Measurement of dark, particle-generated superoxide and hydrogen peroxide production and decay in the subtropical and temperate North Pacific Ocean

Kelly L. Roe^a, Robin J. Schneider^a, Colleen M. Hansel^b, and Bettina M. Voelker^a

^aDepartment of Chemistry and Geochemistry, Colorado School of Mines, Golden, CO 80401, USA

^bDepartment of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA

Corresponding author: Bettina M. Voelker

Abstract

Reactive oxygen species (ROS), which include the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), are thought to be generated mostly through photochemical reactions and biological activity in seawater and can influence trace metal speciation in the ocean. This study reports the results of an intercomparison of two methods to measure particle-generated $[O_2^-]$ in seawater samples, as well as measurements of particle-generated O_2^- and H_2O_2 concentrations, decay kinetics, and dark production rates in seawater samples at Station ALOHA and (O_2^-) only) in the southern California Current Ecosystem. O_2^- was measured using two different methods relying on chemiluminescence detection. The first method measured the difference between steady-state $[O_2^-]$ in filtered and unfiltered seawater, while the second method (standard method) measured O_2^- decay to baseline in freshly filtered seawater. Because both methods detected $[O_2^-]$ relative to the background signal from filtered seawater, both should have measured $[O_2^-]$ generated by particles (presumably biota). However, the O_2^- concentrations determined by the first method were always much smaller than those obtained from the second (standard) method. Follow-up laboratory and field experiments showed that the increased signal in the standard method was due to a filtration artifact that could neither be eliminated nor consistently accounted for under the tested conditions. We therefore recommend the first method for measuring particle-generated $[O_2^-]$. Measured by this method, Station ALOHA had particle-generated O_2^- concentrations that ranged from undetectable to 0.02 nM, with production rates less than 0.6 nM hr^{-1} and decay rate coefficients from 0.003 to 0.014 s^{-1} . The southern California Current Ecosystem had particle-generated O_2^- concentrations that ranged from undetectable to 0.05 nM, with production rates up to 4.7 nM hr^{-1} and decay rate coefficients from 0.006 to 0.017 s^{-1} . H_2O_2 concentrations were measured by chemiluminescence detection, using dark incubations of unfiltered water samples to simultaneously determine production and decay rates. H_2O_2 concentrations at Station ALOHA ranged from 7 to 88 nM. Dark production rates and decay rate coefficients were low (mostly <1.5 nM hr^{-1} and <0.03 hr^{-1} , respectively); higher values were detected when biota were pre-concentrated with net tows. These rates of ROS production are lower than those reported by previous studies in other regions of the Pacific Ocean, but could still be significant compared to photochemical production.

Key words: superoxide, hydrogen peroxide, reactive oxygen species, chemiluminescence

1. Introduction

The reactive oxygen species (ROS) superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are of interest in marine waters because their reactions with trace metals can alter metal speciation and therefore impact metal bioavailability. In addition, H_2O_2 is toxic to the cyanobacterium *Prochlorococcus* at environmentally relevant concentrations (Morris and Zinser, 2011), raising the possibility of an ecological role of H_2O_2 in some parts of the ocean.

Until recently, the detection and measurement of ROS in the ocean has primarily focused on H_2O_2 , which, with typical half-lives on the order of hours to days, is much more stable than O_2^- , whose half-life in seawater ranges from seconds to minutes (Hansard *et al.*, 2010; Heller and Croot, 2010c; Rose *et al.*, 2010; Rose *et al.*, 2008b). Removal of O_2^- from the ocean occurs via multiple pathways. One removal pathway for O_2^- is by a disproportionation reaction to form H_2O_2 and O_2 (Cooper and Zika, 1983; Moffett and Zafiriou, 1990; Petasne and Zika, 1987). Redox reactions with trace metals and a redox cycling component of natural organic matter also occur (Goldstone and Voelker, 2000; Voelker and Sedlak, 1995; Voelker *et al.*, 2000; Zafiriou *et al.*, 1998), which may catalyze O_2^- disproportionation while maintaining a fraction of the metal or organic compound in an unstable oxidation state.

In the ocean, O_2^- and H_2O_2 can be produced by photochemical reactions and by biological processes. Photochemically produced O_2^- and H_2O_2 are attributed to the photo-oxidation of chromophoric dissolved organic matter (CDOM) (Micinski *et al.*, 1993). The photochemical production rate of O_2^- and H_2O_2 is influenced by the concentrations (Fan, 2008) and characteristics (marine vs. terrestrial) of the CDOM (Micinski *et al.*, 1993). Numerous marine phytoplankton species as well as a variety of heterotrophic bacteria have been observed to produce extracellular O_2^- and H_2O_2 under laboratory conditions, even in the absence of light

(e.g. Diaz *et al.*, 2013; Kustka *et al.*, 2005; Marshall *et al.*, 2005a; Rose *et al.*, 2008b). The ROS production rates observed in some culture studies (e.g. Diaz *et al.*, 2013; Rose *et al.*, 2008b) appear to be sufficiently fast to contribute significantly to dark O_2^- and H_2O_2 production rates in some areas of the ocean.

With the recent development of a sensitive chemiluminescence flow-injection system to measure O_2^- in the ocean (Rose *et al.*, 2008a), several studies have attempted to quantify the magnitude of biological O_2^- production by measuring $[O_2^-]$ versus time in freshly filtered seawater samples (Gulf of Alaska: Hansard *et al.*, 2010; Costa Rica Dome upwelling region: Rose *et al.*, 2008b; and the Great Barrier Reef lagoon: Rose *et al.*, 2010). Because sampling occurred at night, or the sample was kept in the dark sufficiently long to eliminate any photochemical contribution to $[O_2^-]$, the $[O_2^-]$ extrapolated backwards to time of filtering was interpreted as the steady-state $[O_2^-]$ signal resulting from the balance of decay and dark, particle-generated (presumably biological) production, allowing calculation of a dark production rate. These studies concluded that significant dark production of O_2^- occurs in the regions that were studied.

Dark H_2O_2 production rates were measured concurrently with O_2^- production rates in only one of these studies, and both rates were of similar magnitude (Hansard *et al.*, 2010; Vermilyea *et al.*, 2010). A 2:1 ratio between O_2^- and H_2O_2 production rates is the expected result if O_2^- forms H_2O_2 by disproportionation and if direct biological formation of H_2O_2 is not significant. However, O_2^- does not necessarily form H_2O_2 in the expected ratio. Rose *et al.* (2010), in their study at the Great Barrier Reef, observed a significant increase in H_2O_2 production rates when superoxide dismutase was added to ensure disproportionation, indicating that in the absence of the added enzyme, the majority of the O_2^- did not form H_2O_2 . In such cases, production rates of

O_2^- may be greater than those of H_2O_2 . However, the opposite may also occur, since direct biological production of H_2O_2 , without O_2^- as a precursor, is also possible (Kim *et al.*, 2007; Palenik *et al.*, 1987). Thus, concurrent measurements of O_2^- and H_2O_2 production rates can provide information on the source of H_2O_2 and the reactions of O_2^- in a water sample.

The main goal of this study was to measure particle-generated O_2^- concentrations and decay rates at Station ALOHA and in the southern California Current Ecosystem, in order to quantify the dark (presumably biological) production rate of O_2^- in these regions. Station ALOHA was the first subtropical, *Prochlorococcus*-dominated region to be examined using these techniques, and the southern California Current ecosystem provided a comparison to a less oligotrophic region. O_2^- measurements were made using two different chemiluminescence flow injection methods, a new method comparing steady-state $[O_2^-]$ in unfiltered and filtered seawater, and the standard method of observation of O_2^- in freshly filtered seawater and extrapolation to time of filtering (Hansard *et al.*, 2010; Heller and Croot, 2010c; Rose *et al.*, 2010; Rose *et al.*, 2008b). The results and potential difficulties of measuring O_2^- by these two different methods are discussed. A secondary goal of the study was to measure H_2O_2 concentrations, production and decay rates, and to compare H_2O_2 dark production rates to those measured for O_2^- .

2. Materials and Methods

2.1 Seawater collection

All seawater was collected at Station ALOHA from July 10-25, 2012 or in the southern California Current Ecosystem from April 9-11, 2013. Three stations were occupied in the California Current; Station 1 (32°57'4.98"N, 117°40'26.28"W), Station 2 (34°19'40.68"N, 120°49'0.60"W) and Station 3 (33°43'35.64"N, 119°14'28.68"W). Seawater was collected with

5L GO-Flo bottles (General Oceanics) attached to a kevlar line. The Station ALOHA casts were conducted ~1 hr before the 0800 CTD cast. The California Current casts took place immediately after the CTD casts, which were 1200-1500 (cast 1 and 2) or 0800 (cast 3). Once the GO-Flo bottles were retrieved, analysis for O_2^- in the samples began 30 min (deep Station ALOHA samples) to 8 hr after collection. Samples were in the dark for at least 10 half lives prior to analysis and were kept in the dark during analysis to ensure that photochemically produced O_2^- did not contribute to the measured values. Depth profiles at Station ALOHA were analyzed from deep to surface samples except on July 24 and 25, when samples were analyzed from shallow to deep. The depth profiles usually consisted of samples from 25m, 75m and the deep-chlorophyll-maximum (105-130m). On alternate days, only a 25 m water sample was collected for analysis. The California Current samples were kept in the dark in a room temperature (~17°C) water bath for a minimum of 30 min prior to analysis to minimize any effects of temperature changes during analysis (Hansard *et al.*, 2010). The California Current profiles were analyzed from surface to deep and consisted of surface (20-25 m) and deeper samples (40- 50 m). Seawater was filtered by slightly pressurizing (< 7 PSI) the GO-Flo bottle or a 500 mL dark Teflon bottle (California Current) with filtered (0.2 μ m PTFE filters, Whatman) compressed air into a 125 mL dark HDPE bottle. The cartridge filter (0.2 μ m Supor cartridge, PALL) was acid washed with 0.1 M trace metal grade HCl, rinsed with Milli-Q and stored in Milli-Q until use. Prior to collection of filtered seawater for analysis, 1 L of seawater was passed through the cartridge filter. At Station ALOHA, the same filter was used for all depths for ~10 days before using a new filter. For the California Current samples, a new cartridge filter was used for each sample.

2.2 Measurement of O_2^- in natural water

O_2^- was detected with a flow injection system (FeLume Mini, Waterville Analytical) with the use of the MCLA (2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one) chemiluminescence probe. The flow injection system was set up to directly pump the seawater sample and MCLA reagent into a homemade flow cell, made of tygon tubing, which sat directly under a PMT (Hamamatsu). The PMT was set to integrate the signal over 20 ms, a collection time of 10 periods, with the default setting for PMT voltage, and two data points were collected every second, except for Cast 3 in the California Current where the integration time was increased to 50 ms. Two different PMT's were used on the cruises, model H9319-11 at Station ALOHA and model HC135-11 in the California Current. The seawater sample and MCLA reagent were each pumped at a flow rate of 3 ml min⁻¹. At this rate it took ~20 s to pump fresh reagent and sample to the flow cell. The MCLA reagent was composed of 30 μ M diethylenetriaminepentaacetic acid (DTPA) (Sigma), 5 μ M MCLA (TCI America), and 0.2 M MES hydrate buffer (Sigma), adjusted to pH 6.0 with ~0.074 M NaOH (Sigma). All measurements were made in a laminar flow clean hood. After each day of sampling, the tubing and flow cell was rinsed with 0.01 M HCl (trace-metal grade) followed by Milli-Q to clean the system.

Method 1. This method examines the difference in steady-state O_2^- in aged filtered and unfiltered water. Either aged 0.2 μ m filtered seawater (aged ≥ 1 hr) or unfiltered seawater was run through the system until a steady-state signal was observed, ~ 5 min. Then a one-point calibration was completed in the sample, described in detail in section 2.3. After the one-point calibration, the sample was spiked with ~0.09 Units mL⁻¹ of SOD to establish a new steady-state signal. Given the effectiveness of SOD measured by Hansard *et al.*, (2010), this was sufficient SOD to decrease the steady-state O_2^- concentration to < 4% of the original value in all of our

154 samples. We therefore assumed that the SOD-amended signal is due only to MCLA auto-
155 oxidation, and calculated the background-corrected O_2^- signal in the sample from:

$$156 \quad R_{\Delta} = R_{SS1} - R_{SS2} \quad (1)$$

157 where R_{Δ} is the calculated change in response (units of counts) from the initial steady-state signal
158 (R_{SS1}) and the steady-state signal in the presence of SOD (R_{SS2}) (Figure S1 A). An average of at
159 least ~600 data points or ~ 5 min measurements was used to calculate R_{SS1} and ~100 data points
160 or ~ 2 min R_{SS2} . There were occasional sharp peaks (duration <8 signal counts) in the unfiltered
161 seawater output signal, likely the result of a bioluminescent organism passing through the flow
162 cell, but these were removed from the data set prior to calculating the averages. R_{Δ} was
163 measured as described above for both unfiltered and aged filtered seawater, and both values were
164 then converted to O_2^- concentrations using one-point calibration as described in Section 2.3.

165 The reported concentration of particle-generated O_2^- measured by Method 1, $[O_2^-]_1$, was
166 then calculated as the difference between the concentrations of O_2^- in aged filtered seawater
167 ($[O_2^-]_{AFSW}$) and in unfiltered seawater ($[O_2^-]_{USW}$):

$$168 \quad [O_2^-]_1 = [O_2^-]_{USW} - [O_2^-]_{AFSW} \quad (2)$$

169 For Station ALOHA, no replicates were measured. In the California Current, two replicates
170 were measured for aged filtered seawater and unfiltered seawater at each depth and the average
171 concentrations from each were used to calculate $[O_2^-]_1$.

172 **Method 2.** This method follows the decaying O_2^- signal as a function of time in freshly
173 filtered seawater. The time required to transport the freshly filtered seawater from the Go-Flo
174 bottle to the clean hood was 5 – 10 s, and the time of filtration and transportation was recorded
175 for every sample. The signal from the freshly filtered seawater was observed for 5 to 12 min until
176 a steady non-decaying baseline was observed. Then a one-point calibration was completed in

each sample, described in detail in section 2.3. Two replicates were measured from every depth at each location.

The O_2^- signal and decay rate in freshly filtered seawater were determined in each sample by observing the change in PMT signal over time (Figure S1 B). The output signal was then fitted to:

$$R_t = R_{BL} + R_{t=0}^{(-k_{loss,O_2^-}t)} \quad (3)$$

where R_t is the measured response (counts) at time t (s), R_{BL} is the baseline response, $R_{t=0}$ is the baseline-corrected response at $t=0$ (time of filtration), and k_{loss,O_2^-} (s^{-1}) is the pseudo-first-order decay rate coefficient. Microsoft Excel's Solver function was used to minimize the sum of the squares of the differences between the actual data and the model to determine best fit values of R_{BL} , $R_{t=0}$, and k_{loss,O_2^-} . $R_{t=0}$ was then converted to the O_2^- concentration at time of filtration as described in section 2.3. Although O_2^- decay kinetics deviating from pseudo-first order behavior have sometimes been observed by others (Rose et al., 2010; Heller and Croot, 2010b; Diaz et al., 2013), especially in the presence of large (>1 nM) additions of O_2^- , all of our decay curves, both those generated by O_2^- initially present in freshly filtered seawater and those generated by added KO_2 spikes during calibration (see Section 2.3), were well described by Equation (3).

2.3 O_2^- Data calibration

To determine the sensitivity of the method, a one-point calibration with a potassium superoxide (KO_2) source was completed in each sample after the initial O_2^- measurements. A one-point calibration was used on every sample since it is known that a O_2^- calibration curve is linear up to 1 nM but that differences in seawater composition may result in changes in sensitivity (Hansard et al., 2010; Rose et al., 2008a).

A primary O_2^- stock solution was made fresh for each sample by adding a small amount of KO_2 powder to a solution of 30 μM DTPA and 0.032 M NaOH (nominal pH 12.5) (Heller and Croot, 2010a). The O_2^- concentration was calculated by measuring the absorbance of the solution before and after addition of 2.4 Units mL^{-1} of Cu/Zn superoxide dismutase (SOD) (Sigma) at 240 nm and dividing the difference in absorbance by $2183 \text{ M}^{-1} \text{ cm}^{-1}$, superoxide's molar absorptivity corrected for absorption of the H_2O_2 formed during decay at pH 12.5 (Table 1b in Bielski *et al.*, 1985). The instrument used for absorbance measurements was a Black-comet UV/VIS spectrometer with a SL5 Deuterium/Halogen light source (Stellar Net Inc) blanked on Milli-Q water. Addition of the small amount of SOD we used did not have a measurable effect on the absorbance of the solution at 240 nm.

A known volume of the primary O_2^- stock solution was withdrawn immediately after the absorbance measurement and transferred into a solution of 10 μM DTPA and 10^{-3} M NaOH to make the working stock. A small volume of the working stock was then spiked into the seawater sample, and the chemiluminescence signal was monitored over time. Prior to adding the O_2^- spike to the seawater sample, the chemiluminescence signal was monitored as shown in Figure S1. A maximum of 5 min passed between the absorbance measurement of the primary O_2^- stock and the addition of the working stock spike to the sample. To ensure that the O_2^- spike did not significantly change the pH of the system, the spike was kept at a maximum ratio of 100 μl per ~100 ml seawater (Hansard *et al.*, 2010). The ranges of concentrations used at Station Aloha and in the California Current, respectively, were 0.023 – 0.392 nM O_2^- (average 0.145 ± 0.076 nM) and 0.314 – 1.334 nM O_2^- (average 0.719 ± 0.256 nM).

The signal from the O_2^- spike was fitted to Equation (3) to determine the initial signal response, of the spike, $R_{t=0,spike}$. The sensitivity (S , units of counts/nM) of the response was then determined as:

$$S = \frac{R_{t=0,spike}}{[O_2^-]_{spike}} \quad (4)$$

where $[O_2^-]_{spike}$ was calculated from the measured $[O_2^-]$ in the primary stock and the appropriate dilution factors.

The measured sensitivity (S) values at Station ALOHA waters ranged from 414 to 5442 counts nM^{-1} with an average $\pm$ standard deviation of 2025 ± 848 counts nM^{-1} . The California Current waters had sensitivities that ranged from 107 to 960 counts nM^{-1} with an average value of 470 ± 247 counts nM^{-1} . The sensitivity measured at Station ALOHA always increased throughout the day with sensitivity increasing to $\sim$ twice the starting value by the end of the day. This was not a function of the water type or depth of the water since measurements at Station Aloha from June 24 and 25 showed the same trend as all the previous analysis days with the samples run in reverse order (i.e. shallow to deep). This observation was not seen in the waters from the California Current.

Because replicate analyses were performed in the California Current samples, we used the average sensitivity from the two replicates of each station, depth, and water type (aged filtered seawater, unfiltered seawater, or freshly filtered seawater) to calculate concentrations as described below. At Station ALOHA, the average sensitivity measured from all three seawater types for each station and depth was pooled and used.

The O_2^- concentration (reported in units of nM) in each seawater sample was then calculated as follows. In Method 1, O_2^- concentrations in unfiltered and aged filtered seawater ($[O_2^-]_{USW}$ and $[O_2^-]_{AFSW}$, respectively) were obtained using:

$$[O_2^-]_{USW} = \frac{R_{\Delta,USW}}{S} \quad (5a)$$

$$[O_2^-]_{AFSW} = \frac{R_{\Delta,AFSW}}{S} \quad (5b)$$

The reported particle-generated O_2^- concentration by Method 1, $[O_2^-]_1$, was then obtained as the difference between the $[O_2^-]$ measured in unfiltered and aged filtered seawater, as explained above (Equation 2). Since SOD eliminates some of the signal generated by MCLA autooxidation, R_{Δ} represents an overestimate of the signal coming from O_2^- in the sample. As long as artifacts of this sort contribute equally to $R_{\Delta,USW}$ and $R_{\Delta,AFSW}$, $[O_2^-]_1$ will accurately represent the particle-generated contribution to the steady-state concentration of O_2^- in the water sample. However, $[O_2^-]_{AFSW}$ should not be interpreted a measurement of the O_2^- in the water due to dissolved sources. This is discussed in more detail in the Discussion, Section 4.1. Because we observed a small amount of drift over time in the SOD baselines and in the measured sensitivity values, calculating $[O_2^-]_{USW}$ and $[O_2^-]_{AFSW}$ separately and using these to calculate $[O_2^-]_1$ was preferable to calculating $[O_2^-]_1$ from the difference between $R_{\Delta,USW}$ and $R_{\Delta,AFSW}$.

The reported O_2^- concentrations by Method 2, $[O_2^-]_2$, were calculated as the concentrations present in freshly filtered seawater immediately after filtration:

$$[O_2^-]_2 = \frac{R_{t=0}}{S} \quad (5c)$$

Since $R_{t=0}$ represents the signal offset from the baseline representing the steady-state signal in the filtered solution, $[O_2^-]_2$, like $[O_2^-]_1$, represents the O_2^- concentrations generated by particles.

The detection limit of the system in nM was calculated as 5 times the standard deviation of the baseline signal divided by the sensitivity (S).

We calculated the production rate of O_2^- in each seawater sample assuming that the measured O_2^- concentration is at steady state, so that:

$$P_{O_2-} \text{ (nM hr}^{-1}\text{)} = [O_2^-] \times k_{loss,O_2-} \times 3600 \text{ s/hr} \quad (6)$$

where the factor 3600 converts s^{-1} , the units of k_{loss,O_2-} , to hr^{-1} . Method 1 particle-generated production rates were calculated as the difference in aged filtered seawater and unfiltered seawater production rates.

2.4 Measurement of H_2O_2 concentrations, decay and production rates

H_2O_2 concentrations were determined using the acridinium ester (AE) method described in detail in King *et al.* (2007) and Cooper *et al.* (2000) with a FeLume flow injection system (Waterville Analytical). Briefly, a slug of sample was pushed into the system by a stream of catalase-treated filtered seawater (10 units mL^{-1}), which then combined with $1 \mu\text{M}$ AE solution (acidified to pH 3 with trace-metal grade HCl) in a mixing tee. The AE/sample solution then entered a flow cell, where it combined with a 0.02 M carbonate buffer (pH 11.2). The flow rate was 2.0 mL min^{-1} for both the sample stream and each of the two reagent streams. Typical standard deviations of replicate H_2O_2 measurements were about 1 nM at H_2O_2 concentrations exceeding 30 nM .

The rate of H_2O_2 production ($P_{H_2O_2}$) and the pseudo-first order decay rate coefficient (k_{loss,H_2O_2}) were analyzed using the method described by Vermilyea *et al.* (2010), which we will refer to here as spiked batch incubations. Each spiked batch incubation measurement consisted of incubation of one bottle filled with an unamended seawater sample and 1-2 others filled with the same sample and spiked with varying amounts of added H_2O_2 (up to $+150 \text{ nM}$). Samples were incubated in acid-washed amber 250 mL HDPE bottles. Total H_2O_2 levels for each bottle were then analyzed over a period of 9-18 hrs. Incubation times were purposely kept below 18 hours because results from a previous cruise (HOT-238, December 18-22, 2011) often showed a

dramatic increase in H₂O₂ decay rates at the 19-20 hr mark, indicating a change in the microbial community. On four days, spiked batch incubations of filtered seawater samples were run alongside spiked batch incubations of unfiltered samples.

2.4.1 Calculation of H₂O₂ production and decay rates

Production and decay were modeled assuming that H₂O₂ dynamics in the different bottles of each set of spiked batch incubation samples are described by the same, constant, production rate P_{H₂O₂} (nM hr⁻¹) and first-order decay coefficient k_{loss,H₂O₂} (hr⁻¹):

$$\frac{d[H_2O_2]}{dt} = P_{H_2O_2} - k_{loss,H_2O_2} [H_2O_2] \quad (7)$$

The solution for this differential equation is:

$$[H_2O_2] = \frac{P_{H_2O_2}}{k_{loss,H_2O_2}} - \left\{ \left(\frac{P_{H_2O_2}}{k_{loss,H_2O_2}} - [H_2O_2]_0 \right) e^{-k_{loss,H_2O_2} t} \right\} \quad (8)$$

Time and concentration data from each spiked batch incubation were fitted to this equation using the Microsoft Excel solver function. Fitting parameters were P/ k_{loss,H₂O₂}, k_{loss,H₂O₂}, and an [H₂O₂]₀ value for each bottle. Note that the parameter P/k_{loss,H₂O₂} represents the steady-state concentration of H₂O₂ (from Equation (8), [H₂O₂] at t=∞).

2.4.2 Calculation of 24-hour average photochemical production rate of H₂O₂ in the mixed layer at Station ALOHA

The rate of photochemical production of H₂O₂ was estimated by assuming that all incident photons are absorbed throughout the top 70 m, which roughly corresponds to the depth of the mixed layer we observed while at Station ALOHA. The depth-averaged production rate, P_{photo}

310 (units of mmol m⁻³) was then calculated using the equation:

311
$$P_{photo} = \frac{\sum E_{o,ave}(\lambda)\Phi(\lambda)}{z} \quad (9)$$

312 where λ is wavelength in nm, $\Phi(\lambda)$ is the wavelength-dependent apparent quantum yield in
313 mmol einstein⁻¹, z is the total depth of 70 m, and $E_{o,ave}(\lambda)$ is the 24-hour average of
314 downwelling (sum of diffuse and direct) spectral irradiance at the sea surface in einstein m⁻² in
315 a 1-nm wavelength range. $\Phi(\lambda)$ values were interpolated from a linear regression of $\ln \Phi$ vs. λ
316 measured by Kieber et al., 2014 at 25 °C; mixed-layer temperatures were 23 °C to 25 °C so
317 temperature corrections were neglected. The linear regression was performed using data for
318 samples from Station ALOHA (using Table A2.2 in Miller, 2000) and yielded the function:

319
$$\Phi(\lambda) = 1.9 \exp(-0.0263 \lambda) \quad (10)$$

320 Hourly values of $E_o(\lambda)$ for July 19 at 22°45' N latitude between 280 and 419 nm were obtained
321 from the National Center for Atmospheric Research Quick total ultraviolet (TUV) calculator
322 using the default settings for overhead ozone column (300 Dobson units) and surface albedo
323 (0.1). The twenty-four hourly values were then averaged to obtain $E_{o,ave}(\lambda)$ values. These
324 $E_{o,ave}(\lambda)$ values represent the clear-sky irradiance in the air immediately above the water; the
325 effect of reflection on the penetration of this light into the water column was neglected in this
326 calculation, but is less than 10% (Zepp and Cline, 1977). The contribution of wavelengths
327 greater than 419 nm in Equation (9) was also neglected since the TUV calculator did not provide
328 these $E_o(\lambda)$ values. This contribution could be as high as 20% of the reported photochemical
329 production rate, based on a visual extrapolation of our action spectrum. However, the
330 wavelength dependence of $\Phi(\lambda)$ was not measured above 400 nm, and there is no a priori reason
331 to assume that it continues to be log linear in this wavelength range; a steeper decay of $\Phi(\lambda)$ with

increasing wavelength than that predicted by Equation (10) would make the contribution of these wavelengths much smaller than 20% (Andrews et al., 2000).

2.5 Filtration and filter tests for O_2^- measurement artifacts

2.5.1 Laboratory tests

Due to the discrepancies in O_2^- concentration measured by our two methods, a series of filters were tested in the lab to examine their potential for producing O_2^- in aged filtered seawater (stored in the refrigerator for >2 months) amended with 10 μ M DTPA. Two cartridge filters (0.2 μ m Supor and 0.8/0.45 μ m Supor cartridge (PALL)) and two disk filters (0.4 μ m polycarbonate 47 mm in a polycarbonate filter holder and a 0.22 μ m polyethersulfone syringe filter both from Millipore) were used in the filter tests. All filters were cleaned with 0.1 M trace metal grade HCl and rinsed with Milli-Q prior to use. Seawater was filtered through the cartridge filters and the 0.4 μ m polycarbonate filter by slightly pressurizing a 1 L HDPE dark bottle with filtered (0.2 μ m PTFE filters, Whatman) compressed air (provided by the house line) into a 125 ml dark HDPE bottle. This technique did not create enough pressure to use the polyethersulfone filter, so the 0.22 μ m polyethersulfone filtered seawater was rapidly (1 mL s⁻¹) syringe filtered into a 125 mL dark HDPE bottle. Before a seawater sample was collected with each filter, 1 L of seawater was passed through the cartridge filters and ~130 mL were passed through the disk filters. Additional controls were conducted with membrane-free 47 mm filter holders and syringes with no syringe filters attached.

To examine if contact with air had an effect on O_2^- concentrations, we also examined the effect of flow rate and of isolating the filtrate stream from air. To examine the effect of flow rate seawater was pushed out of a syringe at ~ 1, 2 or 3 mL s⁻¹ through syringes with no filters

attached. To isolate the filtrate stream from contact with air, a piece of Teflon tubing was attached to the 0.2 μm Supor cartridge outlet. Once the seawater was filtered, the O_2^- signal was monitored the same way as described in Section 2.2, Method 2.

2.5.2 Shipboard tests

On the California Current cruise, filter tests were also conducted at sea by comparing O_2^- concentrations in freshly filtered seawater with those in re-filtered aged filtered seawater. Aged filtered seawater was re-filtered from a 500 mL Teflon dark bottle using the 0.2 μm Supor cartridge by slightly pressurizing with filtered (0.2 μm PTFE filters, Whatman) compressed air, and filling directly into 125 mL HDPE dark bottles. At each depth the same cartridge filter was used for re-filtering as was used for the initial aged filtered seawater sample. Once the seawater was filtered or re-filtered, the O_2^- signal was again monitored the same way as described in Section 2.2, Method 2.

3. Results

3.1. Method intercomparison of O_2^- concentrations measured in the field

Because both $[\text{O}_2^-]_1$ from Method 1 and $[\text{O}_2^-]_2$ from Method 2 are based on the difference in a signal between filtered and unfiltered solutions, both methods should measure the $[\text{O}_2^-]$ that is due to particulate sources. However, the two methods did not produce the same results. $[\text{O}_2^-]_1$ values were typically 90% lower than $[\text{O}_2^-]_2$ values at Station ALOHA and were more than 50% lower in the California Current (compare black bars and white bars in Figure 1; see also bold-faced concentration values in Tables S2 and S3). This discrepancy cannot be attributed to instrumentation error, since the observed output signal during calibration and the instrument

sensitivity in aged filtered seawater and unfiltered seawater were similar to those measured in freshly filtered seawater. We conclude that there was an issue with the method(s) used to measure particle-generated O_2^- .

Detection limits of $[O_2^-]$ in freshly filtered seawater, aged filtered seawater and unfiltered seawater ranged from 0.002 to 0.039 nM in ALOHA samples (average 0.012 ± 0.006 , $n=136$) and from 0.019 to 0.107 nM in the California Current samples (average 0.044 ± 0.016 , $n=30$). The generally higher detection limits in the California Current resulted from the lower method sensitivity S in the California Current. Most samples analyzed were above these detection limits (Tables S2 and S3).

3.2 Filtration and filter tests for O_2^- measurement artifacts in freshly filtered seawater

3.2.1 Laboratory studies

A series of laboratory tests were carried out to determine if filtration can create a transient chemiluminescent signal that would be interpreted as particle-generated O_2^- by Method 2. Four filters were tested: two polyethersulfone (PES) cartridge filters (0.2 and 0.8/0.45 μm), a 0.4 μm 47 mm polycarbonate filter, and a 0.22 μm PES syringe filter. Filtering with all of these filters generated an O_2^- signal in aged filtered seawater amended with DTPA (Table 1). When the filter was removed from the 47-mm filter holder, the O_2^- signal was not statistically different (t-test, two-tailed, $p<0.05$) from that observed when the filter was in place, and when water was dripped from a syringe with no filter attached, the signal was higher than in the presence of the filter (Table 1). We conclude that the signal was not created by contact with the filters. Re-filtering aged filtered seawater amended with SOD did not generate a signal (data not shown).

We performed several experiments to examine whether contact with air during filtration played a role in generating the O_2^- signal. A piece of tubing leading from the end of the 0.2 μm

cartridge filter unit into the bottom of the bottle did not significantly decrease (t-test, two-tailed, $p < 0.05$) the signal (Table 1). We dripped aged filtered seawater from a syringe (with no filter in place) into the sample bottle at varying flow rates. When the flow rate was increased, the amount of signal generated decreased, but only the difference between 1 ml s^{-1} and 3 ml s^{-1} was statistically significant (Table 2). Filtering into a bottle filled with filtered Argon gas did not significantly decrease the O_2^- signal (data not shown, t-test, < 0.05). We were unable to find a filtering technique that generated a sufficiently low and reproducible background signal to be useful for measuring O_2^- concentrations on the order of 100 pM or less by Method 2.

3.2.2 Shipboard studies

We also conducted shipboard studies to more directly compare the magnitude of the O_2^- signal created during filtering to the magnitude of $[\text{O}_2^-]_2$ measured in freshly collected seawater. To do this, several of the aged filtered seawater samples from the California Current were re-filtered and monitored using Method 2. The O_2^- signals created by re-filtering ranged from 0.054 to 0.141 nM , and were not statistically different from the freshly filtered seawater concentrations observed in the same water samples (t-test, two-tailed, $p < 0.05$), except for cast 3 at 20 m (Table 3). These tests indicate that the O_2^- concentration measurements obtained by Method 2 in this study were composed largely of an artificial signal generated during filtration.

3.3 Superoxide decay at ALOHA and in the southern California Current ecosystem

The O_2^- decay rates measured at Station ALOHA were slow, generally ranging from 0.003 to 0.014 s^{-1} (Figure 2, Table S2). On July 16, the decay rates for one replicate of freshly filtered seawater and for unfiltered seawater were abnormally fast. These values are statistical

outliers compared to the other decay data (Q-test, $p < 0.05$). In addition, the other replicate of freshly filtered seawater and the aged filtered seawater sample from the same GO-Flo cast did not show abnormally fast decay rates. We therefore attribute the high rates to contamination in the analysis bottles, and neglect these data in further discussion. Overall, there was little change in the decay rate coefficient with depth and the 25 m water samples showed little day-to-day variability (Figure 2, Table S3).

We did not systematically examine the effect of O_2^- concentration on the measured decay rate coefficients. However, in eight of the duplicates analyses performed on freshly filtered seawater samples from ALOHA station, the concentration of O_2^- from the added KO_2 spikes in the calibrations differed by a factor of two or more. The difference between $k_{\text{loss}, \text{O}_2^-}$ values measured at the higher and lower spike concentration averaged $0.001 \pm 0.003 \text{ s}^{-1}$ (data not shown).

The O_2^- decay rates in the California Current were slightly faster than rates at Station ALOHA with measured $k_{\text{loss}, \text{O}_2^-}$ values ranging from 0.006 to 0.028 s^{-1} (Figure 2, Tables 3 and S3).

There were in general no significant differences between the decay rate coefficient measured in the initial signal vs. in the KO_2 spike used for calibration in freshly filtered seawater, or between decay rate coefficients measured during calibration in aged filtered seawater, unfiltered seawater, and freshly filtered seawater (paired t-tests, $p > 0.05$; Figure 2). The only exception is that the calibration spikes in the freshly filtered and unfiltered California Current samples had significantly higher decay rate coefficients than the calibration spikes in the aged filtered samples (paired t-tests, $p < 0.05$; the average differences in $k_{\text{loss}, \text{O}_2^-}$ values were 0.008 and 0.002 s^{-1} , respectively).

3.4 O_2^- concentrations and production rates at ALOHA and in the southern California Current ecosystem

Because of the Method 2 artifacts discussed above, we discuss here only the particle-generated O_2^- concentrations and production rates measured by Method 1.

Measurements of $[O_2^-]_1$ at ALOHA station ranged from below zero (higher signal in filtered water compared to unfiltered water) to 0.019 nM (Figure 1, Table 4) with one statistical outlier of 0.036 nM (Q-test, $p < 0.05$). In depth profiles, the deepest sample (from the deep chlorophyll maximum) had lower $[O_2^-]_1$ than the 25 m sample in eight out of nine cases; however, the 75 m sample had lower $[O_2^-]_1$ than the 25 m sample in only about half of the depth profiles (Table S2). Average $[O_2^-]_1$ measurements were significantly greater than zero at 25 and 75 m but not at the deep chlorophyll maximum, while particle-generated O_2^- production rates were significantly greater than zero only at 25 m (Table 4). The production rate measured at 25 m was greater than that measured at the deep chlorophyll maximum in seven out of nine depth profiles (Table S2).

In the California Current samples, $[O_2^-]_1$ ranged from undetectable to 0.052 nM (Figure 1), and particle generated production rates were less than 4.7 nM hr^{-1} (Table S3). Although the California Current data set is small and may be too limited to draw any strong conclusions, $[O_2^-]_1$ and production rates were generally higher than at ALOHA station.

3.4.3 Detection limits

$[O_2^-]_1$ was based on the difference between the signal in unfiltered and aged filtered seawater ($[O_2^-]_{\text{USW}}$ and $[O_2^-]_{\text{AFSW}}$, Equation 2), and this difference was generally much smaller than each of these measurements, increasing the relative uncertainty of $[O_2^-]_1$ compared to those

for $[O_2^-]_{USW}$ and $[O_2^-]_{AFSW}$ discussed in Section 3.1. In the California Current, duplicate measurements were performed, and $[O_2^-]_{USW}$ was significantly greater than $[O_2^-]_{AFSW}$ in two out of five of the samples tested (t-test, $p=0.05$), Station 1 50 m and Station 3 20 m, implying detectable $[O_2^-]_1$ in these two samples. Because we were not able to perform replicate unfiltered filtered seawater and aged filtered seawater measurements on the ALOHA samples, we could not determine whether $[O_2^-]_1$ was above detection limit in individual measurements. However, $[O_2^-]$ in unfiltered seawater was greater than $[O_2^-]$ in aged filtered seawater in 31 out of 34 of the ALOHA samples (Table S2), and both the 25 m and 75 m average measurements were significantly greater than zero (Table 4).

3.5 Hydrogen peroxide measurements at Station ALOHA

At Station ALOHA, the seawater H_2O_2 concentrations from the 25 m depth averaged 73 ± 11 nM ($n = 4$) in initial unfiltered seawater. Filtering the seawater did not significantly change the H_2O_2 concentrations, with measured values of 63 ± 7 nM ($n = 4$). (Reported uncertainties in this section represent one standard deviation). On three days, a 3-point depth profile of unfiltered seawater was collected. The H_2O_2 concentrations decreased significantly with depth (two-tailed t-test, $p<0.05$), with measured concentrations of 30 ± 8 nM and 10 ± 4 nM for unfiltered seawater from 75 m and the deep chlorophyll maximum (105-130 m) (Table S4).

Overall, the trend of a decrease with depth also held true for H_2O_2 production and decay rates, but the differences were not significant on a 95% confidence level (two-tailed t-test, $p>0.05$), probably because of the small sample size. Production rates ($P_{H_2O_2}$) were 1.2 ± 0.9 , 0.5 ± 0.2 , and 0.1 ± 0.1 nM hr^{-1} for unfiltered seawater from 25 m ($n=4$), 75 m ($n=3$), and the deep chlorophyll maximum ($n=2$), respectively, while decay rate coefficients (k_{loss, H_2O_2}) for these

depths were 0.021 ± 0.016 , 0.016 ± 0.007 , and $0.006 \pm 0.004 \text{ hr}^{-1}$ (Figure 3, Table S4). For comparison, our estimate of H_2O_2 photoproduction rate, averaged over 24 hours and a depth of 70 m, was 0.6 nM hr^{-1} . There was significantly less variation in both production and decay rates in the filtered samples (F-test, $p < 0.05$); filtered seawater from 25 m had production rates of $0.6 \pm 0.1 \text{ nM hr}^{-1}$ and decay rate coefficients of $0.010 \pm 0.001 \text{ hr}^{-1}$ ($n=4$). There was a strong correlation between production and decay for both filtered and unfiltered seawater from within the mixed layer (25 m samples plus one 45 m sample) (Figure 3).

4. Discussion

4.1 Methodological issues in O_2^- determination

The comparison of $[\text{O}_2^-]$ measured using Method 1 with that measured in freshly filtered seawater using Method 2 (Figure 1, Tables S2 and S3) as well as the filter tests (Tables 1-3) demonstrate that filtering seawater created an initial concentration of $[\text{O}_2^-]$ of up to 0.14 nM , with large variability. (The freshly filtered seawater data at station 2 20 m in the California Current from Table 3 was excluded from this analysis, since this water sample had poor replicates with respect to $[\text{O}_2^-]$.)

While the source of the background signals generated by filtration is unclear, two lines of evidence indicate that the signal was due to the presence of O_2^- . First, the decay rate coefficients of the filtration-generated signal and of the signal from O_2^- spikes were always similar (Figure 2). In addition, re-filtering of aged filtered seawater amended with SOD did not generate a signal. It is important to emphasize that the observed signal from filtering was not a result of cell lysis caused by filtration, since the seawater used for all of our filter tests was already previously filtered. All of the filters produced similar amounts of background signal, as did controls

516 simulating the act of filtering without actual contact of the sample solution with a filter, so the
517 signal cannot be due to contamination of the filters. It is also unlikely to be due to the water's
518 contact with different surfaces (e.g. of the filter holder or the syringe walls), since similar signals
519 were generated by different materials.

520 We considered the possibility that pressure differences and turbulence created in the
521 solutions generated O_2^- . Pushing aqueous solutions through small orifices can create cavitation
522 bubbles (Yan and Thorpe, 1990), which in turn can generate reactive oxygen species (Suslick *et al.*, 1997). However, fluid velocities much higher than those used in our filter tests would be
523 needed for visible formation of cavitation bubbles (Yan and Thorpe, 1990). In addition, we
524 observed that faster flow rates generated a slightly smaller background signal (Table 2), which is
525 inconsistent with a cavitation effect, but may indicate that the background signal arises from
526 contact of the solutions with air. Our other efforts to reduce sample contact with air were less
527 successful in eliminating the background signal.

529 We do not believe that direct partitioning of $HO_2(g)$ from air into our samples can explain
530 the background signals. Although the partitioning is rapid (Sedlak and Hoigne, 1993), at a
531 typical concentration of $HO_2(g)$ ($\sim 2 \times 10^8$ molecules cm^3 , Stone *et al.*, 2012), ~ 30 L of air would
532 be required to supply enough $HO_2(g)$ to increase $[O_2^-]$ in 100 mL of seawater by ~ 0.1 nM. We
533 cannot rule out that other compounds capable of rapid O_2^- generation in water (e.g. $O_3(g)$ or
534 organic radicals) entered the samples from air during filtering.

535 Three previous studies of $[O_2^-]$ in seawater samples relied on analysis of freshly filtered
536 seawater (Method 2) with filter types similar to those we examined here. Two of these studies
537 (Hansard *et al.*, 2010; Rose *et al.*, 2010) reported many $[O_2^-]$ measurements well above our
538 maximum observed artifact value of 0.14 nM; in the third study (Rose *et al.*, 2008b), the reported

values are of a similar magnitude as, or smaller than, our filtration artifact. While it is impossible to determine after the fact how much the $[O_2^-]$ measurements in these previous studies were affected by a filtration artifact, they are likely to be overestimates of the true values. Nevertheless, all three of these studies report internally consistent trends in measured O_2^- concentrations: generally higher values in near-surface samples in Hansard et al. 2010, and a correlation with chlorophyll concentrations in Rose et al., 2010 and Rose et al., 2008. This suggests that at least the higher values measured in these studies exceeded any random signal fluctuations generated by the artifact. It is also possible that the size of the artifact is affected by constituents of the water samples. For example, O_3 introduced into the sample from contact with air during filtration may generate O_2^- during interaction with organic compounds in the water (Staehelin and Hoigne, 1985).

There is only one other published study of marine $[O_2^-]$ (Rusak *et al.*, 2011). Using a methodology similar to Method 1, but without the subtraction of measurements in aged filtered seawater, they detected very high steady-state signals (up to 32 nM) in unfiltered seawater. Rusak *et al.* attributed these high signals either to unusually slow decomposition of O_2^- (contradicting their direct measurements during calibration) or to measurement of high production rates due to detection of O_2^- “inside or near the cell membranes of marine plankton”. We observed nothing comparable in any of our samples.

The filtration artifact discussed here did not affect the results of Diaz et al. (2013), in which the steady-state O_2^- signal was compared before and after bacteria were loaded onto an in-line filter. Some of the results of the present study suggest that contact with air during the filtration procedure causes the Method 2 artifact, but in the set-up used by Diaz et al., the filtrate was directly pumped into the instrument, so there was no contact with air. In addition, the data

reported by Diaz et al. used as a baseline the signal measured with the filter in place but before cells were loaded onto the same filter. Thus, any steady-state signal generated during filtration would have been subtracted out, and any variability introduced by this subtraction would have been accounted for in the replicates, each of which used a fresh filter. Baseline drift with a filter (but no cells) in place was examined over the same time scale as the cell measurements and was found to be negligible compared to most of the measured cell signals; in addition, baseline drift was in a negative direction, which would have resulted in underestimates of the cell signals (Diaz, pers. comm.).

Because the method used by Diaz et al. needs samples with high cell densities, such as laboratory cultures, and because of the problems we observed with Method 2, we recommend Method 1 for measuring the $[O_2^-]$ generated by particulate sources in unfiltered seawater samples. However, some caution in the interpretation of $[O_2^-]_1$ is also warranted, because different possibilities exist for the interpretation of the aged filtered seawater signal ($[O_2^-]_{AFSW}$), which is subtracted from that of unfiltered seawater ($[O_2^-]_{USW}$) to calculate $[O_2^-]_1$. The first possibility is that $[O_2^-]_{AFSW}$ represents a method background, since SOD addition affects the signal generated by the auto-oxidation of MCLA (Hansard et al., 2010 and references cited therein). In this case, $[O_2^-]_1$ would truly represent the concentration of O_2^- in the unfiltered water sample. However, it may also be possible that O_2^- created by dissolved compounds in the water sample contributes to $[O_2^-]_{AFSW}$ (see Section 4.2). For this reason, we emphasize that the values of $[O_2^-]_1$ reported in the present study represent the particle-generated ($>0.2 \mu m$) contribution to the $[O_2^-]$ in the water.

4.2 Rates of H_2O_2 production and decay at Station ALOHA

H₂O₂ concentrations at given depths corresponded well with previously reported data for Station ALOHA (Gasc *et al.*, 2002). Yuan and Shiller (2005) reported a lower value of 54 nM for shallow surface water concentrations, which likely reflected the seasonal variation in H₂O₂, as shown by Gasc *et al.* (2002). We could only find a single previous measurement of H₂O₂ decay rate coefficient at Station ALOHA (0.005 hr⁻¹; Yuan and Shiller, 2005), but because the value was measured over a 4-day incubation time with no attempt to correct for simultaneous production, it is not directly comparable to our measurements.

Because the volume of water required for spiked batch incubations made it impossible to perform replicates on a given day, we could not directly determine whether the large variability in H₂O₂ production and decay rates at 25 m depth was greater than our methodological uncertainty. However, the good correlation ($R^2=0.986$) between H₂O₂ production and decay rates in the unfiltered mixed-layer (25 m and 45 m) samples (filled symbols in Figure 3), as well as the observation that sample-to-sample variability was much smaller in filtered samples, suggest that the range of production rates measured in the 25 m samples reflects natural biological variability.

The slope of the regression line for mixed-layer (25 m and 45 m) samples, 58.5 nM, reflects the average steady-state concentration of H₂O₂ in the absence of photochemical sources, and was very similar to the slope observed in the Gulf of Alaska (64.6 nM, Vermilyea *et al.*, 2010). Vermilyea *et al.* (2010) speculated that the correlation could simply be a result of both production rates and decay coefficients being proportional to cell density, but it could also reflect a biological feedback response. In any case, both the predicted steady-state [H₂O₂] (given by the ratio of production rate to decay rate coefficient, $P_{H_2O_2}/k_{loss,H_2O_2}$, from each incubation) and the H₂O₂ concentrations we measured in the water samples (Table S4) were always well below the 150-200 nM that would be toxic to *Prochlorococcus* (Morris *et al.*, 2011).

The $P_{H_2O_2}$ and k_{loss,H_2O_2} values in filtered samples differed significantly from zero (t-test, $p < 0.05$). These measurements could represent method blanks, but we cannot rule out a contribution from production and decay by natural enzymes or other compounds present in filtered water. The small day-to-day variability in these values could indicate that the compounds producing and breaking down H_2O_2 are relatively long lasting, so that their influence is averaged over time and space.

Past studies have shown that dark H_2O_2 decay in filtered seawater samples is plausible. Learman et al. (2011) observed very rapid decay of H_2O_2 , which was recently attributed to soluble secreted enzymes (H_2O_2 -degrading heme peroxidases) (Andeer et al., in press), in filtrates of cultures of *Roseobacter* sp., a common clade of marine bacteria. While filtration typically reduces or eliminates net H_2O_2 decay (e.g. Petasne and Zika, 1997), a field study in the south Atlantic Ocean showed that net H_2O_2 decay rates in filtered (0.4 μm) and even ultra-filtered (1 kDa) samples were similar to those in the unfiltered sample of surface seawater (Yuan and Shiller, 2001). In addition, ultrafilter retentate (1 kDa < size < 0.4 μm) significantly accelerated net H_2O_2 decay rates, suggesting the presence of soluble enzymes in these water samples.

Dark production of reactive oxygen species in filtered seawater also cannot be ruled out. The majority of past field studies of H_2O_2 did not apply a methodology that could determine gross rather than net production and decay, and typically examined surface samples, which would have contained relatively high initial concentrations of H_2O_2 because of photochemical production. In such samples, only net decay would be observable. Moffett and Zafiriou (1990) measured gross H_2O_2 production rates in 0.2- μm filtered Vineyard Sound surface water of 0.2 ± 0.05 nM hr⁻¹, comparable to the values of 0.5-0.7 nM hr⁻¹ observed in the present study, though it

would be surprising for the oligotrophic waters at ALOHA station to have higher rates than the coastal water examined by Moffett and Zafiriou. In addition, production of O_2^- by soluble secreted enzymes has been observed in cell-free filtrates of *Roseobacter* sp. cultures (Learman et al., 2011; Andeer et al., in press), and production of both O_2^- and H_2O_2 has been observed in filtrates of some algal cultures in our laboratory (unpublished data). In summary, while the present study did not determine how much of the O_2^- and H_2O_2 production in filtered samples should be attributed to a blank signal versus a signal created by dissolved compounds in seawater, future work in this area is warranted.

Whether the filtered samples represent method blanks or real measurements, two observations are puzzling. First, it is difficult to explain why the three lowest production rates and the two lowest decay rates measured in unfiltered samples were significantly lower than those of the pooled filtered samples (t-test assuming equal variances, $p < 0.05$), but this probably indicates that the extremely low variability of the four filtered samples is spurious. In addition, it is unclear why the data from the filtered samples fall on the regression line determined for unfiltered samples (Figure 3), since one would not expect filtering to have the same relative effect on production and decay.

The dark production rates of H_2O_2 observed in the unfiltered 25, 45, and 75 m samples ranged from 0.2 to 2.9 $nM\ hr^{-1}$. The average value for samples from all of these depths, 1.1 $nM\ hr^{-1}$ (or 0.5 $nM\ hr^{-1}$ if one subtracts the 0.6 $nM\ hr^{-1}$ observed in filtered samples), is comparable to our estimate of the 24-hour average photochemical production rate in the mixed layer, 0.6 $nM\ hr^{-1}$, calculated as described in Section 2.4.2. Midday near-surface photochemical production rates will of course be much higher than this 24-hour mixed-layer average. For comparison, Yuan and Shiller (2005) measured a net photoproduction rate of 8 $nM\ hr^{-1}$ in midday shipdeck

irradiations of Station ALOHA water in June 2002. Nevertheless, our calculation indicates that dark production has the potential to affect the total H_2O_2 budget in the mixed layer at Station ALOHA.

4.3 O_2^- production and decay in Pacific Ocean field sites

During the month of July 2012 at Station ALOHA, O_2^- decay rates, concentration and production rates did not vary much from day to day, though slightly higher concentrations and production rates were consistently observed in the 25 m samples (Tables 4 and S2). The data set from the California Current is too small to attempt to determine trends (Table S3).

Our calculation of O_2^- production rates depends on the assumption that the pseudo-first order decay rate coefficients, $k_{\text{loss},\text{O}_2^-}$, determined using KO_2 spikes in unfiltered seawater and aged filtered seawater are the same as those of natural levels of O_2^- . Many of the measured particle-generated O_2^- concentrations were very small (<0.01 nM) compared to the spike concentrations we used (typically >0.1 nM). Rose et al. (2010) showed that $k_{\text{loss},\text{O}_2^-}$ can decrease by over an order of magnitude if the size of the initial O_2^- spike is decreased from 20 nM to 0.2 nM. While we saw no evidence of a similar trend in our samples when comparing $k_{\text{loss},\text{O}_2^-}$ values measured at lower and higher spike concentrations, we did not measure $k_{\text{loss},\text{O}_2^-}$ at natural levels of particle-generated O_2^- . Thus, we cannot entirely rule out that our measured $k_{\text{loss},\text{O}_2^-}$ values, and therefore also our calculated particle-generated production rates (Tables 4, S2 and S3), are overestimates.

The yield of H_2O_2 from O_2^- can theoretically be anywhere from 0 to 1 mol H_2O_2 per O_2^- , with a yield of 0.5 expected if dismutation is the dominant reaction pathway, and a yield of 1.0 if reduction is dominant (see Hansard *et al.*, 2010 and Powers and Miller, 2014, for a more

complete discussion). Comparison of particle-generated O_2^- production rates to H_2O_2 production rates (Table S4) implies that dismutation or reduction of particle-generated O_2^- is not the dominant pathway of H_2O_2 formation, especially in the three samples with the highest H_2O_2 production rates. This indicates the importance of direct biological production of H_2O_2 , such as occurs during algal utilization of organic nitrogen sources (Palenik and Morel, 1987, 1991). In contrast, Hansard et al. (2010) found O_2^- production rates higher than twice the H_2O_2 production rates in most of the samples examined in the Gulf of Alaska, but it is not clear how much the Method 2 artifact contributed to that finding.

Because of the uncertainty in the yield of H_2O_2 from O_2^- , especially during photochemical reactions (Powers and Miller, 2014; Garg et al., 2011), the estimated rate of photochemical H_2O_2 production discussed in the previous section cannot easily be translated to an O_2^- production rate. If we assume a yield of less than 0.5 H_2O_2 per O_2^- , the 24-hour average photochemical production rate in the mixed layer can be estimated as $>1.2 \text{ nM hr}^{-1}$, exceeding the dark particle-generated production rates that we observed at ALOHA station. However, as discussed above, it is unclear whether dissolved sources of O_2^- can also contribute to dark production. Furthermore, in contrast to H_2O_2 , whose long lifetime in seawater ($\sim$ days) means that biota are exposed to a somewhat time- and depth-integrated H_2O_2 signal, O_2^- has a very short lifetime ($\sim$ minutes). Therefore, a steady-state concentration of O_2^- in deeper water (e.g. below the mixed layer) or at night will only be maintained if dark production occurs.

4.4 Future work

Particle-generated O_2^- concentrations and dark production rates in marine systems may be lower than indicated by initial studies, but more measurements in diverse systems are needed to

confirm this. In addition, dark production of ROS by dissolved constituents of seawater may be important and needs to be examined more thoroughly.

Acknowledgements

We would like to thank the C-MORE group and chief scientists Sam Wilson and Jess Fitzsimmons for allowing us to participate on the July 2012 cruise on the R/V Kilo Moana, the CCE-LTER program, and chief scientist Mark Ohman for allowing us to participate on the April 2013 cruise on the R/V New Horizon. This work was enabled by method development that occurred during two previous cruises. We therefore give additional thanks to the HOT program and chief scientist Craig Nosse, who allowed our participation aboard HOT-238 in December 2011, as well as chief scientists Zachary Johnson and Erik Zinser, who allowed us to participate in the POWOW 1 cruise. The crews of the R/V Kilo Moana, R/V Thomas G. Thompson, and R/V New Horizon made this work possible. This work was funded by NSF OCE 1131734/1246174 to BMV and CMH. We are grateful to Andrew Rose and two anonymous reviewers, whose feedback greatly helped us improve this manuscript.

References

- Andeer, P.F., Learman, D.R., McIlvin, M., Dunn, J.A., Hansel, C.M., *in press*. Extracellular heme peroxidases mediate Mn(II) oxidation in a marine *Roseobacter* bacterium via superoxide production. *Environmental Microbiology* (early online: DOI: 10.1111/1462-2920.12893).
- Andrews, S.S., Caron, S., and Zafiriou, O.C., 2000. Photochemical oxygen consumption in marine waters: A major sink for colored dissolved organic matter. *Limnology and Oceanography*, 45 (2), 267-277.
- Bielski, B.H.J., Cabelli, D.E., Arudi, R.L., Ross, A.B., 1985. Reactivity of HO_2/O_2^- radicals in aqueous-solution. *Journal of Physical and Chemical Reference Data* 14 (4), 1041-1100.
- Cooper, W.J., Moegling, J.K., Kieber, R.J., Kiddle, J.J., 2000. A chemiluminescence method for the analysis of H_2O_2 in natural waters. *Marine Chemistry* 70, 191-200.
- Cooper, W.J., Zika, R.G., 1983. Photochemical formation of hydrogen-peroxide in surface and ground waters exposed to sunlight. *Science* 220 (4598), 711-712.
- Diaz, J.M., Hansel, C.M., Voelker, B.M., Mendes, C.M., Andeer, P.F., Zhang, T., 2013. Widespread Production of Extracellular Superoxide by Heterotrophic Bacteria. *Science*.
- Fan, S.M., 2008. Photochemical and biochemical controls on reactive oxygen and iron speciation in the pelagic surface ocean. *Marine Chemistry* 109 (1-2), 152-164.
- Garg, S., Rose, A.L., Waite, T.D., 2011. Photochemical production of superoxide and hydrogen peroxide from natural organic matter. *Geochimica et Cosmochimica Acta* 75, 4310-4320.
- Gasc, A.M.E., Morris, P.J., Karl, D.M., 2002. Sources and Sinks of Hydrogen Peroxide at Station ALOHA. ASLO/AGU Ocean Sciences Meeting, Honolulu, HI.
- Godrant, A., Rose, A.L., Sarthou, G., Waite, T.D., 2009. New method for the determination of extracellular production of superoxide by marine phytoplankton using the chemiluminescence probes MCLA and red-CLA. *Limnology and Oceanography-Methods* 7, 682-692.
- Goldstone, J.V., Voelker, B.M., 2000. Chemistry of superoxide radical in seawater: CDOM associated sink of superoxide in coastal waters. *Environmental Science & Technology* 34 (6), 1043-1048.
- Hansard, S.P., Vermilyea, A.W., Voelker, B.M., 2010. Measurements of superoxide radical concentration and decay kinetics in the Gulf of Alaska. *Deep-Sea Research Part I-Oceanographic Research Papers* 57 (9), 1111-1119.
- Heller, M.I., Croot, P.L., 2010a. Application of a superoxide (O_2^-) thermal source (SOTS-1) for the determination and calibration of O_2^- fluxes in seawater. *Analytica Chimica Acta* 667 (1-2), 1-13.
- Heller, M.I., Croot, P.L., 2010b. Kinetics of superoxide reactions with dissolved organic matter in tropical Atlantic surface waters near Cape Verde (TENATSO). *Journal of Geophysical Research-Oceans* 115.
- Heller, M.I., Croot, P.L., 2010c. Superoxide Decay Kinetics in the Southern Ocean. *Environmental Science & Technology* 44 (1), 191-196.
- Kieber, D.J., Miller, G.W., Neale, P.J., Mopper, K., 2014. Wavelength and temperature-dependent apparent quantum yields for photochemical formation of hydrogen peroxide in seawater. *Environmental Science: Processes & Impacts* 16, 777-791.
- Kim, D., Nakashima, T., Matsuyama, Y., Niwano, Y., Yamaguchi, K., Oda, T., 2007. Presence of the distinct systems responsible for superoxide anion and hydrogen peroxide

764 generation in red tide phytoplankton *Chattonella marina* and *Chattonella ovata*. *Journal of*
765 *Plankton Research* 29 (3), 241-247.

766 King, D.W., Cooper, W.J., Rusak, S.A., Peake, B.M., Kiddle, J.J., O'Sullivan, D.W., Melamed,
767 M.L., Morgan, C.R., Theberge, S.M., 2007. Flow Injection Analysis of H₂O₂ in Natural
768 Waters Using Acridinium Ester Chemiluminescence: Method Development and
769 Optimization Using a Kinetic Model. *Analytical Chemistry* 79 (11), 4169-4176.

770 Kustka, A.B., Shaked, Y., Milligan, A.J., King, D.W., Morel, F.M.M., 2005. Extracellular
771 production of superoxide by marine diatoms: Contrasting effects on iron redox chemistry
772 and bioavailability. *Limnology and Oceanography* 50 (4), 1172-1180.

773 Learman, D.R., Voelker, B.M., Vazquez-Rodriguez, A.I., and Hansel, C.M., 2011. *Nature*
774 *Geoscience* 4, 95-98.

775 Marshall, J.A., de Salas, M., Oda, T., Hallegraeff, G., 2005a. Superoxide production by marine
776 microalgae. *Marine Biology* 147 (2), 533-540.

777 Marshall, J.A., Ross, T., Pyecroft, S., Hallegraeff, G., 2005b. Superoxide production by marine
778 microalgae - II. Towards understanding ecological consequences and possible functions.
779 *Marine Biology* 147 (2), 541-549.

780 Micinski, E., Ball, L.A., Zafiriou, O.C., 1993. Photochemical oxygen activation: superoxide
781 radical detection and production rates in the eastern Caribbean. *Journal of Geophysical*
782 *Research-Oceans* 98 (C2), 2299-2306.

783 Miller, G.W., 2000. Wavelength and Temperature-dependent Apparent Quantum Yields for
784 Photochemical Formation of Hydrogen Peroxide in Seawater, State University of New
785 York, College of Environmental Science and Forestry, Syracuse, NY.

786 Moffett, J.W., Zafiriou, O.C., 1990. An investigation of hydrogen peroxide chemistry in surface
787 waters of Vineyard Sound with H₂¹⁸O₂ and ¹⁸O₂. *Limnology and Oceanography* 35 (6),
788 1221-1229.

789 Morris, J.J., Johnson, Z.I., Szul, M.J., Keller, M., Zinser, E.R., 2011. Dependence of the
790 Cyanobacterium *Prochlorococcus* on Hydrogen Peroxide Scavenging Microbes for
791 Growth at the Ocean's Surface. *PLoS One* 6 (2), e16805.

792 Palenik, B., Zafiriou, O.C., Morel, F.M.M., 1987. Hydrogen peroxide production by a marine
793 phytoplankter. *Limnology and Oceanography* 32 (6), 1365-1369.

794 Palenik, B., Morel, F.M.M., 1991. Amine Oxidases of marine phytoplankton. *Applied and*
795 *Environmental Microbiology* 57(8), 2440-2443.

796 Petasne, R.G., Zika, R.G., 1987. Fate of superoxide in coastal sea water. *Nature* 325 (6104), 516-
797 518.

798 Petasne, R.G., Zika R.G., 1997. Hydrogen peroxide lifetimes in south Florida coastal and
799 offshore waters. *Marine Chemistry* 56, 215-225.

800 Powers, L.C., Miller, W.C., 2014. Blending remote sensing data products to estimate
801 photochemical production of hydrogen peroxide and superoxide in the surface ocean.
802 *Environmental Science--Processes and Impacts* 16 (4), 792-806.

803 Rose, A.L., Godrant, A., Furnas, M., Waite, T.D., 2010. Dynamics of nonphotochemical
804 superoxide production and decay in the Great Barrier Reef lagoon. *Limnology and*
805 *Oceanography* 55 (4), 1521-1536.

806 Rose, A.L., Moffett, J.W., Waite, T.D., 2008a. Determination of superoxide in seawater using 2-
807 methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo 1,2-a pyrazin-3(7H)-one
808 chemiluminescence. *Analytical Chemistry* 80 (4), 1215-1227.

- Rose, A.L., Webb, E.A., Waite, T.D., Moffett, J.W., 2008b. Measurement and implications of nonphotochemically generated superoxide in the equatorial Pacific Ocean. *Environmental Science & Technology* 42 (7), 2387-2393.
- Rusak, S.A., Peake, B.M., Richard, L.E., Nodder, S.D., Cooper, W.J., 2011. Distributions of hydrogen peroxide and superoxide in seawater east of New Zealand. *Marine Chemistry* 127 (1-4), 155-169.
- Sedlak, D.L., Hoigne, J., 1993. The role of copper and oxalate in the redox cycling of iron in atmospheric waters. *Atmospheric Environment Part a-General Topics* 27 (14), 2173-2185.
- Staehelin, J., Hoigne, J., 1985. Decomposition of ozone in water in the presence of organic solutes acting as promoters and inhibitors of radical chain reactions. *Environmental Science and Technology* 19 (12), 1206-1213.
- Stone, D., Whalley, L.K., Heard, D.E., 2012. Tropospheric OH and HO₂ radicals: field measurements and model comparisons. *Chemical Society Reviews* 41 (19), 6348-6404.
- Suslick, K.S., Mdeleni, M.M., Ries, J.T., 1997. Chemistry induced by hydrodynamic cavitation. *Journal of the American Chemical Society* 119 (39), 9303-9304.
- Vermilyea, A.W., Hansard, S.P., Voelker, B.M., 2010. Dark production of hydrogen peroxide in the Gulf of Alaska. *Limnology and Oceanography* 55 (2), 580-588.
- Voelker, B.M., Sedlak, D.L., 1995. Iron reduction by photoproduct superoxide in seawater. *Marine Chemistry* 50 (1-4), 93-102.
- Voelker, B.M., Sedlak, D.L., Zafiriou, O.C., 2000. Chemistry of superoxide radical in seawater: Reactions with organic Cu complexes. *Environmental Science & Technology* 34 (6), 1036-1042.
- Yan, Y., Thorpe, R.B., 1990. Flow regime transitions due to cavitation in the flow through an orifice. *International Journal of Multiphase Flow* 16 (6), 1023-1045.
- Yuan, J.C., Shiller, A.M., 2001. The distribution of hydrogen peroxide in the southern and central Atlantic Ocean. *Deep-Sea Research II* 48(13), 2947-2970.
- Yuan, J., Shiller, A.M., 2005. Distribution of hydrogen peroxide in the northwest Pacific Ocean. *Geochemistry Geophysics Geosystems* 6 (9).
- Zafiriou, O.C., Voelker, B.M., Sedlak, D.L., 1998. Chemistry of the superoxide radical (O₂⁻) in seawater: Reactions with inorganic copper complexes. *Journal of Physical Chemistry A* 102 (28), 5693-5700.
- Zepp, R.G. and Cline, D.M., 1977. Rates of direct photolysis in aquatic environments. *Environmental Science and Technology* 11(4), 359-366.

Table 1. Test for O_2^- generation during filtration and during simulated filtration with no filter present in the housing. Two different filter materials, polyethersulfone (PES) and polycarbonate (PC) were used. The O_2^- concentration with a piece of Teflon tubing attached to the 0.2 μm cartridge filter is also shown. Averages are from triplicate samples with errors showing standard deviations.

Filter	Size	Membrane		Average $[O_2^-]_2$ [nM]	filtration time (s)
Syringe	0.22 μm	PES	Filter	0.062 ± 0.012	95
			No Filter	0.101 ± 0.024	98
47 mm	0.4 μm	PC	Filter	0.039 ± 0.006	84
			No Filter	0.098 ± 0.029	38
Cartridge	0.45 μm	PES	Filter	0.068 ± 0.006	16
Cartridge	0.2 μm	PES	Filter	0.050 ± 0.009	31
			Filter	0.081 ± 0.018	23
			Filter+tubing	0.056 ± 0.004	21

Table 2. Effect of flow rates on O_2^- signal created during expulsion of sample from a syringe. Decay rates of the signal ($k_{\text{loss},\text{O}_2^-}$; see equation 3) are also shown. Tests were completed without a filter attached to the syringe. Averages are from triplicate samples with errors showing standard deviations.

Flow rate (ml s^{-1})	Ave $[\text{O}_2^-]$ (nM)	$k_{\text{loss},\text{O}_2^-}$ (s^{-1})
1	0.215 ± 0.082	0.022
2	0.110 ± 0.047	0.022
3	0.066 ± 0.027	0.023

Table 3. O_2^- concentration by Method 2 and decay in freshly filtered seawater (FFSW) and re-filtered aged filtered seawater (*AFSW) in the southern California Current ecosystem. All samples from the same depth were filtered with the same 0.2 μm cartridge filter. Decay values are from one-point calibration. Values shown are averages from replicate samples with standard deviation.

Day	Depth		$[O_2^-]_2$ [nM]	k_{loss, O_2^-} (s^{-1})	$\Delta Conc$
1	25 m	FFSW	0.090 ± 0.025	0.028 ± 0.001	-0.015
		*AFSW	0.105 ± 0.007	0.013 ± 0.002	
	50 m	FFSW	0.064 ± 0.004	0.010 ± 0.001	0.010
		*AFSW	0.054 ± 0.006	0.011 ± 0.003	
2	20 m	FFSW	0.459 ± 0.379	0.019 ± 0.004	0.338
		*AFSW	0.141 ± 0.021	0.011 ± 0.001	
	40 m	FFSW	0.133 ± 0.028	0.012 ± 0.001	0.000
		*AFSW	0.133	0.010	
3	20 m	FFSW	0.183 ± 0.010	0.016 ± 0.006	0.132
		*AFSW	0.051 ± 0.028	0.020 ± 0.003	
	100 m	FFSW	0.032 ± 0.021	0.015 ± 0.004	-0.012
		*AFSW	0.044 ± 0.004	0.010 ± 0.001	

Table 4. O_2^- concentrations and production rates measured by Method 1 at ALOHA station at 25 m, 75 m, and the deep chlorophyll maximum (DCM). Averages significantly greater than zero (t-test, $p < 0.05$) are marked with an asterisk. The full data set is given in Table S2. This summary excludes the outlier concentration value measured at 125 m (the DCM) on July 11, and the production rate calculated using the outlier decay rate measured at 25 m on July 16.

	$[O_2^-]_1$ (nM)		O_2^- production rate (nM hr ⁻¹)		n
	Range	Average	Range	Average	
25 m	0.004-0.019	0.010±0.004*	0.01-0.43	0.25±0.14*	14
75 m	0.000-0.017	0.010±0.006*	-0.28-0.40	0.11±0.25	10
DCM	-0.011-0.010	0.001±0.007	-0.27-0.53	0.11±0.23	9

912

913

914

915

916

917

918

919

920

921

Figure 1. Comparison of particle-generated O_2^- concentration measured by Method 1 (black) and Method 2 (white) at ALOHA station (A) and in the California Current (B). Station ALOHA samples from 25 m only are shown here and are shown by sampling date (July 10-July 26); data for other depths is given in Table S2. Southern California Current ecosystem (California Current) samples are referred to by cast number; all depths are shown. All California Current values are average values with standard deviation from duplicate samples.

Figure 2. O_2^- decay rate coefficients in the Pacific Ocean. A) O_2^- decay rate coefficients in the freshly filtered seawater from Station ALOHA (25 m, 75 m, and deep chlorophyll maximum or DCM) and the southern California Current ecosystem (CC) determined from initial decay (sample) and during calibration (spike). B) O_2^- decay rate coefficients from calibration spike from Station ALOHA (25 m, 75 m, and DCM) and the California Current compared in freshly filtered and unfiltered samples. Decay rates from Station ALOHA freshly filtered seawater and the CC samples are averages of duplicates, with error bars showing standard deviation. C) O_2^- decay rate coefficients from the calibration spike from Station ALOHA (25 m, 75 m, and DCM) and the California Current (CC) compared in unfiltered and aged filtered samples. Decay rates from CC samples are averages of duplicates with error bars showing standard deviation. Dashed line in all the graphs show a 1:1 relationship.

Figure 3. Correlation of H₂O₂ production rates and decay rate coefficients at Station ALOHA, 7/18/2012 through 7/25/2012. The trendline represents the correlation for unfiltered seawater samples from the mixed layer (black symbols, 25 m and 45 m USW) and has a slope of 58.1 nM.