

# Zooxanthellae expelled from bleached corals at 33°C are photosynthetically competent

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**ABSTRACT:** While a number of factors have been linked to coral bleaching, such as high light, high temperature, low salinity, and UV exposure, the best explanation for recent coral bleaching events are small temperature excursions of 1 to 2°C above summer sea-surface temperatures in the tropics which induce the dinoflagellate symbionts (zooxanthellae) to be expelled from the host. The mechanism that triggers this expulsion of the algal symbionts is not resolved, but has been attributed to damage to the photosynthetic mechanism of the zooxanthellae. In the present investigation we addressed the question of whether such expelled zooxanthellae are indeed impaired irreversibly in their photosynthesis. We employed a Microscopy Pulse Amplitude-Modulated (PAM) fluorometer, by which individual zooxanthellae can be examined to study photosynthesis in zooxanthellae expelled when corals are subjected to a temperature of 33°C. We show that the expelled zooxanthellae from *Cyphastrea serailia* were largely unaffected in their photosynthesis and could be heated to 37°C before showing temperature-induced photosynthetic impairment. These results suggest strongly that the early events that trigger temperature-induced expulsion of zooxanthellae involve a dysfunction in the interaction of the zooxanthellae and the coral host tissue, and not a dysfunction in the zooxanthellae per se.

**KEY WORDS:** Chlorophyll fluorescence · Coral bleaching · Zooxanthellae · PAM

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## INTRODUCTION

Summer temperatures of surface waters in the southern Great Barrier Reef rise to 28.5°C and rarely to 30°C. In 1998, however, summer surface-water temperatures reached 32°C and this event was correlated with bleaching of many species of corals on 80% of the reefs on the Great Barrier Reef (Hoegh-Guldberg 1999). In many instances the corals showed good recovery. In other parts of the world, coral reefs occur in waters where sea surface temperature may be a degree or two higher than this and here the temperatures that trigger bleaching are concomitantly higher. Thus, the temperature where coral bleaching occurs is 30 to 34°C (Hoegh-Guldberg 1999).

The temperatures at which coral bleaching is induced are remarkably low in relation to the general thermal tolerance of tropical organisms. For example, tropical marine organisms generally do not show adverse effects below 40°C (Glynn 1968), and photosynthetic reactions are little affected up to temperatures of 40 to 45°C (Berry & Bjorkman 1980). Nevertheless, it has been clearly demonstrated that *in situ* corals exposed to temperatures above 32°C show reduced fluorescence yield and other effects that point to a significant reduction in photosynthetic activity (Iglesias-Prieto et al. 1992, Fitt & Warner 1995, Warner et al. 1996, Iglesias-Prieto 1997, Lesser 1997, Jones et al. 1998, 2000). This has led to the following assumptions concerning the zooxanthellae, that are damaged by temperatures of 32 to 34°C: that the damage is to the photosynthetic apparatus, that the damage is long-term, and that it is this damage which is the trigger

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leading to the expulsion of the zooxanthellae. We set out to test these assumptions using a Microscopy Pulse Amplitude-Modulated (PAM) fluorometer.

## MATERIAL AND METHODS

Shallow-water specimens of *Cyphastrea serailia* and *Pocillopora damicornis* were collected from Heron Island Lagoon (23° 31' S, 152° 08' E, Great Barrier Reef, Australia) and maintained in continuously flowing seawater for 24 h under shaded conditions ( $<200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) before experimentation.

**Fluorescence measurements.** Fluorescence measurements on single zooxanthellae cells were performed using a microscopy-PAM (Walz GmbH, Effeltrich, Germany). The samples of zooxanthellae were acclimated to a specified temperature (see below) and constant irradiance of  $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for 5 min during the measurement of an induction curve. At the end of the 5 min induction curve, a rapid light curve (Ralph et al. 1998) was performed with a 5 s step-width over the range of irradiance from 0 to  $360 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

Temperatures were controlled using a specially constructed attachment to the microscope stage. The attachment comprised of a copper block ( $85 \times 30 \times 10 \text{ mm}$ ) with an optical window. Rapid thermal exchange was achieved by circulating heated water through a series of tubes in the block, and water temperatures were controlled ( $\pm 0.25^\circ\text{C}$ ) using a digitally controlled water bath (Julabo Labortechnik F20-MH, Seelbach, Germany).

**Isolation of zooxanthellae.** Samples of zooxanthellae were isolated from coral colonies following a modified method (Patton et al. 1977, Lesser & Shick 1989, Gates et al. 1992). Zooxanthellae were collected by gently rubbing the surface of the coral with a toothbrush, filtering the homogenate through a  $125 \mu\text{m}$  mesh, rinsing in  $0.45 \mu\text{m}$ -filtered seawater, and centrifuging at 2000 rpm for 2 min. The pellet was re-suspended in filtered seawater, centrifuged again, and was re-suspended in filtered seawater.

**Expelled zooxanthellae — bleached.** Bleaching was induced by exposure to  $33^\circ\text{C}$  under moderate light conditions ( $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) (Fitt & Warner 1995, Warner et al. 1996). Coral samples ( $n = 30$ ) were placed in fresh seawater in a beaker within a heated water bath (Haake E3, Karlsruhe, Germany). When the temperature was raised to  $33^\circ\text{C}$ , samples of expelled zooxanthellae were collected from the surface of coral. Few zooxanthellae were apparent on the water surface or in mucus at temperatures below  $33^\circ\text{C}$ . Zooxanthellae released abundantly from heat-treated samples were rinsed in filtered ( $0.45 \mu\text{m}$ ) seawater to remove mucus (Gates et al. 1992).

## RESULTS AND DISCUSSION

Specimens of *Cyphastrea serailia* and *Pocillopora damicornis* were bleached in the light when water temperature was raised to  $33^\circ\text{C}$  for 3 h. We use the term 'bleached' in this context to mean that significant numbers of zooxanthellae were released from the coral host in comparison to controls at lower temperature. At  $25^\circ\text{C}$  few zooxanthellae could be sampled ( $60 \text{ cells ml}^{-1}$  in the filtered supernatant), whereas after 3 h at  $33^\circ\text{C}$  the zooxanthellae density in the sampled water was in the order of  $6000 \text{ cells ml}^{-1}$ . The exact nature of this release is not fully understood. We use the term 'expelled zooxanthellae' to describe the zooxanthellae that are released, to signify that there is probably a process of active expulsion by the host. Below  $33^\circ\text{C}$  there was no sign of bleaching, i.e. only a few zooxanthellae were released and these may have been senescent cells. Light was necessary for the bleaching to occur. However, we deliberately chose a rather low light level ( $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) that still elicited bleaching because photoinhibition occurred at higher light levels. Thus, we chose the mildest conditions that would still cause the onset of bleaching to study the primary process(es) of bleaching and not secondary effects due to photoinhibition or heat damage. The bleached corals at  $33^\circ\text{C}$  retained a large number of zooxanthellae at that stage, and therefore it was possible to use PAM fluorometry to probe the photosynthetic characteristics of the *in situ* zooxanthellae. The colonies of *C. serailia* held at  $33^\circ\text{C}$  showed a significant reduction (Student's *t*-test,  $p < 0.05$ ) in the variable/maximum fluorescence ratio,  $F_v/F_m$  ( $0.521 \pm 0.041$ , mean  $\pm$  SEM, as measured by a Diving-PAM: Walz GmbH, Effeltrich, Germany) in comparison to those held at  $25^\circ\text{C}$  which maintained a maximum quantum yield of  $0.616 \pm 0.024$ . These values are similar to those for temperature-induced bleached specimens of *Pleisiastrea versipora* (Jones et al. 2000). As shown in Figs. 1 & 2, the electron transport rate (*ETR*) of both expelled and isolated zooxanthellae from *C. serailia* was strongly inhibited by increased temperature, in agreement with previous studies (Warner et al. 1999, Jones et al. 2000). However, the  $33^\circ\text{C}$  'expelled' zooxanthellae from the same samples of coral showed relatively normal photosynthetic activity (in comparison to the colony), as demonstrated by the rapid light curve (RLC) in Fig. 1. The maximum electron transport rate ( $ETR_{\text{max}}$ ) was approximately  $8 \mu\text{mol electrons m}^{-2} \text{s}^{-1}$ . This rate compared favourably with that of zooxanthellae, which were artificially extracted (isolated) into a physiological suspending medium at  $25^\circ\text{C}$  and which had an  $ETR_{\text{max}}$  of about  $9.5 \mu\text{mol electrons m}^{-2} \text{s}^{-1}$  (Fig. 2). The  $33^\circ\text{C}$ -expelled zooxanthellae (Fig. 1) showed similar RLCs to the  $33^\circ\text{C}$  isolated samples

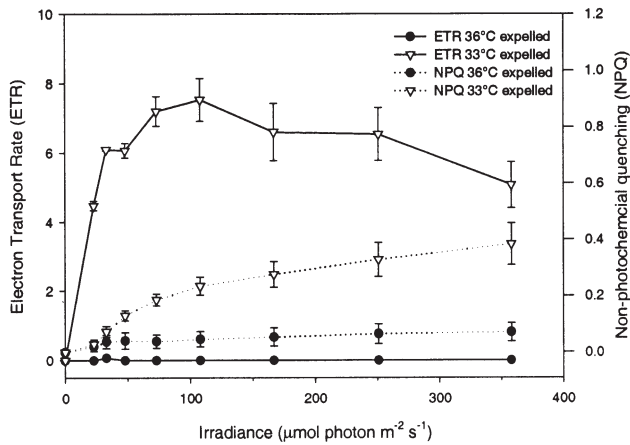


Fig. 1. Series of rapid light curves (RLC) of expelled zooxanthellae from specimens of *Cyphastrea serailia* incubated at 33 and 36°C for 3 h under 150 μmol photons m<sup>-2</sup> s<sup>-1</sup> (mean ± SEM, n = 3). Units of ETR are μmol electron m<sup>-2</sup> s<sup>-1</sup>. Units of NPQ are arbitrary

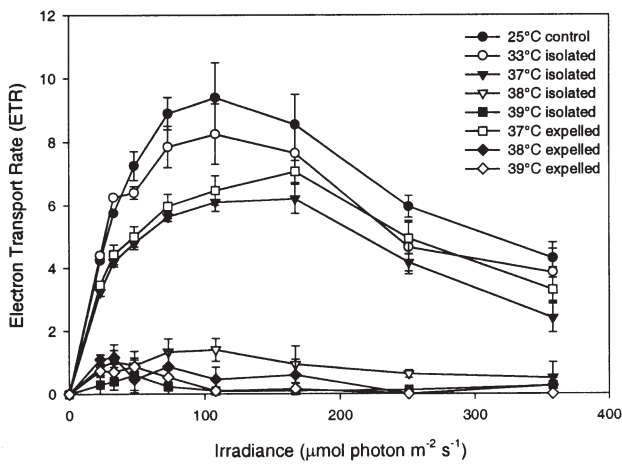


Fig. 2. Series of RLC of expelled zooxanthellae from specimens of *Cyphastrea serailia* incubated at 33°C for 3 h under 150 μmol photons m<sup>-2</sup> s<sup>-1</sup> (mean ± SEM, n = 3), and then exposed to 37, 38 and 39°C on a thermal stage for 5 min before measuring RLC. RLC of the isolated samples were performed after 5 min exposure to 25, 33, 37, 38 and 39°C. Units of ETR is μmol electrons m<sup>-2</sup> s<sup>-1</sup>

(Fig. 2), indicating that the process of *in hospite* thermal exposure does not influence the photosynthetic activity of the zooxanthellae. While it might be argued that isolated zooxanthellae, however carefully isolated, might show some damage, the ETR is relatively high by any standard, and numerous studies in the past have used isolated zooxanthellae to study physiological processes (Iglesias-Prieto et al. 1992). Thus, we conclude that the photosynthetic rate and maximum quantum yield of photosynthesis of zooxanthellae *in hospite* at 33°C were not inhibited.

When specimens of *Cyphastrea serailia* were raised to a temperature of 36°C they also began to bleach and expel zooxanthellae. However, in strong contrast to the situation at 33°C, the expelled zooxanthellae showed very low ETR (Fig. 1). Another means of assessing functionality in photosynthetic systems is to study non-photochemical quenching (NPQ). This mechanism, which involves certain xanthophylls and is known to occur in hermatypic corals (Brown et al. 1999), diverts excess energy to heat rather than to the photochemical processes. When zooxanthellae, either *in hospite* or in isolation, are exposed to elevated irradiance at elevated temperatures, non-photochemical quenching is the main process for energy dissipation of excess absorbed photons. As shown in Fig. 1, NPQ was not functional in zooxanthellae expelled from the 36°C-treated corals. Thus, the loss of NPQ along with the flat ETR response indicated that photosynthesis was completely inactivated at 36°C and that the photosystems were probably damaged (Fig. 1). In strong contrast, the NPQ of the 33°C-expelled zooxanthellae was normal (Fig. 1) and was similar to values for 25 and 33°C-isolated zooxanthellae (Fig. 3): both 33°C expelled and isolated zooxanthellae had NPQ of approximately 0.4 at 360 μmol photons m<sup>-2</sup> s<sup>-1</sup>, which is approximately a 40% dissipation of absorbed light energy as heat. Thus, our findings agree with many others (Berry & Bjorkman 1980, Iglesias-Prieto et al. 1992, Iglesias-Prieto 1997) that exposing coral to an elevated temperature of 36°C results in dysfunctional photosynthesis in zooxanthellae, either *in hospite* or when expelled. However, zooxanthellae that were expelled at 33°C were not dysfunctional.

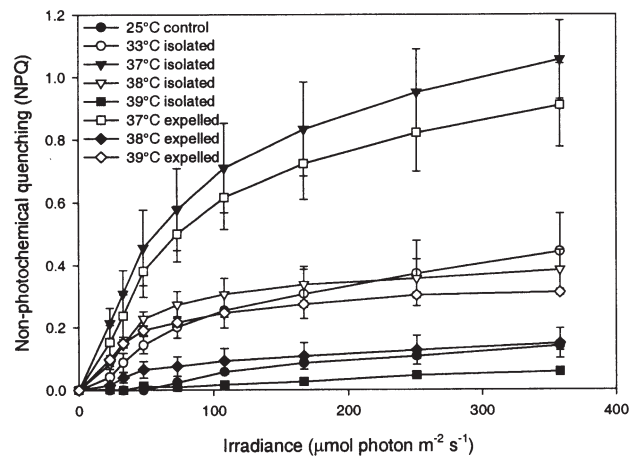


Fig. 3. Series of RLC of expelled and isolated zooxanthellae from specimens of *Cyphastrea serailia* as described in Fig. 2 (mean ± SEM, n = 3). Units of non-photochemical quenching are arbitrary

An important question therefore, was at what temperature do zooxanthellae expelled at 33°C show dysfunction as a result of elevated temperature. As shown in Fig. 2, these zooxanthellae were able to maintain normal photosynthetic functions up to 37°C. The effect of elevated temperature is dose-dependent, and therefore the greater the exposure the greater the photosynthetic damage. Both expelled and isolated zooxanthellae (Fig. 2) show a dramatic loss of photosynthetic activity ( $ETR$ ) between 37 and 38°C. This is in agreement with results of Thebud & Santarius (1982), who demonstrated that spinach leaves and protoplasts lost up to 80% of photosynthesis when temperature was increased from 37 to 38°C. The NPQ response curves for both

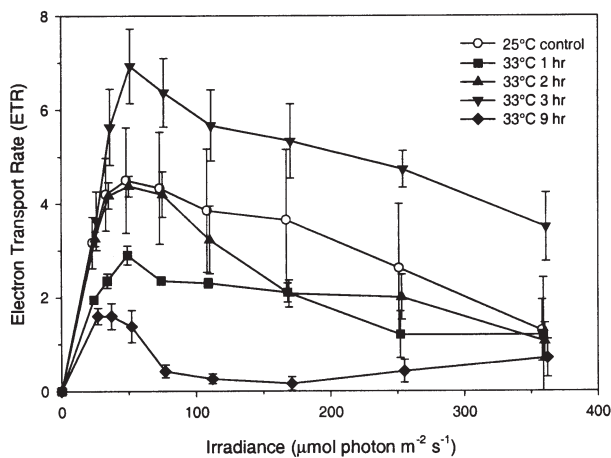


Fig. 4. Series of expelled zooxanthellae from *Cyphastrea serailia* exposed for 1, 2, 3, and 9 h (mean  $\pm$  SEM,  $n = 5$ ). Units of  $ETR$  are  $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$ .

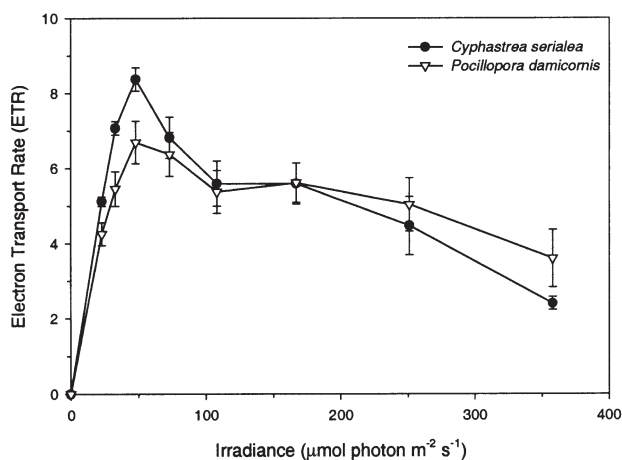


Fig. 5. Comparison of 33°C-expelled zooxanthellae from *Cyphastrea serialea* and *Pocillopora damicornis* after 3 h under 150  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (mean  $\pm$  SEM,  $n = 5$ ). Units of  $ETR$  are  $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$ .

expelled and isolated zooxanthellae (Fig. 3) indicated that dynamic adjustment of NPQ, in response to increasing irradiance, was operational up to 37°C. The pre-conditioning of expelled specimens to 33°C (to initiate expulsion) did not alter the overall photoprotection mechanism of the zooxanthellae. The  $ETR_{\text{max}}$  of the expelled and isolated zooxanthellae at 37°C were not significantly different ( $7.1 \pm 0.73$  and  $6.6 \pm 0.21$  respectively, Student's  $t$ -test  $p = 0.321$ ). The zooxanthellae maintained at 37°C showed some symptoms of photosynthetic stress, with an elevated NPQ response at higher irradiances (Berry & Bjorkman 1980, Hoegh-Guldberg & Jones 1999).

The effect of thermal bleaching is dose-dependent, whereby the period of exposure to 33°C governs the density and condition of expelled zooxanthellae. At room temperature (25°C), few zooxanthellae (60 cells  $\text{ml}^{-1}$ ) were naturally released, and the overall condition was highly variable, as indicated by the larger error bars for the 25°C treatment (Fig. 4). The population of zooxanthellae first expelled at 33°C (1 h exposure) showed a lower overall  $ETR_{\text{max}}$  (ca 3  $\mu\text{mol electron m}^{-2} \text{s}^{-1}$ ). However, this increased progressively with increased exposure period, up to 6 h: at 2 h exposure to 33°C, the average  $ETR_{\text{max}}$  for the population of expelled zooxanthellae had increased to 4.5, and after 3 h exposure the  $ETR_{\text{max}}$  was 7  $\mu\text{mol electron m}^{-2} \text{s}^{-1}$ . Beyond this time, the rates fell and, after 9 h exposure to 33°C, the  $ETR$  of the expelled zooxanthellae had declined dramatically (Fig. 4).

The majority of the experimental data reported here was based on the coral *Cyphastrea serailia*. However, to demonstrate the general applicability of these outcomes to other species of coral, we compared rapid light curves (Ralph et al. 1998, White & Chritchley 1999) for expelled zooxanthellae, induced at 33°C, for *C. serailia* and *Pocillopora damicornis*. The results for both species were very similar (Fig. 5), supporting a common thermal bleaching response for at least these 2 species.

Iglesias-Prieto et al. (1992) obtained results that are not entirely consistent with those presented here. Cultures of *Symbiodinium microadriaticum* (one of the dinoflagellate species which form zooxanthellae) were exposed to temperatures above 30°C for 45 min. At temperatures above 32 to 33°C, photosynthetic efficiency was reduced in a similar manner to *in hospite* zooxanthellae, and complete dysfunction resulted at 36°C, although the cells were not dead as respiration continued. It is these responses that have led many authors to speculate that it is the thermal impact on the algae not the host that is responsible for the breakdown of the symbiotic relationship during bleaching. Clearly those results are not consistent with the demonstration here that expelled zooxanthellae from

33°C-treated corals were photosynthetically competent up to 37°C. This raises the possibility that the expelled zooxanthellae were not truly representative. We have carried out an extensive series of tests on 30 coral specimens. In each of these we examined at least 3 representative expelled zooxanthellae. In no instance did we find a single zooxanthella with strong inhibition of photosynthetic activity or lack of NPQ. We must therefore conclude that the expelled zooxanthellae (at 33°C) showed photosynthetic competency. While it is possible that there is a sub-set of zooxanthellae that are not expelled and that are photosynthetically inhibited, there is no evidence for this at present. Furthermore, the expelled zooxanthellae in our experiments must have survived the treatment at 33°C. Thus, a discrepancy remains and could be the result of species differences (the *S. microadriaticum* of Iglesias-Prieto et al. 1992 were obtained from the jellyfish *Cassiopeia xamachana*) or associated with the use of a different type of fluorescence technique.

We conclude that the upper thermal limit for photosynthesis for *Cyphastrea serailia* zooxanthellae is around 38°C, not 32 to 34°C as might have been concluded previously. These observations are consistent with previous results on the thermal tolerance of photosynthetic systems, and in particular of PS II (Thebud & Santarius 1982, Yamane et al. 1998). Photosystem II has been a particular focus of attention in coral bleaching studies (Warner et al. 1996, 1999) and it has been suggested that dysfunction of this photosystem, and in particular damage to D1 protein, is the trigger for expulsion of these damaged zooxanthellae. However, as indicated by the present results, any explanations of the process of expulsion of zooxanthellae must take into consideration the fact that the photosynthetic apparatus of the expelled zooxanthellae is not permanently damaged. It therefore seems unlikely that the small temperature rise that induces coral bleaching involves a simple effect on any photosynthetic mechanism. It seems much more likely that the effect involves an interaction of the zooxanthellae and the host tissue and that this leads to a dysfunction.

Under saturating or extreme light, zooxanthellae can become carbon-limited, whereby the zooxanthellae would have used all internal inorganic carbon supplies and would depend upon ambient CO<sub>2</sub> and bicarbonate in seawater, the supply of which is limited due to membrane transport and solubility. It has been suggested that thermal dissociation of the symbiosis could be a result of CO<sub>2</sub> limitation (Yonge & Nicholls 1931); this is also supported by the fact that zooxanthellae have an unusual form of RUBisco with a lower affinity for CO<sub>2</sub> than O<sub>2</sub>, an especially important point since the bleaching host cells would be hyperoxic (Berry & Bjorkman 1980) and CO<sub>2</sub> is less soluble in warmer

water. Inorganic carbon supply would be limited under bleaching conditions, whereas expelling the zooxanthellae from the host into seawater would alleviate this sink limitation stress, which could explain the apparent lack of damage to expelled zooxanthellae. Alternatively, dysfunction of other exchange mechanisms between symbiont and host could disturb the physiological balance and lead to expulsion of zooxanthellae.

Various proposals that have been put forward in the past are consistent with the current facts. Jones et al. (1998) described carbon sink limitation under supra-optimal irradiances, whereby the over-reduction of electron transport results in singlet oxygen and other reduced oxygen intermediates that damage PSII. This could result from a temperature-induced dysfunction of the process of carbon exchange between symbiont and host. Lesser (1996) has also proposed that oxygen-free radicals produced as a result of heat treatment are a primary trigger for the expulsion. These proposals are not inconsistent with our observations, but need further substantiation. It is also possible that there is a dosage effect and that longer times at temperatures below 37°C would cause permanent damage to the expelled zooxanthellae. However, this does not alter the conclusion from the present results that under our conditions of mild bleaching, the expelled zooxanthellae are photosynthetically competent. Thus, while the present results shed little light on the mechanism of expulsion of zooxanthellae, they suggest strongly that the early events that must be involved in triggering this mechanism involve a dysfunction in the interaction of the zooxanthellae and the coral host tissue and not just a dysfunction in the zooxanthellae per se.

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