Chemical and Biological Characteristics of Coral Reef Ecosystem at Microscale/Nanoscale: Effect of Multiple and Synergistic Stresses

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Beatriz E. Casareto, Toshiyuki Suzuki, and Yoshimi Suzuki

Abstract

Global environmental changes are recently occurring faster than any other time. The resulting combinations of natural and anthropogenic disturbances are strongly affecting coral reef communities. Coral damage can be caused by both abiotic factors (temperature, sedimentation, nutrients inputs, ultraviolet radiation) and biotic factors (predation, overgrowth of algae, infectious diseases). These factors acting mostly in synergy had resulted in worldwide coral reef deterioration. Coral bleaching is the most impacting process that is affecting coral survival under elevated sea surface temperature and high irradiance scenario. Bleaching is well known to occur around the world; however, its mechanism is not well understood. This is due to the high complexity of the "coral holobiont" (coral in symbiosis with its zooxanthellae and a microbial community that maintains a delicate balance to keep the coral health). In this chapter a novel point of view of bleaching mechanism using micro/nano-size scales is presented: (1) the study of pigment dynamics during thermal-induced bleaching revealed that bleaching is a detoxification strategy to avoid the formation of reactive oxygen species (ROS), (2) the synergistic action of thermal stress with pathogenic bacteria exacerbates the bleaching process, and (3) the synergistic effect of thermal stress in a nitrate-enriched environment can impede the recovery of corals after a bleaching event, turning the corals to be more susceptible to other environmental or anthropogenic stressors.

Keywords

Coral bleaching • Bacteria • Nutrient • Pigment

2.1 New Approach for Understanding Coral Bleaching Mechanism Using Pigment Analysis

2.1.1 Introduction

Previously it was thought that bleaching was the result of expulsion of zooxanthellae by the host (Hoegh-Guldberg and Smith 1989; Gates 1990; Brown et al. 1995; Jones 1997), or it resulted from the degradation of photosynthetic pigments in zooxanthellar cells (Fitt and Warner 1995; Fitt et al. 2001). The loss of zooxanthellae has been linked to several environmental stressors, especially susceptibility to

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B.E. Casareto (✉)
Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Shizuoka, Japan

Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Shizuoka, Japan
e-mail: becasar@shizuoka.ac.jp

T. Suzuki • Y. Suzuki
Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Shizuoka, Japan
e-mail: suzuki.toshiyuki@shizuoka.ac.jp; suzuki.yoshimi@shizuoka.ac.jp

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high water temperature, which damages cells by changing the chloroplast morphology and function (Bhagooli and Hidaka 2002, 2003, 2006). Kuroki and van Woesik (1999) reported that during the massive Okinawan bleaching event in 1998, zooxanthellae retained in coral tissues changed morphologically and lost pigmentation. Also other authors (Mise and Hidaka 2003; Reimer et al. 2007) described diverse forms of zooxanthellae in tissues of naturally bleached coral during summer.

To examine the morphology and abundance of expelled and retained zooxanthellae, temperature stresses were applied to the coral *Montipora digitata*. Expelled algal cells were collected for classification, enumeration, and analysis of their pigments using high-performance liquid chromatography (HPLC). These parameters were used to compare retained and expelled cells, paying particular attention to pigment composition shifts in response to thermal stress.

2.1.2 Degradation of Zooxanthellae in Coral

Branches of *M. digitata* were collected from a single colony growing in the waters of Bise, Motobu, Okinawa, Japan (26°42′N and 127°52′E). Three branches were incubated in glass bottles containing 800 ml of filtered (0.2 μm) seawater at 12-h light and 12-h dark illumination condition. Incubations were done at 27 °C (control) and 32 °C. To make observations on expelled zooxanthellae, water from incubation vessel was collected during daytime and nighttime. Half of the collected water was filtered through 2.0-μm Nuclepore polycarbonate membranes for observation and counting of expelled zooxanthellae; the remaining water was passed through GF/F filters for pigment analyses. Zooxanthellae in tissue were collected by removing coral tissue using Waterpik.

We classified zooxanthellae into three categories: (i) healthy morphology with normally expanded chloroplasts; (ii) shrunken cells of reduced size with partially fragmented, darkened, and shrunken chloroplasts; and (iii) bleached cells with pale or colorless chloroplasts (Fig. 2.1). We observed three zooxanthellar morphologies in coral tissue, but bleached cells were rare (0.39 % of totals at 27 °C and 1.97 % at 32 °C). As indicated in Table 2.1, the density of zooxanthellae in coral branches after 4 days of incubation at 27 °C was similar to the initial value, as were the proportions of shrunken and healthy zooxanthellae. At 32 °C, zooxanthellar density declined significantly (t-test, $p = 0.002$) to 42 % of the initial value, and the number of shrunken cells increased from 3.78×10^4 to 4.25×10^5 cells cm^{-2}, accounting for ~18 % of total density. Zooxanthellar expulsion rates at the two temperatures are detailed in Table 2.2 and Fig. 2.2. At 27 °C, expelled cell numbers ranged from 3.78×10^2 to 2.39×10^3 cells cm^{-2} coral during 12 h of darkness and from 3.06×10^3 to 1.82×10^4 cells cm^{-2} coral during 12 h of illumination (Fig. 2.2). More zooxanthellae were expelled during illumination and most were shrunken. At 32 °C, expelled cell numbers ranged from 2.27×10^2 to 1.41×10^3 cells cm^{-2} coral during 12 h of darkness and from 5.47×10^2 to 9.87×10^2 cells cm^{-2} coral during 12 h of illumination. Total numbers of expelled cells over 4 days were 4.39×10^4 at 27 °C and 6.00×10^3 at 32 °C, accounting for ~1 % of the total zooxanthellae contained in coral tissue at the outset.

Since the number of cells expelled was very low in the incubations, it was proposed that the expulsion of zooxanthellae from coral is a natural physiological phenomenon and may not be the main mechanism underlying coral bleaching. Although zooxanthellae were expelled at a low rate from coral held at 32 °C, the algal cell density greatly decreased inside coral tissues. A decrease in the zooxanthellar mitotic index in corals held at 32 °C (compared to 27 °C) was also observed. Therefore, algal cell division was reduced by thermal stress, but this does not explain the decrease in cell density inside the coral tissue (Table 2.1): there must be a process of zooxanthellar degradation inside the host (Fig. 2.3). Titlyanov et al. (1996) reported that coral hosts commonly digest their algal symbionts, a phenomenon that has also been observed in the sea anemone *Phyllactis flosculi* (Steele and Goreau 1977), in giant clams (Fankboner 1971), and in the marine hydroid *Myrionema amboinense* (Fitt and Cook 1990). In this research, a large number of shrunken zooxanthellae in coral tissue and in the water of the experimental system were found. Zooxanthellae with shrunken cytoplasm and reduced chloroplasts have been observed previously in corals under thermal stress (Fukabori 1998) and several coral species, e.g., *M. digitata* (Titlyanov et al. 1996; Papina et al. 2007), *Stylophora pistillata* (Titlyanov et al. 1996; Kuroki and van Woesik 1999; Titlyanov et al. 2001), *Galaxea fascicularis* (Bhagooli and Hidaka 2002), and *Zoanthus sansibaricus* (Reimer et al. 2007), among others (*Acropora selago*, *Acropora muricata*, *Heliofungia actiniformis*, *Ctenactis echinata*, *Oxypora lacera*, and *Pocillopora eydouxi*; Fujise et al. 2013). Although these zooxanthellae have been classified as degraded (Titlyanov et al. 1998; Downs et al. 2009, 2013), the mechanism responsible for the formation of these shrunken cells is poorly understood.

2.1.3 Finding of Cyclo Enol in Shrunken Zooxanthellae

Shrunken zooxanthellae collected from the seawater in the incubation vessel at 27 °C were compared in their pigments with those of healthy algal cells collected from coral tissue.

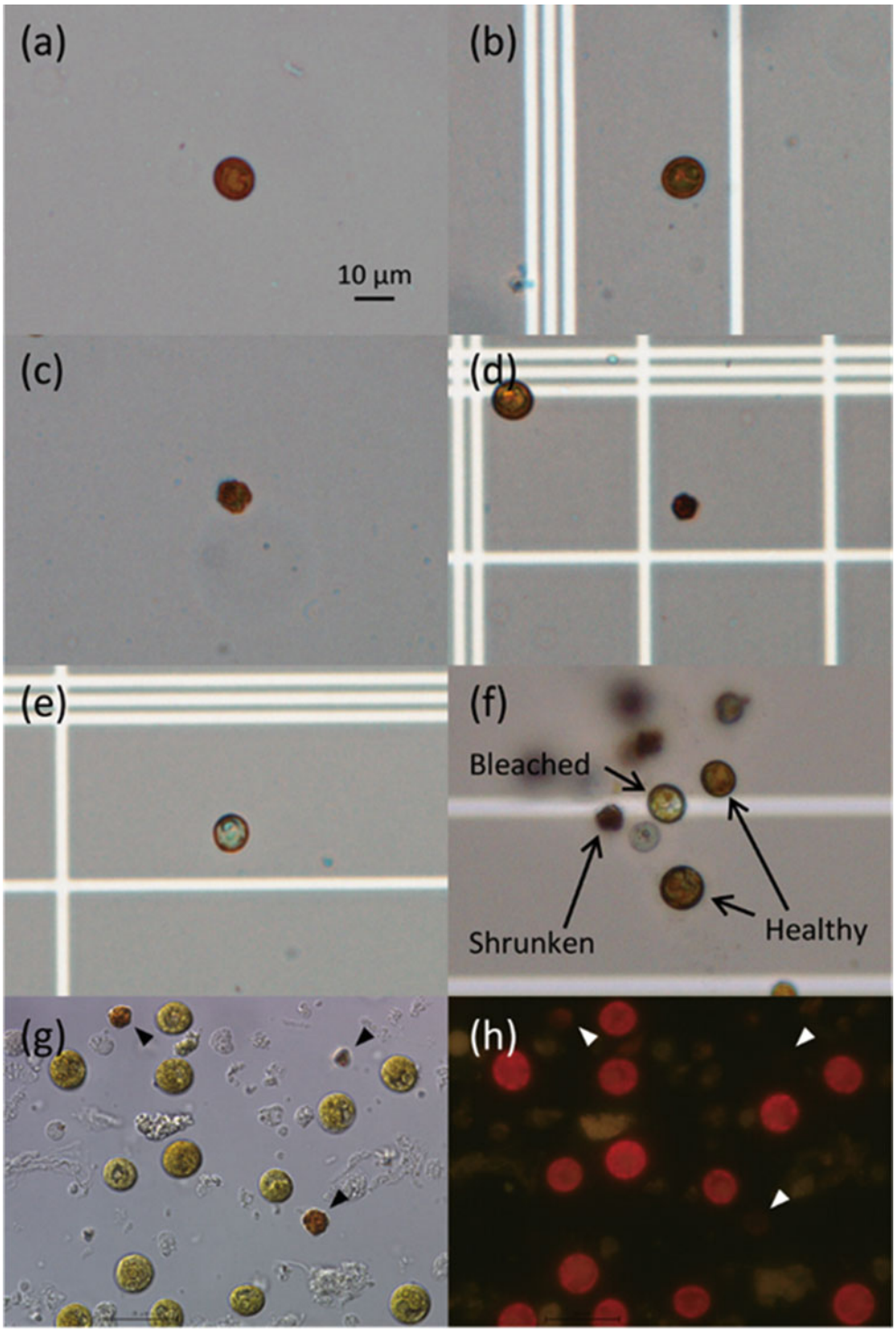

Fig. 2.1 Types of zooxanthellae observed in coral tissue. (**a**, **b**) Healthy cells with a spherical shape and expanded chloroplast. (**c**, **d**) Shrunken, darkly colored cells with reduced sizes and partially fragmented chloroplasts. (**e**) Bleached cells with pale and colorless chloroplasts. (**f**) Three categories of zooxanthellae. (**g**) White light micrograph and (**h**) fluorescence image of healthy and shrunken cells (shrunken cells are indicated by arrowheads) (Reproduced from Suzuki et al. (2015) by permission of John Wiley & Sons Ltd. Copyright © 2014 The Authors. *Journal of Phycology* published by Wiley Periodicals, Inc. on behalf of Phycological Society of America)

Elution profiles of pigments of shrunken and healthy zooxanthellae are depicted in Fig. 2.4. Seven peaks appeared only in samples dominated by shrunken zooxanthellae: pheophorbide *a* and pigments similar to it (17.05-min retention time), a pigment similar to peridinin (22.33 and 22.69 min), a pigment similar to diadinochrome

Table 2.1 Numbers and proportions of zooxanthellae (cells cm^{-2} coral surface) retained and expelled from coral tissue after 4 days of incubation at two temperatures

Zooxanthellae retained			
	Day 0	Day 4	
		(27 °C)	(32 °C)
Total	5.64×10^6	5.65×10^6	2.37×10^6
Healthy	5.60×10^6 (99.3 %)	5.52×10^6 (97.7 %)	1.95×10^6 (82.3 %)
Shrunken	3.78×10^4 (0.7 %)	1.25×10^5 (2.3 %)	4.25×10^5 (17.7 %)
Zooxanthellae expelled over 4 days			
		(27 °C)	(32 °C)
Total		4.39×10^4	6.00×10^3
Healthy		8.55×10^3 (19.4 %)	3.08×10^3 (51.3 %)
Shrunken		3.54×10^4 (80.6 %)	2.92×10^3 (48.7 %)

Table 2.2 Rates of zooxanthellar expulsion (cells cm^{-2} coral surface h^{-1}) at two temperatures during illuminated and dark periods of the day

	Total	Healthy	Shrunken
(27 °C)			
Day	808.2	124.7	683.5
Night	106.5	53.4	53.1
(32 °C)			
Day	61.5	30.6	30.9
Night	63.5	33.6	29.8

(27.76 min), a type of chlorophyll (31.03 min), a pigment similar to alloxanthin (32.13 min), and pyropheophytin *a* (39.93 min). A noticeable pigment peak at 31.03-min retention time had a maximum absorption peak at 686 nm (red band), matching a report by Goericke et al. (2000). The absorption spectrum of this pigment (extracted from zooxanthellae) was compared with an authentic standard of 13^2, 17^3-cyclopheophorbide *a* enol (cyclo enol, cPPB-*a*E) and almost perfect match, and therefore the extracted pigment was identified as cyclo enol (on the basis of retention time and absorption spectrum). Concentrations of chlorophyll *a*, peridinin, and chlorophyll c_2 after 4 days were closely similar at the two temperatures (Fig. 2.5); however, the concentration of cyclo enol was much higher at 32 °C (Fig. 2.5), even though cell numbers had declined at this higher temperature (Table 2.1). Pigment contents of expelled zooxanthellae are shown in Fig. 2.6. Chlorophyll *a* and c_2 concentrations were low at both temperatures. Cyclo enol was the most abundant pigment extracted from zooxanthellae expelled from corals at 27 °C (Fig. 2.6), and the number of expelled shrunken zooxanthellae exceeded the number of healthy cells (Fig. 2.4).

Cyclo enol has been reported as a degradation product of chlorophyll *a* in phytoplankton (Kashiyama et al. 2012), and it is commonly present in aquatic environments: marine and lacustrine sediments (Chillier et al. 1993; Harris et al. 1995; Ocampo et al. 1999; Louda et al. 2000), sponges (Karuso et al. 1986), bivalves (Sakata et al. 1990; Yamamoto et al. 1992; Watanabe et al. 1993; Louda et al. 2008), and protozoa (Goericke et al. 2000). Kashiyama et al. (2012, 2013) found that herbivorous protozoa produce cyclo enol when they graze on and digest microalgae. Cyclo enol is generated from pyropheophytin *a* (Kashiyama and Tamiaki 2014). Several types of phytoplankton are also able to generate cyclo enol (Kashiyama et al. 2014). Yamada et al. (2013) reported the formation of cyclo enol (in small quantities) in zooxanthellae that had been extracted from coral and cultivated in flask in stationary phase. In this research, cyclo enol and pyropheophytin *a* were detected in pigment extracts of shrunken zooxanthellae. Thus, cyclo enol is likely generated from chlorophyll *a* through a degradation pathway leading to the formation of shrunken zooxanthellae.

Degradation of zooxanthellae inside the coral tissues is still a question. It is hypothesized that this process may be a detoxification strategy to avoid reactive oxygen species (ROS) formation. Free chlorophyll *a* released from broken chloroplasts becomes a generator of singlet oxygen when exposed to light, thereby promoting the formation of ROS which results in significant damage of cell structure (Perl-Treves and Perl 2002). Cyclo enol differs from chlorophyll *a* in that it does not have fluorescence; therefore, ROS are not formed (Kashiyama et al. 2012, 2013). Protozoans that feed on microalgae have transparent bodies, and their body contents are therefore always exposed to light during daytime. Accordingly, these organisms have developed a strategy for detoxifying free chlorophyll *a* through degradation to the non-fluorescing product cyclo enol (Kashiyama et al. 2012, 2013). In case of coral, the red fluorescence of chlorophyll was largely quenched in the shrunken algal cells (Fig. 2.1g, h). Kashiyama et al. (2012) also reported that chloroplasts of diatoms grazed on by protozoans were shrunken and had no chlorophyll fluorescence. This loss of fluorescence may indicate that ROS are not produced by chlorophylls freed from damaged chloroplasts. Corals also have transparent bodies and they live symbiotically with zooxanthellae. Therefore, they are always exposed to potential damage caused by the oxidative stress of ROS (Lesser et al. 1990; Dykens et al. 1992; Downs et al. 2002). Oxidative damage becomes more severe as UV radiation and water temperature increase (Lesser et al. 1990). Moreover, damaged chloroplasts are repaired with difficulty during thermal stress episodes, and ROS formation is thereby increased (Bhagooli and Hidaka 2006). It is proposed that corals and zooxanthellae employ the detoxification strategy used by herbivorous protists and phytoplankton, viz., degradation of chlorophyll *a* to cyclo enol. Furthermore, reductions in zooxanthellar numbers within coral tissue caused by

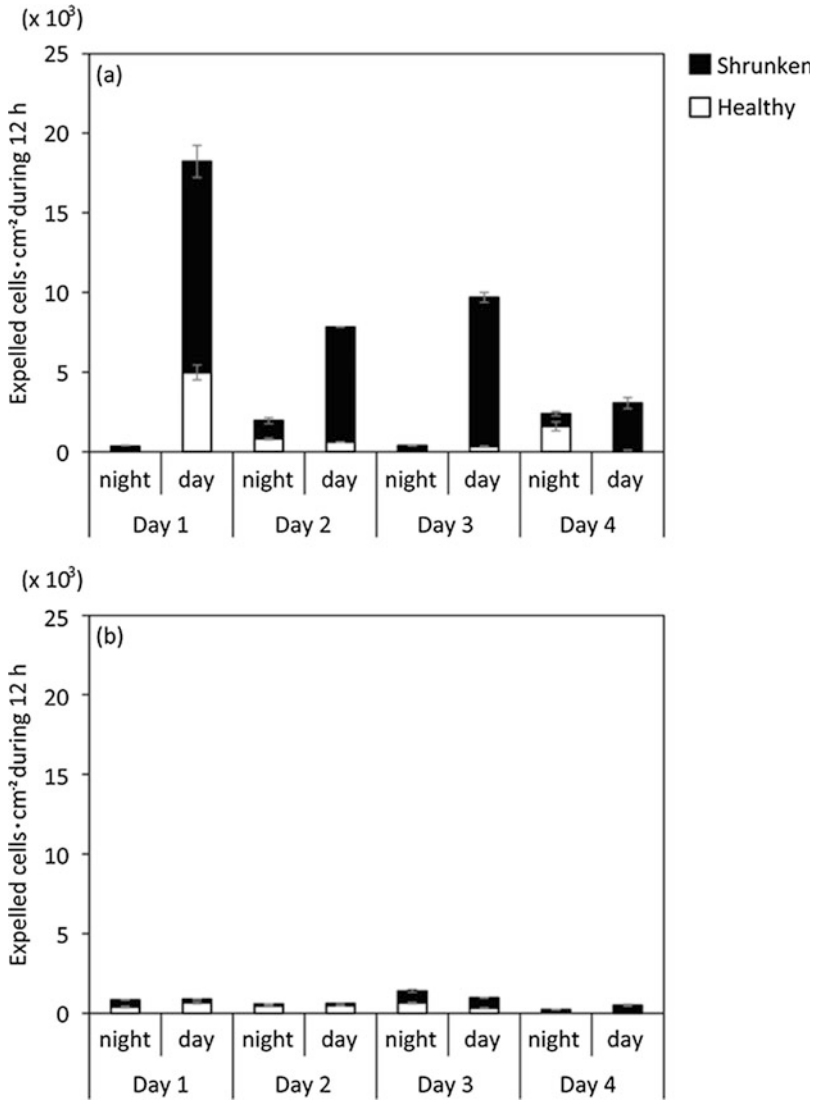

Fig. 2.2 Zooxanthellar density and composition in water from incubation vessel collected over 12-h intervals for 4 days. (**a**) 27 °C and (**b**) 32 °C. Cell numbers are normalized to surface areas of coral branches. Values are means±SE ($n = 3$) (Reproduced from Suzuki et al. (2015) by permission of John Wiley & Sons Ltd. Copyright © 2014 The Authors. *Journal of Phycology* published by Wiley Periodicals, Inc. on behalf of Phycological Society of America)

degradation of algal cells and bleaching may be an important mechanism for reducing the production of ROS (Fig. 2.7). Thus, coral bleaching is a physiological mechanism used as a survival strategy by corals facing oxidative damage.

2.1.4 Summary of Sect. 2.1

The morphology and pigment composition of zooxanthellae were examined in corals subjected to normal temperature (27 °C) and thermal stress (32 °C). Several normal and abnormal morphological types of zooxanthellar cells were observed. Normal cells were intact and their chloroplasts were unbroken (healthy); abnormal cells were shrunken and had partially degraded or broken chloroplasts, or they were bleached without visible chloroplasts. At 27 °C, most healthy zooxanthellae were retained in the coral tissue, whereas shrunken zooxanthellae were expelled. Under thermal stress, the abundance of healthy zooxanthellae declined and the proportion of shrunken/abnormal cells increased in coral tissues. Within the shrunken cells, it was detected the presence of a chlorophyll-like pigment that is not ordinarily found in healthy zooxanthellae. Analysis of the absorption spectrum, absorption maxima, and retention time indicated that this pigment was 13^2, 17^3-cyclopheophorbide *a* enol (cyclo enol, cPPB-*a*E), which is frequently found in marine and lacustrine sediments and in protozoans that graze on phytoplankton. The production of cyclo enol in shrunken

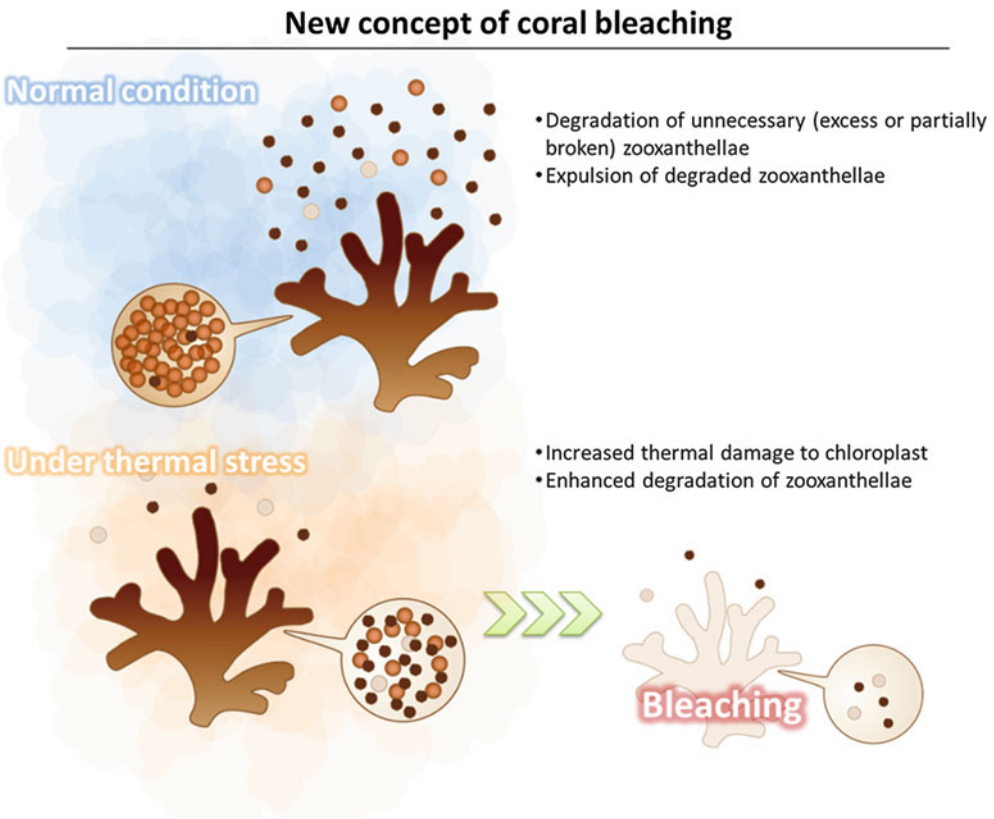

Fig. 2.3 New concept of coral bleaching revealed from counting and observation of zooxanthellae

zooxanthellae suggests that the chlorophylls have been degraded to cyclo enol, a compound that is not fluorescent. The lack of a fluorescence function precludes the formation of reactive oxygen species. Therefore, it was considered that the formation of cyclo enol in shrunken zooxanthellae is a mechanism for avoiding oxidative stress.

2.2 Enhancement of Bleaching Under Synergistic Action of Thermal Stress and Pathogenic Bacteria on the Coral *Montipora digitata*

2.2.1 Introduction

Corals are complex symbiotic systems including the host, a cnidarian, the endosymbiotic algae, prokaryotes (bacteria and archaea), fungi, and virus. Although the role of the microorganisms associated with corals is not yet well defined, they seem to play an important role in the maintenance of the coral health by providing essential nutrients (Agostini et al. 2009) and defense against potential pathogens (Ritchie 2006). Their potential roles in the maintenance of coral health were reviewed in Rosenberg et al. (2007). Beneficial roles of the associated prokaryotes have been reported in the literature, but on the other side, growing evidences show the existence of coral pathogens which can cause severe diseases often resulting in the death of the coral. Bleaching of the coral *Oculina patagonica* in the Mediterranean Sea has been shown to be caused by the coral pathogen *Vibrio shiloi* (Kushmaro et al. 1997). In *Pocillopora damicornis*, the coral pathogen *Vibrio coralliilyticus* causes bleaching followed by tissue necrosis (Ben-Haim et al. 2003). Moreover, there is a higher prevalence of disease following bleaching events (Muller et al. 2007). These results may be due to a shift in the coral-associated microbial community during a bleaching event (Bourne et al. 2008), changes in the coral metabolism under high-temperature stress especially the decrease in primary production (Fujimura et al. 2008), and the release of mucus and ammonia (Suzuki and Casareto 2011). Therefore, the effects of high seawater temperature and potential pathogens on the coral metabolism should be studied to understand the synergistic effects of these two stresses and the sequences that lead from a temperature-induced bleaching event to bacteria/temperature-caused bleaching event along with their differences. Coral bleaching triggered by a thermal stress disrupts the function of the coral metabolism, weakening the coral and allowing opportunistic pathogens to cause damages to the host. Figure 2.8 illustrates

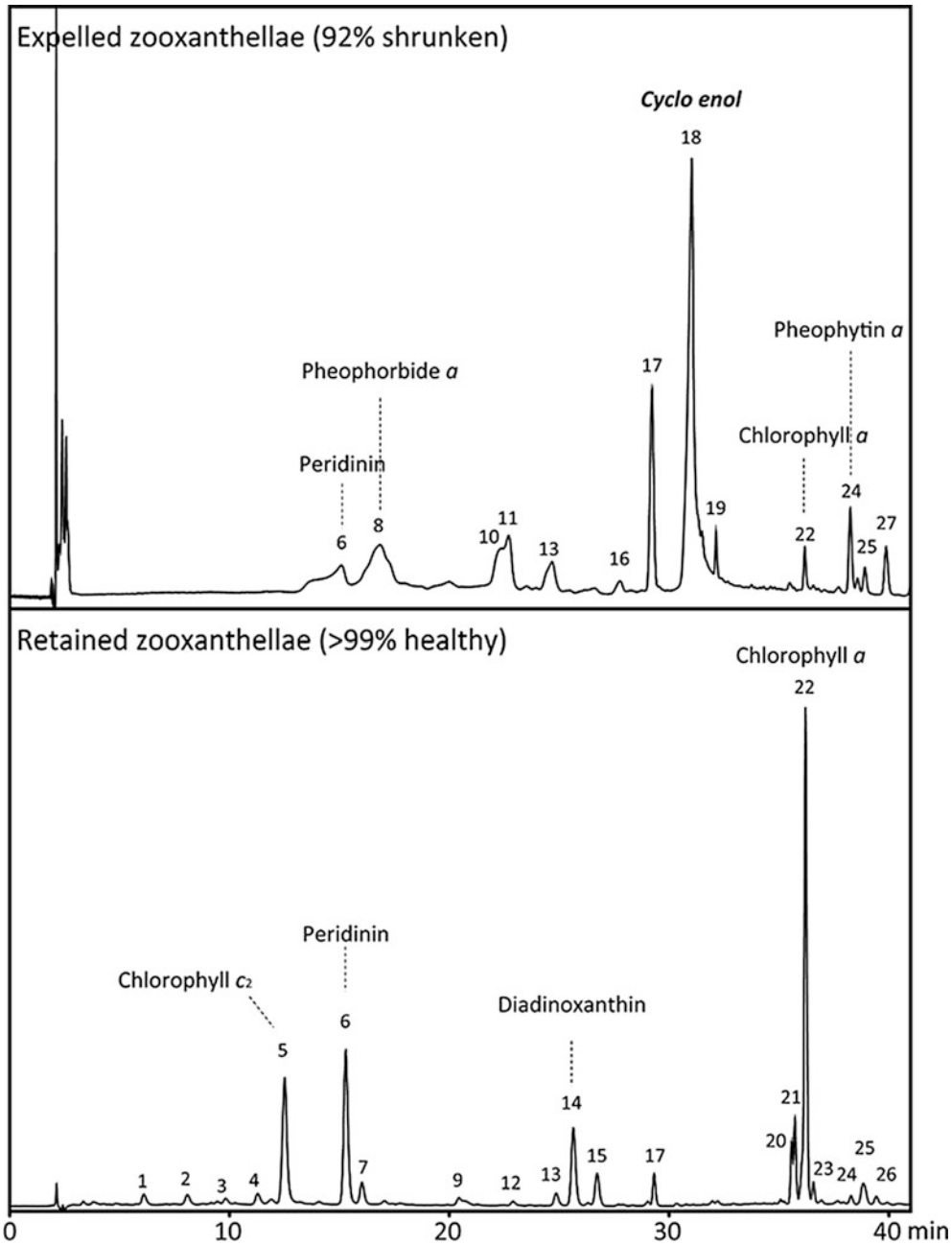

Fig. 2.4 Elution profiles of expelled zooxanthellae (*upper*) and retained zooxanthellae at the outset of the experiment (*lower*) at 27 °C (Reproduced from Suzuki et al. (2015) by permission of John Wiley & Sons Ltd. Copyright © 2014 The Authors. *Journal of Phycology* published by Wiley Periodicals, Inc. on behalf of Phycological Society of America

three possible ways of coral bleaching considering different stages of coral health from a normal stage, passing through a step where bleaching has already started affecting some of the metabolic functions of corals and/or the associated zooxanthellae but still without any visible signals, followed by a step of visible bleaching and a serious compromised tissue necrosis that takes the coral finally to death. All these coral health stages can be imagined through an arbitrary timescale depending on the bleaching type as follows:

(a) Thermal stress-mediated bleaching with a possible recovering if the thermal stress ceases, with a relatively long timescale.
(b) Microbial mediated bleaching (apply for the case studied on the coral *Pocillopora damicornis* triggered by the bacteria *V. coralliilyticus*). This process is almost irreversible and takes the coral to death in rapid timescale.
(c) Synergistic action of thermal stress and pathogenic bacteria infection: this process results from the infection of

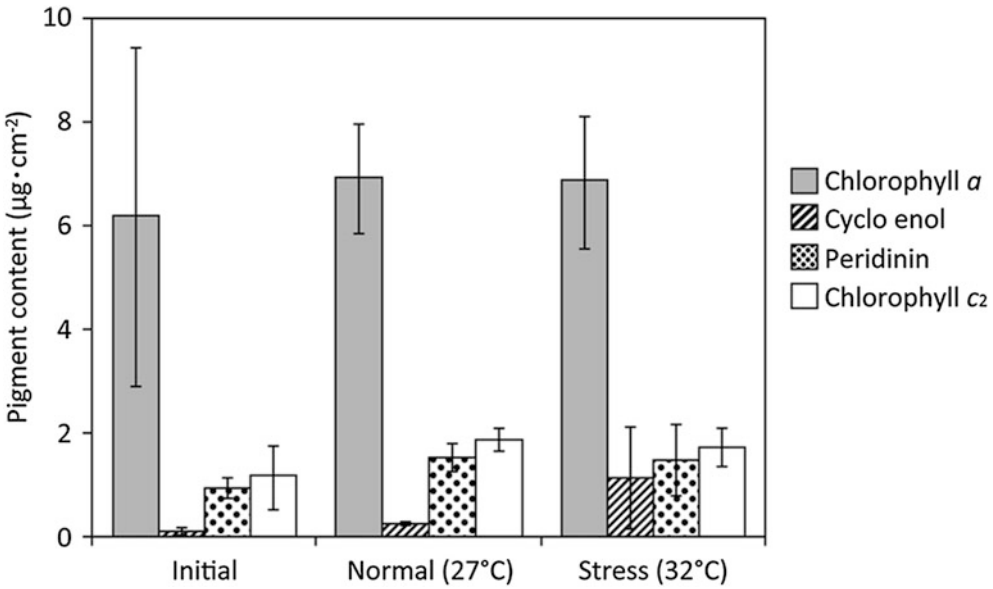

Fig. 2.5 Initial pigment concentrations of zooxanthellae retained in coral tissue compared with values after 4 days of incubation at 27 °C and 32 °C. Pigment contents are normalized to surface areas of coral branches. Values are means±SD ($n = 9$) (Reproduced from Suzuki et al. (2015) by permission of John Wiley & Sons Ltd. Copyright © 2014 The Authors. *Journal of Phycology* published by Wiley Periodicals, Inc. on behalf of Phycological Society of America)

coral during bleaching induced by thermal stress. This can be explained due to the weakness of coral once some changes in metabolic functions start to occur due the thermal stress, facilitating infection of opportunistic pathogens.

The present research tries to describe the (c) type of synergistic actions of thermal stress combined with pathogen bacteria.

2.2.2 Approach for Testing Bacteria Strains in an Incubation

Mixed solutions containing five species of bacteria, *Vibrio coralliilyticus* (AB490821), *Vibrio harveyi* (AB490822), *Paracoccus carotinifaciens* (AB490820), *Pseudoalteromonas* sp. (AB691769), and *Sulfitobacter* sp. (AB691770), were prepared at the concentration of 5.5×10^5 ml^{-1} to 1.2×10^6 ml^{-1} and inoculated to coral branches of the coral *Montipora digitata* kept in incubation vessels (four branches/vessel in triplicates). To confirm the effects of each bacterial species, individual species were also tested, using a final abundance of approximately 10^6 cells ml^{-1}. These bacteria are naturally found in coastal regions of Okinawa, Japan.

Coral branches of the coral *Montipora digitata* were kept in incubation vessels (four branches/vessel in triplicates). Seawater temperatures were set at 27 °C or 32 °C, and two levels of bacterial abundance (no addition and addition of mixed bacteria) were tested in a 2 × 2 factorial design with three replicates per treatment. Continuous flow of seawater was provided to incubation vessels at the rate of 15 ml min^{-1}. Illumination was provided at 12:12-h light/dark cycle. A total of 30 glass bottles were prepared: 15 bottles were kept in a water bath maintained at a constant temperature of 27 °C, and the other 15 bottles were in a water bath at 32 °C. After 4 days, the bacteria were inoculated into six bottles at each temperature, using an additional pump to maintain a total flow rate of 15 ml min^{-1}. Tissue samples and water samples for measurements of coral metabolisms were taken on day 0 (initial), day 4, and day 8. Corals from three replicate bottles under each condition were used for measurements of photosynthetic efficiency (*Fv*/*Fm*) and posteriorly sacrificed for tissue analysis (zooxanthellae counts, primary production, and pigment analysis). Water samples were taken from three replicate bottles under each condition and used for the measurement of dissolved oxygen and alkalinity. The same experimental design was used for individual bacterial testing. Corals were incubated for 4 days at 32 °C in seawater enriched with bacteria at a final total abundance of 10^6 cells ml^{-1}. Five bottles containing one branch each were prepared for each bacterium. The final state of the corals was evaluated by their coloration, their maximum photochemical yield (*Fv*/*Fm*), and the presence or absence of tissue necrosis

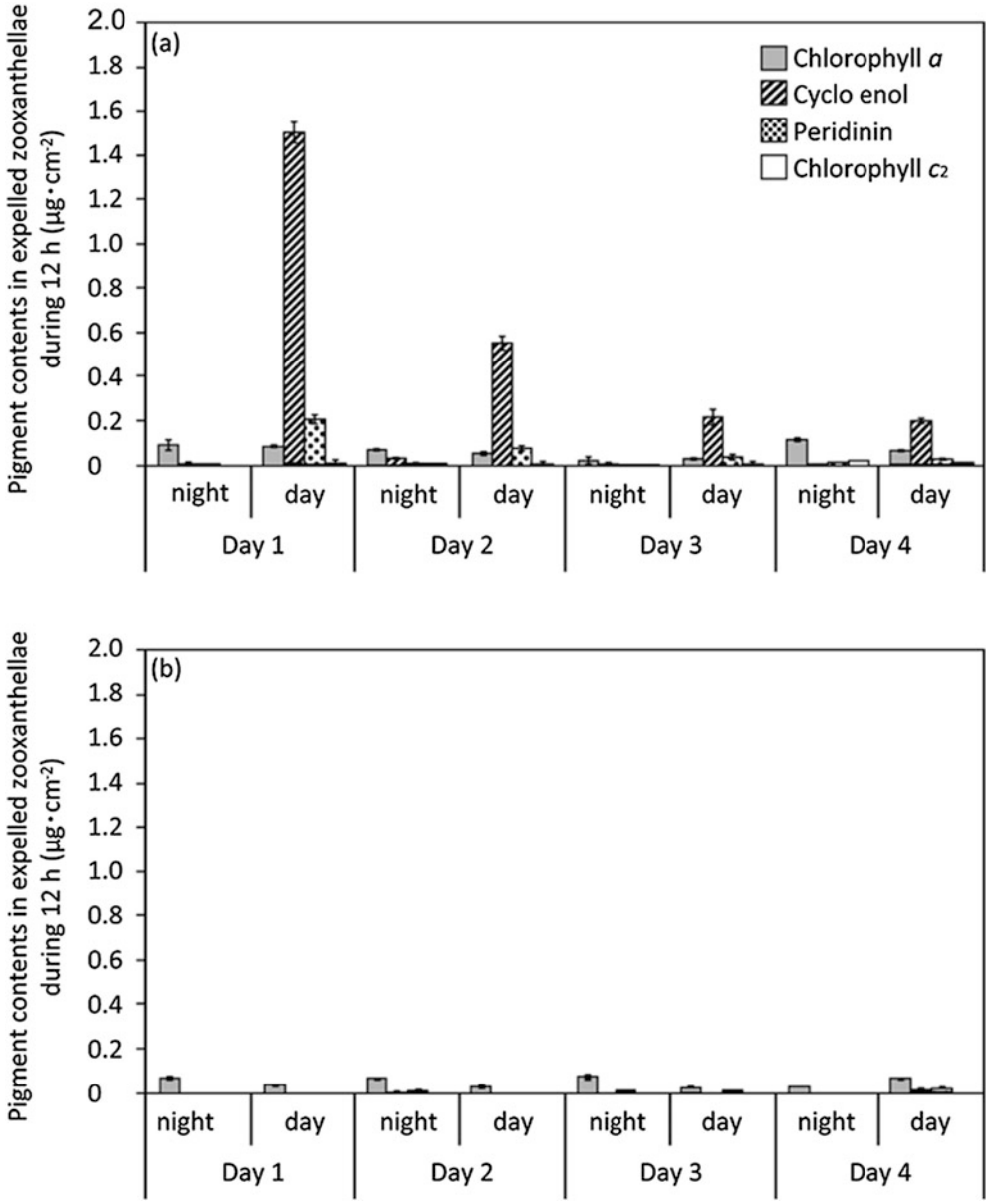

Fig. 2.6 Pigments compositions of zooxanthellae expelled from coral branches held under a 12-h photoperiod over 4 days of incubation. **a** 27 °C and **b** 32 °C. Values are means±SD ($n = 3$) (Reproduced from Suzuki et al. (2015) by permission of John Wiley & Sons Ltd. Copyright © 2014 The Authors. *Journal of Phycology* published by Wiley Periodicals, Inc. on behalf of Phycological Society of America)

2.2.3 Effect of Bacteria and Their Role in Coral Metabolisms

After 8 days of incubation, the loss of zooxanthellae under high temperature was clearly visible especially for those corals under thermal stress. Zooxanthellae density of *M. digitata* did not significantly decrease with the addition of bacteria at 27 °C; however, corals were bleached dramatically by synergistic stresses of high temperature and bacteria. *M. digitata* lost about 45 % of its zooxanthellae under high temperature and about 70 % under combined high temperature and bacteria on day 8 of incubation (Fig. 2.9a–f). This means that combination of thermal stress and bacteria strongly influenced coral bleaching. In terms of chlorophyll *a*, in controls at initial time, it was 2.0 μg cm^{-2} and slightly increased to 2.4 μg cm^{-2} in controls after 8 days of incubations; however, it decreased to 1.7 μg cm^{-2} at 27 °C with addition of bacteria. At stress temperature (32 °C), it decreased to 1.7 μg cm^{-2} and 1.3 μg cm^{-2} in combined stresses of 32 °C and bacteria addition. Moreover chlorophyll *a* content per zooxanthellae varied from 5.2 picograms chlorophyll *a* $cell^{-1}$ to 4.6 picograms $cell^{-1}$ in control after

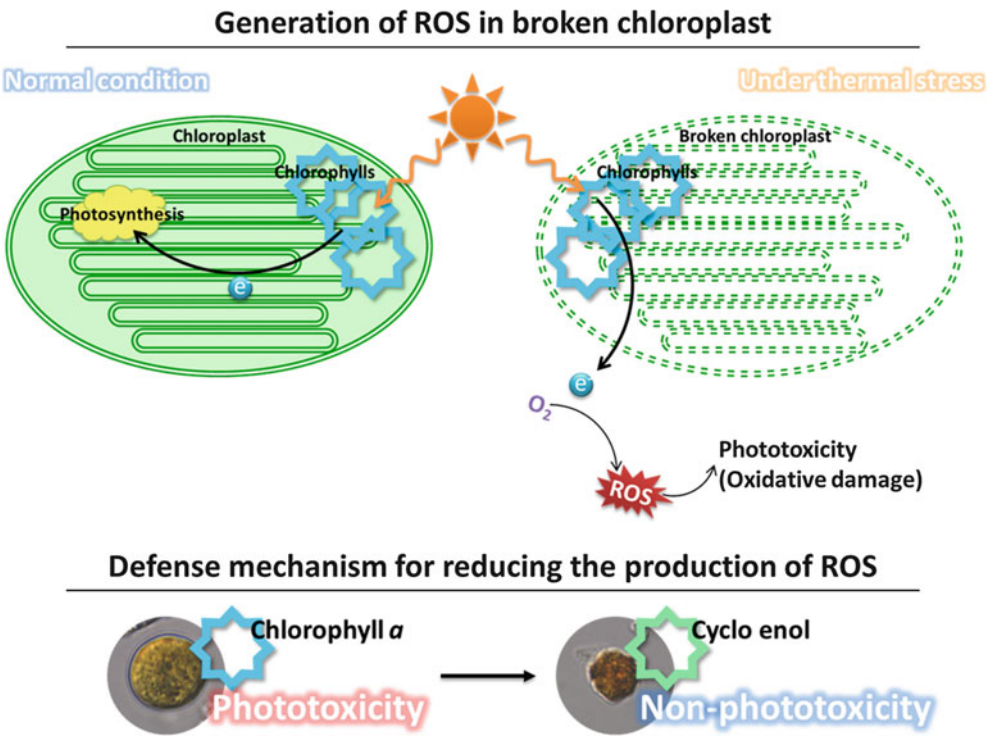

Fig. 2.7 Generation of ROS in broken chloroplast and significance of the conversion of chlorophyll *a* to cyclo enol

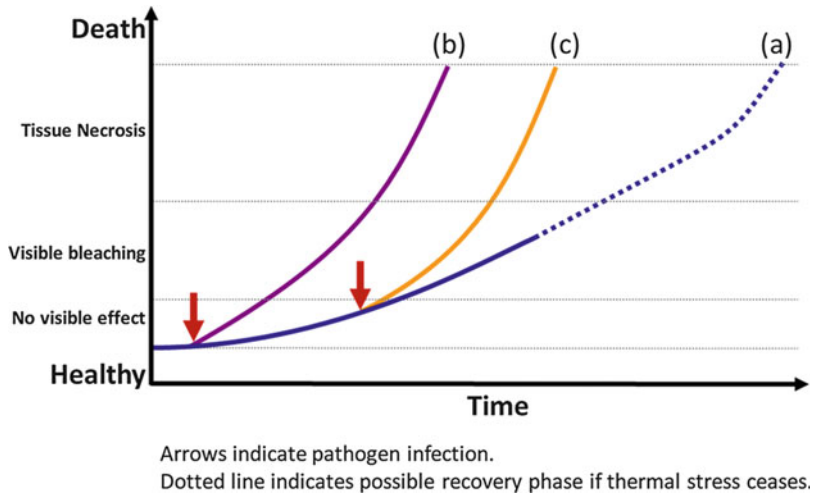

Fig. 2.8 Three possible ways of coral bleaching

8 days of incubations and 3.8 picograms cell^{-1} at 27 °C with addition of bacteria, but it increased to 6.1 picograms cell^{-1} at high temperature treatment and to 8.5 picograms cell^{-1} in combined stresses treatment. This may indicate that corals retain healthy zooxanthellae that contain intact and replete chloroplasts in order to face disadvantages during bleaching events (Fig. 2.9d, e). The *Fv*/*Fm* ratio (Fig. 2.9f) significantly changed at high temperature with bacterial addition (HSD, $P < 0.05$). A two-way ANOVA showed a significant effect of temperature on the maximum photosynthetic yield on day 8 ($P = 0.003$). The *Fv*/*Fm* ratio of coral at high temperature with inoculation was significantly lower than the ratios of corals with the other treatments, with average values of 0.57 ± 0.02 and 0.67 ± 0.05 and 0.77 ± 0.02 and 0.74 ± 0.04 at 32 °C and 27 °C with and without bacteria, respectively. Thus, bacterial inoculation decreased photosynthetic efficiency by about 25 % at 32 °C.

At the same time, synergistic stress of high temperature with bacteria inoculation significantly affected primary production rates (Fig. 2.10a): at elevated temperature (32 °C), primary

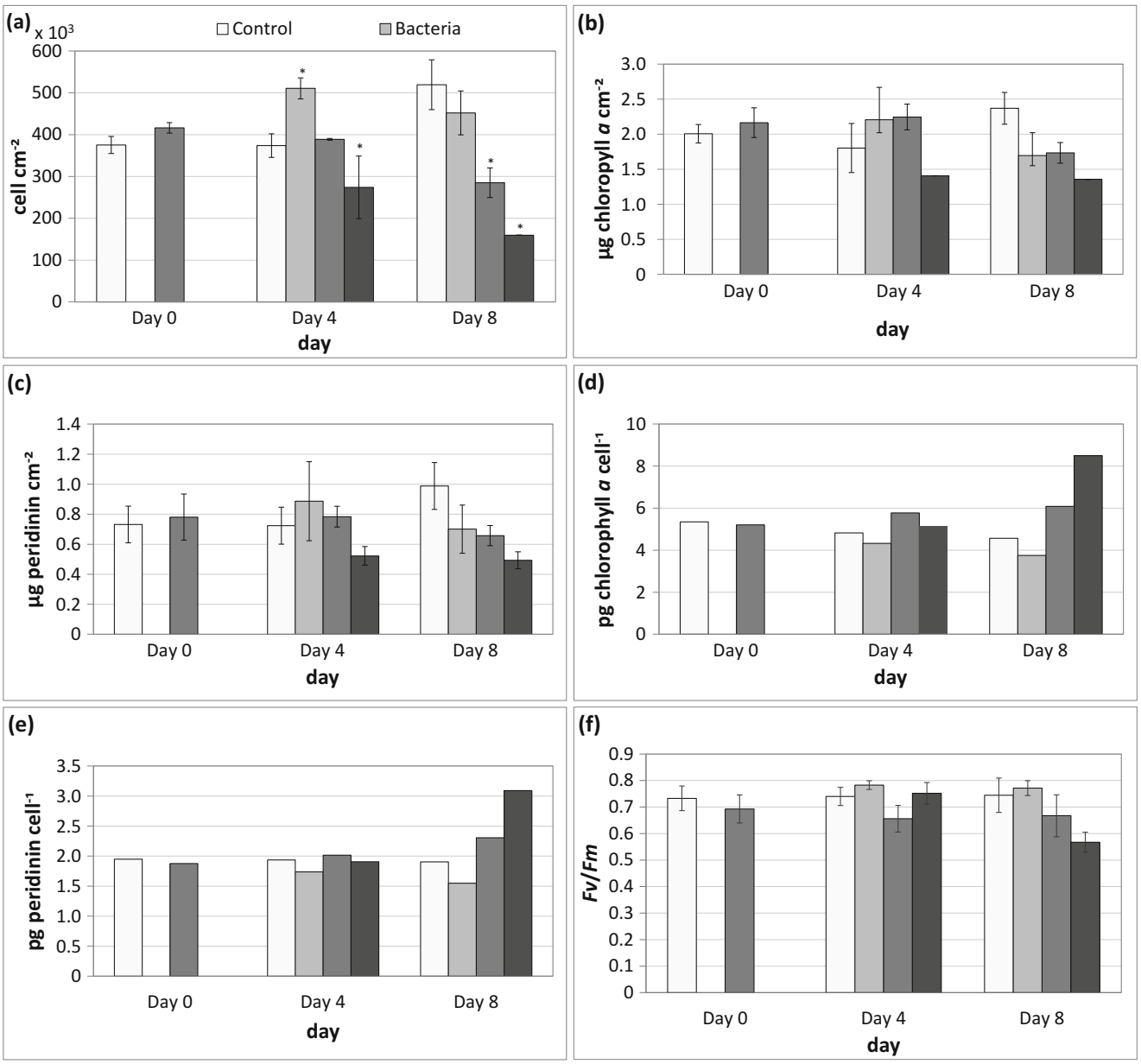

Fig. 2.9 Variation in (**a**) zooxanthellae density in coral tissue, (**b**) chlorophyll *a* content, (**c**) peridinin content, (**d**) chlorophyll *a* per zooxanthellar cell, (**e**) peridinin per zooxanthellar cell, and (**f**) maximum quantum yield (*Fv*/*Fm*) with two levels of bacteria abundance (no addition and addition of five bacterial strains) at ambient (27 °C) and at increased temperature (32 °C). Values are mean values±SE ($n = 3$ for each treatment). *$P < 0.05$ compared to the control according to the Tukey–Kramer HSD test

production of the control (1.03 ± 0.06 µg C cm^{-2} h^{-1}) decreased 28 % to an average value of 0.74 ± 0.04 µg C cm^{-2} h^{-1} (HSD, $P < 0.05$), and with synergistic effects of high temperature and bacteria addition, primary production was also significantly decreased by about 66 % (HSD, $P < 0.05$), to an average of 0.35 ± 0.10 µg C cm^{-2} h^{-1}, while respiration rates (Fig. 2.10b) increased by approximately 30 % under high temperature to reach a value of 0.50 ± 0.03 and 0.49 ± 0.01 µmol O_2 cm^{-2} h^{-1} for high temperature without and with bacteria, respectively. However, addition of bacteria did not affect respiration rates at both high temperature and normal temperature. Respiration, which reflects host metabolism and to a lesser extent zooxanthellae metabolism, followed a similar pattern as photosynthesis variation but with a smaller amplitude and reverse direction. The higher respiration rates shown here may be attributable to higher energy requirements to repair the damage caused by temperature stress.

The calcification rate (Fig. 2.10c) was not significantly decreased by the addition of bacteria at 27 °C, with rates of

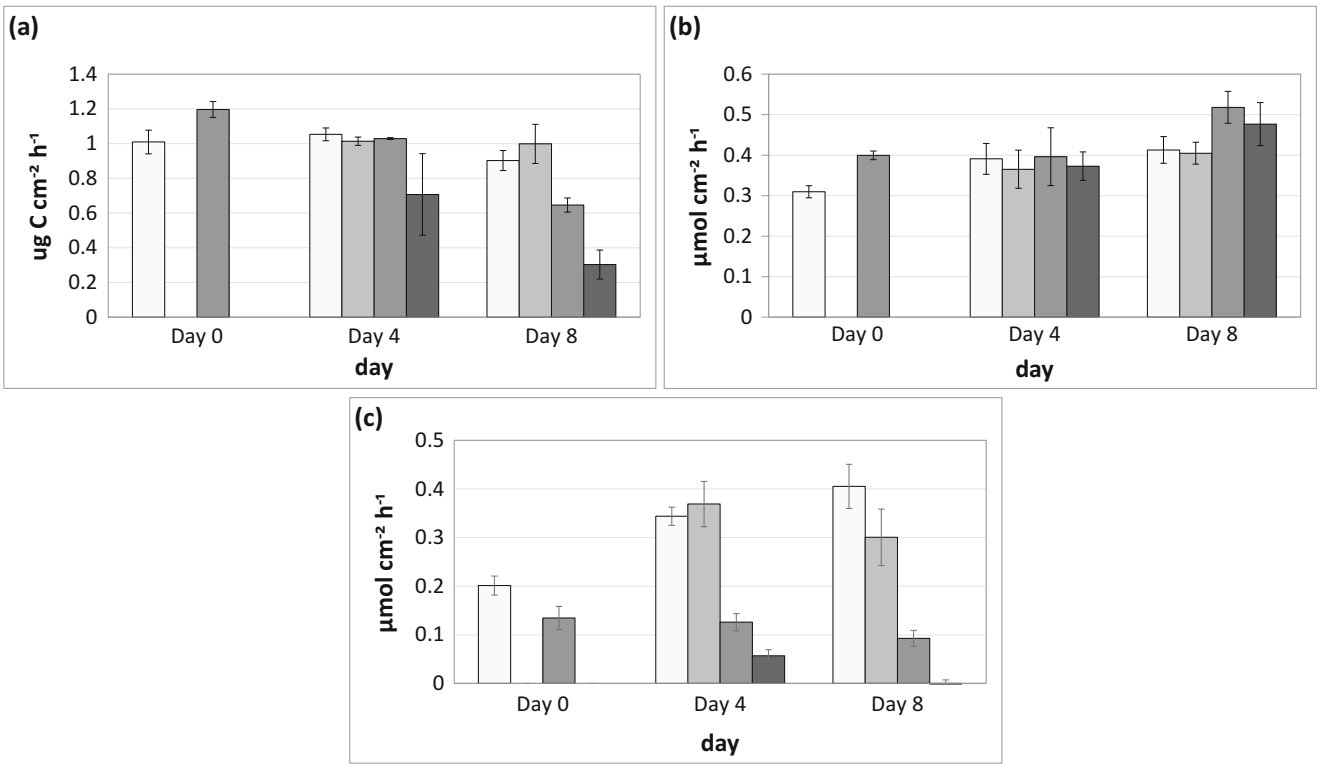

Fig. 2.10 Variation in (**a**) primary production, (**b**) respiration, and (**c**) calcification with two levels of bacteria abundance (no addition and addition of five bacterial strains) at ambient (27 °C) and at increased temperature (32 °C). Values are mean values±SE ($n = 3$ for each treatment). *$P < 0.05$ compared to the control according to the Tukey–Kramer HSD test

0.41 ± 0.05 µmol cm^{-2} h^{-1} for the control treatment and 0.30 ± 0.06 µmol cm^{-2} h^{-1} with the addition of bacteria. However, temperature stress significantly affected the calcification rate (ANOVA, $P < 0.001$), which decreased to 0.09 ± 0.02 µmol cm^{-2} h^{-1} at 32 °C, representing a decrease of about 77 % (HSD, $P < 0.05$). Moreover, the combined stresses of high temperature and bacteria greatly decreased the calcification rate, by about 101 % (HSD, $P < 0.05$), with a net dissolution of calcium carbonate from the coral occurring on day 8 at 32 °C with bacteria (-0.01 ± 0.01 µmol cm^{-2} h^{-1}).

During a challenge with individual bacterial strains, the appearance of branches that were incubated in the presence of *V. coralliilyticus*, *V. harveyi*, *P. carotinifaciens*, or *Pseudoalteromonas* sp. did not change after 4 days of incubation at 32 °C and appeared to be as healthy as the branches shown in Fig. 2.11a, b. The *Fv/Fm* ratios of these branches were relatively stable. All five replicates maintained their coloration and expanded their polyps. However, branches in the presence of *Sulfitobacter* sp. at 32 °C exhibited signs of severe bleaching and some tissue necrosis (Fig. 2.11e), and the *Fv/Fm* ratio was decreased by about 24 % compared with control branches.

2.2.4 Significance of Synergistic Actions of Thermal Stress and Pathogens Inputs

Individually, only *Sulfitobacter* sp. provoked acute bleaching of the coral after 4 days of incubation at 32 °C. The other strains did not cause visible effects on *M. digitata* in these conditions. However, *V. coralliilyticus* (Ben-Haim et al. 2003; Sussman et al. 2008), *P. carotinifaciens* (Casareto, pers. comm.), and *V. harveyi* (Sutherland et al. 2004; Gomez-Gil et al. 2004) were reported as coral pathogens. These three last strains individually, without a trigger other than temperature, may not be responsible for the bleaching of *M. digitata*. Lesions on corals under thermal stress with a mixture of the five bacteria and under thermal stress with *Sulfitobacter* sp. alone showed a loss of coloration and some tissue necrosis.

With *Sulfitobacter* sp. alone, the lesions were more severe. At high temperature without the addition of bacteria, moderate loss of coloration was observed, but there were no signs of necrosis. Tissue necrosis can be interpreted as a more advanced compromised stage following bleaching. Five mixed bacteria did not have any effects at 27 °C. Therefore, *Sulfitobacter* sp. or these bacteria when mixed

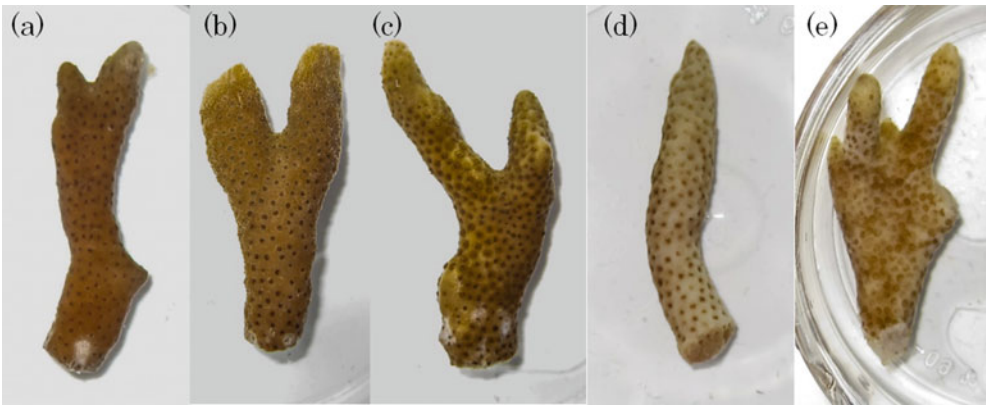

Fig. 2.11 Photograph of branches of *Montipora digitata* after incubation under each condition. (**a**) At 27 °C without addition of bacteria, (**b**) at 27 °C with addition of five mixed bacteria (*Vibrio coralliilyticus*, *Vibrio harveyi*, *Paracoccus carotinifaciens*, *Pseudoalteromonas* sp., and *Sulfitobacter* sp.), (**c**) at 32 °C without addition of bacteria, (**d**) at 32 °C with addition of five mixed bacteria, and **e** at 32 °C with addition of *Sulfitobacter* sp.

required a temperature higher than normal to cause damage to *M. digitata*. This phenomenon may be due to two different mechanisms: the bacteria express some virulence factor only at high temperature as for the *Oculina patagonica*/*V. shiloi* model (Rosenberg and Falkovitz 2004), or the coral immune system was weakened by the heat stress allowing the action by these bacteria (Mylardz et al. 2009). The effect of bacteria as accelerator for coral bleaching is large with high temperature.

Over a short period, moderate thermal stress may not have a strong effect on corals, and if temperatures return to normal levels, it is highly probable that the corals would be able to recover. Under normal temperature, bacteria did not affect coral bleaching and metabolism. However, under high temperature, bacteria caused severe bleaching and decreased photosynthesis and calcification activity, suggesting that potentially pathogenic bacteria can have a dramatic negative impact on corals under thermal stress. However, respiration did not increase with the addition of bacteria under high temperature. The tested bacteria, especially *Sulfitobacter* sp., greatly enhanced and accelerated the bleaching process. These bacteria were isolated from the seawater surrounding the bleached corals and from the corals themselves. They can be found in the seawater around Okinawa. If their populations increase due to still undetermined factors, the damage to coral reefs in this area, which are under thermal stress with increasing frequency, could be dramatic.

2.2.5 Summary of Sect. 2.2

In this research the hypothesis that coral bleaching triggered by thermal stress disrupts coral metabolism, weakening the coral and allowing bacterial challenges to affect the host, was tested. This secondary process of bacterial challenges enhances and accelerates the bleaching process, which involves the loss of zooxanthellae and changes in metabolic processes. Such successional unfavorable events may become quite common under the combined effects of various environmental changes linked to climate change as higher seawater temperature and changes in water quality increasing the number of opportunistic pathogens in reef waters. The hypothesis was tested in the laboratory using the coral *Montipora digitata* and challenges from five bacterial species (*Vibrio coralliilyticus*, *Vibrio harveyi*, *Paracoccus carotinifaciens*, *Pseudoalteromonas* sp., and *Sulfitobacter* sp.) previously isolated from corals and their surrounding seawater.

Bacterial challenges in addition to high-temperature stress resulted in coral bleaching, with a 70 % decrease in zooxanthellae density compared with the control, a 25 % decrease in photosynthetic efficiency (*Fv*/*Fm*), a 66 % decrease in photosynthesis, and a 101 % reduction in calcification activity. Tissue necrosis was observed in the most compromised branches. Among the bacteria examined, *Sulfitobacter* sp. had a greater capacity to enhance and accelerate the bleaching process under thermal stress.

2.3 Synergistic Effects of Thermal Stress and High Nutrient Levels on the Scleractinian Coral *Pocillopora damicornis*

2.3.1 Introduction

Coral reefs, the most diverse marine ecosystem, are known to harbor a wide variety of marine organisms providing important goods and services to coastal communities through income generation such as tourism, fishing, and

building materials (Hoegh-Guldberg et al. 2007; Hughes et al. 2003). Coral reefs support high production even though nutrient levels are very low (oligotrophic). Typically, coral reef waters consist of 0.6 μM of nitrate (NO_3) and 0.2 μM of phosphate (PO_4), which are the main sources of nutrient for organism surviving in these waters (Kleypas 1994). The success of reef-building corals in this oligotrophic environment is attributed to the symbiosis with phototrophic dinoflagellates commonly known as zooxanthellae (Muscatine 1990). In this relationship, the coral host gets photosynthetic organic products from the zooxanthellae to fuel important processes like respiration, tissue growth, and calcification (Muscatine 1990). Disruption of this host–symbiont relationship eventually leads to coral bleaching by loss of algal cells and/or algal pigments (Douglas 2003; Hoegh-Guldberg 1999; Hughes et al. 2003; Suzuki et al. 2015). Several environmental factors like increased sea surface temperature, high light, low water quality, and bacterial infections have been found to be the cause of coral bleaching (Douglas 2003; Fitt et al. 2001; Higuchi et al. 2013; Hoegh-Guldberg 1999). In addition to these factors, excess input of nutrients in the marine ecosystem, due to sewage dumping, pollution, and runoff from agricultural areas, has been shifting the composition of coral reefs by favoring the outgrowth of macroalgae (Birkeland 1987; Fabricius and De'ath 2004; Stimson et al. 2001). However, the effects of high nutrient concentration in combination with higher than normal seawater temperature on corals are still not fully understood due to the complexity of the host–symbiont relationship. Studies showing no adverse effect on coral physiology in regions of high nutrient level, in addition with reports of increase in zooxanthellae cell density due to availability of high external nitrogen level, may seem to indicate that corals can survive in high nutrient areas (Agostini et al. 2012; Atkinson et al. 1995; Bongiorni et al. 2003; Fabricius 2005; Marubini and Davies 1996; Muscatine et al. 1989; Szmant 2002). However, recent studies have shown that anthropogenic nutrient input into the ocean can result in nutrient imbalance and cause unavailability of other nutrients like phosphate (Fabricius 2005; Parkhill et al. 2001; Wiedenmann et al. 2012). This may lead to phosphate starvation of the symbiont, causing a decrease in maximum quantum yield (*Fv/Fm*) and thus making the coral host more susceptible to bleaching (Parkhill et al. 2001; Wiedenmann et al. 2012).

Even though respond of corals to high temperature and high nitrate concentration has been widely researched, very little is known about recovery of these corals after the stress exposure. So, this study was carried out to investigate the effects of high temperature and high nitrate concentration on the coral *Pocillopora damicornis* and the ability of the coral holobiont to recover from these stresses. During the incubation experiment, measurement of maximum quantum yield (*Fv/Fm*) and maximum excitation pressure on photosystem II (*Qm*) was carried out together with analysis of pigment content of the zooxanthellae.

2.3.2 Testing Coral Response to Combined Stresses

Fragments of *Pocillopora damicornis* ($n = 32$) were sampled from Sesoko Beach, Okinawa, Japan (26°38′N 127° 51′E) at a depth of 1–3 m during low tide. After acclimatization for 3 days in a tank with running seawater and ambient light conditions, the coral nubbins were moved to the incubation system. The collected nubbins were placed in 800 ml containers at ambient temperature. The containers were supplied with filtered seawater by peristaltic pumps, and the use of stirrers provided water movement inside each container (Fujimura et al. 2008).

Incubation of *Pocillopora damicornis* ($n = 2$) with different seawater temperature and nitrate conditions was carried out so as to investigate the combined effects of high temperature and nitrate level. High temperature and high nitrate stress (HN32) was carried out by subjecting the nubbins to 32 °C and providing a constant supply of filtered seawater (0.2 μm) with a nitrate concentration of 10 μmol/L. The control (AN27) was kept at 27 °C and constantly supplied with ambient nitrate filtered seawater (without addition of nitrate). Conditions to evaluate the effect of high nitrate concentration (HN27) and high temperature (AN32) separately were also set up. The light level was kept at 200μmol$m^{-2}s^{-1}$ and followed a daily routine of 12-h light and 12-h dark cycle. High temperature and nitrate stress were carried out for 2 days followed by 2 days of recovery at ambient temperature and nitrate concentration. For evaluation on the effect of high nutrients under the thermal stress to the coral symbiont, measurement for chemical and biological parameters, examples PAM data, chlorophyll *a*, pigments, and zooxanthellae density.

2.3.3 Effect of High Nitrate Concentrations Under the Thermal Stress

Variation in photochemical efficiency of PSII (*Fv/Fm*) throughout the experiment indicated photophysiological stress in zooxanthellae of *P. damicornis* and subsequent recovery of the photosynthetic system of the zooxanthellae in different incubation conditions. The coral fragments that were incubated under high temperature (AN32), high nitrate (HN27), and high nitrate combined with high temperature (HN32) showed a significant decrease in *Fv/Fm* after 48 hrs of stress phase when compared with the control (AN27). During the first day of recovery, only corals under HN27

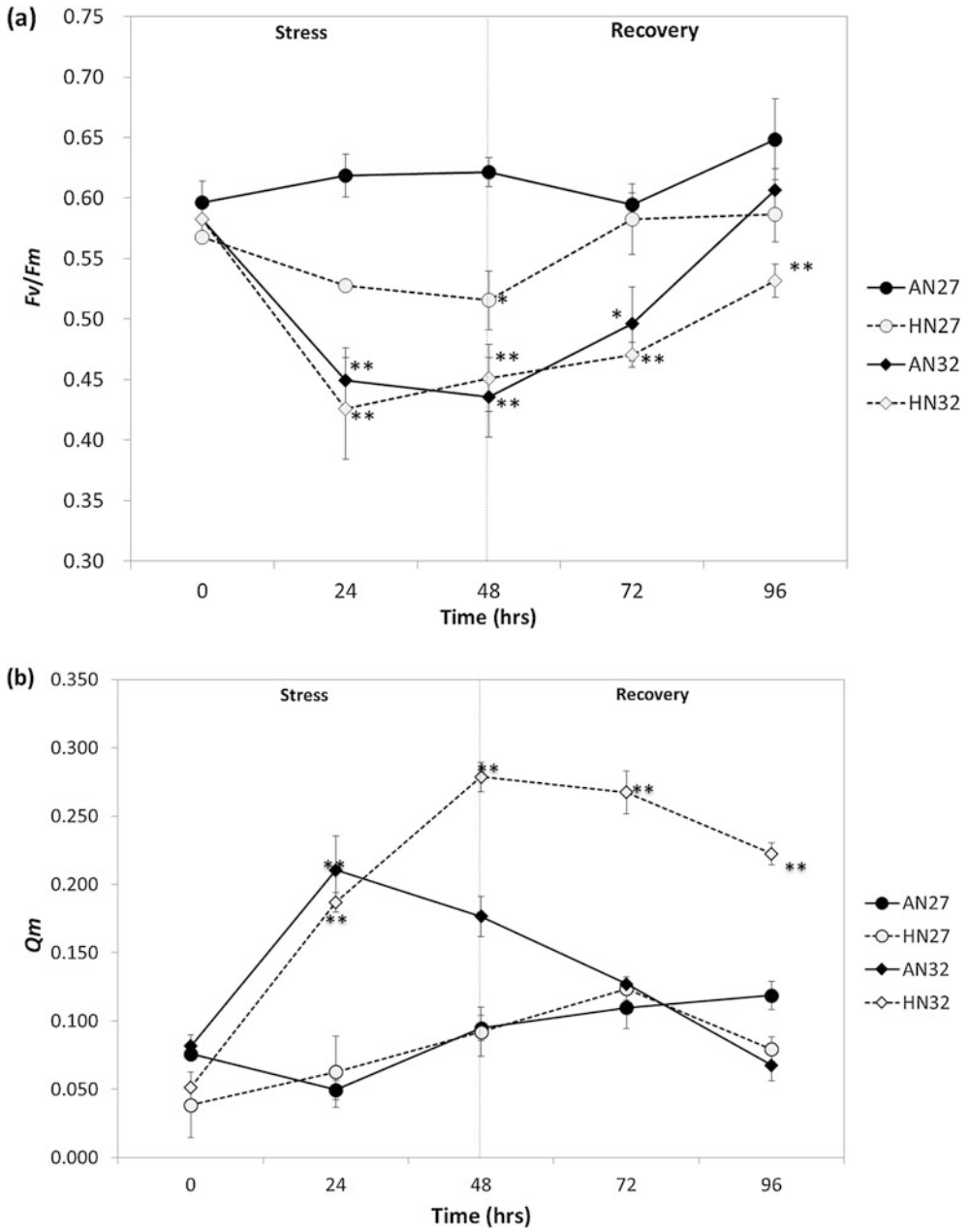

Fig. 2.12 (**a**) Changes in maximum quantum yield of corals during stress and recovery phases. (**b**) Changes in maximum excitation pressure on PSII of corals during stress and recovery phases. Different nitrate and temperature conditions were used during the incubation: ambient nitrate at 27 °C (AN27), high nitrate at 27 °C (HN27), ambient nitrate at 32 °C (AN32), and high nitrate at 32 °C (HN32). A three-way ANOVA test was performed and Tukey HSD post hoc test was then applied to the data for comparison with the control (AN27). Asterisks represent significant differences ($*p < 0.05$, $**p < 0.01$). Vertical bars represent standard deviation from mean value (n = 2)

showed almost complete recovery, whereas corals under AN32 and HN32 still had significantly lower *Fv*/*Fm* values. After 2 days of recovery phase, corals under all the conditions showed good recovery in *Fv*/*Fm*, except those subjected to HN32. For these corals, *Fv*/*Fm* (0.522 ± 0.014; $n = 2$) was significantly lower than the control (0.625 ± 0.033; $n = 2$) even after 48 h of recovery (Fig. 2.12).

Zooxanthellae density varied in the different incubation conditions after stress and recovery phases. After a 2-day stress period, zooxanthellae density of corals in HN27, AN32, and HN32 was significantly lower than the control (AN27) (Fig. 2.13). As for the recovery phase, an increase in zooxanthellae cell density was noted for corals in AN32 and HN32 with a significantly higher zooxanthellae cell density for HN32 when compared to the control (Fig. 2.13). When

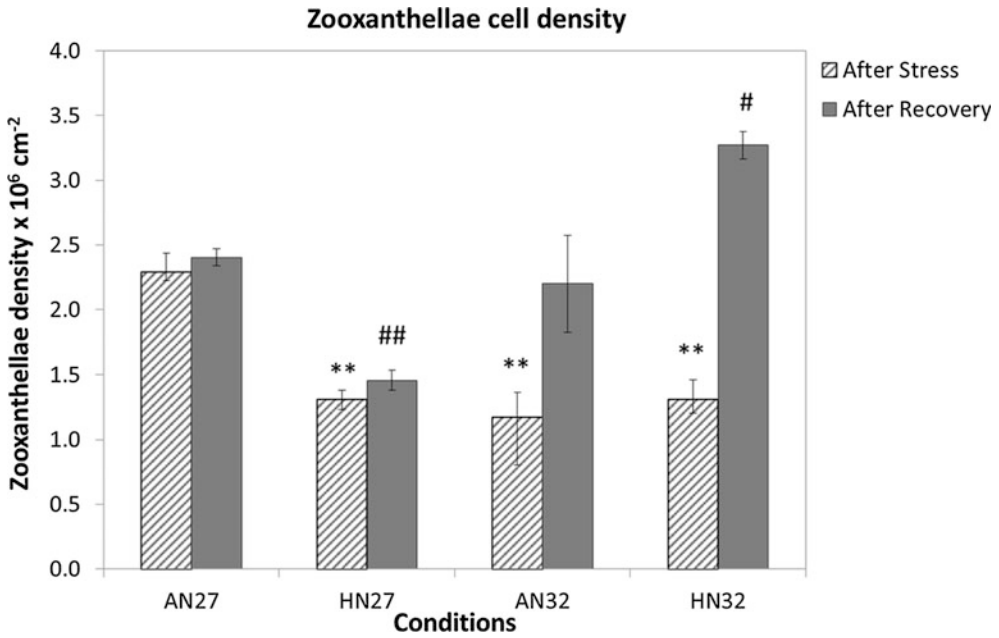

Fig. 2.13 Zooxanthellae cell density after stress and recovery periods. Corals incubated in ambient nitrate (AN27), high nitrate (HN27), high temperature (AN32), and a combination of high nitrate and high temperature (HN32) were tested statistically for significant differences when compared to control (AN27). A three-way ANOVA test and Tukey HSD post hoc test were used. * and # represent significant differences with the control for stress and recovery phases, respectively (*$p < 0.05$, *$p < 0.01$; #$p < 0.05$, ##$p < 0.01$). Vertical bars represent standard deviation from mean value ($n = 2$)

comparison was made with zooxanthellae density from after stress period, corals incubated in AN32 and HN32 showed an 88 % and 150 % increase, respectively. Corals in high nitrate condition (HN27), after the recovery phase, still had significantly lower zooxanthellae cells than the control and showed only an 11 % increase from the stress phase.

Zooxanthellae cells possess both chlorophyll *a* and peridinin as photosynthetic pigments. Contrary to chlorophyll *a*, which is also found in epilithic and endolithic algae associated with the coral, peridinin is specific to zooxanthellae cells and thus gives a more accurate indication of pigment changes in zooxanthellae cells. Chlorophyll *a* and peridinin content per cell in corals during the whole incubation experiment followed a similar trend. After the stress phase, no significant change was observed in chlorophyll *a* and peridinin contents (Fig. 2.14a, b) in all the conditions. After recovery, when compared to the control, both chlorophyll *a* and peridinin per cell decreased significantly for corals in HN32. For this condition, the chlorophyll *a* and peridinin content decreased by 44 % and 46 %, respectively, when compared with the stress phase.

2.3.4 Stress Phase

During the stress phase, zooxanthellae density decreased for all the condition compared to the control (Fig. 2.13). Even though zooxanthellae cell decreased, it was noted that chlorophyll *a* and peridinin concentration per cell was relatively unaffected (Fig. 2.14a, b). This indicated that the corals under nitrate or temperature stress may have released or digested zooxanthellae cells that have been damaged and cannot function properly. This mechanism prevented the formation of excess reactive oxygen species (ROS) by "unhealthy" zooxanthellae cells that can damage the host and remaining zooxanthellae (Lesser and Farrell 2004; Warner et al. 1999; Suzuki et al. 2014). Compared to other studies carried out, an increase in zooxanthellae cell density was not observed after incubation with high nitrate concentration only (Fabricius 2005; Miller and Yellowlees 1989). Nutrient imbalance due to the availability of abundant nitrate could be the cause of this decrease in zooxanthellae density. Under such case, phosphate usually becomes the limiting factor for growth and proliferation (Miller and Yellowlees 1989). Recent studies have shown that under phosphate deficiency, the anionic environment of the thylakoid membrane is disrupted by the excessive conversion of the phospholipid phosphatidylglycerol (PG) to the sulpholipid sulfoquinovosyldiacylglycerol (SQDG) (Frentzen 2004; Wiedenmann et al. 2012). This could explain the decrease of zooxanthellae cells for corals under high nitrate (Fig. 2.13).

Fv/*Fm* and *Qm* values throughout the experiment tend to indicate that the combined effect of high temperature and

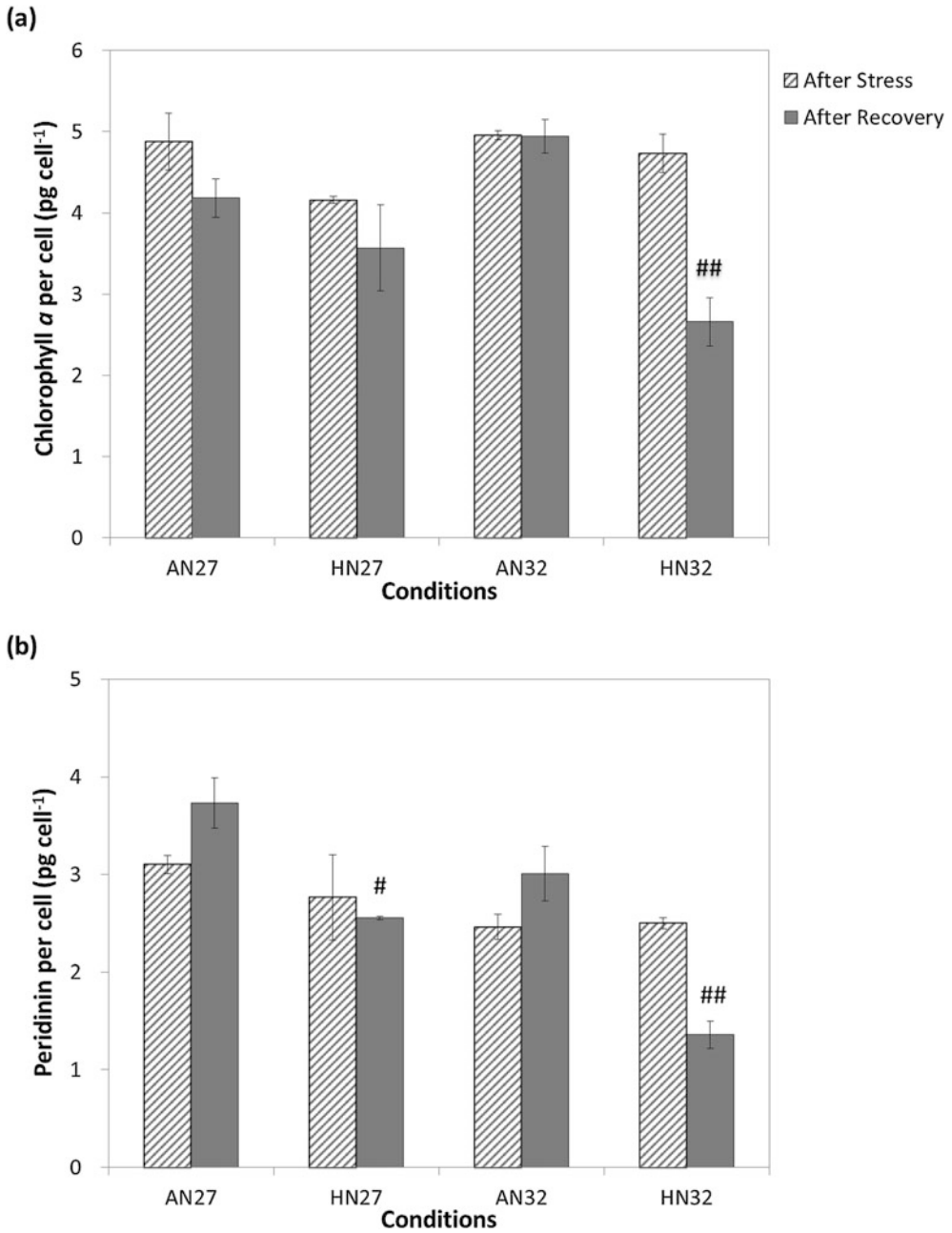

Fig. 2.14 Pigment content in zooxanthellae cells after stress and recovery periods. Chlorophyll *a* per cell **a** and peridinin per cell **b** were monitored for different incubation conditions: ambient nitrate (AN27), high nitrate (HN27), high temperature (AN32), and high nitrate together with high temperature (HN32). A three-way ANOVA test and Tukey HSD post hoc test were used to distinguish significant changes in pigment contents. # shows significant differences with the control for recovery period. No significant differences were found for after stress period (#$p < 0.05$, ##$p < 0.01$). Vertical bars represent standard deviation from mean value ($n = 2$)

high nitrate concentration may produce a more severe stress than high temperature alone (Fig. 2.12a, b). The decrease in *Fv*/*Fm* for both AN32 and HN32 can be attributed to the increase in ROS due to photoinhibition that can be harmful to both host and symbiont (Lesser and Farrell 2004), but higher *Qm* for HN32 after the stress phase may indicate that high nitrate can enhance temperature-mediated stress. In case of HN32, where the coral was already under thermal stress, high concentration of NO_3 could instigate production of nitric oxide (NO). Previous studies carried out on the plant enzyme nitrate reductase showed that this enzyme could reduce NO_3^- to NO, and in addition to this, NO synthesis by nitric oxide synthetase (NOS) in the coral holobiont was found to increase during thermal stress (Perez and Weis 2006; Trapido-Rosenthal et al. 2005; Yamasaki and Sakihama 2000). So, NO_3^- uptaken by zooxanthellae cells

can be reduced to nitrite (NO_2^-) and finally to NO through enzymatic reactions. Even though NO is a natural signaling molecule in animals, it also acts as a cytotoxic molecule. In the presence of ROS, the highly reactive peroxynitrite ($ONOO^-$) can be produced from NO (Weis 2008). The combination of ROS, NO, and $ONOO^-$ could cause damaged to the thylakoid membrane of zooxanthellae, thus increasing *Qm* and decreasing *Fv*/*Fm* for HN32 during the stress phase.

2.3.5 Recovery Phase

Response of corals during the recovery phase varied greatly according to the conditions they experienced during the stress phase. Zooxanthellae density among recovering corals showed an increase for AN32 and HN32, but the pigment contents did not show a similar trend. In case of AN32, the increase in zooxanthellae cells was accompanied with an unchanged concentration of chlorophyll *a* and peridinin per cell, whereas for HN32, a decrease in chlorophyll *a* and peridinin per cell was noted with increasing zooxanthellae density. This decrease in chlorophyll *a* content in zooxanthellae cells for HN32 could be the reason for the partial recovery of *Fv*/*Fm* compared to AN32 that showed almost complete recovery. Even though the zooxanthellae cell density increased more than the control, a below-normal pigment content could have hampered the recovery of corals that experienced both high nitrate concentration and high temperature.

Thermally stressed corals recovered by selective degradation and replacement of damaged proteins to repair affected PSII systems. As photosynthetic efficiency is restored, the coral is able to overcome the effects that occurred due to the stress. Increase in photosynthesis efficiency will allow production of more photosynthetic products by the zooxanthellae that will be used for restoration of damaged PSII sites and proliferation of healthy zooxanthellae cells. These energy-rich products, such as glycerol and glucose, are then translocated to the coral and used by the latter for respiration (Muscatine et al. 1984; Trench 1993). This is the case with AN32 where good recovery in *Fv*/*Fm* was observed. On the other hand, data obtained in this experiment may indicate that the damage caused during high nitrate and high temperature can impede the recovery of these corals. The combined effects of ROS, NO, and $ONOO^-$ could affect not only the thylakoid membrane but also directly degrade DNA content of both host and symbiont which may result in cell apoptosis (Pacher et al. 2007; Weis 2008). So, during the recovery phase, above normal zooxanthellae proliferation could occur to compensate for the damage. High density of low chlorophyll *a* content cells may help the coral to overcome the effects of the stress phase, and when it achieves constant photosynthetic efficiency, these cells are replaced by healthier ones.

This study demonstrated that an increase in nitrate and temperature could be harmful to the coral *Pocillopora damicornis*. Also recovery can be more difficult due to the severity of damage that occurred during the stress period. Short and sudden increases in nitrate and temperature levels can impede recovery of the coral so that they are more susceptible to other environmental or anthropogenic stresses. Care should be taken when judging the impacts of high nitrate and high temperature as acclimatization to these new environmental conditions may require longer exposure time in further experiments.

2.3.6 Summary for Sect. 2.3

Increase in sea surface temperature and excessive input of nutrient in reef waters, due to anthropogenic activities, have been among the various factors responsible for bleaching and mortality of corals around the world. Moreover synergistic effects of these environmental stressors can accelerate and aggravate bleaching process and infections of possible opportunistic pathogens that take advantage of these environmental changes.

In this study the effects of elevated nitrate (NO_3) concentration in combination with high seawater temperature on the coral, *Pocillopora damicornis*, and the ability of the coral holobiont to recover from these stresses were investigated. Coral fragments were incubated for 2 days at different temperatures (27 °C and 32 °C) and NO_3 concentrations (<1 μmol/L and 10 μmol/L). Following 2 days of stress under 32 °C and 10 μmol/L NO_3, the nubbins were moved to 27 °C and ambient (<1 μmol/L) NO_3 levels for 2 days of recovery period. Maximum quantum yield (*Fv*/*Fm*) and maximum excitation pressure (*Qm*) at photosystem II indicated that combined effects of high temperature and high NO_3 were more severe and only corals under these conditions exhibited an incomplete recovery. Furthermore, zooxanthellae density and pigment data showed that the response mechanism of these coral nubbins was different from high temperature or high NO_3 stress only. During the recovery phase, zooxanthellae density was higher (~0.5-fold), whereas chlorophyll *a* per cell was lower (~0.5-fold) than the control for combined stress of high nitrate and temperature. Hence, this study showed that under thermal stress, high nitrate amplifies damage to the zooxanthellae of the coral *Pocillopora damicornis* and recovery of the holobiont is more difficult after the stress.

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References

Agostini S, Suzuki Y, Casareto BE, Nakano Y, Hidaka M, Badrun N (2009) Coral symbiotic complex: hypothesis through vitamin B12 for a new evaluation. Galaxea JCRS 11:1–11

Agostini S, Suzuki Y, Higuchi T, Casareto BE, Yoshinaga K, Nakano Y, Fujimura H (2012) Biological and chemical characteristics of the coral gastric cavity. Coral Reefs 31 (1):147–156

Atkinson MJ, Carlson B, Crow GL (1995) Coral growth in high-nutrient, low-pH seawater: a case study of corals cultured at the Waikiki Aquarium, Honolulu, Hawaii. Coral Reefs 14:215–223

Ben-Haim Y, Zicherman-Keren M, Rosenberg E (2003) Temperature-regulated bleaching and lysis of the coral *Pocillopora damicornis* by the novel pathogen *Vibrio coralliilyticus*. Appl Environ Microbiol 69:4236–4242

Bhagooli R, Hidaka M (2002) Physiological responses of the coral *Galaxea fascicularis* and its algal symbiont to elevated temperatures. Galaxea JCRS 4:33–42

Bhagooli R, Hidaka M (2003) Comparison of stress susceptibility of in hospite and isolated zooxanthellae among five coral species. J Exp Mar Biol Ecol 291:181–197

Bhagooli R, Hidaka M (2006) Thermal inhibition and recovery of the maximum quantum yield of photosystem II and the maximum electron transport rate in zooxanthellae of a reef-building coral. Galaxea JCRS 8:1–11

Birkeland C (1987) Nutrient availability as a major determinant of differences among coastal hard-substratum communities in different regions of the tropics. In: Comparison between Atlantic and Pacific tropical marine coastal ecosystems: community structure, ecological processes, and productivity, UNESCO Reports in Marine Science. UNESCO, Paris, pp 45–97

Bongiorni L, Shafir S, Angel D, Rinkevich B (2003) Survival, growth and gonad development of two hermatypic corals subjected to in situ fish-farm nutrient enrichment. Mar Ecol Prog Ser 253:137–144

Bourne D, Iida Y, Uthicke S, Smith-Keune C (2008) Changes in coral-associated microbial communities during a bleaching event. ISME J 2:350–363

Brown BE, Le Tessier MDA, Bythell JC (1995) Mechanisms of bleaching deduced from histological studies of reef corals sampled during a natural bleaching event. Mar Biol 122:655–663

Chillier XFD, Gulacar FO, Buchs A (1993) A novel sedimentary lacustrine chlorin: characterization and geochemical significance. Chemosphere 27:2103–2110

Douglas AE (2003) Coral bleaching—how and why? Mar Pollut Bull 46:385–392

Downs CA, Fauth JE, Halas JC, Dustan P, Bemiss J, Woodley CM (2002) Oxidative stress and seasonal coral bleaching. Free Radic Biol Med 33:533–543

Downs CA, Kramarsky-Winter E, Martinez J, Kushmaro A, Woodley CM, Loya Y, Ostrander GK (2009) Symbiophagy as a cellular mechanism for coral bleaching. Autophagy 5(2):211–216

Downs CA, McDougall KE, Woodley CM, Fauth JE, Richmond RH, Kushmaro A, Gibb SW, Loya Y, Ostrander GK, Kramarsky-Winter E (2013) Heat-stress and light-stress induce different cellular pathologies in the symbiotic dinoflagellate during coral bleaching. PLoS ONE 8(12):e77173. doi:10.1371/journal.pone.0077173

Dykens JA, Shick JM, Benoit C, Buettner GR, Winston GW (1992) Oxygen radical production in the sea-anemone *Anthopleura elegantissima* and its endosymbiotic algae. J Exp Biol 168:219–241

Fabricius KE (2005) Effects of terrestrial runoff on the ecology of corals and coral reefs: review and synthesis. Mar Pollut Bull 50:125–146

Fabricius KE, De'ath G (2004) Identifying ecological change and its causes: a case study on coral reefs. Ecol Appl 14:1448–1465

Fankboner PV (1971) Intracellular digestion of symbiotic zooxanthellae by host amoebocytes in giant clams (Bivalvia: Tridacnidae), with a note on the nutritional role of the hypertrophical siphonal epidermis. Biol Bull 141:222–234

Fitt WK, Cook CB (1990) Some effect of host feeding on growth of zooxanthellae in the marine hydroid *Myrionema amblonense* in the laboratory and in nature. In: Nardon P, Gianinazzi-Pearson V, Grenier AM, Margulis L, Smith DC (eds) Endocytobiology IV. INRA, Paris, pp 281–284

Fitt WK, Warner ME (1995) Bleaching patterns of four species of Caribbean reef corals. Biol Bull 189:298–307

Fitt WK, Brown BE, Warner ME, Dunne RP (2001) Coral bleaching: interpretation of thermal tolerance limits and thermal thresholds in tropical corals. Coral Reefs 20:51–65

Frentzen M (2004) Phosphatidylglycerol and sulfoquinovosyldiacylglycerol: anionic membrane lipids and phosphate regulation. Curr Opin Plant Biol 7:270–276

Fujimura H, Higuchi T, Shiroma K, Arakaki T, Hamdun AM, Nakano Y, Oomori T (2008) Continuous-flow complete-mixing system for assessing the effects of environmental factors on colony-level coral metabolism. J Biochem Biophys Methods 70:865–872

Fujise L, Yamashiya H, Suzuki G, Koike K (2013) Expulsion of zooxanthellae (*Symbiodinium*) from several species of scleractinian corals: comparison under non-stress conditions and thermal stress conditions. Galaxea JCRS 15:29–36

Fukabori Y (1998) Whitening mechanism of reef building coral. Decomposition and discharge of symbiotic algae. Umiushi Tsushin 18:2–4 (in Japanese)

Gates RD (1990) Seawater temperature and sublethal coral bleaching in Jamaica. Coral Reefs 8:193–198

Goericke R, Strom SL, Bell MA (2000) Distribution and sources of cyclic pheophorbides in the marine environment. Limnol Oceanogr 45:200–211

Gomez-Gil B, Soto-Rodríguez S, García-Gasca A, Roque A, Vazquez-Juarez R, Thompson FL, Swings J (2004) Molecular identification of *Vibrio harveyi*-related isolates associated with diseased aquatic organisms. Microbiology (Reading, England) 150:1769–1777

Harris PG, Pearce GES, Peakman TM, Maxwell JR (1995) A widespread and abundant chlorophyll transformation product in aquatic environments. Org Geochem 23:183–187

Higuchi T, Agostini S, Casareto BE, Yoshinaga K, Suzuki T, Nakano Y, Fujimura H, Suzuki Y (2013) Bacterial enhancement of bleaching and physiological impacts on the coral *Montipora digitata*. J Exp Mar Biol Ecol 440:54–60

Hoegh-Guldberg O (1999) Climate change, coral bleaching and the future of the world's coral reefs. Mar Freshw Res 50:839–866

Hoegh-Guldberg O, Smith GJ (1989) The effects of sudden changes in light, temperature and salinity on the population density and export of zooxanthellae from the reef corals *Seriatopora hystrix* and *Stylophora pistillata*. J Exp Mar Biol Ecol 129:279–303

Hoegh-Guldberg O, Mumby PJ, Hooten AJ, Steneck RS, Greenfield P, Gomez E, Harvell CD, Sale PF, Edwards AJ, Caldeira K,

Knowlton N, Eakin CM, Iglesias-Prieto R, Muthiga N, Bradbury RH, Dubi A, Hatziolos ME (2007) Coral reefs under rapid climate change and Ocean acidification. Science 318:1737–1742

Hughes TP, Baird AH, Bellwood DR, Card M, Connolly SR, Folke C, Grosberg R, Hoegh-Guldberg O, Jackson JBC, Kleypas J, Lough JM, Marshall P, Nyström M, Palumbi SR, Pandolfi JM, Rosen B, Roughgarden J (2003) Climate change, human impacts, and the resilience of Coral Reefs. Science 301:929–933

Jones RJ (1997) Changes in zooxanthellar densities and chlorophyll concentrations in corals during and after a bleaching event. Mar Ecol Prog Ser 158:51–59

Karuso P, Bergquist PR, Buckleton JS, Cambie RC, Clark GR, Rickard CEF (1986) 13^2, 17^3-Cyclopheophorbide enol, the first porphyrin isolated from a sponge. Tetrahedron Lett 27:2177–2178

Kashiyama Y, Tamiaki H (2014) Risk management by organisms of the phototoxicity of chlorophylls. Chem Lett 43:148–156

Kashiyama Y, Yokoyama A, Kinoshita Y, Shoji S, Miyashiya H, Shiratori T, Suga H, Ishikawa K, Ishikawa A, Inouye I, Ishida K, Fujinuma D, Aoki K, Kobayashi M, Nomoto S, Mizoguchi T, Tamiaki H (2012) Ubiquity and quantitative significance of detoxification catabolism of chlorophyll associated with protistan herbivory. Proc Natl Acad Sci U S A 109:17328–17335

Kashiyama Y, Yokoyama A, Shiratori T, Inouye I, Kinoshita Y, Mizoguchi T, Tamiaki H (2013) 13^2,17^3-Cyclopheophorbide *b* enol as a catabolite of chlorophyll *b* in phycophagy by protists. FEBS Lett 587:2578–2583

Kashiyama Y, Yokoyama A, Tamiaki H (2014) 13^2,17^3-Cyclopheophorbide enols as detoxified catabolites of chlorophylls – a widely distributed metabolism among phototrophic/heterotrophic protists. Abstract with peer review of 8th international conference on Porphyrins and Phthalocyanines (ICPP) abs121382.

Kleypas JA (1994) A diagnostic model for predicting global coral reef distribution. In: Bellwood O, Choat H, Saxena N (eds) Recent advances in Marine Science and Technology. PACON International and James Cook University, Townsville, pp 211–220

Kuroki T, van Woesik R (1999) Changes in zooxanthellae characteristics in the coral *Stylophora pistillata* during the 1998 bleaching event. Galaxea JCRS 1:97–101

Kushmaro A, Rosenberg E, Fine M, Loya Y (1997) Bleaching of the coral *Oculina patagonica* by Vibrio AK-1. Mar Ecol Prog Ser 147:159–165

Lesser MP, Farrell JH (2004) Exposure to solar radiation increases damage to both host tissues and algal symbionts of corals during thermal stress. Coral Reefs 23:367–377

Lesser MP, Stochaj WR, Tapley DW, Shick JM (1990) Bleaching in coral reef anthozoans: effects of irradiance, ultraviolet radiation, and temperature on the activities of protective enzymes against active oxygen. Coral Reefs 8:225–232

Louda JW, Loitz JW, Rudnick DT, Baker EW (2000) Early diagenetic alteration of chlorophyll-*a* and bacteriochlorophyll-*a* in a contemporaneous marl ecosystem; Florida Bay. Organic Geochem 31:1561–1580

Louda JW, Neto RR, Magalhaes ARM, Schneider VF (2008) Pigment alterations in the brown mussel *Perna perna*. Comp Biochem Physiol B 150:385–394

Marubini F, Davies PS (1996) Nitrate increases zooxanthellae population density and reduces skeletogenesis in corals. Mar Biol 127:319–328

Miller DJ, Yellowlees D (1989) Inorganic nitrogen uptake by symbiotic marine Cnidarians: a critical review. Proc R Soc Lond B Biol Sci 237:109–125

Mise T, Hidaka M (2003) Degradation of zooxanthellae in the coral *Acropora nasuta* during bleaching. Galaxea JCRS 5:33–40

Muller EM, Rogers CS, Spitzack AS, van Woesik R (2007) Bleaching increases likelihood of disease on *Acropora palmata* (Lamarck) in Hawksnest Bay, St John, US Virgin Islands. Coral Reefs 27:191–195

Muscatine L (1990) The role of symbiotic algae in carbon and energy flux in reef corals. In: Coral reefs, ecosystems of the world. Elsevier, Amsterdam, pp 75–87

Muscatine L, Falkowski PG, Porter JW, Dubinsky Z (1984) Fate of photosynthetic fixed carbon in light- and shade-adapted colonies of the symbiotic coral *Stylophora pistillata*. Proc R Soc Lond B 222:181–202

Muscatine L, Falkowski PG, Dubinsky Z, Cook PA, McCloskey LR (1989) The effect of external nutrient resources on the population dynamics of zooxanthellae in a reef coral. Proc R Soc Lond B Biol Sci 236:311–324

Mydlarz LD, Couch CS, Weil E, Smith G, Harvell CD (2009) Immune defenses of healthy, bleached and diseased *Montastraea faveolata* during a natural bleaching event. Dis Aquat Organ. Special Issue 5; The role of environment and microorganisms in diseases of corals. 87: 67–78

Ocampo R, Sachs JP, Repeta DJ (1999) Isolation and structure determination of the unstable 13^2, 17^3-Cyclopheophorbide *a* enol from recent sediments. Geochim Cosmochim Acta 63:3743–3749

Pacher P, Beckman JS, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87:315–424

Papina M, Meziane T, van Woesik R (2007) Acclimation effect on fatty acids of the coral *Montipora digitata* and its symbiotic algae. Comp Biochem Physiol B 147:583–589

Parkhill J-P, Maillet G, Cullen JJ (2001) Fluorescence-based maximal quantum yield for PSII as a diagnostic of nutrient stress. J Phycol 37:517–529

Perez S, Weis VM (2006) Cnidarian bleaching and nitric oxide: an eviction notice mediates the breakdown of symbiosis. J Exp Biol 209:2804–2810

Perl-Treves R, Perl A (2002) Oxidative stress: an introduction. In: Van Montagu M, Inzé D (eds) Oxidative stress in plants. Taylor and Francis Books Ltd, London, pp 1–32

Reimer JD, Ono S, Furushima Y, Tsukahara J (2007) Seasonal changes in morphological condition of symbiotic Dinoflagellates (*Symbiodinium* spp.) in *Zoanthus sansibaricus* (Anthozoa: Hexacorallia) in southern Japan. South Pacific Stud 27:1–24

Ritchie K (2006) Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. Mar Ecol Prog Ser 322:1–14

Rosenberg E, Falkovitz L (2004) The *Vibrio shiloi*/*Oculina patagonica* model system of coral bleaching. Annu Rev Microbiol 58:143–159

Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I (2007) The role of microorganisms in coral health, disease and evolution. Nat Rev Microbiol 5:355–362

Sakata K, Yamamoto K, Ishikawa H, Yagi A, Etoh H, Ina K (1990) Chlorophyllone *a*, a new phaeophorbide *a* related compound isolated from *Ruditapes philippinarum* as an antioxidative compound. Tetrahedron Lett 31:1165–1168

Steele RD, Goreau NI (1977) The breakdown of symbiotic zooxanthellae in the sea anemone *Phyllactis* (= *Oulactis*) *flosculifera* (Actiniaria). J Zool (Lond) 181:421–437

Stimson J, Larned S, Conklin E (2001) Effects of herbivory, nutrient levels, and introduced algae on the distribution and abundance of the invasive macroalga *Dictyosphaeria cavernosa* in Kaneohe Bay, Hawaii. Coral Reefs 19:343–357

Sussman M, Willis BL, Victor S, Bourne DG (2008) Coral pathogens identified for White Syndrome (WS) epizootics in the Indo-Pacific. PloS ONE 3:e2393. doi:10.1371/journal.pone.0002393

Sutherland K, Porter J, Torres C (2004) Disease and immunity in Caribbean and Indo-Pacific zooxanthellate corals. Mar Ecol Prog Ser 266:273–302

Suzuki Y, Casareto BE (2011) The role of Dissolved Organic Nitrogen (DON) in coral biology and reef ecology. In: Dubinsky Z, Stamler N

(eds) Coral reefs: an ecosystem in transition. Springer, Dordrecht, pp 207–214

Suzuki T, Casareto BE, Shioi Y, Ishikawa Y, Suzuki Y (2015) Finding of 13^2, 17^3–cyclopheophorbide *a* enol as degradation product of chlorophyll in shrunk zooxanthellae of the coral *Montipora digitata*. J Phycol 51(1):37–45. doi:10.1111/jpy.12253

Szmant AM (2002) Nutrient enrichment on coral reefs: is it a major cause of coral reef decline? Estuaries 25:743–766

Titlyanov EA, Titlyanova TV, Leletkin VA, Tsukahara J, van Woesik R, Yamazato K (1996) Degradation of zooxanthellae and regulation of their density in hermatypic corals. Mar Ecol Prog Ser 139:167–178

Titlyanov EA, Titlyanova TV, Loya Y, Yamazato K (1998) Degradation and proliferation of zooxanthellae in planulae of the hermatypic coral *Stylophora pistillata*. Mar Biol 130:471–477

Titlyanov EA, Titlyanova TV, Yamazato K, van Woesik R (2001) Photo-acclimation of the hermatypic coral *Stylophora pistillata* while subjected to either starvation or food provisioning. J Exp Mar Biol Ecol 257:163–181

Trapido-Rosenthal H, Zielke S, Owen R, Buxton L, Boeing B, Bhagooli R, Archer J (2005) Increased zooxanthellae nitric oxide synthase activity is associated with coral bleaching. Biol Bull 208:3–6

Trench R (1993) Microalgal-invertebrate symbioses – a review. Endocyt Cell Res 9:135–175

Warner ME, Fitt WK, Schmidt GW (1999) Damage to photosystem II in symbiotic dinoflagellates: a determinant of coral bleaching. Proc Natl Acad Sci U S A 96:8007–8012

Watanabe N, Yamamoto K, Ishikawa H, Yagi A, Sakata K, Brinen LS, Clardy J (1993) New chlorophyll-*a*-related compounds isolated as antioxidants from marine bivalves. J Nat Prod 56:305–317

Weis VM (2008) Cellular mechanisms of Cnidarian bleaching: stress causes the collapse of symbiosis. J Exp Biol 211:3059–3066

Wiedenmann J, D'Angelo C, Smith EG, Hunt AN, Legiret FE, Postle AD, Achterberg EP (2012) Nutrient enrichment can increase the susceptibility of reef corals to bleaching. Nat Clim Chang 3:160–164

Yamada N, Tanaka A, Horiguchi T (2013) cPPB-*a*E is discovered from photosynthetic benthic dinoflagellates. J Phycol 50(1):101–107

Yamamoto K, Sakata K, Watanabe N, Yagi A, Brinen LS, Clardy J (1992) Chlorophyllonic acid and methyl ester, a new chlorophyll *a* related compound isolated as an antioxidant from short-necked clam, *ruditapes philippinarum*. Tetrahedron Lett 33:2587–2588

Yamasaki H, Sakihama Y (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. FEBS Lett 468:89–92