Regulation and control of intracellular algae (= zooxanthellae) in hard corals

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SUMMARY

To examine algal (= zooxanthellae) regulation and control, and the factors determining algal densities in hard corals, the zooxanthellae mitotic index and release rates were regularly determined in branch tips from a colony of a staghorn coral, *Acropora formosa*, recovering from a coral 'bleaching' event (the stress-related dissociation of the coral–algal symbiosis). Mathematical models based upon density-dependent decreases in the algal division frequency and increases in algal release rates during the post-bleaching recovery period accurately predict the observed recovery period ($\approx$ 20 weeks). The models suggest that (i) the colony recovered its algal population from the division of the remaining zooxanthellae, and (ii) the continual loss of zooxanthellae significantly slowed the recovery of the coral. Possible reasons for the 'paradoxical' loss of healthy zooxanthellae from the bleached coral are discussed in terms of endodermal processes occurring in the recovering coral and the redistribution of newly formed zooxanthellae to aposymbiotic host cells. At a steady-state algal density of 2.1×10^6 zooxanthellae cm^{-2} at the end of the recovery period, the zooxanthellae would have to form a double layer of cells in the coral tissues, consistent with microscopic observations. Neighbouring colonies of *A. formosa* with inherently higher algal densities possess proportionately smaller zooxanthellae. Results suggest that space availability and the size of the algal symbionts determines the algal densities in the coral colonies. The large increases in the algal densities reported in corals exposed to elevated nutrient concentrations (i.e. between a two- and five-fold increase in the algal standing stock) are not consistent with this theory. We suggest that increases of this magnitude are a product of the experimental conditions: reasons for this statement are discussed. We propose that the stability of the coral–algal symbiosis under non-stress conditions, and the constancy of zooxanthellae densities in corals reported across growth form, depth and geographic range, are related to space availability limiting algal densities. However, at these densities, zooxanthellae have attributes consistent with nutrient limitation.

1. INTRODUCTION

Under normal conditions the algal (= zooxanthellae) densities in symbiotic corals are remarkably constant, averaging approximately $1–2.5 \times 10^6$ zooxanthellae cm^{-2} (Drew 1972; Muscatine *et al.* 1989). Neither the zooxanthellae nor the host outgrows the other, suggesting a carefully regulated control. The maintenance of this steady-state algal density in the presence of on-going algal division may be through either pre-mitotic control of the zooxanthellae (i.e. occurring before algal division) or post-mitotic control (occurring after algal division). Possible pre-mitotic control mechanisms include the production of growth-inhibiting factor(s), and/or the limitation of algal nutrient supply. Post-mitotic control mechanisms may involve the digestion of healthy or senescent zooxanthellae, and/or the expulsion of excess or senescent zooxanthellae (Muscatine & Pool 1979). Studies of pre- and post-mitotic algal control are complicated by the inherent stability of the symbiosis. However, corals regaining their zooxanthellae after natural bleaching events (the stress-related dissociation of the coral–algal symbiosis) provide dynamic systems which are useful to explore the control of the algal population (Høegh-Guldberg & Smith 1989*b*).

In January 1994, a coral bleaching event occurred on the fringing reefs of Magnetic Island (Great Barrier Reef region). We used this bleaching event to examine the manner by which a bleached colony of a staghorn coral, *Acropora formosa* (Dana 1846), recovers its algal symbionts, and the processes involved in algal regulation and control. There is little information on how corals recover from bleaching events, although bleached corals have higher zooxanthellae division frequencies, suggesting they may recover from division of the remnant algal population (Yonge & Nicholls 1931; Høegh-Guldberg & Smith 1989*b*; Fitt *et al.* 1993). However, it is tenable that bleached corals recover from an infestation of zooxanthellae from outside the colony (Fitt *et al.* 1993). Indeed, it has recently been suggested that coral bleaching may be an adaptive mechanism for switching algal partners (Buddemeier & Fautin 1993). Repetitive bleaching events could therefore lead to a reassortment of host and algal partners, culminating in combinations which are better suited to changing environmental conditions. An understanding of the method and time-scale for the regeneration of the algal population in bleached corals

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is clearly important, not only for defining the short-term consequences of bleaching events, but also the ecological and co-evolutionary aspects of the response.

In this communication, the rates of zooxanthellae division and release are examined as a coral recovers from a bleaching event. A series of equations are derived to model the repopulation of the bleached coral by zooxanthellae (the first, we believe, of their kind for the coral–algal symbiosis). A description for algal regulation and control in corals is proposed, whereby zooxanthellae are both space- and nutrient-limited at steady-state levels.

2. MATERIALS AND METHODS

In January 1994, a bleaching event occurred on the fringing reefs of Magnetic Island (Great Barrier Reef region), following a period of elevated air temperatures (maximum 44.3 °C) and seawater temperatures (maximum 32.4 °C on the reef slope at 5 m, recorded by *in situ* thermocouples and data loggers). The bleaching event ended abruptly in early February 1994 following a rapid reduction in air and seawater temperatures during storm activity associated with a nearby cyclone (Jones 1995). The zooxanthellae density, algal chlorophyll *a* (hereafter chl *a*) concentration, algal cell size and mitotic index (MI) were determined in a staghorn coral, *Acropora formosa* (Dana 1846), located on the reef slope (5–6 m depth) immediately after the bleaching event, and at 2–5 week intervals for the remainder of 1994. On each sampling occasion the terminal 40–50 mm of five 'brown-tipped' branches (Oliver 1984) were selected. All samples were collected between 0900 and 1000 h, and frozen for 1 h prior to transportation back to the laboratory packed in ice. The coral tissues were stripped from the skeleton with a jet of re-circulated 0.45 μm filtered seawater using a WaterPik (Johannes & Wiebe 1970). Small subsamples of the tissue homogenate were taken for an analysis of zooxanthellae densities (ten replicate counts using a haemocytometer on each of 2×10 ml subsamples), concentration (measured by the method of Jeffrey & Humphrey 1975), and zooxanthellae cell size (the largest linear diameter of 50 zooxanthellae from each of the tips, measured using a calibrated ocular micrometer at $\times 1000$ magnification under oil immersion). In addition, the zooxanthellae mitotic index was determined from the number of cells appearing as doublets in two samples of 1000 algae. For each tip the total number of zooxanthellae and total chl *a* was normalized to the number of polyps, recorded by visual census (Muscatine *et al*. 1991), and surface area estimated using the aluminium foil technique (Marsh 1970).

A number of studies were conducted to examine how the release of zooxanthellae from the coral colony changed as it recovered from the bleaching event. In the first weeks of February, March, June and July 1994, six brown-tipped branches (40–50 mm long) were cut from the colony and mounted with Plasticine in small acrylic holders. The prepared tips were slotted into an acrylic rack and left beside the parent colony for 14–20 d to recover from the collection and preparation procedures. At the end of the recovery period the prepared tips were transported back to the laboratory and placed in individual beakers containing 120 ml filtered (GF/C, Whatman) seawater. The beakers were then partially immersed inside a water bath receiving a continuous supply of seawater pumped from the reef. Each beaker was supplied with air from a series of aquarium pumps. Very slow bubbling of air onto the inside surface of each beaker served to maintain oxygen saturation of the test solutions and to provide a gentle water motion around the corals. A 3 mm thick sheet of Plexiglas was placed above the beakers to protect against UV light and to provide cover against rainfall. Two layers of 80% (absorption) shade cloth were draped around the water bath to reduce the light levels experienced by the corals. During the experiments, the light levels were measured with a Lambda Li-Cor 190 SA underwater sensor, and the mean, maximum and minimum light intensities recorded on a Li-Cor data logger. Maximum light levels experienced by the corals did not exceed 150 μmol photon $m^{-2} s^{-1}$. Light levels were further controlled to give a 12 h light/dark cycle by placing a dark plastic sheet over the tanks between 1800 and 0600 h.

The number of zooxanthellae released from each of the coral tips in 24 h was measured. Release rates were also examined over night-time (1800–0600 h) and daytime (0600–1800) periods. To maintain the corals in a healthy state the incubation water was changed at the end of 6 h periods. Each of the experiments was started at 1800 h, so the first two incubations (6 h each) occurred in the night-time periods, and the second two incubation periods occurred during daylight hours. To collect any released zooxanthellae, the 'incubation' beaker containing a coral tip was removed from the water bath and placed under shade. About 2–3 ml of the incubation water was squirted against the coral holder to resuspend any released zooxanthellae that may have settled. This process was repeated three or four times. The coral was then removed from the test container and transferred to a 500 ml 'holding' beaker containing clean unfiltered aerated seawater. Approximately 20–30 ml of the incubation water was then squirted against the sides of the incubation beaker using a syringe, to remove any zooxanthellae that may have been adhering to the glass surface. This process was repeated two or three times. The incubation water was then poured into a plastic container and fixed by the addition of 5 ml of 40% buffered formalin. A fresh test solution was then placed in each incubation beaker and the coral tip transferred back. The beaker was then placed back in the water bath and the air delivery system reattached. The process was repeated for the next coral tip. At the end of each of the experiments the corals were frozen for 1 h prior to transport back to the laboratory packed in ice. The tips were then WaterPiked and the zooxanthellae densities determined.

Zooxanthellae released from the coral tips during the experiments were concentrated by centrifugation. The containers holding the incubation water were first shaken vigorously to dislodge any zooxanthellae

adhering to the sides, and the incubation water homogenized for 15 s with a small domestic blender to disrupt any algal clumps. A 100 ml aliquot was centrifuged at 2500 rpm for 15 min and the supernatant discarded. This left a pellet of zooxanthellae released from the coral during the 6 h incubation. The algal pellets from all the 6 h incubations were then combined, and resuspended in a known volume of 2 % formalin solution. Ten replicate counts of the zooxanthellae densities were determined with a haemocytometer.

During the first of the four studies on the zooxanthellae release rates, conducted immediately after the bleaching event, the patterns of zooxanthellae release were measured as a function of the day or night over 48 h. Since the experiment was started at 1800 h, the zooxanthellae released in two successive 6 h incubations were combined and counted, to give the total zooxanthellae released from the tips in either a 12 h dark period or 12 h light period. For all experiments the zooxanthellae specific expulsion rate, μ_x (Høegh-Guldberg *et al*. 1987), was calculated using equation (1):

$$\mu_x = \frac{1}{N} \times \frac{\Delta E}{\Delta t}, \quad (1)$$

where N is the standing stock of zooxanthellae and ΔE is the number of zooxanthellae released in Δt (24 h). When the algal densities in the coral were at a steady-state level, an additional experiment was conducted where the mitotic index of the algae released from the tips was compared to the mitotic index of the algae still remaining inside the tips (*in hospite*). Four tips were prepared and incubated in filtered seawater for 24 h as described previously. Every 6 h the water was replaced with a fresh solution. The incubation water from the last 6 h incubation was fixed with formalin, centrifuged, and the mitotic index of the released zooxanthellae determined. The tips were then WaterPiked and the mitotic index of the zooxanthellae in the homogenate determined.

On 9 March 1994, seven 'brown-tipped' branches from the bleached colony were tagged with plastic coated wire about 30 mm from the ends of the branch (Yap & Gomez 1985). On 14 April 1994, and 29 June 1994, the distance from the wire to the end of the branch was measured to the nearest 0.5 mm using calipers.

In August 1994, the relationship between zooxanthellae density and mitotic index in sections along the 'white-tipped' branches (Oliver 1984) of *Acropora formosa* colonies from Magnetic Island was determined. Four white-tipped branches (80 mm long) were cut from each of four colonies at 5–6 m depth. Each branch was then sectioned using a saw into six divisions, comprising 2×5 mm^2 sections, 3×10 mm^2 sections and a terminal 15 mm section (see figure 4). The corresponding sections from each of the four branches for each colony were pooled, and the skeletons dissolved in 5 % HCl. A sub-sample of the digest was centrifuged at 1500 rpm for 5 min, and the supernatant discarded, leaving the algal pellet. The pellet was then resuspended in 0.45 µm filtered seawater (FSW) and the centrifugation and resuspension process repeated until the pH of the solution was approximately 8. Zooxanthellae densities and MI were then determined for the pooled algal pellets as described previously.

In December 1994, the zooxanthellae densities and cell size were determined in nine colonies of *Acropora formosa* at 5–6 m depth on the reef slope at Magnetic Island. Each of the colonies was separated from its nearest neighbour by between 3 and 10 m. Five 'brown-tipped' branches tips were collected from each of the colonies during three sampling trips conducted over the space of 7 d. The coral tips were frozen for 1 h before being transported back to the laboratory packed in ice. All tips were WaterPiked within 3 h of collection and the zooxanthellae densities and algal cell sizes determined within 12 h of collection.

3. RESULTS

Immediately after the bleaching event in January 1994, the study colony appeared a light tan colour. Recovery of the colony occurred quickly, with a discernible colour change from a light tan to a light green/brown colour by the next sampling trip (24 February 1994). By May 1994, the colony had returned to a normal brown coloration. From immediately after the bleaching event until May 1994, both the zooxanthellae density and the algal chl *a* concentration in the colony increased simultaneously (see figure 1), resulting in a rapid increase in the areal chl *a* concentration (see figure 1 inset).

After May 1994, the zooxanthellae density continued to increase, reaching a steady-state level of approximately 1.5×10^5 zooxanthellae polyp^{-1} ($\approx 2.10 \times 10^6$ zooxanthellae cm^{-2}). However, the algal chl *a* concentration fell sharply from 10.6 ± 1.7 pg chl *a* zooxanthella^{-1} (mean $\pm$ 95 % confidence interval (c.i.) $n = 5$) on 28 April 1994, to 4.7 ± 0.6 pg chl *a* zooxanthella^{-1} on 28 November 1994. From May 1994 onwards the

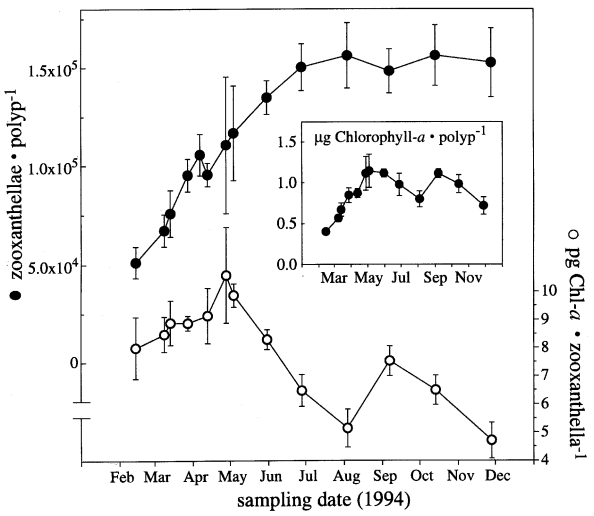

Figure 1. *Acropora formosa*. Mean zooxanthellae polyp^{-1}, pg chl *a* zooxanthella^{-1}, and µg chl *a* polyp^{-1} (inset) in the terminal 40–50 mm of brown-tipped branches of a staghorn coral, *A. formosa*, from February 1994 to December 1994. Each value represents the mean $\pm$ 95 % confidence intervals, $n = 5$ tips.

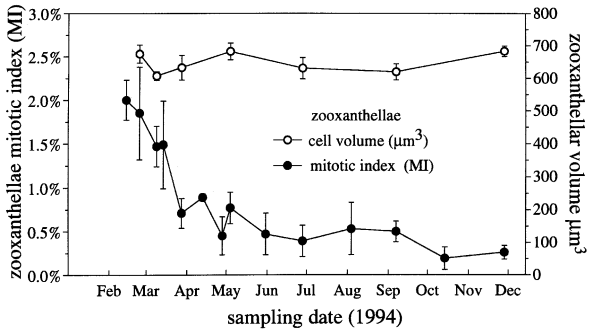

Figure 2. *Acropora formosa*. (*a*) Mean zooxanthellae mitotic index (MI, primary *y*-axis) and zooxanthellae cell size (µm³, secondary *y*-axis) in the terminal 40–50 mm of brown-tipped branches of a staghorn coral, *A. formosa*, from February 1994 to December 1994. Each value represents the mean ± 95 % confidence intervals, $n = 5$ tips.

zooxanthellae chl *a* concentration decreased as the zooxanthellae density increased, the chl *a* concentration per polyp remained at a relatively steady level for the remainder of the year (see figure 1 inset).

No change in the mean zooxanthellae cell size was observed over the course of the year (see figure 2).

The zooxanthellae MI in the colony was highest (2 %) when the zooxanthellae density was at its lowest, immediately after the bleaching event (see figure 2). The average zooxanthellae MI then progressively decreased to approximately 0.45 % by the end of the recovery period.

The mitotic index of the zooxanthellae released from the tips in the last 6 h of a 24 h incubation (3.2 ± 1.5 %, mean ± 95 % c.i., $n = 4$) was significantly higher than

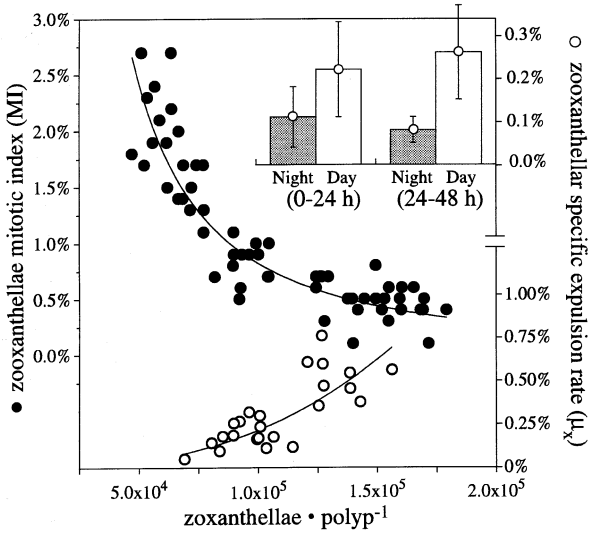

Figure 3. *Acropora formosa*. The relationship between zooxanthellae density (zooxanthellae polyp^{-1}) and zooxanthellae mitotic index (MI) and zooxanthellae specific expulsion rate (μ_x, equation (1), see text) in the terminal 40–50 mm of brown-tipped branches of a staghorn coral, *A. formosa*, immediately after a bleaching event, and in the subsequent ten months. Inset: zooxanthellae specific expulsion rate (μ_x) immediately after the bleaching event during dark (1800–0600 h) and light (0600–1800 h) periods. Each value represents the mean algal release rate ± 95 % confidence intervals, $n = 6$ tips.

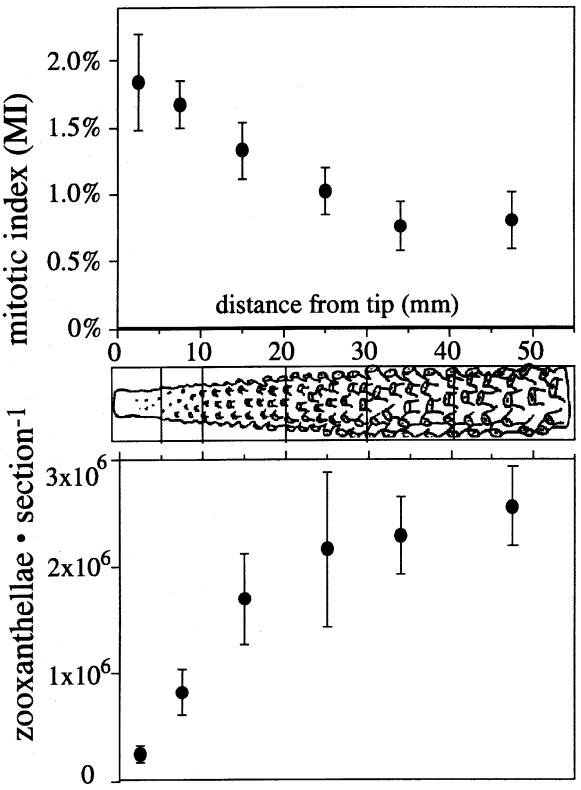

Figure 4. *Acropora formosa*. Zooxanthellae density (zooxanthellae polyp^{-1}) and zooxanthellae mitotic index (MI) along white-tipped branches of four colonies of *A. formosa*. Each value represents the mean in each section from four separate colonies (± 95 % c.i.). For each colony the zooxanthellae density and MI in each section was determined by pooling the respective sections from four separate white-tipped branches.

the mitotic index of the zooxanthellae in the tips at the end of the experiment (0.5 ± 0.2 %, mean ± 95 % c.i., $n = 4$, ANOVA, $p < 0.05$; algal density in the intact tips = $1.4 \pm 0.4 \times 10^5$ zooxanthellae polyp^{-1}).

The zooxanthellae MI decreased, and the zooxanthellae specific expulsion rate increased with increasing zooxanthellae density as the colony recovered from the bleaching event (see figure 3).

Immediately after the bleaching event, the zooxanthellae release rate from small tips excised from the colony was measured during successive light (daytime) and dark (night-time) periods over 48 h. On average, algal release rates were significantly higher during the light period (0600–1800 h) than the previous dark period (ANOVA, $p < 0.05$), corresponding to a 2.5 times difference between daytime and night-time release rates.

In the white-tipped branches of four colonies of *Acropora formosa*, the highest mitotic index, 1.8 ± 0.4 % (mean ± 95 % c.i., $n = 4$ colonies), was recorded in the apical 5 mm of the branch, where the zooxanthellae density was lowest (see figure 4). The mitotic index decreased as zooxanthellae density increased in the sections further back from the tip, eventually reaching a value of approximately 0.7 % in the sections 30–40 and 40–55 mm from the tip.

No linear growth occurred in the brown-tipped branches of the colony over the study period: 9 March

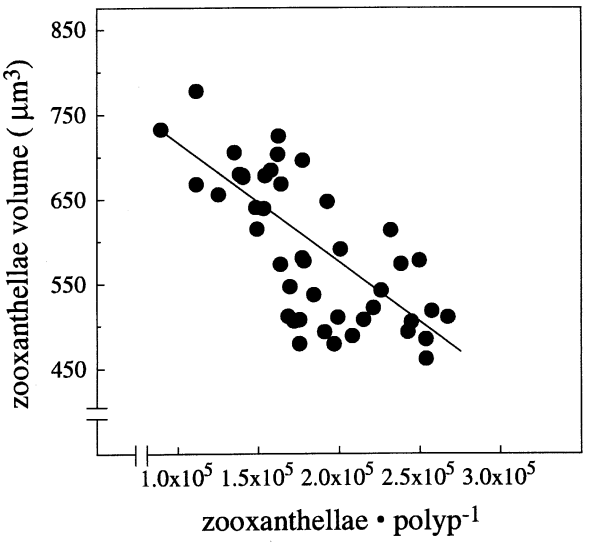

Figure 5. *Acropora formosa*. Zooxanthellar cell size (μm^3) versus density (zooxanthellae $polyp^{-1}$) in branch tips from nine colonies of *A. formosa* from 5–6 m at Magnetic Island (five tips from each colony). The correlation between the two parameters was significant ($r = 0.50$, $p < 0.05$).

1994, 31.5 ± 5.6 mm (mean $\pm$ 95 % c.i., $n = 7$); 14 April 1994, 31.0 ± 5.6 mm; 29 June 1994, 31.5 ± 5.5 mm.

Ten months after the bleaching event, the zooxanthellae densities and algal cell sizes of five branch tips from each of nine colonies of *Acropora formosa* at Magnetic Island were determined. The zooxanthellae densities ranged from 1.3 ± 0.3 to $2.3 \pm 0.2 \times 10^5$ zooxanthellae $polyp^{-1}$ (mean $\pm$ 95 % c.i., $n = 5$ tips from each colony) and the zooxanthellae chl *a* concentration ranged from 3.7 ± 0.4 to 6.0 ± 0.6 pg chl *a* $zooxanthella^{-1}$ (mean $\pm$ 95 % c.i., $n = 5$). Zooxanthellae cell volumes ranged from 498 ± 20 to $698 \pm 60\ \mu m^3$ (mean $\pm$ 95 % c.i., $n = 5$). Overall, there was a significant negative relationship between zooxanthellae cell size and zooxanthellae density in all the tips from the colonies (see figure 5, $p < 0.05$, $r = 0.50$).

4. DISCUSSION

After a bleaching event, the zooxanthellae density in a colony of *Acropora formosa* increased from 0.5×10^5 zooxanthellae $polyp^{-1}$ to a steady-state level of approximately 1.5×10^5 zooxanthellae $polyp^{-1}$ by July 1994. If the algal density in early December 1994 is considered to be representative of the level before the bleaching event (see figure 1), then the colony took approximately 20 weeks to recover from a loss of approximately 66 % of its zooxanthellae complement. The zooxanthellae density at the end of the recovery period, approximately 2.1×10^6 zooxanthellae cm^{-2}, is typical for a healthy coral (Drew 1972; Muscatine *et al.* 1989). The colony recovered from the bleaching event in a two-stage process. The first stage involved a simultaneous increase in both zooxanthellae density and algal chl *a* concentration until the colony had regained its normal coloration and areal chl *a* concentration by May 1994 (see figure 1). The second stage involved a continuing increase in zooxanthellae density from May 1994 to July 1994, but at the same time a concomitant decrease in zooxanthellae chl *a* concentration, which maintained the areal chl *a* concentration (and colony coloration) at a constant level.

The inverse relationship between zooxanthellae density and algal mitotic index described in this study (see figure 3) has also been observed by Høegh-Guldberg & Smith (1989*b*) and Fitt *et al.* (1993) in corals after bleaching events. When the coral had recovered to a steady-state zooxanthellae density, the zooxanthellae mitotic index (≈ 0.45 %, see figure 2) was similar to, or slightly lower than, other values reported for scleractinia: 0.4–0.7 % for *Stylophora pistillata*, from the Red Sea (Wilkerson *et al.* 1983; Muscatine *et al.* 1984; Høegh-Guldberg *et al.* 1987), 2–4 % for *Seriatopora hystrix*, from the Great Barrier Reef (Høegh-Guldberg & Smith 1989*b*).

Zooxanthellae were continually lost from the colony of *Acropora formosa*, even immediately after the bleaching event (see figure 3). The released zooxanthellae appeared as spherical, 8–12 μm diameter, green/brown algal cells. Occasionally released zooxanthellae were observed still enclosed within the host cell membrane (Gates *et al.* 1992). As the colony recovered from the bleaching event there was an increase in the absolute number of zooxanthellae released per polyp day^{-1}, and the relative number of cells released per day (as a fraction of the algal standing stock). This relationship has not been reported before. Algal release rates ranged from 0.05 % of the algal standing stock day^{-1} at the start of the recovery period to approximately 0.75 % when the zooxanthellae density had returned to normal levels. These values fall within the range described for other corals: 0.1 % for *Stylophora pistillata* in the Red Sea (Høegh-Guldberg *et al.* 1987), *S. pistillata* and *Seriatopora hystrix* from the Great Barrier Reef (Høegh-Guldberg & Smith 1989*a*); approximately 1 % for *Pocillopora damicornis* from Hawaii (Stimson & Kinzie 1991); < 1 % for *P. damicornis* from Hawaii (Muscatine *et al.* 1991). Algal release rates occurred in a distinct diel pattern, with release rates during the daylight hours (0600–1800 h) 2.5 times higher than during the night-time (figure 3 inset). A similar diel pattern of algal release, with zooxanthellae release rates occurring two times faster in the daytime compared to the night-time (peaking at midday), has been observed in *P. damicornis* from Hawaii (Stimson & Kinzie 1991).

No change in algal cell size was measured during the recovery period, despite a four-fold decrease in the algal division frequency (see figure 2). This is not entirely unexpected, since given the very low division frequencies of zooxanthellae *in hospite* (< 2.5 %), only a small proportion of the algal cells in the population are likely to have divided recently. Wilkerson *et al.* (1988) report no relationship between algal division frequency and zooxanthellae size in coral species from different depths, hence light availability (although a negative correlation exists only if the data from the different coral species are pooled). Thinh (1991) observed no changes in cell size of zooxanthellae from *Acropora formosa* and *A. cuneata* held in different light conditions (the main environmental gradient in light-dependent

organisms). Høegh-Guldberg & Smith (1989*b*) and Berner & Izhaki (1994) also report no changes in algal cell size in coral colonies exposed to elevated nutrient conditions.

(*a*) *Modelling zooxanthellae regeneration*

The density-dependent decrease in zooxanthellae division frequency, and increase in zooxanthellae specific release rate during the recovery period (see figure 3), suggest the operation of pre- and post-mitotic control mechanisms to limit the algal population growth. To determine whether the colony regained its zooxanthellae from the division of the remaining zooxanthellae, and whether the continual loss of zooxanthellae significantly affected its recovery rate, it is necessary to model the algal repopulation. The algal growth rate is a function of both the zooxanthellae division frequency (mitotic index) and the duration of the division phase. The mitotic index of the algal population at any given zooxanthellae density can be determined from the line of best fit in figure 3. Using an estimate of the duration of the division phase of zooxanthellae (i.e. the duration of cytokinesis, t_d) of 11 h (Wilkerson *et al*. 1983) the algal growth rate can be calculated by

$$\mu_z = \frac{1}{t_d} \ln (1+f), \qquad (2)$$

where f is the zooxanthellae MI at any given zooxanthellae density, estimated from the line of best fit in figure 3.

The algal density (Z) at day $n+1$ can be calculated using equation (3), and the algal repopulation modelled by reiteration.

$$Z_{n+1} = Z_n + (\mu_z \times Z_n) \quad \text{(model 1)}, \qquad (3)$$

where $Z_n$is zooxanthellae polyp^{-1} at day n.

However, healthy zooxanthellae are continually lost from the coral during the recovery period (see figure 3). The number of zooxanthellae released per day at any given zooxanthellae density can be calculated from the zooxanthellae specific expulsion rate (μ_x) determined from the line of best fit in figure 3. Thus, a new model can be constructed which also incorporates the continual release of zooxanthellae from the coral:

$$Z_{n+1} = Z_n + ((\mu_z - \mu_x) \times Z_n) \quad \text{(model 2)}. \qquad (4)$$

Both of the models rely on the fact that no significant growth occurred in the coral tips over the course of the recovery period (measured between 9 March and 29 June 1994). Oliver (1994) also reports a cessation of linear extension in brown-tipped branches of *Acropora formosa* colonies at Magnetic Island. Thus, if more zooxanthellae are produced each day by algal division than are released by the coral, the zooxanthellae density in the tissues will increase. Both models also assume that no changes in the duration of cytokinesis (t_d) occurred over the recovery period. Smith & Høegh-Guldberg (1987) have observed no changes in the duration of cytokinesis in large and small colonies of three scleractinian species from the Great Barrier Reef with markedly different zooxanthellae specific growth rates.

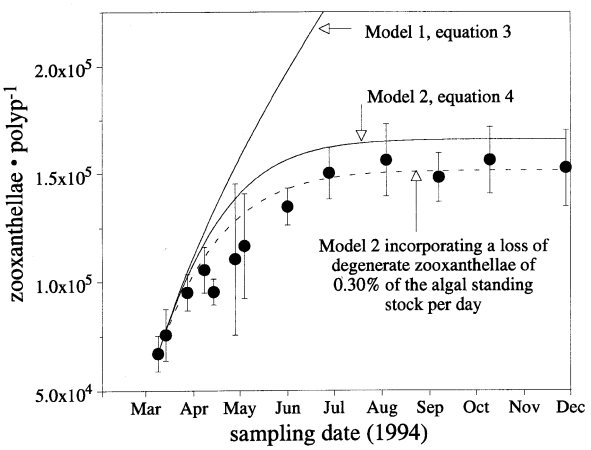

Figure 6. *Acropora formosa*. A comparison of modelled and observed zooxanthellae regeneration rates in a colony of *A. formosa* following a bleaching event. The observed recovery rates are shown as symbols, the modelled recovery rates (lines) are estimated by equation (3) (model 1, excluding the continual loss of zooxanthellae) and equation (4) (model 2, including the continual release of zooxanthellae). Both models use an estimate of the duration of cytokinesis (t_d) of 11 h. Dashed line, model 2, incorporating a rate of release of degenerate algae 0.30 % of the algal standing stock day^{-1} (see text).

The output of the two models is shown in figure 6, together with the measured recovery rate of the coral in the field.

Both models were started on 9 March 1994, approximately one month after the bleaching event ended, when it was clear that the algal densities in the tissues were beginning to increase. The results for the first model (equation (3)) show an increase in zooxanthellae density through algal division immediately after the bleaching event. As the zooxanthellae density increases, the growth rate of the algal population decreases. The algal standing stock eventually ceases to grow when the zooxanthellae division frequency decreases to an insignificant level. However, the model grossly overestimates the measured rate of algal population growth and also the final zooxanthellae density at the end of the recovery period (see figure 6). The second model (equation (4)), which incorporates the continual release of healthy zooxanthellae, provides a far better estimate of algal regeneration rate throughout the recovery period, and the final zooxanthellae density at steady-state levels.

However, the second model also slightly overestimates the recovery of the coral compared to the field data, suggesting an additional loss of zooxanthellae which has not been accounted for. In the absence of host growth, it is likely that this loss occurs by the production of degenerate zooxanthellae. These appear as small misshapen, red–brown objects 3–5 μm in diameter (see Yonge & Nicholls 1931; Steele 1975) and were sometimes observed in the incubation water in the algal release experiments. Degenerate zooxanthellae have also been observed in tissue homogenates of freshly collected coral tips. However, in both cases

their presence was highly variable and could not be accurately quantified. Some of this variability may be related to a behavioural mechanism of the coral. For example, branch tips of *Acropora formosa* maintained in aquaria can sometimes be observed releasing small brown mucus-laden boluses comprising mostly of degenerate zooxanthellae (> 95 %), but also healthy zooxanthellae, remnants of feeding, nematocysts and material of unknown origin and composition (personal observation). During the release process, the boluses emerge simultaneously from many polyps along the branch. If degenerate zooxanthellae are accumulated over a period of time, formed into a bolus and then episodically discharged (as opposed to a continual or diel release pattern), then this may explain the high degree of variability in freshly collected corals and during the algal release experiments. It also follows that determining the percentage of the algal standing stock which occurs as degenerate zooxanthellae in freshly collected corals may under- or overestimate the true number of degenerate zooxanthellae depending on whether the bolus of degenerate cells has been recently discharged.

Since no measurable growth occurred in the brown-tipped branches it is possible to estimate the rate of release of degenerate zooxanthellae as the difference between the observed and the modelled recovery rates. If a loss of approximately 0.3 % of the algal standing stock per day as degenerate zooxanthellae is incorporated into model 2 (equation (4)), then the observed and predicted values match closely (figure 6). However, this indirect method must be treated with caution. If the technique used to estimate algal specific growth rates based on the number of zooxanthellae appearing as doublets (equation (2), see Wilkerson *et al*. 1983) underestimated the true value of μ_z, or if the length of t_d was less than the estimate of 11 h, then this would also lead to an underestimation of the rate of release of degenerate zooxanthellae. The estimate of t_d (11 h) was determined from zooxanthellae from a tropical pelagic jellyfish, *Mastigias* sp. (Wilkerson *et al*. 1983). The value compares favourably with values from scleractinian corals, 13.2–13.7 h for zooxanthellae from *Seriatopora hystrix* (Høegh-Guldberg & Smith 1989*b*), and 9 h for zooxanthellae from *Pocillopora damicornis* (Høegh-Guldberg 1994). It remains unclear whether degenerate zooxanthellae are the product of algal mortality or host digestion, or both. Host digestion has been suggested since Boschma (1925), and its study should be revived.

We have drawn three conclusions from the modelling exercise. First, the recovery of the coral can be accounted for by the division of the zooxanthellae remaining in the coral after the bleaching event. However, these results do not rule out the possibility that 'external' zooxanthellae (of the same or different species) had been recruited during repopulation. Secondly, the continual low level loss of zooxanthellae from the coral slowed the recovery rate and was an integral determinant of the zooxanthellae density at any one time. Thirdly, growth rate and expulsion of zooxanthellae are not sufficient to account for the observed repopulation given a duration of cytokinesis of 11 h without the inclusion of an additional loss of zooxanthellae in the form of degenerate zooxanthellae.

(*b*) *Algal regulation through post-mitotic control*

At a steady-state zooxanthellae density (i.e. after July 1994, see figure 1) the loss of zooxanthellae from the coral can be interpreted as a post-mitotic algal control mechanism, i.e. the release of excess or 'supernumerary' symbionts. However, the loss of zooxanthellae immediately after the bleaching event cannot be interpreted in the same manner. The loss of zooxanthellae clearly affected the rate of the recovery (cf. models 1 and 2, see figure 6) and their loss represents a paradox. Logically, a bleached coral must be continually redistributing zooxanthellae to aposymbiotic endodermal cells, in order to fully recover from the loss. The mechanism by which this process(es) occurs is very poorly understood, although a number of ways can be suggested. In the first instance host cell necrosis, programmed cell death (PCD), or host cell adhesion dysfunction followed by disintegration (Gates *et al*. 1992) may result in zooxanthellae free within the coelenteron. These extracellular zooxanthellae may then be taken up by vacant endodermal cells where they may become established and divide. However, zooxanthellae free within the coelenteron may equally be lost to the surrounding seawater, thus explaining the continual release of algae observed during the study. A number of other processes could also result in zooxanthellae free within the coelenteron. For example, *Acropora formosa* typically has two zooxanthellae per endodermal cell, although occasionally one and three are observed (Harrison 1980; Meek 1982); similar ratios are also observed in *Pocillopora damicornis* (Gates & Muscatine 1992). Once the ratio of zooxanthellae to host cell is exceeded from algal division, the extra zooxanthella(e) may be released from the host cell into the coelenteron (or all zooxanthellae are released and the host cell then reacquires one cell). This process could therefore maintain a constant host to algal cell ratio, and yet produce zooxanthellae free within the coelenteron where they may be taken up by empty host cells (Berner *et al*. 1993) or lost from the coral. Another process for regaining zooxanthellae from a bleaching event is through faster growth of zooxanthellae-bearing host cells, and together with synchronous division of host and symbiont, the outgrowth of algal-free cells (Berner *et al*. 1993).

It seems likely that when a large proportion of host endodermal cells are algal-free (i.e. bleached corals), any extracellular zooxanthellae free within the coelenteron will be quickly acquired by empty host cells. In this situation only a few zooxanthellae may be lost from the coral. As the coral gradually recovers its zooxanthellae, uptake points (vacant host cells) will become saturated, and progressively more zooxanthellae in the coelenteron may be lost from the coral. This may explain the density-dependent release recorded during the recovery period (see figure 3). The presence of healthy zooxanthellae in the coelenteron may also act as a readily accessible reservoir from which the algal-free host cells in the actively growing areas (i.e. the

growing tips of branching colonies, or edges of encrusting colonies) can acquire symbionts.

The release of healthy zooxanthellae into the coelenteron may constitute post-mitotic control which serves two functions for the coral. First, in a steady-state situation it allows for release of supernumerary zooxanthellae, and second, during the recovery from a bleaching event (or during host growth) it provides an accessible pool for (re)population of empty host endodermal cells.

(*c*) *Algal regulation through pre-mitotic control*

The zooxanthellae division frequency was inversely correlated with zooxanthellae density during the post-bleaching recovery period. Similarly, the highest zooxanthellae mitotic index in the actively growing white-tipped branches of the *Acropora formosa* colonies occur in the apical sections where the zooxanthellae densities are at their lowest (see figure 4). In the sections proximal to the tips, the zooxanthellae density progressively increases and the mitotic index decreases.

There are a number of possible explanations for the inverse relationship between algal division frequency and density, involving either direct host control via mitogenic factors (Muscatine & Pool 1979), or a gradual limitation of carbon (Weis *et al*. 1989), light (through algal self-shading, Crossland & Barnes (1977)), or nutrients (Cook & D'Elia 1987; Cook *et al*. 1988; Høegh-Guldberg & Smith 1989*b*; Miller & Yellowlees 1989; Muscatine *et al*. 1989; Falkowski *et al*. 1993). Of these possible limiting factors, nutrient availability has received most attention, since corals exposed to elevated nutrient concentrations often have both higher algal specific growth rates (Høegh-Guldberg 1994), and higher algal densities (Høegh-Guldberg & Smith 1989*b*; Muscatine *et al*. 1989; Dubinsky *et al*. 1990; Stambler *et al*. 1991; Muller-Parker *et al*. 1994*b*). Furthermore, studies conducted after natural bleaching events have found that bleached corals (with lower than normal algal densities) have higher algal chl *a* concentrations (Høegh-Guldberg & Smith 1989*a*; Fitt *et al*. 1993, present study). The chl *a* concentration of an algal cell has been suggested as a good indicator of both nutrient sufficiency (Falkowski 1980; Høegh-Guldberg & Smith 1989*b*; Muscatine *et al*. 1989) and light availability (Falkowski & Dubinsky 1981; Chang *et al*. 1983; Porter *et al*. 1984). The inverse relationship between algal density and chl *a* concentration is opposite to that expected from a photoadaptive response to self-shading as the algal density increases. The relationship therefore most likely signifies gradual nutrient limitation of the zooxanthellae in recovering corals (Høegh-Guldberg & Smith 1989*b*; Fitt *et al*. 1993).

In the study colony, the algal chl *a* concentration actually increased with increasing symbiont density during the first stage of the recovery process, possibly a response to algal self-shading (see figure 1). The algal chl *a* concentration only decreased during the second stage of the recovery process, after the areal chl *a* concentration (and colony coloration) had returned to 'normal' levels (see figure 1 inset). If the algal chl *a* concentration can be used as an index of nutrient sufficiency, then the results suggests that the zooxanthellae were not nutrient limited in the bleached coral (see also Cook *et al*. 1994), but became nutrient limited when the algal chl *a* concentration per unit area had returned to normal levels. In contrast, the algal division frequency decreased systematically over both stages of the recovery period (see figure 3), suggesting that additional factors may be involved in determining when an algal cell will divide. One such factor may be the availability of vacant host cells. Immediately after the bleaching event, more zooxanthellae are located in host cells with room for division, and the algal mitotic index is correspondingly high. As the algal density increases and vacant endodermal cells become filled, the proportion of zooxanthellae with room for division becomes reduced, and the zooxanthellae mitotic index decreases. The remarkable constancy of the relationship between zooxanthellae density and mitotic index (which was generated over the course of ten months despite marked seasonal changes in light levels and seawater temperatures), also suggests that a physical factor (space availability) is involved in controlling the algal division frequency. This is further supported by the relationship between algal division frequency and density along the white-tipped branches (see figure 4). Thus, the nature of the inverse relationship between the algal division frequency and density depicted in figure 3 may be equally related to space availability as to the nutrient sufficiency of the algal symbionts.

Immediately after the bleaching event, and despite having lost $> 60\,\%$ of its zooxanthellae, the zooxanthellae specific growth rates were still markedly lower than those reported for zooxanthellae in culture (see Chang *et al*. 1983). The mitotic index of the zooxanthellae released from the tips during the last 6 h of a 24 h incubation was seven times higher than the mitotic index of the zooxanthellae in the intact tips at the end of the experiment (see also Suharsono & Brown 1992; McCloskey *et al*. 1996). Both results imply a degree of host influence of zooxanthellae division *in hospite*; however, they cannot be used as evidence *per se* for direct host control because light levels and the flux of O_2 and CO_2, and nutrients to free-living cells will be different from that to intracellular zooxanthellae. It remains unclear from these studies what degree of direct pre-mitotic control, indeed if any, the host played in the repopulation of the tissues by the zooxanthellae.

(*d*) *Factors determining algal densities in corals*

The study colony attained a steady-state level of 1.5×10^5 zooxanthellae polyp^{-1} or 2.10×10^6 zooxanthellae cm^{-2}. However, other neighbouring colonies of *Acropora formosa* at the same depth have inherently different algal densities (i.e. range 1.3–2.3 $\times 10^5$ zooxanthellae polyp^{-1}, $n = 9$ colonies). In general algal densities in corals are remarkably constant, irrespective of genus, depth, growth form or location (Drew 1972; Muscatine & Porter 1977; Porter *et al*. 1984). Drew (1972) measured an algal density of 1.45×10^6 cells cm^{-2} (range 0.9–2.5 cells cm^{-2}) in ten genera of

hermatypic corals and two genera of soft corals. Based on these observations he suggested that the area available for the zooxanthellae to occupy directly determines their densities under steady-state conditions, i.e. a 'space limitation' theory. However, Duerden (1902) has pointed out that in corals at depths, the undersides of the tissues often have no symbiotic algae (see also Dubinsky & Jokiel 1994). It follows that light availability may also limit algal densities and restrict zooxanthellae from occupying deeper tissues in corals with highly perforate skeletons. Also, newly formed host cells at the growing points of colonies represent areas for algal colonization which have not yet been exploited. These areas are generally paler (Oliver 1984), and contain fewer zooxanthellae (see figure 4). Thus, under the 'space limitation' theory, the zooxanthellae density may still exist below the maximum possible level.

There are many lines of evidence to support 'space limitation' in the study described here. First, the colony of *Acropora formosa* recovered to a steady-state zooxanthellae density of 2.1×10^6 zooxanthellae cm^{-2}: given that the average zooxanthellae diameter during the recovery period is 10.6 μm, then approximately 1×10^6 zooxanthellae could fit into an area of 1 cm^2 to form a single layer of cells. It follows that, at the measured algal density, the zooxanthellae would have to form two layers of cells in the tissues. This is consistent with microscopic observations, where the endodermal tissues of *A. formosa* contain a double layer of zooxanthellae with typically two algae per host cell (Harrison 1980; Meek 1982). Second, there was a direct linear correlation between zooxanthellae density and algal cell size in the branch tips from neighbouring colonies of *A. formosa* at the same depth at Magnetic Island (see figure 5). Colonies with proportionately smaller zooxanthellae had proportionately higher algal densities. Third, the algal density in the study colony did not change from July 1994 to December 1994 (see figure 1), during a period in which average daily seawater temperatures ranged from 21.2 to 29.0 °C, and which included the coral spawning period at Magnetic Island in October/November. This suggests that seasonal cycles in algal density do not occur, consistent with the idea that algal density is set close to the upper maximum limit determined by space availability. The density-dependent decrease in the zooxanthellae division frequency and increase in zooxanthellae release rate during the recovery period are also consistent with the gradual occupancy of all available host endodermal tissues by algal cells as the coral recovers from the bleaching event.

An apparent contradiction to the 'space limitation' theory, is the reported increases in zooxanthellae densities in corals following exposure to elevated nutrient concentrations (Høegh-Guldberg & Smith 1989*b*; Muscatine *et al.* 1989; Dubinsky *et al.* 1990; Stambler *et al.* 1991; Muller-Parker *et al.* 1994*b*). In the first instance, under the space limitation theory, the algal densities in a whole colony do not exist at the maximum possible level (see above). It follows that under nutrient enrichment, algal-free areas may acquire zooxanthellae faster through an increase in algal specific growth rate (Høegh-Guldberg 1994) or a decrease in release rates (Stimson & Kinzie 1991), resulting in an increase in the areal algal density relative to control colonies. Host growth also represents a sink for newly formed zooxanthellae. If a decrease in host growth occurs during nutrient enrichment (see for example Stambler *et al.* 1991), then increases in algal densities may occur without changes in either the algal specific growth or expulsion rates. Thus, increases in algal densities in whole corals may be measured and this does not controvert the theory that algal densities are determined by space availability. However, in some of these nutrient enrichment studies, algal densities have increased between two- and five-fold. Increases of these magnitudes seem inconsistent with the space limitation theory. On closer inspection of the data, we suggest that the two- to five-fold increases in zooxanthellae densities are a product of the experimental conditions. For example, in none of the experiments has it been determined whether: (i) the zooxanthellae densities in the study corals before the experiment were at steady-state or 'normal' levels, or (ii) the zooxanthellae densities in the nutrient-enriched corals at the end of the experiments were higher than a dedicated set of controls maintained on the reef (to control for the experimental manipulations). Maintaining corals in a healthy state under artificial conditions (inside aquaria or respirometer chambers) for long periods of time is notoriously difficult. A frequent stress response of corals to handling, transportation and experimental enclosure is loss of zooxanthellae (see Stambler *et al.* 1991; Stambler *et al.* 1994*a*). Since decreases in algal density of up to 40–50 % may occur in coral colonies without marked changes in the coral coloration (personal observation in colonies of *Acropora formosa* and *Pocillopora damicornis*), nutrient enrichment programs may begin with, or be conducted with, corals which contain significantly fewer zooxanthellae than normal (i.e. steady-state conditions). It is conspicuous that in none of the nutrient enrichment studies conducted thus far have algal densities been measured which are higher than those typically encountered under 'normal' circumstances. However, many of the control colonies involved in these studies contain algal densities significantly lower than normal, either before the experiment (Høegh-Guldberg & Smith 1989*b*), or afterwards (Dubinsky *et al.* 1990; Stambler *et al.* 1991; Muller-Parker *et al.* 1994*b*).

For example, in the study conducted by Dubinsky *et al.* (1990) the algal density in the control colony, which was maintained inside a regularly flushed respirometer chamber for 18 d, was only 0.06×10^6 zooxanthellae cm^{-2}. This value is approximately 4 % of the algal density reported for the same species of coral at the same location in an earlier study (Falkowski & Dubinsky 1981). The algal density in the nutrient-enriched colony (0.29×10^6 zooxanthellae cm^{-2}) was five times higher than the control colony, but this value is still markedly lower than the normal densities expected for this species at this location. The results suggest either (i) all colonies lost zooxanthellae but the net loss rate in the control colonies was greater, or (ii) having lost zooxanthellae, the algal regeneration rate

under nutrient enrichment was higher, or (iii) both. Similarly, in the nutrient enrichment studies with *Pocillopora damicornis*, described by Stambler *et al.* (1991), a net decrease in the zooxanthellae density in the control corals was reported during the experiment (i.e. from 1×10^6 zooxanthellae cm^{-2} to 0.34×10^6 zooxanthellae cm^{-2} at the end of the experiment). The algal densities in the nutrient-enriched colonies at the end of the 13 d incubation were only markedly higher ($\approx 5\times$) because of the changes in algal densities in the control corals.

In the study conducted by Høegh-Guldberg & Smith (1989*b*), increases in zooxanthellae densities following nutrient enrichment occurred in colonies of *Stylophora pistillata*, but not in colonies of *Seriatopora hystrix*. In their experiment, which was conducted after a natural bleaching event (see Høegh-Guldberg & Smith 1989*a*), the algal densities in the *S. pistillata* colonies (0.5×10^6 zooxanthellae cm^{-2}) were naturally lower at the start of the experiment than the colonies of *S. hystrix* (1×10^6 zooxanthellae cm^{-2}, Høegh-Guldberg & Smith (1989*b*)). The results suggest that marked increases in zooxanthellae densities following nutrient enrichment will only occur if algal densities are significantly lower than 'normal' levels, but not if zooxanthellae densities are at normal levels.

In 1991, one of the most comprehensive studies of the effects of ammonium enrichment on corals was conducted in Hawaii using the coral *Pocillopora damicornis*. Whole colonies were exposed to 20 or 50 μM ammonium concentrations for periods of time ranging from two to eight weeks, and the algal densities compared to those in control colonies exposed to ambient seawater alone for eight weeks (termed $t = 0$; Muller-Parker *et al.* 1994*a*, p. 235). The nutrient-enriched colonies had significantly higher algal densities than the control colonies after only two weeks' exposure. However, the zooxanthellae densities were not higher in colonies exposed to elevated nutrients for longer periods (i.e. four, six and eight weeks), and there was no difference in zooxanthellae density in corals exposed to the 20 and 50 μM treatments. At the end of this study, the zooxanthellae density of one freshly collected colony of *P. damicornis* was also determined. The zooxanthellae density in the coral (0.68×10^6 zooxanthellae cm^{-2}), was the same as those of the nitrogen-enriched corals (Muller-Parker *et al.* 1994*b*; see figure 5, p. 281), and notably higher than those of the control colonies. Although this analysis is only limited to the densities in one colony, the data suggest that exposure of colonies of *P. damicornis* to ammonium concentrations > 50 times higher than those typically encountered in oligotrophic waters (Stambler *et al.* 1994*b*), for a total of two months, did not cause an increase in zooxanthellae densities relative to a coral in the field. It is thus equally tenable that the net algal loss rates (which is a function of both μ_z and μ_x) from the control colonies was higher than from the nutrient-enriched colonies. Without an unambiguous reference point of the algal densities *in situ* at the beginning of the experiment (a field control), the impression that algal densities have increased markedly relative to 'normal' may be inferred.

4. CONCLUSIONS AND PERSPECTIVE

The results of this study suggest that bleached corals recover their algal population by the division of the remaining zooxanthellae. Algal densities reach a steady-state level determined by space availability and the size of algal symbionts, i.e. space limitation. The algal cell size in this study appears invariant of seasonal change, zooxanthellae density, chl *a* concentration and division frequency. However, it follows that if the algal size can be determined by the host or external light conditions or nutrient supply, then it may ultimately determine the whole nature of the association.

At a steady-state algal density, zooxanthellae (both healthy and degenerate) are lost from the coral at a rate balanced by the production of new zooxanthellae from algal division. Thus, at a steady-state zooxanthellae density, it is equally tenable that the algal division frequency is determined by the rate of algal loss (hence the availability of space for algal division), rather than zooxanthellae being released as a result of algal division. The loss of zooxanthellae may represent the release of zooxanthellae supernumerary to the host cell, but may also represent zooxanthellae released following host cell necrosis, programmed cell death, and detachment. Degenerate zooxanthellae may be produced by host digestion, but also by algal autosenescence, or both. The direct intervention by the host in putative pre- and post-mitotic control mechanisms observed in these studies remains equivocal.

The inverse relationship between algal chl *a* concentration and algal density, and the increases in algal specific growth rates and densities in corals exposed to elevated nutrient concentrations suggest nutrients may be limiting to the zooxanthellae. Algal densities may thus be limited by both space availability and nutrient supply at steady-state levels. Only newly formed host cells at growing points and tissues not receiving a sufficient light supply represent areas which have not yet been colonized by zooxanthellae. That a physical factor (space availability) determines the algal densities in corals may therefore account for the remarkable stability of the association under non-stress conditions, and the constancy of algal density between species, growth forms, and over depth and geographic range.

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