Biogeosciences Discuss., 11, C1701–C1731, 2014
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# *Interactive comment on* "Release of hydrogen peroxide and antioxidant by the coral *Stylophora pistillata* to its external *milieu*" *by* R. Armoza-Zvuloni and Y. Shaked

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Dear Reviewers and Editor, Prof. Gattuso

We carefully read the reviewer's comments and made substantial efforts to accommodate them all. We conducted new experiments, reanalyzed much of the data (with new statistics), drafted new figures, and re-wrote large sections of the manuscript.

Our major revisions include:

1. Reorganization and rewriting of the material and methods. Addition of many more

details as requested regarding stirring speed, experiment period, number of replicates etc.

2. New statistical tests were preformed according to the reviewer's suggestions.

3. Three new experiments were accomplished – (i) inhibition of antioxidant activity by 3-amino-1,2,4-triazole, (ii) H2O2 and antioxidant release at reducing stirring speed; (iii) effect of coral size and water volume on antioxidant activity release.

4. Figures were modified or replaced by new figures– Fig. 4 (bleached versus non-bleached); Fig 5 (changing stirring speed), Fig 6b (antioxidant activity characterization)

5. Inclusion of new data (graphs, tables and text) to the appendix – (i) diurnal changes in H2O2 concentrations and release rates, (ii) reversed flow experiment, (iii) coral size vs. antioxidant activity (iv) linear vs. exponential fits to kantiox release kinetics.

6. Rephrasing and correction of misused terms and problematic statements.

We thank you for the time and thought invested to improve our study (both scientifically and presentation wise). Please find below our detailed answers to the reviewers comments.

For your convenience, we also include our response letter as a supplemental file. This file contains the same information but it is easier to follow as our responses are colored differently and the formats are kept.

Sincerely yours,

Rachel Armoza-Zvuloni and Yeala Shaked

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Response to reviews

Referee #1

Comment 1: This is the second time I have reviewed this manuscript that in its original



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form was combined with Shaked and Armoza-Zvuloni (2013). Having read the published part of that contribution and this submission to BGD I find very few, if any, of my original comments were incorporated or rebutted.

Response: On March 2013, we submitted a manuscript to coral reefs that included field work and laboratory experiments. We received four constructive reviews that offered serious changes to the manuscript structure and content. Based on these reviews we went back to the drawing board and lunched a series of additional field measurements and conducted many additional experiments. We have then divided the original manuscript to two manuscripts focusing on ecological and on physiological aspects of the novel hydrogen peroxide and antioxidant release phenomena. The current contribution was re-written entirely and it includes a very different data set compared to the previous manuscript (half of the experiments here were not included in the manuscript submitted to coral reef). It is important to us to emphasize that while apparently we failed to address some of the reviewer's comments, we have invested a lot of time and thought in the manuscript since the last round. Obviously other reviewers gave completely different comments and the outcome reflects an attempt to accommodate many suggestions as well as new ideas.

Comment 2: Most of my concern then, and now, revolves around the Materials and Methods. They are poorly described and render the experiments largely unrepeatable. Additionally, the design that one can decipher strongly suggests that the statistics applied are incorrect making an evaluation of the results and their interpretation in the Discussion difficult to do. As a result, I will confine most, but not all, of my comments to the M+M. First and foremost the manuscript is poorly organized making it difficult to read, as was the original combined manuscript.

Response: The Materials and Methods part was reorganized, the statistics was corrected and additional information on protocols and experiments was included (see detailed description next to specific comments). Organization wise, in the former version, we had two sections that described the experimental setup (sec. 2.1 and 2.5), these



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sections are combined now in section 2.3 (staring in Corals handling and preconditioning moving to Incubation experiment setup and finishing with Experiment types). All statistical tests were corrected according to your suggestion and their description (that appeared in the former version in a separate section -2.6), is now integrated with the relevant experiments. We have added to the appendix pictures, tables and other data that hopefully will help the reader understand and be able to reproduce our experiments and enable better evaluation of our scientific integrity.

Comment 3: Abstract, P 34, line 17; Please define “ventilation”. There is no term that I’m aware of in the hydrodynamic/fluid dynamic literature that uses the term “ventilation”. One assumes the authors are possibly talking about changes in the diffusive boundary layer with flow and subsequent mass flux changes but the readers are left to wonder what the intent of the authors is here.

Response: We accept the reviewer comment. We indeed intended to say that flow reduces the thickness of the diffusive boundary layer and increases mass flux, as the reviewer understood. We changed the term ventilation that appeared in the text. The respective line in the abstract is now: “Stirring was shown to induce the release of H2O2, possibly since the flow reduces the thickness of the diffusive boundary layer of the coral and thus increases H2O2 mass flux”.

Comment 4: Introduction, P 35, line 18; ROS production, largely as a result of hyperoxia, in symbiotic cnidarians has been well known for a long time. The authors references don’t reflect that history. P35, line 19; Here again, using Papina et al. 2003 as a primary reference to describe the nature of the coral algal symbiosis is problematic. What about Muscatine, Dubinsky, Porter Falkowski and others who actually did the original work???

Response: We accept the reviewer comment. We added three references: Jones et al., 1998, Lesser, 1996 and Muscatine, 1990.

Comment 5: Materials and Methods, P 37, line 4; How many fragments?



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Response: We rewrote the Materials and Method. This information appears now in Experiment types in section 2.3. For each experiment we clearly state the number of fragments used.

Comment 6: P 37, line 6; How long is “a short period” for acclimating?

Response: The short incubation period is of 15 min. We added the missing information to the text.

Comment 7: P37, line 8; Water is not “homogenized”, it is mixed.

Response: We accept the reviewer comment. We changed from homogenized to mixed through the text.

Comment 8: P 37, lines 13-14; The issue of keeping the volume constant in the incubation seawater seems to me to require some sort of dilution factor. You are adding fresh seawater to the incubation and that would dilute any measured constituent, however slightly, in the medium.

Response: In order to keep constant water volume in the experiments, the sampled waters were replaces with fresh seawater (8 ml every 15 min). This replacement diluted the water by only 8% (8ml out of 100 ml) and hence diluted the H2O2 and antioxidant activity signals in the water. We think this is negligible and that no corrections are needed. Moreover, if anything this effect slightly underestimates our claims. It is now explained in the text.

Comment 9: P 37, lines 17-18; Irradiance should be expressed as _mole quanta m-2 s-1 which are SI units.

Response: We accept the reviewer comment. We changed from $\mu$E to $\mu$mole quanta m-2 s-1 through the text.

Comment 10: P 37, So it is unclear from this initial description how many replicate corals were used; you have multiple factors in play including time, flow speed, and light



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versus dark and the analysis of these experiments later in the paper doesn't discuss any interactions between the three. The experimental design is completely unclear and as written the analysis appears to be completely inappropriate.

Response: We rewrote the Materials and Methods and explained the experimental setup for each of the three major experiments we conducted. This information includes: fragment numbers, replicate numbers, preconditioning, and the time period when the experiments were run (see also comment below). We did not examine interactions between factors since each set of experiments was independent and only one factor was examined. These factors were time (long experiments at constant stirring speed), bleached vs non-bleached (short experiments at constant stirring speed) and flow speed (long experiments with changing stirring speed). Light was not a factor here since all the experiments (except for the one presented now in appendix A4 and appendix A5) were conducted at the same low light conditions.

Comment 11: Also, later in the paper it appears that multiple experiments were done over a period of a year. The authors have combined these data for analysis but they should be blocked for time to see if the results of experiments done at different times, even under the same supposed conditions, produced different results.

Response: This was indeed unclear; the data presented here were collected over a year (while many preliminary experiments were conducted during 2 additional years). However, each set of experiments was conducted over a relatively short time period. The long (5-7 hrs) incubations were conducted between July and August 2012, the short (1 hr) experiments with bleached and non-bleached corals were conducted between April and June 2013 and the experiments with different stirring speeds were conducted between August and September 2012.

Comment 12: P 38. In my previous review I raised the issue that both POHPPA and catalase are light sensitive. While the authors now say the samples being tested were kept in the dark my concerns were about the stock reagents.



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Response: True, it was not mentioned. All stock reagents were kept in the dark. The POHPPA and catalase vials were covered with aluminum foil and were placed in a dark covered ice bucket. It is now mentioned.

Comment 13: Also, and again from my previous review, no metal chelating agents (e.g., DTPA) were used in these assays/experiments. The authors do understand the importance of chelating redox metals as evidenced from their previous publications but nowhere have those same precautions been taken in this work that this reviewer finds worrisome.

Response: We have previously studied the kinetics of H2O2 (and superoxide) accumulation and degradation (i.e antioxidant activity) in the Gulf of Aqaba seawater, amended with DTPA, Fe and Cu (Shaked et al. 2010). Metals were indeed found to react with H2O2 but at rates substantially slower than those we report here with corals. In further experiments with coral free seawater and DTPA (in the setup used in this study) we did not observe any accumulation of H2O2 or antioxidant activity. Since we are equipped for trace metal clean work, we are routinely keeping everything at pretty low metal levels through acid cleaning (10% HCl), using trace metals certified tubes, low metal reagents etc. We have tested initially the level of "metal cleanness" required for these experiments and observed no differences whether stringent cleaning was done or a more mild cleaning. Due to all these reasons we see no need for DTPA. We do feel that adding a control experiment with no coral (as suggested by the 2nd reviewer) can highlight this point, it is now added to Fig. 4.

Comment 14: Discussion, I can make no substantive comments because I do not know if any of the experiments were analyzed correctly. Response: We have done our best to rewrite the experiment details and improve the statistics. We feel that this comment is out of place as it disregards our established record as solid and careful researchers who pay great attention to experimental and analytical details.

Comment 15: P 47, lines 25-28 +; The notion that there would be a "pool" of hydrogen



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peroxide, in the absence of any direct evidence, defies our current knowledge of ROS biochemistry. It would be extremely dangerous for any cell to maintain a "pool" of hydrogen peroxide as if it isn't broken down enzymatically can cause damage itself such as DNA damage and it is very susceptible to reduction to more toxic species of ROS especially if any transition metals (e.g., Fe) are available where Fenton chemistry could occur. The source of the hydrogen peroxide produced is likely mitochondrial in origin.

Response: We accept the reviewer comment. At this stage we can not support with our data the presence of hydrogen peroxide pool and these sentences were removed. (Just to clarify that this in not complete nonsense, what we had in mind are low "safe" levels 10-20 uM H2O2, which are sufficient to generate the measured external signals).

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Referee #2

Comment 1: I have a first concern about the term "antioxidant" used throughout the article (including in the title). Although the authors try to make a -very basic- characterization of this so-called antioxidant activity, the nature of the involved compounds is not known yet. In fact, it is probably a mix of very different molecules released by either the coral nubbin or associated microorganisms, and the observed H2O2 decrease could be due -at least in part- to the conversion of H2O2 to much more oxidative molecules (in particular hydroxyl radicals). That is not just a play on words, and I would recommend to use a more neutral term like "H2O2 degradation" rather than "antioxidant". This point (pro-oxidative conversion) should also be evoked when discussing about the nature of the possible "antioxidant" molecules.

Response: Please see below, together with our response to comment 2

Comment 2: On this same aspect, if the authors really suspect some extracellular catalase release (very interesting point), why have they not used a specific inhibitor



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like 3-amino- 1,2,4-triazole? This sounds much more relevant than a 80_C incubation! What's about the time-course variation in the seawater protein content (as a proxi of mucus production) and correlation with stirring speed?

Response: Addressing the reviewer 2nd comment, we conducted additional experiments for characterizing the antioxidant activity using the catalase inhibitor 3-Amino-1,2,4-Triazole (3- AT). In these experiments, three coral fragments were incubated for 1h under stirring conditions. The corals were removed and the coral water split to subsamples, some of which were incubated for 40 min with 3- AT at a range of concentrations (0.01-6 mM). The coral water with and without 3- AT was then assayed for antioxidant activity. Following the literature (see table below) and few preliminary experiments, we chose to apply a range of inhibitor concentrations to ensure a more specific inhibitory effect. We also verified in control experiments that the inhibitor at the concentration used does not react with H2O2 and POHPPA, and does not interfere with the fluorometric measurements. The results show that 33- AT inhibits the antioxidant activity in a dose dependant manner (see new panel b in Fig 6). Moreover, a marked (98%) inhibition was observed at a 0.1 3- AT, a relatively low concentrations (see table below). We thus conclude that the antioxidant activity is enzymatic by nature (based on our previous findings) and that catalases are most likely the major enzymes at play. We therefore: 1. Prefer to keep the term anti-oxidant activity rather than H2O2 degradation. 2. Pro-oxidants – having established that the H2O2 is degraded by catalase-like enzymes, we can assume is fate is water and oxygen and not other radicals. We thus chose not to explore this option in the text. In addition, since metals are not important players here (see response below #3 and that to reviewer #1), it is unlikely to invoke metal catalyzed hydroxyl radical or superoxide conversion of hydrogen peroxide.

Table with experimental conditions for the use of the inhibitor now added to appendix (A8) The last point you raised in comment 2 refers to protein quantification in the coral water, and the presence of mucus. We have made few attempts to follow POC and DOC as well as protein levels in these experiments and were always below detection



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limit. We tried different mucus stains, and again the samples were too dilute. We had a master student who focused specifically on the antioxidant activity of the mucus. Which he collected in such incubations, as well as by milking (following air exposure), with syringe from the coral surface etc. . .. We can assure that coral mucus has antioxidant activity and that some mucus is released when the corals are stirred, but at current we can not unequivocally claim that the observed antioxidant activity is exclusively released with the mucus.

Comment 3: All measurements and calculations should be presented with their respective controls: seawater incubated under same conditions without coral nubbins! This was the case in the 2013 paper, why not here? This is absolutely necessary to assess the implication of both metallic (Fe, Cu, Al etc.) and organic contaminants (including microorganisms) present in the "natural seawater".

Response: Control experiments were carried out frequently but were not included in the manuscript. We now added representative control experiments to Fig 4 (both for antiox and H2O2). Overall, in the control experiments conducted at different conditions H2O2 concentrations were always low and did not change with time, and antioxidant activity was always below detection limit throughout the experiment (see also comment to reviewer #1 on DTPA).

Comment 4: It is very frustrating that the presented data are only obtained under dim light and that there is no comparison between photoactive (bight light) and non photoactive (dim light) coral nubbins. The bleached vs symbiotic state comparison is very interesting and relevant, but this does not solve out what is going on in a photoactive symbiotic coral! This very straightforward experiment (ligh/dark cycle) would help the reader to get convinced about the validity of the methodology, in a much more relevant manner than with the effects of variable stirring speeds!

Response: Indeed, we are also frustrated but not lazy. In the last two years we have made large efforts to study this important question using different setups. The current



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setup was not appropriate for oxygen based evaluation of photosynthesis and hence we switched to metabolic cells. In the metabolic cells we measured simultaneously oxygen and H2O2 production (as well as antioxidants) at increasing light intensities (PI curves). While oxygen gave the expected response with light we had different problems with H2O2 and antioxidants measurements. Specifically we had to replace all the water from the cells when increasing the light level due to accumulation of antioxidants (that prevented H2O2 accumulation). It seems that this intensive washing resulted in a gradual decline in the rate of H2O2 release with time. In another setup we conducted long term diurnal cycle experiment that tested H2O2 release in semi natural conditions (using flow-through systems and natural sunlight in water tables). We observed higher H2O2 releases at noon, which corresponded to high irradiance (measured) and probably photosynthesis (not measured). Following your comment we decided to add one such preliminary experiment to the appendix (appendix A5) with an acknowledgment in the discussion that this project needs further study.

Comment 5: The authors should consider deleting the fig 5, which bring the exact same data as fig 4.

Response: The data in figure 4 and 5 was combined and re-ploted. This figure now shows the kinetics of release using representative (bleached and non-bleached) experiments with their respective representative controls (Fig 4a-f). the additional panels of the figure 4g-i, present the averages values ($\pm$SE) after 1 hour for all experiments conducted (n=15 for the non-bleached and n=10 for the bleached). This part of the figure demonstrates the variation within and between groups. New statistical analysis was performed using parametric (two sample Student's T-Test) and nonparametric (2 sample Mann Whitney U Test) tests.

Comment 6: In the text (lines 9-10 pg 40) it is explained that "Kantiox increases linearly with time" and that Kantiox value is then calculated "using a linear equation that was fitted through the data", but no data are further presented in the result session to show this correlation. I tried to make some calculations from the slopes presented fig 1d, and



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I found an r2 of 0.91 for a linear and an r2 of 0.97 for an exponential correlation! If fig-1 results are representative, kantiox seems to increase exponentially and not linearly, which has major consequences considering long incubation times (400 min as in Fig-3). This point really needs clarifications by showing the mean variations with standard errors and corresponding statistical test of linearity (or exp.) and not just few points of one experiment, as it is the case Fig-2.

Response: The point raised by the reviewer was addressed at different levels. To test whether kantiox increase over time is linear or exponential, we conducted curve-fitting tests in 15 long experiments (100-240 min). We compared the R2 values of linear and exponential correlations and found that R2 values of linear correlations ($0.84\pm0.15$) are significantly higher ($p=0.019$, paired T-Test) then those of exponential correlation ($0.77\pm0.13$). This information suggests that using linear equation for calculating kantiox value is fine. We added this data analysis to the revised manuscript (please note appendix A1). Due to the linear relationship we kept using linear equation to calculate the expected kantiox values. However, for short experiments (including stirring speed), we chose based on your concern to change the calculation method for missing data. Rather than applying a single equation for the whole set we calculated kantiox for the 1-3 missing time intervals (depending on the spacing of the H2O2 measurements), by averaging the starting and final values of that time interval and correcting for the elapsed time. This calculation still assumes linear increase in kantiox but for only 20 min increments, and hence it prevents large deviations from the measured values. These changes are now described in the methods and we have added to the appendix (A1) a description of the kantiox calculation.

Last, since in all cases the antioxidant increased with time, but not always in a linear fashion we changed some of the phrases describing it (such as in the abstract): “H2O2 and antioxidant activity linearly steadily increased in the water surrounding the coral over short periods of 1-2 h”.

Comment 7: Why the Kantiox and H202 variations over time presented in the Fig-2



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are so different(40 times higher), than the one published previously (Fig 8 of the 2013 paper)?

Response: in our 2013 paper we present an experiment where kantiox valuses after 60 min incubation is 1 h-1, in the current MS kantiox values after 60 min incubation is 4 h-1(Fig. 2), this is 4 times higher not 40. We would like to point out that there is some variation in kantiox and H2O2 release rates between coral fragments. This variation is apparent from the data in Fig. 4g-i.

Comment 8: Is it due to stylophora species or just to a coral size/beaker volume ratio? If size/volume ratio is correct, that means that the indicated values should really be normalized by the relevant factor to allow further comparisons. Similarly, to me it is necessary in such a methodology paper to verify that the kinetics parameters are well correlated to the nubbin size/surface

Response: We agree with the reviewer that normalization factor can be neat. However, the appropriate normalization factor in this case is not conclusive (it could be protein content, surface area, zooxanthellae count, mucus thickness and more) because the source of H2O2 and antioxidants are yet unclear. Using inappropriate normalization factor might change the data significantly. For this contribution, it is important to note that we maintained similar sized coral fragments and similar water volume and hence the experiments are comparable.

Based on our previous findings with anti-superoxide activity (which was correlated to coral size, Saragosti et al. 2010) and following the reviewer comment, we tested the effect of coral size on antioxidant activity release. Six coral fragments of different size (volume) were used and following 1 hr incubation, we examined how much antioxidant activity was released to the water (results were normalized to water volume). In general, antioxidant activity increased with coral size however, linear correlation was very weak (appendix A2). At this stage, we think that water volume to coral volume should be kept constant when comparing different conditions, but that normalization to coral



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size (or other parameter) is a potential source of error.

Comment 9: The authors should give indications about the min/max limits in the biological material amount needed to achieve relevant measurements.

Response: Our experiments were conducted using Ìť 20cm2 surface area coral fragments and water volume of 100ml. In order to achieve reliable measurements we recommend to keep the ratio of coral size to water volume as described above. note that H2O2 concentration in the sample is influenced by the coral H2O2 releases rates, the antioxidant activity that degrade H2O2 simultaneously and the flow speed in the beaker. In addition, the quality of the measurements is also influenced by the assay accuracy and sensitivity as well as reliable blanks and trustful calibration curve. If one can develop sensitive and accurate H2O2 assays, then a smaller ratio of coral size to water volume can be used. So this is clearly not a clear cut answer, but we added more information in the appendix (A2) on that issue.

Comment 10: Many method precisions should be given in the changing flow experiments: - slow/moderate/fast are not scientifically relevant terms if not connected to values expressed either in rpm or even better in water movement speed.

Response: We accept this comment. We examined a more precise stirring speed term, the magnate Rounds per Minute (RPM) using high-resolution video camera. Analyzing the videos in slow motion, we counted the number of rounds in ten-second time interval and added the missing data to the manuscript, including clearer explanation of the experiment. This information might help the readers in repeating our experiments however; it is important to note that it does not provide a good evaluation of the flow field around the coral fragment, considering the complexity of the branching coral morphology.

Comment 11: since the same coral nubbin is consecutively submitted to the three flows, the statistical test should be a repeated measures ANOVA (at least if ANOVA test conditions are respected... which I doubt concerning the homoscedasticity). –



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Response: We accept the comment. We reanalyzed the data using repeated measures ANOVA and Tukey post hoc tests (with R software). In addition, we present the results in new manner after standardization. Since the experiments and later on the data analysis were preformed on coral fragments that were subjected to three different stirring speeds continuously, we present the relative change over time. In slow flow, we standardized the results to 1 and at faster stirring speeds (moderate and fast) we calculated the relative difference in each parameter compare to slow.

Comment 12: at the highest speed, did the vortex formation created some foaming, which could probably interfere with the observed results? since coral nubbins were suspended by a Nylon thread, the fragments should also rotate due to stirring, modifying the relative flow to which the coral surface was actually submitted in the beaker.

Response: No signs of physical damage to the corals were observed at the high stirring speed, corals did not rotate and foam was not formed. At all stages, corals had their polyps extended and did not show signs of stress (please note appendix A3). We added these explanations to the relevant section in the methods (increasing speed experiments)

Comment 13: since these experiments are done in such a small volume (100ml), without water renewal, authors should not compare their findings to the effects of open-sea currents (line 6, pg 49).

Response: Acknowledging Nakamura and van Woesik, and Smith and Birkeland works aimed to connect between our observations of flow induced H2O2 release and their hypothesis of rapid ROS release under strong currents. Since no hard evidence was provided, so far, on external ROS release, our lab-based data (although conducted under lab conditions) is important indication that corals are capable of this release. Based on these and many others (including large volumes, short term, flow-through experiments etc..) we are certain that the H2O2 release is not a result of stress that caused by minimal water volume or small water renewal (as may be inferred from the



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reviewer comment). We took great care to assure that the corals were not stressed before and during the experiments (as explained in more detail now in methods). We think that our findings are relevant to coral reefs ecology, at least qualitatively therefore, discussing large scale behaviors is not problematic.

Comment 14: Are the observed effects reversible with further decreasing speeds?

Response: Yes, the effect of flow is observed also in reversible flow regime. To address your comment we preformed a complimentary experiment with reversible stirring regime and presented the data in the appendix (appendix A6). At the beginning of the experiment, fast flow speed was applied and after 60 min the flow was reduced dramatically to slow speed. The results show high increase in H2O2 concentration in the first stage and immediate decrease after the change of flow. Calculation of the total amount of H2O2 been released for each time interval (considering the H2O2 been degraded by antioxidant activity) shows a rapid release of H2O2 at the first stage and lower release under lower stirring speed.

Comment 15: Although I understand the need of it for calculation, presentation of data corresponding to exogenous H202 addition in the bleached coral is very confusing (Fig 4-d).

Response: Thanks. We added a small insert that highlight the external H202 addition.

Comment 16: In the method session we should know how long is the incubation in heating water bath (is it 10 min. as for azide?).

Response: Yes, the water were pre-incubated for 10 min at different temperatures and decay analysis was preformed after water cooled down and reached room temperature. We added the missing data to the manuscript.

Comment 17: The end of this paragraph (lines 24-25 pg 40) is very confusing, since no further data are presented about these “highly active samples”.

Response: We rewrote the paragraph, and explained better that in order to know how



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much antioxidant activity the coral incubation water have, we compared between our decay assay and a standard enzymatic activity assay (of catalase). Both assays were preformed on the same water samples (the "highly active samples") and then compared. In the text, we write: "The antioxidant activities measured here can not be simply converted to standard units of enzymatic activity. Catalase activity, for example, is measured using 10.3 mM H2O2 at pH 7 and at 25°C. Where, one catalase unit is defined as the amount of enzyme decomposing 1 $\mu$mol of H2O2 in 1 min at these conditions. To allow this conversion we assayed coral incubation water with high antioxidant activity in two different procedures. Few subsamples were diluted and assayed with our procedure as described above. Other subsamples were amended with 10.3 mM H2O2 (at pH 7 and 25°C), and its decay was followed at 240 nm using a Varian Cary 50 spectrophotometer."

Comment 18: Technical corrections, In the Fig-6, the half of the H2O2 concentration figures is not visible.

Response: We corrected the Figure (please note fig 5)

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Comments by Daniel Sher:

1- Reorganization of the methods.

Please note response to comment 2, reviewer #1.

2- Addition of control at no flow conditions.

Please note response to comment 3, reviewer #2.

3- Examination of H2O2 and antiox release after the flow is stopped.

Please note response to comment 14, reviewer #2.

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Comments by Andrew Rose:

1- I agree that use of the term “antioxidant” is ill-defined and not the most appropriate term; as anonymous reviewer #2 suggests, H2O2 degradation would seem a much more accurate description of the process.

Please note response to comments 1 and 2, reviewer #2.

2- I also found the methods description to be inadequate in several cases, as discussed by both anonymous reviewers. I believe that the methods themselves are appropriate for the study, but the clarity of presentation could be greatly improved by additional details.

Please note response to comment 2, reviewer #1.

3- If at all possible, a more quantitative description of stirrer speeds (and potentially some calculations of associated mass transport rates) could really improve the paper. For example, calculation of mass transport rates of hydrogen peroxide might allow a more quantitative test of the proposition that stirring promotes release of hydrogen peroxide from some kind of intracellular “pool”, which is a relatively contentious idea as noted by anonymous reviewer 1.

Please note response to comment 10, reviewer #2 and comment 15, reviewer #1.

Please also note the supplement to this comment:
http://www.biogeosciences-discuss.net/11/C1701/2014/bgd-11-C1701-2014-supplement.pdf

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Interactive comment on Biogeosciences Discuss., 11, 33, 2014.





Figure 4

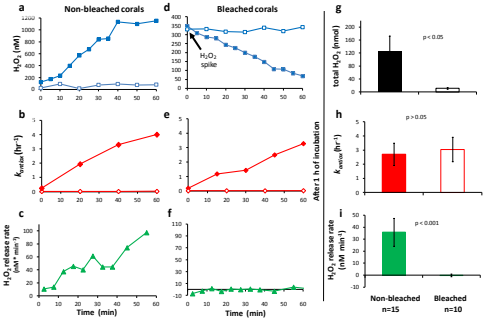


**Figure 4. $H_2O_2$ and antioxidant activity release from non-bleached and bleached *S. pistillata*.** Line plots show changes with time in $H_2O_2$ concentrations (a, d), antioxidant activity (b, e), and calculated $H_2O_2$ release rates (c, e) from individual experiments with bleached (a-c) and non-bleached (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 total $H_2O_2$ released during 1 h incubation (g), antioxidant activity at 1 h (h), and $H_2O_2$ release rates at 1 h (i). Differences between treatments were tested using Mann Whitney U Test (g, i) and Students T-test (h). Note that $H_2O_2$ spike was added to the bleached coral (d), see text for details.

**Fig. 1.** Figure 4



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Figure 5

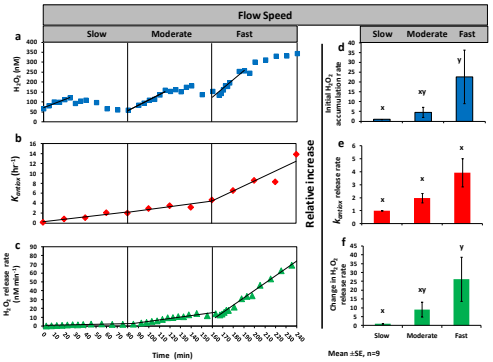


**Figure 5. Release of $H_2O_2$ and antioxidant activity by *S. pistillata* at variable stirring speeds.** Line plots show changes with time in $H_2O_2$ concentrations (a), antioxidant activity (b), and calculated $H_2O_2$ release rates (c) obtained at increasing stirring speeds in an individual experiment. The rate of change in each of the parameters (i.e. the slopes drawn in a-c) was calculated for each of the stirring speeds. For each parameter, these rates were then expressed relative to the rate at slow stirring, and 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 of $H_2O_2$ release rates (f). Letters in panels d-f refer to significant differences of $p < 0.05$ between the relative release rates of each of the studied parameters at the different stirring speeds, tested with Tukey's multiple comparison test.

**Fig. 2.** Figure 5



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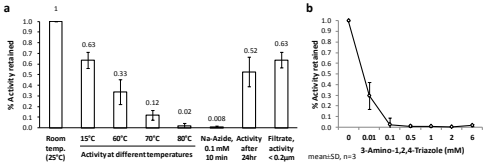


**Figure 6. Basic biochemical characterization of the coral released antioxidant activity.** The effect of temperature (15, 60, 70, and 80°C), haem-enzymes inhibitor (0.1 mM Na Azide), filtration (0.2 µm), and storage (24 h) on antioxidant activity from coral incubation water (a). The effect of increasing 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± SD) is presented as percentage of the non-treated samples (n=3).

**Fig. 3.** Figure 6



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**Appendix A1. Changes with time in $k_{antiox}$ and its calculation for missing time points**

In all experiments $k_{antiox}$ values increased with time. The release kinetics could be fitted with a linear or an exponential function. To determine the more appropriate function we fitted both of these functions to data from 15 long incubations (100-240 min) and compared the resulting $R^2$ values of the fits (Table A1). We found that $R^2$ values of linear correlations (0.84±0.15) are significantly higher (p=0.019, paired T-Test) then those of exponential correlations (0.77±0.13; see Table A1).

The change with time in the antioxidant activity and its curve fitting is important for our understanding of this phenomenon and for practical reasons of calculating missing data points. The later issue of missing data points is of significance for the calculation of $H_2O_2$ release rates according to equation 5. This calculation is conducted for small time intervals 5-10 min, while the antioxidant activity was determined only every 20 min. Hence we had to calculate 1-3 $k_{antiox}$ values (depending on the spacing of the $H_2O_2$ measurements) to allow frequent calculations of $H_2O_2$ release rates. For long experiments (with many $k_{antiox}$ assays) we used linear equation to calculate the missing $k_{antiox}$ values. For short experiments, including the different stirring speed experiments (when few $k_{antiox}$ assays are available and linearity could not be determined), we calculated $k_{antiox}$ for the missing time intervals by averaging the starting and final values of that time interval and correcting for the elapsed time. This calculation still assumes linear increase in $k_{antiox}$ but for only 20 min increments, and hence it prevents large deviations from the measured values.

**Fig. 4.** Appendix A1



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**Appendix A1. Changes with time in $k_{antiox}$ and its calculation for missing time points**

**Table A1. Equation fitting through the antioxidant activity data.** Curve-fitting for the increase in $k_{antiox}$ over time for 15 long incubations (100-240min). $R^2$ values of linear and exponential correlations were calculated and compared. $R^2$ values of linear correlations are significantly higher (p=0.019, paired T-Test) then those of exponential correlations.

| Date | Exp. Duration (min) | Decayes assays (number) | Linear fit $R^2$ | Exponential fit $R^2$ |
|---|---|---|---|---|
| 22.7.2012 | 120 | 7 | 0.76 | 0.83 |
| 22.7.2012 | 120 | 7 | 0.95 | 0.89 |
| 22.7.2012 | 120 | 7 | 0.94 | 0.58 |
| 23.7.2012 | 240 | 13 | 0.91 | 0.84 |
| 23.7.2012 | 240 | 13 | 0.87 | 0.71 |
| 23.7.2012 | 240 | 13 | 0.84 | 0.77 |
| 25.7.2012 | 100 | 6 | 0.78 | 0.81 |
| 25.7.2012 | 100 | 6 | 0.96 | 0.92 |
| 25.7.2012 | 100 | 6 | 0.96 | 0.91 |
| 25.7.2012 | 100 | 6 | 0.61 | 0.63 |
| 25.7.2012 | 100 | 6 | 0.69 | 0.64 |
| 25.7.2012 | 100 | 6 | 0.46 | 0.48 |
| 8.8.2012 | 100 | 6 | 0.96 | 0.87 |
| 8.8.2012 | 100 | 6 | 0.97 | 0.88 |
| 8.8.2012 | 100 | 6 | 0.98 | 0.76 |
| Average | | | 0.84 | 0.77 |
| SD | | | 0.15 | 0.13 |
| n= | | | 15 | 15 |
| paired T-Test | | | p = 0.0195 | |

**Fig. 5.** Appendix A1 (2)



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**Appendix A2. How does coral size and water volume influence the obtained rates?**

Normalization of the measured rates to coral related parameters

A clear benefit to our newly described phenomenon can stem from normalization of the rates of $H_2O_2$ and antioxidant activity release to some parameters of the coral that generate them. However, at current, the "appropriate" parameter of normalization is not yet known since the source of $H_2O_2$ and antioxidants are not yet fully resolved. Several parameters can be considered such as coral size (surface area, volume, and weight), protein content, zooxanthellae density, tissue or mucus thickness etc. The data set described in the paper was conducted using similar sized coral fragments and similar water volumes, in an attempt generate comparable experiments. It is however possible that some of the variability observed between experiments reflect coral parameters that were not measured.

Release of antioxidant activity by corals with different sizes

A preliminary attempt towards obtaining a normalization factor was done by incubating six coral fragments of different sizes in containers with different water volumes. Following 1 h of incubation the coral water was assayed for antioxidant activity. These results were normalized to a constant water volume (to avoid dilution effect) and were plotted against coral size expressed in volume (Fig. A2). The coral volume was estimated by a simple technique of water displacement. In this technique corals are introduced to a beaker completely filled with seawater and the overflowing water are collected and weighted. This simple technique is rather accurate (given sufficient repetitions) and imposes minimal stress on the corals. In our case, we allowed the corals to recover for one weak between this measurement and the incubation experiment. The experiment show a general trend of increase in antioxidant activity with the coral (Fig. A2). However we observed a rather weak linear correlation ($R^2 = 0.44$). Considering these results, we think that that normalization of the observed rates to coral size could introduce large noise to the data.

**Fig. 6.** Appendix A2



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**Appendix A2. How does coral size and water volume influence the obtained rates?**

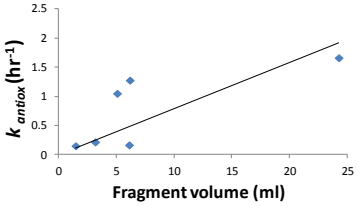


**Figure A2. The effect of coral size on antioxidant activity release**. Following 1 h of incubation that antioxidant activity in the incubation water was determined for six coral fragments of varying sizes. The coral volumes were examined by the water displacement technique and the antioxidant activities were normalized to a constant water volume.

Recommended optimal coral size for our experiments

Our experiments were conducted using~20$cm^2$ surface area coral fragments and water volume of 100ml. In order to achieve reliable measurements we recommend keeping this ratio of coral size to water volume. Note that $H_2O_2$ concentration in the sample is influenced by the coral $H_2O_2$ releases rates, the antioxidant activity that degrade $H_2O_2$ simultaneously and the flow speed in the beaker. In addition, the quality of the measurements is also influenced by the assay accuracy and sensitivity as well as reliable blanks and trustful calibration curve. If one can develop sensitive and accurate $H_2O_2$ assays, then a smaller ratio of coral size to water volume can be used. We did manage to measured $H_2O_2$ and antioxidants from much smaller corals (~2 $cm^2$) with a similar water volume, but this required an experienced personal. For larger corals, larger water volumes will be required and rates are expected to be kept on the same order of magnitude.

**Fig. 7.** Appendix A2 (2)

**Appendix A3. Pictures of the experimental setup and of *S. pistillata* coral fragments**

To enable readers to repeat our experiments we included few photos of the experimental setup for measuring $H_2O_2$ and antioxidant activity release rates.


**Figure A3 a.** Three *Stylophora pistillata* coral fragments are shown suspended in the incubation beakers. The beakers are placed on a stirrer where a 1 cm long magnet bar ensures complete water mixing. The coral polyps were extended and the corals did not show signs of stress.

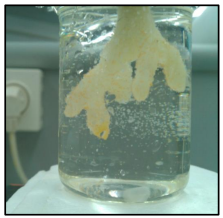

**Figure A3 b.** Bleached *Stylophora pistillata* coral fragment, pictured during its feeding with a one-day-old *Artemia salina* nauplii.

**Fig. 8.** Appendix A3



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**Appendix A4. $H_2O_2$ release by *S. pistillata* fragments under complete darkness and low light intensity.**

In general, our experiments were conducted under fluorescence laboratory light of ~10 µmole quanta $m^{-2}$ $s^{-1}$. This low illumination is sufficient for only minimal photosynthesis (or none at all), as it is far below the compensation light intensity of these corals. Several experiments were conducted in complete darkness to allow comparison with our standard low light conditions. In these experiments, the initial $H_2O_2$ accumulation rates calculated in nmol per min were similar between dark and low light conditions (Fig. A3). These results suggest that the $H_2O_2$ released during our standard experiments was not produced via photosynthesis.

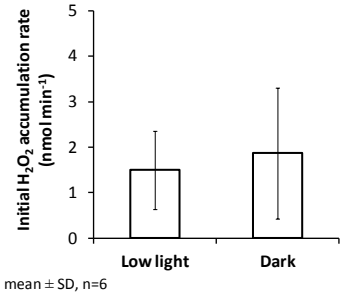


**Figure A4. $H_2O_2$ release by *S. pistillata* coral fragments at low light intensity and complete darkness.** Initial $H_2O_2$ accumulation rates (calculated in nmol $min^{-1}$) are compared between experiments conducted at complete darkness (n=6) and low light intensity of ~10 µmole quanta $m^{-2}$ $s^{-1}$ (n=6). Rates are not corrected for $H_2O_2$ decay since at this stage of the experiment $k_{antiox}$ values are low.

**Fig. 9.** Appendix A4



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**Appendix A5. $H_2O_2$ release from *S. pistillata* under high natural irradiance**.

The experiments reported in the manuscript are restricted to low light conditions, where photosynthesis is negligible. Since the symbiotic algae are the source of the released $H_2O_2$ (Fig. 4), it is highly feasible that upon illumination and the commencement of photosynthesis corals will release more $H_2O_2$. We are currently investigating these issues and have been establishing a different experimental setup to examine the effect of light on $H_2O_2$ release dynamics. This setup involves a flow-through system in a water table where constant water exchange enables long experiments under natural irradiance. Analytically it is rather challenging to obtain an appropriate water exchange rate that will not wash out the coral produced $H_2O_2$ nor allow too much antioxidants to accumulate. Such an experiment was run with a large coral fragment of ~ 100 $cm^2$, in a 600 mL glass beaker, with the water flow rate of 13.5ml/min and moderate stirring, in Dec 2013. The coral was placed over night in the experimental conditions for long acclimation . Shown are $H_2O_2$ measurements and calculated $H_2O_2$ release rates (which take into consideration dilution and decays; Fig. A5). This experiment indeed shows higher $H_2O_2$ concentrations and release rates at noon, when high solar irradiation was measured. We have not measured the coral photosynthesis rate in this experiment, and can relate at current only light and $H_2O_2$ release. However, this is a promising first step in studying the link between $H_2O_2$ release dynamics and photosynthesis.

**Fig. 10.** Appendix A5



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**Appendix A5. $H_2O_2$ release from *S. pistillata* under high natural irradiance.**

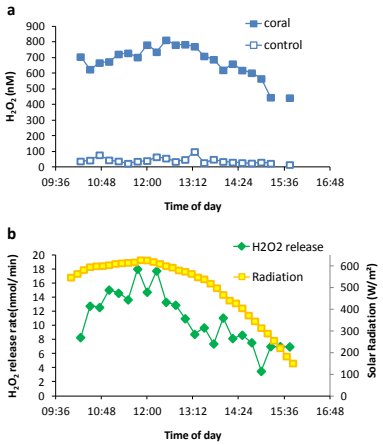


**Figure A5. Diurnal variation in $H_2O_2$ release by *S. pistillata*.** (A) Changing $H_2O_2$ concentration over 5 h experiment at midday in the coral surrounding water and in a control experiment (water alone). (b) Calculated $H_2O_2$ release rates and solar radiation measurements. The experiment was conducted in a water table under natural daily cycle using flow through system.

**Fig. 11.** Appendix A5 (2)



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**Appendix A6. A complimentary experiment with decreased stirring speed**

An experiment with reversed stirring regime (compared to those in Fig. 5) was conducted to test the effect of reducing flow speed on $H_2O_2$ and antioxidants release. At the beginning of the experiment, fast flow speed was applied and after 60 min the flow was reduced dramatically to slow speed. $H_2O_2$ concentrations increased initially and then dropped immediately when the flow was reduced (Fig, A6 a). Antioxidant activity on the other hand continued accumulating throughout the experiment (Fig. A6 a). From these parameters we calculated the amount of $H_2O_2$ released during each time interval. We then present the cumulative $H_2O_2$ released (Fig. A6 b). It is apparent from the slopes, that the rate of change, that is the rate of $H_2O_2$ accumulation is faster when stirring speed is rapid.

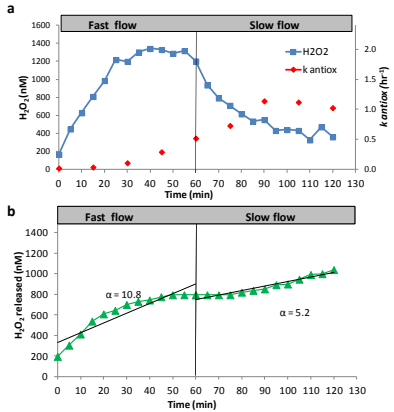


**Figure A6. The effects of reversed flow regime on $H_2O_2$ and antioxidants release.** (a) Changes with time in $H_2O_2$ concentrations and antioxidant activity obtained at fast (0-60 min) and slow (60-120min) stirring speeds. (b) Cumulative $H_2O_2$ that was released to the water by the coral. The slopes plotted through the data indicate the rate of $H_2O_2$ release in each of the stirring speeds.

**Fig. 12.** Appendix A6



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**Appendix A8. Summary of 3-Amino-1,2,4-Triazole experiments.**

| Citation | Organism and Tissue type | 3-Amino-1:2:4-Triazole concentration and time of incubation | Catalase activity retained |
|---|---|---|---|
| Margoliash and Novogrodsk, Biochem. J. Mar., 68, 468–475, 1958. | Mammals- rat-kidney suspension | 20mM<br>80 min | 10% |
| Tephly et al., JPET, 134, 77-82, 1961. | Mammals- crystalline beef liver | 24mM<br>30 min | 5% |
| Cohen and Somerson, J. Bacteriol., 98, 543-546, 1969. | Bacteria- *Mycoplasma pneumoniae* | 50 mM<br>60 min | 25% |
| Havir, Plant Physiol. 99, 533-537, 1992. | Plants- Leaves of *Nicotiana sylvestris* | 20mM<br>13 hours | 15% |
| Merle et al., Free Radical Biology & Medicine, 42, 236–246, 2007. | Cnidaria- Whole sea anemones | 0.5 mM<br>6 days | 25% |

**Table A8. A summary of experimental conditions used to inhibit Catalase activity with 3-Amino-1,2,4-Triazole.** 3-Amino-1,2,4-Triazole have been used to inhibit catalase activity in various tissues, (including cell suspentions, leaves and whole sea anemones) and various organisms (from mammals to bacteria). Incubations performed at tens of millimolars, inhibited 95-75% of the antioxidant activity. In our experiment, 3-Amino-1,2,4-Triazole inhibited the antioxidant activity a low concentration of 0.1 millimolar with high efficiency of 98%.

**Fig. 13.** Appendix A8



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