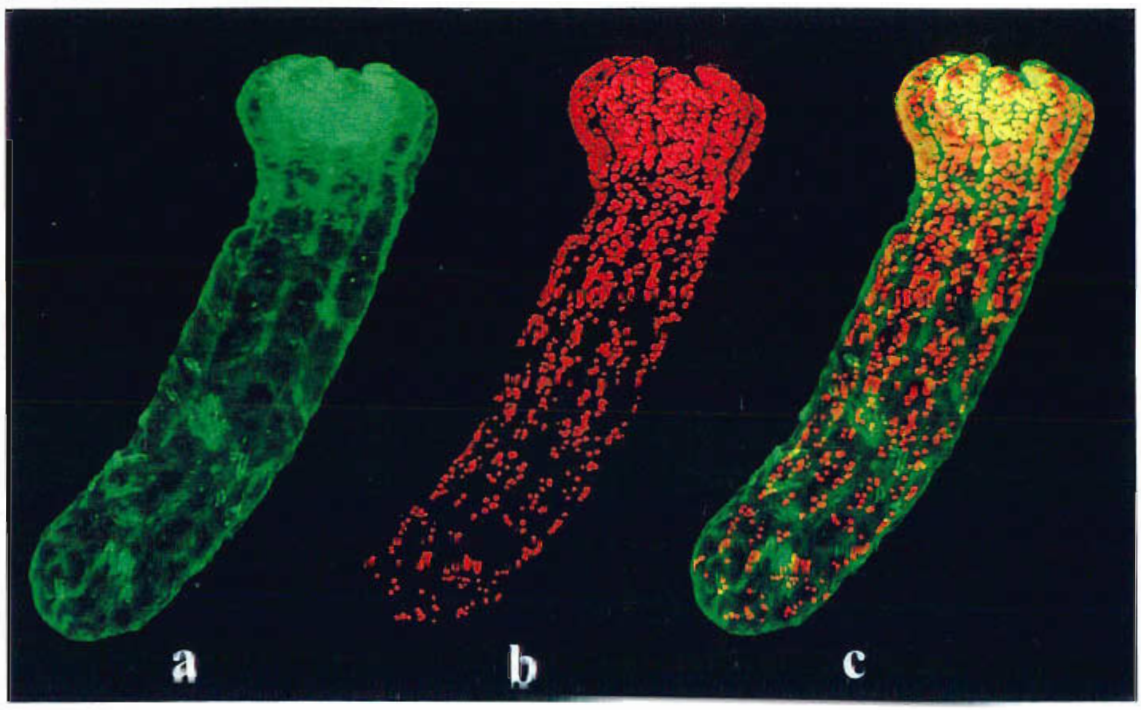


Figure 5.2. Confocal microscopy images of a single three months old *Lobophytum compactum* polyp, infected with zooxanthellae derived from *Cassiopeia xamachana*. This technique allows a separation of animal and plant tissue, with the green colour denoting animal tissue (**a**), while red represents the symbiotic algal cells (**b**). The superimposed image (**c**) clearly shows that zooxanthellae have populated most of the polyp after 3 months, with highest densities found in the light-exposed tentacles. (Confocal micrograph courtesy of A. Salih and the Electron Microscopy Unit, University of Sydney).

5.4.3. Polyp growth

Significant differences in growth rates were found between polyps infected with different strains of zooxanthellae (2-factorial ANOVA, $F = 13.65$, $df = 4$, $p < 0.0001$) (Figure 5.3.). Tanks (nested in treatment) had no effects on the polyp growth (2-factorial ANOVA, $F= 0.63$, $df = 2$, $p = 0.535$). Thus all treatment effects were independent from the tanks in which they were carried out. Post-hoc comparisons of polyps infected with the maternal strain

strain (*L. compactum*) versus zooxanthellae derived from either of the two other soft corals species (*S. flexibilis* and *S. trocheliophorum*), revealed that all colonies grew at similar rates (Tukey's HSD test, p = 0.992, p = 0.999 for *S. flexibilis* and *S. trocheliophorum* respectively). Those individuals infected with the jellyfish (*C. xamachana*) or the clam-derived (*T. squamosa*) strains, however, attained significantly lower growth rates than the control polyps (Tukey's HSD test, p = 0.05 for the former and p < 0.0001 for the latter species) (Figure 5.3).

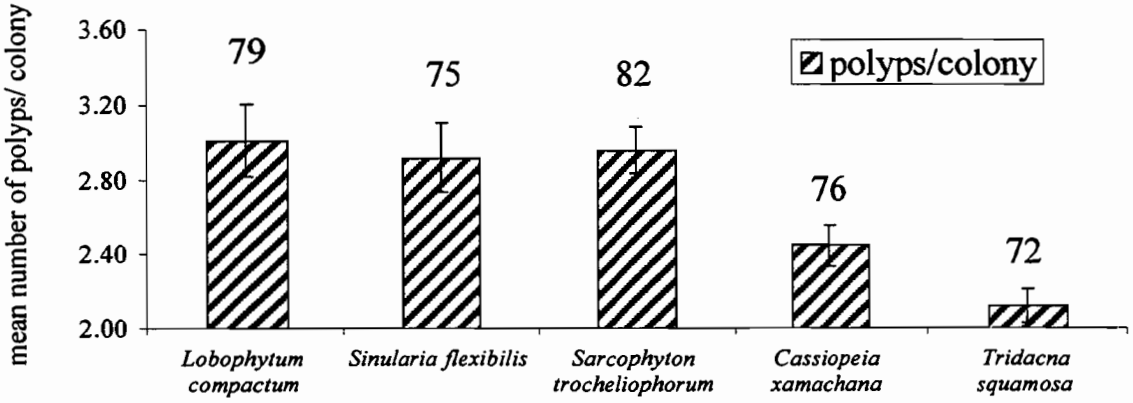


Figure 5.3. Mean number of polyps per colony in *Lobophytum compactum* inoculated with different strains of zooxanthellae five months after settlement. *Lobophytum compactum, Sinularia flexibilis* and *Sarcophyton trocheliophorum* strains are derived from soft coral hosts, whereas *Cassiopeia xamachana* and *Tridacna squamosa* strains are strains derived from a jellyfish and a clam respectively. Figures on top of columns represent the respective number of surviving colonies at five months. Error bars represent the standard error.

The differences in growth are further substantiated by the fact that a number of colonies infected with the maternal strain and with strains derived from other alcyonacean soft corals had reached the 8-polyp stage after 5 months. Primary polyps infected with the jellyfish and clam-derived strains, however, never reached this stage during the monitoring period. Polyps infected with the clam-derived strain of zooxanthellae did not reach the 4-polyp stage during the five months.

5.4.4. Secondary metabolite chemistry

NMR analysis of 6 week old polyps (pooled extracts, n = 8) inoculated with each of the five different strains of zooxanthellae, showed no detectable amounts of isolobophytolide (Figure 5.4, shows example for polyps inoculated with the maternal strain).

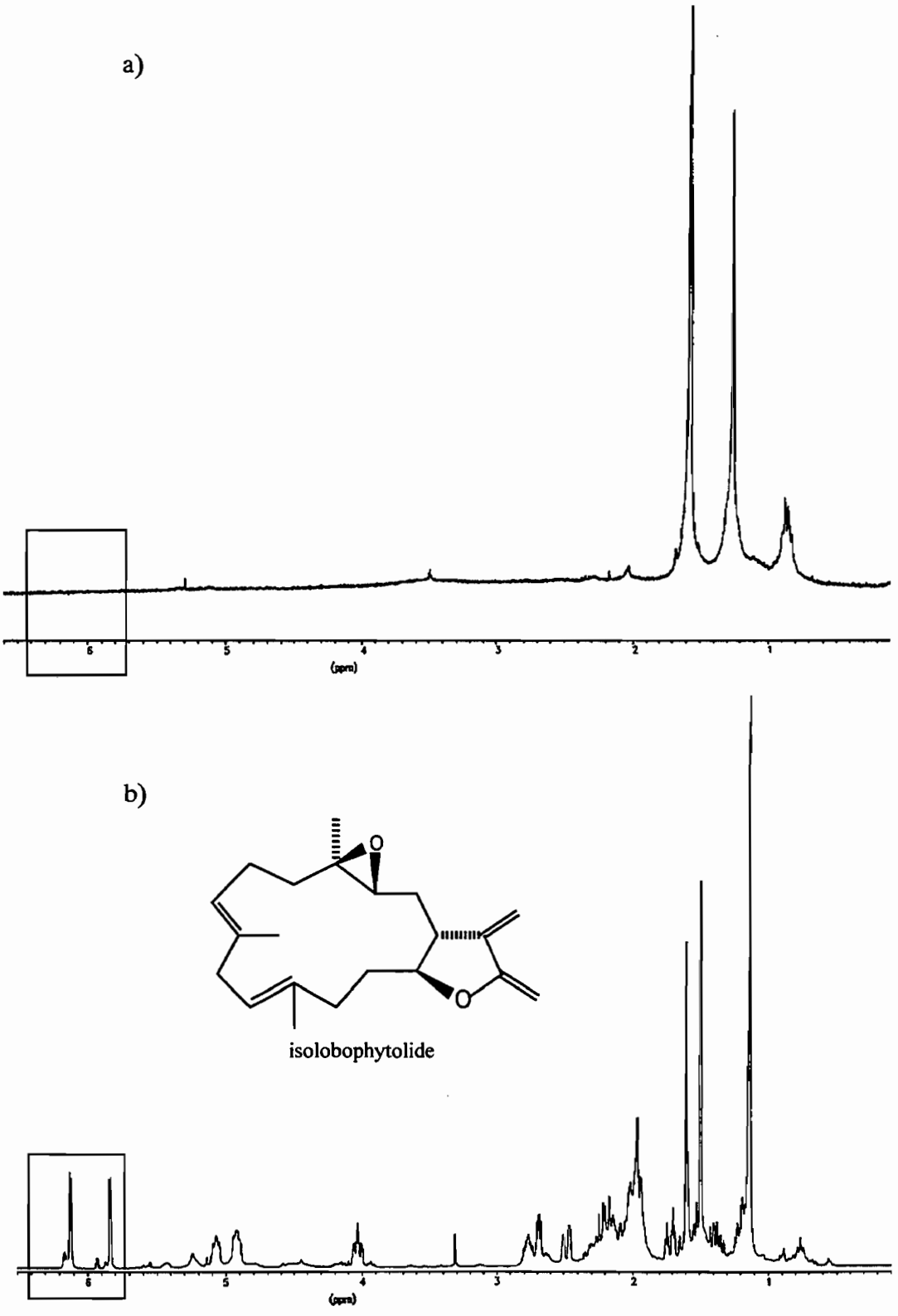


Figure 5.4. ^{1}H - n.m.r. spectra a of **a)** six week old inoculated polyps of *Lobophytum compactum* (pooled sample, n = 8) showing no traces of isolobophytolide or other methylene lactones and that **b)** of adult polyps with signals for the methylene lactone protons of isolobophytolide (see structure within) clearly visible at δ5.94 and δ6.23 (n = 8). Signals observed in the six weeks old polyp extract were assigned to lipids.

After 3 months, however, all zooxanthellae-host combinations resulted in the expression of the diterpene isolobophytolide, that is the typical *Lobophytum compactum* secondary metabolite. The calculated exact mass of the sodium ion complex of isolobophytolide ($C_{20}H_{28}NaO_3^+$) is 339.19363 and corresponds well with the observed mass, which ranged from 339.1906 to 339.1956 in the polyps. The compound, however, was not expressed in all polyps of each cross-infection, with higher percentage expression in polyps inoculated with either of the soft corals strains (Table 5.1).

Table 5.1. Percentage of individuals expressing typical *Lobophytum compactum* chemistry (isolobophytolide) after infection with different strains of zooxanthellae (n = 8 polyps per treatment). (No chemical analysis was carried out with polyps that were not successfully infected with the *Zoanthus sp.* and *Lobophytum microlobulatum* strain).

Treatment	***% polyps with isolobophytolide present in tissue (n = 8 polyps)***
Lobophytum compactum (maternal soft coral strain, Orpheus Island, GBR)	87.5
Sinularia flexibilis (soft coral strain, Orpheus Island, GBR)	100
Sarcophyton trocheliophorum (soft coral strain, Orpheus Island, GBR)	87.5
Cassiopeia xamachana (jellyfish strain, Florida)	75
Tridacna squamosa (clam strain, Great Barrier Reef)	50
Control (no zooxanthellae)	75

Note that the group that had no access to zooxanthellae (control) also produced isolobophytolide. No minor or atypical terpenoid products other than those found in the "mother" and "father" coral were produced in any of the symbiotic associations infected with different strains of zooxanthellae.

5.4.5. Correlation between growth and expression of secondary metabolite chemistry

Regression analysis indicated that the growth rate of colonies (number of polyps per treatment after 5 months) was strongly (R^2 = 0.864) and significantly (df = 1, F = 16.54, p = 0.02) correlated with the number of polyps that expressed the secondary metabolite isolobophytolide (Figure 5.5).

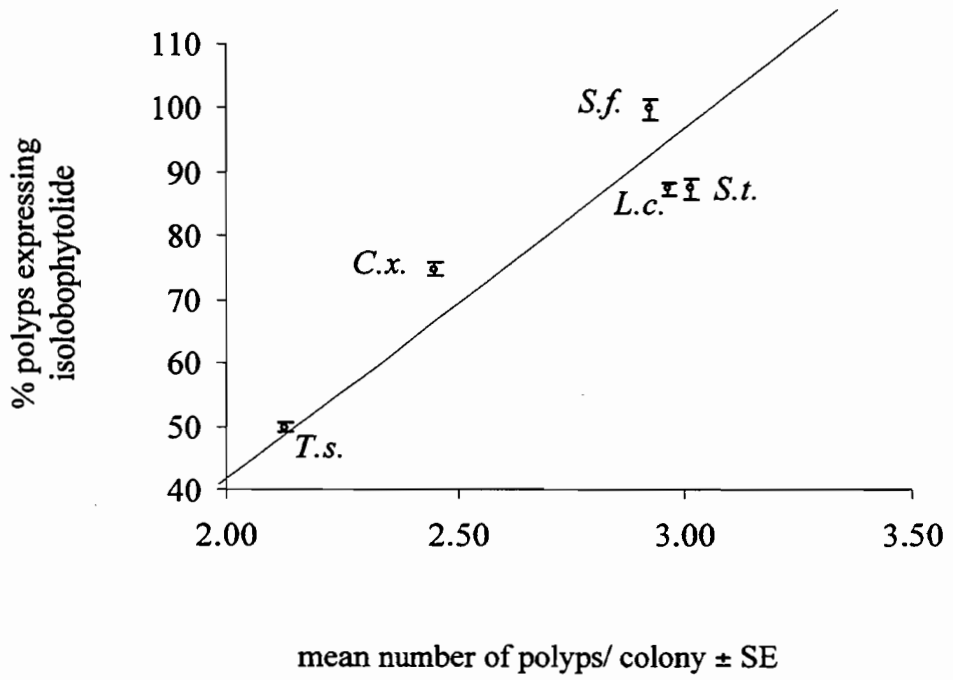


Figure 5.5. Relationship between growth of colonies inoculated with different strains of zooxanthellae (as mean number of polyps/colony) and the percentage of polyps expressing isolobophytolide. Zooxanthellae strains were derived from *Tridacna squamosa* (= *T.s.*), *Cassiopeia xamachana* (= *C.s.*), *Lobophytum compactum* (maternal strain, = L.c), *Sarcophyton trocheliophorum* (= *S.t.*) and *Sinularia flexibilis* (= *S.f.*). Error bars denote the standard error.

5.5. DISCUSSION

This study unequivocally demonstrates for the first time, that control over production of terpenoid secondary metabolites lies with the coral host and that zooxanthellae are not essential for the production process. However, links between polyp growth and investment into production of the secondary metabolite suggest that zooxanthellae influence the quantity of the production via primary metabolism.

5.5.1. Control and production of terpenoid coral secondary metabolites

During the 30 year debate about control and production of terpenoids in corals, a number of studies have provided evidence for host control and/or production of terpenoids. Evidence has been either indirect (Kokke et al., 1984; Ciereszko, 1989) or experimental designs have not accounted for possible dietary sources (Schwartz and Scheuer, 1981; Bandurraga et al., 1982) and thus the origin of the compounds has never been conclusively established. By excluding dietary access to terpenoids and testing experimentally inoculated primary polyps that had no residual isolobophytolide (maternal supplies had been used up) I have provided direct evidence for host control and the independence of the animal from its algal symbiont for terpenoid production.

Bacteria are known to be prolific producers of secondary metabolites (Fenical, 1993; Pain, 1998) and are commonly associated as epifauna on *Lobophytum compactum* and on soft corals in general (Ducklow, 1979; Rublee, 1980; K.M-W unpublished). Therefore bacterial origin of terpenoids has also to be taken into consideration. Bacterial involvement in the production of the diterpene isolobophytolide, however, is highly unlikely for a number of reasons. Firstly, bacteria are predominantly associated with coral mucus and are not intracellular symbionts, therefore translocation of the compound to the host tissue, which harbours about 70% of the isolobophytolide (K.M-W unpublished) would be extremely difficult. Furthermore, polyps were washed 3 times with distilled water prior to chemical analysis, which would have removed most of the mucus and its associated epifaunal bacteria, including the bacterial terpenoids. Finally, despite intensive research into bacterial secondary metabolites, to date no terpenoid products have been reported from marine bacteria (reviewed in Fenical, 1993). Thus, I believe that the animal host is most likely the producer of isolobophytolide in *Lobophytum compactum*.

Maternal provision of eggs with secondary metabolites is well established for *L. compactum* with epi-thunbergol, alcyonal-A and isolobophytolide as typical terpenoid egg products

(Heaton, 1988; Almeida de Leone, 1993). Fast metabolisation (within days) of some of these egg - specific compounds has been reported in freshly metamorphosed *L. compactum* polyps (Heaton, 1988). By the time of settlement, epi-thunbergol and alcyonal-A, are no longer detectable and concentrations of isolobophytolide have greatly decreased within one week old planulae (Heaton, 1988). Therefore, it was not surprising that I did not detect any traces of isolobophytolide (detectable by ^{1}H-n.m.r) in the six week old polyps. This absence of terpenoids in the six week old polyps and the lack of isolobophytolide in some of the three month old polyps is an important confirmation that the products I observed were not just the remains of parental provision to eggs, but produced by the progeny themselves.

While all cross-infected groups tested in this study expressed the typical *Lobophytum compactum* chemistry, not every polyp of each treatment contained a detectable amount of isolobophytolide in its tissue at three months of age. The reason for the absence of isolobophytolide in the three month old polyps is most likely due to insufficient energy reserves to divert to the production of secondary metabolites. The animals tested were very young and thus resource allocation to primary metabolism and growth was obviously favoured in some individuals as an early life history strategy. It is also possibly that the establishment of high densities of algal symbionts initially requires host energy reserves and nutrients, and only when a certain density of algal symbionts is reached, does a positive energy budget for the host follow. If this interpretation was correct, only once zooxanthellae cells reach a certain density within their juvenile host (primary polyp) sufficient energy can be diverted to primary and secondary metabolism. While this is speculative, this interpretation is supported by the results of the regression analysis, which showed that the groups with high growth rates also contained the highest number of individuals that expressed isolobophytolide (Figure 5.1). Thus, while these results have demonstrated that different algal symbionts do not influence the type of terpenoid compound that is produced, they indirectly impact on the production via provision of energy through primary production. This has important implications for soft coral colonies affected by bleaching. While the production of e.g. terpenoid algaecides is controlled by the host, with reduced energetic contribution from the algal symbiont, energy reserves may be insufficient to maintain the production of threshold concentrations. This is consistent with the highly reduced levels of isolobophytolide I observed in experimentally bleached *Lobophytum compactum* (Chapter 4; Figure 4.5.) which only returned to background levels with zooxanthellar recovery. Moreover, those soft coral colonies affected by the 1998 mass-bleaching event, which were fouled and overgrown also had the lowest levels of their specific algaecide (Chapter 4; Figure 4.8) further corroborating the importance of zooxanthellae to the primary metabolism of the association.

5.5.2. The impact of different strains of zooxanthellae on colony growth

The type of zooxanthellae a host becomes inoculated with, has substantial impact on the association, given the significant variations in polyp growth rates. Polyps living symbiotically with strains derived from other alcyonacean soft corals species did not experience significantly different growth rates from polyps inoculated with the maternal zooxanthellae (Figure 5.5). Those living symbiotically with the jellyfish or clam strains, however, featured significantly lower growth rates. My findings are consistent with those of a number of studies, in which the possibility for cross-infection of coelenterate and mollusc hosts with various zooxanthellar strains has been demonstrated (Kinzie, 1974; Kinzie and Chee, 1979; Fitt, 1985). Those studies found significant differences in the growths rates of an anemone (Fitt, 1985) and gorgonian soft coral hosts (Kinzie, 1974; Kinzie and Chee, 1979) depending on the zooxanthellar strain used for inoculation.

The ranking of growth rates of the coral-alga associations tested in this study showed that polyps that were symbiotic with zooxanthellae isolated from certain alcyonacean hosts performed best, followed by those of *Cassiopeia xamachana* (jellyfish). The lowest growth rates were found in individuals inoculated with the mollusc strain derived from *Tridacna squamosa*. It appears that strains isolated from closely related hosts (*L. compactum, S. flexibilis, S. trocheliophorum,* phylum: Cnidaria, class: Alcyonaria,) perform better than those from either less closely related (*Cassiopeia xamachana*, phylum: Cnidaria, class: Scyphozoa) or unrelated strains (*Tridacna squamosa*, phylum: Mollusca, class: Gastropoda). Given that the strain isolated from the most closely related host, *Lobophytum microlobulatum*, however, did not result in the establishment of a symbiosis in the first place, suggests that phylogenic closeness cannot be the only factor involved in regulating host-symbiont interactions. Surface recognition phenomena, which are known to play an important part in the establishment of the symbiosis (Trench, 1997), may be one factor responsible for the failure to form symbiotic associations with the *Zoanthus sociatus* or *Lobophytum microlobulatum* derived strains. In the case of the latter strain, however, major differences in the secondary metabolite chemistry (Coll et al., 1986) and subsequent chemical incompatibility may also be responsible. Unlike *L. compactum*, which produces cembranoid diterpenes, *L. microlobulatum* produces mainly lobanes together with germacrene and eudesmane-derived diterpenoids (Coll et al., 1986). Some soft coral secondary metabolites have been demonstrated to have negative impacts on zooxanthella motility, with total immobilisation of the alga in some cases (Ciereszko and Guillard 1989). Thus, a chemical incompatibility between the zooxanthellae of *L. microlobulatum* and the polyps of *L. compactum*, could have been the basis for the unsuccessful inoculation.

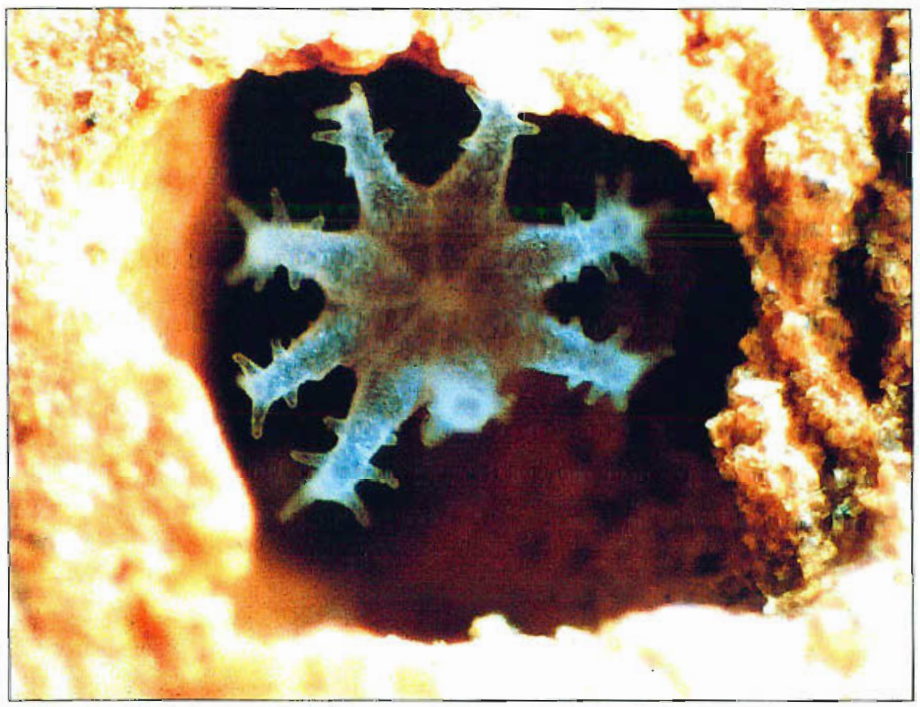
There is now overwhelming evidence that there are algal strains that are more susceptible to light and temperature stress than other strains (Dustan, 1982; Masuda et al., 1993) suggesting that inoculation with a "suitable" strain is crucial for the fitness of the symbiosis. The micro-environment from which a particular zooxanthellar strain is derived has been suggested to be the key-factor to determine the susceptibility to e.g. a certain photic regime (Berner et al., 1987; Rowan and Knowlton, 1995; Rowan et al., 1995; 1997). All soft coral zooxanthellar strains tested in this study were derived from the same site and depths as the maternal strain at Orpheus Island and thus the environmental conditions, that is the photic and temperature regime, were very similar for the three strains. In contrast, the *Cassiopeia xamachana* strain, for example, stems from a host that is pelagic for part of the time, and thus has most likely been adapted to different light, temperature and salinity levels. This could make it a less than ideal symbiotic partner, which is consistent with the observation of reduced growth rates of the juvenile soft corals.

5.5.3. Summary and conclusions

This study has provided unequivocal evidence that the algal partner is not directly required for the synthesis of terpenoid secondary metabolites in soft corals. The strong correlation between polyp growth and investment into production of secondary metabolites suggests, however, that via primary production zooxanthellae have the capacity to indirectly influence secondary metabolism. This has important implications for the soft corals affected by bleaching. With or at reduced energetic contribution from the algal symbiont, energy reserves in bleached colonies may be insufficient to maintain the production of threshold levels of ecologically important terpenoids such as algaecides, facilitating the entry of pathogens into already weakened colonies.

Chapter 6

The impacts of bleaching on the soft coral *Lobophytum compactum*. I. Fecundity, fertilisation and offspring viability



A freshly metamorphosed primary polyp of *Lobophytum compactum*

6.1. ABSTRACT

In this study I document long term effects of a simulated bleaching event on the reproductive output and offspring viability of the soft coral *Lobophytum compactum*. Corals were subjected to temperature and UV radiation treatments to produce both moderately (48-60%) and heavily bleached (90-95%) colonies. Although bleached colonies recovered their zooxanthellae within 4 months, impacts on reproductive output were significant for at least two spawning seasons. In the first year, both polyp fecundity and mean oocyte diameters were inversely correlated with the degree of bleaching, with complete failure of fertilisation in the group of heavily bleached colonies. Survival and growth of sexual offspring did not differ significantly between moderately bleached and unbleached treatments. Although no further reductions in zooxanthellae densities were recorded throughout the subsequent year, egg size and fecundity of the heavily bleached soft corals were still significantly reduced 20 months later. Severe bleaching clearly has long-term sublethal impacts, reducing overall reproductive output for at least two spawning seasons.

6.2. INTRODUCTION

The evolutionary success of shallow tropical coral reefs in a highly oligotrophic system is generally attributed to the predominance of symbiotic relationships between coral hosts and their algal symbionts (Odum, 1971). Tight nutrient recycling of crucial plant nutrients (ammonia and phosphate) from the coral's waste metabolism and the translocation of photosynthates (i.e. glycerol, lipids, amino acids, small peptides) are the fundamental processes contributing to the associations' success (Trench, 1979; Miller and Yellowlees, 1989; Sutton and Hoegh-Guldberg, 1990; Wang and Douglas, 1998; Swanson and Hoegh-Guldberg, 1998).

Under ideal conditions, it is estimated that the autotrophic production of zooxanthellae can provide as much as 143% of the daily energy budget of the coral host (Davies 1991). Thus, disruption of the symbiosis due to extensive bleaching, will translate into nutritional constraints for the coral association. Consequently, extensive bleaching has been shown to result in decreased lipid and protein concentrations in host tissues (Fitt et al., 1993), reduced calcification (Clausen and Roth, 1975; Leder et al.,, 1991), reduced growth (Szmant and Gassman, 1990; Goreau and Macfarlane, 1991) and, in extreme cases, in the coral's death due to starvation (Fabricius 1999).

The loss of zooxanthellae not only results in decreased autotrophic input to the association, but may also require catabolisation of storage lipids. In extreme cases, catabolisation of structural lipids and proteins, which has been reported for recovery after (mechanical) injury in corals may be required (Meesters et al., 1994; Ward, 1995). Furthermore, heterotrophic feeding, which has been suggested to play an integral role in soft coral diets (more so than in hard corals) (Fabricius, 1995a) is highly reduced in bleached soft coral colonies (Fabricius, 1999). Devoid of both principal modes of nutrition, i.e. autotrophic input through zooxanthellae and heterotrophic feeding, and possibly having to re-allocate remaining resources towards recovery, corals affected by bleaching may not have sufficient energy for reproduction.

To date, only one pioneering study, carried out by Szmant and Gassman (1990) has investigated the possible effects of bleaching on gametogenesis and provided the first, if limited evidence, that bleaching has the potential to disrupt the reproductive cycle. Given the important role that sexual reproduction plays in the maintenance and replenishment of reefs, studies of the possible longer-term, sub-lethal impacts of bleaching on reproduction are clearly required.

In this study I simulated moderate (approx. 50% reduction in zooxanthellae densities) and strong (90-95% reduction) bleaching events and monitored the effects of the disturbance on

reproductive output in the common, dioecious soft coral *Lobophytum compactum* for the subsequent two spawning seasons. This chapter documents the impact of the disturbance on adult soft corals (fecundity and egg size), their gametes (fertilisation) and their offspring (survival and growth of juveniles). Chapter 7 documents the impact of the disturbance on biochemical parameters associated with fitness and reproduction in the experimental adults and their gametes.

6.3. MATERIALS AND METHODS

6.3.1. Site description

This study took place between October 1995 and December 1997 in Pioneer Bay (for location, see figure 2.1 in Chapter 2). All corals were collected from the reef slope at 3-4m depth and subsequently returned to the same site during the recovery phase.

6.3.2. Species description

Lobophytum compactum Tixier-Durivault, 1956, a common reef flat coral on the inner and mid-shelf reefs of the Great Barrier Reef (Dinesen 1983), was used in experimental manipulations described below. As found in some soft corals (Achituv and Benayahu, 1990) and typical for this genus, polyps of *L. compactum* are dimorphic with autozooids functioning as feeding polyps and bearing gonads, whereas siphononzoids are reduced in size and function solely to circulate water throughout the colony (Tixier-Durivault, 1958; Versefeldt, 1983). *L. compactum* is gonochoric and has a two-year oogenic cycle (Aliño and Coll, 1989) so that large and small eggs, representing two different year cohorts, are present simultaneously within autozooids (Aliño and Coll 1989). In contrast, spermatogenesis requires only one year (Aliño and Coll 1989; K.M-W, unpublished), i.e. mature oocytes are produced every year in synchrony with the annual cycle of sperm production in male colonies, but mature females carry two generations of oocytes at any one time, as described for *Sarcophyton glaucum* (Benayahu and Loya, 1986). Staging of oocytes was carried out after Yamazato et al. (1981). The species typically spawns on the 4th day after the November full moon at Orpheus Island, with egg release starting around 18:30 and ending around 20:30 (Aliño and Coll 1989; K.M-W, unpublished).

6.3.3. Collection and preparation of experimental corals

Ten randomly-selected female colonies of *Lobophytum compactum* were divided *in situ* into 4 approximately equal-sized pieces. Each fragment was randomly assigned to a different group (a, b, c, or d) and each group subjected to a different experimental treatment. Only female colonies of a similar initial diameter were used. To avoid reversed puberty associated with reduction in colony size below a minimum threshold, all females were approximately 1 m^2 in size and

fragments were between 36-40 cm in diameter. After chiselling and tagging, all coral pieces were left to recover at their site of origin for 5 months. Group "a" were chiselled colonies that remained *in situ* for the duration of the study (handling controls) whereas group "b" corals were transported to flow-through tanks where they were maintained at ambient temperatures and light (tank controls). Group "c" and "d" corals were submitted to increased solar radiation and temperature to simulate a moderate and severe bleaching event respectively. In addition, ten random colonies (group "e" corals) from the same site and depth as experimental colonies were tagged and left *in situ* as unchiselled field controls (chiselling controls).

6.3.4. Bleaching treatment

In order to simulate a moderate bleaching event (approx. 50% loss of zooxanthellae), group "c" corals were submitted to an enhanced solar radiation and temperature treatment for 8 days. The simulation of a comparatively strong bleaching event (90-95% loss of zooxanthellae) was accomplished by treating group "d" corals to the same experimental treatment for 12 days. Temperatures in flow-through experimental tanks at Orpheus Island Research Station were increased from 29-30° C (ambient) to 31° C ± 0.5° C (see Berkelmans and Willis, 1999, for details of the experimental set-up). Photosynthetically active radiation (PAR) and ultraviolet radiation (UVR) were increased through translocation of corals from 3-4 m *in situ* to 50 cm in the experimental tanks. This translocation was approximately equivalent to an increase of 50% in PAR and 33% in UVR respectively according to light profiles recorded in Pioneer Bay using a Li-Cor light sensor and UV sensor (calculations based on data provided by W. Dunlap, unpublished). The timing of the experiment, March 1996, was set to coincide with the most likely timing for a natural bleaching event.

6.3.5. Sampling regime and analysis of biochemical and reproductive parameters

At the conclusion of the bleaching treatment, corals were returned to the site of collection and their recovery monitored initially at 2-weekly and then at monthly intervals between March 1996 and November 1997. Each month, tissue samples (5 lobes in the range of 1-2 g wet weight / colony) were collected and frozen at –20° C prior to determination of zooxanthellae densities.

1. Quantification of zooxanthellae tissue concentrations: Zooxanthellae densities were monitored as indicators of bleaching and recovery of host tissues and quantified as described in Chapter 2, section 2.3.2).

2. Gamete capture: Eggs were quantitatively collected by placing egg nets fitted with collecting jars on top of each experimental and control female colony. Egg nets were deployed approximately 1 hour prior to spawning (17:00) and retrieved 3 hours later (20:00). To collect sperm, male colonies were placed in well-aerated flow-through aquaria at Orpheus Island Research Station 4 days prior to spawning and placed individually in highly aerated containers 2 hours prior to spawning. Four male colonies of *L. compactum* were collected randomly and thus their bleaching histories were unknown. Sperm water was decanted immediately after spawning and diluted to concentrations found to optimise fertilisation (10^6 sperm/ml, Willis et al., 1997).

3. Fecundity and egg size determination: Immediately prior to spawning, tissue samples at least 3 cm long were collected from ridges located at the centre of each female colony in all experimental treatments. Samples were fixed in the field with 10% seawater formalin for 24 hours, rinsed in freshwater and then stored in 70% ethanol (Jokiel and Coles, 1977; Szmant, 1991). Staging of oocytes was carried out after Yamazato et al. (1981). Ten polyps per colony were dissected to estimate polyp fecundity. In each polyp, the diameters of ten stage III oocytes (mature oocytes) were measured using a calibrated ocular micrometer. Only oocytes over 580 μm were scored as mature.

4. Fertilisation rates: Fertilisation experiments were only carried out during the 1996 breeding season. Spawned eggs from all corals (including the minute quantity of eggs spawned by the heavily bleached group) were collected in egg nets and washed 3 times in millipore filtered seawater (0.45 μm). Eggs were combined with sperm from each of the four males, that is 4 egg-sperm combinations per female. For each combination, eggs were incubated in quadruplicate vials with sperm at a concentration of 1.2×10^6 sperm/ml. After 6 hours, sperm-water was discarded, the fertilised eggs washed in millipore filtered seawater, fixed in Bouins solution for 24 hours and then stored in 70% ethanol. One hundred eggs per vial were examined and those that had undergone cleavage were scored as fertilised.

6.3.6. Larval rearing

Approximately 500 larvae from the moderately bleached group and each of the control groups (combined from all female colonies within each group) were reared in separate 10 litre aquaria supplied with 1 μm filtered flow-through seawater and maintained under temperature controlled (25-29° C) conditions. Metal-halide lights provided light in the PAR region (approximately 800 $\mu Em^{-2}sec^{-1}$) to each aquarium and were set on a 12 hour light: 12 hour dark cycle. Previously conditioned clay tiles served as settlement substratum for the coral larvae. After larvae had metamorphosed, gymnodinoid swarmers and non-motile coccoid zooxanthellae derived from *Lobophytum compactum* (maternal strain) and kept in culture at James Cook University were added to each of the four treatment tanks on a daily basis for two weeks.

6.3.7. Juvenile growth

Colony growth was assessed on a two-weekly basis for five months after settlement of larvae and recorded as the number of polyps per colony.

6.3.8. Statistical analysis

Differences in fertilisation rates, fecundity, egg sizes and growth of polyps were tested for significance by one way ANOVA followed by multiple comparisons (Tukey's HSD test) using the SPSS 7.5 software package. Regression analyses were carried out in order to establish the relationships between polyp fecundity and both the degree of bleaching and time for recovery of zooxanthellae densities.

6.4. RESULTS

6.4.1. Coral survival and recovery of zooxanthellae

Comparison of zooxanthellae densities before and after experimental treatment revealed that the moderately and strongly bleached corals lost approximately 49% and 93% of their zooxanthellae populations respectively (Table 6.1). In contrast, no colonies in any of the control groups (chiselling, handling or tank controls) underwent significant bleaching during the experimental period (Table 6.1) and no visible effect of handling could be discerned. No mortality of test corals, regardless of whether bleached or unbleached, handled or not handled, was recorded during the experiment.

Table 6.1. Mean zooxanthellae densities/ g tissue wet weight (± SE) before and after experimental treatments in *Lobophytum compactum*. N = 10 colonies (each density is a mean of 2 subsamples per colony x 4 replicate assays per subsample).

Treatment	**Mean zooxanthellae densities**	
	before treatment (n = 10)	**after treatment** (n = 10)
Handling control "a"	33.03 ± 1.21	33.13 ± 0.79
Tank control "b"	34.08 ± 1.03	33.39 ± 0.62
Chiselling control "e"	33.05 ± 1.24	34.40 ± 1.09
Moderately bleached corals "c"	33.06 ± 0.85	16.83 ± 0.71
Heavily bleached corals "d"	32.63 ± 0.81	2.33 ± 0.21

Regression analysis indicated that the length of time required for zooxanthellae to return to background densities was strongly correlated with the degree of bleaching (R^2 = 0.96). While the moderately bleached group recovered within 10 weeks, zooxanthellae densities in colonies that were strongly bleached returned to background levels only after 18 weeks (Figure 6.1). With the exception of a slight loss of zooxanthellae during the summer of 1997 (February/ March; < 7 % decrease), no other natural loss of zooxanthellae occurred during the 20 months.

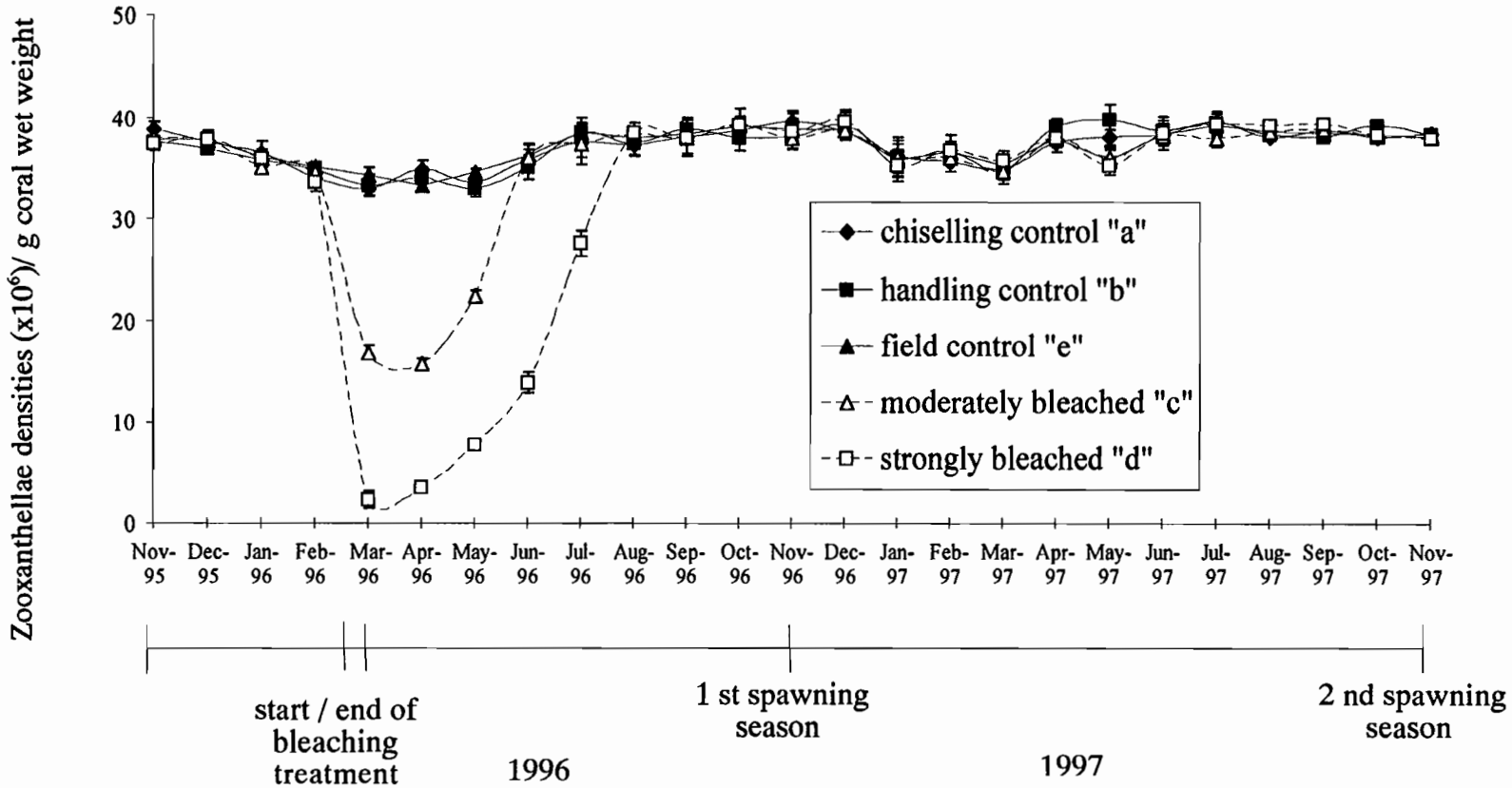


Figure 6.1. Mean zooxanthellae densities (± SE) in three control and two experimental groups of *Lobophytum compactum* between November 1995 and November 1997. N = 10 colonies (i.e. each density is a mean of 2 subsamples per colony x 4 replicate assays per subsample). Vertical bars on the time scale denote the initiation of the experiment, the beginning (1 March 96) and end (15 March 96) of the bleaching treatment and the spawnings in November 1996 and 1997 respectively.

6.4.2. Oogenic cycle

I found gonads only in the autozooids of *Lobophytum compactum* but not in the siphonozooids. All autozooids examined in both the colony centre and 3 cm from the tips of colony ridges were fertile. Autozooids bore gonads on up to six of the eight mesenteries. Both immature stage II oocytes (Figure 6.2a) and mature stage III oocytes (Figure 6.2b) were present in autozooids just prior to spawning in both 1996 and 1997. Oocytes were borne along the length of mesenteries with stage II oocytes developing proximally and stage III oocytes distally relative to the oral disc of the polyp (Fig 6.2b).

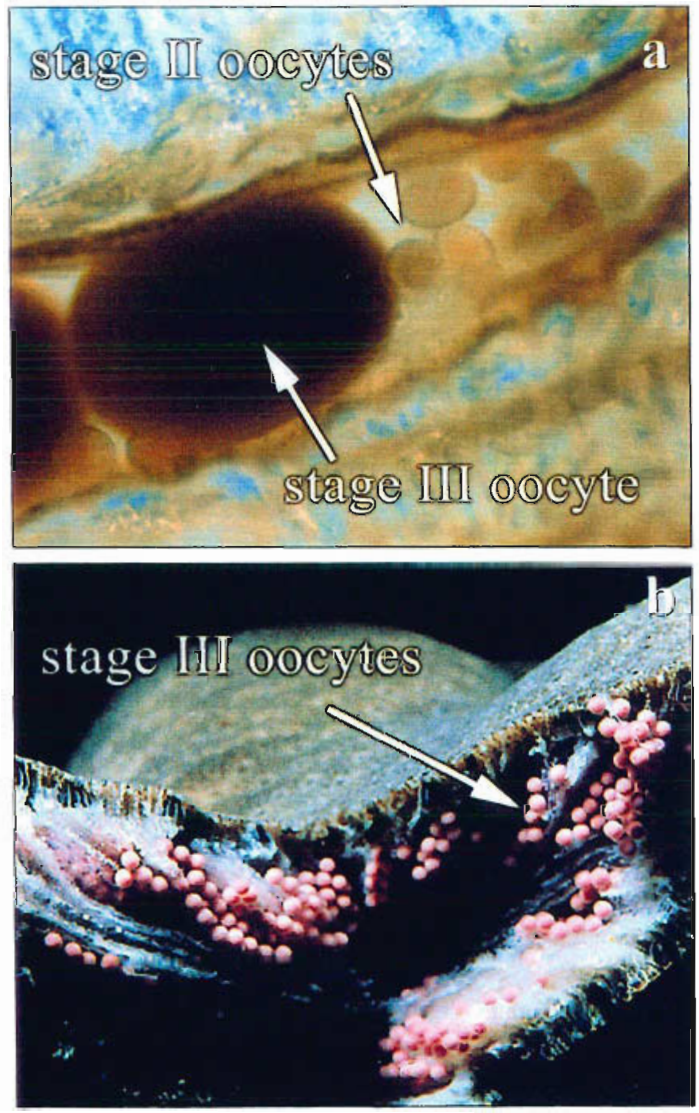


Figure 6.2. Oocytes in the tissue of *Lobophytum compactum*. **a)** Histological section showing stage II (immature) and stage III (mature) oocytes, **b)** Dissected colony from the control group showing stage III oocytes prior to spawning.

By the 1996 November spawning, gametes of severely bleached females were at a stage normally reached in May, which means that their development lagged behind controls by five months. Interestingly, the unspawned eggs did not change in size or resume development until April of 1997, when they were once more in phase with the normal oogenic cycle (per. obs.). Maturation of all other oocytes in their second year of development was accompanied by a gradual colour change from white to bright pink (Figure 6.2b), which is due to a protein bound carotenoid in the eggs (B. Bowden, pers. com.). Oocyte maturation was found to occur synchronously within and between colonies. Mature stage III oocytes (diameter of 600-620 μm) were recorded around September, that is 2-3 months prior to spawning and 22-23 months after first appearing as stage II oocytes with an initial diameter of 50 μm (data not shown). The mean diameters of the large and small oocytes at spawning were 630 ± 1.45 μm (n = 1000) and 110 ± 1.26 μm (n = 1000) respectively. No parental provision of zooxanthellae was detected in eggs of *L. compactum*. Uptake of zooxanthellae and establishment of a symbiosis occurred only after metamorphosis.

6.4.3. Polyp fecundity

Chiselling and handling of coral colonies had no significant effect on their fecundity (Figure 6.3, cf. handling and tank controls *vs* undisturbed field (= chiselling controls)).

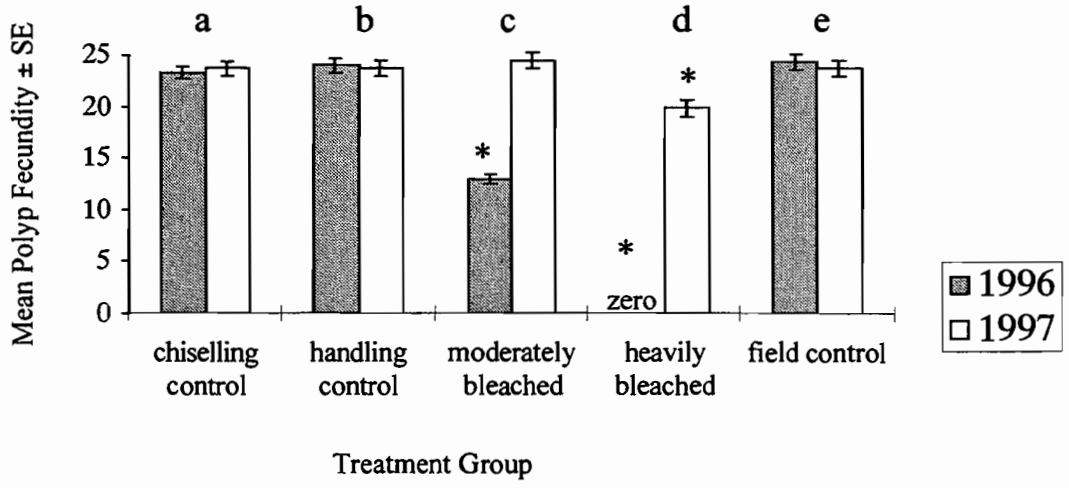


Figure 6.3. Mean polyp fecundity (± SE) in bleached and unbleached colonies of *Lobophytum compactum*, one (1996) and two (1997) spawning seasons after experimental treatment. Fecundity is expressed as total number of mature oocytes (> 580μm)/ polyp. N = 100 polyps per group. Asterisks denote significantly different means for comparisons between treatments and controls in each year.

In 1996 the mean fecundity of the moderately bleached colonies (13 ± 0.4, n =100) was approximately half that of the chiselling controls (23 ± 0.6, n =100). The heavily bleached group failed to produce any mature oocytes at all in the first spawning season (Fig 6.3). In the second year following experimental treatment fecundity of the moderately bleached group was equivalent to that of the controls (Tukey's HSD test, p = 0.95). Reproductive output, of the heavily bleached group was still significantly reduced (Tukey's HSD test, p = 0.003) by approximately 20% (Figure 6.3).

A strong negative relationship was found between the degree of bleaching (in March 1996) and the number of mature eggs (stage III oocytes) in the gonads of *Lobophytum compactum* immediately prior to the first spawning season (in November 1996) (Figure 6.4). An analysis of variance of the regression of fecundity (numbers of stage III oocytes/polyp) on zooxanthellae density confirmed that the relationship was highly significant (R^2 = 0.92, F = 587.46, $p < 0.0001$).

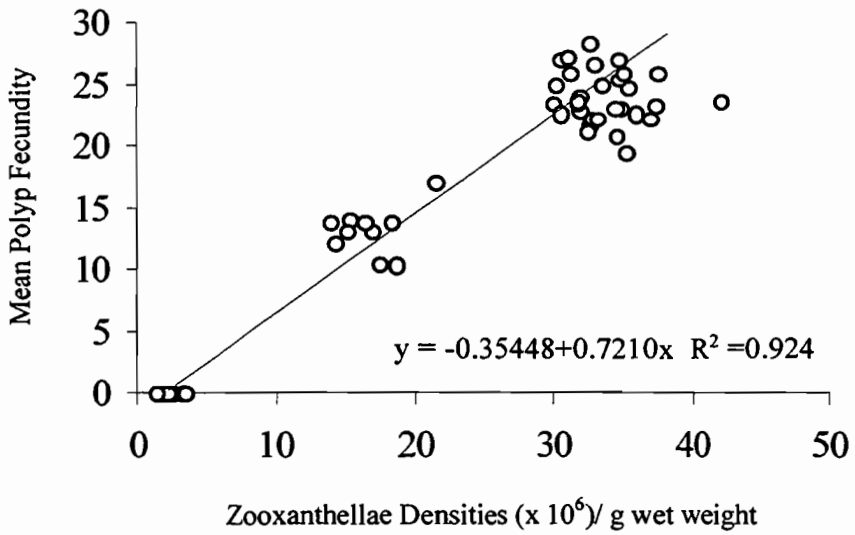


Figure 6.4. Relationship between polyp fecundity and zooxanthellae densities in *Lobophytum compactum*. Mean values were used to calculate the correlation coefficient, N = 10 colonies) for determinations of zooxanthellae densities (i.e. each density is a mean of 2 subsamples per colony x 4 replicate assays per subsample); N = 100 polyps for fecundity estimates.

6.4.4. Egg size comparisons

The diameters of stage III oocytes immediately prior to spawning followed a normal distribution in all experimental treatments, with the majority of eggs from females in control groups falling into the 605-655 μm size class (Figure 6.5). I found a highly significant difference in egg size, however, between control and experimental groups in the first (1996) spawning season (one-factorial ANOVA, $F = 1101.27$, $p < 0.0001$). Egg sizes of the moderately and severely bleached groups differed both from the controls (Tukey's HSD test, both $p < 0.0001$) and from each other (Tukey's HSD test, $p < 0.0001$). Chiselling and handling of the colonies did not significantly affect egg size, as no statistical difference in egg size could be discerned between the undisturbed field colonies (chiseling controls) and the handling or tank controls in either year (Tukey's HSD test for 1996 comparisons: $p = 0.18$, and 0.19 for field *vs* handling and field *vs* tank controls respectively; Tukey's HSD test for 1997 comparisons: $p = 0.97$, and 0.72 respectively). The most striking difference in the size-frequency distribution of eggs from moderately bleached colonies in comparison to control colonies was the strong decrease in the number of eggs in the two largest size classes. Despite this loss of large eggs, the modal size class of 605-655 μm was the same as that found in distributions for all of the control groups. In contrast, the modal size class for corals that had been heavily bleached decreased to 455-505 μm. Moreover, I found no eggs greater than 555 μm in diameter (Figure 6.5). Typically, a diameter of approximately 580 μm is the minimum size required for eggs to be spawned. With a mean diameter of 468.1 ± 1.47 μm, the eggs from heavily bleached corals were approximately 25% smaller than control eggs (mean diameter = 633.4 ± 1.86 μm; Figure 6.5).

I continued to detect a significant difference in mean egg size among control and experimental groups in the second spawning season following treatment (one-factorial ANOVA, $F=60.182$, $p< 0.0001$). Eggs from heavily bleached colonies were still significantly smaller than those from control colonies (Tukey's HSD test, $p < 0.0001$) by approximately 5% (Figure 6.5). Eggs from moderately bleached corals, however, were not significantly different from those of control colonies (Tukey's HSD test, $p = 0.94$) by the second breeding season.

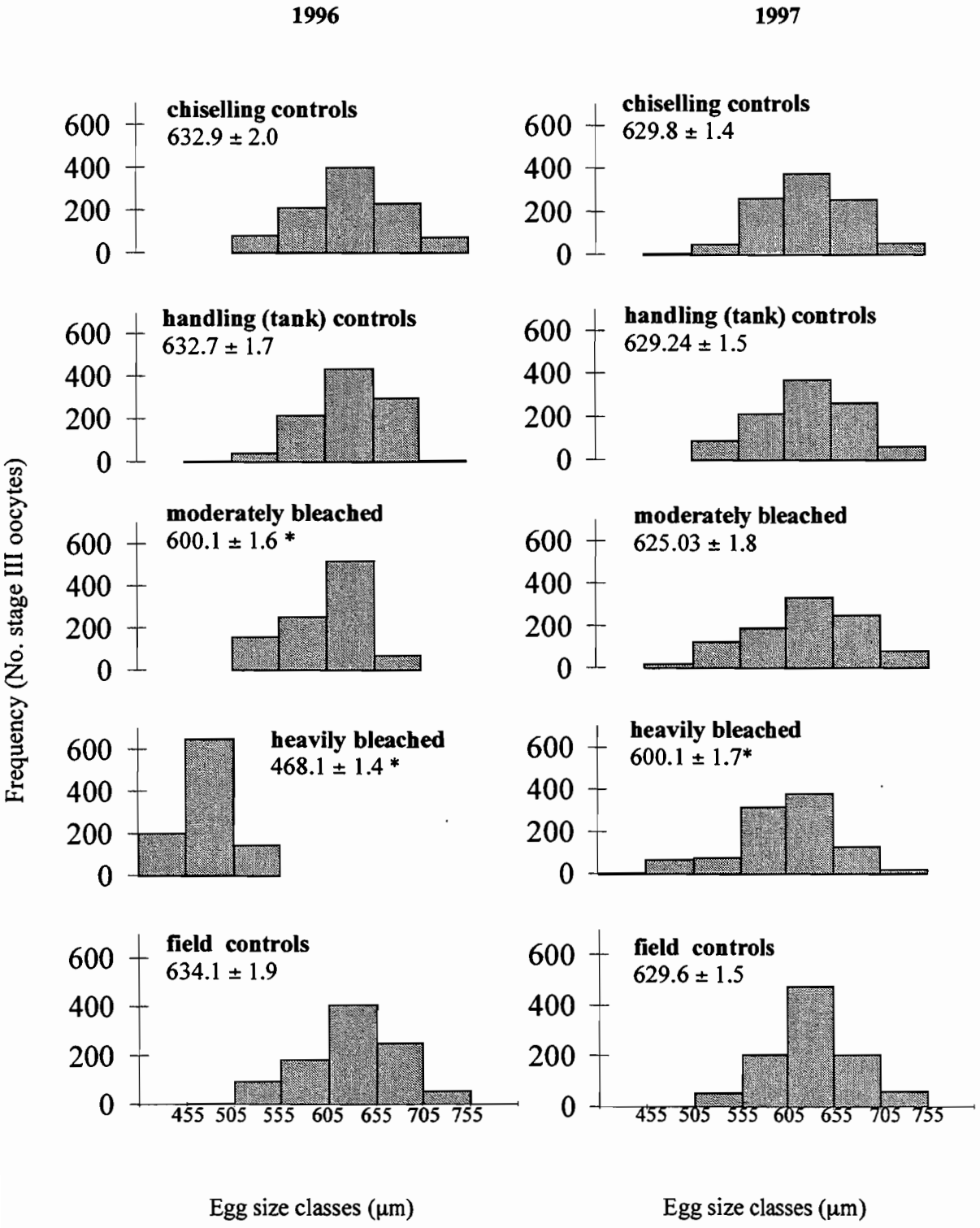


Figure 6.5. Size - frequency distributions of stage III eggs derived from bleached and unbleached colonies of *Lobophytum compactum* immediately prior to the 1996 and 1997 spawning seasons. N = 1000 eggs per treatment (i.e. 10 eggs x 10 polyps x 10 colonies). Numbers in the top right corner of each graph denote mean egg diameter (μm) ± SE. Asterisks denote a significant difference from the field control.

6.4.5. Spawning in 1996 and 1997

With the exception of the heavily bleached colonies of *Lobophytum compactum*, colonies in all experimental and control groups released all of their stage III oocytes in November 1996. In the heavily bleached group, 8 of the 10 colonies released a few eggs in November, but the rest did not spawn. In November 1997, all but three of the experimental colonies in the heavily bleached group released eggs (data not shown). Surprisingly, even though gonads of these three colonies contained mature-sized pink eggs, no spawning was detected in the following three months.

6.4.6. Fertilisation rates

Mean fertilisation success differed significantly among the two experimental and three control groups (one-factorial ANOVA, $F = 21.59$, $p < 0.0001$). This was entirely due to the fact that eggs collected from the heavily bleached group did not fertilise (Figure 6.6). No statistical differences in fertilisation rates were found between gametes derived from the moderately bleached corals (93 ± 2 mean percent fertilisation, $n = 40$) and those of the three control groups (92 ± 2, 92 ± 2 and 93 ± 2 mean percent fertilisation for the chiselling, handling and tank controls respectively, $n = 40$ for all groups, one-factorial ANOVA, $df = 3$, $F = 0.49$, $p = 0.69$).

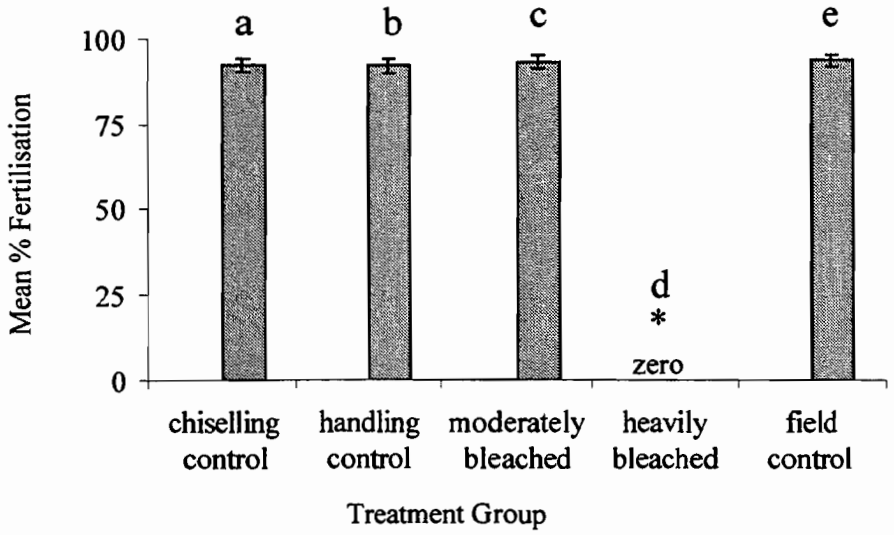


Figure 6.6. Mean fertilisation success (± SE) for eggs retrieved from bleached and unbleached colonies of *Lobophytum compactum* in the first spawning season following experimental treatment. N = 400 eggs per treatment. The asterisk denotes significantly different fertilisation success.

6.4.7. Survivorship and growth of offspring

No differences in survivorship or growth were detected between juveniles originating from the moderately bleached group and any of the control groups of corals (Table 6.2). Larvae (Figure 6.7a) started to settle on the clay tiles 4 days after fertilisation and approximately 80% had settled after one week. Metamorphosis and development of the primary polyp followed within 3-4 days after settlement. Post-settlement development began with the opening of the "mouth" (actinopharynx) and subsequent development of the tentacles and pinnules (Figure 6.7b). Juveniles reached the four-polyp stage after approximately 4 months (Figure 6.7c). Uptake of zooxanthellae followed immediately after metamorphosis and significant densities were visible within two weeks.

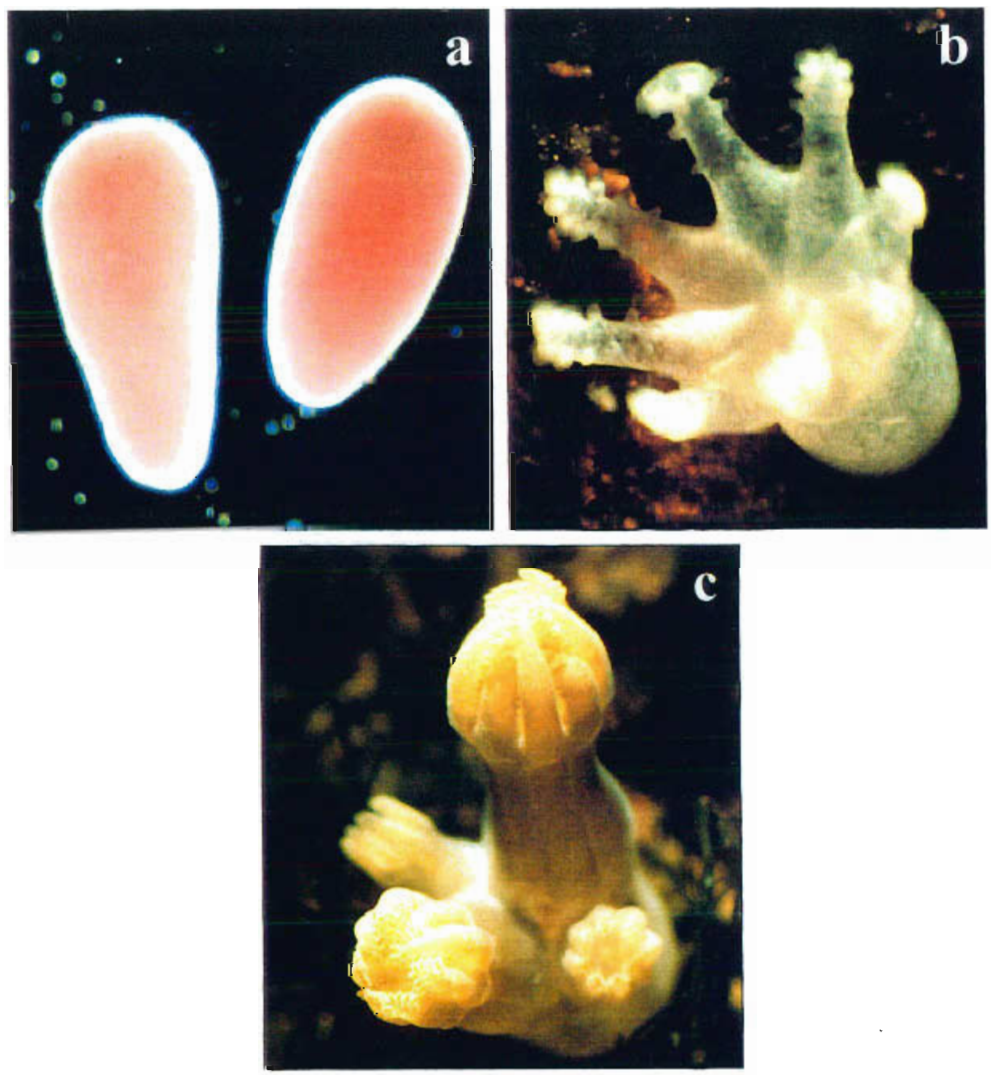


Figure 6.7. Developmental stages of *Lobophytum compactum:* **a)** planulae 48 hours after fertilisation; **b)** one-month old primary polyp; **c)** four-month old juvenile (4-polyp stage).

Table 6.2. Juvenile survival and growth measured as number of polyps per colony five months after spawning. A total of 500 larvae were monitored in each treatment. Multiple polyp stage refers to the number of polyps/ colony. Heavily bleached corals did not produce larvae.

Treatment	*Number of surviving juveniles after 5 months*	*Multiple polyp stage*				*Total number of polyps / treatment*	*Mean number of polyps / colony (± SE)*
		1	*2*	*3*	*4*		
Chiselling control "a"	36	2	7	10	17	114	3.16 ± 0.16
Handling control "b"	30	1	4	7	18	102	3.18 ± 0.16
Field control "e"	33	1	4	9	19	112	3.39 ± 0.14
Moderately bleached colonies "c"	34	3	2	8	21	115	3.38 ± 0.16
Strongly bleached colonies 'd"	*Heavily bleached corals did not produce larvae*						

6.5. DISCUSSION

This study shows clearly, for the first time, that the reproductive output of soft corals may be affected by bleaching events and that the degree of impact increases with the intensity of the disturbance. Moderate bleaching reduced both the number and size of mature oocytes in experimental colonies of the soft coral *Lobophytum compactum*. Given that lipids comprise up to 65% of the egg dry weight, smaller mean egg sizes mean that larvae are energetically less well-equipped for survival once eggs are fertilised (chapter 7; Michalek-Wagner and Willis, 2000b). Severe bleaching reduced mean size of mature oocytes below an apparent viability threshold so that they were retained for an entire year beyond the normal time of release. Although mature-sized eggs developed in all severely bleached females in the second breeding season following experimental treatment, lack of spawning in 30% of these females demonstrates that the impact of such disturbances can extend beyond 20 months.

I also demonstrate that the rate of zooxanthella recovery depends on the degree of bleaching experienced by the coral host. Recovery of algal stocks in the moderately and strongly bleached groups of experimental soft corals took approximately 70 and 126 days, respectively. These results compare well with the approximate 120-day zooxanthellae recovery period I found for experimentally (> 90%) bleached soft corals reported in chapter 4. Davies (1991) estimated that corals could survive periods of decreased photosynthetic productivity for 28 to 114 days through metabolisation of lipid reserves. Although lipid reserves were sufficient to allow the recovery of all severely bleached colonies of *Lobophytum compactum* in this study without any (even partial) mortality, the reduced fecundity of these colonies indicates that recovery occurred at a significant cost to resources normally allocated to reproduction.

The 45% reduction in fecundity of *Lobophytum compactum* in the moderately bleached group and complete failure to produce mature eggs in the severely bleached group in the first year clearly demonstrate that fecundity is a good indicator of sublethal stress in *Lobophytum compactum*. Similarly, differences in egg sizes between bleaching treatments suggest that egg size can also be used to gauge impact severity in natural bleaching disturbances. It has been well-established that reproductive failure can be induced in corals by stresses such as turbidity, sedimentation (Kojis and Quinn, 1984), high seawater temperatures (Jokiel and Guinther, 1978) and anthropogenic pollution (Loya and Rinkevich, 1979). Although a number of authors have suggested that fecundity could be used as an indicator of sublethal stress (Kojis and Quinn, 1984; Harrison and Wallace, 1990), it is generally not easy to separate sublethal impacts on reproduction from natural variations in fecundity due to factors such as differing size and age of

colonies sampled (Wallace, 1985; Szmant, 1991; Sier and Olive, 1994; Slattery et al., 1999). I reduced potential sources of natural variation in fecundity by standardising the size of experimental colonies and the location of sampling within a colony. The low variation in fecundity I found between colonies within a treatment or between the two years, suggesting that standardisation of sampling may allow fecundity to be used more widely as a sublethal indicator of stress.

The reduced fecundities of moderately bleached *Lobophytum compactum* (13 ± 0.44 mature eggs/polyp) in comparison to control colonies (24 ± 0.67), despite the bleaching event occurring after the typical complement of oocytes had developed, (i.e. midway through the oogenic cycle), suggest that some early stage oocytes had been resorbed to allow development of the remaining ones. Thus, energy allocated to reproduction was apparently directed towards maintaining fewer eggs than normal to ensure that they attained a mature size. This interpretation is consistent with observations of Yamazato and co-workers (1981) who noted that some reduction in the number of oocytes commonly occurs in early oogenesis in *L. crassum,* even in the absence of detectable stress, and is presumably linked to re-allocation of energy to support remaining oocytes.

Although resorption of some immature eggs also occurred in strongly bleached *Lobophytum compactum* (19.6 ± 1.2 mature eggs/polyp), such redirection of reserves was still not sufficient to allow production of mature eggs in the first year. Eggs that were produced had a mean size 25 % smaller than those of controls and were apparently neither spawned nor resorbed but retained until the next (1997) spawning season. The almost complete failure of all strongly bleached females to spawn in 1996, eight months after experimental treatment is consistent with the interpretation that developmental delays associated with recovery from the disturbance prevented oocytes from attaining maturity by the time of the breeding season. The fact that a few under-sized eggs were spawned during the 1996 breeding period demonstrates the pervasiveness of seasonal proximate cues that trigger spawning in mass spawning soft corals.

The retention of most stage III oocytes for a full year beyond their expected release suggests that there are inherent benefits in retaining nearly mature eggs until the next mass spawning period (e.g. increased sperm availability and reduced mortality because of increased potential for predator satiation (Babcock and Heyward, 1986; Johnson, 1992). Although a number of studies have demonstrated that mature oocytes may be resorbed following incomplete spawning in hard corals (Rinkevich and Loya 1979; Szmant and Gassman 1990; Sier and Olive, 1994), this may not always be the optimal solution. Resorption of a full complement of oocytes, relatively advanced in their development, followed by *de novo* gametogenesis is unlikely to be

as energetically favourable as retention of late stage oocytes for a full year. Lack of development of under-sized oocytes in severely bleached corals until oogenesis was in phase with field populations suggests that energy allocation strategies of soft corals are highly plastic. Both mature and immature oocytes may be resorbed to provide energy for remaining oocytes, or the soft coral may suspend oogenesis altogether until it is once more in phase with annual gametogenic cycles.

Differences in fertilisation success of eggs between severely bleached and control colonies in the first spawning season following experimental treatment were most likely due to differences in mean sizes of gametes and associated maturity factors. Eggs of severely bleached females were 26% smaller than those of control females (468.1 ± 1.47 μm in the heavily bleached group vs. 634.1 ± 1.94 μm in controls) and resulted in zero fertilisation. Sperm-egg histo-incompatibility can be ruled out as an explanation for the lack of fertilisation because all crosses were replicated using the same clone combinations in all treatments. Since heavily bleached females retained the majority of their eggs for a further year, it is unlikely that eggs had undergone the final maturation steps required to become receptive to sperm. Although eggs produced by moderately bleached females were 5% smaller than eggs of control females, they had similar fertilisation success (93% versus 92% fertilisation success, respectively). Despite an approximate 45% reduction in the number of spawned eggs in moderately bleached colonies, eggs that were spawned were able to fertilise and develop normally. Survival and growth rates of offspring did not differ significantly between moderately bleached and control colonies, thus, the strategy of allocating energy towards a smaller number of eggs to ensure that they attain maturity was successful for moderately bleached *Lobophytum compactum*. The fewer number of eggs available for fertilisation and development from bleached corals is consistent with observations of reduced recruitment following natural bleaching events (Glynn et al., 1996) and disproportionately reduced representation of families in recruitment assemblages that are most affected by bleaching events (Gleason, 1996).

In conclusion, these results demonstrate that bleaching disturbances have the potential to decrease reproductive output or suspend gametogenesis entirely in the subsequent breeding season. They also show that soft corals may recover from bleaching by re-allocating energy reserves that would normally be directed to reproduction towards recovery. Although zooxanthella recovery was completed within a few months, reproductive output was negatively affected for at least another 15 months in cases of severe bleaching. Thus, although zooxanthellae concentrations track the immediate response and recovery of the coral host following a bleaching disturbance, they do not give an accurate picture of sublethal impacts. I believe that zooxanthellae densities should only be used as an indicator of coral health in

conjunction with other indicators of sublethal stress, such as fecundity. If bleaching episodes occur with increasing intensity and at shorter intervals in the future (see Williams and Bunkley-Williams, 1990; Jones et al., 1997, Hoegh-Guldberg, 1999), then the longer term implications of this study are that sustained reductions in reproductive output and fertilisation success may impede replenishment of coral assemblages and, ultimately, natural reproductive rhythms of corals with long oogenic cycles may be completely disrupted.

Chapter 7

The impacts of bleaching on the soft coral *Lobophytum compactum*. II. Biochemical changes in adults and their gametes

Experimentally bleached colony of *Lobophytum compactum* after relocation to the reef

7.1. ABSTRACT

In this chapter I demonstrate that experimental bleaching reduces important biochemical parameters in adult tissues of the soft coral *Lobophytum compactum*. These losses are subsequently passed on to their gametes, possibly jeopardising their viability. Although zooxanthellae densities of experimental corals recovered within 4.5 months, protein, lipid, MAA and carotenoid concentrations were reduced for at least eight months in adult tissues. Reductions in tissue concentrations were amplified when they were passed on to gametes, with the greatest reductions occurring in lipids and proteins. Although reductions in MAAs were relatively smaller when passed on to gametes, even minor proportional reductions have significant implications for larval survival, given that I found MAA levels to be approximately three times higher in eggs than in maternal tissues. By the second spawning season (20 months after experimental bleaching), the biochemical composition of adult tissues and their gametes was indistinguishable from that of control (unbleached) corals.

7.2. INTRODUCTION

It is established to date that nutritional constraints associated with bleaching have negative effects on the primary metabolism of affected corals (Porter et al., 1989; Leder et al., 1991; Fitt et al., 1993). Reductions in levels of important primary metabolites, such as proteins and lipids, and secondary metabolites, such as carotenoids and MAAs could have dire consequences for the capacity to recover after a bleaching event. Moreover, reductions in levels of certain biochemical parameters could be passed on to the next generation, thereby potentially reducing the fitness and survival of the progeny.

In this chapter I focus on the effects of bleaching on important indicators of the biochemical status of adult soft corals and their gametes, in particular, on tissue concentrations of lipids, proteins, UV protecting mycosporine-like amino acids and anti-oxidative carotenoids. Reductions in these parameters help to explain decreases in the reproductive output and gamete viability of the soft coral, *Lobophytum compactum* that I reported in Chapter 6.

Comparisons of adult and larval tissues show that lipids comprise up to 70% of the dry weight of coral larvae (Arai et al., 1993; Richmond, 1996), whereas concentrations rarely exceed 30 - 40% in adult tissues (Patton et al., 1977; Stimson, 1987; Ward, 1995). As the majority of both hard and soft corals spawn eggs that lack zooxanthellae and, with few exceptions, coral larvae are not known to feed (Tranter et al., 1982; Wright, 1986), parental provision of lipid reserves is essential. Biochemical changes in adult tissues undoubtedly affect the next generation and significant changes may compromise the survival and dispersal of larvae of most coral species.

Investment into biochemical defence systems for protection against high irradiance stress of the highly exposed early planktonic larval stages is a life-history trait not exclusive to corals. Parental provision of both MAAs (Aliño, 1989; Carefoot et al., 1998; Chiocarra et al., 1986; Adams and Shick, 1996) and carotenoids (Almeida de Leone, 1993; Dall et al., 1995; Tsushima et al., 1995) has been documented for a number of invertebrates with pelagic larval stages. The presence of the former compounds has also been shown experimentally to decrease mortality of larvae due to UVR (Gleason and Wellington, 1995). The established efficacy of carotenoids to quench singlet oxygen and scavenge deleterious free radicals and reactive oxygen species (Goodwin, 1976; Paerl 1984; Ben-Amotz and Avron, 1983; Shick et al., 1996) makes them an important part of the diverse photo-protective system of corals (Ambarsari et al., 1997). Carotenoids cannot be synthesised *de novo* by the animal host and available evidence suggests that the zooxanthellae partner is the most likely candidate for the production of MAAs (Dunlap and Chalker, 1986). Bleaching therefore has the potential to reduce the fitness of gametes

directly through reductions in lipid and protein reserves available for gamete development or indirectly through reduced production of photo-protective MAAs and carotenoids.

In this Chapter I describe the impact of a bleaching event, occurring approximately midway through gametogenesis, on the subsequent biochemical composition of adult females and their gametes for 20 months, a period that spanned two spawning seasons. To determine the longer term, sublethal impacts of reductions in zooxanthellae densities on the biochemical constitution of the coral host, I simulated a moderate (approx. 50% loss of zooxanthellae) and severe (90-95% loss) bleaching event (for details see Chapter 6). I then compared a series of biochemical parameters between bleached and unbleached colonies of the soft coral, *Lobophytum compactum*. Here I demonstrate that biochemical data provide important information for understanding the longer-term, sublethal effects of bleaching and ultimately for understanding how, and to what extent, bleaching affects the fitness of corals and the viability and dispersal potential of their offspring.

7.3. MATERIAL AND METHODS

7.3.1. Experimental design and sampling regime

The same colonies of *Lobophytum compactum* that were collected in March 1996 and exposed to elevated seawater temperature and solar radiation treatments to monitor the impacts of experimental bleaching on reproduction (see Chapter 6) were sampled for biochemical analyses as described below. After the bleaching treatment, corals were returned to their reef of origin and recovery of zooxanthellae densities monitored at monthly intervals (see Chapter 6). Tissue samples (5 lobes of 1-2 g wet weight/ colony) of all 50 test corals (i.e. 10 colonies per treatment) were collected immediately prior to spawning for two subsequent years (November 1996 and 1997) and frozen at -20° C until analysed biochemically. For each of the four biochemical parameters outlined below, comparisons were made among colonies in moderately bleached, heavily bleached and unbleached treatments. Colonies representing the unbleached treatment included all those used as chiselling, handling and field controls (see Chapter 6). Gametes were captured with egg nets or dissected from the gonads of test corals.

7.3.2. Biochemical analyses

*1. **Lipid analysis:*** Samples of adult females and their eggs were collected for lipid analyses immediately prior to spawning in November 1996 and 1997 and frozen at -20° C. Samples were freeze-dried, weighed (to 0.001g), crushed with mortar and pestle, and immersed for 24 h in a 2:1 (v:v) chloroform:methanol solution to extract the lipids. Extracts were then washed once with a 0.88% potassium chloride solution, three times with a 1:1 (v:v) methanol : water solution and dried for 24 hours before being re-weighed. The total lipid content is expressed as % lipid per gram tissue dry weight (Stimpson, 87; Harland et al., 1992).

*2. **Carotenoid analysis:*** The quantification of the carotenoid peridinin in adult colonies, and that of a closely related peridinin derivative (altered by only one functional group and indistinguishable from peridinin by spectro-photometric analysis, B. Bowden pers. com.) found in the eggs were carried out as described in Chapter 2, section 2.3.2.

*3. **Analysis of MAAs:*** MAAs were analysed as described in Chapter 2, section 2.3.2.

*4. **Protein determination:*** Proteins were analysed as described in Chapter 2, section 2.3.2.

7.3.3. Statistical analysis

The significance of differences in lipid, protein, MAA and carotenoid tissue concentrations among moderately bleached, severely bleached and control colonies were tested by one-way ANOVA followed by multiple comparisons using Tukey's HSD test. Significance of correlations between zooxanthellae densities immediately following experimental treatment and each of the biochemical parameters eight months later were tested by analysis of variance of regression. The SPSS 7.5 software package was used for analyses

7.4. RESULTS

Relevant measures of all biochemical parameters assessed in this study differed between experimentally (heavily) bleached and control (unbleached) colonies, both in adult females and their eggs (Figure 7.1, see following sections for test results).

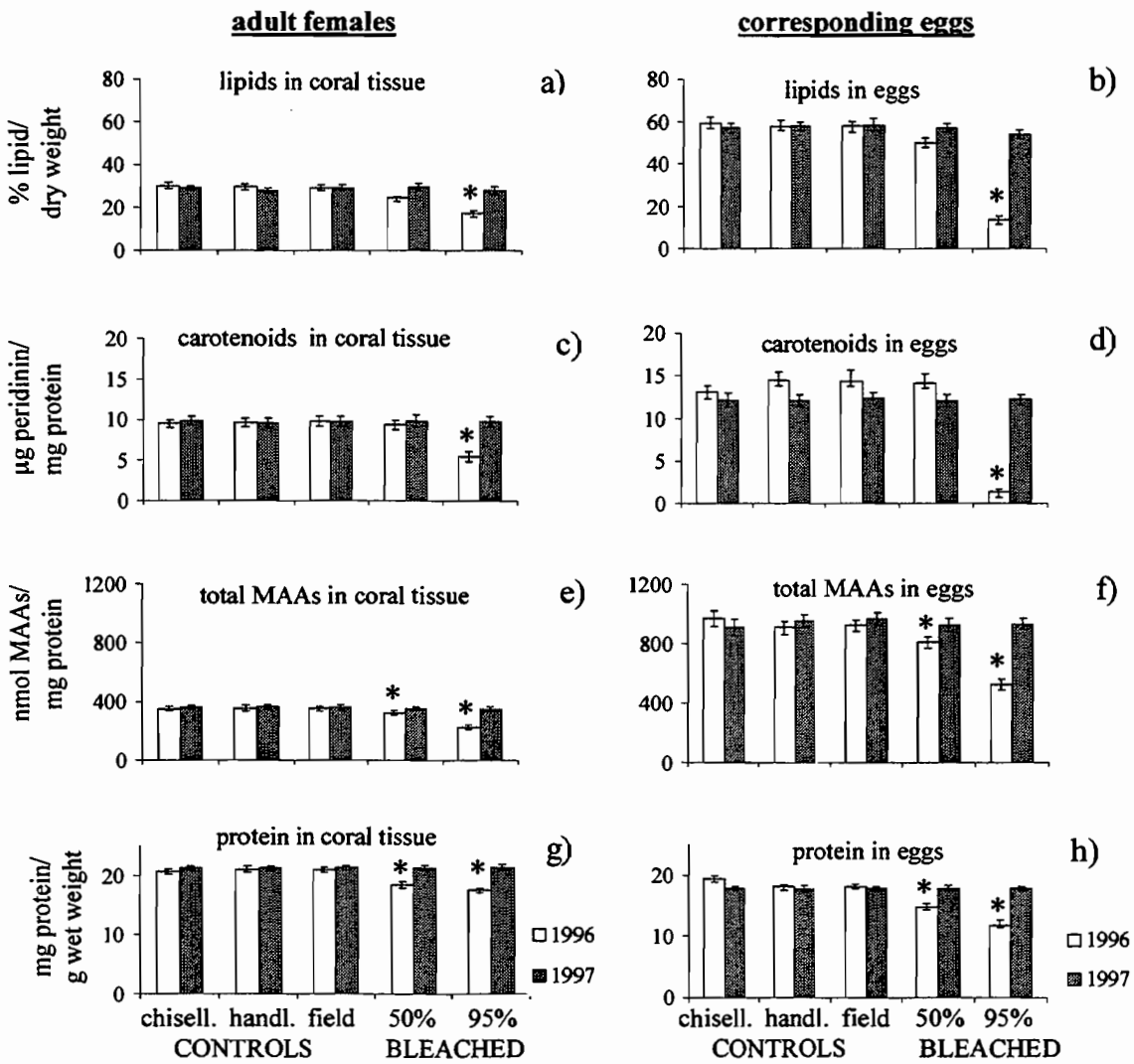


Figure 7.1. Mean tissue concentrations (± SE) of various biochemical parameters immediately prior to spawning in 1996 (white histograms) and 1997 (solid histograms) for adult females of *Lobophytum compactum* (left hand column) and their eggs (right hand column): **a-b** lipids, **c-d** carotenoids, **e-f** total MAAs, **g-h** proteins. N = 20 or 40 determinations, i.e. 10 colonies per treatment x 2 subsamples per colony (for lipid, protein and carotenoid determinations) or x 4 subsamples (for MAA analysis). Asterisks denote significant differences.

It is noteworthy that these differences were detected eight months after experimental treatment. Reductions in lipids, carotenoids, MAAs and proteins were significant for severely bleached corals but only reductions in proteins and MAAs were significant for moderately bleached corals.

7.4.1. Lipid analysis

Lipid concentrations in tissues of adult females immediately prior to spawning (November 1996) and their spawned eggs were both positively correlated with zooxanthellae densities in female tissues just after experimental bleaching (March 1996) ($r^2_{females} = 0.41$, $p < 0.0001$; $r^2_{eggs} = 0.62$, $p < 0.0001$; Figure 7.2). Thus, the lower the density of zooxanthellae after experimental bleaching, the longer the time required for recovery of zooxanthellae numbers and the lower the subsequent lipid concentrations in both females and their corresponding eggs (Figure 7.2).

Eggs derived from moderately bleached soft corals in the first spawning season after impact contained on average 14% less, while eggs of heavily bleached females contained 76% less total lipid than those of control corals (Figure 7.1b). Reduced concentrations of total lipids were also found in the maternal tissues of corals in the moderately and severely bleached corals (i.e. 12% and 41% reduction in lipids respectively) in comparison to control corals (Figure 7.1a). Note that concentrations of lipids in eggs are typically twice as high as those in maternal tissue. While the lower lipid concentrations in both the females and their gametes in the heavily bleached group were significantly lower than controls (one-factorial ANOVA, females: $F = 17.19$ $df = 4$, $p < 0.0001$; gametes: $F = 60.71$, $df = 4$, $p < 0.0001$), the trend for lower lipid concentrations in moderately bleached females and their eggs was not statistically significant.

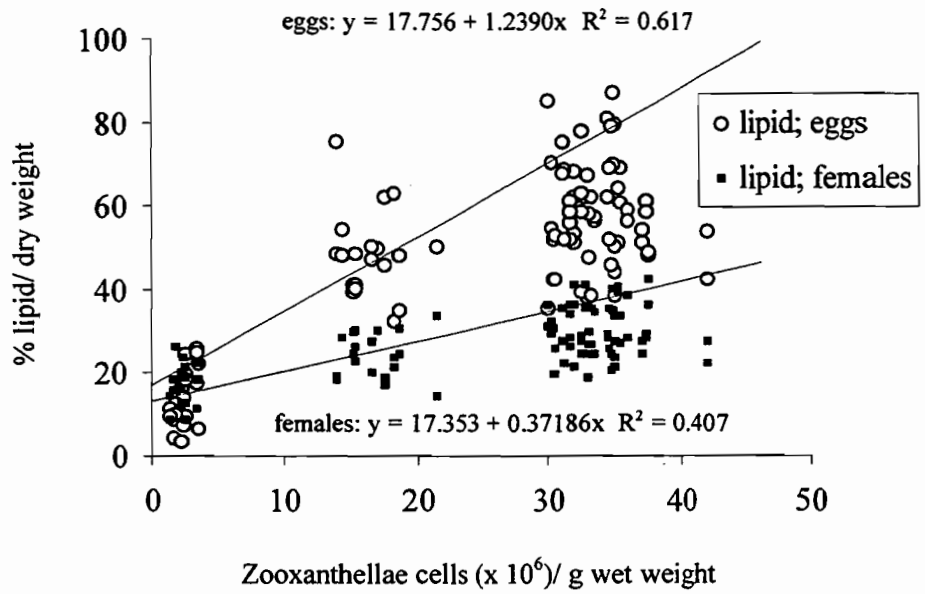


Figure 7.2. Relationship between zooxanthellae densities in adult females immediately following experimental bleaching and tissue concentrations of lipids eight months later (1996 spawning), in both eggs and females of *Lobophytum compactum*, n = 100.

Lipid concentrations in tissue samples of both moderately and heavily bleached corals and their eggs taken immediately prior to the 1997 spawning (i.e. 20 months after the bleaching treatment), were not significantly different from those of control corals (one-factorial ANOVA, $F = 0.30$, $df = 4$, $p = 0.88$ for females; $F = 0.57$, $df = 4$, $p = 0.67$ for eggs).

7.4.2. Carotenoids

The major carotenoid found in *Lobophytum compactum* is peridinin, accompanied by small amounts of diadinoxanthin, diatoxanthin and β-carotene, a complement similar to that found in dinoflagellate symbionts in general (Jeffrey et al., 1997). Carotenoid levels in tissues of adult females immediately prior to spawning (November 1996) and their spawned eggs were both positively correlated with zooxanthellae densities in female tissues just after experimental bleaching (March 1996) ($r^2_{females} = 0.20$, $p < 0.0001$; $r^2_{eggs} = 0.42$, $p < 0.0001$). Immediately prior to the first spawning season (8 months after experimental bleaching), concentrations of peridinin in corals from the severely bleached group were reduced by 90% in eggs (one-factorial ANOVA, $F = 31.17$, $df = 4$, $p < 0.0001$) (Figure 7.1d) and by 43% in adult females (one-factorial ANOVA, $F = 9.82$, $df = 4$, $p < 0.0001$) (Figure 7.1c) in comparison to concentrations

in eggs and females from the control group. I detected no discernible effect of the elevated temperature and solar radiation treatment on the levels of peridinin in the moderately bleached group of corals 8 months after experimental bleaching, either in adult female tissues (Tukey's HSD test, $p = 0.99$) or their eggs (Tukey's HSD test, $p = 0.99$). In 1997, the second spawning season following the disturbance, no significant differences in peridinin levels were detected between experimental and control corals for either adult females (one-factorial ANOVA, $F = 0.05$, $df = 4$, $p = 0.99$; Figure 7.1c) or their eggs (one-factorial ANOVA, $F = 0.23$ $df = 4$, $p = 0.99$; Figure 7.1d).

7.4.3. Mycosporine-like amino-acids (MAAs)

The typical MAAs I identified in adult *Lobophytum compactum* and their eggs were shinorine, porphyra-334, palythine, asterina, palythinol and traces of palythene. Palythine represented by far the largest fraction, comprising 90-95% of the total MAA content in both eggs and adult female tissue. Levels of MAAs in eggs were approximately 3 times those in the maternal tissue (cf. Figs. 7.1e, 7.1f), revealing a high level of parental provision of UV protectants.

MAA levels in tissues of adult females immediately prior to spawning (November 1996) and their spawned eggs were both positively correlated with zooxanthellae densities in female tissues just after experimental bleaching (March 1996) ($r^2_{females} = 0.22$, $p < 0.0001$; $r^2_{eggs} = 0.39$, $p < 0.0001$). In the first spawning season following experimental bleaching, elevated seawater temperature and UV treatments significantly reduced levels of MAAs, by 8% in moderately bleached adult females (one-factorial ANOVA, $F = 8.92$, $df = 4$, $p < 0.0001$; Figure 7.1e) and by 13% in their eggs (one-factorial ANOVA, $F = 19.5$, $df = 4$, $p < 0.0001$; Figure 7.1f) in comparison to control corals. Reductions in MAAs were even greater in the heavily bleached group, ranging from 35% ($p < 0.001$) for females to 44% ($p < 0.001$) for their corresponding gametes. By the second spawning season following the event, MAA concentrations of experimental corals did not differ significantly from controls for either adult females (one-factorial ANOVA, $F = 0.14$, $df = 4$, $p = 0.97$; Fig 7.1e) or their gametes (one-factorial ANOVA, $F = 0.23$, $df = 4$, $p = 0.92$; Fig 7.1f).

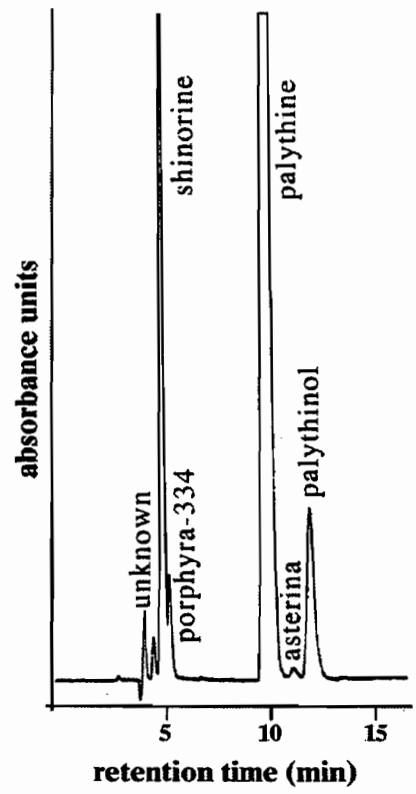


Figure 7.3. Typical HPLC spectrum of MAAs extracted from eggs of *Lobophytum compactum*.

7.4.4. Proteins

As for lipids, I found a positive correlation between zooxanthellae densities after experimental treatment and protein levels eight months later at the time of spawning ($r^2_{females} = 0.34$, $p < 0.0001$; $r^2_{eggs} = 0.53$, $p < 0.0001$; Figure 7.4). The impact of experimental bleaching was not as extreme for proteins as it was for lipids, average protein reductions in eggs being 17% for moderately and 38% for heavily bleached corals (see Figure 7.1h). I found a similar though less extreme trend in adult female tissues, with reductions of approximately 11% and 17% in moderately and heavily bleached corals respectively (Figure 7.1g). In samples collected immediately prior to the second (1997) spawning I found no significant differences in protein concentrations between bleached and unbleached treatments for either eggs (one-factorial ANOVA, $F = 0.02$, $df = 4$, $p = 0.99$; Fig 7.1h) or adult female tissues (one-factorial ANOVA, $F = 0.10$, $df = 4$, $p = 0.98$; Fig 7.1g).

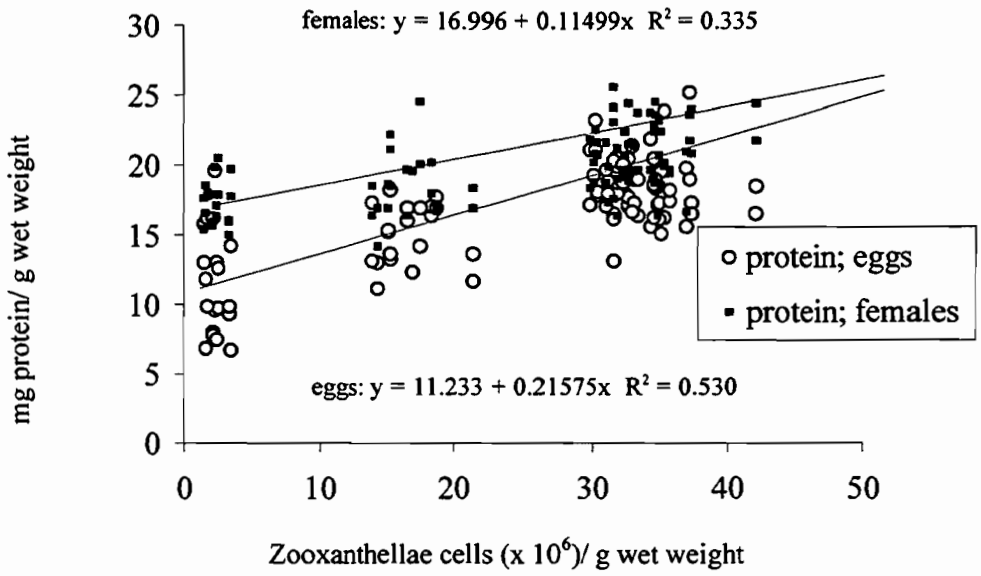


Figure 7.4. Relationship between zooxanthellae densities in adult females following experimental bleaching and protein concentrations eight months later in both eggs and females of *Lobophytum compactum*, n = 100.

7.5. DISCUSSION

The results presented here clearly show that both moderate and severe bleaching disturbances reduce the levels of proteins and MAAs in female colonies of *Lobophytum compactum* and their eggs for up to eight months after a disturbance. In addition, severe bleaching disturbances also reduce lipids and carotenoids in both adult females and their eggs for a similar period of time.

It is well established that photosynthetic rates of corals are high in shallow water and that the amount of carbon translocated from symbiotic algae easily meets the host's requirements for respiration and growth (Muscatine et al., 1984). With the loss of zooxanthellae following bleaching disturbances, however, this situation can change dramatically. I found that corals suffered reductions of up to 17% in tissue protein in comparison to unbleached corals, eight months after bleaching. Reductions in lipids of up to 41% in heavily bleached corals were even more dramatic. Given that zooxanthellae are estimated to comprise only 5-12 % of the biomass of corals (Muscatine et al., 1984), the reductions found in this study could not have been due to the loss of the algal partner, but reflect real changes in the tissue of the coral host. Porter et al. (1989) estimated that the carbon contribution of zooxanthellae towards the animal's metabolic requirements changed from 112% in unbleached *Montastrea annularis* to 51% in corals that had lost approximately 86% of their zooxanthellae population in a natural bleaching event. As a consequence, host levels of tissue protein, lipid and carbohydrates eight weeks after the disturbance had dropped by 38.8%, 66.3% and 44.2 % respectively (Porter et al., 1989). This study demonstrates that the impact of such a natural disturbance, which was similar to the impact experienced by the heavily bleached group of soft corals, may continue for at least eight months. Even more critical was the finding that both protein and lipid levels were reduced in mature eggs immediately prior to spawning. Thus nutritional perturbations experienced by the host were passed on to their gametes and affected their viability eight months later (see Chapter 6).

I found that reductions in tissue levels of protein and lipids eight months after experimental bleaching were correlated with reductions in zooxanthellae densities immediately after the disturbance. Thus, not only does the magnitude of the bleaching directly affect the time required for recovery of zooxanthellae densities (see Chapter 6); it also determines the biochemical composition of the host and their gametes for an extended period of time. Reductions in both proteins and lipids in tissues of severely bleached females were approximately doubled in magnitude when passed on to their offspring (cf. 17% reductions in tissue protein in adult females versus 38% in their eggs; 41% reductions in lipids of females versus 76% reductions in their eggs). Protein and lipid reductions of this magnitude, prevented

eggs from attaining maturity during the ensuing spawning season (see Chapter 6). Although the 11-12% reductions in proteins and lipids in moderately bleached females reduced both their fecundities and egg sizes, the consequent 17% and 14% reductions in proteins and lipids passed on to their eggs did not apparently affect their ability to be fertilised (ibid.). By the following spawning season (20 months after the disturbance), both protein and lipid levels had recovered to background levels. This is consistent with findings of Fitt et al. (1993) that reduced protein and lipid levels in *Montastrea annularis* persisted for 10 months after an intense natural bleaching event.

The large size (632μm mean diameter) and high lipid composition (65%) of eggs of *Lobophytum compactum* combined with lack of either zooxanthellae or feeding in larvae prior to metamorphosis indicate that planulae of *L. compactum* are lecithotrophic. The 65% lipid content I found for eggs of *L. compactum* was similar to the 60 to 70% lipid compositions reported for eggs of acroporids and other broadcast spawning species (Arai et al., 1993). Wax esters (mainly cetyl palmitate) constitute the largest fraction of the energy reserves of both corals and their eggs and represent a high-energy source because they are metabolised at a slower rate than other lipids. Thus wax esters can function as long term energy sources and support long distance dispersal (Benson and Muscatine, 1974; Benson et al., 1978).

Larvae developing from lecithotrophic eggs are dependent on their mothers for energy reserves for survival. Studies of the competence and longevity of coral larvae (Richmond, 1987; Havenhand, 1993) highlight the correlation between stored energy reserves and dispersal potential. Accordingly, maternal investment has been found to be up to an order of magnitude greater in azooxanthellate in comparison to zooxanthellate larvae (Ben-David-Zaslow and Benayahu, 1996; 1998). I found that lipids, the primary energy reserve of coral larvae, suffered the greatest reductions of the four parameters analysed following experimental bleaching. If mothers bleach to the extent that their lipid reserves are decreased during gametogenic development, azooxanthellate larvae are more likely to suffer reduced viability and dispersal than zooxanthellate larvae, which may derive 13-27% of their requirements from photosynthates translocated from their algal partner (Richmond, 1987).

In addition to direct impacts of bleaching associated with reductions in lipids and proteins, bleaching can also indirectly reduce the fitness of both adults and their larvae by interfering with the production of secondary metabolites. I found significant reductions in the carotenoid peridinin in both heavily bleached females (by 43%) and their eggs (by 90%). Given that carotenoids are primarily acquired from zooxanthellae or partially through heterotrophic feeding in corals (Davies, 1976; Frank and Cogdell, 1996), reduced concentrations following bleaching

are not surprising. Kleppel and co-workers (1989) found similar reductions in carotenoids immediately following a natural bleaching event. Such reductions are likely to have far-reaching effects, because carotenoids function as: accessory pigments in the form of a peridinin-chlorophyll-protein complex (Prézelin, 1976); photoprotectants due to their capacity to quench chlorophyll excited states; scavengers of singlet oxygen molecules; and dissipaters of excess energy (Frank and Cogdell, 1996). Increased concentrations of some carotenoids (i.e. xanthophylls) found by Ambarsani et al. (1997) following a solar bleaching event, presumably relate to their role as photoprotectants.

Peridinin-chlorophyll-protein complexes constitute more than three-quarters of the carotenoid pool in dinoflagellates (Jeffrey and Haxo, 1968), thus their presence in corals is generally assumed to be related to their role as a major component of the light harvesting apparatus of symbiotic dinoflagellates (Iglesias-Prieto, et al., 1992; Iglesias-Prieto and Trench, 1997). Their role as accessory photosynthetic pigments in eggs that are devoid of zooxanthellae, however, is not as clear. I suggest that it is likely that peridinin functions as a photoprotectant in eggs and subsequently in larvae. Although only diatoxanthin and diadinoxanthin, which are present in small quantities in dinoflagellates and are known to be light inducible (Ambarsani et al., 1997; Brown et al., 1999), are typically described as photoprotectants, peridinin also has the structural basis to act in this capacity. The potency of carotenoids as effective quenchers of reactive oxygen species is correlated to the number of conjugated double bonds in these compounds (Shimidzu et al., 1996). The eight double bonds of peridinin (in comparison to ten and eleven in diadinoxanthin and diatoxanthin respectively) certainly suggest that it could act as a photo-protectant. This role would be consistent with my finding that carotenoid levels were approximately one-third greater in eggs than in their mothers in both bleached and unbleached *L. compactum*. Many invertebrate larvae are similarly equipped with high levels of carotenoids, which would counteract increased UV levels encountered during their pelagic stage (Dall et al., 1995; Tsushima et al., 1995). If this is the primary role of peridinin in eggs, then the 90% loss I found for carotenoids in the eggs of heavily bleached *L. compactum* would have resulted in severe oxidative stress.

It is well established that MAAs are an important line of defence against damage due to high solar irradiance and particularly UVR in corals and many other invertebrates and algae (Chapter 3; reviewed in Dunlap and Shick, 1998). The 8 % and 35 % reductions I measured in tissues of moderately and strongly bleached females eight months after bleaching implies that they might have experienced oxidative stress and possibly cell and tissue damage, particularly as MAA losses were undoubtedly higher during the earlier portions of the experimental period. While such losses did not result in mortality of adults in this study, reductions in MAA

concentrations were amplified as they were passed on to the next generation (to 13 and 44% reductions in eggs derived from moderately and heavily bleached corals respectively). Since UVR is potentially much higher for coral eggs and larvae, which typically spend their first 24 hours at the sea surface and then continue to develop in the water column (Willis and Oliver, 1988; 1990), substantial reductions in MAAs in eggs are potentially lethal. The occurrence of reduced MAA levels as a longer-term effect of bleaching is particularly intriguing with respect to my findings of highly increased MAA levels during and immediately after a bleaching disturbance (Chapter 3). This suggests that immediate resource allocation towards MAA production after bleaching translates into a longer-term metabolic burden for soft corals, i.e. sub-optimal MAA levels months after the disturbance.

The conclusion that the potential for UV damage is greater for pelagic larvae than for adults is corroborated by my finding that MAA levels were approximately three times higher in eggs of *Lobophytum compactum* in comparison to maternal tissues. Parental provision of MAAs in larvae is not exclusive to corals, but has been demonstrated to be a common strategy for avoiding UV damage in the early life history stages of other taxa with planktonic larvae (e.g. sea hares (Carefoot et al., 1998) and sea urchins (Chiocarra et al., 1986; Adams and Shick, 1996)). While I did not compare the effects of UVR on gametes with low versus high MAA levels in this study, evidence from other studies clearly demonstrate the negative impact of UVR on invertebrate larvae with reduced MAA levels (Gleason and Wellington, 1995; Adams and Shick, 1996). Behavioural modifications, vertical mixing of the water column or early cryptic settlement may mitigate UV-induced mortality in marine larvae (Gleason and Wellington, 1995). However, the surface development and dispersal of many coral larvae (Willis and Oliver, 1988; 1990) preclude such avoidance strategies and expose developing coral larvae to extreme levels of UVR.

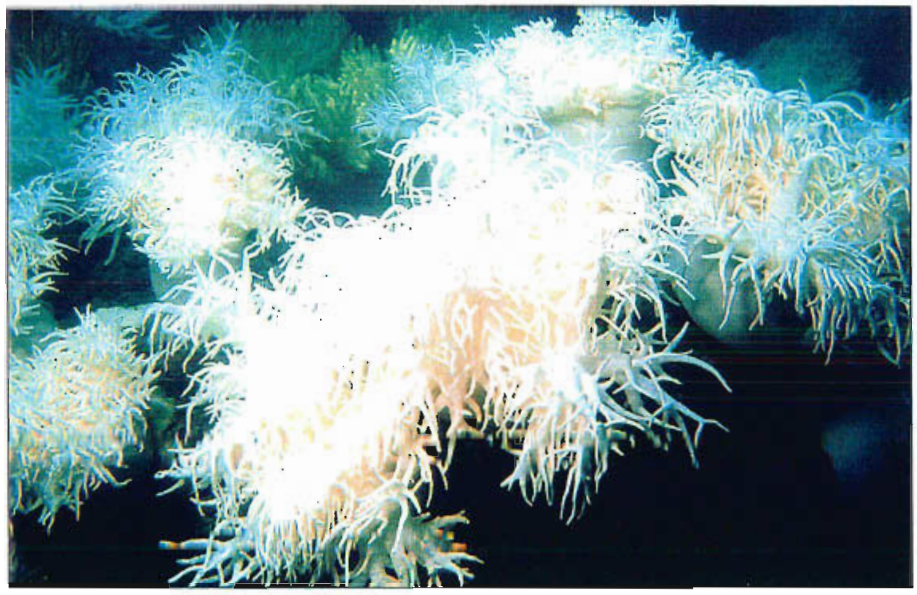
While it is clear that carotenoids are either produced by the algal partner (Davies, 1976; Frank and Cogdell, 1996) or acquired through heterotrophy, the origin of MAAs in the association has not been established unequivocally. In terrestrial plant and fungi systems, MAAs have been shown to be produced via the shikimic acid pathway (Favre-Bovin, 1987) but this pathway is absent in metazoans. Therefore, except for possible acquisition through the diet, zooxanthellae may be the primary supplier of MAAs in the coral association (Dunlap and Chalker, 1986). Since zooxanthellae levels had returned to background levels by June and August for the moderately and heavily bleached group respectively (see chapter 6; Michalek-Wagner and Willis, 2000a), normal production of MAAs and carotenoids could have been resumed for up to five months prior to sampling. The reduced levels of both MAAs and carotenoids at the time of sampling suggest, however, that energy was allocated elsewhere. Since reductions in lipid and

protein levels were not as great as reductions in MAAs and carotenoids in adult females or their eggs eight months after the experimental bleaching, it is possible that whatever resources were available were allocated towards recovery of these primary biochemical constituents, at the cost of secondary metabolites. If this indeed was the case, then it suggests that the coral host exerts considerable control over zooxanthellae metabolism and/or the products translocated. Such host control has been established for a number of zooxanthella-host interactions (Cook and D'Elia, 1987).

In summary, this study demonstrates that bleaching events may alter the biochemical composition of soft corals for an extended period of time. In addition, not only does bleaching have the potential to reduce reproductive output in *Lobophytum compactum* (see Chapter 6; Michalek-Wagner and Willis, 2000a), but reductions in key biochemical parameters may also be passed on to their gametes. As a consequence larvae may be biochemically less well-equipped, and potentially suffer reduced fitness and survival. Thus, even if corals survive a bleaching disturbance, their reproductive outputs are likely to be reduced or totally impaired for a significant period of time and the effects of bleaching are likely to be carried into the next generation. Given that a number of recent bleaching events have been associated with high mortality (Glynn et al., 1996; Fabricius, 1999; reviewed in Hoegh-Guldberg, 1999), sexual reproduction plays an integral part in the replenishment of a reef. An important longer term implication of this study is that gamete development may be impaired for a significant period of time after a bleaching event, even when corals recover their zooxanthellae, and thus inhibit recovery processes dependent on replenishment of coral populations.

Chapter 8

Summary and general discussion



Colonies of *Sinularia flexibilis* during a natural bleaching event at Orpheus Island, March 1998

8. Summary and General Discussion

8.1. Brief summary of major findings

Soft corals employ a number of biochemical defence systems, including photo-protective MAAs (Chapters 2 & 3) or antimicrobial and antifouling agents (Chapters 4 & 5) to prevent and counteract damage associated with bleaching. Through nutritional constraints associated with bleaching, however, production of these compounds can be compromised in adult soft corals, and changes subsequently passed on to the next generation via reduced numbers of less-well equipped progeny (Chapters 6 & 7). Before discussing the future implications of results synthesised from these studies, I will next briefly highlight the major findings of each study.

8.1.1. Seasonal variations in MAA concentrations in male and female soft corals in relation to annual solar irradiance and temperature cycles (Chapter 2)

My findings of MAA production so closely linked to environmental conditions suggests that the compounds are an integral component of the biochemical defence system and protect soft corals against stresses associated with high irradiance and/or temperature stress which induce bleaching. Further support for this notion comes from the observation of higher MAA production in females than males prior to spawning (Chapter 2), presumably to protect the eggs and progeny from high solar radiation.

8.1.2. The effects of high irradiance and temperature on tissue concentrations of UV absorbing MAAs in soft corals (Chapter 3)

High irradiance and elevated temperature have the potential to act synergistically, and thus cause highly acerbated bleaching in *Lobophytum compactum* and *Sinularia flexibilis*. The key to understanding this synergy, however, is not a thermal degradation of MAAs as previously assumed, given that MAAs are not thermolabile at temperatures typically experienced during bleaching disturbances. Conversely, my findings of active up-regulation of MAA levels in response to increased temperature, both in experimentally and naturally bleached soft corals, suggest other functions for MAAs and/or different regulating factors.

Subsequent studies (Chapter 3) have shown that higher MAA levels in *Lobophytum compactum* do not confer an ecological advantage with respect to bleaching, if bleaching is solely triggered

by increased temperature. In contrast, when UVR and temperature act as stress factors simultaneously, increased MAA levels also translate into higher protection against bleaching.

8.1.3. The effects of bleaching on the secondary metabolite chemistry of alcyonacean soft corals (Chapter 4)

This study has provided evidence that the soft corals *Lobophytum compactum* and *Sinularia flexibilis* are capable of surviving a severe short-term bleaching event, but lipid stores may be reduced as a consequence. Most importantly, rapid regulation and/or investment into species-specific algaecides and antimicrobial agents, as found in experimentally and naturally bleached soft corals, suggests that secondary metabolites may aid in the prevention of fouling of weakened corals after a bleaching episode.

8.1.4. The impact of different strains of zooxanthellae on the secondary metabolite chemistry and growth of the soft coral host *Lobophytum compactum* (Chapter 5)

The major finding of this study is that control over production of terpenoid secondary metabolites lies clearly with the animal host and that the algal partner is not essential for the production of terpenoids in the association. Substantial differences in colony growth in hosts inoculated with different algal strains and a positive correlation between growth and investment into production of secondary metabolites, suggest that via primary production, zooxanthellae may still exert a strong impact on secondary metabolite chemistry. This means that while the host controls the production of ecologically important terpenes, when energetic contribution from the algal symbiont is reduced or absent because of a bleaching event, energy reserves may be insufficient to maintain the production of significant concentrations.

8.1.5. Impacts of bleaching on the soft coral *Lobophytum compactum:* I. Fecundity, fertilisation and offspring viability; II. Biochemical changes in adults and their gametes (Chapters 6 & 7).

The results of these studies have shown that a strong short-term bleaching event has the capacity to reduce reproductive output in *Lobophytum compactum* via reduced fecundity and fertilisation success for two consecutive spawning seasons after a disturbance. Furthermore, bleaching caused reductions in key biochemical parameters (lipids, MAAs, proteins,

carotenoids) in adult tissues, which were subsequently passed on to their gametes, possibly jeopardising their fitness and survival.

8.2. General discussion

Soft corals, like most shallow reef dwelling organisms, have evolved a range of biochemical strategies to prevent, counteract and repair damage associated with solar stress. The requirement for phototrophy in the symbiotic coral association has precluded morphological forms of protection, such as scales, hair or feathers, and thus this challenge has been overcome to some degree by the production of MAAs (which allow photosynthesis while screening UVR) (reviewed in Dunlap and Shick, 1998). Other components of a soft coral's complex biochemical defence system against solar stress also include antioxidants, oxygen detoxifying enzymes (Lesser et al., 1990), fluorescent pigment granules (Salih et al., 1998) and possibly the light inducible pocilloporin pigment (Dove et al., 1995; reviewed in Shick et al., 1996). Given the highly unpredictable nature of stress or disturbances, it would be energetically too costly for an organism to invest into all avoidance/repair systems at all times. Induction of secondary metabolites under stress conditions, when benefits of production are realised, is a well-established phenomenon in both terrestrial (reviewed in Greshenzon, 1994) and marine systems (Paul and Fenical, 86; Coll et al. 1987; Paul and Van Alstyne, 1988; 1992). My research has shown that variable investment into MAA production in response to variable environmental conditions also occurs in soft corals (Chapter 2). The lack of whole colony mortality observed in either experimentally bleached colonies of *Lobophytum compactum* or *Sinularia flexibilis* suggests that such (host) control over MAA production contributes to a certain degree of tolerance to strong fluctuations of irradiance and temperature (20-31° C), the temperatures typically experienced by reef flat populations. Yet exposure to temperatures only 1 to 2° C above the normal summer maximum over a number of weeks (as experienced in summer 1998) killed every single colony in shallow depths, highlighting that the potential for tolerance limits to be exceeded by only small temperature excursions above typical maxima with potentially fatal consequences.

Although MAAs have received substantial attention for their possible role as photoprotectants in recent years (reviewed in Dunlap and Shick, 1998), until now it was not clear whether higher metabolic investment into MAAs also translates into increased protection against bleaching. The results of my research demonstrate that when solar stress is involved in the bleaching process, increased levels of MAAs do indeed provide some protection against bleaching (Chapter 3). Yet, it is important to recognise that even high MAA levels cannot protect fully

against solar bleaching and provide no direct protection against thermally induced bleaching, although MAAs may compensate for increasing light-sensitivity of thermally damaged zooxanthellae (Chapter 3).

To date all major bleaching events have been linked to thermal anomalies or a combination of temperature and light (Glynn, 1993; Glynn, 1996; Hoegh-Guldberg and Salvat, 1995; Berkelmans and Oliver, 1999), with the interaction between elevated temperature and light thought to be the key to understanding the severity of mass bleaching events (Coles and Jokiel, 1978; Lesser et al., 1990; Reaka-Kudla, 1993; Berkelmans and Oliver, 1999). Prior to my research it was believed that thermal destruction of MAAs and subsequent high exposure to UVR represented a possible molecular level at which a light–temperture interaction could take place (Lesser et al., 1990; Glynn et al., 1992). The conclusion derived from my studies, however, is that MAAs are not thermolabile, but conversely, MAAs actually increase during exposure to elevated temperature (i.e. 32 °C), which have been frequently recorded during natural bleaching events (reviewed in Brown 1997a; reviewed in Hoegh-Guldberg, 1999) (Chapter 3). Hence, MAAs are not the key to understanding the temperature – light interaction. Recently, aided by technical development of PAM (pulse-amplitude modulation fluorometry), an underlying model for the interaction of light and temperature has been provided by Jones et al. (1998). It appears that primary damage caused by elevated temperatures results in a decreased capacity of zooxanthellae to process the excitation energy coming from the dark reactions of photosynthesis, which leads secondarily to an increased sensitivity to light, even under normal light conditions. Under the scenario of possible global climate change (Williams and Bunkley-Williams, 1990), minute changes in temperature could thus push corals closer to not only their upper thermal, but also their irradiance limit, leaving them more exposed to bleaching despite some defence against solar stress. Thus, the significance of MAAs with respect to bleaching could be the avoidance of a fatal combination of stresses, by reducing additional high radiation hitting cellular targets. Alternatively, MAAs could diminish the probability of enhanced impacts due to synergistic interactions between temperature and light (Chapter 3) before thermal thresholds are reached and damage to the zooxanthellae photosynthetic apparatus occurs.

Terpenes have long been recognised as multifunctional vectors in the chemical defence of terrestrial plants (reviewed in Gershenzon, 1994) and soft corals (reviewed in Coll, 1992), but this study provides the first evidence that secondary metabolites may also be involved in overcoming the medium-term secondary impacts associated with bleaching. Coral recovery after mechanical injury or disturbance is typically accompanied by re-allocation of resources away from growth (Bak et al., 1977; Chadwick and Loya, 1990; Van Veghel and Bak, 1994;

Ward, 1995) and reproduction (Rinkevich, 1996; Hall, 98) towards regeneration of injured polyps. Similar trade-offs have also been established for the recovery of corals after bleaching (Porter et al., 1989; Leder et al., 1991; Szmant and Gassman, 1990). Increased production of secondary metabolites is one process potentially requiring extra resources in the weeks and months after bleaching, in order to prevent secondary effects such as fouling by opportunistic bacteria and algae (Chapter 4). The results of my research suggest that soft corals regulate and increase investment into specific terpenoid secondary metabolites after bleaching (Chapter 4). Energy budgets calculated for higher plants suggest that the production of terpenoids requires higher resources than the biosynthesis of many primary metabolites and other classes of secondary metabolites, due to their often high degree of reduction and requirement for enzymes that cannot be shared with other pathways (reviewed in Gershenzon, 1994). In the same way that the magnitude, type and intensity of injury (Oren et al., 1997) may limit how much energy remains for investment into the prevention of secondary infection, e.g. through increased mucus production (Hall, 1998), the severity of bleaching may limit investment into secondary metabolites. This notion is supported by the fact that algal overgrowths after the intensive natural bleaching event in summer 1998 (around 3-4 weeks), occurred only in soft corals with significantly reduced algaecide levels. Given the high degree of uncertainty as to the exact impact of pathogens on corals following bleaching (Kushmaro et al., 1996, 1997; Toren et al. 1998) and the fact that coral diseases (e.g. black band and white band disease, coral plague, aspergillosis) involving a variety of pathogens have increased dramatically during the past few years (Negelkerken et al., 1997; Richardson, 1998) this is an area of research that should become the focus of much greater research effort.

On a longer time scale, I also found a strong correlation between the strength of bleaching and the degree to which energy was diverted away from reproduction (Chapter 6). I discerned two strategies by which energy was diverted from reproduction, firstly by reduced egg numbers and mean egg size for two spawning seasons after the disturbance (Chapter 6), and secondly by reductions in key biochemical parameters such as lipids, MAAs, carotenoid and proteins (Chapter 7). Reductions in these parameters continued for at least 8 months after the disturbance in adult colonies and were also passed on to progeny, possibly compromising their fitness and survival (Chapter 7). No coral mortality occurred in any experimental colonies within two years, lending support to the notion that coral reefs can recover and flourish when disturbances are intermediate in intensity. If bleaching episodes occur with increasing intensity and at shorter intervals in the future (see Williams and Bunkley-Williams, 1990; Jones et al., 1997, Hoegh-Guldberg, 1999), then the longer term implications of this study are that complete recovery of corals might not be possible and ultimately, natural reproductive rhythms may be

completely disrupted. Paradoxically, sexual reproduction may become increasingly important for the replenishment of reefs, since a number of recent bleaching events have been associated with high mortality (Glynn et al., 1996; Fabricius, 1999; Berkelmans and Oliver, 1999). Moreover, it is important to evaluate the effects of bleaching within the context of the multitude of other natural disturbances such as injury, storms, crown-of-thorns outbreaks, or anthropogenic disturbances, such as increased sediment loads, eutrophication, overfishing, pollution and the possible introduction of pathogens (Hughes, 1994; reviewed in Brown, 1997a; Connell et al., 1997). While corals may be resilient enough to overcome any one of these disturbances, in combination it may result in the large-scale degradation of coral reefs.

8.3. Final conclusions

Given that coral bleaching is currently perceived as one of the greatest threats to the health of coral reefs world-wide (reviewed in Hoegh-Guldberg, 1999), the results of this research substantially increase our understanding of sub-lethal effects of bleaching in soft corals.

This is the first detailed study on bleaching in soft corals and provides new insights into the mechanisms that protect soft (and potentially hard) corals against bleaching and on the longer-term effects of bleaching. Specifically, my research has provided substantial understanding of the function of photo-protective MAAs both under natural conditions and during the bleaching process in soft corals. Furthermore it has shown that specific secondary metabolites may be involved in the prevention of fatal bacterial and/or algal fouling on soft corals after a bleaching disturbance. These two studies have shown that soft corals have a repertoire of photo-protective and antifouling defence strategies to counteract damage related to short-term bleaching events. However, observations of mass mortality of soft corals following the 1998 GBR mass bleaching event have made it clear that their capacity to withstand major bleaching events is limited. Thirdly, my findings of substantial effects of both moderate and severe bleaching disturbances on the reproductive output of soft corals highlight the need to extend research into bleaching well beyond zooxanthellae recovery. Thus, this is one of the first pieces of evidence to provide environmental managers with information on the longer-term effects of bleaching. These findings will enable environmental managers to set ecologically meaningful criteria for protection of reefs against additional damage, which could prove fatal in the wake of bleaching events.

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