



Rapid Hydrogen Peroxide Release during Coral-Bacteria Interactions

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Ocean warming has exacerbated the severity of coral diseases, many of which are mediated by pathogenic bacteria. In response to the presence of pathogens various organisms activate an oxidative burst response, involving strong and rapid generation of reactive oxygen species (ROS) that serve both as bactericides and as signals for other defense systems. While many components of the coral immune systems are being unveiled, an oxidative burst response, triggered by bacteria proximity, has not yet been reported. Hydrogen Peroxide (H₂O₂), one of the ROS produced during the oxidative burst, is often monitored as an indicator of this pathway. Here we report a yet undescribed release of H₂O₂ from the coral *Stylophora pistillata* upon contact with bacteria. In a series of short term experiments we monitored H₂O₂ concentrations in the vicinity of *S. pistillata* prior to and following local administration of five bacteria isolates belonging to the genus *Vibrio* (obtained from healthy *S. pistillata*). In most experiments, rapid H₂O₂ release was recorded at the site of interaction within one min from *Vibrio* addition and persisted for the remaining 5 min of the experiment. In some experiments, discrete or continuous drop of H₂O₂ to below background levels were seen following *Vibrio* addition. This H₂O₂ loss was quantitatively accounted for by the bacterial antioxidants and implies that *Vibrio* may at times offset the coral released H₂O₂. The total H₂O₂ released by the coral from the small interaction site (~10 mm²) was 200–600 pmol, which may build up to a concentration of ~20 μM in the coral diffusive boundary layer. H₂O₂ concentrations above 10 μM resulted in significant mortality of the coral pathogen *Vibrio coralliilyticus*, suggesting that the released H₂O₂ may act as a bactericide. It is thus far unclear if this H₂O₂ release is part of an oxidative burst response, but it's occurrence strongly indicates that corals sense and chemically react toward bacteria.

Keywords: hydrogen peroxide, *Stylophora pistillata*, coral-bacteria interactions, *Vibrio*, oxidative burst response, coral surface layer

INTRODUCTION

Coral reefs are highly diverse ecosystems, containing thousands of invertebrates and fish species that live in complex symbiotic relationships (Bellwood and Hughes, 2001). On top of these interactions, reef organisms also harbor complex associations with microorganisms that live in their tissue, on their surfaces, in the water surrounding them and in the sediments (DiSalvo and Gundersen, 1971; Moriarty, 1979; Rohwer et al., 2002).

During the last few decades, especially following major worldwide coral disease outbreaks, intense research has been conducted to explore the different factors controlling the onset of coral

diseases. So far, half of the 18 recognized coral syndromes have been strongly linked to fungal and bacterial infection with six of them associated with bacteria from the genus *Vibrio* (Bourne et al., 2009; Arotsker and Kushmaro, 2015; Munn, 2015). Infectious coral diseases, caused by specific pathogen or microbial assemblages, can be transmitted from infected to susceptible corals by different mechanism such as, vector mediation (e.g., butterflyfish; Aeby and Santavy, 2006) and water-borne infection (Zvuloni et al., 2009). Due to global warming, infectious diseases are expected to become more prevalent with higher transmission rates (Zvuloni et al., 2015). This may result in a severe threat to coral reefs worldwide.

The search for causative agents of coral diseases has also revealed the huge diversity of coral associated microorganisms (Rohwer et al., 2002) and shed light on various coral-bacteria interactions (Ritchie, 2006). In addition to acting as pathogens, many coral associated bacteria were found to be beneficial to the coral (Ritchie, 2011), producing different organic and antibacterial compounds (Shnit-Orland and Kushmaro, 2009) and assisting in nutrient recycling (Raina et al., 2009; Rådecker et al., 2015). Bacterial populations associated with corals are now thought to play important roles in the stability of the coral animal as a whole (termed holobiont; Reshef et al., 2006).

The community composition of the coral associated bacteria often changes along with changes in environmental conditions (Webster et al., 2016), physiological state of the coral (Ng et al., 2015), and exposure to anthropogenic impacts (Ziegler et al., 2016). These changes might generate open spaces for virulent bacteria to proliferate and establish pathology, taking advantage of the unstable state of the coral holobiont (Mydlarz et al., 2010). Severe pathology will appear if bacteria are capable of carrying out a few critical stages including: first interaction, adhesion, penetration, proliferation in the coral tissue, and damage to physiological functions (Rosenberg and Ben-Haim, 2002).

To prevent the pathology caused by bacterial infection, corals make use of different mechanisms; many of which rely on the coral associated bacteria (Shnit-Orland and Kushmaro, 2009). The coral external mucus layer is densely populated with dynamic and rich microbial communities. These microbial populations compete for resources and space and produce antibiotics to fight each other. This competitive behavior eventually reduces the chances of new, potentially pathogenic, species to establish in the coral tissue (Rypien et al., 2010). The corals themselves combat invading bacteria with immune defenses (Mydlarz et al., 2009), cilia-mediated water flow that prevent bacteria adhesion (Shapiro et al., 2014), or by secreting chemicals that inhibit microbial growth, attachment, and behavior (Ritchie, 2006). Such chemicals could include compounds that regulate bacteria communication or have antimicrobial activity (Geffen et al., 2009).

Reactive oxygen species (ROS) have been assigned some roles in antibacterial defense in corals. Banin et al. (2003) showed that the bacterium *Vibrio shiloi*, which causes bleaching in the stony coral *Oculina patagonica*, cannot establish itself in the coral during winter. They suggested that the coral protects itself from invading bacteria with the aid of high levels of ROS in the coral tissue. In winter when the bacterial superoxide dismutase activity

is low, the pathogen is highly susceptible to ROS. Mydlarz and Jacobs (2006) found that injury by sonic sound cavitations and heat stresses were capable of inducing the release of ROS in four gorgonian species. They suggested that ROS release may be the first line of defense in gorgonian corals as well as acting as a signal to induce additional immune and wound healing responses.

Indeed, rapid release of ROS, known as “oxidative burst” (transient ROS generation within time periods from several minutes up to hours), is activated in plants and mammalian immune cells under stress conditions and as a defense against microorganisms (Lamb and Dixon, 1997). The steep increase in internal and often external ROS is mediated by enzymatic ROS production as well as the inhibition of the ROS depletion systems (Schmitt et al., 2014). The elevated ROS, including superoxide, hydrogen peroxide, hydroxyl radicals and nitrous oxide, are harmful to the invading bacteria but also act as signals for activating stress-response and defense pathways (Schmitt et al., 2014). In plants the oxidative burst is part of the hypersensitive response, where rapid death of cells occurs in the local region surrounding a microbial infection to prevent it from spreading. The H₂O₂ burst play multiple roles in the hypersensitive response, damaging the plant cell membrane and acting as a diffusible signal that activates the programmed cell death pathway (Levine et al., 1994).

H₂O₂ is moderately reactive and is a relatively long-lived molecule with a half-life of 1 ms within cells and ~1 d in coastal water (Shaked et al., 2010; Sharma et al., 2012). As the only ROS that can diffuse through aquaporins in the membranes and over larger distances within the cell, H₂O₂ received particular attention as a signal molecule which induces molecular, biochemical, and physiological responses (Gough and Cotter, 2011). The cytotoxic activity of H₂O₂, involving oxidative damage to proteins and lipids is based on the highly reactive hydroxyl radical produced during H₂O₂ interactions with superoxide (Haber-Weiss's reaction) or Fe(II) (Fenton's reaction; Sharma et al., 2012). Oxidative damage to DNA by H₂O₂ is thought to occur through ferryl radical formed when H₂O₂ reacts with DNA-associated iron (Linley et al., 2012). H₂O₂ may also initiate lethal cellular cascades such as lipid peroxidation and apoptotic cell death (Apel and Hirt, 2004; Repetto et al., 2012), and is often used in interactions between organisms as a grazer and pathogen deterrent (McDowell et al., 2014).

Recently we identified and characterized a rapid release of H₂O₂ from the stony coral *Stylophora pistillata*, which occur on time-scales of minutes in response to various triggers (Armoza-Zvuloni et al., 2016). In incubation experiments, with healthy and bleached coral fragments, we documented rapid H₂O₂ release during feeding on zooplankton prey and in response to physical and chemical stimuli. By applying localized stimuli and a frequent sampling procedure we found that the response is limited to the site of stimulus (rather than whole colony response) and its duration is limited to a few minutes.

Here, in a follow-up study, we applied the same setup as in Armoza-Zvuloni et al. (2016) to test whether corals sense the presence of bacteria and respond to it by locally releasing H₂O₂. We used *Stylophora pistillata* coral fragments and cultures of five *Vibrio* isolates obtained from healthy

S. pistillata. We characterized H₂O₂ release kinetics by measuring H₂O₂ concentrations next to the coral surface before and after local administration of concentrated bacteria, and detected a significant, persistent and local release of H₂O₂. The bacteria did not contribute to this H₂O₂ production; on the contrary, their antioxidants degraded some of the H₂O₂ released by the coral. By probing for H₂O₂ toxicity to the coral pathogen *Vibrio coralliilyticus*, and estimating H₂O₂ accumulation in its site of release, we obtained comparable concentrations, suggesting it may act as a bactericide.

MATERIALS AND METHODS

Coral Handling and Preconditioning

Thirty similarly sized *S. pistillata* coral fragments (with a surface area of ~20 cm²) were collected from the Interuniversity Institute for Marine Sciences (IUI) coral nursery located in the Gulf of Aqaba (29°30'6.84"N, 34°55'4.09"E). Coral sampling and handling was carried out in accordance with the recommendations of the Israeli Nature and Park Authority. The fragments were glued to plastic lids facing upright using underwater epoxy glue (Propoxy 20, Hercules Chemicals), and underwent an acclimation period of 6 months in shaded water tables with running seawater at ambient temperatures of 21–27°C.

Vibrio Culturing

Non-pathogenic *Vibrios* were isolated from mucus of three healthy *S. pistillata* coral fragments. The coral fragments were collected from the coral nursery, brought to the lab, washed shortly with filtered seawater and centrifuged for 1 min at maximal speed to remove mucus. The mucus was then diluted and plated on *Vibrio*-selective TCBS agar (4.5% Difco TCBS agar, 0.9% marine broth, 0.45% NaCl, and 1.2% Difco Bacto agar). After serial dilution and plating, five phenotypically different isolates were screened and isolated. A pure culture was obtained by sub-cultivation under the same conditions. The *Vibrio* isolates were assigned numbers from 1 to 5 and were maintained as viable cultures on plates at room temperature or stored as 20% (v/v) glycerol suspensions at –80°C. Prior to the H₂O₂ release experiments, one *Vibrio* colony was inoculated in conical flasks containing 5 mL marine broth medium (1.8% marine broth, Difco MA 2216, and 0.9% NaCl), and incubated at 30°C under shaking (160 rpm) for 24–36 h. Growth was monitored by measuring OD at 590 nm. Cultures were collected by centrifugation at 3000 g for 5 min and re-suspended in 500 μl FSW. Centrifugation and re-suspension was repeated five more times to ensure the complete removal of culture media. Bacteria density was examined using hemocytometer and diluted to final concentration of 1 × 10⁶ cells mL⁻¹. We lack genetic information on the isolates due to failure of the freezer and sample loss.

H₂O₂ Measurements

H₂O₂ concentrations were measured using the POHPAA (4-hydroxyphenylacetic acid) technique detailed in Shaked and Armoza-Zvuloni (2013). The reagent stock, consisting of 0.25 mM POHPAA, 70 units mL⁻¹ of horseradish peroxidase, and

0.25 M Tris at pH 8.8, was added to the samples at a 1:50 dilution. All stocks were kept on ice in the dark. The fluorescence of the dark-kept samples was read within 30 min of sampling using a Varian spectrofluorometer (Cary Eclipse) set at an excitation of 315 ± 10 nm and an emission of 408 ± 10 nm. Calibration curves were run daily using filtered seawater spiked with freshly made H₂O₂ standards and catalase treated seawater as blanks.

H₂O₂ Release Experiments

The release of H₂O₂ from the stony coral *S. pistillata* in response to bacteria proximity was studied in high temporal resolution in localized experiments. We utilized a container fitted with a narrow Teflon tube (0.03 inch internal diameter). The tube carried water by gravitation from the coral surface to cuvettes for H₂O₂ analysis. The cuvettes, containing 20 μL of POHPAA reagent stock (see H₂O₂ measurements), were placed outside the container. The tube position was adjustable and the opening of the tube was set at ~0.2 cm from the coral surface, adjacent to the site of stimulus. No stirring was applied during the experiment and care was taken to minimize turbulence while administering the *Vibrio* culture. The tubes constantly transferred water at a rate of 2 mL min⁻¹ from the coral surface to cuvettes that were replaced every 0.5 min. The experiments started with 2.5 min of background H₂O₂ measurements. Then, 100 μl *Vibrio* culture, containing 1 × 10⁶ cells mL⁻¹, was applied on the coral surface near the tube opening over a small area of 10 mm². Following the addition of bacteria, H₂O₂ readings were taken every 0.5 min from 3.5 to 8 min. To rule out the possibility that the water motion rather than the bacteria addition induced the release of H₂O₂, these experiments were repeated with similar volumes of filtered seawater (FSW). All experiments were conducted under fluorescent laboratory lighting (~10 μmol quanta m⁻² s⁻¹) at a constant temperature (25 ± 1°C). Coral fragments were usually used for one experiment every week. Prior to the experiments initiation, corals were allowed to recover from their preparative handling for 30–60 min under running seawater. Experiments commenced once the coral polyps were extended. Coral fragments did not show any signs of pathology, tissue lysis or bleaching during the period of the experiments and afterwards.

Quantification of the Amount of H₂O₂ Released from the Corals

To convert the H₂O₂ concentrations measured next to the coral to amounts of H₂O₂ released by the coral we conducted the following steps. We first calculated the H₂O₂ released in each discrete time interval, by subtracting the minimal H₂O₂ concentration detected prior to stimulation from all measurements. These minimal values reflect the basal levels of H₂O₂ occurring naturally in seawater. We then converted the nM concentrations in to moles of H₂O₂ according to the collected volumes. In order to get the total H₂O₂ released before and after stimulation we summed up all the relevant time intervals. To account for the shorter duration of the experiment prior to stimulus we multiplied the amount of H₂O₂ released prior to stimulus by 2.5 (the time ratio between the two experimental sections). Note that our sampling procedure leads to removal of all the H₂O₂ released by the coral in each time interval. Therefore,

any addition of H₂O₂ above background levels represents in fact a continuous release of H₂O₂ as explained below.

Sensitivity of *Vibrio coralliilyticus* to H₂O₂

H₂O₂ toxicity was studied according to the assay of Geffen and Rosenberg (2005) using the bacteria *V. coralliilyticus* YB2 (LMG 21348), a virulent coral pathogen (Ben-Haim and Rosenberg, 2002). *Vibrio coralliilyticus* cultures were donated by Prof. Eugene Rosenberg (Tel-Aviv University, Israel). The bacteria were incubated at a final density of 1×10^5 cells mL⁻¹ with increasing concentrations of H₂O₂ for 30 min at 30°C and with filtered seawater as controls. Then 5 µl aliquots ($n = 10$) were spotted on TCBS agar at two dilutions (undiluted and 10-fold). Plates were incubated for 24 h at 30°C. The toxicity of H₂O₂ to *V. coralliilyticus* is evaluated based on the number of colonies growing in each spot on the plates (termed CFU -colony forming units). The experiments were repeated twice at different range of H₂O₂ concentrations using independently grown cultures.

RESULTS

Release of H₂O₂ from Corals in Response to *Vibrios*

We probed for coral H₂O₂ release in response to a local administration of $\sim 10^5$ *Vibrio* cells suspended in 100 µl FSW, by monitoring H₂O₂ concentrations in the coral vicinity prior to and following the bacteria addition. We conducted 103 experiments using 5 different *Vibrio* isolates (*Vibrios* 1-5), with *Vibrio* 4 tested most frequently and hence shown first.

Average Changes in H₂O₂ Concentrations on *Stylophora pistillata* Surface in Response to Interaction with *Vibrios*

Figure 1 shows the mean H₂O₂ concentrations measured in all 48 experiments with *Vibrio* 4 and in 10 control experiments using *Vibrio*-free filtered seawater. Before the stimulus, the water next to the coral had stable H₂O₂ concentrations of 27 ± 2.2

nM (mean \pm SE, $n = 48$). A significant increase in H₂O₂ was recorded at the site of stimulus following *Vibrio* addition reaching 42 ± 7.3 nM ($p = 0.03$, paired t -test). This localized H₂O₂ release occurred rapidly, within 1 min, and persisted for the remaining 5 min of the experiment. To assure that the coral responded specifically to *Vibrio* and not the jet of water from the pipette, we repeated the experiments with a similar volume (100 µl) of filtered seawater (FSW). Here, mean H₂O₂ concentrations on the coral surface were similar before (20 ± 2.7 nM, mean \pm SE, $n = 10$; 1–2.5 min) and after (21 ± 3.8 nM; 3.5–8 min) FSW addition ($p > 0.05$, paired t -test; Figures 1A,B). Testing whether the bacteria themselves are a source of H₂O₂, we measured the H₂O₂ levels of the bacteria suspension used in the experiments. In all five *Vibrios* cell suspensions, H₂O₂ concentrations were similar or lower than in FSW (not shown). Mean H₂O₂ concentrations measured in similar experiments with *Vibrios* 1–3 and 5 are shown in Figure SI-1.

It is important to note that while sampling the water next to the coral with the collection tube, all the H₂O₂ released by the coral was also removed from the experiment. This is based on our recent study showing no diffusion of H₂O₂ from the site of stimulus to other areas of the beaker (Armoza-Zvuloni et al., 2016). Hence, the elevated concentrations measured in response to bacteria represent, in fact, a continuous and localized release of H₂O₂.

Variations in the Patterns of H₂O₂ Release in Response to *Vibrio* 4

Figure 1 shows the average behavior of all corals but hides information on variations in the H₂O₂ release patterns and amounts between individual experiments. While in many experiments rapid H₂O₂ accumulation was observed, in other experiments H₂O₂ concentrations did not change and even dropped below background levels (see Figure SI-2 for representative individual experiments).

To better understand patterns of H₂O₂ release we calculated the changes in H₂O₂ in individual experiments following

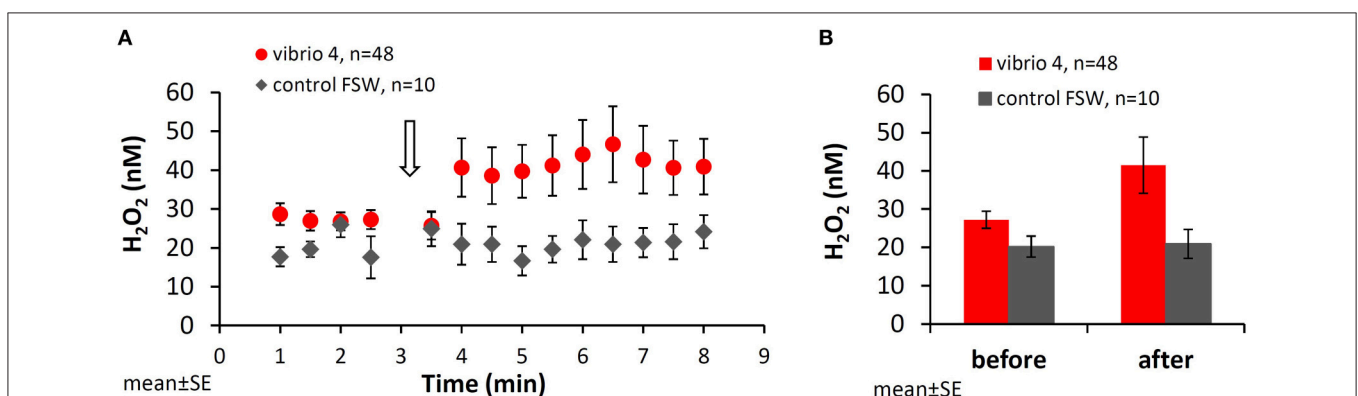


FIGURE 1 | Changes in H₂O₂ concentrations in the vicinity of *Stylophora pistillata* following interaction with *Vibrio*. (A) H₂O₂ concentrations next to the coral surface prior to and following addition of bacteria (10^5 cells of *Vibrio* 4; red dots) or filtered seawater as a control (gray diamonds). *Vibrio* 4 culture, originally isolated from healthy *S. pistillata*, was added after 3 min (open arrow) and the coral response was examined for additional 5 min. (B) Mean H₂O₂ concentrations measured before (between 1.0 and 2.5 min) and after (between 3.5 and 8.0 min) interaction with *Vibrio* 4. All the data is averaged over the entire set of 48 experiments.

stimulus in two ways and presented them on a scatter plot (Figure 2). The first calculation examines the immediate response to bacterial addition using the ratio of two single H₂O₂ measurements taken immediately after and before the stimulus (=Immediate after/before). The second calculation examines the longer term response to bacteria using the ratio of averaged H₂O₂ concentrations measured after and before the stimulus (=Averaged after/before). The resulting ratios conveniently demonstrate the net change in H₂O₂ and its degree following bacteria addition and allow us to split the experiments into four categories, as shown in Figure 2.

In our data set, 15 out of 48 experiments with *Vibrio* 4 plot on the upper right corner, indicating both an immediate and a longer term H₂O₂ accumulation (i.e., ratios > 1). The average increase in H₂O₂ concentration in response to bacteria of these 15 experiments (shown on the upper right corner of Figure 2), is naturally higher than the average change observed in all the experiments (Figure 1). Eleven of the remaining experiments plot on the lower right corner, indicating an immediate decrease in H₂O₂ but a longer term H₂O₂ increase (as shown also by their average changes in time). Together the 26 experiments with “averaged after/before” larger than 1 (the right hand side of the plot), represent overall H₂O₂ accumulation, despite an immediate drop in H₂O₂ in almost half of them. Interestingly, 17 of the 48 experiments plot on the lower left corner, showing both an immediate and a longer term H₂O₂ loss. The controls with *Vibrio*-free FSW addition had ratios close to 1 on both axes with 6 out of the 10 experiments showing mild positive H₂O₂ release (Figure 2; upper right corner).

H₂O₂ Degradation by *Vibrios*

The common pattern in 28 of 48 experiments of an immediate or a longer term drop in H₂O₂ following bacteria addition (i.e., the lower side of the plot with an Immediate after/before < 1;

Figure 2), may stem from bacterially mediated H₂O₂ degradation. We therefore measured the rates of H₂O₂ degradation in water sub-samples removed from the corals through the collection tube in a few of the experiments. We followed the procedure described in detail in Armoza-Zvuloni and Shaked (2014), of adding 1 μM H₂O₂ to the sample of interest and monitoring its loss over time (see Figure SI-3 for details on the methods). We found high H₂O₂ degradation rates in water collected from the coral surface immediately after bacteria addition, which dropped back to background within few minutes (Figure SI-3).

To further verify that the bacteria themselves and not the corals are responsible for H₂O₂ degradation we tested the rates of H₂O₂ degradation by diluted *Vibrio* 4 cell suspension. H₂O₂ degradation rates in the diluted cell suspension were high, ~6-fold higher than those measured in water collected from corals following the bacteria addition (Figure SI-3).

Quantifying the Amount of H₂O₂ Released from Corals in Response to *Vibrios*

The H₂O₂ concentrations presented so far do not accurately reflect the actual H₂O₂ release from corals in response to bacteria. This is due to our experimental setup where H₂O₂-enriched water from the coral surface is continuously removed through the collection tube. Hence, when estimating H₂O₂ release in each time interval, as presented below, we should take into account the fact that the coral repeatedly replenishes this H₂O₂. We calculated the number of H₂O₂ moles released in each time interval by multiplying the water volume collected in each fraction by the concentrations of H₂O₂ above that of ambient seawater (see Methods for calculation details). Here we present the experiments with all *Vibrios* but include only experiments with positive H₂O₂ release (i.e., Averaged

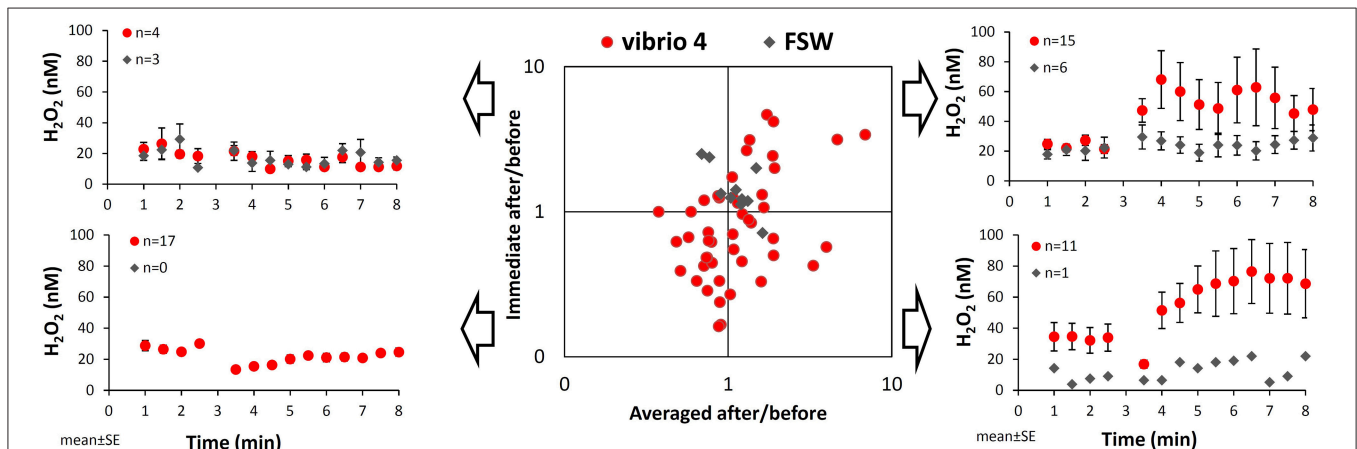


FIGURE 2 | Variations among individual experiments in the pattern of H₂O₂ release from *Stylophora pistillata* following interaction with *Vibrio*. Taking a closer look at the changes in H₂O₂ concentrations over time in individual experiments we noted four distinct patterns, which are illustrated on the side panels. The scatter plot conveniently distinguishes between these patterns according to the ratio of H₂O₂ concentrations after and before the interaction with bacteria. These ratios are calculated separately for mean changes in H₂O₂ concentrations (x-axis) or for immediate changes in H₂O₂ concentrations (y-axis; i.e., at 3.5 min over 2.5 min). Here, values below 1 represent a drop in H₂O₂ concentrations following interaction, while values above 1 represent an increase in H₂O₂ concentrations. The red dots are all 48 experiments conducted with *Vibrio* 4 and the gray diamonds are the 10 seawater controls.

after/before >1) to eliminate the H₂O₂ degradation effect and probe the maximal strength of this phenomenon. This analysis, presented in **Figure 3** for the five *Vibrio* isolates and a control (A–F), reveals an active H₂O₂ release by the corals from the time of interaction and throughout the experiment.

Summing up all these fractions and separately averaging the experiments with each of the *Vibrios*, we obtain an overall release of 200–600 pmol H₂O₂, with the highest release in the presence of *Vibrio* 5 and the lowest in the presence of *Vibrio* 2 (**Figure 3G**). When looking at individual experiments, the range of values increase and the overall H₂O₂ release vary between 100 and 1000 pmol after stimulus (data not shown). The FSW control experiments had lower and more moderate range of H₂O₂ release (**Figure 3**).

Sensitivity of the Pathogenic *Vibrio Coralliilyticus* to H₂O₂

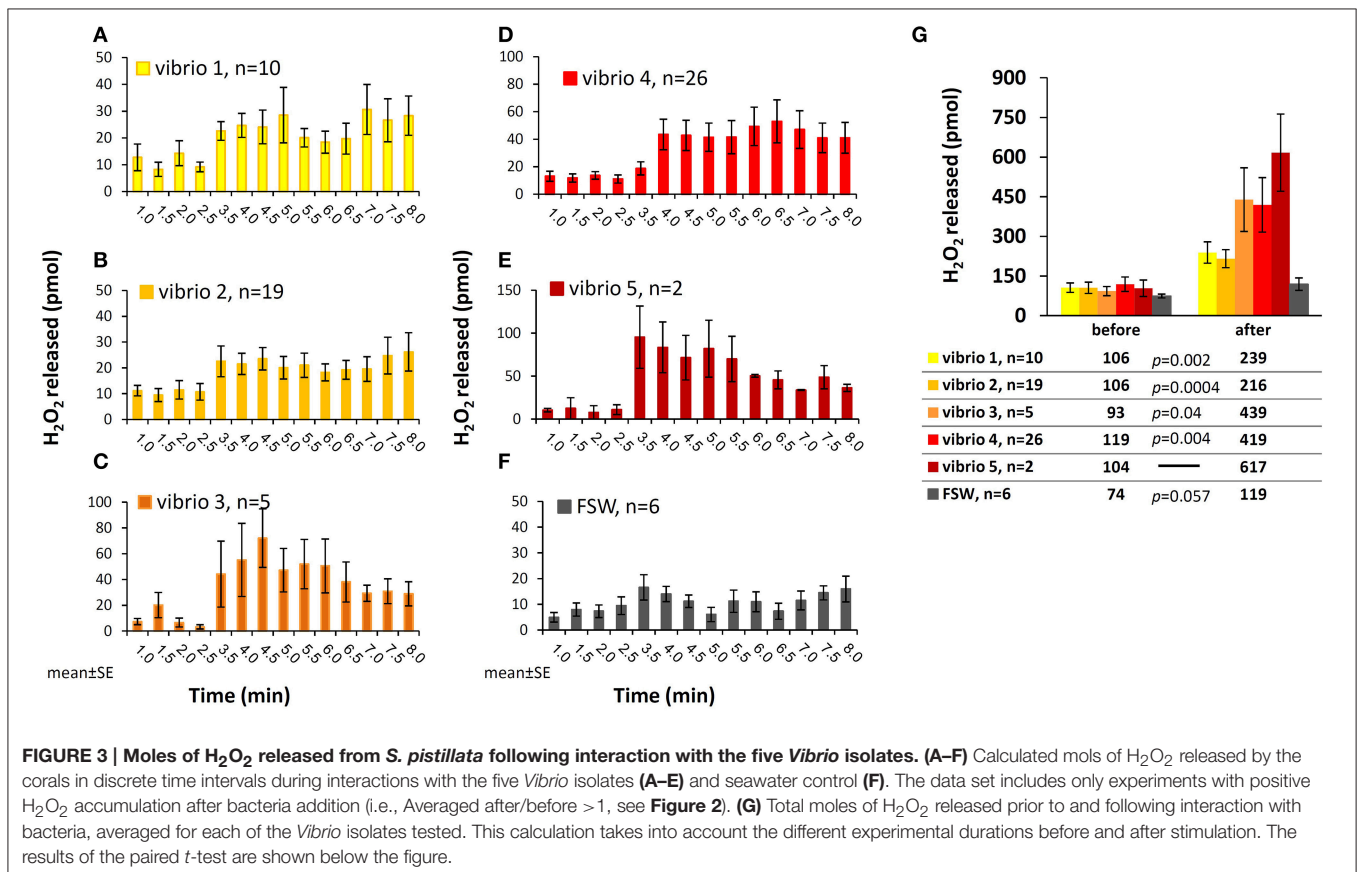
The released H₂O₂ may play multiple roles in the coral recognition and defense against bacteria. Testing for its direct role as a bactericide, we conducted toxicity assays with a strain of pathogenic *Vibrio*–*Vibrio coralliilyticus*. We chose this strain over our *Vibrio* isolates due to its environmental significance and frequent use in toxicity assays (Munn et al., 2008; Geffen et al., 2009). Incubating *V. coralliilyticus* in FSW with different H₂O₂ concentrations we obtained the expected trend of decreasing bacterial survival with increasing H₂O₂ (**Figure 4**). In the first

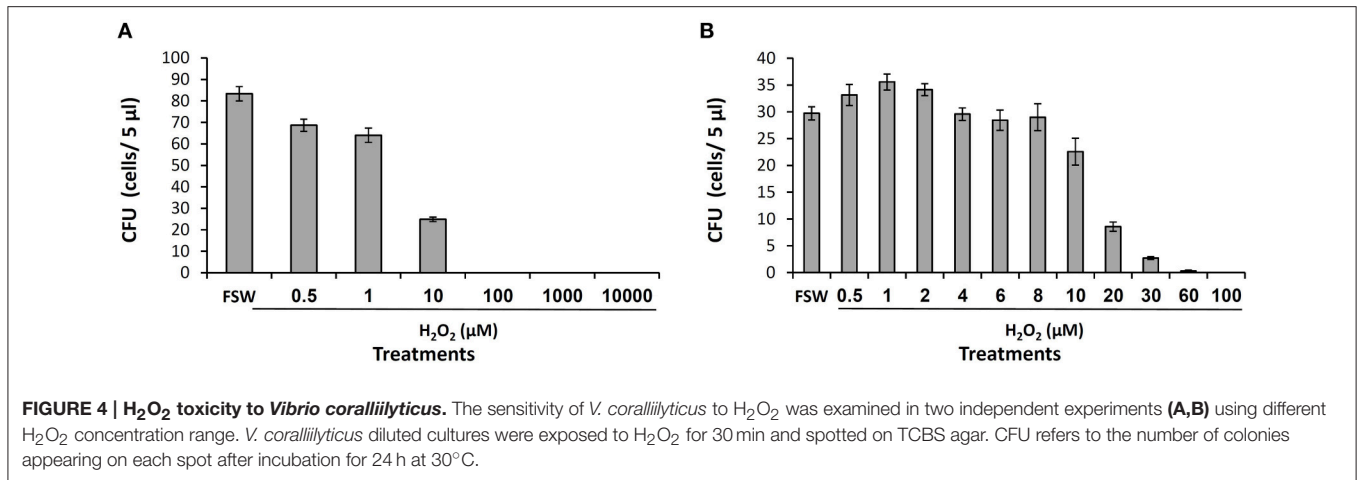
experiment, using a large range of H₂O₂ concentrations, we crudely established that 10 μM of H₂O₂ is highly toxic to *V. coralliilyticus* (**Figure 4A**). Then, using a finer concentration range we examined the toxicity of H₂O₂ at micromolar levels. The bacteria colonies decreased to 75, 30, and 10% of the controls at H₂O₂ concentrations of 10, 20, and 30 μM, respectively and at 60 μM H₂O₂ only 1% of the bacteria survived (**Figure 4B**).

DISCUSSION

Corals Actively Release H₂O₂ during Interaction with *Vibrio* Cells

Our study reports a previously unstudied phenomenon of H₂O₂ release from the stony coral *S. pistillata* in response to interactions with different *Vibrio* isolates (previously obtained from healthy *S. pistillata*). The release occurred locally and rapidly, within 1 min from bacteria addition and persisted for the remaining 5 min of the experiment (**Figures 1–3**). Given our experimental system, which removes the coral released H₂O₂ through a collection tube, the elevated and relatively constant H₂O₂ concentrations observed following bacteria addition indicate that, in fact, an active and continuous release of H₂O₂ by the coral is required to replenish and maintain these concentrations. The rapid and persistent response suggests that the corals recognize the presence of the bacteria and actively release H₂O₂ at the site of interaction. As corals released





H₂O₂ following stimulation with all five *Vibrio* isolates tested (Figure 3), we postulate that the coral response is general to interactions with *Vibrios*.

We can compare our current findings with H₂O₂ release patterns we recently reported for other stimuli using the same coral species and experimental system (Armoza-Zvuloni et al., 2016). When subjected to physical or chemical stimuli the corals rapidly released H₂O₂ within 1–2 min from the time of stimulation at comparable concentrations. However, the H₂O₂ release period was shorter in response to physical and chemical stimuli and H₂O₂ concentrations dropped to background levels within 2–3 min from the stimuli. Considering the ability of pathogenic *Vibrios* to adhere to the coral's surface layer before penetrating the coral tissue (Toren et al., 1998), it is possible that after stimulation, some bacteria remained attached to the coral surface and hence were recognized by the coral, leading to continues H₂O₂ release.

In coral focused literature, there are no previous reports of rapid H₂O₂ production or release in response to contact with bacteria. However, short term elevated production and release of H₂O₂ (and other ROS), referred to as an oxidative burst response, is well-documented in plants, marine micro- and macro algae and few marine invertebrates, when challenged with pathogens or various chemical elicitors (e.g., Levine et al., 1994; Coteur et al., 2002; Weinberger, 2007). The oxidative burst often involves the enzyme NADPH oxidase that generates superoxide in the cell or on its membrane (Schmitt et al., 2014). Since this superoxide is rapidly converted to the more stable H₂O₂, activity of a cell surface NADPH oxidase is a likely precursor for an external H₂O₂ flux. Our group has reported on continuous superoxide release from *S. pistillata* to its external milieu, which is inhibited by the NADPH oxidase inhibitor DPI (Diphenyleneiodonium chloride; Saragosti et al., 2010). Such a pathway, if activated upon contact with bacteria, may enable corals to generate an oxidative burst response. Indeed, an up-regulation of transcripts linked to ROS production, including three subunits of the enzymatic complex NADPH oxidase, were reported in the coral *Acropora cervicornis* infected with the pathogen related White Band Disease (Libro et al., 2013). Further research is required to

identify the enzymes and ROS that play the major role in this pathway, promoting oxidative burst response in corals during interactions with bacteria.

Influence of Bacterial Antioxidants on Observed H₂O₂ Release

In many of the experiments, a sharp decline in H₂O₂ concentrations occurred immediately following the addition of bacteria and, in many cases, a net drop in H₂O₂ was observed throughout the whole experiment (Figure 2). Measuring the rates of H₂O₂ degradation in the experiment water and in *Vibrio* cell suspension (Figure SI-3), we assigned this effect to the bacteria themselves, holding a variety of antioxidant enzymes and molecules. This effect is lost in many of the experiments, where H₂O₂ does accumulate, since most of the bacteria are washed off of the coral surface and sucked into our collection tube. Similarly, Munn et al. (2008) found high catalase and superoxide dismutase activity in pathogenic and non-pathogenic *Vibrio* cultures. Considering the antioxidant activity of *Vibrios* and the drop in H₂O₂ levels following interaction, it is reasonable to assume that the actual H₂O₂ release by the coral is higher than measured here. On the other hand, the significant capability of bacteria to degrade H₂O₂ may imply that *Vibrios* in nature can offset the coral released H₂O₂. The actual ability of natural bacteria to counteract the coral released H₂O₂ will depend on their densities, antioxidant levels, H₂O₂ fluxes, and concentrations of trace metals that react with H₂O₂ to form the highly toxic hydroxyl and ferryl radicals (Linley et al., 2012).

Possible Role of the Released H₂O₂ in Coral Protection from Bacteria

Corals secrete various chemicals for preventing bacterial attachment, colonization and invasion (Ritchie, 2006). H₂O₂ and other ROS, which are co-released with it or form as it reacts with trace metals, may act as antimicrobial agents when released at high enough concentrations (Linley et al., 2012). In a rather crude calculation, we can estimate H₂O₂ concentrations in the diffusive boundary layer (DBL) at the

coral–water interface prior to dilution in the medium. For this calculation we take the overall moles of H₂O₂ released following stimulus as presented in **Figure 3G** (340 ± 47 pmol, mean \pm SE $n = 63$). We then divide the averaged moles of H₂O₂ by the volume into which H₂O₂ is released ($20 \text{ mm}^3 = 10 \text{ mm}^2$, site of stimulus \times 2 mm DBL). This results in an average H₂O₂ concentration of $17 \pm 2.4 \mu\text{M}$ at the site of stimulus. Considering all 103 experiments reported here, H₂O₂ concentrations of 10–20 μM are calculated for 15% of the experiments, and H₂O₂ concentrations higher than 20 μM are calculated for additional 14% of the experiments.

Are these concentrations high enough to be toxic for bacteria? In our assays with *V. coralliilyticus*, 10 and 20 μM H₂O₂ were found to induce 30 and 70% mortality, respectively (**Figure 4B**). Based on these assays, the estimated average value of 17 μM H₂O₂ released to the DBL is high enough to induce some bacterial mortality. Furthermore, H₂O₂ concentrations exceeding 17 μM , which were estimated in several individual experiments, are high enough to induce significant mortality. It is important to note however, that the bacterial sensitivity to H₂O₂ obtained in such assays vary substantially, depending on the bacterial density and growth phase, experimental medium, incubation length etc. For example, Munn et al. (2008) reported a higher resistance to H₂O₂ of various coral associated bacteria including *V. coralliilyticus*, with toxic effects seen only at mM H₂O₂ concentrations. Another complication of the H₂O₂ toxicity assays is that they fail to probe for the toxicity of other ROS (and possibly other antibacterial compounds) that may be released together with H₂O₂ to the coral surface. These ROS in combination with trace metals in the medium or in the bacterial cells can become lethal even at moderate concentrations (Sharma et al., 2012). Hence, while our data hints at the potential role of the released H₂O₂ as a bactericide, more research is required to determine if H₂O₂ indeed acts as a bactericide *in situ*.

In plants, the rapid ROS release in response to pathogens, often result in programmed cell death of the cells surrounding the pathogen, preventing it from penetrating into the plant tissue (Levine et al., 1994). In analogy to plants, we speculate that the rapid and local H₂O₂ release we measured in response to bacteria may result in a localized programmed cell death (apoptosis) of a single polyp. Such a mechanism may protect the whole colony from proliferation of the pathogen. Since each of the polyps in the coral colony is performing all life functions, a local damage to a single polyp may not reduce

the coral fitness. Corals were reported to actively cut off nutritional supply to injured parts of the colony if the coral was suffering from bleaching (Fine et al., 2002). Moreover, since corals can stop apoptotic pathways (Kvitt et al., 2011) apoptosis can be contained within a small part of the colony. Nowadays, when infectious coral diseases are becoming more prevalent in concert with the rise in sea surface temperature; it is worth examining whether colonial corals can apply ROS induced programmed cell death in order to protect themselves from pathogens.

CONCLUSIONS

Our novel findings of local, rapid release of H₂O₂ by *S. pistillata* upon contact with *Vibrio*, implies that the corals chemically sense the bacteria and respond to their presence. The released H₂O₂ can build up to 10–20 μM in the coral diffusive boundary layer; high enough to induce mortality of the coral pathogen *V. coralliilyticus*. The H₂O₂ release observed here, together with our recent reports of H₂O₂ release by corals under flow (Armoza-Zvuloni and Shaked, 2014), during feeding, and in response to different chemical and physical stimuli (Armoza-Zvuloni et al., 2016), demonstrate the broad use of H₂O₂ during interactions of corals with their surroundings. The actual roles of H₂O₂ in these interactions remain to be elucidated.

AUTHOR CONTRIBUTIONS

YS, RA, and AS designed the research; RA and AS performed the experiments; RA analyzed the data. YS and RA wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmars.2016.00124>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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