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Effect of nutrient enrichment on the complementary (secondary) metabolite composition of the soft coral *Sarcophyton ehrenbergi* (Cnidaria: Octocorallia: Alcyonaceae) of the Great Barrier Reef

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Abstract A long-term study of the effects of nutrient enrichment on coral reefs (ENCORE Experiment) was carried out at One Tree Island (23°30′S; 152°96′E), Great Barrier Reef, between 1992 and 1996. The experiment involved the addition of water-soluble nutrients to 12 microatolls which contained a range of organisms and were situated within the larger lagoon. Three replicates of each of three nutrient treatments (nitrogen, phosphorus, nitrogen plus phosphorus) and an untreated set of three control atolls were prepared using the 12 selected microatolls. As part of the larger ENCORE experiment, changes in the chemical composition of the alcyonacean soft coral *Sarcophyton ehrenbergi* Marenzeller (Octocorallia: Alcyonacea), placed in the treatment microatolls, were monitored for a 1 yr period in an attempt to detect any responses attributable to nutrient enrichment. Multivariate analyses were performed to determine whether there were any patterns of response in the different nutrient treatments. At the level of individual metabolites, there were no clear treatment effects. However, the ratio of bioactive or stress metabolites (terpenes) to energy storage metabolites (lipids), referred to as the "physiological-change indicator", revealed effects of nutrient enrichment. Nitrogen enrichment resulted in a trend towards higher physiological-change indicators than control or phosphorus treatments in the majority of cases, while phosphorus enrichment significantly decreased the ratio relative to controls. In most cases, the physiological-change indicator increased in soft soft corals relocated into contact with the scleractinian *Pocillopora damicornis*. The potential of soft corals to serve as indicators of a changed nutrient regime is discussed.

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Introduction

Coral reefs accomodate the most diverse and complex communities in the marine environment. They flourish in tropical waters that are generally low in nutrient concentrations and hence low in phytoplankton, but affording high water-quality and clarity (Levinton 1995). The input of nutrients from fertilizer run-off and sewage from cities into coastal waters has been implicated in causing direct disturbances to reef ecosystems within the Great Barrier Reef (GBR) lagoon (Brodie and Furnas 1993).

Stress in coral reefs is generally poorly understood. Nevertheless, there is concern that chronic stress from elevated nutrient levels on reefs may cause them to become more effective primary producers, resulting in a gradual shift towards extreme algal dominance (Hawker and Connel 1989). Recent studies (e.g. Belda et al. 1993; Rasmussen 1994) have revealed that increased phosphates in seawater can affect the calcification processes of coral reef organisms. Disturbance of the crystallization of calcium carbonate in the coral skeleton arises from "crystal poisoning" (Simkiss 1964) and results in fragile calcareous structures (Kinsey and Davies 1979; Barnes and Chalker 1990; Belda et al. 1993). More detailed studies about chemical and physical factors and how each affects corals from the biochemical level through to the population level, however, are required

for a better understanding of the eco-physiological processes operating on coral reefs.

Because of the lack of detailed scientific information about eutrophication on the GBR, the Great Barrier Reef Marine Park Authority (GBRMPA), Australia, coordinated a long-term in situ experiment to investigate the response of coral reefs to nutrient enrichment (ENCORE) within the One Tree Island (OTI) lagoon (Steven and Larkum 1993; Larkum and Steven 1994). As part of that project, this study attempted to determine whether the biosynthesis of complementary (secondary) metabolites (Sammarco and Coll 1997) in the soft coral *Sarcophyton ehrenbergi* Marenzeller would be affected by increased levels of ammonium and phosphate added to the natural environment of microatolls at OTI over a 12 mo period. There are no reports in the literature regarding the use of quantitative data on the chemical composition of soft corals as an indicator of changed nutrient regimes.

Sarcophyton spp. colonies are rich sources of natural products (Tursch et al. 1978) that generally belong to the cembranoid diterpene class (e.g. cembrene-C, an ant-trail pheromone: Coll 1992). Several have been shown to possess significant bioactivities, of which sarcophytoxide, an ichthyotoxin (Tursch et al. 1978) is probably the most studied (see Kobayashi et al. 1983). Colonies of *Sarcophyton* spp. have also been involved in studies of allelopathy (Sammarco et al. 1985; Maida et al. 1995), and are commonly observed to be superior to other species in interspecific interactions in the field in both contact and non-contact situations (JCC personal observations).

Materials and methods

Study site

Field experiments were conducted in One Tree Island lagoon at the southern end of the GBR, Australia (23°30′S; 152°06′E). OTI was selected as the study site because of the facilities provided by the One Tree Island Research Station and the guarantee of no human interference.

Twelve microatolls (12 to 25 m diam, 0.6 to 1.0 m depth) in the northern end of the lagoon were selected to examine the effects of added nitrogen (N) and phosphorus (P), both separately (N, P) and combined (NP). Each microatoll was randomly allocated to one of four nutrient treatments in three replicates: addition of N, P and NP, or no nutrient addition (control, C).

Addition of nutrients commenced in September 1993 after nearly a year of preparation and baseline studies. Between September 1994 and January 1996, nutrient solutions were added three times during each 4 h period that the microatolls were ponded, commencing at low tide. Loadings within the microatolls were 20 μM N and 4 μM P respectively, $\simeq$20 times background concentrations on One Tree Reef and on the GBR in general (Furnas 1992) for the majority of the ponded period. Nutrients were added using radiocontrolled automatic dispensers (Larkum and Steven 1994), nitrogen as NH_4Cl, and phosphorus as KH_2PO_4. Both substances are in a biologically available form and readily soluble in seawater.

Data were compared with *Sarcophyton ehrenbergi* colonies from the natural environment, i.e. non-relocated controls on the reef wall adjacent to the entrance of the lagoon ("E" in Table 1).

Collection and relocation

Seventy-two colonies of *Sarcophyton ehrenbergi* Marenzeller were collected by SCUBA diving near the entrance to the lagoon within an area of 50 m on a reef wall at ~4 m depth at low tide. The colonies were removed without damage, together with hard substrate, and were placed in a test microatoll for acclimatisation for

Table 1 *Sarcophyton ehrenbergi*. Total terpene and fatty ester content (mean % dry wt and physiological-change indicator (ratio of total terpenes:fatty esters) of *S. ehrenbergi* with self ($S \times S$) or with scleractinian coral *Pocillopora damicornis* ($S \times P$) at One Tree Island as a function of nutrient treatment and season [*m* March; *j* July; *s* September; *f* February; *C* control *N* nitrogen; *P* phosphorus; *NP* nitrogen and phosphorus; *E* entrance (collection site); *i* = *S. ehrenbergi* in interaction with *P. damicornis*]. Indicator data are means (SD) of ratios calculated for each individual coral

Interaction $S \times S$				Interaction $S \times P$			
Treatment	Total terpenes	Fatty ester	Indicator (SD)	Treatment	Total terpenes	Fatty ester	Indicator (SD)
mC	2.73	1.11	3.37 (1.90)	mCi	2.92	0.95	4.52 (3.59)
mN	2.91	0.80	3.93 (1.37)	mNi	2.38	0.50	5.27 (1.75)
mP	2.82	1.38	2.55 (1.25)	mPi	2.62	1.12	2.85 (1.44)
mNP	2.24	1.05	3.03 (2.01)	mNPi	2.28	0.47	4.94 (0.9)
jC	1.35	1.13	1.15 (0.47)	jCi	1.88	0.59	4.00 (2.02)
jN	1.59	0.84	2.14 (1.27)	jNi	1.28	0.46	2.64 (1.65)
jP	2.10	1.35	1.78 (0.76)	jPi	1.97	1.02	2.36 (1.31)
jNP	2.35	0.79	3.0 (0.36)	jNPi	1.48	0.71	2.74 (1.49)
sC	2.56	1.05	2.96 (1.68)	jE	2.81	0.88	3.61 (1.88)
sNP	2.08	0.83	2.79 (1.17)	sCi	2.60	0.53	5.10 (0.93)
sP	2.49	1.10	2.54 (0.65)	sNI	2.40	0.51	4.80 (0.92)
sNP	2.29	1.15	2.06 (0.54)	sPi	2.95	1.20	2.75 (0.93)
fC	2.26	1.03	2.52 (1.01)	sNPi	2.04	0.77	3.05 (1.42)
fN	3.20	1.11	2.96 (0.80)	sE	3.79	1.01	4.11 (2.02)
fP	2.73	1.64	1.76 (0.51)	fCi	1.90	0.48	4.97 (3.50)
fNP	1.76	1.13	1.57 (0.17)	fNi	2.23	0.44	5.06 (0.90)
				fPi	2.57	1.30	2.72 (1.69)
				fNPi	2.63	0.59	4.38 (0.66)
				fE	3.80	2.27	1.84 (1.07)

3 mo prior the experiment. Two sets of three colonies each were attached to plastic duckboards, labelled, and placed in each of the 12 microatolls. One set of three colonies was placed where nearest-neighbour contacts were only with *S. ehrenbergi*, while the other set was placed such that nearest-neighbour contacts were with *Pocillopora damicornis* Linnaeus (Hexacorallia: Scleractinea), a common hard coral in the OTI microatoll environments. Six colonies were left in situ as non-relocated controls ("E" in Table 1) at the collection site. Sampling the colonies involved cutting wedge-shaped slices $\simeq$20 mm from the edge towards the middle of the disc of the colony and 20 mm wide. The coral samples were collected in labelled plastic bags approximately every season, in March (late summer), July (winter) and September (spring) 1995 and February (summer) 1996, and frozen until analysed at Central Queensland University in Rockhampton.

Chemical procedures

For each collection, samples from the colonies on each duckboard were combined, making two representative samples from each treatment microatoll; non-relocated control samples were also collected. The coral samples were freeze-dried, weighed, and cut in small pieces, and extracted with dichloromethane; the solvent was removed in vacuo. Quantitative ^{1}H NMR analyses of the crude samples were performed on a Bruker AM 300 MHz nuclear magnetic resonance (NMR) spectrometer, using *m*-dinitrobenzene as internal standard (details in: Leone 1993; Leone et al. 1995); $\sim$16 mg of the standard were dissolved in 25 ml $CDCl_3$. An aliquot (0.5 ml) this solution was mixed with $\sim$10 mg crude extract in a vial for ^{1}H NMR analysis. The distinguishable signals (non-overlapping) used for analysis were at δ8.52 (standard), δ4.05 (saturated fatty ester lipid), and δ4.49 (sarcophytoxide); the signals for cembrene-C were at δ5.85, 5.88, 5.93, 5.96 (overlapping signals) and were deconvoluted (using the analog integration trace) from the complex of peaks at δ5.75 to 6.40 (total conjugated cembrane-dienes).

Data analyses

The quantity of diterpene and lipid in each sample was calculated using the relationship between areas of signals for the diterpenes or lipid and the standard in the NMR spectra, leading to the number of moles of diterpene and lipid. The concentrations of components were calculated as percentages of the dry coral weight (Leone 1993; Leone et al. 1995).

All data were arcsine-transformed prior to graphing for the purposes of normalization (Zar 1984, pp 239–241). Chemometric analyses were performed by multivariate methods: principal component (PC) and the cluster analyses were derived from the Karlov and Hier programs, respectively, both included in the Arthur Package (Bruns and Scarminio 1987). The similarity coefficient used was the euclidian average distance between pairs. This program produced a hierarchical dendrogram (equal sample-weight pair-group) plot from the data sets (Bruns and Scarminio 1987).

The principal-component method permits the projection of higher-order space in two or three dimensions with a minimal loss of statistical information (Geyer et al. 1990). The matrix had 132 data points, including replicate samples (March, July, September and February), different boards or experiments (*Sarcophyton ehrenbergi* with or without *Pocillopora damicornis*) from each atoll and the collection site (March 1995 data not available). These data were separated into five categories (nitrogen, N; phosphorus, P; nitrogen + phosphorus, NP; relocated control, C; and non-relocated control, E), and four variables (sarcophytoxide, cembrene-C, others cembrenes, and saturated fatty ester).

A quantity which we term the "physiological-change indicator" was determined for each situation. It is defined as the ratio of the total terpenoid (sarcophytoxide + cembrene-C + other cembrenes; % dry wt) divided by the total lipid composition (% dry wt) for each coral. It reflects the relative expenditure of energy on defensive/competitive type compounds (terpenes) relative to energy storage compounds (lipids), and serves as an indicator of the level of stress experienced by the colonies as a function of treatment. Data were not arcsine-transformed prior to calculation of this ratio, as the ratio is a dimensionless quantity.

Comparisons of the values of the physiological-change indicator for different treatments were made using one-way ANOVA (Zar 1984, pp 239–241).

Results

Chemical analyses

Extracts from collected *Sarcophyton ehrenbergi* colonies contained several cembranoid diterpenes: cembrene-C (Vanderah et al. 1978), sarcophytoxide (Bowden et al. 1987), which were fully identified, and another three conjugated cembrane-dienes of undetermined structures (total cembrene). In addition, some fractions contained mixtures of saturated fatty esters, predominately cetyl palmitate, the major wax ester in coral tissue (Kung and Ciereszko 1977).

Field observation of *Sarcophyton ehrenbergi* colonies during one year

Field observation of the *Sarcophyton ehrenbergi* colonies (n = 72) during one year (March, July, September 1995 and February 1996), revealed that colonies relocated from the collection site to the microatolls adapted very well to the new environment. In general, many buds or small *S. ehrenbergi* colonies were present, and they showed remarkable regenerative ability even after several months of sampling. Survival of the soft coral was independent of its interaction with *Pocillopora damicornis*; all soft corals thrived throughout the experiment, irrespective of treatment regime. It was not possible to monitor growth in the soft coral colonies, because their size varies significantly with their state of turgidity.

Chemometric analysis

Principal-component analysis (PCA)

PCA using complementary metabolites (% dry wt) as variables, [i.e. sarcophytoxide (Variable 1), cembrene-C (Variable 2), others cembrenes (Variable 3) and fatty ester (Variable 4)] indicated that several factors may affect the concentration of individual metabolites, including seasonal variations, nutrient enrichment (N, P, NP), relocation of colonies from the natural environment, and competitive interaction with *Pocillopora damicornis*.

The coordinate axes (Principal Components 1 and 2) represented 79.7% of the total variance, of which PC1 represents 52.5%. This result shows the responsiveness of % cembrene-C (Variable 2) and others

cembrenes (Variable 3) to environmental changes. A reasonably defined region was apparent for non-relocated control colonies. There was some tendency for division into two regions on the basis of interaction of *Sarcophyton ehrenbergi* with *Pocillopora damicornis*. However, the control points are generally not distinguishable from the treatments.

Similarity analysis

Analyses based on the euclidian distances of the ratio data revealed two small subgroups: non-relocated controls and interaction of *Sarcophyton ehrenbergi* with *Pocillopora damicornis*.

Physiological-change indicator

The data are listed in Table 1 as a function of treatment and season. In summary: *Sarcophyton ehrenbergi* colonies interacting with *Pocillopora damicornis* had higher ratios than non-interacting colonies i.e. colonies interacting only with self as nearest neighbour (15 of 16 significant at $p < 0.001$, ANOVA). Enhanced phosphorus (P treatment) produced lower ratios (7 of 8 significant at $p < 0.05$, ANOVA). Enhanced nitrogen (N or NP treatments) tended to generate higher ratios (5 of 8 not significant, $p > 0.05$, ANOVA). This effect was strongest in February and March (summer), when growth is greatest and enhanced nitrogen exerts the greatest stress on the symbiosis, and hence the largest effect on terpene biosynthesis.

Discussion

The multivariate cluster-analyses and the cladistic approach tell us nothing about the effect of nutrients on the complementary metabolite composition of the soft coral *Sarcophyton ehrenbergi*. These are only revealed by the physiological-change indicator.

The PC analysis revealed the interaction between and the high variability of the data for the three classes of cembranoid diterpenes. By summing these quantities as the numerator of a ratio, some of this variability was reduced. Even for this aggregated data, there was a high level of variability in the physiological-change indicator between treatments and across sampling periods. We have shown that within soft corals in general, terpenoids play a range of physiological roles (Sammarco and Coll 1990; Maida et al. 1993), and in *Sinularia flexibilis*, individual diterpenes are ichthyotoxins, algicides, corallicides and feeding deterrents (Coll 1992). The summation of the concentrations of all the terpenes is thus a good indicator of a soft coral's response to stress. The lipid concentration reflects the energy stored by the coral in reserve. The ratio of terpenes to lipids reflects the balance between the energy invested in response to stress and the energy placed in reserve.

The important finding that the physiological-change indicator is significantly higher ($p < 0.001$) for colonies interacting with hard corals than with self is consistent with the expectation that stress levels in the soft coral will be higher under these competitive conditions. The experiment further revealed that phosphorus enrichment significantly lowered the indicator ratio ($p < 0.05$), while nitrogen enrichment tended to increase it (however, $p > 0.05$).

Zooxanthellae in symbiotic coral associations are nutrient-limited in respect to nitrogen (Rees 1987; Muscatine et al. 1989; Falkowski et al. 1993). Because of the low in vivo levels of nitrogen in the symbiosis, zooxanthellae have higher C:N ratios under normal nutrient conditions than are found in most phytoplankton (Cook and D'Elia 1987; Rees 1991). It is assumed (Dubinsky and Jokiel 1994) that in the absence of added nitrogen, zooxanthellae produce more carbon compounds than they can use for growth and cell division, and they translocate the excess carbon-rich metabolites to their host. If the ambient nitrogen levels are increased, zooxanthellae are able to utilize more of the products of their carbon fixation and undergo growth (Muscatine et al. 1989; Stambler et al. 1991; Falkowski et al. 1993; Dubinsky and Jokiel 1994). This reduces the amount of carbon metabolites that they can translocate, and causes stress to the symbiotic association (Stimson 1993). In response to this stress, it is likely that the coral redirects its energy expenditure into its control systems, i.e. into terpenoids which are known to play roles in algal growth modification (Coll et al. 1986), at the expense of lipid storage. Our experiments revealed an increase in the physiological-change indicator in conformity with the above supposition, albeit only in an indicative way.

Increasing levels of phosphorus have not been found to affect algal metabolism and growth significantly (see Muscatine et al. 1989; Stambler et al. 1991). They do however affect the calcification process in corals (Walker and Ormond 1982; Rasmussen 1994) and tridacnid clams (Belda et al. 1993), presumably by interfering with crystal deposition on poisoned facies (Simkiss 1964; Burton and Walker 1990; Belda et al. 1993). Increased availability of phosphorus may enhance the deposition of phospholipids in membranes and the biosynthesis and storage of acylated glycerol derivatives and wax esters in vesicles at the expense of terpene biosynthesis. Biosynthesis of these complex lipid classes depends on phosphate-derived intermediates (Garrett and Grisham 1999, pp 819–823). As predicted from the foregoing discussion, enhanced phosphorus levels in the present study reduced the physiological-change indicator for the soft coral colonies significantly.

A series of associated studies found that changes in nutrient regimes failed to cause quantitative changes in the C:N:P ratios of the soft coral *Sarcophyton ehrenbergi* (Tentori et al. 1997), while the present study failed to

detect nutrient-driven changes to individual metabolites. The value of the physiological-change indicator is thus reinforced as representing the most sensitive indicator of enhanced nutrient levels in the environment.

In general, enhancement of nutrients in ambient waters by a factor of three or more times normal ambient levels appears to constitute a major stress for coral reef communities (Hawker and Connell 1989). Our results provide good evidence, however, that the majority of the *Sarcophyton ehrenbergi* colonies adapt well to relocation, showing fast-regeneration and producing large quantities of carbon-based compounds. The non-relocated control colonies produced significantly higher amounts of the diterpene sarcophytoxide than those in the treatment atolls, a fact attributable to increased interspecific competition in the more competitive natural environment (Fleury et al. 2000).

These findings are consistent with earlier studies in the GBR, which revealed the adaptability of soft corals to increased nutrient loads. Thus, the octocoral *Clavularia inflata* displayed enhanced growth rates and competitive advantage over the faster-growing scleractinian coral *Acropora longicyathu*s when the two species were relocated from an outer reef to naturally higher nutrient levels in the inshore central region of the GBR (Aliño et al. 1992). Furthermore, *Sarcophyton ehrenbergi* colonies were the first re-colonizers of the Keppel Bay reefs after they suffered almost 100% mortality during the severe floods in 1990/1991 (Tentori unpublished results), attesting to their resilience in the face of high nutrient levels. Although the soft corals survive and apparently thrive under elevated nutrient conditions, the physiological-change indicator is able to detect subtle changes in their metabolism as a result of the environmental stress. Soft corals are prominent among marine organisms in the quantitative investment they make in terpenoid-based defence and competition, and also in the amount of lipid stored in their tissues. We have shown that the balance between these classes of compounds can reveal the presence of stress in a system.

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