Zooxanthellae loss as a bioassay for assessing stress in corals

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ABSTRACT: Branch tips from colonies of a staghorn coral *Acropora formosa* were exposed to elevated concentrations of copper in order to examine the potential of the 'bleaching' response as a means of assessing stress in corals. Two identical toxicity tests were conducted over 48 h using 2 colonies with naturally different algal (zooxanthellae) densities. The tips from both colonies bleached in proportion to the copper concentrations, through loss of zooxanthellae to the surrounding water. A higher incidence of mortality was observed in tips from the colony with fewer zooxanthellae. During the tests, control corals lost zooxanthellae at a rate of <1 % of the algal standing stock d^{-1}, zooxanthellae release from both control and experimental corals occurred 2× faster during the daytime than night time, and the number of zooxanthellae appearing as doublets (the mitotic index) was 5 to 8× higher in zooxanthellae released from the corals' tips than inside the tips (*in hospite*) at the end of the experiment. The implications of these findings for future toxicity tests using the bleaching response, and for using changes in the number of zooxanthellae appearing as doublets to assess stress are discussed. Results suggest (1) that loss of zooxanthellae can be used within the framework of a standardised laboratory-based bioassay with universal integrity for assessing stress in zooxanthellate corals, and (2) that inherent differences in the densities of zooxanthellae between coral colonies are likely to affect the outcome of experiments using the bleaching response and of other biochemical or physiological stress responses.

KEY WORDS: Bleaching · Copper · Stress assessment · Zooxanthellae · *Acropora formosa*

INTRODUCTION

In a review of stress assessment in hard corals, Brown & Howard (1985) conclude '...there is little convincing evidence in the literature reviewed so far of a sensitive laboratory bioassay to any pollutant tested...'. If a suitable technique is available, then it can provide (1) water quality criteria for reefal areas (e.g. for heavy metals), (2) an initial assessment or 'screening' of the toxicity of potential toxicants which may be introduced to reefal waters (e.g. anti-foulants, oil dispersants, drilling fluids and produce formation water from the oil and gas industry), and (3) matrices of hard data useful for reef management strategies and to underpin decision making processes.

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Coral bleaching has been suggested as a potential physiological response which can be used to assess stress (Brown 1988). Corals gain most of their colouration from the photosynthetic pigments of the symbiotic algae (zooxanthellae) in their tissues. The term 'bleaching' has been used to describe the change of colouration as the white skeleton becomes visible through the relatively transparent animal tissues. This may occur following a decrease in the number of algae in the tissues (Yonge & Nicholls 1931, Hoegh-Guldberg & Smith 1989a), a decrease in the photosynthetic pigments of the algae (Hoegh-Guldberg & Smith 1989a), or both (Kleppel et al. 1989, Porter et al. 1989). Corals may also discolour if the tissues become strongly retracted exposing the naked coral skeleton (Brown et al. 1994). Such bleaching may occur without any loss of photosynthetic pigments from the coral's tissues.

Bleaching (as a decrease in photosynthetic pigments in the tissues) is an ecologically significant response,

resulting in a loss of photosynthetic potential (Porter et al. 1989), a cessation or reduction of growth (Jokiel & Coles 1977, Porter et al. 1989, Goreau & MacFarlane 1990), and a decrease in reproductive output (Szmant & Gassman 1990).

Bleaching has been observed in response to a diverse range of stresses, see Brown & Howard (1985) for a review. In more recent studies loss of zooxanthellae has been documented in corals exposed to elevated concentrations of iron (Harland & Brown 1989), lowered seawater temperatures (Muscatine et al. 1991, Gates et al. 1992), ultraviolet (UV) light (Gleason & Wellington 1993), terpenoids (secondary metabolites of soft corals, Aceret et al. 1995) and cyanide (author's unpubl. results).

Bleaching is usually considered to be the initial response to a stress that may eventually kill a coral. There are numerous observations of recovery of pigmentation by bleached corals (Yonge & Nicholls 1931, Coles & Jokiel 1978, Hayes & Bush 1990, Szmant & Gassman 1990, Fitt et al. 1993). Consequently, bleaching may provide a tool for grading the response of corals to natural and un-natural stress in a range between 'no effect' and 'lethal'.

In the present study the bleaching response is evaluated as a means of assessing stress in corals within the framework of a standardised laboratory-based bioassay. The heavy metal copper was chosen for the tests as it is a common marine pollutant. Some inputs to reefal environments include mining and smelting operations (Harland & Brown 1989, Denton & Heitz 1990), coastal run-off (Guzmán & Jiménez 1992), sacrificial anodes on boats (McConchie & Harriott 1992), and the marine disposal of municipal solid waste incinerator ash (Knap et al. 1991). Small branch tips of a staghorn coral *Acropora formosa* were exposed to elevated copper concentrations and the nature, processes and patterns of bleaching examined. Two identical toxicity tests were conducted using tips from neighbouring colonies containing different zooxanthellae densities (Jones 1995) to examine intraspecific variability of bleaching. The number of zooxanthellae appearing as doublets (the zooxanthellae mitotic index, MI) is also examined as a potential means of assessing stress.

MATERIALS AND METHODS

Experiments were conducted using 2 colonies of a staghorn coral *Acropora formosa* (Dana 1846) located at 5 to 6 m depth on the inshore fringing reefs of Nelly Bay, Magnetic Island, Australia (146° 51' E, 19° 10' S). Colonies were separated by a distance of ~4 m. The terminal 40 to 50 mm portion of 35 brown-tipped branches (see Oliver 1984) were cut from each of the colonies using surgical bone forceps. The basal 2 to 5 mm of each tip was wrapped with a thin layer of modelling clay. Tips were then inserted into small acrylic cylinders, and slotted into acrylic racks next to the parent colonies for a 14 to 20 d recovery period. Experiments commenced when the tips had begun to deposit a small 1 to 2 mm 'skirt' of lightly calcified coenosarc tissue around the modelling clay.

Five branch tips from the pool of prepared tips from each colony were exposed to copper concentrations of 5, 10, 20, 40 or 80 µg l^{-1} for 48 h in individual 150 ml glass beakers. Both experiments (Tests 1 and 2) were started at 12:00 h. In a subsequent experiment (Test 3) branch tips from 1 of the colonies were exposed to 5 µg l^{-1} copper for 96 h. This experiment was started at 18:00 h. Three separate controls were used during the toxicity tests. In the first control, referred to as the parent colony control (PC), 5 brown-tipped branches tips were excised from each of the parent colonies at 3 to 5 wk intervals for 6 mo after the tests. In the second control, referred to as the handling controls (HC), 5 brown-tipped branches were randomly selected from the pool of prepared coral tips immediately before each experiment. In the third control, referred to as the test control (TC), 5 brown-tipped branches were exposed to ambient seawater only during the tests. Algal densities were determined in each of the separate controls.

Seawater used for making test solutions was collected from the reef each morning and filtered through Whatman GF/C filters. Required test concentrations were made by adding a stock solution of Cu_2SO_4 in distilled water. 120 ml of either copper or control solution was placed in individual acid washed glass beakers which were then placed inside a water bath receiving a continuous supply of seawater pumped from the reef. Each beaker was supplied with air from a network of aquarium pumps. A 3 mm thick sheet of plexiglass (cut off ~400 nm, Lesser & Shick 1989) was placed above the beakers to protect against UV light, and to provide cover against rainfall. A double layer of 80% (absorption) shade cloth was used to reduce light levels experienced by the corals (Fig. 1). Light levels during the tests were recorded with a Lambda Li-Cor 190 SA underwater sensor mounted above the test containers. Mean light intensity over 10 min intervals was recorded onto a Li-Cor data logger (Fig. 1). Water temperatures were measured with a thermometer immersed within the water bath and compared to seawater temperatures on the reef slope (5 m) measured by *in situ* waterproof platinum RTD thermocouple sensors (accuracy ± 0.1°C) and recorded onto data loggers (Stobart 1994). Water temperatures during both 48 h toxicity tests ($\bar{x}$ = 24.6°C during Test 1, 23.0°C during
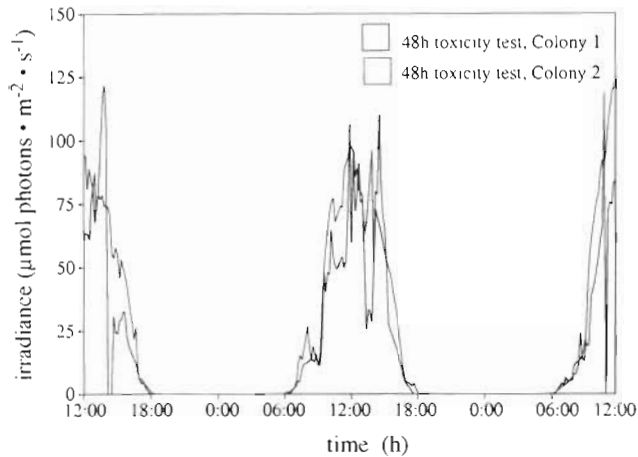
Fig. 1 Mean irradiance (μmol photons m^{-2} s^{-1}) during 48 h toxicity tests

Test 2) were similar to *in situ* temperatures on the reef slope at Magnetic Island ($\bar{x}$ = 24.9°C during Test 1, 22.3°C during Test 2, Stobart 1994).

At the start of each experiment the prepared coral tips were randomly allocated to the beakers containing the test solutions. Every 6 h the incubation water in each beaker was renewed. During this process 2 to 3 ml of the incubation water was squirted against the coral holder to resuspend any released zooxanthellae that may have settled. The coral was then transferred to a 500 ml 'holding' beaker containing clean unfiltered aerated seawater Approximately 20 to 30 ml of the incubation water was then squirted against the sides of the incubation beaker to remove zooxanthellae that may have adhered to the glass surface. The incubation water (containing any released zooxanthellae) was poured into containers and fixed with 5 ml of 40% buffered formalin. A fresh test solution was then placed in each incubation beaker and the coral tip transferred back.

At the end of the second 48 h toxicity test (Test 2), 3 randomly selected coral tips from each test concentration were transferred back to the reef and placed in the acrylic racks beside the parent colonies. The tips were examined for colour and appearance after 1, 2, 3, 6 and 10 wk. All other tips were frozen.

Zooxanthellae released from the corals during Test 2 were concentrated by centrifuging. The containers holding the fixed incubation water were shaken vigorously, and the incubation water was then homogenised for 15 s with a blender. A 100 ml aliquot was centrifuged at 3000 × g for 15 min and the supernatant discarded, leaving an algal pellet. The algal pellet was then re-suspended in a 2% formalin solution and the number of zooxanthellae counted with a hemacytometer (10 replicate counts) The number of zooxanthel-

lae released during the incubation was determined after correcting for the volume centrifuged and the addition of formalin.

Rates of release of zooxanthellae from the coral tips were analysed over 6, 12 and 24 h periods (see 'Results'). When rates of release were examined over 12 and 24 h periods, algal pellets produced after centrifuging the incubation water from successive 6 h incubations were pooled and then counted. Rates of release over different time periods were normalised to a 24 h basis, and then expressed as zooxanthellae released polyp^{-1}. The zooxanthellae specific expulsion rate (μ_x) was calculated using the equation described in Hoegh-Guldberg et al. (1987).

Tissues of the corals were removed from the skeleton with a jet of re-circulated 0.45 μm membrane-filtered seawater (FSW) using a WaterPik™ (Johannes & Wiebe 1970). Two separate washes of 60 to 80 ml FSW were used on each tip, and 20 ml was used to rinse the coral skeleton, WaterPik™ reservoir, and flush the WaterPik™ pump after each wash. Successive washes produced from the stripping and cleaning processes were pooled, homogenised for 10 to 20 s, and the volume recorded. The number of zooxanthellae in each of two 10 ml subsamples of the homogenate was determined from 10 replicate counts using a hemacytometer. An average of the 2 subsamples was used to determine the total zooxanthellae in the tips. Zooxanthellae were normalised to the number of polyps, recorded by visual census of calices on the branch (Muscatine et al. 1991).

The number of zooxanthellae appearing as doublets (mitotic index, MI) in the tips (*in hospite*) at the end of the first 48 h toxicity test (Test 1), and the zooxanthellae released into the incubation water during the last 6 h incubation period of the test, was determined from each of 2 samples of 1000 algae. Zooxanthellae were examined at 400× magnification and scored as dividing if they were present as doublets. The zooxanthellae specific growth rate (μ_z) in the control tips at the end of the test was calculated using the equation outlined in Wilkerson et al. (1983).

For chlorophyll analyses, two 10 to 20 ml subsamples of the tissue homogenate were filtered through Whatman GF/C glass fibre filters. Each filter was then cut into small pieces with dissecting scissors and homogenised for 30 s using a tissue homogeniser. Chlorophylls were extracted from the filters with 10 ml of 90% acetone for 24 h in a freezer. The extracts were then centrifuged at 3000 × g for 3 min to remove filter fibres from suspension, and the absorbances of the supernatant solutions were read on a Hitachi UB2000 spectrophotometer. The equations of Jeffrey & Humphrey (1975) were used to calculate the weight of chlorophyll a (hereafter chl a) and an average of the 2

subsamples used to provide a final estimate of the total chl *a* in each tip.

The ability of the tissue stripping process to remove the coral tissues from the skeleton (hence extract the zooxanthellae) was determined with a number of bleached and unbleached coral tips. After the tissues had been stripped the coral skeletons were digested in 2 to 3 ml of 5% HCl and the digest centrifuged at 3000 × *g* for 5 min leaving a small algal pellet (representing zooxanthellae still remaining in the skeleton after tissue stripping). The supernatant was then discarded, the algal pellet re-suspended in 0.45 µm FSW, and the process repeated until the pH of the solution approximated 8. Zooxanthellae were then counted with a hemacytometer (10 replicate counts), and compared to the algal densities stripped from the skeleton with the WaterPik™.

Data are presented as means ± 95% confidence intervals (CI). Type I ANOVA ($\alpha = 0.05$, JMP 1994) was used to examine treatment effects. Dunnett's test of significance was used to compare the nature of significant differences by comparing treatment and control means. Prior to all analysis assumptions of normality (Shapiro-Wilks test) and homogeneity of variance (Welch's test) were tested. MI data were first subjected to arcsine transformation before statistical analysis.

RESULTS

Toxicity tests conducted over 48 h

Branch tips of *Acropora formosa* from Test 1 exposed to 80 µg l^{-1} copper turned from a normal brown colour to a lighter colour within 6 h, and to a light tan colour by 24 h. Tips exposed to 40 µg l^{-1} copper began to discolour after 24 h. Between 24 and 36 h, all tips exposed to 80 µg l^{-1} copper died, and 4 of the 5 tips in the 40 µg l^{-1} treatment died. In Test 2 (conducted with tips from a separate neighbouring colony), the tips also bleached at the highest (80 µg l^{-1}) copper concentration. However, discolouration appeared to be less extensive, and no mortalities were observed at any test concentration.

Colour loss was uniform across the tips during the bleaching process. However, in some corals exposed to 10 and 20 µg l^{-1} copper preferential loss of colour was observed from corallites, giving the corals a speckled appearance. In the 40 and 80 µg l^{-1} treatments zooxanthellae were observed settled on the modelling clay surrounding the bases of the coral tips. Coral polyps were partially contracted within the corallites during the tests and only the ends of the tentacles could be observed protruding from the calices. Polyps responded to tactile stimuli during the bleaching process and fully retracted when touched with a pipette tip.
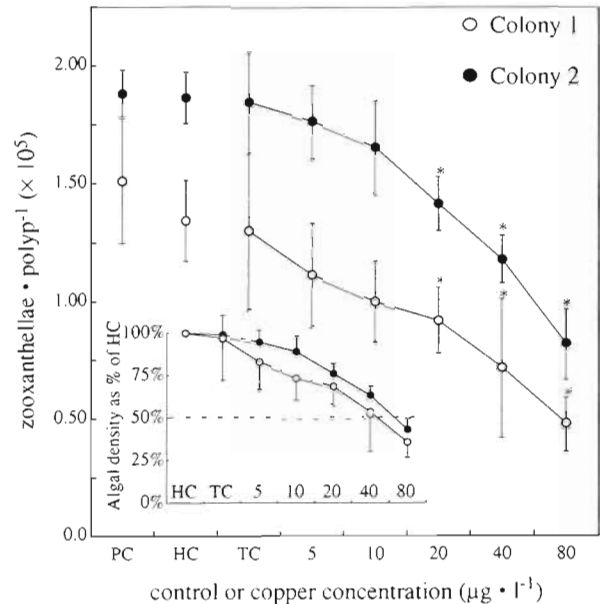

Fig. 2. *Acropora formosa*. Zooxanthellae density at the end of the 48 h toxicity tests, expressed as $\bar{x}$ zooxanthellae polyp^{-1} ± 95% confidence intervals, CI (n = 5 tips). HC: handling control, TC: test control, PC: parent colony (see 'Materials and methods' for explanation). For the first copper toxicity test (Test 1) (open circles) the zooxanthellae density at 80 µg l^{-1} represents those present after 30 to 36 h at which point the corals died. *Significant differences from the HC (ANOVA, $p < 0.05$). Inset, zooxanthellae densities in each treatment concentration expressed as a % of the densities in the respective HC

The tissue stripping process with the WaterPik™ removed 98.3 ± 0.5% ($\bar{x}$ ± 95% CI, n = 18 branch tips) of the available zooxanthellae from both bleached and unbleached branch tips.

The zooxanthellae density in the first colony (PC, 1.5 ± 0.3 × 10^5 zooxanthellae polyp^{-1}, $\bar{x}$ ± 95% CI, n = 6 sampling times) was significantly different from that in the second colony (1.9 ± 0.1 × 10^5 zooxanthellae polyp^{-1}, Student's *t*-test, $p < 0.05$, Fig. 2). There were no significant differences in the algal densities among the PC, HC and TC for each colony during the tests (ANOVA, $p > 0.05$, Fig. 2). In both experiments, algal densities in tips exposed to 20, 40 and 80 µg l^{-1} copper were significantly different from the HC (ANOVA, $p < 0.05$, Fig. 2).

There were no significant differences in the algal chl *a* concentration between treatments in the experiment with the first colony (Fig. 3). In the experiment with the second colony, the algal chl *a* concentration was significantly different from the HC in the 40 and 80 µg l^{-1} treatments (ANOVA, $p < 0.05$, Fig. 3, lower graph).

Corals returned to the reef after the second toxicity test regained their colouration within 6 wk, except 2
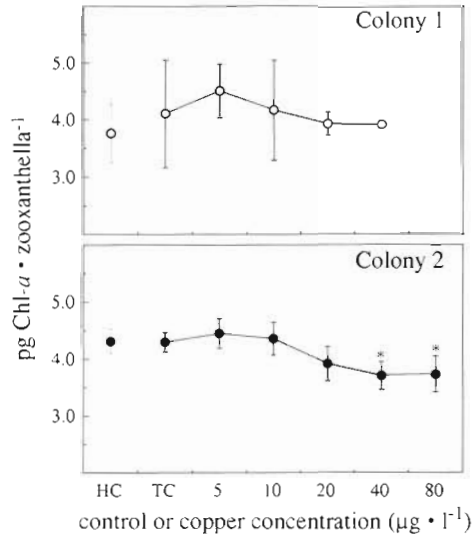
Fig. 3. *Acropora formosa*. Algal chl *a* concentration (pg chl *a* zooxanthella^{-1}, $\bar{x}$ ± 95% CI, n = 5 tips) in the first colony (upper graph; Test 1) and second colony (lower graph; Test 2) at the end of the 48 h toxicity tests. Due to mortalities during the first copper toxicity test (see text) no data is available for the 80 µg l^{-1} copper treatment, and data from only 1 coral is available for the 40 µg l^{-1} treatment. *Significant difference from TC (ANOVA, p < 0.05)

of the tips exposed to the 40 µg l^{-1} solutions which still appeared lighter than the parent colony after 10 wk.

The rate of release of zooxanthellae from the TC averaged 4.1 ± 3.6 × 10^2 cells polyp^{-1} 24 h^{-1} ($\bar{x}$ ± 95% CI, n = 5), similar to rates of release from tips exposed to 5 µg l^{-1} copper (Fig. 4). Rates of release of zooxanthellae were significantly different than from the TC in corals exposed to 20 and 40 µg l^{-1} copper (ANOVA, p < 0.05, Fig. 4).

The rate at which zooxanthellae were released from the corals (μ_x) exposed to ambient seawater (TC) was 40% of the rate at which zooxanthellae were added to the population from algal division (μ_z).

The rate of release of zooxanthellae was examined over 6 h intervals during the last 24 h of Test 2. In all treatments the rate of release of zooxanthellae fell during the first dark period (18:00 to 0:00 h), fell again during the second dark period (0:00 to 06:00 h), and then increased markedly during the morning 6 h light period (06:00 to 12:00 h, Fig. 5).

There were no significant differences between the MI of zooxanthellae in the control corals (TC) and those in the copper treatments (ANOVA, p > 0.05, Fig. 6). The MI of zooxanthellae released from the corals during the last 6 h incubation period was 5 to 8× higher than the MI of zooxanthellae *in hospite* at the end of the experiment.
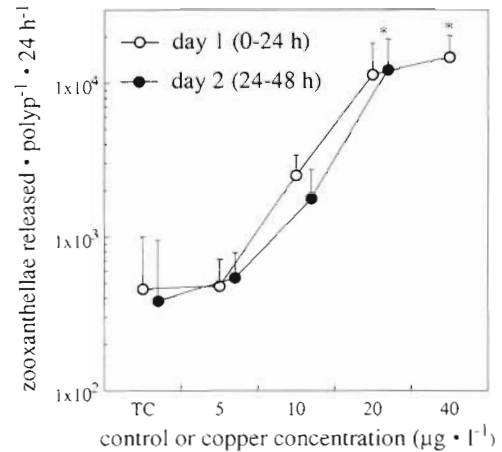

Fig. 4. *Acropora formosa*. Rate of release of zooxanthellae from coral tips over 24 h periods, expressed as $\bar{x}$ cells released polyp^{-1} 24 h^{-1} ± 95% CI (n = 5 tips). Note log scale on *y*-axis. *Significant difference from TC control (ANOVA, p < 0.05)
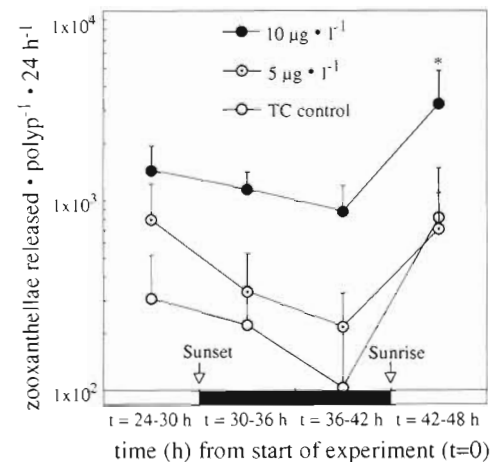

Fig. 5. *Acropora formosa*. Rate of release of zooxanthellae over 6 h periods during the last 24 h of Test 2, expressed as $\bar{x}$ cells released polyp^{-1} 24 h^{-1} ± 95% CI (n = 5 tips). Note log scale on *y*-axis. *Significant difference from TC control (ANOVA, p < 0.05). Each 6 h period occurred either in the light or dark, the first period lasting from 12:00 to 18:00 h, etc. Black bar indicates hours of darkness

Toxicity test conducted over 96 h

There was no significant difference in the colour of the corals during the experiment (Test 3), or in the algal densities between treatments at the end of the experiment [PC = 1.5 ± 0.3 × 10^5 zooxanthellae polyp^{-1} ($\bar{x}$ ± 95% CI, n = 6 sampling times), HC = 1.3 ± 0.2 × 10^5 zooxanthellae polyp^{-1}, TC = 1.1 ± 0.2 zooxanthellae polyp^{-1}, 5 µg l^{-1} = 1.3 ± 0.2 zooxanthellae polyp^{-1}, ANOVA, p > 0.05]. Polyps appeared partially expanded during the experiment and contracted when touched with a pipette tip.
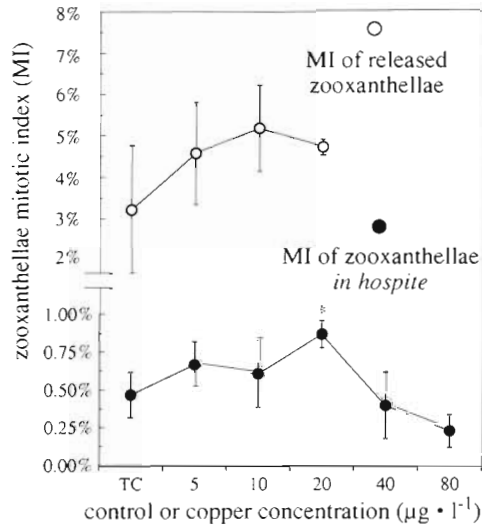
Fig. 6. *Acropora formosa.* Mitotic index (MI) of zooxanthellae remaining in coral tissues at the end of the 48 h toxicity test, and of zooxanthellae released from the corals in the last 6 h of the test. Each value represents the $\bar{x}$ cells released polyp^{-1} 24 h^{-1} ± 95% CI (n = 5 tips). *Significant difference from TC (ANOVA, p < 0.05)

Fig. 7 *Acropora formosa.* Rate of release of zooxanthellae over 12 h periods during the 96 h toxicity test, expressed as $\bar{x}$ cells released polyp^{-1} 24 h^{-1} ± 95% CI, n = 6 tips. Note log scale on primary y-axis. Each 12 h period occurred exclusively in the light or the dark, the first dark period lasting from 18:00 to 06:00 h, etc. Secondary y-axis shows irradiance levels (μmol photon m^{-2} s^{-1}) during the toxicity test

The mean daily rate of release of zooxanthellae from corals exposed to 5 μg l^{-1} copper ($3.4 \pm 1.5 \times 10^3$ cells polyp^{-1} 24 h^{-1}, $\bar{x} \pm 95\%$ CI, n = 6) was significantly different from the rate of release from control corals ($0.9 \pm 0.3 \times 10^3$ cells polyp^{-1} 24 h^{-1}, $\bar{x} \pm 95\%$ CI, n = 6, ANOVA, p < 0.05, Fig. 7). In the control corals the rate of release of zooxanthellae during the daytime periods was 2× higher than during the night time periods. In corals exposed to 5 μg l^{-1} copper, a high initial release of zooxanthellae was observed in the first dark period ($7.5 \pm 5.0 \times 10^3$ cells polyp^{-1} 24 h^{-1}); however, there was a gradual return to a day/night rhythm of algal release as the experiment progressed (Fig. 7). Algal release rates were also analysed over 6 h intervals during the daytime periods of the 96 h test.

DISCUSSION

Branch tips of a staghorn coral *Acropora formosa* bleached (discoloured) during exposure to elevated concentrations of the heavy metal copper. The discolouration occurred through loss of zooxanthellae to the surrounding water, although decreases in algal chl a concentrations were measured in one of the tests in corals exposed to the higher copper concentrations. Bleached tips returned to the reef after the experiment gradually recovered their colouration, suggesting the loss of zooxanthellae observed during the studies was a sublethal response.
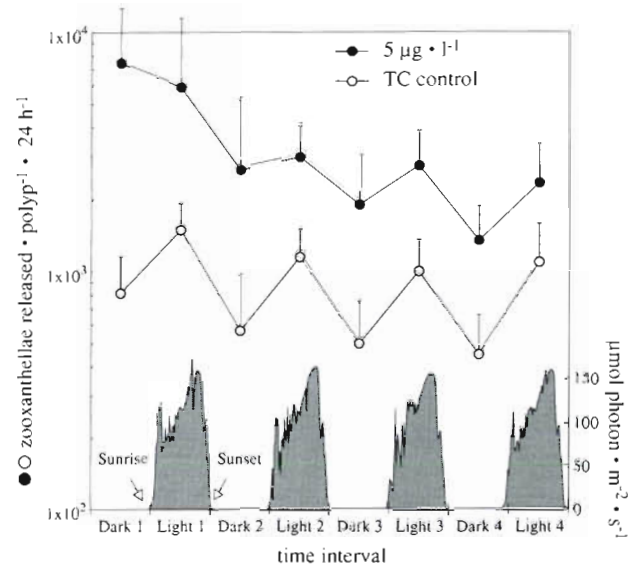
Bleaching of corals following exposure to heavy metals has been described before. *Pocillopora damicornis* and *Montiporra verrucosa* discoloured during exposure to 10 μg l^{-1} copper, and died during exposure to 100 μg l^{-1} copper (Evans 1977). A visual gradation of colour loss has also been observed in *M. verrucosa* following exposure to copper concentrations ranging from 10 to 50 μg l^{-1} (Howard et al. 1986). Decreases in algal density have been measured in a colony of *Porites lutea* exposed to elevated iron concentrations (Harland & Brown 1989); the algal pigment concentration was not measured during that study.

The zooxanthellae released from the corals appeared morphologically intact, with no evidence of vacuolisation or disintegration of organelles. A significant proportion of the zooxanthellae in the expelled population were in the process of dividing (see below), also suggesting the zooxanthellae were functionally intact when released. Occasionally, mis-shapen red-brown objects 3 to 4 μm in diameter were observed being expelled with the healthy zooxanthellae. These have been interpreted as degenerate (pycnotic) zooxanthellae (Yonge & Nicholls 1931, Trench 1974). Due to inherent variability in the presence and number of pycnotic cells, degenerate zooxanthellae were not counted.

Zooxanthellae were released from control corals at a rate of less than 1% of the algal standing stock d^{-1}. Similar algal release rates have been described for

other corals: 0.1% of the algal standing stock d^{-1} for *Stylophora pistillata* in the Red Sea (Hoegh-Guldberg et al. 1987), and *S. pistillata* and *Seriatopora hystrix* from the GBR (Hoegh-Guldberg & Smith 1989a), and ~1% and >1% for *Pocillopora damicornis* from Hawaii (Muscatine et al. 1991, Stimson & Kinzie 1991). The rate at which zooxanthellae were released from the coral tips (μ_x) was less than the rate at which cells were added to the populations by algal division (μ_z). Zooxanthellae produced from algal division in excess of those released from the coral may either be taken up by newly formed endodermal cells (i.e. from radial growth or growth at the base of the coral tips), or used to balance the loss of pycnotic zooxanthellae. The low level loss of zooxanthellae may represent the release of excess or 'supernumerary' zooxanthellae. Alternatively, zooxanthellae may be released as a result of necrosis, apoptosis (programmed cell death), or adhesion dysfunction of algal bearing host endodermal cells (Gates et al. 1992, Jones & Yellowlees 1996).

The release of zooxanthellae from control corals occurred 2× faster during the daytime than night time (Fig. 7). Diel patterns of zooxanthellae release, also with twice as many zooxanthellae released during the daytime than night time, have been reported for colonies of *Pocillopora damicornis* in Hawaii (Stimson & Kinzie 1991). It is unlikely that the diel patterns of algal release observed in these studies are related to contraction and expansion patterns of the polyps, as they remained partially contracted throughout the experiments. One possibility is that the diel release pattern is associated with diel patterns of algal division. Pre-dawn peaks in algal division frequencies have been observed in *Stylophora pistillata*, *P. damicornis* (Hoegh-Guldberg & Smith 1989b), *Montastrea annularis* (Fitt et al. 1993) and *Acropora formosa* (Jones 1995). Dividing zooxanthellae may be separating into daughter cells during the light period and released.

Assessing changes in the zooxanthellae division frequency (measured by the number of cells appearing as doublets, MI) has been suggested as a potential means of assessing stress in corals (Brown 1988). The number of doublet cells *in hospite* was not significantly different between control and test solutions (Fig. 6). However, the number of cells appearing as doublets was 5 to 8× higher in the expelled population than in the tips at the end of the experiment (Fig. 6). A higher algal MI has also been observed in zooxanthellae expelled from sea anemones (*Anemonia viridis*, Zamani 1991, Suharsono & Brown 1992; *Anthopleura elegantissima*, McCloskey et al. 1996).

If the algal MI increases markedly when the zooxanthellae are free from the host endodermal cells, then

this introduces a considerable problem with the tissue stripping technique: using a WaterPik™ to remove the tissues from a coral skeleton will produce an homogenate including both intracellular zooxanthellae, and extracellular zooxanthellae still within the coelenteron awaiting release (Suharsono & Brown 1992). Corals in the process of losing zooxanthellae will have higher ratios of extracellular (more doublets cells) to intracellular zooxanthellae which may increase the MI of the algal population determined from an homogenate of the coral tissues. For example, in *Anemonia viridis* exposed to elevated water temperatures, the MI of zooxanthellae determined from slide preparations of tissues (i.e. a measurement of intracellular zooxanthellae division frequencies) was significantly lower than the MI of zooxanthellae determined from an homogenate technique (Suharsono & Brown 1992).

Using the number of cells appearing as doublets to assess stress in corals using homogenate techniques clearly has a number of experimental and methodological problems. The algal MI is also higher in corals which have lost zooxanthellae following an external stress (Hoegh-Guldberg & Smith 1989b, Fitt et al. 1993, Jones 1995) Thus both increases and decreases in the algal MI can occur during, and/or after an external stress, producing a generally obscured picture of the stress response.

Branch tips from the colony with lower algal densities (Test 1) discoloured to a greater extent during the 48 h toxicity tests, consistent with them having fewer zooxanthellae. However, a number of the branch tips exposed to 40 µg l^{-1} copper died, and all tips exposed to 80 µg l^{-1} copper died. No mortalities were observed during the toxicity test with the tips from the other colony (Test 2). Zooxanthellae have been suggested as sites for metal uptake in cnidarians (Buddemeier et al. 1981). Copper, cadmium and zinc accumulated in the zooxanthellae of the symbiotic anemone *Anemonia viridis* at levels 2 to 2.5× higher than those in the body of the anemone as a whole (Harland & Nganro 1990, Harland et al. 1990). Zooxanthellae expulsion has also been suggested as a mechanism by which *A. viridis* could excrete metals and regulate metal concentrations (Harland & Nganro 1990).

No measurements were made of the uptake and partitioning of copper during the toxicity tests described here. However, if metals preferentially accumulate in zooxanthellae, then corals with inherently higher algal densities may be more tolerant of elevated metal concentrations, or have a greater capacity for metal regulation through algal release. Interestingly, Harland & Brown (1989) have observed that colonies of *Porites lutea* collected from a reef-flat adjacent to a tin smelter and ore dressing plant in Thailand had higher algal

densities than did control colonies collected away from any localised metal enrichment. Following exposure to elevated iron concentrations under laboratory conditions, the colonies with naturally higher densities did not lose zooxanthellae and had higher overall body burdens of iron. The results were interpreted as an example of acquired metal tolerance in colonies adjacent to the ore dressing plant. Differences in algal densities in colonies of *Acropora formosa* at Magnetic Island have been attributed to differences in the size of the algal symbionts between colonies (Jones 1995, Jones & Yellowlees in press). Inherent differences in the algal densities between coral colonies, which may not necessarily be related to prior exposure to elevated metal levels, may therefore give the impression of an acquired metal tolerance.

During the present study, the effect of copper was only examined on 2 colonies (of 1 species), and consequently it is too early to recommend water quality criteria based upon the loss of zooxanthellae. However, the study has indicated that measuring loss of zooxanthellae from corals has considerable potential as a means of assessing stress. Significant effects were detected at copper concentrations of 5 ppb, a level similar to those reported in polluted tropical marine environments (Harland & Brown 1989). The sensitivity of the bioassay compares favourably with other coelenterate bioassays, e.g. hydroid bioassays (Stebbing & Brown 1984), where significant changes in reproductive output, enzyme activity and growth rates have been detected at copper concentrations of 0.1 to >10 ppb. The technique can be used under controlled laboratory conditions and loss of zooxanthellae can be quantified using simple techniques. Day/night patterns of zooxanthellae loss observed during these studies indicate that starting times, and light levels, during future toxicity tests should be standardised. Intraspecific differences in algal densities between neighbouring colonies are likely to affect the outcome of experiments using loss of zooxanthellae to assess stress (or indeed any other physiological or biochemical responses) and must be adequately controlled for.

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