Biogeosciences Discuss., 11, 33–59, 2014
www.biogeosciences-discuss.net/11/33/2014/
doi:10.5194/bgd-11-33-2014

Biogeosciences Discussions
Open Access

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

R. Armoza-Zvuloni[1,2] **and Y. Shaked**[1,2]

[1]Interuniversity Institute for Marine Sciences, Eilat, 88103, Israel
[2]Institute of Earth Sciences, The Hebrew University, Jerusalem, 91904, Israel

Received: 30 November 2013 – Accepted: 13 December 2013 – Published: 2 January 2014

Correspondence to: R. Armoza-Zvuloni (rachelarmoza@gmail.com)

Published by Copernicus Publications on behalf of the European Geosciences Union.

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 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 methodology for resolving the actual rates of H_2O_2 release by the corals.

H_2O_2 and antioxidant activity linearly 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 ~ 1 µM by 2–3 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 slow down and stop by 5–7 h. Stirring was shown to induce the release of both H_2O_2 and antioxidant activity, possibly due to ventilation of the coral by the flow. Antioxidant activity was released at similar rates by bleached and non-bleached corals, suggesting that the antioxidant did not originate from the symbiotic algae. H_2O_2, however, was only minimally 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 possibly assist in preventing coral bleaching under conditions of elevated temperature and irradiance.

BGD
11, 33–59, 2014

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 (Bartozs, 2009) However, ROS production and accumulation beyond the capacity of an organism to efficiently quench them or to repair the resulting damage, a state known as oxidative stress, results in extensive damage to cellular components including proteins, lipids, 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 a 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 (Saragosti et al., 2010; Suggett et al., 2008). When conditions are optimal, the symbiotic algae of the genus *Symbiodinium* that live within the coral endodermal cells provide the coral host with energy (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

BGD
11, 33–59, 2014

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 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 antioxidants (anti-O_2^-) to its surrounding water (Saragosti et al., 2010). We observed that *S. pistillata* and six other Red-Sea coral genera also release H_2O_2 and antioxidants (anti-H_2O_2) to the incubation water (Shaked and Armoza-Zvuloni, 2013). In the field, we documented elevated antioxidant activities 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 to study the release of H_2O_2 and antioxidant activity from intact, non-stressed *Stylophora pistillata* fragments in 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 antioxidants 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 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.

BGD
11, 33–59, 2014

2 Materials and methods

2.1 Experimental setup for measuring H_2O_2 and antioxidant release rates

General – the release rates of H_2O_2 and antioxidants by the branching coral *Stylophora pistillata* was tested by incubating individual fragments in a glass beaker with 100 mL seawater for 1–7 h. Coral fragments were gently transferred and suspended in the incubation beakers and were allowed to acclimate for a short period 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 homogenization. H_2O_2 concentrations were determined in sub-samples every 2–5 min throughout the incubation. The 1 mL sub-samples were mixed with 20 µL of the POHPPA reagent stock in cuvettes and were read within 2 h (see Sect. 2.2). Antioxidant activities were determined in 5 mL sub-samples every 15–20 min. Assaying for antioxidant activity involved spiking with 1 µM H_2O_2 and following its decay rate (see Sect. 2.2). The water level was kept constant by replacing the sampled volume with fresh seawater. Physical contact with the coral or exposure to air were found to invoke high H_2O_2 and antioxidant fluxes, respectively, and were minimized as possible. All incubations were conducted at constant temperature ($25 \pm 1\,^{\circ}C$) and under fluorescence laboratory light. This low illumination ($\sim 10\,\mu E$) is sufficient for only minimal photosynthesis (or none at all), as it is far below the compensation light intensity of these corals of 25 µE (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.

Changing flow experiments – the effect of flow on H_2O_2 and antioxidant activity release rates was studied in a similar setting with a 1 cm long magnet and a stirrer at three stirring speeds. These long (240 min) incubations included three stages of minor, moderate, and fast stirring speeds. At the first 80 min, the stirrer operated at minimal speed for only 30 s prior to sampling, ensuing complete water homogenization but slow flow. In the next 80 min moderate flow was set by constant and low stirring speed, while in the last stage fast stirring speed was applied. No signs of physical damage to the

BGD
11, 33–59, 2014

corals were observed at the high stirring speed. At all stages, corals had their polyps extended and did not show signs of stress.

Bleached corals – no H_2O_2 release from bleached corals was observed in preliminary experiments. Since antioxidants are released simultaneously, low H_2O_2 release rates may be missed. To account for this possibility 250–300 nM H_2O_2 was added to the water at the start of the experiment, enabling the calculation of H_2O_2 release rates (Eq. 5).

2.2 H_2O_2 and antioxidant activity determination

H_2O_2 measurements – H_2O_2 concentration was measured with a Varian spectrofluorometer (Cary Eclipse) using the POHPPA technique as detailed in Shaked and Armoza-Zvuloni (2013). Briefly, the POHPPA reagent stock consisting of 0.25 mM POHPPA (4-hydroxyphenylacetic acid), 70 mL^{-1} of horseradish peroxidase, and 0.25 M Tris at pH 8.8, was added to the samples at a 1 : 50 dilution. The sample florescence was read using excitation of 315 ± 10 nm and emission of 408 ± 10 nm, within 2 h of sampling (keeping the samples in the dark). 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 the anti-H_2O_2 activity, released by the corals was determined experimentally by following the loss kinetics of H_2O_2 spikes added to sub-samples collected over the course of the incubation. The 5 mL sub-samples 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 sub-sampled 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 group together all antioxidants and assign them with a composite efficiency in the form of a rate constant (k_d

BGD
11, 33–59, 2014

with units of M^{-1} time^{-1}). We can then describe the rate of H_2O_2 degradation:

$$H_2O_2 \text{ degradation rate} = \frac{\partial[H_2O_2]}{\partial t} = -k_d \cdot [\text{Antioxidants}] \cdot [H_2O_2] \quad (1)$$

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 = $k_d \cdot$[Antioixdants]) is constant throughout the assay. Considering the renewable (i.e. enzymatic) nature of many antioxidants, the short assay duration and 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}):

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

Drawing on first-order law principles, the antioxidant activity of a sample (k_{antiox}) can be presented graphically as a linear slope when plotting the natural log of H_2O_2 vs. time, where steeper slopes refer to stronger antioxidant activity (Fig. 1). The value of k_{antiox} in a sub-sample 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 sub-samples increase with time since more antioxidants are released to the water (Fig. 2).

2.3 H_2O_2 release rates 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:

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

BGD
11, 33–59, 2014

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

$$\frac{\mathrm{d}[H_2O_2]}{\mathrm{d}t} = H_2O_2 \text{ release rate} - k_{\text{antiox}} \cdot [H_2O_2] \quad (4)$$

Which can be rearranged as follows:

$$H_2O_2 \text{ release rate} = \frac{\mathrm{d}[H_2O_2]}{\mathrm{d}t} + k_{\text{antiox}} \cdot [H_2O_2] \quad (5)$$

Having conducted frequent H_2O_2 measurements, we can calculate the values of $\frac{\mathrm{d}[H_2O_2]}{\mathrm{d}t}$ for subsequent time intervals of 5 to 10 min throughout the experiment. Similarly, for each 5–10 min interval we can calculate H_2O_2 degradation rates using the experimentally obtained k_{antiox}. In practice, since k_{antiox} increases linearly with time, we calculated the k_{antiox} value for each interval using a linear equation that was fitted through the data.

2.4 Biochemical characterization

Basic biochemical characterization of the antioxidant activity was conducted using the heam-enzyme inhibitor sodium azide (NaN_3, applied at 0.1 mM for 10 min). Samples were also filtered through 0.2 µm Sartorius syringe filter, stored overnight at room temperature, and incubated at a range of temperatures in heating water baths. The antioxidant activities were calibrated with a standard assay for catalase activity (one unit of catalase is defined as the amount of enzyme decomposing 1 µmol of H_2O_2 in 1 min at pH 7.0 and 25 °C, with initial $[H_2O_2]$ of 10.3 mM). Highly active samples were assayed under these conditions with a Varian Cary 50 spectrophotmer at 240 nm and also with our method (following dilution).

2.5 Corals handling and preconditioning

The presented incubation experiments were conducted over a year period with 30 similarly sized *Stylophora pistillata* fragments (with a surface area of ~ 20 cm^2) from differ-

BGD
11, 33–59, 2014

ent genotypes. The fragments were kept in water tables with running natural seawater prior to and throughout the experiment period. To avoid contact with the container wall the corals were suspended on PVC stands using fine nylon threads. After one month of acclimation, 10 coral fragments were placed in a dark room to generate bleaching. After one month, the dark kept corals turned white and a complete loss of the algal chlorophyll was measured using PAM (pulse amplitude modulation chlorophyll fluorescence techniques). It is important to note that the bleached corals did not experience thermal or irradiance stress. Bleached corals were feed twice a week with one-day-old *Artemia salina* nauplii. The non-bleached coral fragments were kept outdoors under partial shade, experiencing natural dark/light regime.

2.6 Statistical analysis

Statistical analyses were carried out using Statistica 8® software. Prior to analysis, the data were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Levene's test). The differences between bleached and non-bleached *S. pistillata* coral fragment were tested using two tailed Students *T* test. The effect of stirring speed on the coral release rates was tested using one-way ANOVA (analyses of variance) and Tukey HSD test for post hoc comparisons.

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

BGD
11, 33–59, 2014

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 calculate 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.

In Fig. 2, using a representative experiment we demonstrate these calculations. Initially, the incubation water contains neither H_2O_2 nor antioxidants, and later on the levels of both increase. While the antioxidant activity accumulates with time, H_2O_2 concentrations rise in the first 40 min and then remain stable (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 generates larger differences between the measured and released H_2O_2 concentrations (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).

3.2 Kinetics of H_2O_2 and antioxidant activity release

Ten long (5–7 h) incubations conducted to evaluate the kinetics and duration of these releases, reveled similar patterns (although at varying rates and slightly different timing; see Supplement S1.). A representative experiment (Fig. 3), shows a steady accumulation with time of the antioxidant activity, yielding a good linear fit ($R^2 = 0.91$). In the first two h, H_2O_2 concentrations also increased steadily with time. Then, H_2O_2 concentrations reached maximal values of $\sim 1\,\mu M$ that were maintained for at least 60 min. 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 are low and fluctuating in the first hour. Then, H_2O_2 release rates increased, peaked at 80 nM min^{-1} ($= 0.4\,nmol\,min^{-1}\,cm^{-2}$) around 3.5 h, and declined afterwards. Note that early on even

BGD
11, 33–59, 2014

low H_2O_2 release rates result in H_2O_2 accumulation. As the experiment progresses, even high H_2O_2 release rates can only maintain H_2O_2 concentrations constant. In the final stages the high antioxidant activity outcompetes the H_2O_2 release rate and H_2O_2 concentrations decline. The total amount of H_2O_2 released by the 20 cm^2 coral fragment during this experiment is 1400 nmol or ~ 70 nmol cm^{-2} coral.

3.3 H_2O_2 and antioxidant release by bleached vs. non-bleached *S. pistillata*

Six short (1 h) incubations with bleached and non-bleached *S. pistillata* fragments were conducted to identify the sources of the released H_2O_2 and antioxidants, and their averaged data is presented in Figs. 4 and 5. All studied parameters steadily increased with time in the non-bleached corals (Fig. 4a–c). The antioxidant activity accumulated in a similar manner in the bleached coral incubations (Fig. 4e), but H_2O_2 did not (Fig. 4d). To discern whether bleached corals do not release H_2O_2 or that its degradation exceeds its release, we added H_2O_2 to enable H_2O_2 release calculation. Following a minor initial increase this added H_2O_2 declined exponentially (Fig. 4d). The resulting rate of H_2O_2 release by bleached corals was minimal and occurred only in the first time interval (Fig. 4f).

To facilitate an easy comparison between the bleached and non-bleached corals we looked at the same data at the end of the experiment (1 h; Fig. 5). At this stage the antioxidant activity is similar between the treatments ($p > 0.05$, student T test, Fig. 5a), while H_2O_2 is released only by non-bleached corals ($p < 0.01$, student T test, Fig. 5b). The overall amount of H_2O_2 released during 1 h by non-bleached corals is 10 fold higher than that released by bleached corals ($p < 0.01$, student T test, Fig. 5c).

3.4 The effect of stirring speed on H_2O_2 accumulation

Six, long (3–4 h), incubations were conducted to test the effect of stirring rate, as representative of flow speed, on H_2O_2 and antioxidant activity release rates. Faster release of all studied variables was observed at higher stirring speeds, as shown in a typical

BGD
11, 33–59, 2014

experiment (Fig. 6a–c) and in compilation of six experiments (Fig. 6d and e). In accord with other experiments, the antioxidant activity was released continuously in each experimental stage. Here, in addition, its rate of release increased with the flow ($p < 0.05$, One-way Anova, Fig. 6b and e). Similarly, the rate of H_2O_2 release increased with time in each experimental stage, and flow further accelerated the rate of change ($p < 0.001$, One-way Anova, Fig. 6c and f). H_2O_2 accumulated initially in each experimental stage, but with time its concentrations leveled off due to the antioxidant activity (Fig. 6a). The computed initial H_2O_2 accumulation rates also increased with stirring speed ($p < 0.001$, One-way Anova, Fig. 6d).

3.5 Biochemical identity of the antioxidant activity

Basic biochemical characterization reveals that the antioxidants released by the coral are anti-H_2O_2 enzymes (Fig. 7). The antioxidant activity was optimal at 20–30 °C and decreased at low (4–15 °C) and high (60–70 °C) temperature. Heating to 80 °C for 10 min resulted in a nearly complete loss of activity. Sodium azide, an inhibitor of haem enzymes such as catalase and peroxidase (Nizhnikov et al., 2007), completely inhibited the antioxidant activity. About half of the activity remained following an overnight storage at room temperature. Filtration through 0.2 µm membrane resulted in 35 % loss of the activity, indicating that the antioxidants are mostly in the dissolved phase. To allow comparison with commonly used measures of antioxidant activity we measured highly active sub-samples in a standard catalase assay (see Sect. 2.4). The antioxidant activities released by the coral were found to range between 10^{-4}–10^{-3} catalase-like units.

BGD
11, 33–59, 2014

4 Discussion

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

In this study, a major emphasis was placed on developing methodological and conceptual approaches for following H_2O_2 and antioxidants release by corals in experimental settings. 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 low H_2O_2 concentration, longer degradation curves (up to 4 h) and the use of a degradation rate constant (k_{antiox}) rather than initial H_2O_2 degradation rate. This approach enables the detection of minute concentrations of antioxidants and allows studying the behavior of biochemically uncharacterized compounds that react with H_2O_2. The use of k_{antiox} as a measure of antioxidant activity allows evaluating 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 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 our findings to the coral physiology and compare between treatments (such as, bleached vs. non-bleached or different flow speeds).

BGD
11, 33–59, 2014

4.2 Antioxidants nature and source

The antioxidants released by the corals were partially characterized as dissolved anti-H_2O_2 enzymes, probably resembling catalase and/or peroxidase (Fig. 7). Possible sources for these enzymes are the coral host and the microorganisms that live in the mucus layer covering the coral, both of which posses an array of antioxidants (Levy et al., 2006; Munn et al., 2008). The symbiotic algae do not seem to contribute to the released antioxidants since bleached corals released comparable antioxidant activity to non-bleached corals (Figs. 4 and 5). 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, 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. 7; Saragosti et al., 2010).

Since production of antioxidant enzymes is metabolically costly, the observed release may be considered a wasteful process. Nonetheless, the actual amount of released enzyme is rather modest, ranging between 10^{-4}–10^{-3} catalase-like units. These activities are substantially than in the coral tissue, which we estimate range between 10–1000 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 minute enzyme quantities is likely if one of the enzymes at play is catalase. The turnover numbers of catalase are one of the highest of 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 6 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

BGD
11, 33–59, 2014

water collected from surfaces of individual and knoll corals and from the reef lagoon. In nature, these 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 source and mode of release

The potentially largest source of H_2O_2 within the coral consortium is 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 (Figs. 4 and 5), 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 are capable of only minimal H_2O_2 release, implying that the symbiotic algae are indeed the major source of the released H_2O_2.

Photosynthesis is considered as 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 µE) enabled no or only minimal photosynthesis (as confirmed by oxygen electrode, data not shown). Moreover, statistically similar H_2O_2 release rates were obtained in a series of experiments conducted in complete darkness (see Supplement S2). 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). Alternatively, the symbiotic algae may have generated an internal H_2O_2 pool prior to the experiment, which is released to the water upon stirring and ventilation of the coral. This idea of a finite H_2O_2 pool, present within the coral prior to the experiment, is supported by

BGD
11, 33–59, 2014

the long incubations showing gradual decline in H_2O_2 release rates to very low rates toward the end of the experiment (Fig. 3). Given the complexity of the coral consortium, our data are not sufficient to discern between these options. Further research into the effect of light and photosynthesis on H_2O_2 release kinetics can help explore the pathways at play.

We recently reported that *S. pistillata* also release 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 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 (Figs. 4 and 5), the release of O_2^- was statistically indistinguishable between bleached and non-bleached corals.

We now turn to address the mode of H_2O_2 release, which was experimentally linked to stirring or flow speed. In the changing flow experiment, H_2O_2 release rates were found to increase with stirring speed (Fig. 6c and f). 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). We thus suggest that stirring ventilates the coral and releases algal produced H_2O_2 (either stored or freshly generated). Further research is required to resolve the kinetics of H_2O_2 release and whether this 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 being expelled from the coral tissue to the surrounding water. This extracellular H_2O_2 release may result in deple-

BGD
11, 33–59, 2014

tion of internal H_2O_2 and as such, be considered as 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 conditions, provide the first "hard evidence" for 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). "False" oxygen signal from H_2O_2 breakdown can mostly effect 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 S1), can generate half that 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 experimental waters, higher concentrations may 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}; appendix S1) crosses a thin 100 µm layer that surrounds the coral, it will amount in a local flux of 100 µM min^{-1}. These 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

BGD
11, 33–59, 2014

resistance of the coral to infection in winter, when its pathogen *Vibrio shiloi* had only minimal extracellular SOD activity (Rosenberg and Falkovitz, 2004).

5 Summary

By means of incubation experiments with *Stylophora pistillata* and kinetics approach, we characterized a yet unexplored phenomenon 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 temperatures. Flow was found to induce the release of both H_2O_2 and antioxidant activity from the coral. The symbiotic algae were identified as the source of the released H_2O_2 but not of the antioxidants. The flow induced H_2O_2 release may be viewed as an exogenous mechanism for lowering internal H_2O_2 concentrations that may be of significance during oxidative stress.

Supplementary material related to this article is available online at http://www.biogeosciences-discuss.net/11/33/2014/bgd-11-33-2014-supplement.pdf.

References

Apel, K. and Hirt, H.: Reactive oxygen species: metabolism, oxidative stress, and signal transduction, Annu. Rev. Plant Biol., 55, 373–399, 2004.

Avery Jr, G. B., Cooper, W. J., Keiber, R. J., and Willey, J. D.: Hydrogen peroxide at the Bermuda Atlantic Time Series Station: temporal variability of seawater hydrogen peroxide, Mar. Chem., 97, 236–244, 2005.

Bartosz, G.: Reactive oxygen species: destroyers or messengers?, Biochem. Pharmacol., 77, 1303–1315, 2009.

BGD
11, 33–59, 2014

Downs, C. A., Fauth, J. E., Halas, J. C., Dustan, P., Bemiss, J., and Woodley, C. M.: Oxidative stress and seasonal coral bleaching, Free Radical. Bio. Med., 33, 533–543, 2002.
Ferrier-Pagès, C., Witting, J., Tambutté, E., and Sebens, K. P.: Effect of natural zooplankton feeding on the tissue and skeletal growth of the scleractinian coral Stylophora pistillata, Coral Reefs, 22, 229–240, 2003.
Gechev, T. S. and Hille, J.: Hydrogen peroxide as a signal controlling plant programmed cell death, J. Cell Biol., 168, 17–20, 2005.
Halliwell, B., Clement, M. V., and Long, L. H.: Hydrogen peroxide in the human body, FEBS Lett., 486, 10–13, 2000.
Hansard, S. P., Vermilyea, A. W., and Voelker, B. M.: Measurements of superoxide radical concentration and decay kinetics in the Gulf of Alaska, Deep-Sea Res. Pt. I, 57, 1111–1119, 2010.
Hoegh-Guldberg, O.: Climate change, coral bleaching and the future of the world's coral reefs, Mar. Freshwater Res., 50, 839–866, 1999.
Jones, R. J., Hoegh-Guldberg, O., Larkum, A. W. D., and Schreiber, U.: Temperature-induced bleaching of corals begins with impairment of the CO_2 fixation mechanism in zooxanthellae, Plant Cell Environ., 21, 1219–1230, 1998.
Lesser, M. P.: Oxidative stress causes coral bleaching during exposure to elevated temperatures, Coral Reefs, 16, 187–192, 1997.
Lesser, M. P.: Coral bleaching: causes and mechanisms, in: Coral Reefs: an Ecosystem in Transition, Springer, New York, 405–419, 2011.
Levy, O., Achituv, Y., Yacobi, Y. Z., Dubinsky, Z., and Stambler, N.: Diel "tuning" of coral metabolism: physiological responses to light cues, J. Exp. Biol., 209, 273–283, 2006.
Munn, C. B., Marchant, H. K., and Moody, A. J.: Defenses against oxidative stress in vibrios associated with corals, FEMS Microbiol. Lett., 281, 58–63, 2008.
Nakamura, T.: Importance of water-flow on the physiological responses of reef-building corals, Galaxea Journal of Reef Studies, 12, 1–14, 2010.
Nakamura, T. and van Woesik, R.: Water-flow rates and passive diffusion partially explain differential survival of corals during the 1998 bleaching event, Mar. Ecol.-Prog. Ser., 212, 301–304, 2001.
Nii, C. M. and Muscatine, L.: Oxidative stress in the symbiotic sea anemone Aiptasia pulchella (Calgren, 1943): contribution of the animal to superoxide ion production at elevated temperature, Biol. Bull., 192, 444–456, 1997.

BGD
11, 33–59, 2014

Nizhnikov, M. E., Molina, J. C., and Norman, E.: Spear Central reinforcing effects of ethanol are blocked by catalase inhibition, Alcohol, 41, 525–534, 2007.
Palenik, B. and Morel, F. M. M.: Amino acid utilization by marine phytoplankton: a novel mechanism, Limnol. Oceanogr., 35, 260–269, 1990.
Pamatmat, M. M.: Non-photosynthetic oxygen production and non-respiratory oxygen uptake in the dark: a theory of oxygen dynamics in plankton communities, Mar. Biol., 129, 735–746, 1997.
Papina, M., Meziane, T., and van Woesik, R.: Symbiotic zooxanthellae provide the host-coral Montipora digitata with polyunsaturated fatty acids, Comp. Biochem. Physiol. B. Biochem. Mol. Biol., 135, 533–537, 2003.
Rosenberg, E. and Falkovitz, L.: The vibrio shiloi/Oculina patagonica model system of coral bleaching, Annu. Rev. Microbiol., 58, 143–159, 2004.
Saragosti, E., Tchernov, D., Katsir, A., and Shaked, Y.: Extracellular production and degradation of superoxide in the coral Stylophora pistillata and cultured Symbiodinium, PLoS One, 5, e12508, doi:10.1371/journal.pone.0012508, 2010.
Shaked, Y. and Armoza-Zvuloni, R.: Dynamics of hydrogen peroxide in a coral reef: sources and sinks, J. Geophys. Res.-Biogeo., 118, doi:10.1002/2013JG002483, 2013.
Shaked, Y., Harris, R., and Klein-Kedem, N.: Hydrogen peroxide photocycling in the Gulf of Aqaba, Red Sea, Environ. Sci. Technol., 44, 3238–3244, 2010.
Sharma, P., Jha, A. B., Dubey, R. S., and Pessarakli, M.: Reactive oxygen species, oxidative damage and antioxidative defense mechanism in plants under stressful conditions, J. Bot., 2012, 217037, doi:10.1155/2012/217037, 2012.
Smith, D. J., Suggett, D. J., and Baker, N. R.: Is photoinhibition of zooxanthellae photosynthesis the primary cause of thermal bleaching in corals?, Glob. Change Biol., 11, 1–11, 2005.
Smith, L. W. and Birkeland, C.: Effects of intermittent flow and irradiance level on back reef Porites corals at elevated seawater temperatures, J. Exp. Mar. Biol. Ecol., 341, 282–294, 2007.
Suggett, D. J., Warner, M. E., Smith, D. J., Davey, P., Hennige, S., and Baker, N. R.: Photosynthesis and production of hydrogen peroxide by Symbiodinium (Pyrrhophyta) phylotypes with different thermal tolerances, J. Phycol., 44, 948–956, 2008.
Warner, M. E., Fitt, W. K., and Schmidt, G. W.: Damage to photosystem II in symbiotic dinoflagellates: a determinant of coral bleaching, P. Natl. Acad. Sci. USA, 96, 8007–8012, 1999.

BGD
11, 33–59, 2014

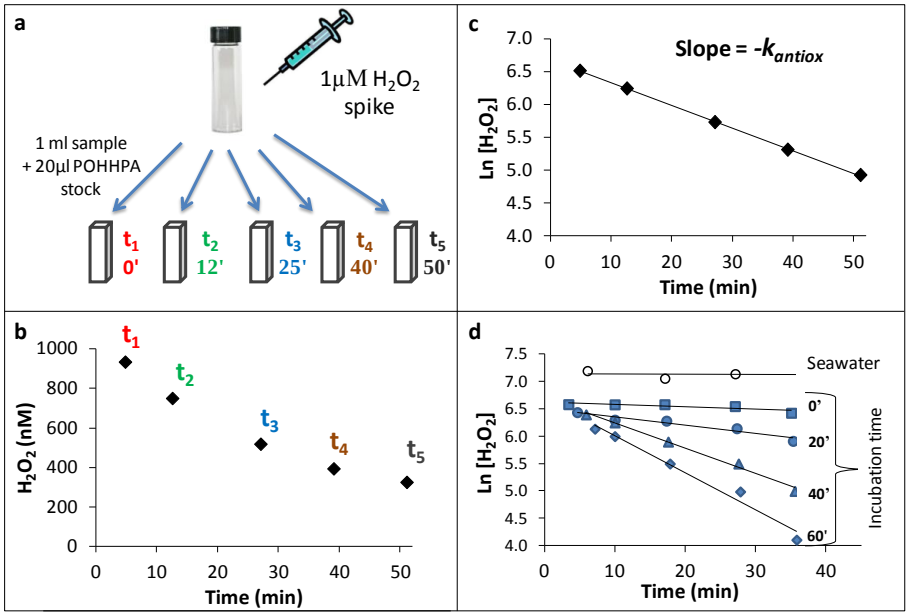

Fig. 1. Determination of antioxidant activity in coral incubation water. **(a)** H_2O_2 spike is added to a sample, mixed, and sub-sampled into cuvettes containing PHOPPA reagent at time intervals ranging from a few minutes to few hours. **(b)** H_2O_2 concentrations decline with time due to the antioxidant 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 H_2O_2 concentration vs. time. **(d)** The antioxidant activity released from the coral accumulate 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}).

BGD
11, 33–59, 2014

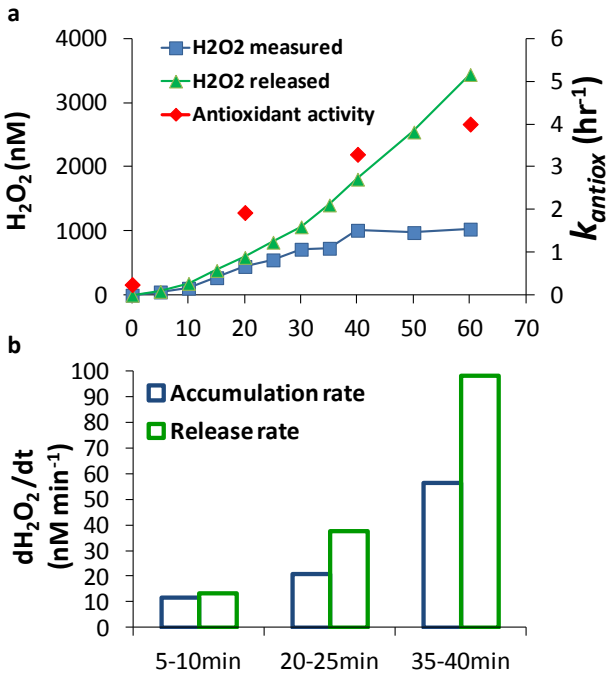

Fig. 2. Quantification of H_2O_2 release by *Stylophora pistillata*. Data from a representative experiment is plotted to demonstrate the corrections made to account for H_2O_2 loss due to antioxidant activity. **(a)** The measured variables are H_2O_2 concentrations (blue squares) and antioxidant activities (red diamonds). Since H_2O_2 is degraded by the antioxidants, its measured concentrations are lower than that released by the coral (green triangle). The coral released H_2O_2 is calculated for small time intervals using Eq. (5) and 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.

BGD
11, 33–59, 2014

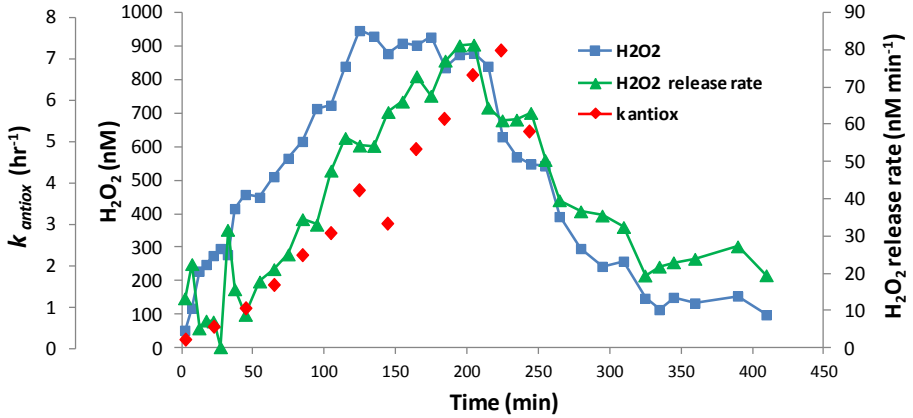

Fig. 3. H_2O_2 and antioxidant activity release kinetics in a long incubation. A representative, seven hours long experiment, showing accumulation of antioxidant activity (red diamonds) and changes in H_2O_2 concentrations (blue squares) over time in the coral incubation water. These frequent measurements enable the calculation of actual H_2O_2 release rates by the corals (green triangle).

BGD
11, 33–59, 2014

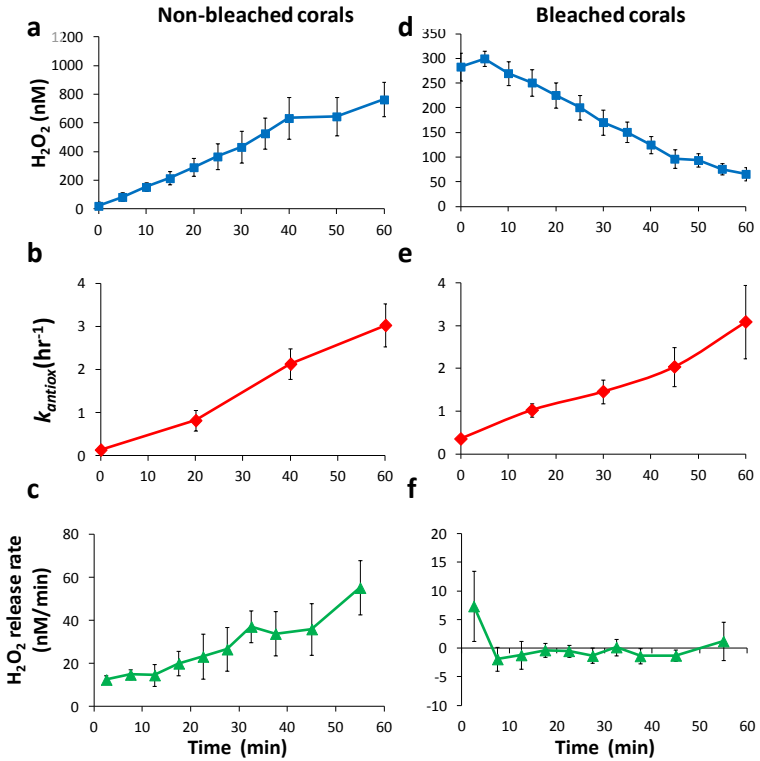

Fig. 4. H_2O_2 and antioxidant release kinetics by non-bleached **(a–c)** and bleached *S. pistillata* **(d–f)**. Release and accumulation patters of H_2O_2 concentrations **(a, d)**, antioxidant activity **(b, e)**, and calculated H_2O_2 release rates **(c, e)**. Presented data are mean ± SE of 6 bleached and 6 non-bleached corals.

BGD
11, 33–59, 2014

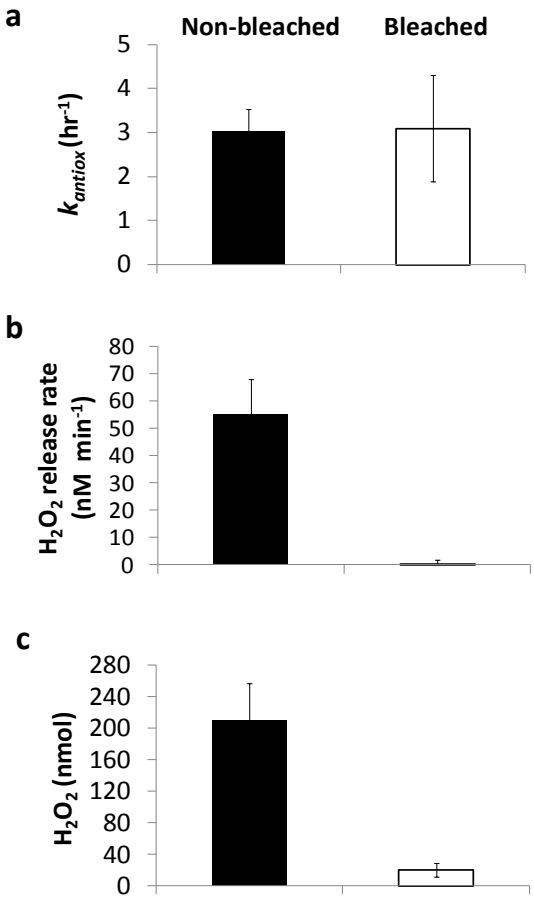

Fig. 5. Overall H_2O_2 and antioxidant release by non-bleached and bleached *S. pistillata*. To facilitate quantitative comparison between treatments, data from Fig. 4 is re-plotted, showing antioxidant activity at 1 h **(a)**, H_2O_2 release rates at 1 h **(b)**, and overall H_2O_2 released during the experiment **(c)**. Presented data are mean ± SE of 6 bleached and 6 non-bleached corals.

BGD
11, 33–59, 2014

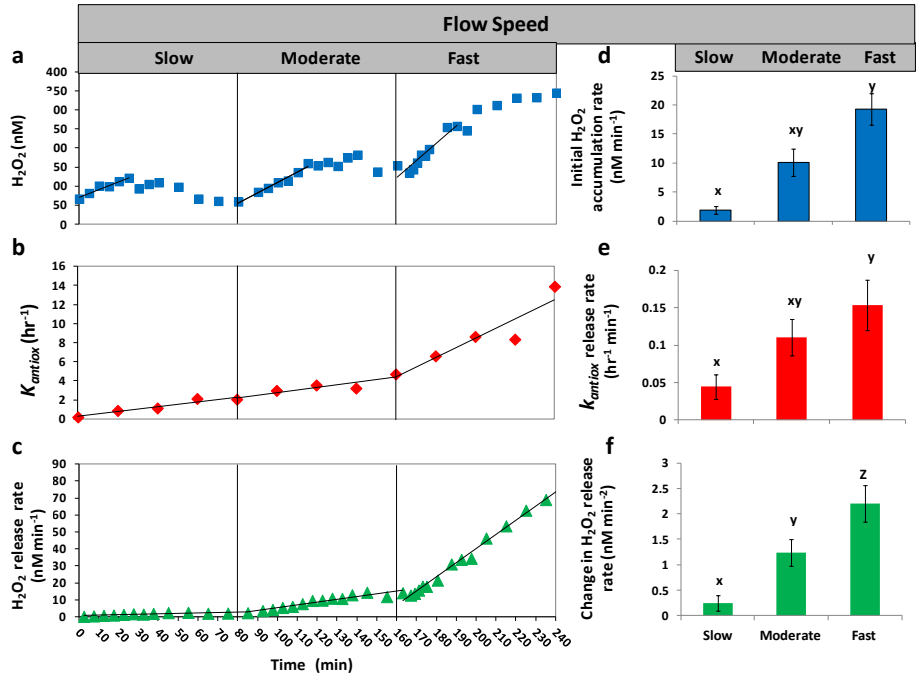

Fig. 6. Release of H_2O_2 and antioxidant activity by *S. pistillata* at variable stirring speeds. Change with time in H_2O_2 concentrations **(a)**, antioxidant activity **(b)**, and calculated H_2O_2 release rates **(c)** obtained at increasing stirring speeds in an individual experiment. For a given stirring speed, we calculated the rate of change in each of the parameters (i.e. the slopes drawn in **a–c**) and compiled them with six other experiments, to yield the bar graphs in panels **(d–f)**. Letters in panels **(d–f)** refer to significant differences of $p < 0.05$ between the release rates of each of the studied parameters at the different stirring speeds, tested with One-way Anova followed by Tukey HSD test.

BGD
11, 33–59, 2014

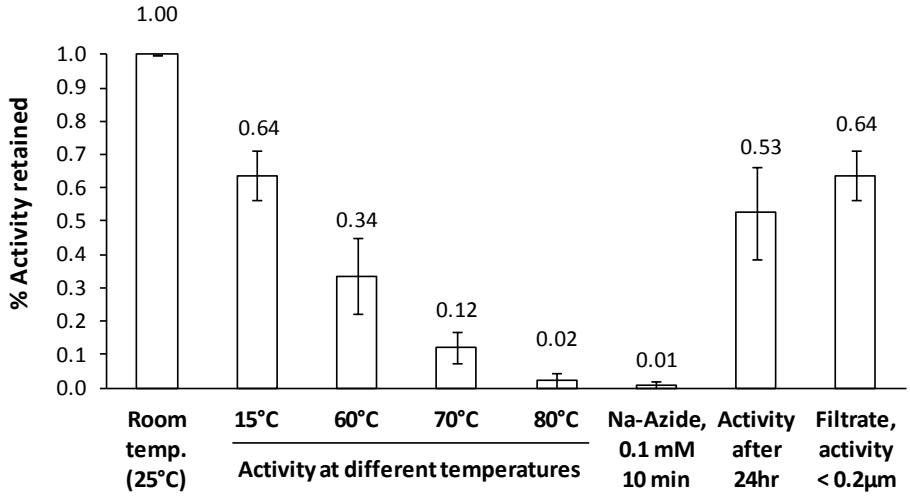

Fig. 7. Basic biochemical characterization of the coral released antioxidants. Effect of temperature (15, 60, 70, and 80 °C), haem-enzymes inhibitor (0.1 mM sodium azide for 10 min), filtrations (0.2 µm), and storage (24 h) on the antioxidant activity in coral incubation waters. The antioxidant activity of the treated samples ($n = 3$; mean ± SD) is presented as percentage of the non-treated samples ($n = 3$).

BGD
11, 33–59, 2014