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Aquat. Living Resour. 15 (2002) 107–117

Aquatic Living Resources

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Effect of increased irradiance and thermal stress on the symbiosis of *Symbiodinium microadriaticum* and *Tridacna gigas*

Bela Hieronymus Buck [a,*], Harald Rosenthal [b], Ulrich Saint-Paul [a]

[a] *Center for Tropical Marine Ecology, Fahrenheitsstraße 6, 28359 Bremen, Germany*
[b] *Institute for Marine Science, University of Kiel, Düsternbrooker Weg 20, 24105 Kiel, Germany*

Received 15 January 2001; accepted 28 November 2001

Abstract

The impetus for this study was the mass bleaching event of giant clams in 1997–1998 at several reefs in the Great Barrier Reef (GBR, Australia). From September until December 1999, the study investigated the effects of high light intensities and increased temperature accompanied by a nutrient limitation on the metabolism of *Tridacna gigas*, to test, if these environmental changes can induce bleaching. In a 50-day trial similar conditions of the mass bleaching event were imitated stressing clams, collected from Orpheus Island and Nelly Bay, by a two- to threefold light intensity and an increased temperature of 4–6 °C. The objectives of the experiments were to determine whether high light intensities can induce changes in chlorophyll content or alter zooxanthella cell sizes and populations in the tissue. After 50 days of exposure to high light intensity the mantle tissue of stressed clams exhibited a decreased number of zooxanthellae per unit area from 19.8 ± 0.8 ($\times 10^7 \cdot cm^{-2}$) to 0.2 ± 0.2 ($\times 10^7 \cdot cm^{-2}$) (mean ± CL). Additionally, the average cell size of zooxanthellae were downsized from 7.4 ± 0.1 µm to 5.3 ± 0.1 µm (mean ± CL). Subsequently, the chlorophyll content of both, chl *a* and chl c_1, declined as well, chl *a* from 192 ± 4 to 0.1 ± 0.1 µg·ml^{-1} and chl c_1 from 145 ± 6 to 0 µg·ml^{-1} (mean ± CL). This study shows that increased light intensity and temperature are the main causes for bleaching in giant clams. Thus, the study confirmed the four major aspects involved in bleaching: (1) loss of symbiotic algae, (2) decrease of chl a/c_1 in the remaining symbiotic algae, (3) retention of small zooxanthellae in the tissue and (4) release of ammonium (NH_4^+) into the water column while nutrient uptake of ammonium was largely blocked.

Résumé

Effet d'une irradiance croissante et d'un stress thermique sur la symbiose de *Symbiodinium microadriaticum* et *Tridacna gigas*. Cette étude a été lancée, suite au phénomène de blanchiment en masse de bénitiers, en 1997-1998, sur plusieurs récifs de la Grande Barrière (GBR, Australie). De septembre à décembre 1999, nous avons étudié les effets de fortes intensités lumineuses et de températures croissantes, accompagnés par une limite en nutriments, sur le métabolisme de *Tridacna gigas*, pour tester si des changements environnementaux peuvent induire le blanchiment. Dans une expérimentation de 50 jours, des conditions similaires du phénomène de blanchiment ont été imitées et appliquées aux mollusques, récoltés de l'île Orpheus à Nelly Bay, par 2-3 d'intensité lumineuse et une température plus élevée de 4-6 °C. L'objectif de ces expériences était de déterminer si de fortes intensités lumineuses peuvent induire des changements en chlorophylle ou altérérer la taille et les populations des cellules de zooxanthelles dans les tissus. Après 50 jours d'exposition à des intensités lumineuses élevées, les tissus du manteau ont montré un nombre décroissant de zooxanthelles par unité de surface de $19{,}8 \pm 0{,}8$ ($\times 10^7 \cdot cm^{-2}$) à $0{,}2 \pm 0{,}2$ ($\times 10^7 \cdot cm^{-2}$) (moyenne ± LC). De plus, la taille moyenne des cellules de zooxanthelles s'est réduite de $7{,}4 \pm 0{,}1$ µm à $5{,}3 \pm 0{,}1$ µm (moyenne ± LC). Par la suite, les contenus en chlorophylle, chl *a* et chl c_1, diminuèrent également, la chl *a* de 192 ± 4 à $0{,}1 \pm 0{,}1$ µg·ml^{-1} et la chl c_1 de 145 ± 6 à 0 µg·ml^{-1} (moyenne ± LC). Cette étude montre que l'augmentation de l'intensité lumineuse et de la température sont les causes principales du blanchiment chez les bénitiers. Elle confirme les quatre aspects majeurs impliqués dans le blanchiment : (1) perte de l'algue symbiotique, (2) diminution du rapport chl a/c_1 dans l'algue symbiotique restante, (3) rétention de petites zooxanthelles dans les tissus et (4) dégagement d'ammonium (NH_4^+) dans la colonne d'eau, tandis que l'absorption d'ammonium est largement bloquée.

Keywords: Symbiosis; Bleaching; Ammonium assimilation; Chlorophyll; Zooxanthellae; *Tridacna gigas*

* Corresponding author.
E-mail address: buck@zmt.uni-bremen.de (B.H. Buck).

PII: S0990-7440(02)01159-2

1. Introduction

Giant clams of the family Tridacnidae are familiar and conspicuous residents of shallow coral reefs throughout most regions of the tropical Indo-Pacific (Rosewater, 1965). Like corals and anemones, these clams live in symbiosis with microalgae, such as *Symbiodinium microadriaticum* (Dinophyceae), commonly called 'zooxanthellae'. These zooxanthellae live extracellularly in a branched tubular system originating from the stomach which splits into small and very thin secondary and tertiary tubes dorsally into the root of the siphonal mantle. The tertiary tubes are directly under the surface of the mantle tissue (Norton et al., 1992; Norton and Jones, 1992), allowing sufficient light to penetrate for algal photosynthesis. The symbionts are capable of transferring parts of their photosynthetic products to the clam, such as glucose and glycerol (Muscatine, 1967; Streamer et al., 1988; Fitt, 1993). Although filtration is the common origin of nutrition of bivalves, most giant clams, such as *Tridacna gigas*, obtain their major nutritional source through their symbiotic partners (Klumpp et al., 1992). Thus the availability of light seems to be a critical factor affecting the growth of giant clams (Lucas et al., 1989).

Environmental changes, such as increasing temperature, higher irradiance, changes in salinity and pollution (Brown, 1997), can cause the loss of the symbiont (Hoegh-Guldberg and Smith, 1989; Kleppel et al., 1989), which provokes the whitening of the clam's mantle tissue (Estacion and Braley, 1988). Either the giant clams or the zooxanthellae end the symbiosis in a process known as bleaching.

At present, there is great concern about the environmental integrity and future of coral reefs and their inhabitants, as the frequency and magnitude of mass bleaching events have increased drastically since the El Niño event in 1982–1983. Just three mass bleaching events have ever been reported until 1979, but 60 major coral bleaching events occurred between 1979 and 1990 (Glynn, 1993; Grice, 1999).

Late in 1997 to February 1998 a substantial portion of corals within the Great Barrier Reef (GBR) in Australia were bleached (Berkelmans and Oliver, 1999). Extensive bleaching of cultured clams and wild stocks in the central region of the GBR was observed at White Lady Bay, Pioneer Bay and Nelly Bay on Magnetic Island, at Kelso Reef, and at James Cook University Orpheus Island Research Station (Grice, 1999). More than 8 000 *Tridacna gigas* out of a total of 9 000 individuals were bleached (Leggat, pers. comm.). Different parts of the clams' mantle had various kinds of whitening, some had a complete loss of symbionts in all parts of the mantle (Grice, 1999), other clams were only affected in the central part of the mantle between the siphonal orifices or at the margins of the mantle tissue, as was observed personally from clams at Nelly Bay.

This mass bleaching accompanied with high sea surface temperatures (SST; GBRMPA, 1998) was the first case where bleaching of clams had occurred on such a grand scale (Braley, pers. comm.).

This research covers various aspects of experimentally induced bleaching through altering environmental factors, such as light intensity and temperature, and by monitoring the progression of bleaching in specific individuals under varying exposure conditions. Furthermore, the study investigates the nature of the symbiosis between *T. gigas* and *S. microadriaticum* in relation to chlorophyll a/c_1 contents as well as the size and the density of the algal cells within the mantle tissue. Therefore, the population of zooxanthellae living in bleached giant clams and the loss of those in faeces pellets in the progress of the bleaching process are studied. Additionally, ammonium concentrations (NH_4^+) in the haemolymph and in the water column surrounding the clams was determined in order to identify its uptake or release.

2. Materials and methods

2.1. Origin of experimental animals

The giant clams used in this study originated from Orpheus Island (OI; 146° 30' E, 18° 37' S) and Nelly Bay/Magnetic Island (NB; 146° 51' E, 19° 09' S). Animals used had a shell length between 50 to 67 cm (57.1 ± 5 cm; mean ± SD) and were taken from a minimum water depth of 2 m. Clams from this shallow water habitat around Magnetic Island were adapted to lower light intensities due to moderate currents, which caused a suspension of the fine sediments and a high sedimenation load, respectively. Clams from shaded reef areas were preferred to assure that no previous exposure to high sunlight could have caused bleaching and that the clams were adapted to lower light intensities. Water visibility was measured at all reefs taking Secchi-depth readings.

Twenty clams were lifted from the sea bed on board the vessel, carefully secured in containers and transported 'dry' to the laboratory where they were transferred to four outdoor tanks (240 cm × 120 cm × 60 cm; water volume 1 700 l each) at the Reef Headquarters of the Great Barrier Reef Marine Park Authority (GBRMPA) in Townsville/Australia. Clams originating from OI were placed in the Reef Aquarium of the Reef Headquarters (4.5 m deep) several months prior to the start of the experiment.

The clams were measured and weighed, thus allowing the determination of changes of these parameters during the bleaching experiment. The displacement volume of the species was calculated by using the formula of Braley (1992). All clams were kept in the prepared tanks (five per tank) for acclimatisation for 2 weeks before experimental use.

2.2. Experimental design and operation system

The tank system was designed to simulate stress conditions by varying light and temperature. Tanks were covered

with shadecloth to simulate initial reef conditions and supplied with natural seawater at 26.6 ± 0.7 °C (mean ± SD) (n = 70 at 09:00), obtained from the general circulation system of the Reef Aquarium. During the experiments all tanks were operated independent from each other while also being disconnected from the general circulation system. It was possible to connect tanks to the general circulation system of the Reef Aquarium, permitting flushing with new sea water (approx. 1 400 $l \cdot h^{-1}$) every 2 to 3 days. Two airlifts (compressed air) were used in each tank to support water circulation and to prevent thermal stratification. Protein skimmers were needed to separate dissolved and particulate organic matter, especially to capture the mucus which was released by stressed clams (Sander, 1998).

During the 50-day trial (October-December 1999), three of the tanks containing five clams each were exposed to conditions similar to those known to occur during the mass bleaching event in 1997–1998 with a two- to threefold increase in irradiance above ambient levels in combination with a temperature increase of 4–6 °C. This was achieved by removing the shadecloth. Clams adapted to lesser light intensities in their natural environment were now exposed to a relatively higher irradiance during the experiment. This change in light exposure pertained to the range of the Photosynthetic Active Radiation (PAR) as well as the component of UV-A and UV-B. The water temperature levels in tanks 1–3 were controlled simply by various methods of insolation (no heaters were required). The fourth tank remained shaded at all times and was used as control tank. The light intensity in the control tank corresponded with the ambient light regime in the reef environment of the clams (see below).

Growth of algae on tank walls and bottoms was regularly removed via a suction pipe. Every 2–3 days about 15% of the tank water volume was drained and the tanks were flushed with fresh seawater taken from the general circulation system of the institute. Detritus (e.g., dead algae and faeces) was also removed from the bottom of the tank on a regular basis. This was combined with a sampling of the zooxanthellae for later analysis. Before the clams were placed in the tanks the entire epifauna and epiflora was wiped away from the clam shells with a common brush. Extreme resident biofouling, such as Porifera or Polychaeta, was removed using hydrogen peroxide.

2.3. Determining environmental conditions

2.3.1. UV, light intensity and temperature

At reef sampling sites, from which clams were collected, and in experimental tanks UV and ambient light (PAR) conditions were measured. For PAR determinations, an ‘Underwater Quantum Sensor’ was used (LI.COR, UWQ 3485/3917; [$\mu E \cdot m^{-2} \cdot s^{-1} \approx mmol \cdot m^{-2} \cdot s^{-1}$]), which recorded the light intensity near the water surface and underwater at the level of the siphonal mantle of host animals (depth: 10 cm). For the UV measurement, a UV-Dosimeter (Eluv-14 Version: 2.007, ESYS GmbH [$mW \cdot m^{-2}$]) was deployed, which registered the UV radiation at the height of the mantle of test animals. Additionally, the UV-Dosimeter recorded the water temperature. On the reefs and in all experimental tanks continuous recordings of light conditions and water temperature were performed and lasted for 1 week. Temperatures were recorded three times daily at 08:00, 12:00, and 16:00 h in all experimental tanks (Optic-Base-Station, *Onset, TidbiT Data Logger*). Fouling on instruments was eliminated every second day.

The pH-value was determined on a daily basis. Oxygen was not determined but was assumed to be close to saturation because of the intensive aeration of the system at all times and the low stocking density.

2.3.2. Water quality analysis

Water samples were taken daily to determine nitrite, nitrate, ammonium and phosphate. Water samples were in general analysed immediately. If this was not possible 40 µl of saturated mercury chloride ($HgCl_2$) was added to the sample (to stabilise the nutrient concentration) and stored deep frozen. Samples were analysed based on methods given by Hansen and Koroleff (1999) using an autoanalyser (Lachat Instruments QuickChem 8 000 and Flow Injection Analysing System-FIAS with Scalar-Deluter and Matrix-Photometer). After the determination of the total-ammonia, the un-ionised ammonia fraction was calculated and subtracted to obtain the level of the dissolved ammonium ion (NH_4^+), which is used by algae in photosynthesis. Calculations were done by using the tables given by Trussel (1972) and Emerson et al. (1975).

2.4. Isolation and measurement of Zooxanthellae from Tridacna gigas

Zooxanthellae were extracted from clam tissue to estimate population size and determine cell sizes as well as chlorophyll a/c_1 content. Three tissue fragments of the mantle tissue of *T. gigas* were isolated from each specimen by using a biopsy punch (ϕ = 0.8 cm). Each subsample was homogenised in a test tube with 15 ml filtered seawater (0.45 µm Whatman) for 10 s using a blender (Black and Decker, Slender Blender). The homogenate was filtered through three layers of wet gauze cloth to remove the suspended algae cells from the clam tissue fragments. The suspension was centrifuged (1.5×10^3 rpm, 3 min) and washed with 10 ml filtered seawater at least three times until the supernatant was clear. The dark brown pellet with the Freshly Isolated Zooxanthellae (FIZ) was resuspended in filtered seawater, giving a final volume of 20 ml. The algal cell density was determined by diluting 2 ml of algal suspension with 8 ml of filtered seawater and counted under a microscope (magnification of 400) with four replicate subsamples using a haemocytometer (Neubauer, Marienfeld/Germany). Ten algae cells were randomly cho

sen and measured, in order to determine the size of the zooxanthellae. For the measurement, an objective-micrometer (Olympus 0.01 mm) was employed at a magnification of 1 000.

2.5. Analysis of chlorophyll a/c_1 content in zooxanthellae

In order to determine the concentration of chlorophyll a/c_1 a 10 ml subsample from the algal suspension was used. Some drops of magnesium carbonate ($MgCO_3$) were added to the rest of the suspension to avoid acidity of the filter surface (Parson et al., 1985) and filtered under vacuum pressure through a dried glassfibre-microfilter (Whatman GF/C 0.45 µm). These filters were then wrapped in foil and frozen in total darkness for later analysis. The filters were cut into small pieces and bathed in the dark with 10 ml acetone (90%) at 4 °C for 24 hours, then centrifuged (1 500 rpm, 8 min). The chlorophyll content was then measured at 630, 647, 663, 664, and 750 m (Parson and Strickland, 1963). Chlorophyll concentrations were then calculated according to the equation of Jeffrey and Humphrey (1975). Chlorophyll levels were expressed as µg·cm^{-1} and pg·cell.

2.6. Sampling and analysis of ammonium in the haemolymph

Samples of the haemolymph were taken to examine the concentration of ammonium (NH_4^+). The haemolymph was collected by the method described in Rees et al. (1993b). Samples were taken along the entire longitudinal axis by piercing a syringe (19 gauge, 2.75 inch) in the haemal sinus or into the haemal sack. The haemal sack extends most and thins out towards the shell endings, thus, the best place was the area between the incurrent and the excurrent siphonal orifice. After inserting the syringe carefully, approx. 15 ml of haemolymph were extracted. To ensure that the sample was not seawater, the syringe with its content was held against a black disc. In general, haemolymph fluid has a cloudy, light milky precipitation. The syringe was then closed, extracting all air, and analysed or frozen immediately. Before analysing the fresh or thawed haemolymph samples were centrifuged (1 500 rpm, 2 min) to remove cells and debris. Aliquots of the supernatant were analysed using a modification (Gravitz and Gleye, 1975; Wilkerson and Trench, 1986) of the method of Liddicoat et al. (1975), which uses phenol-hypochlorite to estimate the ammonia concentrations. Samples were then measured at an absorbance of 640 nm. Calculations of the concentration of the dissolved ammonium ion (NH_4^+) part was done as described previously.

2.7. Faeces pellets

Faeces of clams were investigated to test the presence of algal cells. Fresh faeces released at the day of sampling were siphoned (exhauster) once a week from all tank bottoms. They were dissolved in test tubes with 10 ml of filtered seawater. The suspension was centrifuged (1 500 rpm, 3 min) and washed at least three times with filtered seawater. Zooxanthellae counts and diameter measurements were performed using a haemocytometer.

In order to count the cells in the faeces, faecal pieces of approx. 5 cm length were taken each time. The 5-cm piece is considered as one unit to recalculate the number of cells per faeces unit (cells·unit^{-1}). This method was used to document the rate of zooxanthellae release without being able to access quantitatively the zooxanthellae population size in clams.

2.8. Data analysis

From all results using subsamples, the use of means was tested by an outlayer test from Nalimov (P = 95%) and/or Grubbs (P = 95%) (Kaiser and Gottschalk, 1972). To calculate the zooxanthellae population in the clams, three tissue fragments with each four subsamples were analysed. In that case calculating means of means were required. F-tests (one dimensional, f = n-1) were done to prove homogeneity of variance as a prerequisite for applying the t-test. For all data, standard deviations (SD), standard errors and confidence limits (CL; f = n-1, t-factor = 95%) were calculated and applied in graphs as appropriate; these are shown in bars. Significance levels were determined by using the student t-test ($P < 0.05$).

3. Results

Measurements from all clams (shell length, weight, volume and circumference) taken at the beginning and at the end of the bleaching experiment did not show any relevant level of variations.

3.1. Light intensity and temperature profiles

Photosynthetic Active Radiation (PAR) measured in open air showed the highest irradiance with 2 428 ± 18 µE·m^{-2}·s^{-1} (mean ± SD) around noon, followed by the profile from the clam holding tank (stress) with 2 400 ± 11 µE·m^{-2}·s^{-1} (Fig. 1). Because of the relatively low underwater depth positioning of the light sensors (10 cm = level of mantle tissue of test animals), both the profile measured in air and in the stress tanks, were similar. The light intensities in the Reef Aquarium (4.5 m depth) were generally lower (1 204 ± 5 µE m^{-2}·s^{-1}) due to shadowing effects of the tank walls and the shadecloth covering the Reef Aquarium. The lowest irradiance profile occurred in the holding tank (controls, 10 cm depth) and Nelly Bay observation site (4 m depth) reaching maxima of 1 112 ± 3 µE·m^{-2}·s^{-1} and 1 009 ± 7 µE·m^{-2}·s^{-1}, respectively. The low irradiance at

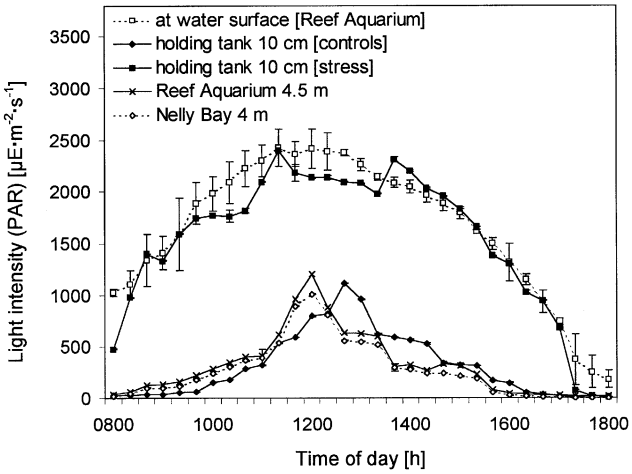

Fig. 1. Studying light effects (photosynthetic active radiation, PAR) on giant clams. Daily light cycle at site of origin where test animals were taken (Nelly Bay, depth 4 m; Reef Aquarium, depth 4.5 m) and in experimental tanks at 10 cm depth. The low depth in the holding tanks was due to the depth of the siphonal mantle of test animals. Daily cycles were taken at 5-min intervals on sunny days (Oct. 1999); means and standard deviations (vertical bars; $n = 3$).

Nelly Bay was the consequence of high turbidity occurring at the reef during the observation period.

UV light curves of all observation points at field stations, in bays and at reefs as well as in tanks showed a comparable profile to those of PAR: minima in the morning and late evening, maxima around midday. In the clam holding tanks (stress tanks) the highest underwater UV-light intensity occurred with 361 ± 3 mW·m^{-2} (mean ± SD) at noon, in the holding tank (control) of 126 ± 4 mW·m^{-2} around 14:00, in the Reef Aquarium of 119 ± 1 mW·m^{-2} around 14:00, and at Nelly Bay of 84 ± 1 mW·m^{-2} at noon. The low UV-light intensity in these locations is due to the depth of the measurement (Fig. 2).

The temperature at all sites showed a 24-h cycle with a maximum in the early afternoon (15:00), declining thereafter towards the night. Variations in temperatures at the

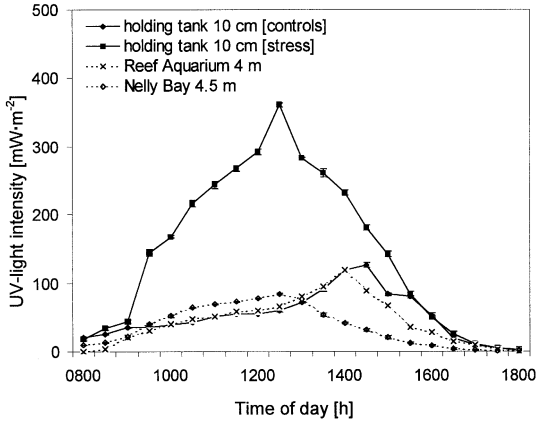

Fig. 2. Daily UV light exposure cycles at natural sites (Nelly Bay, depth 4 m; Reef Aquarium, depth 4.5 m) and in tank exposures (holding tanks, depth 10 cm). Daily cycles taken at 5-min intervals on sunny days (Oct. 1999).

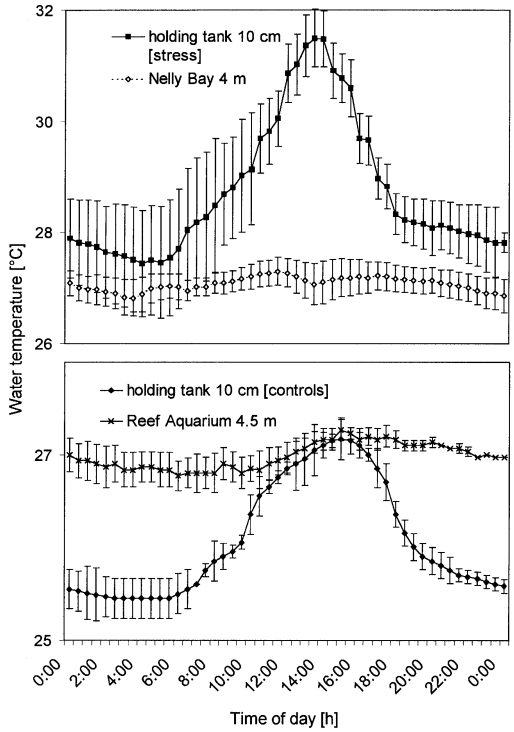

Fig. 3. Daily temperature cycles at natural sites (Nelly Bay, depth 4 m; Reef Aquarium, depth 4.5 m) and in tank exposures (holding tanks, depth 10 cm). Daily cycles taken at 5-min intervals on sunny days (Oct. 1999); means and standard deviations (vertical bars; $n = 3$).

assumed daily peak time of 15:00 did not show relevant differences for either field sites or experimental units (Nelly Bay: 27.3 ± 0.2 °C; mean ± SD; Reef Aquarium: 27.3 ± 0.1 °C; holding tank controls: 27.2 ± 0.2 °C) except the holding tanks (stress) with 31.5 ± 0.5 °C (Fig. 3).

3.3. Variation in nutrient concentrations

All analysed nutrients (nitrite, nitrate, ammonium, phosphate) did not vary greatly, except in the stress tank. Table 1 shows the ammonium concentrations in seawater surrounding the aposymbiotic clams which increased progressively during the experiment from 12.0 ± 0.6 µmol·l^{-1} (mean ± SD) to 37.0 ± 3.2 µmol·l^{-1}, representing a 36-fold increase over control tanks or ambient nutrient concentrations at coral reefs (< 1 µmol·l^{-1} Kelso Reef). The nutrient concentration in the seawater of control tanks remained stable during the entire duration of the experiment. The ammonium concentration in control tank water was between 0.5–1.0 µmol·l^{-1}.

The ammonium concentrations in the haemolymph of control clams and in bleached clams were slightly different at the end of the experiment: about 3.8 ± 0.9 µmol·l^{-1} in controls and 4.6 ± 1.1 µmol·l^{-1} in bleached clams (Table 1).

Although 15% of tank water exchanged every 2–3 days, total ammonia concentrations increased with time, except for week 6 (Table 1), when an unexplained decrease occurred, indicating that dilution during most of time was

Table 1
Weekly check on ammonium (NH_4^+) concentrations in the water surrounding the bleached clams and in the clams' haemolymph during the bleaching trial. Sampling time of (1) water: 10h00 ($\bar{x} \pm$ SD; $n = 3$) and (2) haemolymph: 10h00–11h00 ($\bar{x} \pm$ CL; $n = 3$)

Weeks	1	2	3	4	5	6	7	8
Haemolymph NH_4^+ ($\mu mol \cdot l^{-1}$)	3.7 ± 0.1	3.9 ± 0.3	3.9 ± 0.3	4.2 ± 0.4	4.5 ± 0.4	4.4 ± 0.5	4.5 ± 0.8	4.6 ± 1.1
Tank water NH_4^+ ($\mu mol \cdot l^{-1}$)	12.0 ± 0.6	15.4 ± 1.1	18.5 ± 1.4	22.0 ± 2.4	37.0 ± 3.7	19.2 ± 3.9	27.1 ± 4.8	31.2 ± 3.2

insignificant and other factors besides release from the clams may have (at interval) contributed to the overall increase in total ammonia concentrations.

3.4. Zooxanthellae population and densities

During the first experimental week there was no significant difference in zooxanthellae counts in the mantle tissue of clams kept under high light intensity and those under control conditions. Significant differences ($P < 0.001$) were detected however, 2 weeks after the beginning of exposure to high light intensity. At this time the clams started to lose the colour at the margins of their mantle tissue. Zooxanthellae counts from control clams were $19.8 \pm 0.8 \times 10^7$ cells·cm^{-2} (mean ± CL), while in exposed clams cell densities started to decline during the first 3 weeks to a level about 75% of those in controls, which were within their average cell range (ACR; see below). Thereafter, the decline was rapid and continuous, with $10^7 \cdot (0.2 \pm 0.2)$ cells·cm^{-2} left 8 weeks after the exposure started (Fig. 4).

3.5. Cell sizes

Initial average size of the zooxanthella cells of all giant clams from all origins sampled in this study was 7.4 ± 0.1 μm (mean ± CL, $n = 60$) with a range between 2.7 and 10.8 μm (Fig. 5). The cell size did not vary greatly in the control clams throughout the experiment. Stressed clams expelled cells and apparently released the bigger ones first (7–10 μm), reducing average values by about 5% for the first few weeks. Consequently, the proportion of small-size zooxanthella cells increased initially in these experiments as revealed from measurements in test samples of faeces with 8.4 ± 0.4 μm at week four. Thereafter, average cell sizes continued to decline gradually ($P < 0.05$ after 5 weeks until the end of the experiment). At the end of the bleaching experiment, all stressed clams showed within their mantle tissue a cell sizes variation of about 2.7–8 μm with an average size of 5.3 ± 0.2 μm.

The results have to be taken with some caution as it was not possible to determine the exact algal species composition. One of the cell types obviously belonged to the genus *Symbiodinium* and dominated. However, other species of dinoflagellates with green pigments or even microalgal species from different systematic categories may have been present, but could not be identified.

3.6. Discharged cells in faeces

The quantity of zooxanthella cells discharged in the faeces was exceptionally high at the end of the bleaching experiment. Stressed clams lost $10^5 \cdot (10.5 \pm 0.47)$ cells·unit^{-1} (mean ± SD) at the end of the experiment whereas control clams lost only $10^5 \cdot (4.7 \pm 0.33)$ cells·unit^{-1} (cells·unit$^{-1} \approx 5$ cm piece).

3.7. Chlorophyll content

Chlorophyll content in those algal cells remaining in the mantle tissue was affected by high light intensity, and this holds for both chl *a* and chl c_1. The chlorophyll concentrations declined with increasing light intensity ($P < 0.05$ for chl a/c_1, after 1 week until the end of experiment) (Fig. 6). At the beginning of the bleaching experiment, all clams exhibited an average chlorophyll content of about 192 ± 4 μg·ml^{-1} chl *a* (mean ± CL) and 145 ± 6 μg·ml^{-1} chl c_1. At

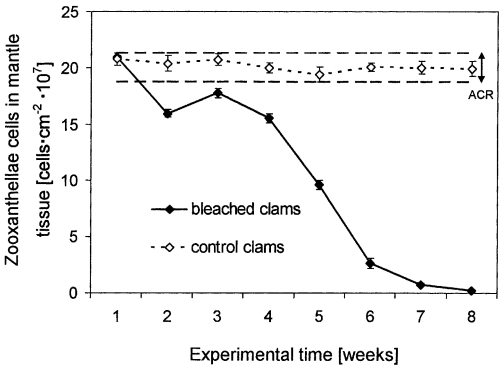

Fig. 4. Counts of zooxanthella cells in mantle tissue of giant clams during the bleaching experiment over a period of 8 weeks (means ± confidence limit [95%]); controls ($n = 20$) and bleached ($n = 60$). Hatched lines show the average cell range (ACR) in clam tissues. It shows the natural variation of the algae population according to environmental changes (see text).

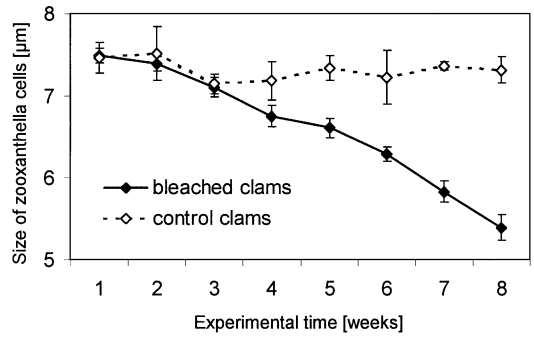

Fig. 5. Cell size of zooxanthellae in mantle tissue of giant clams during the experiment over a period of 8 weeks (means ± confidence limit [95%]); controls ($n = 20$) and bleached clams ($n = 60$).

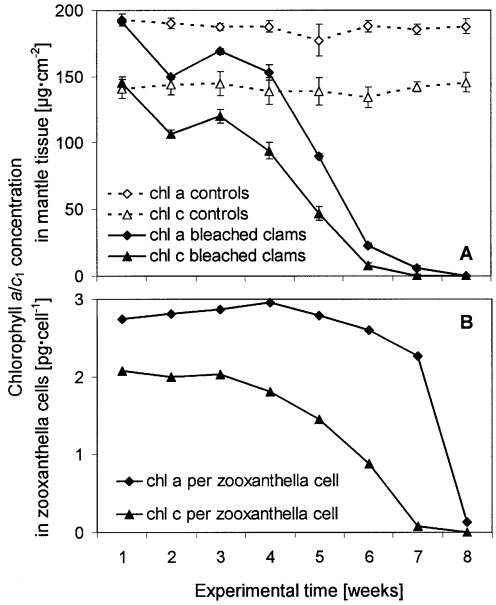

Fig. 6. Chlorophyll a/c_1 concentrations in zooxanthella cells inhabiting the mantle tissue of giant clams during the experiment over a period of 8 weeks. **(A)** shows the chl concentration per mantle area and **(B)** per zooxanthella cell; (means ± confidence limit [95%]); controls (n = 20) and bleached clams (n = 60).

the end of the bleaching experiment, chlorophyll concentrations were reduced to 0.1 ± 0.1 µg·ml^{-1} chl *a* and 0 µg·ml^{-1} chl c_1, respectively. In contrast, the chlorophyll contents in mantle tissues of control clams remained stable throughout the experiment.

By calculating the average chlorophyll content per zooxanthella cells of stressed clams, the concentration in both, chl *a* and chl c_1, declined during the experiment from 2.7 to 0.2 pg chl *a* and 2.1 to 0 pg chl c_1 (Fig. 6).

4. Discussion

This study presents some insight into factors affecting bleaching of *Tridacna gigas*. The process of bleaching in giant clams can be described as follows: (1) loss of most of the symbiotic algae; (2) decrease of chlorophyll content in the remaining symbiotic algae; (3) retention of small zooxanthellae in the tissue; and (4) release of ammonia into the water column by bleached clams while nutrient uptake of ammonia was largely blocked.

4.1. Environmental factors

Tridacna gigas has the ability to respond to a wide range of temporary changes in environmental factors, such as sea surface temperatures (SST), salinity, and light intensities. Exposure to extremes of these factors happened between January and February 1998 at the GBR, when temperatures increased from 22 °C to 32 °C within several weeks, accompanied by a heavy rainfall with 770 mm·day^{-1}, which lowered the salinity of the seawater to 18 (Yellowlees, pers. comm.). In the beginning, rain clouds prevented direct sun exposure, but when the sky cleared, high light intensities occurred, causing a mass bleaching event. Some clams apparently adapted to the situation while most of the clams died most likely because of nutrient limitations. High temperatures and high light intensities had already been seen to cause bleaching and mortality in juvenile *T. gigas* (Braley, 1992).

The light intensity profiles in the holding stress tank showed a higher irradiance in PAR and UV light. The radiant energy of PAR increased in these tanks by two times and of UV light by three times over ambient values, respectively. The overlapping of the curves derived from continuous recording measured in air and water as well as the sudden decrease around midday are due to clouds, which may have covered the light meter. The temperature increased in the early afternoon (15:00) by 1.5 degrees effected by solar radiation. However, this caused stress levels which exceeded the tolerance range of those clams adapted to low light intensities and temperatures around 27 °C. The clams expelled their symbiotic partners in response to this environmental change. The response of zooxanthellae to changes in irradiance and temperature in the present study concurs with the natural distribution of the symbionts in the mass bleaching event in 1998.

From our data it can be concluded that the combination of both high light intensities and elevated temperature was the primary cause of the bleaching of the siphonal mantle.

4.2. Zooxanthellae: population, sizes, and chlorophyll

This study clearly shows that increased temperature and high light intensities affect the population density and sizes of algal symbionts in *T. gigas*. The density of zooxanthella cells in the mantle tissue of stressed clams decreased over time to just 1% of the normal value (0.22·10^{7}cells·cm^{-2}). Ammonium-enriched water should result in a growing population of zooxanthellae in clam tissue (Grice and Bell, 1999; Belda-Baillie et al., 1998). Despite the high ammonium concentrations in the test tanks algal cells were expelled. The symbionts did not adapt to this short-term changes in their photic environment, whereby the host had to oscillate between autotrophy and heterotrophy, as already illustrated for several corals (Mingoa, 1988). The distribution pattern of zooxanthellae in the siphonal tissue suggests that populations of algae are also influenced by environmental conditions associated with a limitation in nutrient supply. Margins of the siphonal mantle were predominantly discoloured. These thinner mantle areas were most exposed to high light intensities and increased temperature which presumably caused the whitening.

A reduction of zooxanthella cells per mantle area between colour patterns on the siphonal mantle is only one option to explain the discoloration of the mantle. Another possibility is the decreasing concentration of chlorophyll *a*

and c_1 per zooxanthella cell. Furthermore, Hough (pers. comm.) observed more chlorophyll *a* located in algal cells of darker tissue colours. Similar results were reported in juveniles of *T. gigas* where the chlorophyll *a* concentration in shade-adapted clams was more than twice that of light-adapted ones (Mingoa, 1988). Braley (pers. comm.) suggested that this may be caused by accommodating different strains of algae. This may be caused by different abilities of pigments to effectively utilise light, suggesting that algal performance characteristics may determine species selection while the role of the clam is more passive, partly contradicting the observations made in this study. Light attenuation also occurs within animal tissue. In giant clams the thickness of the expanded mantle inhabiting the symbionts in dense populations increases with increasing animal size. Furthermore, while small clams reach maximum photosynthetic rates at one quarter of maximum sunlight intensities in air, algal cells in larger clams never reach those rates. Cells located lower in the tissue show a tendency towards shade behaviour (Ralph et al., 1999) which may be due to the extensive shading by those around them. Fisher et al. (1985) found 97% shading in 24-cm clams compared to 34% of 1-cm juveniles. In that case, cells must adapt to these different light and shade conditions within the clam and accordingly adjust their chlorophyll concentrations. Besides, Ralph et al. (1999) described the light absorbing properties of the mantle through its pigments or its iridophores (Griffiths et al., 1992). These pigments effectively filter much of the incident solar radiation which influences the symbionts to photosynthesise.

The loss of algal cells ceased 1 week after the start of exposure and remained low for the next 2 weeks. At present, we are not certain if changes in weather conditions were involved or if this behaviour is entirely due to the capability of some zooxanthellae strains (most likely the smaller ones) to undergo a short-term adaptation to be more resistant to stress factors. Several studies have been done to determine different types of zooxanthellae within corals (Rowan and Knowlton, 1995) or giant clams (e.g., see Baillie et al., 1998; Baillie et al., 2000). This diversity in zooxanthellae populations could result in an adaptation of the giant clam association to changes of environmental conditions, such as increased temperature or solar radiation (Belda-Baillie et al., 1999). The remaining cells in the mantle of the above-mentioned stressed clams could be a more resistant type of zooxanthellae.

It seems that the algal cell population within the mantle tissue of control clams undergoes permanent changes. The variation of zooxanthellae cells within the tissue of control clams is less than 7% and could be due to the location in the mantle where samples were taken, such as margins, or the existence and quantity of filtering pigments (Ralph et al., 1999). We suggest that these variations were apparently caused by the ambient weather conditions in Townsville and characterise the range within the algal population as average cell range (ACR).

Decreased chlorophyll contents in mantle tissue were mainly caused by the loss of zooxanthellae. The decline of the chlorophyll contents per zooxanthella cell may be due to the high irradiance during the bleaching trial. The proportion of both chlorophyll types remained constant up to the point were hardly any zooxanthella cells were left in the tissue.

The average sizes of cells within the tissue declined during the bleaching experiment. It was not possible to determine why the stressed clams favourably expelled the larger algal cells first. Exposure to high UV light or increased temperature can result in photoinhibition of photosynthesis in zooxanthella cells (Lesser and Shick, 1989). The continuous high absorption of energy combined with a reduced energy transport will damage or inactivate the photosystem 2 (PS 2) (Osmond, 1981). When absorption happens in the presence of oxygen, active oxygen radicals are produced (Asada and Takahashi, 1987). The elevated active oxygen can induce membrane changes in the thylakoids that further disrupt primary photochemistry (Kyle, 1987). The resulting oxidative stress can be proposed as a possible mechanism of inhibition of photosynthesis in zooxanthellae leading to bleaching in symbiotic invertebrates (Lesser and Shick, 1989; Lesser et al., 1990), such as the sea anemone *Anthopleura elegantissima* (Dykens and Shick, 1984). One possible reason, that stressed clams expelled the larger cells may be the higher amount of chlorophyll, which could produce a substantial amount of active oxygen radicals. Additionally, the PS 2 reaction centre is known to be directly vulnerable to UV-radiation (Renger, 1989) or high light (PAR) intensities (Powles, 1984). That could explain the loss of larger cells, but assumes that the zooxanthellae were expelled in a moribund state.

4.3. Ammonium concentrations

Rees et al. (1993a/b) revealed that the haemolymph of *T. gigas* appears to be the immediate source of DIN for zooxanthellae, which were able to assimilate ammonium for the synthesis of amino acids (Muscatine, 1980). The high ammonium concentrations in the water surrounding the aposymbiotic clams compared to the ammonium conditions in the water column of control clams indicated that the reduced zooxanthellae population within the aposymbiotic clams were responsible for the missing depletion of ammonium. Clams maintained in seawater containing elevated ammonium concentrations normally show an increase of ammonium in their haemolymph (Shepherd et al., 1999) and this influences the photobiology of the zooxanthellae (Ambariyanto and Hoegh-Guldberg, 1999). Because of the decreasing population of zooxanthellae within the mantle of stressed clams, little ammonium was required. This may explain the slightly increased ammonium concentration in the haemolymph. Symbiotic and partly symbiotic clams were unable to assimilate nutrients from the tank water. In

contrast these clams constantly released ammonium into the tank water.

A comparison of the total ammonia levels in the control tanks indicate that the concentration in the haemolymph was in osmotic balance with the seawater (Grice, 1999). Symbiotic clams which were maintained in the control tank did not release ammonium into the water.

4.4. Faeces

The zooxanthellae population released by the clams during bleaching was approximately 5×10^7 cells·cm$^{-2}_{\text{tissue}}$·week^{-1}, resulting at the end of the bleaching experiment in a discharge of approximately 1.1×10^6 cells per 5-cm faeces pellet^{-1}. This cell loss can be considered as relatively low (nearly 2.2% of cell release) compared to the high loss of cells out of the tissue. Belda et al. (1993) estimated specific growth rates of zooxanthellae in *T. gigas* in natural seawater of 0.04%·day^{-1}. Although the cells grow and divide daily under stress situations (Hough, pers. comm.), the majority of cells was not found in the faeces. Maruyama and Heslinga (1997) found approximately 10% of the zooxanthellae cells in the mantle of *T. derasa* to divide every day (an in situ doubling time was calculated to be 5.0–8.3 days). In small clams (shell length 5.6 cm) it was estimated that 4.9×10^5 cells were excreted in the faeces daily, reaching a level of up to 16% of newly formed cells in a clam. It was also observed by the same authors that the percentage of daily increased zooxanthellae in the mantle and discharged with the faeces was equivalent to 11–36% of the newly formed zooxanthellae population. Therefore, 64-89% of the newly formed zooxanthellae population were missing, which may be due to the stress conditions.

Some zooxanthellae might have been lost from the faeces by differentiation into 'swimming cells' with a flagellum. Fitt et al. (1981) observed that motile cells appear only for a limited time at the end of the light period in a light/dark cycle, and no motile zooxanthellae were observed during the dark period. This observation coincides with the sampling time of faeces in this experiment.

Another possibility why low counts of zooxanthellae cells occur in the faeces pellets may be the fact that the zooxanthella cells were digested by the host to compensate for a temporary lack of nutrient supply. Digestion of the cells by the host has been suggested by Fankboner (1971) and Yonge (1980). Trench et al. (1981) determined intact zooxanthella cells in the rectum of *T. derasa* in addition to cells in various stages of disorganisation. This may indicate at least partially the digestion of zooxanthellae in the clam. Fitt et al. (1986) observed that juvenile *Hippopus hippopus* ingested and partly retained zooxanthella cells, but no trace of their digestion was recognised. Nearly 76% of the ^{14}C-labelled cells were detected in the faeces. Maruyama and Heslinga (1997) described that zooxanthellae discharged in the faeces obviously pass intact the digestive tract, but nearly 80% of the newly formed algal cells are digested by the clam. All other defecated algae, which were photosynthetically functional, must have passed from the siphonal tissues through the digestive tract undamaged.

The mechanism by which the algae pass from the tubular system to the alimentary tract or stomach is unknown. It is unlikely that motile cells can move within the tubular system. Firstly, cells with a flagellum have never been seen in the tertiary system. Secondly, the tubes seem to be too narrow; there would not be enough space to move. Morton (1978) proposed that digested zooxanthellae are discharged from the amoebocytes in the visceral mass to the lumina of the diverticulum.

5. Conclusion

In 1998 bleached clam cohorts were observed from Nelly Bay, White Lady Bay, Kelso Reef, Pioneer Bay, and Orpheus Island (GBR) and these were due to environmental changes. Bleaching was never seen before on a large scale (Braley, pers. comm.) This bleaching, however, did not occur in isolated cohorts of clams living at particularly exposed habitats. Long-term effects of such occurrences are difficult to predict and there are presently not much data available for comparative analysis. The problem requires urgent attention by scientists and resource managers and the present study aims to contribute to the understanding of some of the processes involved.

The mass expulsion of zooxanthellae by corals seems to be an important constituent of nutrition for *Tridacna* (Fankboner and Reid, 1990). It may, therefore, be important that those corals surviving a mass bleaching event are situated near or among clams. Clams are more robust than corals and can stand several environmental stress factors much longer (Grice, 1999). Currently, it is unclear whether the clams are actively expelling the zooxanthellae in response to certain environmental signals or the zooxanthellae themselves are responding to these signals by terminating the symbiosis. Recently, Perez et al. (2001) supported the findings of previous studies that increased temperature causes the bleaching in a zooxanthellae-sea anemone association, but also reveals that some types of zooxanthellae can partly cope with this stress factor. Studies on corals by Fitt and Warner (1993) indicate a substantial resistance of some strains of zooxanthellae to high irradiance and increase in sea surface temperature.

Our results illustrate that the bleaching of clams can be induced by changing environmental conditions and endorse similar observations made by Estacion and Braley (1988). The algal species complement of *T. gigas* was altered by changes in light and temperature. The fact that only the combination of UV light and high temperature induced bleaching clearly shows the importance of these environmental changes in light of global warming. There are many large-scale events that may in general be linked to changes in performance of the species and certainly the declining

ozone layer and the increasing frequency of El Niño-Southern Oscillations (and La Niña effects) are among those that enter the debate (Glynn et al., 1988; Glynn and D'Croz, 1990; Hoegh-Guldberg et al., 1997; McClanahan et al., 2001).

Several suggestions on future research priorities can be inferred from the overall observations on bleaching and on the basis of the results of the present study. We would endorse the view of Belda-Baillie et al. (1999) that the diversity of symbionts living in giant clams needs to be elucidated to better understand the dynamics of the algae and how these symbionts adapt to a change of environmental conditions. The factors affecting reestablishment of the algal populations in giant clams and corals need more multifunctional studies and this subject should be of high priority in future research. Understanding the mechanisms that cause bleaching may allow the development of mitigation strategies, at least in culture, to help re-build healthy giant clam populations which might become severely damaged through bleaching. Consolidated knowledge will then also be applicable in the commercial giant clam mariculture to prevent the loss of juvenile clams otherwise harmed by events that may cause bleaching.

Acknowledgements

The authors are grateful to Paul Hough, Great Barrier Reef Marine Park Authority in Townsville/Australia, and to Prof. Dr. David Yellowlees and Dr. William Leggat, James Cook University in Townsville/Australia, for supplying equipment and facilities during the studies. We are also indebted to Dr. Richard Braley, Aquasearch on Magnetic Island/Australia, for providing clams and scientific advises. The ELUV Quantum Sensor used in this study was provided by Prof. Dr. Gunther Krause, Alfred Wegener Institute-Foundation of Polar and Marine Research in Bremerhaven/Germany.

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