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Research

Symbiodinium sp. cells produce light-induced intra- and extracellular singlet oxygen, which mediates photodamage of the photosynthetic apparatus and has the potential to interact with the animal host in coral symbiosis

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Summary

• Coral bleaching is an important environmental phenomenon, whose mechanism has not yet been clarified. The involvement of reactive oxygen species (ROS) has been implicated, but direct evidence of what species are involved, their location and their mechanisms of production remains unknown.

• Histidine-mediated chemical trapping and singlet oxygen sensor green (SOSG) were used to detect intra- and extracellular singlet oxygen ($^1O_2$) in *Symbiodinium* cultures.

• Inhibition of the Calvin–Benson cycle by thermal stress or high light promotes intracellular $^1O_2$ formation. Histidine addition, which decreases the amount of intracellular $^1O_2$, provides partial protection against photosystem II photoinactivation and chlorophyll (Chl) bleaching. $^1O_2$ production also occurs in cell-free medium of *Symbiodinium* cultures, an effect that is enhanced under heat and light stress and can be attributed to the excretion of $^1O_2$-sensitizing metabolites from the cells. Confocal microscopy imaging using SOSG showed most extracellular $^1O_2$ around the cell surface, but it is also produced across the medium distant from the cells.

• We demonstrate, for the first time, both intra- and extracellular $^1O_2$ production in *Symbiodinium* cultures. Intracellular $^1O_2$ is associated with photosystem II photodamage and pigment bleaching, whereas extracellular $^1O_2$ has the potential to mediate the breakdown of symbiotic interaction between zooxanthellae and their animal host during coral bleaching.

Introduction

Reef-building corals, which belong to the class Anthozoa (Cnidaria), form an endosymbiosis with dinoflagellate algae from the genus *Symbiodinium* (also known as zooxanthellae), which reside within the endodermal tissue of the anthozoan host. In this symbiotic relationship, the algae provide organic carbon in the form of photosynthates to their host to support metabolic energy requirements, and the zooxanthellae are given protection and receive inorganic compounds, such as CO_2 and nitrogen compounds, from the host, which creates a sustainable environment for coral physiology (see Yellowless *et al.*, 2008). As a result of environmental stressors, the symbiosis between the coral host and its symbiotic partner may break down, leading to the expulsion of the zooxanthellae and giving rise to the phenomenon known as coral bleaching (Brown, 1997; Hoegh-Guldberg *et al.*, 2007).

The mechanisms that trigger the expulsion of the symbiotic partner are not yet fully understood. However, the involvement of reactive oxygen species (ROS), such as superoxide, singlet oxygen and hydroxyl radicals, which are formed under combined heat and light stress, has been implicated based on a number of different lines of evidence. One key process, first suggested by Lesser *et al.* (1990), is the leakage of ROS from the symbiont to the host, which then overwhelms the antioxidant capacity of the host tissue under stressful conditions (Lesser, 1996; Brown *et al.*, 2002; Downs *et al.*, 2002; Richier *et al.*, 2005; Weis, 2008; Hawkins *et al.*, 2015). Recent studies have also revealed the details of the pathomorphogenesis and photo-oxidative damage of the symbiont algae under stressful conditions (Downs *et al.*, 2013).There is accumulating evidence for differing antioxidant capacities and stress tolerances linked with different *Symbiodinium* genetic types (Suggett *et al.*, 2008; Krueger *et al.*, 2015). There is also evidence for elevated antioxidant capacity of the host under thermal stress, alongside unaltered photosynthetic capacity in the symbiont. This suggests a decoupling of host redox mechanisms from the symbiont photophysiology, which

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may prevent the triggering of bleaching (Hawkins *et al.*, 2015; Krueger *et al.*, 2015; Wietheger *et al.*, 2015; Slavov *et al.*, 2016). Nitric oxide (NO), which is a ubiquitous signaling molecule, has also been implicated in the events leading to coral bleaching by inducing cytotoxic effects alone or via interaction with ROS, especially superoxide (Bouchard & Yamasaki, 2008; Hawkins & Davy, 2013; Hawkins *et al.*, 2014).

One of the most reactive oxygen forms is singlet oxygen, which, in photosynthetic systems, can be formed via interaction of ground-state molecular oxygen ($^3\Sigma_gO_2$) with chlorophyll (Chl) triplets that are produced either in the reaction center of the photosystem II (PSII) complex or in the light-harvesting antenna. When ground-state molecular oxygen ($^3\Sigma_gO_2$) interacts with ^{3}Chl, the singlet excited state of oxygen ($^1\Delta_gO_2$, which is abbreviated as $^1O_2$) is formed via energy transfer. $^1O_2$ is a highly reactive species, which oxidizes proteins, lipids and pigments (Davies, 2003) and induces photo-oxidative damage of the photosynthetic apparatus (Krieger-Liszkay, 2005; Krieger-Liszkay *et al.*, 2008). Although $^1O_2$ is obviously a potential candidate among the ROS forms that could be generated in the zooxanthellae and interact with the animal host, no direct evidence has been provided so far for the formation of $^1O_2$ in *Symbiodinium* cells. This has mainly been because of the lack of suitable $^1O_2$ detection methods that can be applied in intact microalgal cells. The measurement of luminescence at 1270 nm specifically arising from $^1O_2$ (Macpherson *et al.*, 1993; Telfer *et al.*, 1999), and which is used frequently in nonpigmented cells and *in vitro* systems, is hampered in zooxanthellae by the influence of background Chl fluorescence (Li *et al.*, 2012). Electron paramagnetic resonance (EPR) spin traps, such as 2,2,6,6-tetramethylpiperidine (TEMP) (Hideg *et al.*, 1994a,b; Fufezan *et al.*, 2007) and 2,2,6,6-tetramethyl-4-piperidone hydrochloride (TEMPD-HCl) (Fischer *et al.*, 2006; see also Hideg *et al.*, 2011), work well in isolated thylakoid membrane preparations, but do not penetrate into intact photosynthetic cells, such as zooxanthellae, and have limited sensitivity. In addition, the sensitive fluorescent $^1O_2$ trap, DanePy (3-[*N*-(β-diethylaminoethyl)-*N*-dansyl]aminomethyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole), which can be applied in intact leaves (Hideg *et al.*, 1998, 2007), does not penetrate through the cell wall of microalgae (E. Hideg, pers. comm.). There is apparently a similar limitation with singlet oxygen sensor green (SOSG), which can be applied in intact leaves (Flors *et al.*, 2006; Driever *et al.*, 2009), but has limited penetration into microalgal cells (see Rehman *et al.*, 2013). However, recent success with the application of histidine (His)-mediated chemical trapping of $^1O_2$ in intact cyanobacteria (Rehman *et al.*, 2013; Bersanini *et al.*, 2014; Hakkila *et al.*, 2014; Sedoud *et al.*, 2014) has opened the way to providing a suitable means for $^1O_2$ detection in *Symbiodinium* cells.

In the present work, we aimed to detect and characterize $^1O_2$ production in cultured *Symbiodinium* cells by His-mediated chemical trapping and SOSG imaging. We were able to successfully detect $^1O_2$ inside *Symbiodinium* cells by application of the His method, and showed that $^1O_2$ production is increased under heat and light stress. We also showed that SOSG does not penetrate the *Symbiodinium* cells. However, both SOSG and His-mediated $^1O_2$ trapping indicate the presence of external $^1O_2$ sensitizer(s), which is (are) excreted from the cells into the culture medium and produce(s) $^1O_2$ on illumination. The implications of these findings for coral bleaching are discussed.

Materials and Methods

Symbiodinium cultures

The *Symbiodinium* sp. culture (strain code CS-156) was obtained from the Commonwealth Scientific and Industrial Research Organization (CSIRO) Australian National Algae Culture Collection (ANACC). *Symbiodinium* sp. CS-156 was originally derived from the coral *Montipora verrucosa* (source locality: Hawaii, USA, North Pacific). Strain CS-156 is an F_1 type based on the nuclear internal transcribed region 2 (ITS2) (Suggett *et al.*, 2015). Cells were grown in artificial seawater (known as f/2 medium) at 26°C at a light intensity of 20 µmol photons m^{-2} s^{-1}, with a light to dark ratio of 12 h : 12 h. The cells were collected during their logarithmic growth phase by centrifugation at 6500 ***g*** for 5 min and resuspended in the same volume of fresh f/2 medium at the same concentration of cells for the experiments. Chl concentration was determined by a UV-1601 (Shimadzu, Kyoto, Japan) spectrophotometer as described in Jeffrey & Humphrey (1975). Measurements were performed in cultures with *c.* 10 µg Chl ml^{-1} (Takahashi *et al.*, 2008; Hill *et al.*, 2014).

Variable Chl fluorescence measurements

The photosynthetic activity of *Symbiodinium* cells was assessed by measuring the variable Chl fluorescence transient during the application of a 2-s saturating pulse (Strasser *et al.*, 1995; Hill *et al.*, 2004) using a fluorometer (FL-3000, Photon Systems Instruments, Brno, Czech Republic). F_v/F_m was obtained by calculating $(F_m - F_o)/F_m$, where F_o and F_m represent the minimum fluorescence in dark-adapted cells and the maximal fluorescence yield under continuous saturating light, respectively.

Oxygen evolution measurements

Steady-state oxygen evolution rates were measured with a Hansatech (Norfolk, UK) DW2 O_2 electrode at 30°C and a light intensity of 2300 µmol photons m^{-2} s^{-1} in the presence of 0.5 mM (2,6-dimethylbenzoquinone) as an artificial electron acceptor. A 2-µl suspension of *Symbiodinium* cells at 10 µg Chl ml^{-1} was used in each measurement, and three replicates were measured.

$^1O_2$ production in intact *Symbiodinium* cells

Singlet oxygen production was detected by the His-mediated chemical trapping technique, as described earlier for *Synechosystis* 6803 (Rehman *et al.*, 2013). *Symbiodinium* cells were collected

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by centrifugation at 6500 ***g*** for 5 min and resuspended at 10 μg Chl ml^{-1} in fresh f/2 medium. The cells were kept under growth conditions for at least 1 h before $^1O_2$ measurement. $^1O_2$ production was detected by measuring the rate of light-induced oxygen uptake in the presence and absence of 5 mM His used as chemical quencher. As the His method traps $^1O_2$ via an oxidation reaction, this process decreases the amount of dissolved oxygen and leads to an apparent decrease in the rate of light-induced oxygen production (Supporting Information Fig. S1), which is proportional to the intracellular concentration of $^1O_2$ (Rehman *et al.*, 2013). The difference between the rates of O_2 evolution in the absence and presence of His gives the rate of $^1O_2$ production ($v(^1O_2) = v(O_{2\text{-His}}) - v(O_{2+\text{His}})$). This method implies that His, in itself, does not inhibit PSII activity, which was confirmed by variable Chl fluorescence measurements (Fig. S2), which were performed using a Hansatech DW2 O_2 electrode at a light intensity of 2300 μmol photons m^{-2} s^{-1}. For verification that light-induced O_2 uptake indeed reflects $^1O_2$ production, heavy water (D_2O) and sodium azide (NaN_3) were used as $^1O_2$ enhancer and scavenger, respectively. As the lifetime of $^1O_2$ is increased in the presence of D_2O (Ogilby & Foote, 1982; Egorov *et al.*, 1989) and decreased in the presence of NaN_3 (Hall & Chignell, 1987), these compounds enhance and ameliorate, respectively, the $^1O_2$-induced effects. For D_2O treatment, cells were resuspended in f/2 medium which was produced using D_2O instead of H_2O, and were incubated for 30 min before the measurements in order to equilibrate the cells with D_2O. For NaN_3 treatment, cells were incubated for 1 min in the presence of 10 mM NaN_3 before the O_2 uptake measurements.

Glycolaldehyde and potassium cyanide treatment for *Symbiodinium* cells

Symbiodinium cells were collected by centrifugation at 6500 ***g*** for 5 min, and freshly resuspended cells were transferred into a conical flask without addition or in the presence of 10 mM glycolaldehyde (GA) or 10 μM potassium cyanide (KCN). The flasks were placed in the dark for 15 min for control measurements and later shifted to a light intensity of 45 μmol photons m^{-2} s^{-1} to see the effect of GA. The KCN-treated cells were incubated in darkness for 30 min. PSII activity was monitored by measuring the maximum quantum yield (F_v/F_m) of control and GA- or KCN-treated samples. $^1O_2$ production was monitored as described earlier.

Detection of $^1O_2$ in cell-free medium of *Symbiodinium* cultures

The cell-free medium of *Symbiodinium* cultures was collected by centrifugation at 6500 ***g*** for 5 min. The His-mediated O_2 uptake method was used to monitor the extent of $^1O_2$ production in the cell-free medium, and its absorption spectrum was determined by a UV-1601 (Shimadzu) spectrophotometer. For the light and temperature treatment, cell-free medium was placed in separate glass tubes and kept at different light and temperature conditions for 2 h: growth light (GL, 20 μmol photons m^{-2} s^{-1}), high light (HL, 100 μmol photons m^{-2} s^{-1}), growth temperature (GT, 26°C) and high temperature (HT, 34°C).

Singlet oxygen imaging in *Symbiodinium*

The SOSG reagent (Molecular Probes Inc., Eugene, OR, USA) was used for $^1O_2$ imaging in cultured *Symbiodinium* cells. SOSG was added to a culture of resuspended *Symbiodinium* cells at a concentration of 250 μM. The cell suspensions containing SOSG were illuminated with a visible light intensity of 2300 μmol photons m^{-2} s^{-1} for 5 min, either directly or through a filter which transmits only in the 380–450-nm range. Imaging of green fluorescence, which is emitted by SOSG after capturing $^1O_2$, was performed by a confocal laser scanning microscope (Leica SP5; Leica Microsystems, Wetzlar, Germany) using excitation at 504 nm and emission detection at 525 nm. The images were obtained by a transmitted light detection module with 488-nm excitation using a laser diode (LD). Simultaneously, Chl fluorescence was also visualized using excitation by the 488-nm line of an argon laser and fluorescence detection through a 650–750-nm filter. The images were analyzed using computer software from Leica.

A fluorescence stereomicroscope (Leica Microsystems) was also used for the detection of $^1O_2$ production in cell-free medium employing SOSG imaging. Cells incubated with SOSG were illuminated with a light intensity of *c.* 2300 μmol photons m^{-2} s^{-1} for 3 or 5 min as indicated in the figure. A custom-made MATLAB program was used to analyze the fluorescence signals from the images obtained.

Heat and light treatment of *Symbiodinium* cells

Cells were collected by centrifugation at 6500 ***g*** for 5 min and resuspended in fresh medium. A 5-μl cell suspension was placed in a glass tube and a total Chl concentration of *c.* 10 μg Chl ml^{-1} was maintained in the absence and presence of 5 mM His to observe any possible protection against pigment bleaching. The samples were kept at different light and temperature conditions in an automated incubator, defined as control (C), growth light (GL, 20 μmol photons m^{-2} s^{-1}) and growth temperature (GT, 26°C); high light (HL), 45 or 100 μmol photons m^{-2} s^{-1} and GT; high temperature (HT), 34°C and GL; high light + high temperature (HL + HT). The maximum quantum yield of PSII (F_v/F_m) was measured from each sample at time zero and after 24 h of treatment using an FL-3000 fluorometer. After 24 h of treatment, 1 ml of cells from each sample was harvested for spectral analysis of pigmentation. Chl concentration was determined as described earlier.

Results

Light induces intracellular $^1O_2$ production in *Symbiodinium* cells

Our recent findings have demonstrated that light-induced $^1O_2$ production can be conveniently studied in intact unicellular microalgae by His-mediated chemical trapping (Rehman *et al.*,

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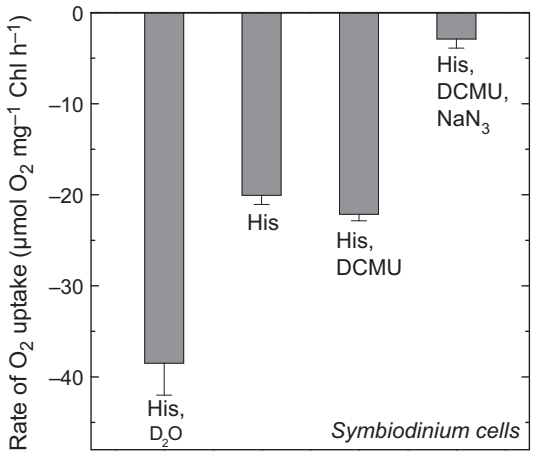


Fig. 1 Production of singlet oxygen ($^1O_2$) in *Symbiodinium* cells. Histidine (His)-mediated oxygen uptake as a measure of $^1O_2$ production was obtained without further addition, in the presence of the $^1O_2$ lifetime enhancer heavy water (D_2O) and in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) with or without 10 mM sodium azide (NaN_3) applied as $^1O_2$ scavenger. The light intensity during the oxygen measurements was 2300 µmol photons m^{-2} s^{-1}. The data represent mean values from three independent measurements with the indicated SE.

2013; Bersanini *et al.*, 2014; Hakkila *et al.*, 2014; Sedoud *et al.*, 2014). As *Symbiodinium*, which is an oxygenic photosynthetic dinoflagellate, contains very efficient $^1O_2$-producing molecules in the form of Chl pigments, it seemed highly likely that *Symbiodinium* would also produce a significant amount of intracellular $^1O_2$. We tested this hypothesis by applying His-mediated chemical trapping for $^1O_2$ detection, which indeed resulted in His-mediated O_2 uptake (Fig. 1). The increase in O_2 uptake in the presence of the $^1O_2$ enhancer D_2O, as well as the decrease in O_2 uptake in the presence of the $^1O_2$ scavenger NaN_3 (Fig. 1), provides firm support for the idea that His-mediated O_2 uptake arises from oxidation of His by $^1O_2$ in the *Symbiodinium* cells.

Intracellular $^1O_2$ production is enhanced under light and temperature stress

Stress conditions, which induce coral bleaching, include increases in light intensity and temperature to levels at which these have synergistic, harmful effects on cultured or *in hospite* *Symbiodinium* cells. In order to check whether intracellular $^1O_2$ production is affected by combined light and heat stress, *Symbiodinium* cells, which were grown at 26°C (GT) and 20 µmol photons m^{-2} s^{-1} (GL) were exposed to 34°C (HT) and 45 µmol photons m^{-2} s^{-1} (HL) for several hours. Incubation of cells under HT + HL leads to a gradual increase in the rate of His-mediated O_2 uptake, showing increased $^1O_2$ production (Fig. 2a). During a 7-h incubation period at HT + HL, the amount of detectable $^1O_2$ doubled from that observed under GT + GL conditions. Under natural conditions, *Symbiodinium* cells *in hospite* experience significantly higher light intensities than the HL applied here. Therefore, we wanted to clarify whether our HT + HL conditions indeed represent a stressful

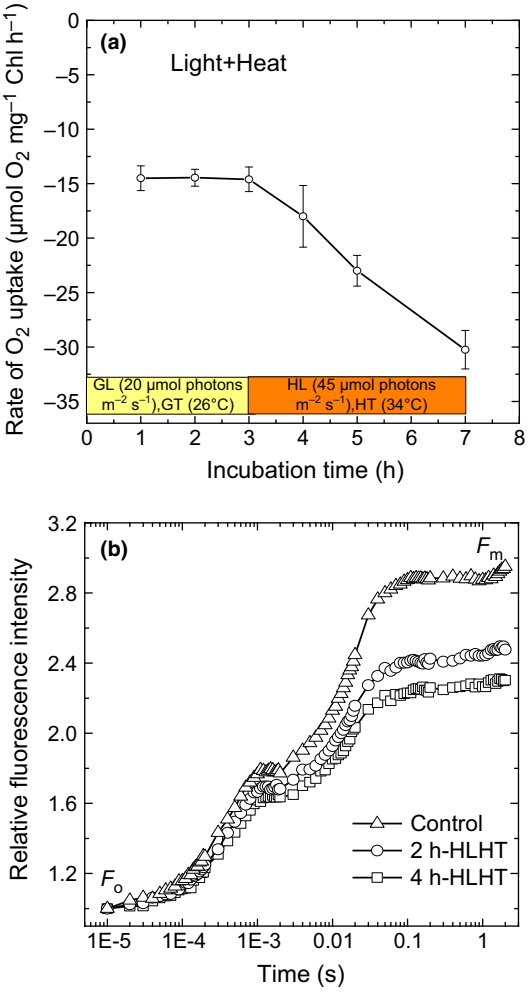


Fig. 2 Effect of heat and light stress on the production of singlet oxygen ($^1O_2$) in *Symbiodinium* cells. (a) Histidine (His)-mediated O_2 uptake, as a measure of $^1O_2$ production, was obtained after shifting cells from growth temperature (GT) and growth light (GL) (26°C and 20 µmol photons m^{-2} s^{-1}, respectively) to high light (HL) plus high temperature (HT) (34°C and 45 µmol photons m^{-2} s^{-1}, respectively) conditions. The light intensity during the oxygen measurements was 2300 µmol photons m^{-2} s^{-1}. The data represent mean values from three independent experiments with the indicated ± SE. (b) Variable chlorophyll (Chl) fluorescence traces of *Symbiodinium* cells under growth conditions (open triangles) and after 2 h (open circles) and 4 h (open squares) of high temperature and high light treatment. The curves are presented after shifting them to the same initial fluorescence level F_o.

situation to the cells by measuring variable Chl fluorescence transients. These data showed a significant loss in the F_m level (Fig. 2b) and, consequently, in the maximal quantum yield of PSII (F_v/F_m); thus, they confirm that, during the HT + HL incubation, which led to enhanced $^1O_2$ production, the cells experienced a stressful situation.

Intracellular $^1O_2$ production is enhanced by inhibition of the Calvin–Benson cycle

Recent results have demonstrated that inhibition of the Calvin–Benson cycle in *Symbiodinium*, either by thermal stress or chemical treatments, can trigger coral bleaching

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(Lilley *et al.*, 2010; Bhagooli, 2013; Hill *et al.*, 2014). CO_2 fixation by the Calvin–Benson cycle acts as a terminal sink for electrons originating from photosynthetic light reactions. Therefore, limited function of the terminal electron sink leads to the reduction of the photosynthetic electron transport chain, which is expected to enhance $^1O_2$ production. This hypothesis was tested using KCN and GA, which both block the Calvin–Benson cycle (Wishnick & Lane, 1969; Sicher, 1984). Incubation of *Symbiodinium* cells with 10 mM GA or 5 μM KCN has been shown to lead to a gradual loss of photosynthetic activity (Hill *et al.*, 2014), and here it was found that identical inhibitor treatments were accompanied by an enhanced level of $^1O_2$ production (Fig. 3).

Trapping of intracellular $^1O_2$ by His protects against heat and light stress-induced pigment bleaching

Under conditions of HL and HT, which lead to the release of zooxanthellae from corals, *Symbiodinium* cells suffer Chl pigment bleaching (Takahashi *et al.*, 2013), as well as a loss of PSII activity. If $^1O_2$ were the causal damaging agent, its trapping by externally added His could ameliorate these effects. In order to test this hypothesis, cells were incubated under HL, HT or HL + HT conditions in the absence and presence of 5 mM His. Exposure of the cells in the absence of His to HL or HT, or to the combination of the two stress conditions, induced a significant decrease in the level of F_m fluorescence (Fig. 4a) and in the maximal quantum yield of PSII (F_v/F_m, Fig. 4b). In addition, Chl bleaching was observed, as shown by the loss of absorbance in the red band of Chl (Fig. 4c). Importantly, the addition of 5 mM His partially prevented all of these effects, as shown by the respective data in Fig. 4(a–d), as well as in Fig. S3.

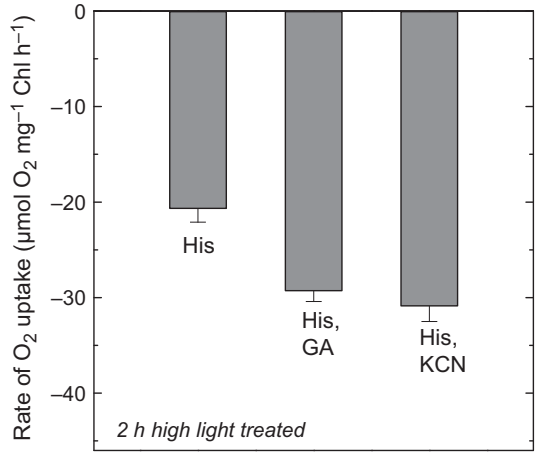


Fig. 3 Effect of glycolaldehyde (GA) and potassium cyanide (KCN) on the extent of singlet oxygen ($^1O_2$) production in *Symbiodinium* cells. $^1O_2$ production, quantified by Histidine (His)-mediated O_2 uptake, was measured without further addition and in cultures incubated with 10 mM GA or 10 μM KCN for 2 h under illumination with a light intensity of 45 μmol photons m^{-2} s^{-1}. The data represent mean values from three independent experiments with the indicated SE.

Symbiodinium cells excrete extracellular $^1O_2$-sensitizing compounds

The data presented earlier strongly indicate the production of $^1O_2$ inside *Symbiodinium* cells. We were also interested to determine whether $^1O_2$ can be detected in the extracellular space, as there is a precedent for the production of $^1O_2$-sensitizing metabolites in cyanobacteria (Shunmugam *et al.*, 2014), whose release to the cell exterior can provide a mechanism for the presence of extracellular $^1O_2$. In order to check the capacity of *Symbiodinium* cells to produce extracellular $^1O_2$ sensitizers, we monitored the level of $^1O_2$ in cell-free medium of *Symbiodinium* cultures. Importantly, we observed light-induced $^1O_2$ production in cell-free culture medium, which was separated from *Symbiodinium* cells by centrifugation (Fig. 5). This effect was typically observed in well-grown cultures of 2–3 wk of age. When the separated cells were resuspended in fresh culture medium, > 30% of the $^1O_2$ production rate was attained in the extracellular medium in a few hours, whereas the rest of the extracellular $^1O_2$-producing activity was restored with a *c.* 3 d half-time (Fig. S4).

These data clearly demonstrate that *Symbiodinium* cells excrete one or more metabolites, which are capable of $^1O_2$ production under illumination. It is important to note that, when the intracellular $^1O_2$ production was assayed, the *Symbiodinium* cells were always resuspended in fresh culture medium before the measurements in order to minimize the effect of the extracellular $^1O_2$-sensitizing metabolite(s).

In order to check the effect of environmental conditions on the excretion of $^1O_2$-sensitizing metabolite(s), *Symbiodinium* cultures, which were grown under 20 μmol photons m^{-2} s^{-1} and 26°C, were separated into two parts. One-half of the culture was grown further under the original conditions, whereas the other half was transferred to 100 μmol photons m^{-2} s^{-1} and 34°C, that is, HL + HT conditions. In both cultures, the absorbance spectrum and the rate of $^1O_2$ production were assayed daily during an 8-d period in the cell-free medium, which was separated from the cells by centrifugation. As shown in Fig. 6(a), the absorbance spectrum of the cell-free medium, which reflects absorption by the excreted metabolite(s), shows the main peak in the UV-C region at *c.* 250 nm, and a shoulder peak at 330 nm, which has a broad and gradually declining tail extending into the visible region. As we used a visible light source for the detection of $^1O_2$ in the His-mediated O_2 uptake assay, these data show that $^1O_2$ sensitization can be induced by the low absorbance of the metabolites in the cell-free medium in the visible part of the spectrum. However, under natural conditions, the UV-A and, perhaps, UV-B part of sunlight can also contribute to $^1O_2$ production. After transferring half of the culture to HL/HT conditions, the absorbance in the cell-free medium increased faster than in the other half of the culture, which remained in the original growth conditions. After 7 d, the absorbance of the HL/HT culture medium was almost two-fold higher at 250 nm than in the GL/GT culture. In parallel with the increase in absorbance, the $^1O_2$-producing efficiency was also increased faster in the HL/HT culture medium, when compared with the cell-free medium

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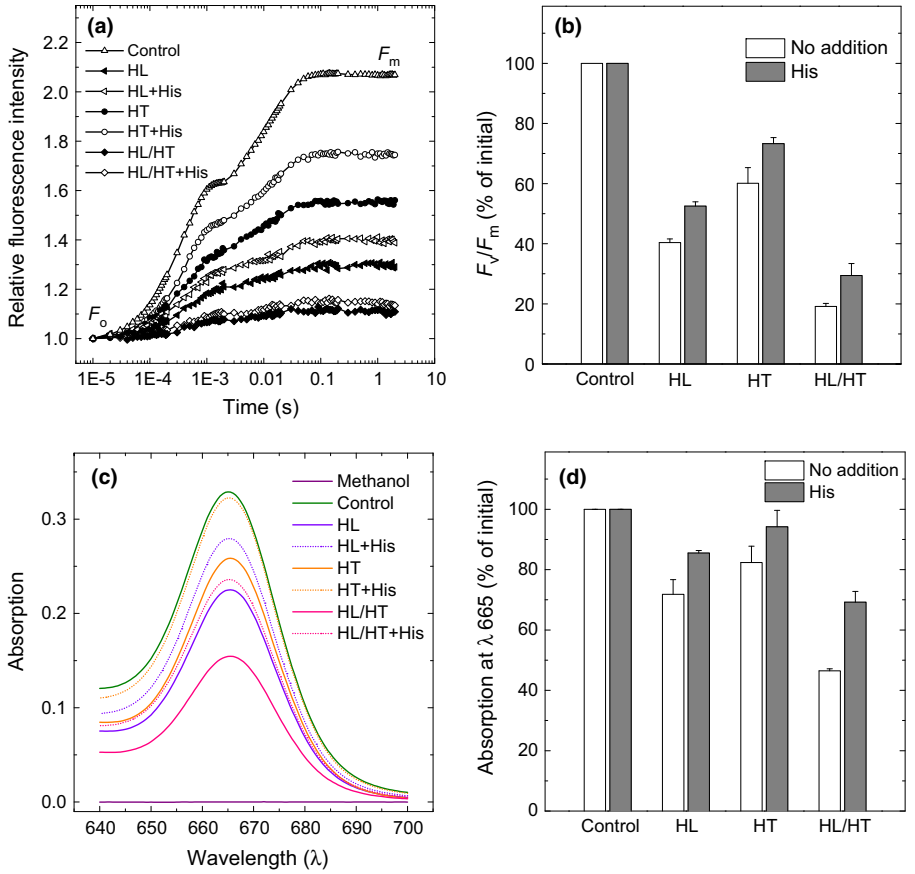


Fig. 4 Effect of histidine on heat and light stress induced photosystem II (PSII) photoinactivation and pigment bleaching in *Symbiodinium* cells. (a) Variable Chl fluorescence traces were measured in cells exposed to growth conditions (open upright triangles), or to high light (HL, 100 μmol photons m^{-2} s^{-1}, closed arrows), high temperature (HT, 34°C, closed circles) and high light/high temperature (HL/HT, closed squares). The traces were also measured in the presence of 5 mM histidine under high light (HL + His, open arrows), high temperature (HT + His, open circles) and high light/high temperature (HL/HT + His, open squares). The curves are presented after shifting them to same initial Fo fluorescence level. (b) F_v/F_m values calculated from data in (a), and shown after normalization to their initial control values. (c) Absorbance spectrum of methanolic extract of *Symbiodinium* cells after 24 h HL, HT and HL/HT treatment with and without 5 mM histidine. (d) Optical density at 665 nm. The data presented in (b, d) are mean values obtained from three independent experiments, with the indicated (SE).

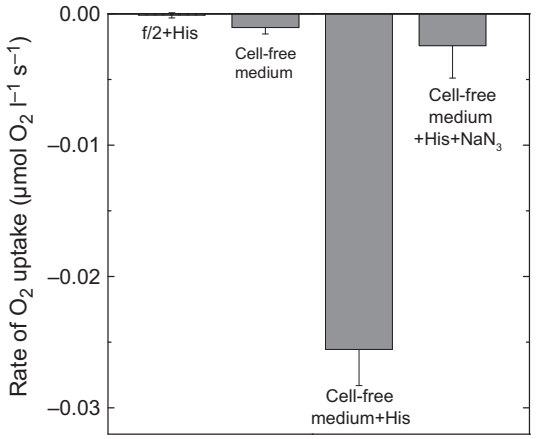


Fig. 5 Singlet oxygen ($^1O_2$) production in cell-free medium of *Symbiodinium* cultures. Histidine (His)-mediated oxygen uptake, as a measure of $^1O_2$ production, was obtained without further addition and in the presence of 10 mM NaN_3 acting as a $^1O_2$ quencher. The data are mean values obtained from three independent experiments, with the indicated SE.

of the GL/GT culture (Fig. 6b). These data demonstrate that HL/HT conditions enhance the excretion of the $^1O_2$-sensitizing extracellular metabolite.

Extracellular $^1O_2$ production is verified by SOSG fluorescence imaging

We also used SOSG, which is a $^1O_2$-responsive sensor molecule whose fluorescence yield increases after interaction with $^1O_2$ (Flors *et al.*, 2006), to check whether $^1O_2$ production occurs in the cell-free medium of *Symbiodinium* cultures. As shown in Fig. 7, in the absence of pre-illumination, both the fresh f/2 medium and the cell-free culture medium showed only very weak green fluorescence when an SOSG-stained drop was imaged under a fluorescence microscope. This weak background fluorescence arose from the SOSG molecules in the absence of interaction with $^1O_2$. When the SOSG-stained drops were illuminated with visible light for 3 or 5 min before microscopic imaging, the fluorescence signal was enhanced in both the fresh f/2 and cell-free medium of an older culture (Fig. 7a). As the fresh f/2

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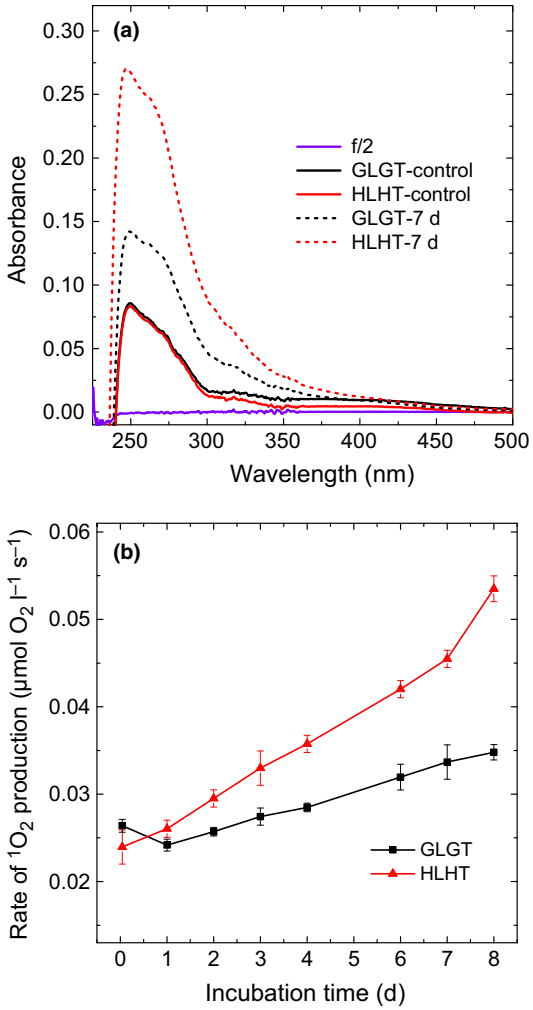


Fig. 6 Effect of light and heat stress on extracellular singlet oxygen ($^1O_2$) production in *Symbiodinium* cultures. Cells which were grown under 20 µmol photons m^{-2} s^{-1} and 26°C were separated into two parts. One-half of the culture was grown further under the original conditions of growth light and growth temperature (GLGT), whereas the other half was transferred to high light and high temperature (HLHT) conditions (100 µmol photons m^{-2} s^{-1} and 34°C, respectively). From both cultures, 2-ml aliquots were withdrawn daily and (a) the absorbance spectrum and (b) the rate of $^1O_2$ production were assayed in the cell-free medium, which was separated from the cells by centrifugation. In (a), representative curves obtained at the beginning and on the 7th day of the experiment are shown. In (b), mean values obtained from three experiments are shown with the corresponding ± SE.

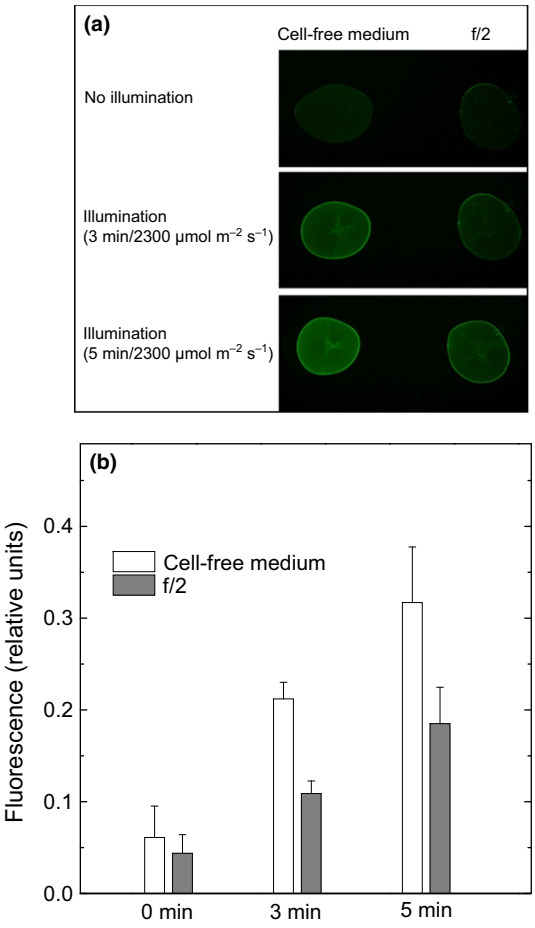


Fig. 7 Microscopy imaging with singlet oxygen sensor green (SOSG) in *Symbiodinium* culture medium. (a) Images of drops of culture medium separated from the cells by centrifugation after 2 wk of culture, or freshly prepared and illuminated in the presence of SOSG for 0, 3 and 5 min by a visible light intensity of 2300 µmol photons m^{-2} s^{-1}. (b) Quantitative analysis of fluorescence intensities from (a) which were quantified by a custom-made image analysis software. Data in (b) are means obtained from three independent experiments with the indicated SE.

medium did not show $^1O_2$ production when the His-mediated chemical trapping method was used, the enhanced SOSG signal probably arises from trapping of $^1O_2$ which is produced by SOSG itself during the illumination, and this is in agreement with earlier data showing $^1O_2$ photosensitization by SOSG (Ragás *et al.*, 2009). However, the stronger fluorescence signal in the cell-free medium (Fig. 7a), which is significantly higher than in the f/2 medium (Fig. 7a,b), reflects $^1O_2$ production by the so far unknown sensitizing compounds, which are excreted into the medium from the *Symbiodinium* cells. These data confirm the applicability of the SOSG technique for the detection of $^1O_2$ in the medium, but also show the limitation of this approach as a result of the reaction of SOSG with self-produced $^1O_2$, leading to an elevated SOSG signal, even in the absence of external $^1O_2$.

Our next question was the applicability of $^1O_2$ imaging by SOSG in *Symbiodinium* cultures using confocal microscopy. When imaging was performed in cultures which contained *Symbiodinium* cells in the medium in which they were grown, $^1O_2$ was shown to be present in a more or less uniform way in the whole medium, but with an increased level in the outer layer of the cells (Fig. 8a). When cells were centrifuged and resuspended in fresh f/2 culture medium before SOSG imaging, the uniform $^1O_2$ oxygen signal disappeared from the medium, but the high SOSG fluorescence in the outer cell layer remained (Fig. 8b). It is important to note that there is no detectable SOSG fluorescence in the cell interior, where Chls, the main intracellular $^1O_2$ sensitizers, are located (Fig. 8a,b). This finding is in agreement with previous results showing the inability of SOSG to penetrate inside the cells of the cyanobacterium *Synechocystis* sp. PCC 6803 (Rehman *et al.*, 2013).

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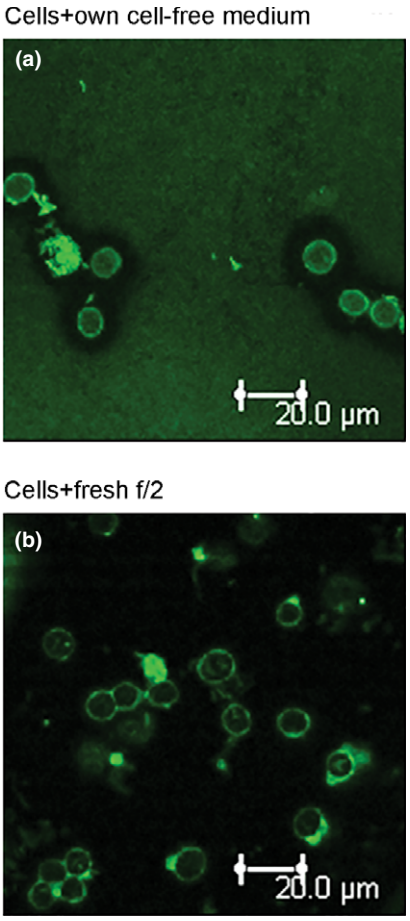


Fig. 8 Confocal fluorescence imaging of singlet oxygen sensor green (SOSG)-stained *Symbiodinium* cultures. The cells were kept either in (a) their original medium in which they were cultured for 2 wk, or (b) were resuspended in fresh culture medium before SOSG imaging. In order to trap singlet oxygen ($^1O_2$), the cells were illuminated by a visible light intensity of 2300 µmol photons m^{-2} s^{-1} in the presence of SOSG for 5 min.

To obtain more detailed information on the distribution of $^1O_2$ production inside *Symbiodinium* cells, SOSG and Chl fluorescence were recorded in parallel with transmission images on the same cells at higher resolution (Fig. 9). Only a minor amount of SOSG fluorescence could be detected if cells were kept in darkness after SOSG addition (Fig. 9a) (the light green halo around the cells in the green fluorescence channel (Fig. 9ai) arises from SOSG, which shows low fluorescence yield when it does not interact with $^1O_2$); in addition, the green laser light, which is used for confocal imaging, also induces self-SOSG fluorescence as a result of trapping of $^1O_2$ produced by the excited SOSG molecules; similarly, the bright green spot outside the cell in Fig. 9(ai) is related to SOSG fluorescence induced by the scanning laser beam in an extracellular location in which SOSG is accumulated. When cells were illuminated with red light, which produces intracellular $^1O_2$ from excited Chls, SOSG fluorescence increased only in the outer layer of the cells (Fig. 9bi), but not in the interior of the cells, where Chl-containing thylakoid membranes are located (Fig. 9bii). As $^1O_2$ production inside *Symbiodinium* cells is demonstrated by His-mediated chemical trapping (Fig. 1), these data can be explained only by the inability of SOSG to reach the interior of the cells, and especially into the plastids. A similar limitation of SOSG entering into microalgal cells was observed in the case of the cyanobacterium *Synechocystis* 6803 (Rehman *et al.*, 2013). The SOSG fluorescence increase appears close to the cell surface, but apparently more inside than outside of the cell wall and at a clearly distinct position from the Chl fluorescence (Fig. 9biv,e). This indicates that SOSG can penetrate into the compartment between the cell wall and the inner membrane, and also that $^1O_2$ is either produced here, from non-Chl sensitizers, or diffuses out from the cell interior. As the previous experiments were performed with red light and as the absorption coefficient of the cell-free medium, which is capable of $^1O_2$ production in the absence of Chls, is very small in the red wavelength range (Fig. 5), we also used illumination through a bandpass filter with a transmission maximum at 425 nm (i.e. violet–blue light), and a half-bandwidth of 120 nm, to induce $^1O_2$ production. This light, although still exciting Chls in the Soret band, shows a larger overlap with the absorbance of the cell-free medium and therefore is expected to produce more $^1O_2$ from non-Chl sensitizers than does red light. As shown in Fig. 9(c), $^1O_2$ production under violet–blue preillumination occurs with the same pattern, that is, outside or in the outer cell layer, as under red preillumination. Although this finding is not conclusive with regard to the origin of $^1O_2$ detected here, it shows that $^1O_2$ is present in a relatively higher amount in the outer layer of *Symbiodinium* cells, under these conditions, which has potential importance for interactions with the coral host.

Discussion

$^1O_2$ is an important ROS, which has been demonstrated to cause light-induced damage to the photosynthetic apparatus (Krieger-Liszkay, 2005; Krieger-Liszkay *et al.*, 2008; Vass, 2011, 2012; Rehman *et al.*, 2013), as well as to mediate signaling events in bacterial, plant and animal systems (Meskauskiene *et al.*, 2001; Laloi *et al.*, 2004; Wagner *et al.*, 2004; Kim *et al.*, 2008; Shao *et al.*, 2013; Schmitt *et al.*, 2014). Although various ROS forms have been implicated as triggering agents leading to the release of zooxanthellae from coral tissue under coral bleaching conditions (Lesser *et al.*, 1990; Lesser, 1996; Brown *et al.*, 2002; Downs *et al.*, 2002; Richier *et al.*, 2005; Weis, 2008), the production of $^1O_2$ in *Symbiodinium* cells has not been studied hitherto. This was mainly because of the lack of suitably sensitive, as well as selective, $^1O_2$ detection methods, which could be applied to microalgae. As our recent results have paved the way for the study of $^1O_2$ formation in intact microalgal cells (Rehman *et al.*, 2013; Bersanini *et al.*, 2014; Hakkila *et al.*, 2014; Sedoud *et al.*, 2014), in the present work we aimed to investigate the occurrence of $^1O_2$ inside and outside of cultured *Symbiodinium* cells, as well as to characterize the physiological consequences of $^1O_2$ formation.

Intracellular $^1O_2$ production in *Symbiodinium*

By using the His-mediated chemical trapping approach, we demonstrated that $^1O_2$ is produced in *Symbiodinium* cell cultures

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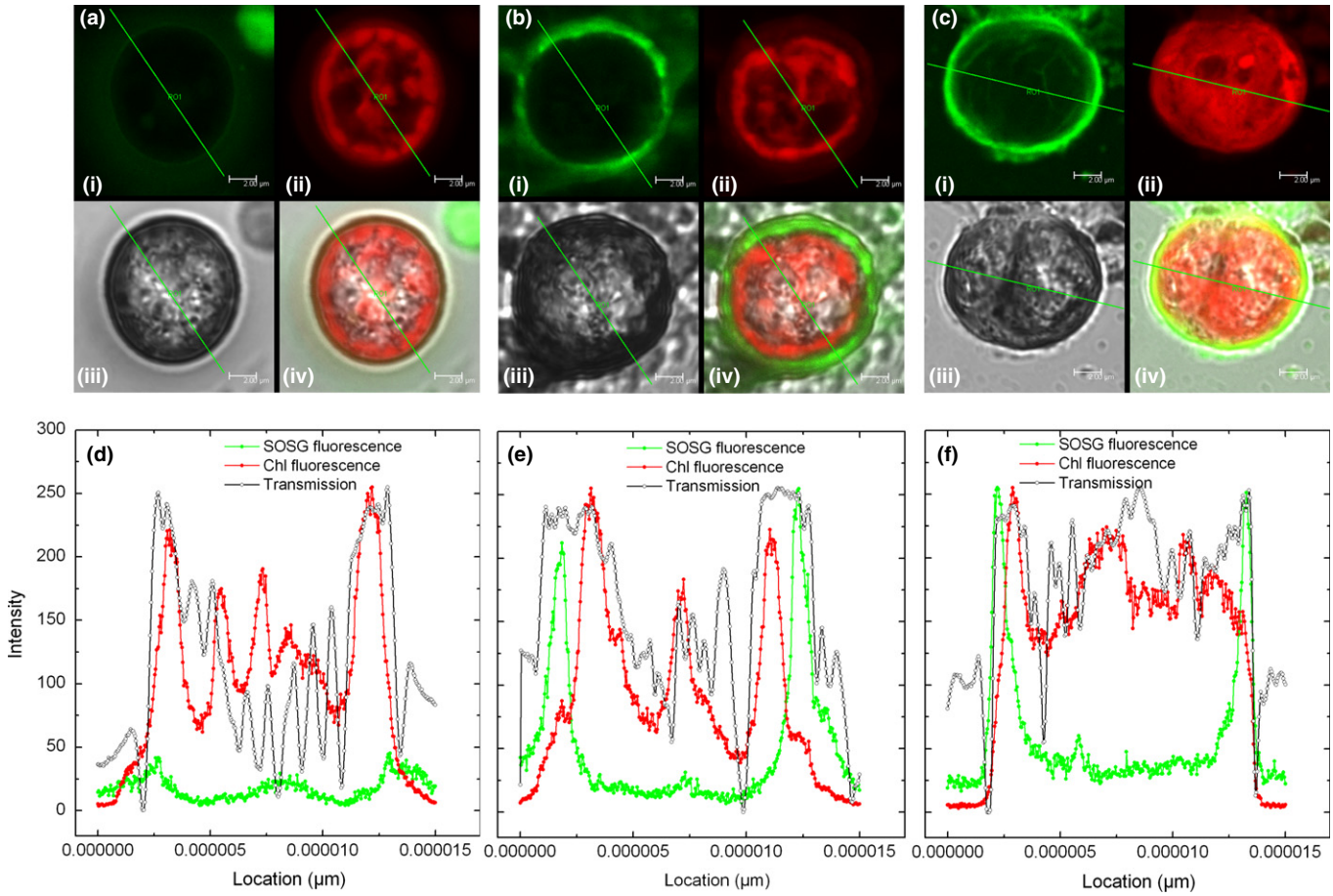


Fig. 9 Confocal microscopy imaging of *Symbiodinium* cells stained with singlet oxygen sensor green (SOSG). Four detection channels are represented: (i) SOSG fluorescence; (ii) chlorophyll (Chl) fluorescence; (iii) transmission; (iv) merged images of the three signals (bars, 200 μm). (a) Laser scanning microscopy (LSM) imaging of cells without light treatment. (b) LSM imaging of cells which were exposed to red light (2300 μmol photons m^{-2} s^{-1} through a filter which transmits light only above 600 nm) for 5 min. (c) LSM imaging of cells which were exposed to violet–blue light (2300 μmol photons m^{-2} s^{-1} through a bandpass filter with a transmission maximum at 425 nm and half-bandwidth of 120 nm) for 5 min. (d), (e) and (f) show the intensity distribution of the three detection channels at the indicated transect lines for (a), (b) and (c), respectively.

during illumination (Fig. 1). As a result of its short lifetime, the diffusion distance of $^1O_2$ is in the range of 70–250 nm in the cellular environment (Moan, 1990; Skovsen *et al.*, 2005), which limits its presence close to the site of production in the thylakoid membranes. Therefore, the observation of His-mediated O_2 uptake in *Symbiodinium* cultures, which were resuspended in fresh f/2 medium before the measurements, demonstrates that $^1O_2$ is produced inside the cells.

With regard to the regulation of $^1O_2$ production, we were especially interested in the effect of environmental conditions, which are known to trigger the release of zooxanthellae from coral tissue. These include high light and elevated temperatures, as well as inhibitors of the Calvin–Benson cycle. Our data indeed show that the level of intracellular $^1O_2$ is increased when cells are shifted from normal growth conditions to increased light intensities and elevated temperatures (Fig. 2). However, trapping of intracellular $^1O_2$ by externally added His ameliorates the inhibition of PSII electron transport, as well as the bleaching of Chls (Fig. 4), which both occur under conditions of coral bleaching (Hill *et al.*, 2004, 2014). These data demonstrate that $^1O_2$, which is formed inside *Symbiodinium* cells, is involved in the loss of PSII activity and also in the oxidative damage of the Chl pigments.

The exposure of cells to inhibitors of the Calvin–Benson cycle, such as GA or KCN, also induced increased intracellular $^1O_2$ production (Fig. 3). This phenomenon can best be explained by the increased reduction level of electron carriers located at the acceptor side of photosystem I (PSI), as well as of the plastoquinone (PQ) pool located between PSII and PSI. This situation is induced by the absence of functional CO_2 fixation in the Calvin–Benson cycle, which serves as the final electron sink in the photosynthetic process. The increased reduction level of the PQ pool enhances charge recombination in the reaction center of PSII, which, in turn, facilitates $^1O_2$ formation (Vass & Cser, 2009; Vass, 2011, 2012). GA treatment has been shown to induce the release of zooxanthellae from *Stylophora pistillata* (Bhagooli, 2013), but not from *Pocillopora damicornis* (Bhagooli, 2013; Hill *et al.*, 2014). However, KCN did induce zooxanthellae release from *Pocillopora damicornis*, but Hill *et al.* (2014) concluded that this was not an effect of Calvin–Benson cycle inhibition, but caused by an additional effect on the stimulation of ROS. Therefore, these data indicate

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that, although intracellular $^1O_2$ production is enhanced in the presence of both Calvin–Benson cycle inhibitors, a direct effect of Calvin–Benson cycle inhibition on the release of zooxanthellae is unlikely (cf. Hill *et al.*, 2014). In particular, the effect of GA might have additional species-specific features, which might lead to the inhibition of photosynthesis and $^1O_2$ production in *Pocillopora damicornis*, without causing the release of zooxanthellae, as suggested by Hill *et al.* (2014). This idea is supported by our earlier findings, which show that the incubation of *Symbiodinium* cells with GA for several hours, which was applied in the coral experiment, induces the inhibition of photosynthetic activity, even in darkness, when light-induced ROS production does not occur (Hill *et al.*, 2014). Therefore, our data are more in favor of an additional effect of GA, which prevents the actual release of zooxanthellae in spite of Calvin–Benson cycle inhibition and concomitant enhancement of ROS production (cf. Hill *et al.*, 2014).

Extracellular production of $^1O_2$ in *Symbiodinium* cultures

In order to interact with the animal host, $^1O_2$ should be present either at the surface of zooxanthellae or at extracellular locations. Our data support both of these possibilities. Confocal microscopy data showed an enhancement of SOSG-trapped $^1O_2$ at the outer layer of *Symbiodinium* cells, where it can interact with the animal host. We should note, however, that the enhancement of SOSG fluorescence can also arise from the increased concentration of SOSG in the outer cell layer. Such hotspots of SOSG accumulation can also be observed at extracellular locations that could originate from detached parts of the exopolysaccharide layer, which is known to be present in dinoflagellates (Kumar *et al.*, 2011), and constitutes part of the complex cell covering system, known as the extracellular matrix in *Symbiodinium* (Xiang *et al.*, 2015). It is unlikely that these extracellular hotspots would contain a high concentration of $^1O_2$ sensitizers. It is more probable that they represent the accumulation of SOSG and that the high fluorescence level arises from the $^1O_2$-sensitizing ability of the SOSG molecules.

A highly important finding of our work was the detection of $^1O_2$ production in the extracellular medium of *Symbiodinium* cultures by both His-mediated chemical trapping and SOSG imaging. This finding demonstrates the presence of $^1O_2$-sensitizing metabolite(s) in the cell-free medium of *Symbiodinium* cultures. As fresh f/2 medium does not sensitize $^1O_2$ production (Fig. 5), the sensitizer(s) is(are) probably excreted from the *Symbiodinium* cells. The excretion is a relatively slow process, with a *c.* 2–3-d half-time (Fig. S4); however, it is clearly accelerated under conditions of increased light intensity and elevated temperature (Fig. 6). Therefore, the so far unknown $^1O_2$ sensitizer(s) represent(s) a potential candidate(s) for a signaling component(s) that can carry information from the intercellular space of *Symbiodinium* to the exterior of these cells. With regard to the possible structure of the excreted $^1O_2$ sensitizer(s), our study provided only the absorbance spectrum of the cell-free medium, which contains the sensitizer(s). The absorbance has its main peak at *c.* 250 nm and a shoulder at 340 nm (Fig. 6a), which could arise from numerous compounds. So far, the only characterized $^1O_2$-sensitizing secondary metabolite, M22, which originates from a *Nostoc* cyanobacterium, has the main absorbance peak below 255 nm with two smaller peaks at *c.* 270 and 375 nm; its molecular mass is 1627, and contains hydroxybenzyl groups, but its detailed structure is not known (Shunmugam *et al.*, 2014). Based on the dissimilarity of the absorbance spectra, it is unlikely that the cyanobacterially produced M22 would be the metabolite that is responsible for extracellular $^1O_2$ sensitization in *Symbiodinium.*

It should be noted that SOSG has been used to observe singlet oxygen in a sea anemone system, which showed an increase in $^1O_2$ levels after heat stress in darkness, conditions which led to the release of *Symbiodinium* from the anemone host (Tolleter *et al.*, 2013). The experimental details presented in the paper by Tolleter *et al.*, 2013 do not allow an unambiguous conclusion as to whether the observed SOSG signal was caused by intra- or extracellular $^1O_2$, but, based on the data presented here, it is highly likely that they also observed extracellular $^1O_2$.

Potential physiological roles of intra- and extracellular $^1O_2$ in *Symbiodinium*

In the present work, we were able to demonstrate, for the first time, that *Symbiodinium* cells form intracellular $^1O_2$ via Chl excitation, and also that they excrete metabolites, which sensitize extracellular $^1O_2$ production. Partial protection against light-induced loss of PSII activity and Chl bleaching by the addition of the chemical $^1O_2$ trap, His, demonstrates that intracellular $^1O_2$ mediates the photodamage of thylakoid-embedded components of the photosynthetic apparatus.

Our observation that intracellular $^1O_2$ levels are enhanced under bleaching conditions that are known to trigger the expulsion of zooxanthellae from coral tissues (high light + high temperature, inhibitors of the Calvin–Benson cycle) point to the possibility that $^1O_2$, which is produced inside the *Symbiodinium* cells, may also participate in the events that lead to coral bleaching.

$^1O_2$ produced from extracellular sensitizers can influence the physiological functions of other living organisms, as shown for the cyanobacterial metabolite (M22) which arises from a *Nostoc* species and exerts a toxic effect on other cyanobacteria (Shunmugam *et al.*, 2014). In the case of *Symbiodinium* cells living in the coral host, the external $^1O_2$ sensitizer(s) has the potential to interact with the animal host and could be involved in the triggering of the release of *Symbiodinium* cells during coral bleaching. However, the causal relationship between $^1O_2$ and coral bleaching requires further investigations, as $^1O_2$ production may occur not only in light via the long-lived triplet states of excited Chls and other sensitizers, but also via light-independent processes, such as the Russel-type mechanism (Pospísil, 2012), and also because the redox status of the host cells might be independent of the endosymbiont antioxidant activities (Krueger *et al.*, 2015).

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Author contributions

A.U.R. performed $^1O_2$ measurements in *Symbiodinium* and cell-free medium, and contributed to writing of the paper. M.S. designed part of the experiments and contributed to the interpretation of the data and writing of the paper. Z.D. contributed to the establishment of $^1O_2$ measurements in *Symbiodinium* as well as in cell-free medium, and measured the absorption spectra of cell-free medium. L.S. analyzed the confocal microscopic photographs. A.L. contributed to the experimental design, analysis of the confocal microscopic photographs (regarding extracellular $^1O_2$ formation) and writing of the paper. P.R. contributed to the interpretation of data regarding extracellular $^1O_2$ formation and writing of the paper. I.V. coordinated the work and contributed to the experimental designs, interpretation of the data and writing of the paper.

References

Bersanini L, Battchikova N, Jokel M, Rehman AU, Vass I, Allahverdiyeva Y, Aro E-M. 2014. Flavodiiron protein Flv2/Flv4-related photoprotective mechanism dissipates excitation pressure of PSII in cooperation with phycobilisomes in cyanobacteria. *Plant Physiology* **164**: 805–818.

Bhagooli R. 2013. Inhibition of Calvin–Benson cycle suppresses the repair of photosystem II in Symbiodinium: implications for coral bleaching. *Hydrobiologia* **714**: 183–190.

Bouchard JN, Yamasaki H. 2008. Heat stress stimulates nitric oxide production in *Symbiodinium microadriaticum*: a possible linkage between nitric oxide and the coral bleaching phenomenon. *Plant and Cell Physiology* **49**: 641–652.

Brown BE. 1997. Coral bleaching: causes and consequences. *Coral Reefs* **16**: S129–S138.

Brown BE, Downs CA, Dunne RP, Gibb SW. 2002. Exploring the basis of thermotolerance in the reef coral *Goniastrea aspera*. *Marine Ecology Progress Series* **242**: 119–129.

Davies MJ. 2003. Singlet oxygen-mediated damage to proteins and its consequences. *Biochemical and Biophysical Research Communications* **305**: 761–770.

Downs CA, Fauth JE, Halas JC, Dustan P, Bemiss J, Woodley CM. 2002. Oxidative stress and seasonal coral bleaching. *Free Radicals in Biology & Medicine* **33**: 533–543.

Downs CA, McDougall KE, Woodley CM, Fauth JE, Richmond RH, Kushmaro A, Gibb SH, 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**: e77173.

Driever SM, Fryer MJ, Mullineaux PM, Baker NR. 2009. Imaging of reactive oxygen species *in vivo*. In: Pfannschmidt T, ed. *Plant signal transduction*. New York, NY, USA: Humana Press, a part of Springer Science+Business Media LLC, 109–116.

Egorov SYu, Kamalov VF, Koroteev NI, Krasnovsky AA Jr, Toleutaev BN, Zinukov SV. 1989. Rise and decay kinetics of photosensitized singlet oxygen luminescence in water. Measurements with nanosecond time-correlated single photon counting technique. *Chemical Physics Letters* **163**: 421–424.

Fischer BB, Eggen RIL, Trebst A, Krieger-Liszkay A. 2006. The glutathione peroxidase homologous gene Gpxh in *Chlamydomonas reinhardtii* is upregulated by singlet oxygen produced in photosystem II. *Planta* **223**: 583–590.

Flors C, Fryer MJ, Waring J, Reeder B, Bechtold U, Mullineaux PM, Nonell S, Wilson MT, Baker NR. 2006. Imaging the production of singlet oxygen *in vivo* using a new fluorescent sensor, Singlet Oxygen Sensor Green®. *Journal of Experimental Botany* **57**: 1725–1734.

Fufezan C, Gross CM, Sjödin M, Rutherford AW, Krieger-Liszkay A. 2007. Influence of the redox potential of the primary quinone electron acceptor on photoinhibition in Photosystem II. *Journal of Biological Chemistry* **282**: 12492–12502.

Hakkila K, Antal T, Rehman AU, Kurkela J, Wada H, Vass I, Tyystjärvi E, Tyystjärvi T. 2014. Oxidative stress and photoinhibition can be separated in the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochimica et Biophysica Acta* **1837**: 217–225.

Hall RD, Chignell CF. 1987. Steady-state near-infrared detection of singlet molecular oxygen: a Stern–Volmer quenching experiment with sodium azide. *Photochemistry and Photobiology* **45**: 459–464.

Hawkins TD, Davy SK. 2013. Nitric oxide and coral bleaching: is peroxynitrite generation required for symbiosis collapse? *Journal of Experimental Botany* **216**: 3185–3188.

Hawkins TD, Krueger T, Becker S, Fisher PL, Davy SK. 2014. Differential nitric oxide synthesis and host apoptotic events correlate with bleaching susceptibility in reef corals. *Coral Reefs* **33**: 141–153.

Hawkins TD, Krueger T, Wilkinson SP, Fisher PL, Davy SK. 2015. Antioxidant responses to heat and light stress differ with habitat in a common reef coral. *Coral Reefs* **34**: 1229–1241.

Hideg É, Kálai T, Hideg K, Vass I. 1998. Photoinhibition of photosynthesis *in vivo* results in singlet oxygen production. Detection via nitroxide-induced fluorescence quenching in broad bean leaves. *Biochemistry* **37**: 11405–11411.

Hideg É, Kós PB, Vass I. 2007. Photosystem II damage induced by chemically generated singlet oxygen in tobacco leaves. *Physiologia Plantarum* **131**: 33–40.

Hideg É, Spetea C, Vass I. 1994a. Singlet oxygen and free radical production during acceptor- and donor-side-induced photoinhibition. Studies with spin trapping EPR spectroscopy. *Biochimica et Biophysica Acta* **1186**: 143–152.

Hideg É, Spetea C, Vass I. 1994b. Singlet oxygen production in thylakoid membranes during photoinhibition as detected by EPR spectroscopy. *Photosynthesis Research* **39**: 191–199.

Hideg É, Deák Zs, Hakala-Yatkin M, Karonen M, Rutherford AW, Tyystjarvi E, Vass I, Krieger-Liszkay A. 2011. Pure forms of the singlet oxygen sensors TEMP and TEMPD do not inhibit Photosystem II. *Biochimica et Biophysica Acta* **1807**: 1658–1661.

Hill R, Larkum AWD, Frankart C, Kühl M, Ralph PJ. 2004. Loss of functional Photosystem II reaction centers in zooxanthellae of corals exposed to bleaching conditions: using fluorescence rise kinetics. *Photosynthesis Research* **82**: 59–72.

Hill R, Szabó M, Rehman AU, Vass I, Ralph PJ, Larkum AWD. 2014. Inhibition of photosynthetic CO_2 fixation in the coral *Pocillopora damicornis* and its relationship to thermal bleaching. *Journal of Experimental Biology* **217**: 2150–2162.

Hoegh-Guldberg O, Mumby PJ, Hooten AJ, Steneck RS, Greenfield P, Gomez E, Harvell CD, Sale PF, Edwards AJ, Caldeira K *et al.* 2007. Coral reefs under rapid climate change and ocean acidification. *Science* **318**: 1737–1742.

Jeffrey SW, Humphrey GF. 1975. New spectrophotometric equations for determining chlorophylls *a, b,* c_1 and c_2 in higher plants, algae and natural phytoplankton. *Biochemie und Physiologie der Pflanzen* **167**: 191–194.

Kim C, Meskauskiene R, Apel K, Laloi C. 2008. No single way to understand singlet oxygen signalling in plants. *EMBO Journal* **9**: 435–439.

Krieger-Liszkay A. 2005. Singlet oxygen production in photosynthesis. *Journal of Experimental Botany* **56**: 337–346.

Krieger-Liszkay A, Fufezan C, Trebst A. 2008. Singlet oxygen production in photosystem II and related protection mechanism. *Photosynthesis Research* **98**: 551–564.

Krueger T, Fisher PL, Becker S, Pontasch S, Dove S, Hoegh-Guldberg O, Leggat W, Davy SK. 2015. Transcriptomic characterization of the enzymatic antioxidants FeSOD, MnSOD, APX and KatG in the dinoflagellate genus *Symbiodinium*. *BMC Evolutionary Biology* **15**: 48.

Kumar MS, Singh RP, Patel V. 2011. Isolation and characterization of exopolysaccharide secreted by a toxic dinoflagellate, *Amphidinium carterae*

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Hulburt 1957 and its probable role in harmful algal blooms (HABs). *Microbial Ecology* **62**: 527.

Laloi C, Apel K, Danon A. 2004. Reactive oxygen signalling: the latest news. *Current Opinion in Plant Biology* **7**: 323–328.

Lesser MP. 1996. Elevated temperatures and ultraviolet radiation cause oxidative stress and inhibit photosynthesis in symbiotic dinoflagellates. *Limnology and Oceanography* **41**: 271–283.

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.

Li H, Melo TB, Arellano JB, Naqvi KR. 2012. Temporal profile of the singlet oxygen emission endogenously produced by photosystem II reaction centre in an aqueous buffer. *Photosynthesis Research* **112**: 75–79.

Lilley RM, Ralph PJ, Larkum AWD. 2010. The determination of activity of the enzyme Rubisco in cell extracts of the dinoflagellate alga *Symbiodinium* sp. by manganese chemiluminescence and its response to short-term thermal stress of the alga. *Plant, Cell & Environment* **33**: 995–1004.

Macpherson AN, Telfer A, Barber J, Truscott TG. 1993. Direct detection of singlet oxygen from isolated photosystem II reaction centres. *Biochimica et Biophysica Acta* **1143**: 301–309.

Meskauskiene R, Goslings D, Kessler F, op den Camp R, Apel K. 2001. FLU: a negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **98**: 12826–12831.

Moan J. 1990. On the diffusion length of singlet oxygen in cells and tissues. *Journal of Photochemistry and Photobiology B: Biology* **6**: 343–347.

Ogilby PR, Foote CS. 1982. Chemistry of singlet oxygen. 36. Singlet molecular oxygen (^1D_g) luminescence in solution following pulsed laser excitation. Solvent deuterium isotope effects on the lifetime of singlet oxygen. *Journal of the American Chemical Society* **104**: 2069–2070.

Pospísil P. 2012. Molecular mechanisms of production and scavenging of reactive oxygen species by photosystem II. *Biochimica et Biophysica Acta* **1817**: 218–231.

Ragás X, Jiménez-Banzo A, Sànchez-García D, Batllori X, Nonell S. 2009. Singlet oxygen photosensitisation by the fluorescent probe Singlet Oxygen Sensor Green®. *Chemical Communications* **45**: 2920–2922.

Rehman AU, Cser K, Sass L, Vass I. 2013. Characterization of singlet oxygen production and its involvement in photodamage of Photosystem II in the cyanobacterium *Synechocystis* PCC 6803 by histidine-mediated chemical trapping. *Biochimica et Biophysica Acta* **1827**: 689–698.

Richier S, Furla P, Plantivaux A, Merle P-L, Allemand D. 2005. Symbiosis-induced adaptation to oxidative stress. *Journal of Experimental Biology* **208**: 277–285.

Schmitt F-J, Renger G, Friedrich T, Kreslavski VD, Zharmukhamedov SK, Los DA, Kuznetsov VV, Allakhverdiev SI. 2014. Reactive oxygen species: re-evaluation of generation, monitoring and role in stress-signaling in phototrophic organisms. *Biochimica et Biophysica Acta* **1837**: 835–848.

Sedoud A, Lopez-Igual R, Rehman AU, Wilson A, Perreau F, Boulay C, Vass I, Krieger-Liszkay A, Kirilovsky D. 2014. The cyanobacterial photoactive orange carotenoid protein is an excellent singlet oxygen quencher. *Plant Cell* **26**: 1781–1791.

Shao N, Duan GY, Bock R. 2013. A mediator of singlet oxygen responses in *Chlamydomonas reinhardtii* and *Arabidopsis* identified by a luciferase-based genetic screen in algal cells. *Plant Cell* **25**: 4209–4226.

Shunmugam S, Jokela J, Wahlsten M, Battchikova N, Rehman AU, Vass I, Karonen M, Sinkkonen J, Permi P, Sivonen K *et al.* 2014. Secondary metabolite from *Nostoc* XPORK14A inhibits photosynthesis and growth of *Synechocystis* PCC 6803. *Plant, Cell & Environment* **37**: 1371–1381.

Sicher RC. 1984. Glycolaldehyde inhibition of photosynthetic carbon assimilation by isolated chloroplasts and protoplasts. In: Sybesma C, ed. *Advances in photosynthesis research. Proceedings of the VIth International Congress on Photosynthesis, Brussels, Belgium, August 1–6, 1983, vol. 3*. The Hague, the Netherlands: Springer, 413–416.

Skovsen E, Snyder JW, Lambert JDC, Ogilby PR. 2005. Lifetime and diffusion of singlet oxygen in a cell. *Journal of Physical Chemistry B* **109**: 8570–8573.

Slavov C, Schrameyer V, Reus M, Ralph PJ, Hill R, Büchel C, Larkum AWD, Holzwarth AR. 2016. "Super-quenching" state protects *Symbiodinium* from thermal stress – implications for coral bleaching. *Biochimica et Biophysica Acta* **1857**: 840–847.

Strasser RJ, Srivastava A, Govindjee. 1995. Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. *Photochemistry and Photobiology* **61**: 32–42.

Suggett DJ, Goyen S, Evenhuis C, Szabó M, Pettay DT, Warner ME, Ralph PJ. 2015. Functional diversity of photobiological traits within the genus *Symbiodinium* appears to be governed by the interaction of cell size with cladal designation. *New Phytologist* **208**: 370–381.

Suggett DJ, Warner ME, Smith DJ, Davey P, Hennige S, Baker NR. 2008. Photosynthesis and production of hydrogen peroxide by *Symbiodinium* (Pyrrhophyta) phylotypes with different thermal tolerances. *Journal of Phycology* **44**: 948–956.

Takahashi S, Whitney S, Itoh S, Maruyama T, Badger M. 2008. Heat stress causes inhibition of the *de novo* synthesis of antenna proteins and photobleaching in cultured *Symbiodinium*. *Proceedings of the National Academy of Sciences, USA* **105**: 4203–4208.

Takahashi S, Yoshioka-Nishimura M, Nanba D, Badger MR. 2013. Thermal acclimation of the symbiotic alga *Symbiodinium* spp. alleviates photobleaching under heat stress. *Plant Physiology* **161**: 477–485.

Telfer A, Oldham TC, Phillips D, Barber J. 1999. Singlet oxygen formation detected by near-infrared emission from isolated photosystem II reaction centres: direct correlation between P680 triplet decay and luminescence rise kinetics and its consequences for photoinhibition. *Journal of Photochemistry and Photobiology B: Biology* **48**: 89–96.

Tolleter D, Seneca FO, DeNofrio JC, Krediet CJ, Palumbi SR, Pringle JR, Grossman AR. 2013. Coral bleaching independent of photosynthetic activity. *Current Biology* **23**: 1782–1786.

Vass I. 2011. Role of charge recombination processes in photodamage and photoprotection of the photosystem II complex. *Physiologia Plantarum* **142**: 6–16.

Vass I. 2012. Molecular mechanisms of photodamage in the photosystem II complex. *Biochimica et Biophysica Acta* **1817**: 209–217.

Vass I, Cser K. 2009. Janus-faced charge recombinations in photosystem II photoinhibition. *Trends in Plant Science* **14**: 200–205.

Wagner D, Przybyla D, op den Camp R, Kim C, Landgraf F, Lee KP, Würsch M, Laloi C, Nater M, Hideg E *et al.* 2004. The genetic basis of singlet oxygen-induced stress responses of *Arabidopsis thaliana*. *Science* **306**: 1183–1185.

Weis VM. 2008. Cellular mechanisms of Cnidarian bleaching: stress causes the collapse of symbiosis. *Journal of Experimental Biology* **211**: 3059–3066.

Wietheger A, Fisher PL, Gould KS, Davy SK. 2015. Sensitivity to oxidative stress is not a definite predictor of thermal sensitivity in symbiotic dinoflagellates. *Marine Biology* **162**: 2067–2077.

Wishnick M, Lane MD. 1969. Inhibition of ribulose diphosphate carboxylase by cyanide. Inactive ternary complex of enzyme, ribulose diphosphate, and cyanide. *Journal of Biological Chemistry* **244**: 55–59.

Xiang T, Nelson W, Rodriguez J, Tolleter D, Grossman AR. 2015. *Symbiodinium* transcriptome and global responses of cells to immediate changes in light intensity when grown under autotrophic or mixotrophic conditions. *Plant Journal* **82**: 67–80.

Yellowless D, Rees TAV, Leggat W. 2008. Metabolic interactions between algal symbionts and invertebrate hosts. *Plant, Cell & Environment* **31**: 679–694.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Histidine (His)-mediated O_2 uptake in *Symbiodinium* cells.

Fig. S2 The effect of histidine on photosystem II (PSII) activity in intact *Symbiodinium* cells as measured by fast chlorophyll fluorescence rise.

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Fig. S3 The effect of histidine on *Symbiodinium* pigment bleaching.

Fig. S4 Kinetics of extracellular $^1O_2$ formation in a resuspended *Symbiodinium* culture.

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