Biogeosciences, 11, 4587–4598, 2014
www.biogeosciences.net/11/4587/2014/
doi:10.5194/bg-11-4587-2014

Biogeosciences Open Access

Release of hydrogen peroxide and antioxidants by the coral *Stylophora pistillata* to its external *milieu*

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Received: 30 November 2013 – Published in Biogeosciences Discuss.: 2 January 2014
Revised: 24 July 2014 – Accepted: 25 July 2014 – Published: 1 September 2014

Abstract. Hydrogen peroxide (H_2O_2), a common reactive oxygen species, plays multiple roles in coral health and disease. Elevated H_2O_2 production by the symbiotic algae during stress may result in symbiosis breakdown and bleaching of the coral. We have recently reported that various Red Sea corals release H_2O_2 and antioxidants to their external *milieu*, and can influence the H_2O_2 dynamics in the reef. Here, we present a laboratory characterization of H_2O_2 and antioxidant activity release kinetics by intact, non-stressed *Stylophora pistillata*. Experimenting with bleached and non-bleached corals and different stirring speeds, we explored the sources and modes of H_2O_2 and antioxidant release. Since H_2O_2 is produced and degraded simultaneously, we developed a methodology for resolving the actual H_2O_2 concentrations released by the corals.

H_2O_2 and antioxidant activity steadily increased in the water surrounding the coral over short periods of 1–2 h. Over longer periods of 5–7 h, the antioxidant activity kept increasing with time, while H_2O_2 concentrations were stabilized at $\sim$ 1 µM by 1–3 h, and then gradually declined. Solving for H_2O_2 release, corals were found to release H_2O_2 at increasing rates over 2–4 h, and then to slow down and stop by 5–7 h. Stirring was shown to induce the release of H_2O_2, possibly since the flow reduces the thickness of the diffusive boundary layer of the coral, and thus increases H_2O_2 mass flux. Antioxidant activity was released at similar rates by bleached and non-bleached corals, suggesting that the antioxidants did not originate from the symbiotic algae. H_2O_2, however, was not released from bleached corals, implying that the symbiotic algae are the source of the released H_2O_2. The observed flow-induced H_2O_2 release may aid corals in removing some of the internal H_2O_2 produced by their symbiotic algae, and may possibly assist in preventing coral bleaching under conditions of elevated temperature and irradiance.

1 Introduction

Hydrogen peroxide (H_2O_2) is a commonly occurring reactive oxygen species (ROS) of great biological importance. In the marine environment, H_2O_2 originates from photochemical and biological reactions, redox cycling of metals, and rain, and its concentrations range from tens to hundreds of nanomolars (Avery et al., 2005; Hansard et al., 2010; Shaked et al., 2010). In aerobic organisms, H_2O_2 is generated during photosynthesis, respiration and numerous other pathways, and its concentrations can reach tens of micromolars (Halliwell et al., 2000; Apel and Hirt, 2004). At low levels, H_2O_2 and other ROS serve as important signaling molecules (Bartosz, 2009). However, ROS production and accumulation beyond the capacity of an organism to quench them efficiently or to repair the resulting damage, a state known as oxidative stress, results in extensive damage to cellular components, including proteins, lipids, and DNA, all of which may eventually lead to cell demise (Apel and Hirt, 2004; Gechev and Hille, 2005). Whether ROS will act as a damaging or signaling molecule depends on the delicate equilibrium between ROS production and degradation (Sharma et al., 2012).

Residing in shallow, warm, and strongly illuminated water, corals are likely subjected to elevated concentrations of photochemically produced ROS (Shaked and Armoza-Zvuloni, 2013). Corals may also experience high ROS fluxes from within – from their symbiotic algae (Jones et al., 1998; Lesser, 1996; Suggett et al., 2008; Saragosti et al., 2010).

When conditions are optimal, the symbiotic algae of the genus *Symbiodinium* that live within the coral endodermal cells provide the coral host with energy (Muscatine, 1990; Papina et al., 2003). However, when corals experience elevated temperature and/or irradiance, ROS generated primarily by the symbiotic algae may result in oxidative stress, symbiosis breakdown, and bleaching (Nii and Muscatine, 1997; Downs et al., 2002; Lesser, 2011). The susceptibility of corals to oxidative stress and bleaching is influenced by physiological and phylogenic factors of the coral host and its symbionts as well as by environmental factors (Hoegh-Guldberg, 1999). Corals subjected to strong currents were found to be less susceptible to bleaching, presumably due to favorable removal of ROS through their diffusive boundary layer (Nakamura and van Woesik, 2001; Nakamura, 2010). Experimental addition of exogenous antioxidants to thermally stressed corals was shown to improve photosynthesis and to ameliorate bleaching (Lesser, 1997). These studies suggest that corals can exchange ROS and antioxidants with their external *milieu* and that these processes may be beneficial during oxidative stress.

In recent years, we adopted sensitive methods and kinetic approaches to measure the dynamics (that is, the production and release) of ROS in the external *milieu* of intact corals in laboratory and natural settings. We found that the coral *Stylophora pistillata* produces and releases superoxide (O_2^-) and enzymes, degrading superoxide to its surrounding water (Saragosti et al., 2010). Later we found that *S. pistillata* and six other Red Sea coral genera also release H_2O_2 and compounds that degrade H_2O_2 (termed here, for simplicity, antioxidants) to the incubation water (Shaked and Armoza-Zvuloni, 2013). In the field, we documented elevated antioxidant activities (or high H_2O_2 degradation rates) next to individual and knoll corals and in the reef lagoon, which were accompanied by lowered H_2O_2 concentrations in the coral vicinity (Shaked and Armoza-Zvuloni, 2013).

Here, we set out to study the release of H_2O_2 and antioxidant activity from intact, non-stressed *Stylophora pistillata* fragments in a laboratory setting. Experimenting with bleached and non-bleached corals and different stirring speeds under low light, we explored the sources of the released H_2O_2 and antioxidant activity and their mode of release. An inherent difficulty in quantifying the dynamics of H_2O_2 is that it is influenced concurrently by two opposing reactions – release from the coral and degradation by antioxidants. By applying sensitive kinetic antioxidant assay and frequent measurements, we were able to resolve the actual concentrations and rates of H_2O_2 release from the coral. This in turn allows a comparison between conditions and treatments, and can serve for future probing of coral response to oxidative stress.

2 Materials and methods

2.1 H_2O_2 and antioxidant activity determination

H_2O_2 measurements – H_2O_2 concentration was measured with a Varian spectroflurometer (Cary Eclipse), using the POHPPA (4-hydroxyphenylacetic acid) technique as detailed in Shaked and Armoza-Zvuloni (2013). Briefly, the POHPPA reagent stock consisting of 0.25 mM POHPPA, 70 units mL^{-1} of horseradish peroxidase, and 0.25 M Tris at pH 8.8, was added to the samples at a 1 : 50 dilution. All stocks were kept on ice in the dark. The florescence of dark-kept samples was read using an excitation of 315 ± 10 nm and an emission of 408 ± 10 nm, within 2 h of sampling. Calibration curves were run daily using filtered seawater spiked with freshly made H_2O_2 standards and catalase-amended seawater as blanks.

Antioxidant activity – Antioxidant activity, or more precisely H_2O_2 degradation activity released by the corals, was determined experimentally by following the loss kinetics of H_2O_2 spikes added to subsamples collected over the course of the incubation. The 5 mL subsamples were stored for 1 h prior to spiking with external H_2O_2, to allow complete degradation of the coral-released H_2O_2. Then, H_2O_2 was added at a final concentration of 1 µM, mixed thoroughly, and subsampled into cuvettes containing the POHPPA reagent stock at time intervals ranging from a few minutes to a few hours (Fig. 1). The antioxidant activity is determined by the concentration and efficiency of the antioxidant. Corals probably release different antioxidants with varying efficiencies. Nonetheless, we can generate a simplified kinetic term that groups together all antioxidants, and assign them with a composite efficiency in the form of a rate constant (k_d with units of $M^{-1}\,time^{-1}$). We can then describe the rate of H_2O_2 degradation:

$$\begin{aligned} H_2O_2 \text{ degradation rate} &= \frac{\partial [H_2O_2]}{\partial t} \\ &= -k_d \times [\text{Antioxidants}] \times [H_2O_2]. \end{aligned} \quad (1)$$

While Eq. (1) predicts a second-order reaction, we observed exponential decline in H_2O_2 concentrations with time (Fig. 1). This pseudo-first-order behavior suggests that the antioxidant activity (the product of the antioxidant efficiency and its concentration: $k_d \times$ [Antioixdants]) is constant throughout the assay. Considering the renewable (i.e., enzymatic) nature of many antioxidants, the short assay duration, and the low $[H_2O_2]$ applied, it is expected that the antioxidant activity will remain unchanged over the course of its determination. We can thus assign a single constant for describing the antioxidant activity of a sample (k_{antiox} with units of $time^{-1}$):

$$H_2O_2 \text{ degradation rate} = -k_{antiox} \times [H_2O_2] \quad (2)$$

Drawing on first-order law principles, the antioxidant activity of a sample (k_{antiox}) can be presented graphically as a linear

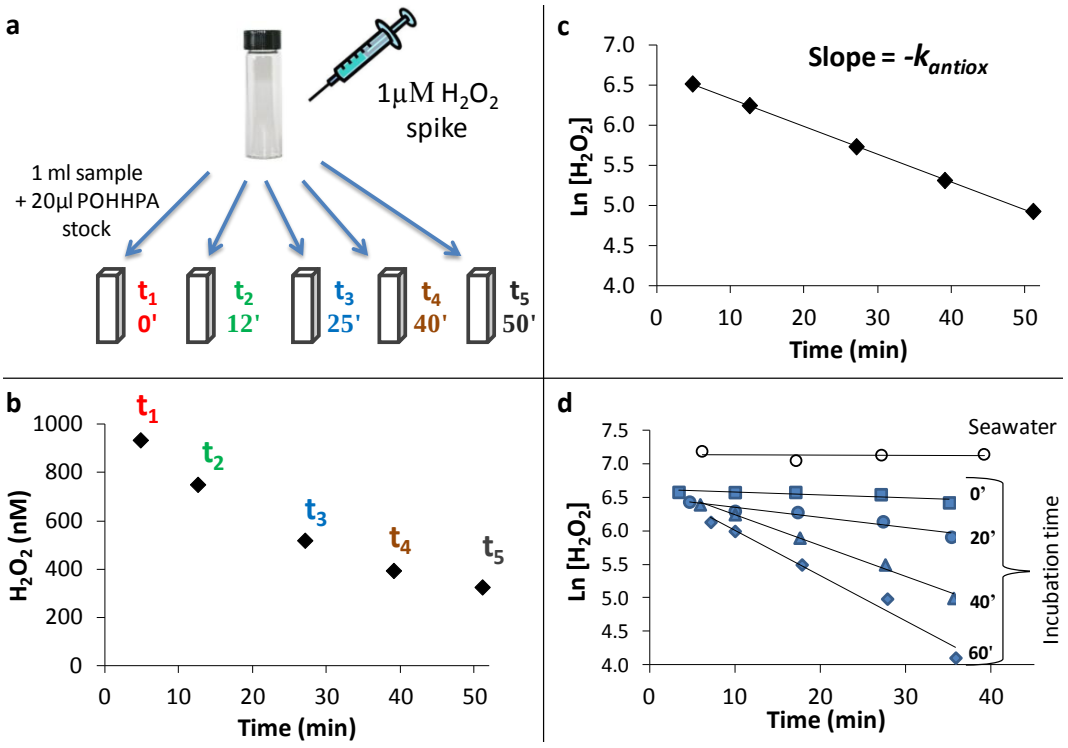

Figure 1. Determination of antioxidant activity in coral incubation water. **(a)** H_2O_2 spike is added to a sample, mixed, and subsampled into cuvettes containing PHOPPA reagent at time intervals ranging from a few minutes to a few hours. **(b)** H_2O_2 concentrations decline with time due to the antioxidant activity. The exponential decline is typical of pseudo-first-order kinetics. **(c)** The antioxidant activity of a sample is evaluated from the decay constant (k_{antiox}) obtained by plotting the natural log of the H_2O_2 concentration versus time. **(d)** The antioxidant activity released from the coral accumulates with time in the incubation water. Subsequently, samples collected throughout the incubation show faster H_2O_2 degradation rates and higher decay constants (k_{antiox}).

slope when plotting the natural log of H_2O_2 versus time, where steeper slopes refer to stronger antioxidant activity (Fig. 1). The value of k_{antiox} in a subsample is fixed, and can be repeatedly measured by re-spiking the sample with H_2O_2. However, throughout an incubation experiment, k_{antiox} values of the subsamples increase with time, since more antioxidants are released to the water (Fig. 2).

2.2 H_2O_2 release calculation

The changes with time in H_2O_2 concentrations in an incubation experiment reflect the balance between two opposing processes – H_2O_2 release from the coral and H_2O_2 degradation by the antioxidants released from the coral:

$$\frac{\partial [H_2O_2]}{\partial t} = H_2O_2 \text{ release rate} - H_2O_2 \text{degredation rate}. \quad (3)$$

For small time intervals (dt), this differential equation can be solved numerically. Plugging Eq. (2) into the degradation rate, we get the following expression:

$$\frac{d[H_2O_2]}{dt} = H_2O_2 \text{ release rate} - k_{antiox} \times [H_2O_2], \quad (4)$$

which can be rearranged as follows:

$$H_2O_2 \text{ release rate} = \frac{d[H_2O_2]}{dt} + k_{antiox} \times [H_2O_2]. \quad (5)$$

Having conducted frequent H_2O_2 measurements, we can calculate the values of $\frac{d[H_2O_2]}{dt}$ for subsequent time intervals of 5–10 min throughout the experiment. Similarly, for each 5–10 min interval, we can calculate H_2O_2 degradation rates using k_{antiox} values. Since k_{antiox} was measured only every 20 min and Eq. (5) solved for 5–10 min intervals, we had to calculate some of the k_{antiox} values. Missing k_{antiox} values were calculated either by averaging the two nearest measured k_{antiox} (for short incubations) or by using a linear equation fitted through the data (for long incubations, see Supplement S1 for more information). The calculation output of discrete H_2O_2 release rates can then be converted to a H_2O_2 concentration released by the coral by multiplying each H_2O_2 release rate by the time it represents, and then summing them up.

2.3 Experimental setup for measuring H_2O_2 and antioxidant release rates

Coral handling and preconditioning – 30 similarly sized *Stylophora pistillata* coral fragments were collected from an in situ coral nursery at a 5 m depth. The fragments had a surface area of $\sim 20\,cm^2$, and they were chosen to fit a 100 mL glass beaker. The surface area was measured on five representative coral fragments with the aluminium foil technique (Marsh, 1970; see Supplement S2 for more information on coral sizes and beaker volumes). The coral fragments originated from different coral colonies, and therefore represent different genotypes. The fragments were kept prior to and throughout the experimental period on outdoor water tables with running natural seawater. The corals experienced natural dark/light regimes and were shaded with a black mesh. The corals were suspended on PVC stands using fine nylon threads to avoid contact with the container walls. Following one month of acclimation, 10 coral fragments were placed in a dark room. These dark-kept corals turned white (bleached) after a month, and a complete loss of the algal chlorophyll was confirmed using PAM (pulse amplitude modulation) chlorophyll fluorescence techniques. It is important to note that these bleached corals did not experience thermal or irradiance stress. The bleached corals were fed twice a week with 1-day old *Artemia salina* nauplii (see the picture in Supplement S3).

Incubation experiment setup – The release rates of H_2O_2 and antioxidants by *S. pistillata* coral fragments were tested by incubating individual fragments in a glass beaker with 100 mL seawater for various time periods (see below). Coral fragments were gently transferred and suspended in the incubation beakers, and were allowed to acclimate for 15 min with running seawater. Water exchange was then stopped, and the beaker was placed on a stirrer, where a 1 cm long magnet bar ensured complete water mixing (as pictured in Supplement S3). The magnet stirring speed, reported as rounds per min (rpm), was enumerated using a high-resolution video camera. A constant stirring speed of 400 rpm was applied in all experiments (except those with increasing stirring speed; see below). Physical contact with the coral and exposure to air were found to invoke high H_2O_2 and antioxidant release, and were minimized where possible. H_2O_2 concentrations were determined in subsamples every 5–10 min throughout the incubation. The 1 mL subsamples were mixed with 20 µL of the POHPPA reagent stock in cuvettes, and were read within 2 h. Antioxidant activities were determined in 5 mL subsamples collected every 15–20 min, as described above (see Sect. 2.1). The water level was kept constant by replacing the sampled volume with fresh seawater (8 mL every 20 min). No correction for this minor dilution with fresh seawater was made (if anything, such a correction should slightly increase the observed values). All incubations were conducted at constant temperature ($25 \pm 1\,°C$) and under fluorescence laboratory light. This low illumination (~ 10 µmole quanta $m^{-2}\,s^{-1}$) is sufficient only for minimal photosynthesis (or none at all), as it is far below the compensation light intensity of these corals of 25 µmole quanta $m^{-2}\,s^{-1}$ (established from oxygen-based PI curves; data not shown). Several experiments in complete darkness were conducted to examine the resemblance of these low-light conditions to darkness (Supplement S4). In addition, a preliminary experiment was conducted at high natural irradiation in a flow-through mode to test the effect of light on H_2O_2 release (see Supplement S5).

Experiment types

(1) Long incubations – Ten long (5–7 h) incubations were conducted on ten different fragments between July and August 2012 to evaluate the kinetics and duration of H_2O_2 and antioxidant activity release.

(2) Bleached versus non-bleached corals – Short (1 h) experiments were conducted between April and June 2013 using 10 bleached fragments and 15 non-bleached fragments. In preliminary experiments, no H_2O_2 release was observed from bleached corals. Since antioxidant activity is released simultaneously, low H_2O_2 release rates may be missed. To account for this possibility, 250–300 nM of H_2O_2 was added to the water at the beginning of the experiment, enabling the calculation of H_2O_2 release rates (Eq. 5). Differences between bleached and non-bleached *S. pistillata* fragments were tested using a student *t* test (for parametric data) and the Mann–Whitney *U* test (for nonparametric data; Statistica 8® software).

(3) Different stirring speeds – Nine long incubations at increasing steering speeds were conducted between August and September 2012, with nine different coral fragments. In all the experiments, identical stirrers and 1 cm long magnet bars were used. Three stirring speeds were tested over the course of 4 h: slow, moderate and fast. In the first 80 min (slow flow), the stirrer was operated for only 30 s prior to sampling (at a speed of 400 rpm) to mix the incubation water. In the next 80 min (moderate flow), the stirrer was operated continuously at 400 rpm. In the last stage (fast flow), the stirrer was operated continuously at 1000 rpm. At all stages, the coral polyps were extended and the corals did not show signs of stress. The fast stirring speed did not change the position of the suspended corals, nor did it generate foam. Statistical analyses were conducted using R software. In order to assess the effect of stirring speed (slow, moderate, fast) on release rates from corals, repeated measure ANOVA was applied using the linear mixed effect (lme) function in R (http://CRAN.R-project.org/package=nlme). Tukey's test for multiple comparisons was applied using the general linear hypothesis testing

(glht) function (http://www.crcpress.com/product/isbn/9781584885740).

A complementary flow experiment was conducted to examine the effect of stirring speed on release rates by corals under reversed conditions. Here, a fast stirring speed (1000 rpm) was applied initially, and after 60 min, stirring was reduced dramatically to a slow speed (see Supplement S6).

2.4 Biochemical characterization

Three coral fragments were incubated in stirred seawater for 1 h. The coral was then removed, and the incubation water was used for basic biochemical characterization of the antioxidant activity. Coral incubation water was subsampled (5 mL) and treated with the heme-enzyme inhibitor sodium azide (NaN_3, applied at 0.1 mM for a 10 min incubation period) and the catalase inhibitor 3-Amino-1,2,4-Triazole (applied at 0.01–6 mM for a 40 min incubation period). Samples were also filtered through a 0.2 µm Sartorius syringe filter, stored overnight at room temperature, and incubated at a range of temperatures in heating water baths (10 min incubation period).

The antioxidant activities measured here cannot simply be converted to standard units of enzymatic activity. Catalase activity, for example, is measured using 10.3 mM H_2O_2 at pH 7 and at 25 °C, where one catalase unit is defined as the amount of enzyme decomposing 1 µmol of H_2O_2 in 1 min under these conditions. To allow this conversion, we assayed coral incubation water with high antioxidant activity in two different procedures. A few subsamples were diluted and assayed with our procedure, as described above. Other subsamples were amended with 10.3 mM H_2O_2 (at pH 7 and 25 °C), and its decay was followed at 240 nm using a Varian Cary 50 spectrophotometer.

3 Results

3.1 Quantifying H_2O_2 release rates

Release of antioxidants and H_2O_2 from *Stylophora pistillata* to the surrounding water was observed in all experiments, when the coral fragments were subjected to stirring. Since H_2O_2 is simultaneously released from the coral and degraded by antioxidants, its measured concentrations provide rather limited information on the actual H_2O_2 released by the coral. In order to quantify the rates and amount of H_2O_2 released to the water, we frequently measured H_2O_2 concentrations and antioxidant activities throughout the experiment. From these measurements, using Eq. (5), we corrected for H_2O_2 loss due to antioxidant activity and calculated H_2O_2 release rates at discrete 5–10 min intervals. Then, summing up these intervals, we can get at the total amount of H_2O_2 released by the coral in the incubation.

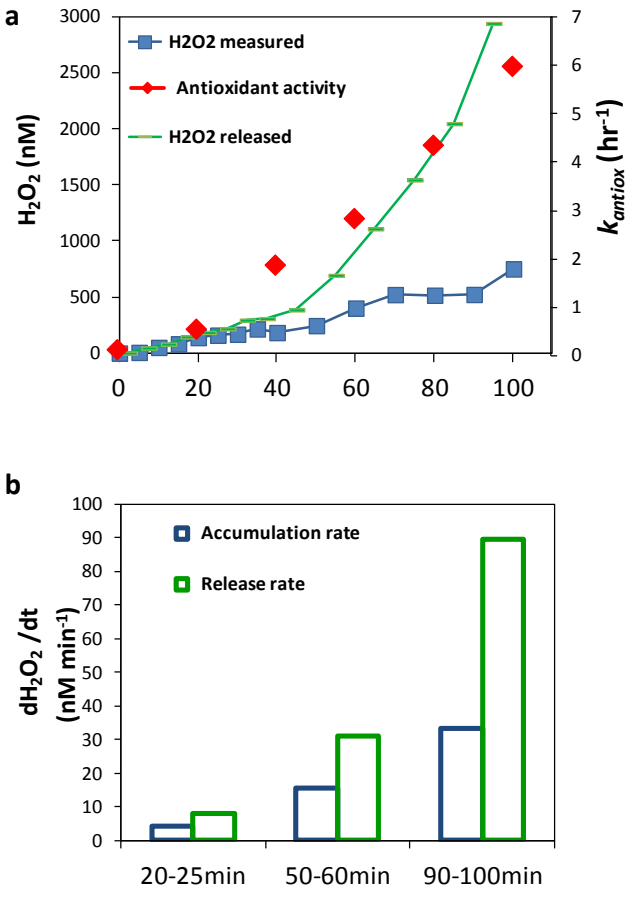

Figure 2. Quantification of H_2O_2 release by *Stylophora pistillata*. Data from a short-term experiment are plotted to demonstrate the corrections made to account for H_2O_2 loss due to antioxidant activity. **(a)** The measured variables are H_2O_2 concentrations (blue squares) and antioxidant activities (red diamonds). Since H_2O_2 is degraded by the antioxidants, its measured concentrations are lower than those released by the coral (green lines). The coral-released H_2O_2 is calculated for small time intervals using Eq. (5), and is then added up. **(b)** Rates of H_2O_2 release (green bars, Eq. 5) are calculated for each time interval from H_2O_2 accumulation rates (blue bars, Eq. 4) and k_{antiox}. These rates grow apart with time due to increased antioxidant activities.

In Fig. 2, using a short-term experiment with non-bleached coral, we demonstrate these calculations. Initially, the incubation water contains neither H_2O_2 nor antioxidants, and with time, their levels increase (Fig. 2a). The actual amount of H_2O_2 released by the coral is greater than its measured concentration, since it is degraded by the antioxidants. As the experiment progresses, the rate of degradation, which is the product of H_2O_2 concentration and the antioxidant activity (k_{antiox}, Eq. 2), increases, and the measured and released H_2O_2 concentrations grow apart (Fig. 2a). Similarly, the rates of H_2O_2 accumulation and release calculated for each 5–10 min time interval (Eq. 5) grow apart with time due to higher H_2O_2 degradation rates (Fig. 2b).

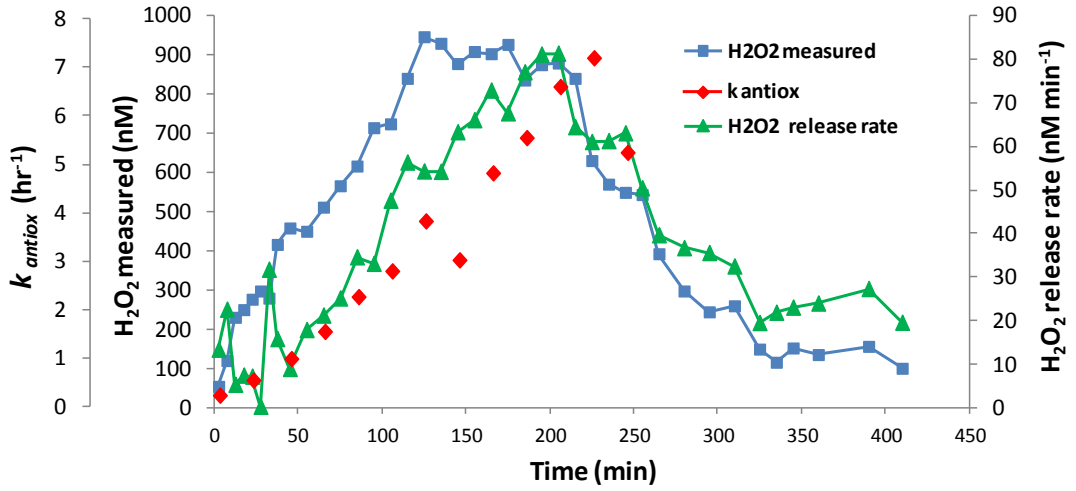

Figure 3. H_2O_2 and antioxidant activity release kinetics in a long incubation. A representative 7 h long experiment showing the accumulation of antioxidant activity (red diamonds) and changes in H_2O_2 concentrations (blue squares) over time in the coral incubation water. These frequent measurements enabled the calculation of actual H_2O_2 release rates by the coral (green triangle, Eq. 5).

3.2 Kinetics of H_2O_2 and antioxidant activity release

Ten long (5–7 h) incubations were conducted to evaluate the kinetics and duration of H_2O_2 and antioxidant release. One such experiment is shown here (Fig. 3), while others appear in Supplement S7, to demonstrate the variability among experiments. In this experiment, both H_2O_2 concentrations and antioxidant activity increased steadily with time initially. The antioxidant kept on increasing throughout the experiment in a linear manner ($R^2 = 0.91$). H_2O_2 concentration reached maximal values of $\sim 1\,\mu M$ at about 2 h, and this concentration was maintained for an additional hour. In the last 2 h of the experiment, H_2O_2 concentration declined with time: at a steep slope initially, and more gradually later on. The rates of H_2O_2 release were low and fluctuating in the first hour. Then, H_2O_2 release rates increased, peaked at $80\,nM\,min^{-1}$ ($= 0.4\,nmol\,min^{-1}\,cm^{-2}$ coral) around 3.5 h, and declined afterwards. Note that with time, as the antioxidant activity increases, it becomes the dominant term in the calculation of H_2O_2 release rates (Eq. 5), and hence the assumptions made when extrapolating k_{antiox} to missing points may influence the predicted H_2O_2 release. Here, using a linear fit for k_{antiox}, we calculated that the total amount of H_2O_2 released by the 20 cm^2 coral fragment during this experiment was 1400 nmol (14 000 nM), or $\sim 70\,nmol\,cm^{-2}$ coral.

3.3 H_2O_2 and antioxidants release by bleached versus non-bleached *S. pistillata*

Incubations with bleached ($n = 10$) and non-bleached ($n = 15$) *S. pistillata* fragments were conducted to identify the sources of the released H_2O_2 and antioxidants. Representative experiments with non-bleached, bleached and coral-free water (control) demonstrate these release patterns (Fig. 4). In incubations with non-bleached coral, all studied parameters steadily increased with time (Fig. 4a–c). In incubations with bleached coral, H_2O_2 concentrations dropped (Fig. 4d), while the antioxidant activity accumulated with time (Fig. 4e). To discern whether bleached corals do not release H_2O_2, or whether its degradation exceeds its release, we added H_2O_2 to enable a H_2O_2 release calculation. The added H_2O_2 rapidly declined in the incubation water (Fig. 4d), and the calculated H_2O_2 release by the bleached coral was minimal (Fig. 4f). To facilitate the comparison between bleached and non-bleached corals, we looked at the data obtained at the end of the experiment at 1 h (Fig. 4g–i). This analysis includes all experiments from each treatment, and the variability among individual experiments is apparent from the large error bars. Despite this variability, the differences between bleached and non-bleached corals are clear ($p < 0.05$, Mann–Whitney U test, Fig. 4g). Non-bleached corals released an average of $1253 \pm 481\,nM$ or $125 \pm 48\,nmol\,H_2O_2$ in 1 h, while bleached corals released only negligible amounts (Fig. 4g). In contrast, both bleached and non-bleached corals release comparable antioxidant activity, as the level accumulated by 1 h in both experiments was statistically indistinguishable ($p > 0.05$, student t test, Fig. 4h).

3.4 The effect of stirring speed on H_2O_2 accumulation

Nine long (3–4 h) incubations were conducted to test the effect of the stirring rate, as a representative of flow speed, on H_2O_2 and antioxidant activity release rates. The experiment time course is shown for a representative experiment (Fig. 5a–c), and changes in release rates with stirring speed are presented for a compilation of all the experiments (Fig. 5d–f). Following an increase in the stirring

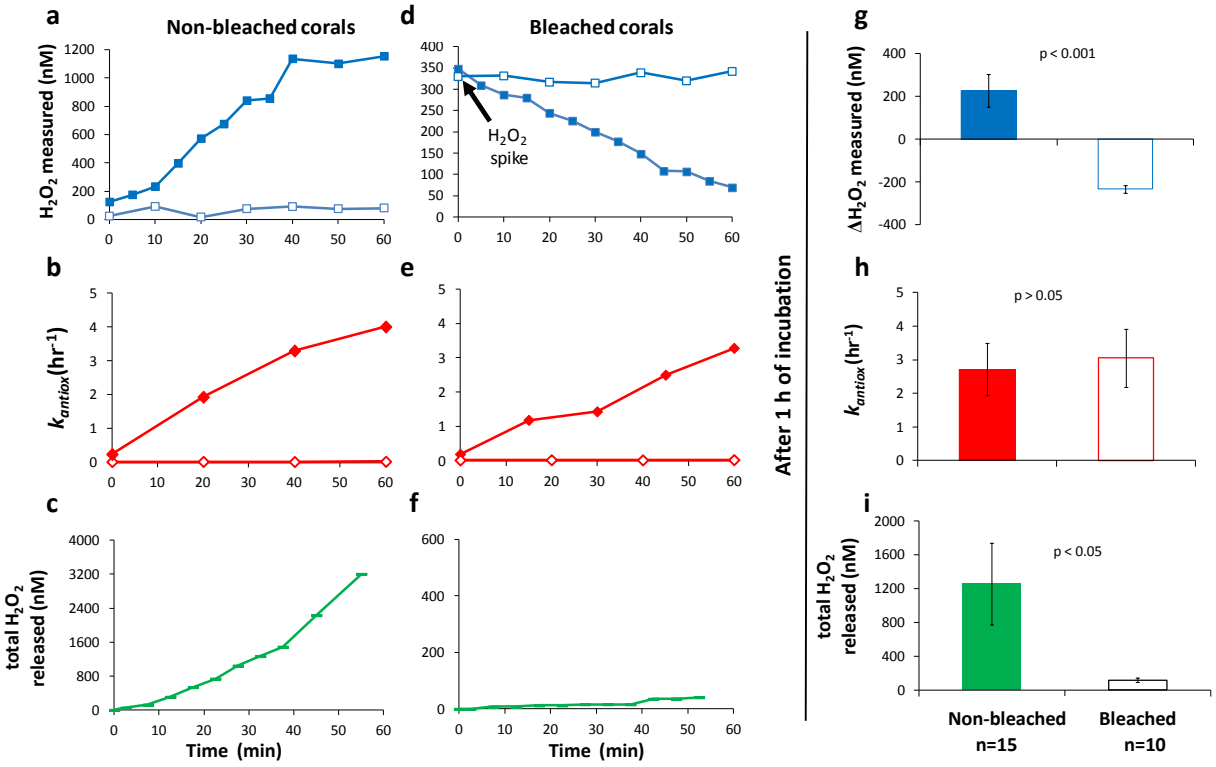

Figure 4. H_2O_2 and antioxidant activity release from non-bleached and bleached *S. pistillata*. Line plots show changes with time in measured H_2O_2 concentrations **(a, d)**, antioxidant activity **(b, e)**, and calculated H_2O_2 release **(c, e)** from individual experiments with bleached **(a–c)** and non-bleached **(d–f)** corals. Parallel measurements in coral-free seawater of H_2O_2 concentrations **(a, d)** and antioxidant activity **(b, e)** are shown using open symbols. Bar graphs show averaged values (mean ± SE) obtained from non-bleached ($n = 15$) and bleached ($n = 10$) coral fragments. These include the delta H_2O_2 measured (i.e., H_2O_2 at 1 h minus the initial H_2O_2) **(g)**, antioxidant activity at 1 h **(h)**, and total H_2O_2 released over 1 h **(i)**. Differences between treatments were tested using the Mann–Whitney U test **(g, i)** and a student t test **(h)**. Note that the H_2O_2 spike was added to the bleached coral **(d)**; see text for details.

speed, H_2O_2 concentrations immediately increased, but with time leveled off due to the antioxidant activity (Fig. 5a). The initial H_2O_2 accumulation rate (calculated from the initial linear increase in the H_2O_2 concentration) was found to increase significantly with stirring speed ($p < 0.05$, repeated measure ANOVA, Fig. 5d). Antioxidant activity was continuously released during the entire experiment (Fig. 5b). No statistical differences between stirring speeds in the rates of antioxidant activity release were obtained ($p > 0.05$, repeated measure ANOVA, Fig. 5b, e). The calculated H_2O_2 release did respond to the increase in stirring speed, and its levels increased (Fig. 5c). When comparing the rate of change in H_2O_2 release, it is significantly faster at higher stirring speeds ($p < 0.05$, repeated measure ANOVA, Fig. 5f). In an additional experiment where stirring was reduced from fast to slow, less H_2O_2 was released, but antioxidant activities kept increasing (Supplement S6).

3.5 Biochemical characterization of the antioxidants

Basic biochemical characterization reveals that the antioxidants released by the corals are anti-H_2O_2 enzymes probably resembling catalase (Fig. 6). The antioxidant activity was optimal at 20–30 °C, and decreased at low (4–15 °C) and high (60–70 °C) temperatures (Fig. 6a). Heating to 80 °C for 10 min resulted in a nearly complete loss of activity. Sodium azide, an inhibitor of heme enzymes such as catalase and peroxidase (Nizhnikov et al., 2007), completely inhibited the antioxidant activity (Fig. 6a). The catalase inhibitor 3-Amino-1,2,4-Triazole (3-AT; Margoliash and Novogrodsky, 1958; Merle et al., 2007; González-Sánchez et al., 2013; Supplement S8) inhibited the antioxidant activity in a dose-dependent manner (Fig. 6b). At low levels (0.01 mM 3-AT), 30 % of the antioxidant activity was retained, and at higher levels (≥ 0.1 mM 3-AT), 98% of the activity was inhibited. Filtration through a 0.2 µm membrane resulted in a 35 % loss of the activity (Fig. 6a), indicating that the antioxidants are mostly in the dissolved phase. The antioxidant activity was relatively stable, as about half of the activity remained following overnight storage at room temperature. To allow comparison with commonly used measures of antioxidant activity, we measured a few highly active samples in a standard catalase assay (see Sect. 2.4). The antioxidant activities

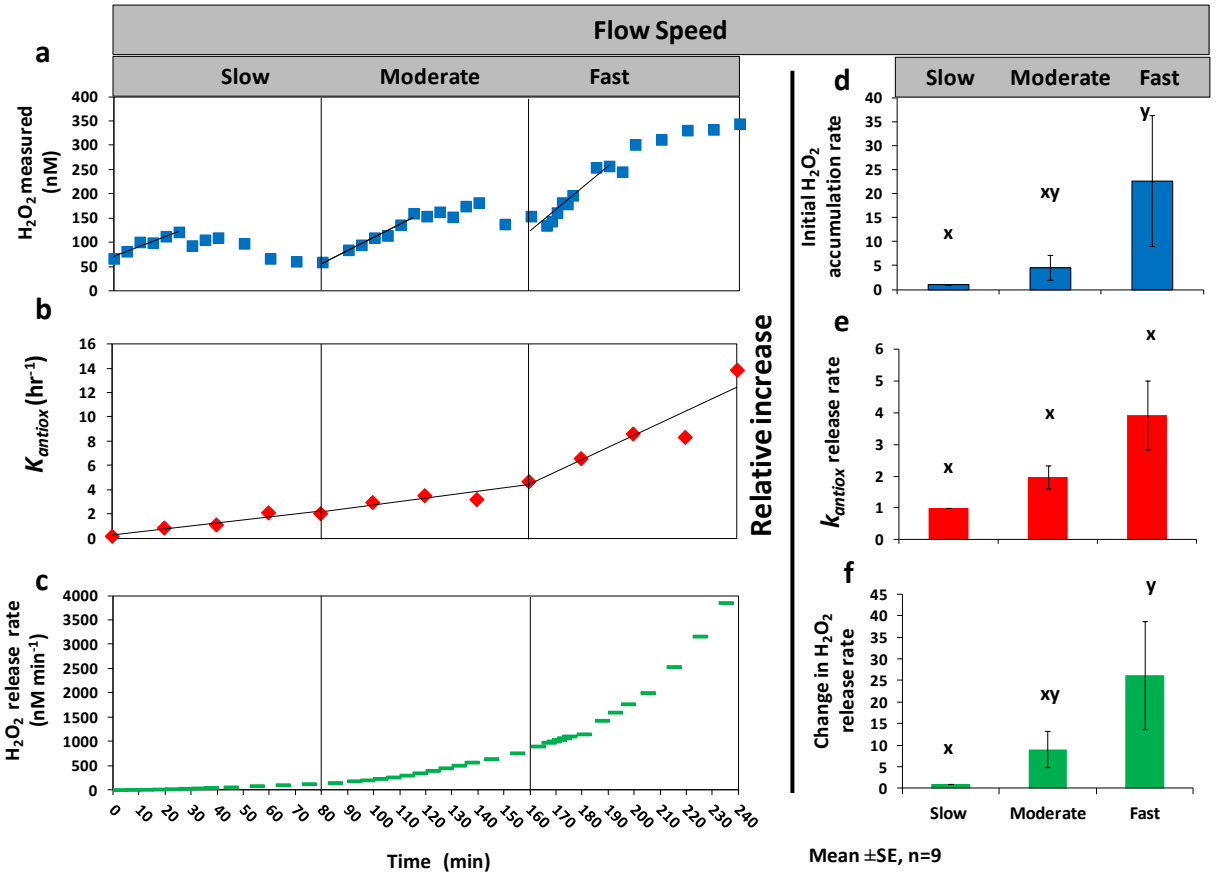

Figure 5. Release of H_2O_2 and antioxidant activity by *S. pistillata* at variable stirring speeds. Line plots showing changes with time in measured H_2O_2 concentrations **(a)** and antioxidant activity **(b)**, and calculated H_2O_2 released **(c)** and obtained by increasing stirring speeds in an individual experiment. The rate of change in each of the parameters (i.e., the slopes drawn in **a–c**) was calculated for every stirring speed. These rates were then expressed relative to those at slow stirring. These relative changes in each parameter were averaged among all nine experiments. Bar graphs show averaged relative changes of initial H_2O_2 accumulation rates **(d)**, antioxidant activity release **(e)**, and change in H_2O_2 release rates **(f)**. The letters in **(d–f)** refer to significant differences of $p < 0.05$ between stirring speeds for each of the studied parameters, tested with Tukey's multiple comparison test.

released by the coral were found to range from 10^{-4} to 10^{-3} catalase-like units.

4 Discussion

4.1 Methodology and concepts of studying H_2O_2 dynamics in the coral's external *milieu*

In this study, major emphasis was placed on developing methodological and conceptual approaches for following H_2O_2 and antioxidant release by corals in experimental settings. A sensitive, fluorometric-based method was applied to measure small changes in H_2O_2 concentrations over time. An accurate and reproducible approach was adopted to assess H_2O_2 degradation kinetics in the water surrounding the coral, termed here antioxidant activity (Fig. 1). This approach differs from standard antioxidant assays by the use of a low H_2O_2 concentration, longer degradation curves (up to 4 h), and the use of a degradation rate constant (k_{antiox}) rather than an initial H_2O_2 degradation rate. This approach enables the detection of minute concentrations of antioxidants and allows for studying of the behavior of biochemically uncharacterized compounds that react with H_2O_2. The use of k_{antiox} as a measure of antioxidant activity allows for evaluation of the actual rate of H_2O_2 degradation in the incubation (Eq. 2).

An inherent difficulty in quantifying the dynamics of H_2O_2 is that it is influenced concurrently by two opposing reactions – release from the coral and degradation by antioxidants. As a result, a differential equation is required to describe the changes in H_2O_2 concentrations with time (Eq. 3). By conducting numerous frequent measurements throughout the incubations, we were able to solve numerically this equation for small time intervals and to calculate the rates and total amount of H_2O_2 released from the corals (Eq. 5). These in turn allow us to examine the quantitative significance of

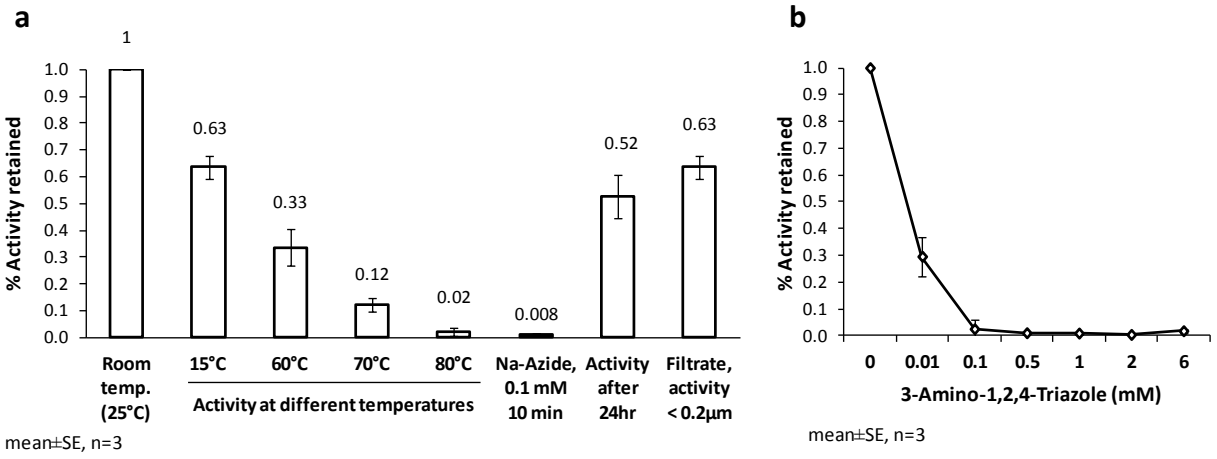

Figure 6. Basic biochemical characterization of the coral-released antioxidant activity. The effect of temperature (15, 60, 70, and 80 °C), the heme-enzyme inhibitor (0.1 mM Na Azide), filtration (0.2 µm), and storage (24 h) on antioxidant activity from coral incubation water **(a)**. The effect of increasing concentrations of the catalase inhibitor 3-Amino-1,2,4-Triazole (0.01–6 mM) on coral-released antioxidant activity **(b)**. The antioxidant activity of the treated water samples ($n = 3$; mean ± SE) is presented as the percentage of the non-treated samples ($n = 3$).

our findings to the coral physiology, and to compare treatments (such as bleached versus non-bleached, or different flow speeds).

4.2 Antioxidants nature and source

Our basic biochemical examination revealed that the antioxidant activity released by the coral holobiont is enzymatic (Fig. 6). Possible sources of these enzymes are the coral host and the microorganisms that live in the mucus layer covering the coral, both of which have an array of antioxidants (Levy et al., 2006; Munn et al., 2008). The symbiotic algae do not seem to contribute significantly to the released antioxidants, since bleached corals released comparable antioxidant activity to non-bleached corals (Fig. 4). Previously, we reported that *S. pistillata* releases anti-superoxide (O_2^-) enzymes to its external *milieu* (Saragosti et al., 2010). These enzymes were also suggested to originate from the coral and/or its associated microbes, but not from the symbiotic algae (based on cultured algae; Saragosti et al., 2010). Both the anti-H_2O_2 and the anti-O_2^- enzymes accumulated with time in a linear fashion (Supplement S1), perhaps hinting at a similar release mode. However, their biochemical properties, such as stability with time and the fraction retained on a 0.2 µm membrane, differ (Fig. 6; Saragosti et al., 2010).

Further characterization of the coral-released anti-H_2O_2 enzymes, including a dose-dependent inhibition of the antioxidant activity by 3-Amino-1,2,4-Triazole (3-AT; Fig. 6), a specific catalase inhibitor (Margoliash and Novogrodsky, 1958, Supplement S8), indicates that catalase-like enzymes are responsible for H_2O_2 degradation. Interestingly, the introduction of 3-AT via seawater to the sea anemone *Anemonia viridis* was found to inhibit catalase activity and result in symbiont expulsion due to oxidative stress (Merle et al., 2007). Anemones are closely related to corals (both anthozoans), and hence the finding of catalase inhibition by 3-AT in anemones strengthens our identification of the antioxidant activity as catalase-like enzymes, at least in part. It is important to note that these catalase-like enzymes are probably a mixture of different compounds, which differ in their sources and physico-chemical properties. Some are soluble enzymes, while others are associated with the readily released coral mucus. Additional antioxidant activity assays and fine proteomic analyses can provide more insight into this suite of antioxidants.

Since the production of antioxidant enzymes is metabolically costly, the observed release may be considered a wasteful process. Nonetheless, the actual number of released enzymes is rather modest, ranging from 10^{-4} to 10^{-3} catalase-like units (U). These activities are substantially lower than in the coral tissue, which we estimate to range from 10 to 1000 catalase units (taking 3–100 U mg^{-1} from Levy et al., 2006, and 0.3–0.8 mg protein cm^{-2} coral from Ferrier-Pagès et al., 2003). An efficient degradation of H_2O_2 by small enzyme quantities is expected for the catalase-like enzymes observed here. The turnover numbers of catalase are one of the highest among all enzymes; one catalase molecule can convert millions of H_2O_2 molecules to water and oxygen each second (Sharma et al., 2012).

In addition to *S. pistallata*, we recently reported that six other Red Sea stony coral genera release anti-H_2O_2 activity to their external *milieu* in similar experimental settings (Shaked and Armoza-Zvuloni, 2013). Anti-H_2O_2 activities were also detected in situ in water collected from surfaces of individual and knoll corals, and from the reef lagoon. In nature, the antioxidant activities were found to lower H_2O_2 concentrations in the coral vicinity. Moreover, we observed during low tide high anti-H_2O_2 activity in a shallow reef

lagoon. This activity was sufficiently high to degrade photochemically produced H_2O_2, and may protect corals from external H_2O_2 fluxes (Shaked and Armoza-Zvuloni, 2013). These findings hint at some of the environmental and physiological implications of the anti-H_2O_2 activity release reported here.

4.3 H_2O_2 sources and modes of release

The potentially largest sources of H_2O_2 within the coral consortium are the symbiotic algae (Warner et al., 1999; Jones et al., 1998; Smith et al., 2005), which in culture were shown to generate high external H_2O_2 fluxes (Suggett et al., 2008). Here, by comparing algae-containing (non-bleached) and algae-free (bleached) corals (Fig. 4), we examined whether the symbiotic algae are the source of the H_2O_2 released to the incubation water. We find that bleached (dark-kept) corals release negligible amounts of H_2O_2, thus implying that the symbiotic algae are indeed the major source of the released H_2O_2.

Photosynthesis is considered the primary H_2O_2 generating pathway in algae (e.g., Suggett et al., 2008). In our experiments, however, the released H_2O_2 was probably produced by the algae through pathways other than photosynthesis. This is so since the low light levels (10 µmole quanta m^{-2} s^{-1}) enabled no or only minimal photosynthesis (as confirmed by an oxygen electrode; data not shown). Moreover, statistically similar H_2O_2 release rates were obtained in a series of experiments conducted in complete darkness (Supplement S4). The pathways responsible for H_2O_2 generation may hence be respiration (Pamatmat, 1997) and extracellular enzymatic activities such as NADPH oxidase, amine oxidase, and L-amino acid oxidase (e.g., Palenik and Morel, 1990). Note that, although the experiments reported here probed for non-photosynthetic H_2O_2, it is likely that illuminated corals will release more H_2O_2 on top of the rates measured here (Supplement S4 and S5). The effect of light on H_2O_2 release may be complicated by possible concomitant changes in the antioxidant activity in the coral tissue, and it calls for more research.

We recently reported that *S. pistillata* also releases superoxide (O_2^-) to its external *milieu* (Saragosti et al., 2010). This superoxide, which is quickly converted to H_2O_2, can potentially account for some of the H_2O_2 release observed here. Such a pathway is quantitatively feasible, based on a rate comparison between the studies. However, the discrepancies between O_2^- and H_2O_2 release by bleached corals rule it out. While bleached corals do not release H_2O_2 (Fig. 4), the release of O_2^- was statistically indistinguishable between bleached and non-bleached corals.

We now turn to addressing the mode of H_2O_2 release, which was experimentally linked to stirring or flow speed. In the changing flow experiments, H_2O_2 release was found to increase with stirring speed (Fig. 5). In a reversed experiment, a lower H_2O_2 release rate was observed when stirring speed was decreased (Supplement S6). In the absence of flow in a similar experimental setting, *S. pistillata* did not release H_2O_2 (Shaked and Armoza-Zvuloni, 2013). Lacking transport systems, corals are dependent on external flow that decreases the thickness of the diffusive boundary layer and accelerates the diffusive exchange of gases and solutes across the tissue–water interface (Nakamura, 2010). Hence, we suggest that stirring generates flow that decreases the thickness of the boundary layer and facilitates mass flux, thus causing corals to release the algal-produced H_2O_2. Another pathway by which flow and mass flux can influence H_2O_2 generation is through the removal of oxygen. High oxygen concentrations that accumulate in the coral tissue due to photosynthesis were shown to drive the symbiotic algae to photorespiration (Mass et al., 2010). Flow was found to remove this excess oxygen, diminish photorespiration, and increase photosynthesis (Mass et al., 2010). It is thus possible that when the symbiotic algae increase their photosynthesis due to flow, they also generate more H_2O_2, which is released to the coral vicinity. Further research is required to resolve the kinetics of H_2O_2 release and the question of whether it is an "active" (regulated) or "passive" (diffusive) mechanism.

4.4 Implications

To date, most experiments examining oxidative stress and bleaching in corals assumed that all H_2O_2 is treated endogenously. Our results show that some of the algal H_2O_2 is not degraded by intracellular antioxidants, but is released from the coral tissue to the surrounding water. This extracellular H_2O_2 release probably results in depletion of internal H_2O_2, and as such can be considered an additional mechanism by which corals can regulate their internal H_2O_2. Under the conditions tested here, the corals did not experience oxidative stress or excessive internal H_2O_2 concentrations. It is possible, however, that under light and/or temperature stress, this mechanism may act even faster, release more H_2O_2, and assist corals in removing some of their internal H_2O_2.

Corals subjected to strong currents were shown to be less susceptible to bleaching (Nakamura and van Woesik, 2001; Smith and Birkeland, 2007). This observation was taken to suggest that, under strong currents, the coral thin diffusive boundary layer enables rapid ROS release and prevents oxidative damage (Nakamura, 2010). To the best of our knowledge, our findings of flow-induced H_2O_2 release from corals, although measured under no-stress laboratory conditions, provide the first "hard evidence" of this hypothesized flow-induced ROS removal.

Conversion of the H_2O_2 released from the coral by catalase to oxygen may also influence O_2 production and consumption measurements (Pamatmat, 1997). A "false" oxygen signal from H_2O_2 breakdown can mostly affect the transition from light to dark by diminishing respiration rates, and should be considered. For example, the observed maximal H_2O_2 release rates of 10 nM min^{-1} for 1 cm^2 of coral

surface area (using 200 nM min^{-1}; Supplement S7) can generate half as much O_2 (since catalase converts H_2O_2 to water and O_2). This rate is ∼ 10 % of the dark respiration rates of ∼ 40 nM min^{-1} cm^{-2} measured for the same corals (data not shown).

The release of H_2O_2 may serve as part of the coral chemical warfare against microorganisms and potential pathogens. Despite the moderate H_2O_2 concentrations and fluxes detected in the experiment waters, higher concentrations will prevail in the diffusive boundary layer at the coral–water interface prior to dilution in the medium. Assuming that the observed H_2O_2 release rate of 20 nmol min^{-1} (equivalent 200 nM min^{-1}; Supplement S7) crosses a thin 100 µm layer that surrounds the coral, it will amount in a local flux of 100 µM min^{-1}. Such fluxes are considered toxic to many coral pathogens (Munn et al., 2008). Involvement of ROS in protection from pathogens was previously suggested for the coral *Oculina patagonica*. This was based on the resistance of the coral to infection in winter, when its pathogen *Vibrio shiloi* had only minimal extracellular SOD activity (Rosenberg and Falkovitz, 2004).

4.5 Summary

By means of incubation experiments with *Stylophora pistillata* and a kinetic approach, we characterized as yet unexplored phenomena of H_2O_2 and antioxidant activity release to the external *milieu*, which is likely shared by other corals. Using intact, non-stressed corals under room light, we established the baseline rates of these releases, allowing future comparison with conditions of increasing light intensities and elevated temperature. Flow was found to induce the release of H_2O_2 from the coral. The symbiotic algae were identified as the source of the released H_2O_2, but not of the antioxidants.

The Supplement related to this article is available online at doi:10.5194/bg-11-4587-2014-supplement.

Acknowledgements. We wish to thank Boaz Lazar, Tamar Lotan, Daniel Sher, Alon Angert, and Victor China for assistance in experimental design and data analysis. We acknowledge the support by the editor Jean-Pierre Gattuso and the valuable comments from three reviewers, Andrew Rose and Daniel Sher. This study was supported by Israel Science Foundation grant 248/11 (www.isf.org.il).

Edited by: J.-P. Gattuso

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