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The combined effects of nitrate with high temperature and high light intensity on coral bleaching and antioxidant enzyme activities

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HIGHLIGHTS

- We studied the multiple effects of high nitrate, high temperature and high light on coral bleaching.
- Nitrate alone was not enough to induce stress with ambient temperature and low light.
- Enrichment of nitrate accelerated coral bleaching under high light or high temperature.
- The negative correlation between zooxanthellae density and antioxidant enzyme activities was found.

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ABSTRACT

The frequency and severity of coral bleaching events have increased in recent decades. Regionally, human-attributed nutrient pollution, particularly nitrate, has increased in coastal areas due to inflow from rivers and groundwater. The combined effects of increased nitrate concentrations with high temperature or high light on bleaching events and reactive oxygen species levels (ROS) were tested. Coral fragments of *Montipora digitata* were incubated for 3 and 6 days at different light intensities (200 and 600 μmol m^{-2} s^{-1}), temperatures (27 and 32 °C), and nitrate concentrations (<1 and 10 μM). Quantum yields of the photosynthetic electron transport system were significantly decreased under high-temperature conditions. Because there were strong correlations between zooxanthellae density and antioxidant enzyme activities (superoxide dismutase and catalase), we hypothesized that a decrease in zooxanthellae occurred due to oxidative stresses. Our results show that enrichment of nitrate can accelerate coral bleaching under high light or high temperature conditions compared with single stress conditions. If the concentration of nitrate increases damage to coral reefs in coastal areas could significantly increase under high temperature or high light stress.

1. Introduction

Environmental factors such as increased seawater temperature, high light, high UV radiation levels, and low water quality have been shown to cause coral bleaching (Hoegh-Guldberg, 1999; Fitt et al., 2001; Yuyama et al., 2012). Bleaching can be defined as the loss of coral pigmentation, which can result from decreased numbers of zooxanthellae in the host tissue and/or the degradation of photosynthetic pigments in symbiotic algae (Hoegh-Guldberg and Smith, 1989). The frequency and severity of bleaching events have increased in recent decades (Baker et al., 2008). Under stressful conditions, reactive oxygen species (ROS) are produced (Dykens et al., 1992; Higuchi et al., 2010), disrupting the symbiotic relationship between the coral host and its photosynthetic symbiont (Lesser et al., 1990; Lesser, 1997). High temperatures reduce the threshold for photoinhibition, and a decrease in the Fv/Fm ratio during stress is a measure of damage to the photosystem II of zooxanthellae (Bhagooli and Hidaka, 2004). The cellular response to the formation of ROS includes many antioxidant mechanisms (Shick et al., 1995). Enzymes such as superoxide dismutase (SOD) and catalase (CAT) are responsible for detoxifying ROS, and their elevated activities indirectly indicate increased ROS production in

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corals as a result of environmental stresses, such as temperature, irradiance, and UV radiation (Lesser et al., 1990).

In certain regions, nutrient pollution, especially nitrate, has increased in coastal areas due to inflow from rivers and groundwater (Umezawa et al., 2002; Higuchi et al., 2014). Human activity has increased nutrient pollution in multiple ways, most notably through the increased use of chemical fertilizers in agriculture (Umezawa et al., 2002). High nutrient levels have an indirect effect on coral. High nutrient inputs lead to competitive algal blooms, which block sunlight; this, in turn, restricts coral growth and induces a shift from a coral to a macroalgae community (McCook et al., 2001; Bellwood et al., 2004). In the field, a strong correlation between coral bleaching severity and nutrient enrichment in seawater has been found (Wagner et al., 2010). Despite these findings, other studies have shown that high nutrient levels have no adverse effects on coral physiology, adding to the confusion on this subject (Atkinson et al., 1995; Szmant, 2002; Bongiorni et al., 2003). Marubini and Davies (1996) reported that nitrate increases zooxanthellae population density and reduces skeletogenesis, both of which are direct effects of nutrient enrichment. In addition to studies reporting that high nitrate alone can negatively affect coral (Fabricius, 2005), coral bleaching has been seen under conditions that combined high nitrate levels with high temperatures (Nordemar et al., 2003; Wiedenmann et al., 2012). Tanaka et al. (2014) reported that high-temperature-induced bleaching is not accelerated under enrichment of both nitrate and phosphate. However, the effect of high nutrient concentrations on coral under multiple stress conditions is still not fully understood due to the complexity of the host–symbiont relationship. In coastal areas, nitrate enrichment (around 10 μM) has been reported, although levels of both ammonium and phosphate ions have not increased (Meekaew et al., 2014). Therefore, this study determined the combined effects of various environmental changes linked to climate change (e.g., high temperature and high light) with increased nitrate concentrations in reef waters on bleaching events and ROS levels in corals.

2. Materials and methods

2.1. Coral specimens

Colonies of the branching coral *Montipora digitata* were collected from a coastal region off Okinawa Island, Japan, with permission from the Okinawa prefectural government (No. 24-49). Coral branch tips (ca. 4 cm long) from large coral colonies were cut and attached to a polyethylene net. Branches were kept for 2 weeks in an outdoor aquarium with running seawater to allow for recovery from sampling stress.

2.2. Experimental design

A continuous-flow complete-mixing (CFCM) experimental system described by Fujimura et al. (2008) was used for incubation of the coral colonies. Two levels of nitrate concentration (<1, 10 μM) were tested with two different temperatures (27, 32 °C) and two different levels of photon flux density (200, 600 μmol m^{-2} s^{-1} during a 12:12-h light:dark cycle), for 3- and 6-day periods. Seawater was pumped into the incubation bottles by peristaltic pumps, and metal halide lamps were used as a light source. Three coral branch tips were used in each treatment. Coral branch tips were placed into 400-ml glass incubation bottles, with a flow rate of 10 ml min^{-1} during incubation periods.

2.3. Sample treatments and measurements

After sampling the branches, the maximum quantum efficiency of Photosystem II (Fv/Fm) was measured using a junior PAM (Walz, Germany) after 30 min of dark adaptation. Coral tissues were treated according to Higuchi et al. (2009). Tissue homogenates were prepared using the air-jet method, in which 100 mM phosphate buffer was sprayed to strip the tissue from the coral skeleton. Then, 15 ml of the non-fixed sample was separated into host coral tissue and endosymbiont zooxanthellae (Zoox) by centrifugation (time and speed as in Higuchi et al., 2009). The zooxanthellae fraction was used for counting zooxanthellae density. Zooxanthellae were lysed by sonication in phosphate buffer with 0.025% Triton X-100. SOD activity, which reflects changes in superoxide anion concentrations, was assayed spectrophotometrically as described by Elstner and Heupel (1976) and Oyanagui (1984). Standards for activity were prepared using bovine erythrocytic SOD (Sigma) for each set of samples. CAT activity, which is responsible for inactivating hydrogen peroxide (H_2O_2), was measured by determining depletion of H_2O_2 at 240 nm (Beers and Sizer, 1952). All assays were conducted at 25 °C immediately after sampling, and enzyme activity is expressed as units (U) per mg protein. Protein content was determined by the Bradford assay (Bradford, 1976). Statistical analyses were performed using three-way analysis of variance (ANOVA) and Tukey–Kramer honestly significant difference (HSD) tests (JMP 8.0, SAS).

3. Results

3.1. Effect on zooxanthellae

The density of zooxanthellae in *M. digitata* did not change significantly over time under high nitrate (10 μM) only (N) or high light (600 μmol m^{-2} s^{-1}) only (HL) conditions for 6 days, although zooxanthellae density slightly increased under high nitrate conditions, and slightly decreased under high light conditions (Fig. 1(a)). With high temperature (32 °C) only (HT), zooxanthellae density was not significantly changed by day 3, but had significantly decreased by day 6, compared with the control (C) ($P < 0.05$). Combining both high light and high temperature with high nitrate levels (HLN and HTN) significantly decreased zooxanthellae density by day 3 ($P < 0.05$). After 6 days of incubation, about 70% of zooxanthellae were lost under HLN, HT, and HTN conditions compared with the control, although no significant difference was found among these three conditions. A significant difference was observed between high light conditions with nitrate and high light conditions without nitrate on days 3 and 6. A significant difference was also found under high temperature conditions by day 3, both with and without nitrate.

The Fv/Fm ratio (Fig. 1(b)) decreased significantly ($P < 0.05$) with HT and HTN treatments compared with control (C). The Fv/Fm under high temperature was equal to about 70% of control on day 6. On the other hand, the Fv/Fm values were not changed with N, HL, and HLN treatments.

3.2. Antioxidant enzymes (SOD and CAT)

Fig. 2(a) and (b) show the effects of high light, high temperature, and nitrate levels on the SOD activity of host coral and zooxanthellae. SOD activities of both the host and zooxanthellae were significantly increased under HL, HLN, HT, and HTN conditions ($P < 0.05$) compared with control. However, SOD activities were not changed by high nitrate alone.

Fig. 3 shows the effects of high light, high temperature, and nitrate levels on CAT activity of host coral. In host tissue, CAT activities, compared with control, were significantly increased ($P < 0.05$) by HL, HLN, HT, and HTN treatments, but were not changed by N treatment alone.