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Extracellular superoxide production by key microbes in the global ocean

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ABSTRACT

Bacteria and eukaryotes produce the reactive oxygen species superoxide both within and outside the cell. Although superoxide is typically associated with the detrimental and sometimes fatal effects of oxidative stress, it has also been shown to be involved in a range of essential biochemical processes, including cell signaling, growth, differentiation, and defense. Light-independent extracellular superoxide production has been shown to be widespread among many marine heterotrophs and phytoplankton, but the extent to which this trait is relevant to marine microbial physiology and ecology throughout the global ocean is unknown. Here, we investigate the dark extracellular superoxide production of five groups of organisms that are geographically widespread and represent some of the most abundant organisms in the global ocean. These include *Prochlorococcus*, *Synechococcus*, *Pelagibacter*, *Phaeocystis*, and *Geminigera*. Cell-normalized net extracellular superoxide production rates ranged seven orders of magnitude, from undetectable to 14,830 amol cell^{-1} h^{-1}, with the cyanobacterium *Prochlorococcus* being the lowest producer and the cryptophyte *Geminigera* being the most prolific producer. Extracellular superoxide production exhibited a strong inverse relationship with cell number, pointing to a potential role in cell signaling. We demonstrate that rapid, cell-number–dependent changes in the net superoxide production rate by *Synechococcus* and *Pelagibacter* arose primarily from changes in gross production of extracellular superoxide, not decay. These results expand the relevance of dark extracellular superoxide production to key marine microbes of the global ocean, suggesting that superoxide production in marine waters is regulated by a diverse suite of marine organisms in both dark and sunlit waters.

Reactive oxygen species (ROS) are oxygen-containing radicals and compounds present at low concentrations, with half-lives ranging from nanoseconds to hours in aquatic systems. The most common forms of ROS in marine systems are hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}/HO_2$), hydroxyl radical ($HO^{\bullet}$), singlet oxygen ($^1O_2$), and carbonate radical ($CO_3^{\bullet-}$). The formation of many ROS within aqueous systems occurs via sequential one-electron transfer reactions (Fridovich 1998). For instance, the ROS $O_2^{\bullet-}$, H_2O_2, and $HO^{\bullet}$ are the intermediates of the sequential one-electron reduction of molecular oxygen to water. ROS play a key role in the remineralization of carbon and the cycling of numerous metals within the ocean (Heller and Croot 2010; Rose 2012; Wuttig et al. 2013*a*).

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The ROS superoxide is ubiquitous in marine environments (Rose et al. 2008*b*; Hansard et al. 2010; Rusak et al. 2011; Diaz et al. 2016; Roe et al. 2016). Concentrations of superoxide in marine environments range from picomolar to hundreds of nanomolar, with higher concentrations typically observed in high-productivity waters and shallow coastal environments (Rose et al. 2008*b*; Hansard et al. 2010; Rusak et al. 2011; Diaz et al. 2016; Roe et al. 2016). In sunlit surface waters, superoxide forms as a photochemical product from the photolysis of colored dissolved organic matter (Heller et al. 2016). Historically, photochemical processes have been viewed as the primary source of superoxide to the ocean, but recent work demonstrates that microbes are a significant source of superoxide to marine environments (Diaz et al. 2013). In sunlit and dark waters alike, microbes appear to be prolific producers of extracellular superoxide (Rose et al. 2008*b*; Diaz et al. 2013; Hansel et al. 2016; Schneider et al. 2016). The diversity of microorganisms contributing to the oceanic superoxide flux is just beginning to come to

light with a broad taxonomic representation already evident (Kustka et al. 2005; Marshall et al. 2005; Rose et al. 2008*b*; Learman et al. 2011; Diaz et al. 2013, 2016; Hansel et al. 2016; Zhang et al. 2016*b*; Diaz and Plummer 2018).

Within microbial systems, superoxide is produced intracellularly as a byproduct of photosynthesis and respiration and extracellularly by transmembrane or secreted enzymes belonging generally to reduced form of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases and peroxidases (Fridovich 1983; Asada 2006; Dickinson and Chang 2011; Diaz et al. 2013; Andeer et al. 2015; Diaz and Plummer 2018). Intracellular and extracellular superoxide production has both detrimental and beneficial impacts on life. As a radical with a half-life on the order of minutes (Heller and Croot 2011), superoxide reacts with biomolecules and redox active metals such as iron within the cell (Fridovich 1998). Thus, high concentrations of superoxide within cells can be toxic, leading to oxidative stress and apoptosis (Buetler et al. 2004). To eliminate oxidative stress caused by excess intracellular superoxide, cells produce antioxidants such as superoxide dismutase (SOD) to keep superoxide at healthy physiological levels (Korshunov and Imlay 2002). As a singly charged anion under physiological pH, superoxide diffusion across the cell membrane is limited (Korshunov and Imlay 2002). Indeed, permeability of superoxide across lipid bilayer membranes is entirely insufficient to explain extracellular superoxide fluxes from microbes (Gus' kova et al. 1984; Diaz et al. 2013). There is an increasing recognition that superoxide is an essential molecule required for basic cellular physiology and growth of other plant, animal, and microbial cells (Saran 2003; Buetler et al. 2004). For instance, extracellular superoxide has been shown to play an important role in cell signaling and growth stimulation in eukaryotes and cell differentiation in fungi (Buetler et al. 2004; Dickinson and Chang 2011). In higher plants, oxidative bursts play a beneficial role in multiple physiological responses, including antimicrobial defense, oxidative cross-linking of cell walls preceding transcription-dependent defenses in wound repair, and gene activation of various stress responses (Lamb and Dixon 1997).

The trade-offs of extracellular superoxide production between the harmful effects of oxidative damage by ROS and the potential benefits of signaling and growth promotion (or other helpful effects) are still very much an active area of study. Although the reasons and mechanisms of extracellular superoxide production within marine microbes have only been minimally explored and remain unclear, a wide diversity of photoautotrophic and heterotrophic microbes have been shown to produce superoxide outside their cells under both light and dark conditions in natural waters (Rose et al. 2008*b*; Diaz et al. 2013; Hansel et al. 2016). Nevertheless, many key marine organisms have not been previously explored. In this study, we set out to better understand the role of extracellular superoxide within the ocean by examining its production by some of the ocean's most abundant organisms. We examine the extracellular production by cyanobacteria *Synechococcus* and *Prochlorococcus*, the two most abundant photosynthesizing organisms in the global ocean. We also measured extracellular superoxide production by SAR11, the most abundant group of marine heterotrophic bacterioplankton, and two marine phytoplankton abundant in coastal Antarctica, *Phaeocystis antarctica* and *Geminigera cryophila*. We test the influence of cell density on extracellular superoxide production and demonstrate a strong inverse relationship between cell number and the cell-normalized superoxide production rate. We show that the cell-normalized extracellular superoxide rate responds to changes in cell number on the order of seconds to hours. This dependence of extracellular superoxide rate to cell number is apparent regardless of whether cells are concentrated or diluted. These data build upon previous studies highlighting the widespread nature of extracellular superoxide production across microbial life and provide essential rates for improved modeling of superoxide distributions within the global ocean.

Methods

Measurement of extracellular superoxide

Extracellular $O_2^{\bullet-}$ production was measured with a FeLume (Waterville Analytical) using a previously described method (Diaz et al. 2013). The FeLume system is a flow-cell reactor designed to measure chemiluminescence, which, in this case, results from the mixture of a superoxide-containing sample and the superoxide-specific chemiluminescent probe methyl *Cypridina* luciferin analog (MCLA, TCI America; Rose et al. 2008*a*). The FeLume system is composed of two separate fluid lines, one being dedicated to the analyte solution and the other to the MCLA reagent. Both solutions are independently flushed through the system at an identical flow rate using a peristaltic pump until they converge in a spiral flow cell immediately adjacent to a photomultiplier tube. The spiral flow cell and photomultiplier tube are housed within an opaque box to eliminate any incidental ambient light. Extracellular superoxide production rates by cells were measured by placing the cells in-line with the FeLume system using either 0.22 or 0.1 μm syringe filters (the latter being used for *Prochlorococcus* and *Pelagibacter* cells). Extracellular superoxide was measured by running artificial seawater (ASW, recipe below) past the filter-supported cells directly into the instrument, where it was mixed with MCLA. Samples of *Phaeocystis* and *Geminigera* were kept on ice to prevent stress to the cells and lysis. All measurements, including calibrations, were collected under dark conditions, which were maintained by covering sample tubing and filter with aluminum foil. Similar systems have been used to generate high-sensitivity measurements of natural superoxide concentrations and decay rates (Rose et al. 2008*a*; Hansard et al. 2010), as well as extracellular superoxide production by bacteria (Diaz et al. 2013), phytoplankton isolates (Kustka et al. 2005; Rose et al. 2008*b*), and natural *Trichodesmium* colonies (Hansel et al. 2016).

For calibration, primary standard solutions of potassium dioxide (KO_2, ACROS Organics, superoxide content > 215 cc g^{-1}) were prepared in NaOH (pH = 12.5) amended with 90 μmol L^{-1} diethylene-triaminepentaacetic acid (DTPA, Sigma > 99%) in order to sequester trace contaminants that would otherwise significantly reduce the lifetime of superoxide. Superoxide

concentrations in primary standards were quantified by measuring the difference in absorbance at 240 nm before and after the addition of 2 U mL^{-1} SOD (SOD from bovine erythrocytes ≥3000 U mg^{-1}, Sigma, stock prepared in distilled water to 4000 U mL^{-1}) and then converting to molar units based on the molar absorptivity of superoxide corrected for the absorption of hydrogen peroxide formed during decay at the same wavelength (Bielski et al. 1985). In order to create secondary standards for analysis on the FeLume, these solutions were further diluted in TAPS-buffered ASW (481 mmol L^{-1} NaCl, 27 mmol L^{-1} $MgCl_2 \bullet 6H_2O$, 10 mmol L^{-1} $CaCl_2 \bullet 2H_2O$, 9 mmol L^{-1} KCl, 6 mmol L^{-1} $NaHCO_3$, $MgSO_4 \bullet 7H_2O$, 3.75 mmol L^{-1} *N*-[tris (hydroxymethyl)methyl]-3-aminopropanesulfonic acid, pH = 8.0, and 75 μmol L^{-1} DTPA, all major salts are Sigma BioXtra grade, TAPS is ARCOS Organics 99+% for biochemistry). Superoxide standards were run with an in-line filter without cells to provide consistency with biological experiments and account for any possible artifacts of filtration. The carrier solution was allowed to pass across the filter and react with the MCLA reagent (4.0 μmol L^{-1} MCLA, 50 μmol L^{-1} DTPA, and 0.10 mol L^{-1} MES, pH = 6.0, reagent grades same as listed above, 2-(N-Morpholino) ethanesulfonic acid hydrate (MES hydrate) is Alfa Aesar 99+%) until a stable baseline (< 5% coefficient of variation) was achieved for ~ 1 min. Then the secondary standards were pumped directly through the analyte line across the in-line filter. The analyte and reagent were typically pumped at a flow rate of 3.0–4.0 mL min^{-1}, which was confirmed gravimetrically. Because superoxide is unstable, both primary and secondary standards were prepared immediately before each measurement.

To prepare calibration curves, the chemiluminescence signal generated from the secondary standards was baseline-corrected for chemiluminescence signal arising from the auto-oxidation of the MCLA reagent. Baseline correction was achieved by subtracting the average background signal generated from the carrier solution passing over the in-line filter (without KO_2) and reacting with the MCLA reagent for at least 1 min. Baseline-corrected chemiluminescence data collected over several minutes of superoxide decay in standard solutions were log-linear with respect to time, and therefore modeled using pseudo-first-order decay kinetics. The half-life of superoxide in most calibrations was typically 2.5 min or less.

Daily calibration curves were generated from three paired observations of time-zero superoxide concentration (dependent variable) and extrapolated chemiluminescence (independent variable) using linear regression. Because chemiluminescence values were baseline-corrected, regression lines were forced through the origin. Calibrations yielded highly linear curves (typically $R^2 > 0.9$), with a typical sensitivity of 1 chemiluminescence unit per pmol L^{-1} superoxide.

As in calibration experiments, each biological experiment began with the placement of a clean syringe filter downstream of the peristaltic pump and upstream of the flow cell in the analyte line. Stable baseline signals (< 5% coefficient of variation) were generated in biological experiments from carrier solution passing through the in-line filter and reacting with MCLA for at least 1 min prior to the addition of cells. The pump was temporarily stopped and cells were added by syringe to the in-line filter to achieve the desired cell number. The presence of cells did not alter flow rates during the experiment. Extracellular superoxide produced by the organisms supported on the in-line filter and released into the carrier solution was detected downstream upon mixing with the MCLA reagent in the flow cell. These signals were corrected for background chemiluminescence by subtracting the average baseline obtained immediately before the addition of cells and converted to steady-state concentration measurements using the calibration function determined on that day. The detection limit for these measurements, calculated assuming that the minimum detectable baseline-corrected signal was three times the standard deviation of the baseline, typically ranged from 25 to 50 pmol L^{-1}. Net superoxide production rates were then calculated as the product of the steady-state superoxide concentration and flow rate (pmol h^{-1}). Production rates of superoxide by each culture sample were normalized to the total number of cells added to provide cell-normalized rates (in units of amol $cell^{-1}$ h^{-1}).

Superoxide decay rates were determined by standard additions of superoxide to cell cultures of a subset of organisms in this study. After stable chemiluminescence signals were achieved using the carrier solution, secondary standards ranging from 3 to 60 nmol L^{-1} were prepared in an aliquot of identical carrier solution, as described above, and pumped across the cells deposited onto the in-line filter. Standard additions were prepared at concentrations chosen to represent a significant (but not excessive) addition to the cell signal. As in calibration experiments, baseline-corrected chemiluminescence data collected over at least 1 min of decay were log-linear. However, in this case, the stable, cell-derived signal measured immediately before the standard addition was used as the baseline. Time-zero chemiluminescence values were then determined by modeling the log-transformed decay data with pseudo-first-order kinetics. The extrapolated chemiluminescence values thus represent the difference in signal due to the added superoxide standard. These were converted to a concentration using the daily calibration factor. These "recovered" concentrations were finally expressed as a percentage of the actual added superoxide concentration. Net superoxide production rates were divided by these standard recoveries to generate gross production rates.

To verify that the signal produced by the cells was due to superoxide, SOD was added to the buffer at the end of each individual run to produce a final SOD concentration of 0.8 U mL^{-1}. SOD always caused a rapid drop in signal, to a final baseline that was typically below the initial baseline measured before cells were loaded. The difference in the initial and final baselines (~ 200 chemiluminescence units) was of the same magnitude as the drop in baseline observed when the same amount of SOD was added to the carrier solution in

the absence of cells. The baseline drop reflects either a small yet nonzero concentration of superoxide in the carrier solutions and/or (more likely) an effect of SOD on the background chemiluminescence produced by the auto-oxidation of MCLA (Hansard et al. 2010). To provide the most conservative value for the superoxide production rates, the higher baseline (without SOD) was used in biological superoxide production calculations.

Culturing and cell counts

Cultures of each organism were grown to mid-exponential phase to late exponential phase for extracellular superoxide production measurement. Axenic cultures of *Synechococcus* WH8102 were grown in 0.2 μm filtered sterile Vineyard Sound water amended with SN nutrients (Waterbury et al. 1986) at 18°C in 14 : 10 h light : dark cycles (35 μmol photons m^{-2} s^{-1}). Growth was monitored via optical density at 750 nm (Molecular Devices SpectraMax M3 microplate spectrophotometer). Cultures of two strains of *Pelagibacter* isolated from contrasting oceanic environments (HTCC1062, Oregon Coast; HTCC7211, Sargasso Sea) were grown at 16°C (HTCC1062) or 20°C (HTCC7211) in 12 h light : dark cycles using sterile ASW amended with 100 μmol L^{-1} pyruvate, 50 μmol L^{-1} glycine, 10 μmol L^{-1} methionine, and 1X vitamin mix (Carini et al. 2013). Four strains of *Prochlorococcus*, each representing a different ecotype, *Synechococcus* WH8102, and *Synechococcus* WH7803 cells were grown in 0.2 μm filtered sterile Sargasso Sea water amended with Pro99 nutrients prepared as previously described (Moore et al. 2007). As *Synechococcus* was cultured in both SN and Pro99 media as part of this study, we will refer to the media conditions when referring to *Synechococcus* throughout the study (SN or Pro99). Cells were grown in a 13 : 11 light : dark cycle with simulated dawn and dusk (Zinser et al. 2009) at 24°C. Near-optimal peak light levels for maximizing growth rate were used for all *Prochlorococcus* strains involved and included the following combinations: MED4 (74 μmol photons m^{-2} s^{-1}), MIT9312 (80 μmol photons m^{-2} s^{-1}), NATL2A (39 μmol photons m^{-2} s^{-1}), and MIT9313 (26 μmol photons m^{-2} s^{-1}). *Synechococcus* WH8102 and *Synechococcus* WH7803 grown in Pro99 media were both grown at peak light levels of 70 μmol photons m^{-2} s^{-1}. To monitor cell growth, cells were monitored via bulk chlorophyll fluorescence (10 AU model, Turner Designs). Cultures used in all experiments were axenic and were tested for purity using three broths ProAC, ProMM, and MPTB (Saito et al. 2002; Morris et al. 2008; Berube et al. 2015), as well as by flow cytometry. *P. antarctica* and *G. cryophila* were grown in f/2+Si media and maintained at 2–4°C in a 14 : 10 light : dark cycle (Guillard 1975).

Cultures were harvested and measured for extracellular superoxide concentration and production while cells were actively growing in their respective growth media. *Synechococcus*, *Prochlorococcus*, and *Pelagibacter* were harvested when the log of cell density (as measured by fluorescence, optical density, and/or flow cytometry) vs. time was linear, indicating exponential growth phase. *Geminigera* and *Phaeocystis* are both relatively slow-growing Antarctic strains that take approximately 1 month to complete a growth cycle. *Geminigera* was harvested when cell counts reached approximately half of the maximum cell count observed at stationary phase (max is ~ 6.5×10^5 under the growth conditions described). Quantifying *Phaeocystis* cell counts can be challenging in the late stages of cell growth due to a mixture of individual and colonial organisms. Therefore, cells were harvested 2 weeks after inoculation, and the absence of colonial cells was confirmed with flow cytometry. The range of growth media requirements, organism size, and organism physiology meant that cells were harvested at different cell densities. Although culture density varied, organisms were measured at similar cell number by varying the volume of culture that was passed over through the filter prior to analysis. Cell densities at the time of analysis were as follows—*Synechococcus* WH8102 (Vineyard Sound SN media): 9.0×10^5 cells mL^{-1}, *Synechococcus* WH8102 (Pro99): $6.3–9.8 \times 10^5$ cells mL^{-1}, *Synechococcus* WH7803 (Pro99): $6.1–6.3 \times 10^5$ cells mL^{-1}, *Prochlorococcus* MED4: $1.0–2.2 \times 10^8$ cells mL^{-1}, *Prochlorococcus* MIT9312: $1.9–2.3 \times 10^8$ cells mL^{-1}, *Prochlorococcus* NATL2A: $6.9 \times 10^7 – 1.2 \times 10^8$ cells mL^{-1}, *Prochlorococcus* MIT9313: $2.3 \times 10^7–4.1 \times 10^7$ cells mL^{-1}, *Pelagibacter* HTCC1062: $4.9–5.0 \times 10^7$, *Pelagibacter* HTCC7211: $4.8–5.1 \times 10^7$, *P. Antarctica*: 2.7×10^5 cells mL^{-1}, and *G. Cryophila*: 3.2×10^5 cells mL^{-1}.

Cell counts of each organism were collected using flow cytometry. Concentrations of *Synechococcus* sp. (cells mL^{-1}) were found by processing 200 μL aliquots of sample as well as 0.01 μm filtered seawater blanks on a Guava easyCyte flow cytometer (Millipore Sigma) at a low flow rate (0.24 μL s^{-1}) for 3 min. Data analyses were performed using Guava InCyte 3.1 software. Populations of *Synechococcus* spp. were identifiable on plots of orange fluorescence vs. forward scatter. Particle concentrations of seawater blanks were subtracted from *Synechococcus* spp. concentrations. The Guava easyCyte flow cytometer was calibrated with instrument-specific beads. Growth of *Pelagibacter* cells was measured by enumerating cells every 48 h. Cell counts were conducted by staining with SYBR Green I (Molecular Probes), and counting with a Guava Technologies flow cytometer as described elsewhere (Carini et al. 2013). *Prochlorococcus* cell abundance measurements were run on a Guava easyCyte flow cytometer. Cells were excited with a blue 488 nm laser analyzed for chlorophyll fluorescence (692/40 nm) and size (forward scatter). *P. antarctica* and *G. cryophila* were counted on an Accuri C6 flow cytometer (BD Biosciences) using the chlorophyll (excitation 640:670/LP) and forward scatter channels. Counts were made using the fast setting (66 μL min^{-1}) with a 2 min run time.

The target cell counts in sequential loading experiments were meant to approach that of a typical milliliter of surface ocean water ($10^5–10^6$ cells); however, in the case of *Prochlorococcus* and *Pelagibacter*, lower net superoxide production rates necessitated higher cell counts to produce a signal above the detection limit of the method. As population dynamics were not readily determined for four strains of *Prochlorococcus*, the extracellular superoxide concentration and net production are determined from two replicates.

Results and discussion

Extracellular superoxide by key marine microbes

Extracellular superoxide production has long been established as a characteristic of fungi and higher plants, yet it is only recently that this phenomenon has been recognized as a widespread phenomenon in heterotrophic bacteria (Lamb and Dixon 1997; Buetler et al. 2004; Diaz et al. 2013) and phytoplankton (Marshall et al. 2002, 2005; Rose et al. 2008*b*; Schneider et al. 2016; Diaz and Plummer 2018). Here, we show that this phenomenon is also widespread among some of the most abundant and ecologically important microorganisms of the global ocean. Extracellular superoxide production was detected and quantified by all nine microbes explored here, including *Synechococcus* (WH8102 and WH7803), *Phaeocystis* (*P. antarctica*), and *Geminigera* (*G. cryophila*), two *Pelagibacter* ecotypes (HTCC1062 and HTCC7211), and four *Prochlorococcus marinus* strains including high-light (HL) and low-light (LL) ecotype representatives (MIT9312, MED4, NATL2A, and MIT9313; Table 1-). Overall, these mid-exponential phase to late exponential phase cultures showed a large range in dark extracellular superoxide production, with steady-state superoxide concentrations ranging from < 35 to 21,768 pmol L^{-1} and cell-normalized superoxide production rates spanning from undetectable levels to 14,830 amol $cell^{-1}$ h^{-1}.

The two Southern Ocean algal representatives were prolific superoxide producers, with *G. cryophila* producing far more superoxide than the other organisms studied here under these laboratory conditions. Average steady-state superoxide concentrations and corresponding cell-normalized superoxide production rates were 15,170 pmol L^{-1} and 6088 amol $cell^{-1}$ h^{-1} for *Geminigera* and 5332 pmol L^{-1} and 3019 amol $cell^{-1}$ h^{-1} for *P. antarctica* (Table 1-). *G. cryophila* is a cryptophyte widespread within surface waters of the Southern Ocean (Gast et al. 2014), whereas its distribution outside of the Southern Ocean is not well characterized. *G. cryophila* is a mixotrophic protist capable of carbon acquisition by oxygenic phototrophy and bacterial ingestion (McKie-Krisberg et al. 2015). *Phaeocystis* spp., a marine haptophyte genus, is typically found at high latitudes (> 50°) in both the Northern and Southern Hemisphere (Vogt et al. 2012). *Phaeocystis* spp. play a particularly important role in the Southern Ocean biological pump, where it is responsible for > 10% of primary productivity and > 30% of the sinking particle flux in some regions (Alvain et al. 2008; Wang and Moore 2011). *Phaeocystis* forms large seasonal blooms in the Southern Ocean that rapidly draw down nutrients in the surface water and can have deleterious effects on other marine organisms (Schoemann et al. 2005; Vogt et al. 2012). Despite their ecological relevance in the biogeochemistry of the Southern Ocean, these are the first measurements of ROS production by *Phaeocystis* and *Geminigera* or haptophytes and cryptophytes in general.

The extracellular superoxide production rates of these two organisms are well within the range of values previously measured for eukaryotic algae, which spans ~ 60 to > 10^7 amol $cell^{-1}$ h^{-1} (Marshall et al. 2002, 2005; Diaz and Plummer 2018). With respect to eukaryotic phytoplankton, *G. cryophila* and *P. antarctica* extracellular superoxide production rates are intermediate to those of Raphidophytes involved in harmful algal blooms (HAB) and diatoms (Diaz and Plummer 2018). Elevated ROS production is a common feature among species that produce harmful algal blooms, which typically occupy the higher end of this range (Diaz and Plummer 2018). Raphidophytes belonging to the genus *Chattonella* produce extracellular superoxide at rates between 6.6×10^6 and 1.6×10^7 amol $cell^{-1}$ h^{-1} (Marshall et al. 2002). Diatoms occupy the lower part of this range, with net extracellular superoxide production rates between 60 and ~ 1300 amol $cell^{-1}$ h^{-1} (Rose et al. 2008*b*; Schneider et al. 2016). The high extracellular superoxide production rates that appear common among many eukaryotic algae point to their potential importance in regulating superoxide levels within marine surface waters, particularly in regions where eukaryotic algae contribute significantly to primary production (e.g., Southern Ocean).

The three globally representative picoplankton explored here produced extracellular superoxide at a wide range of rates, with *Synechococcus* producing the greatest and *Prochlorococcus* the least extracellular superoxide (Table 1-). Average steady-state superoxide concentrations and corresponding cell-normalized superoxide production rates were 5838 pmol L^{-1} and 337 amol $cell^{-1}$ h^{-1} for *Synechococcus* grown in Vineyard Sound SN media, 87 pmol L^{-1} and 23 amol $cell^{-1}$ h^{-1} for *Synechococcus* WH8102 and WH7803 grown in Sargasso Sea Pro99 media, 289 pmol L^{-1} and 0.16 amol $cell^{-1}$ h^{-1} for two ecotypes of *Pelagibacter*, and 380 pmol L^{-1} and 0.026 amol $cell^{-1}$ h^{-1} for four strains of *Prochlorococcus* (Table 1-). The concentrations of extracellular superoxide measured from the *Prochlorococcus* strains were below the method detection limit until the number of cells analyzed on the filter was greater than 10^8 (Table 1-).

The superoxide production rates from the current study represent the first measurements of superoxide by *Prochlorococcus*. All four *Prochlorococcus* strains measured in this study exhibit extremely low extracellular superoxide production rates relative to the other organisms in this study. The four strains of *Prochlorococcus* represent both HL and LL adapted ecotypes. HL adapted *Prochlorococcus* strains MED4 (HLI) and MIT9312 (HLII) are more abundant in surface waters, and the LL adapted strains NATL2A (LLI) and MIT9313 (LLIV) are more abundant at the mixed layer or deeper (Johnson et al. 2006; Kettler et al. 2007). Regardless of light adaptation, the strains did not exhibit any monotonic trend in net or gross superoxide production (Table 2-). *Prochlorococcus* MED4 was the lowest superoxide producer among these *Prochlorococcus* strains. In fact, the net extracellular superoxide production by *Prochlorococcus* MED4 is among the lowest values measured of any marine microbe to date (Diaz et al. 2013; Diaz and Plummer 2018). Net extracellular superoxide production rates among the HLII, LLI, and LLIV ecotypes are not dissimilar, but determining whether light tolerance adaptations and extracellular ROS production are related is not readily tractable without knowledge of a superoxide production

Table 1- Summary of extracellular superoxide concentrations and production rates from cell addition experiments.

Organism	Cell number*	Steady state [$O_2^{\bullet-}$] (pmol L^{-1}) average (range)†	Net $O_2^{\bullet-}$ Production (amol $cell^{-1}$ h^{-1}) average (range)†	Surface-area–normalized net $O_2^{\bullet-}$ production (amol μm^{-2} h^{-1})
Prochlorococcus MIT9312	9.0×10^5–4.5×10^9	920 (ND–1368)	0.041 (ND–0.064)	0.017–0.057
Prochlorococcus MED4	9.0×10^5–4.4×10^9	100 (ND–131)	0.005 (ND–0.007)	0.003–0.006
Prochlorococcus NATL2A	9.0×10^5–2.7×10^9	382 (ND–417)	0.030 (ND–0.037)	0.021–0.033
Prochlorococcus MIT9313	9.0×10^5–8.2×10^8	120 (ND–205)	0.029 (ND–0.050)	0.007–0.045
Synechococcus sp. WH8102 (Vineyard Sound SN media)	9.0×10^5–4.5×10^6	5838 (4135–8777)	337 (114–551)	36–176
Synechococcus sp. WH8102 (Sargasso Sea Pro99 media)	1.6×10^5–6.3×10^6	92 (49–466)	23.1 (4.8–56.0)	1.4–17.7
Synechococcus sp. WH7803 (Sargasso Sea Pro99 media)	1.5×10^5–6.1×10^6	82 (56–146)	22.8 (4.3–67.2)	1.4–21.2
Pelagibacter HTCC1062	2.5×10^8–7.5×10^8	281 (217–339)	0.15 (0.11–0.21)	0.38–0.73
Pelagibacter HTCC7211	2.5×10^8–7.6×10^8	297 (238–349)	0.16 (0.11–0.23)	0.38–0.79
Phaeocystis antarctica	6.7×10^4–6.0×10^5	5332 (2290–8324)	3019 (1657–4173)	33–83
Geminigera cryophila	8.0×10^4–1.3×10^6	15,170 (9907–21,768)	6088 (1673–14,830)	3.1–28

ND values are not included in averages.
*Number of cells added to filter during analysis; cell density of cells within culture are provided in the Methods section.
†Values are not included in averages.

mechanism. One possibility for the significantly lower rates seen in *Prochlorococcus* may be related to a lack of genes encoding superoxide-producing proteins due to genomic streamlining (Giovannoni et al. 2005). It is interesting to note that the lowest producer of extracellular superoxide also contains the smallest genome among these *Prochlorococcus* strains (Hess et al. 2001). Further mechanistic study is needed to adequately address these observations.

Previous measurements of extracellular superoxide production by *Synechococcus* have demonstrated overlapping rates ranging from ~ 10 amol $cell^{-1}$ h^{-1} to in excess of 100 amol $cell^{-1}$ h^{-1} (Rose et al. 2008*b*). The rates we measure for *Synechococcus* also overlap with the range in extracellular superoxide production rates of another cyanobacterium. *Trichodesmium*, a nitrogen-fixing cyanobacterium present in oligotrophic surface waters, produces extracellular superoxide at rates that range from 5 to 20 pmol L^{-1} $colony^{-1}$ h^{-1}, which, corresponds to approximately 250–1000 amol $cell^{-1}$ h^{-1} (assuming ~ 200 trichoms per colony and ~ 100 cells per trichome; Carpenter et al. 2004). *Lyngbya majuscula*, a coastal marine cyanobacterium, also demonstrates extracellular superoxide production, although the cell-specific rate was not quantified (Rose et al. 2005). These measurements suggest extracellular superoxide production is indeed a common trait among marine cyanobacteria. *Synechococcus* is second only to *Prochlorococcus* in cyanobacterium abundance in the ocean and is similarly globally distributed (Flombaum et al. 2013). Its widespread nature and relatively high extracellular superoxide production point toward *Synechococcus* as perhaps the most significant producer and biological regulator of extracellular ROS in the surface ocean.

As with *Prochlorococcus*, these are the first superoxide measurements of organisms belonging to the ubiquitous SAR11 clade. The average superoxide production rate of strains of HTCC1062 and HTCC7211 (0.16 amol $cell^{-1}$ h^{-1}) falls within previous

Table 2- Summary of extracellular superoxide decay among picoplankton.

Organism	Average net $O_2^{\bullet-}$ production (amol cell^{-1} h^{-1})	Pseudo-first-order decay rate constant ($\times 10^{-3}$ s^{-1})	Average standard recovery (%)	Average gross $O_2^{\bullet-}$ Production (amol cell^{-1} h^{-1})
Prochlorococcus MIT9312	0.019	13.9	27.5	0.070
Prochlorococcus MED4	0.003	8.5	40.8	0.007
Prochlorococcus NATL2A	0.023	8.8	37.2	0.061
Prochlorococcus MIT9313	0.050	9.6	54.2	0.091
Synechococcus sp. WH8102 (SN media)	103	20.7	13.1	786
Pelagibacter HTCC1062	0.15	7.6	83.5	0.18
Pelagibacter HTCC7211	0.16	5.9	74.4	0.22

measurements of marine heterotrophic bacteria. One previous survey of heterotrophic bacteria demonstrated that nearly all heterotrophic bacteria produce extracellular superoxide, with net production rates ranging from 0.003 to 13.1 amol cell^{-1} h^{-1} (Diaz et al. 2013). When we consider only previous measurements of marine bacteria in exponential phase belonging to the same phylum as *Pelagibacter*, *Alphaproteobacteria*, this range narrows to 0.04–1.7 amol cell^{-1} h^{-1}. *Roseobacter* spp., which can account for over 20% of marine bacteria in coastal waters (Brinkhoff et al. 2008), exhibits net extracellular superoxide at a rate of 0.09–0.3 amol cell^{-1} h^{-1}. The net extracellular superoxide production exhibited by *Pelagibacterales* is remarkably similar to previously characterized *Alphaproteobacteria* despite quite significant differences in metabolic lifestyles (i.e., oligotroph vs. copiotroph). The two *Pelagibacterales* ecotypes we examined in this study are found in contrasting regions of the surface ocean; HTCC1062 is a member of group Ia.1, which is found in colder high latitude regions, whereas HTCC7211 is a member of group Ia.3, which is found in warm stratified oceans (Giovannoni 2017). SAR11 cells are present and abundant throughout the dark ocean as well, providing a source of superoxide below the photic zone (Giovannoni 2017). *Pelagibacterales*, which contains the most highly conserved genome of any free-living bacteria, is thought to represent approximately 25% of cells in the global ocean (Morris et al. 2002; Grote et al. 2012; Giovannoni 2017). Although it has been shown that dark, extracellular superoxide production among marine heterotrophic bacteria is widespread, the confirmed production of superoxide by *Pelagibacterales*, the most abundant marine, heterotrophic bacterial group, suggests that superoxide production is ubiquitous across a diverse array of oceanic ecosystems (Diaz et al. 2013).

Given the wide range in cell size and hence surface area explored here, trends in extracellular superoxide production were also compared by normalizing rates to cell surface area (Table 1-). Surface-area estimates are derived from various sources as follows: *Synechococcus* (Olson et al. 1990), *Phaeocystis* (Moisan and Mitchell 1999), *Prochlorococcus* (Partensky et al. 1999), *Geminigera* (Johnson et al. 2009), and *Pelagibacter* (Zhao et al. 2017). The order of magnitude difference in net extracellular superoxide production between *Synechococcus* and the two algae, *Phaeocystis* and *Geminigera*, collapses to a much narrower range when normalized to cell surface area (Table 1-). *Synechococcus* (SN media) surface-area–normalized rates ranged from 36 to 176 amol μm^{-2} h^{-1}, *Synechococcus* (Pro99 media) ranged from 1.4 to 21.2 amol μm^{-2} h^{-1}, *Phaeocystis* produced between 33 and 83 amol μm^{-2} h^{-1}, and *Geminigera* produced between 3.1 and 28 amol μm^{-2} h^{-1}. Extracellular superoxide production rates by *Prochlorococcus*, however, are not reconciled with those of other organisms when normalized to surface area. The typical *Synechococcus* cell has about three times the surface area of the typical *Prochlorococcus* cell, (Olson et al. 1990; Partensky et al. 1999) but the measured extracellular superoxide production rates differ by more than two orders of magnitude. The net extracellular superoxide production rate by *Pelagibacter* falls toward the low end of that of previously observed in marine heterotrophs when normalized to cell number, but when normalized to cell surface area, *Pelagibacter* exhibits a net superoxide production rates that are well within the range of other marine heterotrophs (between 0.38 and 0.79 amol μm^{-2} h^{-1}) (Diaz et al. 2013). *G. cryophila*, the organism in this study that is capable of phototrophy and heterotrophy, produced extracellular superoxide at rates in a range that falls in between the phototrophs and heterotrophs in this study. The differences between phototrophs and heterotrophs with respect to extracellular superoxide production and the similarity of surface-area–normalized extracellular superoxide production rates among many phototrophs and heterotrophs suggest that extracellular superoxide production may be fundamentally related to carbon acquisition. However, more information about the pathways of extracellular superoxide production is needed to make such a determination.

Overall, these findings highlight significant potential sources of ROS to the surface and deep ocean. Although these measurements were collected under laboratory conditions with nutrient amended artificial or natural seawater, the maintenance of extracellular superoxide concentrations ranging from ~ 100 pmol L^{-1} to > 10 nmol L^{-1} is consistent with observations of dark

extracellular superoxide production in natural waters (Rose et al. 2008*b*; Hansard et al. 2010; Rusak et al. 2011; Diaz et al. 2016; Roe et al. 2016). The similarity between extracellular superoxide concentrations observed in culture studies and natural waters is consistent with the ability of marine microbes to regulate extracellular ROS in aquatic systems.

Cell number effects on superoxide production

Steady-state net extracellular superoxide production rates varied as a function of cell number (Fig. 1). Generally speaking, as cell number increased, the cell-normalized net extracellular superoxide production decreased. The effects of decreasing per-cell net production rate were apparent in the measured steady-state superoxide concentrations (Fig. 2; representative FeLume and corresponding concentration data for *Phaeocystis*). As cell number increased, the steady-state superoxide concentration also increased. However, this increase was not proportional to the number of cells; the marginal increase in extracellular superoxide production diminished with increasing cell numbers. In some cases, this trend reached a maximum steady-state superoxide concentration, and subsequent cell additions led to a small decrease in superoxide concentration. These cell-number–dependent trends were observed for all organisms investigated in this study with the exception of *Prochlorococcus*. Due to the high cell numbers required to obtain a detectable superoxide signal for *Prochlorococcus* using this methodology, sequential loading experiments did not allow us to determine what, if any, population effects may be at work in *Prochlorococcus* cultures.

To test whether the same trend was observed when cells were diluted prior to analysis, as opposed to sequential addition during analysis, superoxide production was measured for serially diluted cultures of *Synechococcus* WH8102 (Fig. 3). Exponential phase cells of *Synechococcus* were diluted 10- and 100-fold into sterile seawater, and extracellular superoxide production was analyzed by loading a single aliquot after 3 and 7.5 h following dilution. We found similar cell-density trends for dilution series and sequentially loaded *Synechococcus* cells. Although the per-cell superoxide production rates did not change significantly between the undiluted and the 10-fold dilution ($p = 0.11$ and $p = 0.30$ for 3 and 7.5 h time point, respectively, using two-sample *t*-test), the 100-fold dilution demonstrated a significantly higher cell-normalized superoxide production rate at both time points ($p = 0.02$ and $p = 0.004$ for the 3 and 7.5 h time point, respectively). Additionally, the 100X dilution at the 7.5 h time point yielded nearly twice the cell-normalized superoxide production rate than the 3 h time point.

The low cell-normalized net superoxide production rate by *Prochlorococcus* under the conditions of this study made it difficult to draw any direct comparisons between *Synechococcus* and *Prochlorococcus* at the same cell number. To account for differences in extracellular superoxide production that may arise from growth conditions, we measured extracellular superoxide production rates by *Synechococcus* and *Prochlorococcus* controlling for growth media. Figure 4 shows the net extracellular superoxide production rate of *Synechococcus* and *Prochlorococcus* grown in Sargasso Sea Pro99 media at similar cell numbers ranging from ~ 10^5 to ~ 10^7 cells. *Synechococcus* grown under these conditions produced net superoxide ranging from 4.3 amol cell^{-1} h^{-1} at 6.1 × 10^6 cells to 67 amol cell^{-1} h^{-1} at 1.5 × 10^5 cells, whereas *Prochlorococcus* produced net extracellular superoxide at undetectable levels. The cell number trend seen for *Synechococcus* grown in Vineyard Sound SN media is still quite apparent for *Synechococcus* grown in Sargasso Sea Pro99 media. Net extracellular superoxide production rates were similar between *Synechococcus* WH8102 and *Synechococcus* WH7803, which we discuss below. Although both strains of *Synechococcus* produce significantly more extracellular superoxide than *Prochlorococcus*, the more nutrient-poor Pro99 media influenced the net extracellular superoxide produced by *Synechococcus*. At the same cell density, *Synechococcus* WH8102 grown in Sargasso Sea Pro99 media produced extracellular superoxide at a rate of one to two orders of magnitude less than that observed in Vineyard Sound SN media. Such a difference suggests that nutrient replete coastal waters may be greater sources of extracellular superoxide to the ocean than their nutrient deprived counterparts. Additionally, this suggests that growth rate may be a factor in determining extracellular superoxide production rate.

The cell-number trends that we see across this suite of disparate organisms have been previously recorded for several HAB-forming algae and the cyanobacterium *Trichodesmium* (Marshall et al. 2005; Hansel et al. 2016; Diaz et al. 2018). Our work and these previous works establish cell abundance control on ROS as a common trait among extremely diverse groups of marine microorganisms. This cell number response is consistent with cell signaling behavior and may point to extracellular superoxide production as a key physiological process within these microbes. Although a more general response to changing environmental conditions cannot be ruled out, the only systematic change between measurements of a given microbe was the number of cells loaded on the filter. Therefore, any change in environmental conditions is most likely cell-density–dependent. At the very least, we can rule out a systematic effect of cell biomass and growth stage as cells were added from the same culture. The extracellular concentration of ROS is likely set by a consortium of microbial members that produce and degrade extracellular superoxide, whereby superoxide levels likely evolve toward some community optimum.

Superoxide decay and gross superoxide production

To determine the role of superoxide decay on measured net superoxide production rates, a subset of measurements was conducted on *Synechococcus*, *Pelagibacter*, and *Prochlorococcus* using standard superoxide additions (Table 2-; Fig. 5). The recovery of standard KO_2 spikes provides insight as to the general mechanisms by which organisms modulate extracellular superoxide concentration, specifically via production or decay. The pseudo-first-order decay rate constants among these three picoplankton

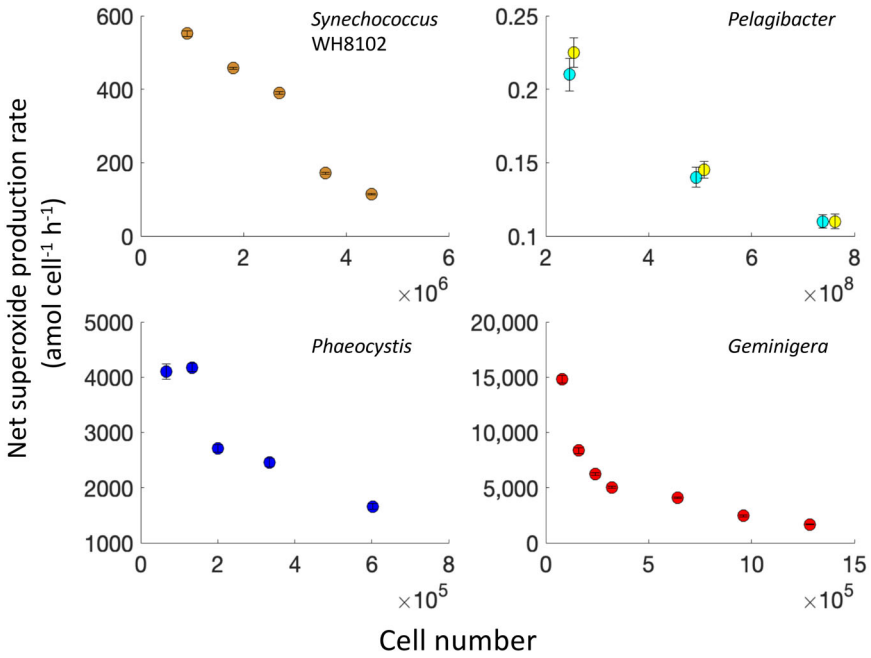

Fig. 1- Net extracellular superoxide production rates (amol cell^{-1} h^{-1}) of *Synechococcus* sp. WH8102 grown in SN media (top left, orange), *Pelagibacter* strain HTCC1062 (top right, cyan) and strain HTCC7211 (top right, yellow), *P. antarctica* (bottom left, blue), and *G. cryophila* (bottom right, red). Production rates are shown as a function of different number of cells added to the filter during analysis (added as sequential aliquots of a cell culture with cell densities provided in the Methods section). Error bars represent 1 standard error (SE) of the mean superoxide production rate of a single biological sample of the duration of the measurement.

ranged from 0.0059 to 0.0207 s^{-1}, with *Pelagibacter* exhibiting the lowest rate and *Synechococcus* exhibiting the highest (Table 2-). These decay rate constants of extracellular superoxide are similar to rates observed in culture and in natural seawater (Rose et al. 2008*b*; Diaz et al. 2013; Roe et al. 2016). For *Synechococcus*, the average recovery for a superoxide standard addition (i.e., the fraction of added superoxide that was not degraded by cells) was 13.1% (SEM = 5.5%) and did not correlate with the number of cells used in the measurement (R^2 = 0.03, cell number range: 1.5×10^5 to 3.6×10^6 cells). Both strains of *Pelagibacter* produced an average yield of 78.9% (SEM = 11.1%), and similarly did not correlate with cell number (R^2 = 0.002, cell number range: 2.5×10^8 to 7.5×10^8 cells). The four *Prochlorococcus* strains had standard recoveries ranging from 27.5% to 54.2%. The HL strain MIT9312, which represents the most abundant *Prochlorcoccus* ecotype, exhibited the highest superoxide decay rate constant and lowest recovery of exogenous superoxide among other *Prochlorococcus* strains, 27.5% (Table 2-). This elevated superoxide decay rate constant may be related to an inherent capacity to degrade photochemically generated superoxide in the uppermost surface waters (Powers and Miller 2014). These values highlight the elevated ability for *Synechococcus* and to a lesser extent *Prochlorococcus* to eliminate extracellular superoxide. The relative insensitivity of extracellular superoxide degradation to cell number also suggests that variation in the extracellular superoxide concentrations observed here is primarily a function of rapid changes in production.

Gross extracellular superoxide production, much like net extracellular superoxide production, exhibited a wide range among the picoplankton analyzed in this study (Table 2-). Average gross extracellular superoxide production rates were 786 amol cell^{-1} h^{-1} for *Synechococcus*, 0.057 amol cell^{-1} h^{-1} for four *Prochlorococcus* strains, and 0.20 amol cell^{-1} h^{-1} for two ecotypes of *Pelagibacter*. The relatively high cell-normalized superoxide production rate by *Synechococcus* has been previously reported, but its elevated capacity to degrade exogenous superoxide indicates that its gross production is nearly 10 times higher than measurements of net superoxide production would suggest (Rose et al. 2008*b*). Its ability to degrade nearly 90% of the exogenous superoxide also suggests that *Synechococcus* may play an important role in controlling superoxide levels within the surface ocean via antioxidant pathways.

Insights into marine ROS formation

Despite a widespread ability of marine microbes to produce extracellular superoxide, the reasons for this process and underlying mechanisms remain unclear. Extracellular superoxide production rates are dependent upon physiological and environmental factors, such as cell growth stage, cell density, light intensity, iron availability, and overall nutrient availability (Rose et al. 2008*b*; Diaz et al. 2013, 2018; Hansel et al. 2016; Schneider et al. 2016; Hansel et al. 2019). The data we present in this study expand the number of organisms known to display cell-density–dependent superoxide production to

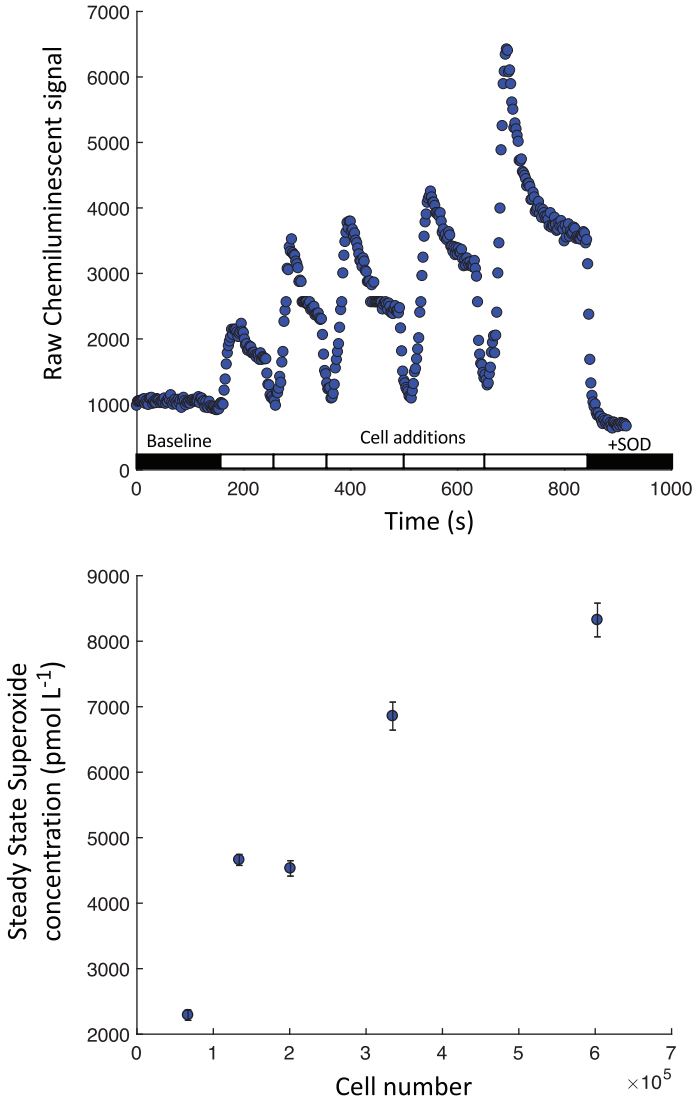

Fig. 2- (top) FeLume trace collected from *P. antarctica* cells. Baseline, sequential cell additions, and SOD additions are indicated in the black and white boxes below the trace. (bottom) Steady-state superoxide concentration (pmol L^{-1}) produced by cells of *P. antarctica* harvested at exponential growth phase. Data shown from one bio replicate; error bars represent 1 SE of FeLume signal stability.

include additional eukaryotic algae, picocyanobacteria, and picobacteria. Although we cannot make broad claims about the functionality of extracellular superoxide in these organisms based on these observations, the apparent upregulation of biological superoxide production at low cell densities is certainly consistent with a role for extracellular superoxide in cell signaling and/or cell growth (Buetler et al. 2004). In fact, recent studies found that extracellular superoxide within the widespread *Roseobacter* clade is tightly regulated via both production and decay processes over the course of a life cycle (Hansel et al. 2019). Removal of this extracellular superoxide greatly inhibited cell growth, pointing to an essential role for superoxide in growth by this ubiquitous bacterial group. For the eukaryotic microbes explored here (*Geminigera* and *Phaeocystis*), extracellular superoxide may be produced by NAD(P)H oxidases, the widespread eukaryotic enzyme that is involved in fungal and plant extracellular superoxide production (Lara-Ortíz et al. 2003). Similar enzymes have also been implicated in superoxide production by the coral algal symbiont *Symbiodinium* (Saragosti et al. 2010), the toxic raphidophyte *Chattonella* (Kim et al. 2000), and the diatoms *Thalassiosira weissflogii* and *Thalassiosira pseudonana* (Kustka et al. 2005). The enzymes responsible for extracellular superoxide production have not been established in *Synechococcus* or *Prochlorococcus*, hindering predictions of the mechanisms at play in these microorganisms. Within heterotrophic bacteria, heme peroxidases are responsible for the formation of extracellular superoxide production by a bacterium within the common marine *Roseobacter* clade (Andeer et al. 2015). Similar heme peroxidases are not annotated within current *Pelagibacter* genomes. Considering that the heme peroxidase in *Roseobacter* sp. is large (~ 3500 amino acids; Andeer et al. 2015) and the fact that *Pelagibacter* possesses a streamlined genome, alternative enzymes are clearly responsible for extracellular superoxide production in this organism. In addition to enzymes responsible for superoxide production, the superoxide scavenger SOD is responsible for regulation of extracellular superoxide levels in some organisms, including the bacteria *Escherichia coli* and *Salmonella typhimurium* (Carlioz and Touati 1986; Storz et al. 1987) and is likely responsible at least in part for the superoxide decay observed here. Clearly, further insight into the processes at play in these superoxide dynamics is needed, and future investigations will specifically target the biochemical process(es) responsible for superoxide production within these key marine microbes.

Prochlorococcus appears to be an outlier among both picoplankton and marine oxygenic phototrophs with respect to extracellular superoxide production. One study observed the expression of genes related to ROS protection and detoxification through a diel cycle for *Synechococcus* and *Prochlorococcus* and found that oxidative stress from both external sources and internal sources (such as the Mehler reaction) can arise from excess visible and ultraviolet light (Mella-Flores et al. 2012). *Synechococcus* exhibited strongly upregulated SOD production during the most intense sunlight hours, whereas *Prochlorococcus* exhibited weakly downregulated SOD production during the most intense sunlight hours and slightly upregulated SOD production in the dark (Mella-Flores et al. 2012). Although *Synechococcus* and *Prochlorococcus* contain different SOD genes (*sodB* and *sodC* in *Synechococcus* vs. *sodN* in *Prochlorococcus*), the divergent behavior with respect to the elimination of superoxide cannot be readily explained. If extracellular superoxide production is involved in cell growth promotion, this divergent behavior could arise from the inability of *Prochlorococcus* to produce optimal extracellular superoxide for its own growth, thus relying on exogenous sources. However, such a conclusion cannot be drawn from this study.

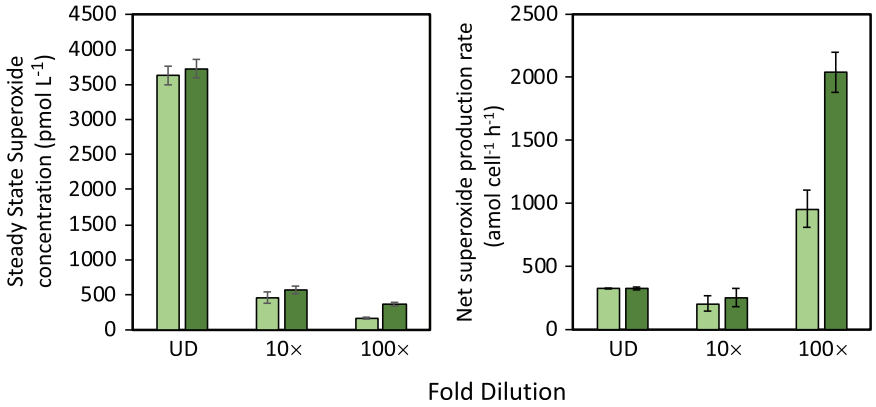

Fig. 3- Steady-state extracellular superoxide concentration (left) and cell-normalized net superoxide production rate (right) after 3 hours (light green) and 7.5 hours (dark green). Cell numbers range from 1.3 × 10^6 cells in the undiluted cultures to 2.2 × 10^4 cells in the 100X dilution at the end of the 7.5 h time point. Error bars represent 1 SE of two biological replicates.

The mechanisms of extracellular superoxide production are not well known, so the low relative extracellular ROS production by *Prochlorococcus* cannot be readily explored with genomic or transcriptomic tools at this time. It would be consistent with the genomic streamlining in *Prochlorococcus* to lose much of the ability to produce extracellular superoxide in the presence of abundant sources in the water column (Biller et al. 2015). *Prochlorococcus* is known to take advantage of the ROS degradation capacity of other organisms in the water column to compensate its own ROS degradation deficiencies (Morris et al. 2008, 2011). Itself lacking the ability to produce catalase, *Prochlorococcus* relies on passive diffusion of hydrogen peroxide across the cell membrane where other organisms can degrade it (Morris et al. 2011). Superoxide production and degradation via a reductive pathway or dismutation will lead to hydrogen peroxide formation (Wuttig et al. 2013*b*). Thus, extracellular superoxide production may ultimately be a disadvantage for *Prochlorococcus* if it adds additional oxidative stress in the form of intracellular hydrogen peroxide. It is also important to note that the *Prochlorococcus* cell numbers added to the filters during superoxide analysis in this study far exceed those of

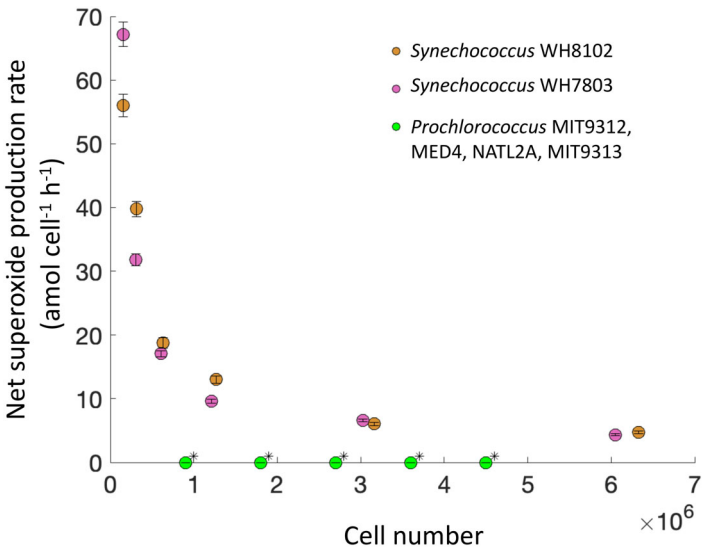

Fig. 4- Direct comparison of net extracellular superoxide production rates as a function of cell number by *Synechococcus* WH8102 (orange), *Synechococcus* WH7803 (pink), *Prochlorococcus* MIT9312 (green), *Prochlorococcus* MED4 (green), *Prochlorococcus* NATL2A (green), and *Prochlorococcus* MIT9313 (green), all grown in Sargasso Sea Pro99 media (see Methods section). All *Prochlorococcus* strains were measured at the same cell numbers and produced net extracellular superoxide below the detection limit (indicated with *), thus each green circle represents four independent measurements.

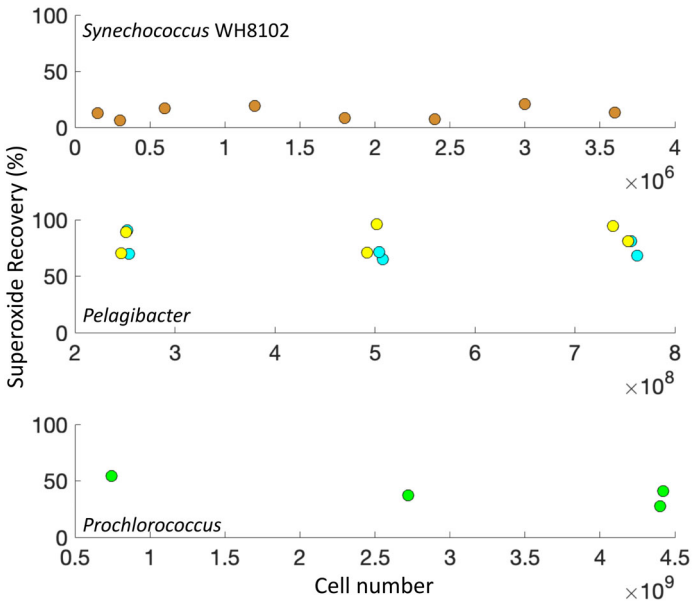

Fig. 5- Recovery of standard superoxide spikes (as KO_2) through filter-supported cells. Average superoxide recovery for *Synechococcus* sp. WH8102 (orange) is 13.1% ± 5.5% (R^2 = 0.03). Average recovery for *Pelagibacter* strain HTCC1062 (cyan) and strain HTCC7211 (yellow) is 78.9% ± 11.1% (R^2 = 0.002). Average recovery for four *Prochlorococcus* strains (light green) is 39.9% ± 11.0% (R^2 = 0.68).

natural waters because of its low extracellular superoxide production rate; extracellular superoxide dynamics may differ at lower cell densities. We also must consider the possibility that *Prochlorococcus* in axenic culture may produce less extracellular superoxide to manage hydrogen peroxide concentrations, which may not be representative of the natural environment, where ROS-degrading microbial associates are likely present. The notion that *Prochlorococcus* produces less extracellular superoxide for the sole purpose of managing hydrogen peroxide stress becomes less tractable, however, when comparing the net extracellular superoxide production rates by *Synechococcus* WH8102 and *Synechococcus* WH7803 (Fig. 4), which are catalase negative and catalase positive, respectively (Scanlan et al. 2009). These two strains produce net extracellular superoxide at similar rates, suggesting that upstream regulation of hydrogen peroxide levels via modulation of extracellular superoxide production does not occur. More studies are needed to detail the mix of extracellular and intracellular superoxide production and the spatial allocation of SOD within cells to better understand the economy of ROS in and around cells. Coculture studies and culture studies with an artificial superoxide source are potential ways to address the potential role of extracellular superoxide in *Prochlorococcus* physiology.

Summary and conclusions

Here, we report dark, extracellular superoxide production by five widespread marine microbe groups: *Synechococcus*, *Prochlorococcus*, *Pelagibacter*, *Phaeocystis*, and *Geminigera*. All organisms produced measurable dark extracellular superoxide, exhibiting a large range in steady-state superoxide concentrations and cell-normalized production rates. *Synechococcus*, *Pelagibacter*, *Phaeocystis*, and *Geminigera* decreased their per-cell net superoxide production rate with increasing cell number. Recovery of standard superoxide additions in the form of KO_2 did not vary significantly with cell number, suggesting that changes in the net generation of superoxide as a function of cell number are driven by changes in gross production.

Extracellular superoxide production was markedly lower for all four strains of *Prochlorococcus* compared with other organisms assessed in this study. The data presented here and previous studies interrogating ROS degradation by *Prochlorococcus* suggest that *Prochlorococcus* has a different relationship altogether with extracellular superoxide (Mella-Flores et al. 2012). Should *Prochlorococcus* indeed have a physiological need for extracellular superoxide, as appears to be the case for some microbes that utilize it as an autocrine growth promotor or as a means to influence iron bioavailability (Buetler et al. 2004; Rose 2012), it is possible that it relies upon extracellular superoxide produced by other organisms and/or abiotic processes to meet this need.

The data we collected in this study highlight the dynamic nature of ROS cycling in representative microorganisms that are present throughout the global ocean. The suite of globally significant marine organisms in this study demonstrates a significant flux of superoxide that is similarly globally distributed. Four of the five organisms in this study are oxygenic phototrophs or mixotrophs that live in the surface ocean. Previous work has shown that these photosynthetic organisms may produce significant internal ROS from light-dependent processes, but we show here that these organisms are also prolific producers of superoxide in the dark. Furthermore, light has been shown to increase extracellular superoxide levels by some phytoplankton (Hansel et al. 2016; Schneider et al. 2016; Zhang et al. 2016*a*; Diaz et al. 2018), suggesting that these organisms may be even greater sources of marine superoxide, depending on prevailing light conditions. The significant superoxide flux suggested by our measurements has further implications on trace nutrient cycling within the ocean. Superoxide has been suggested to play a role in nutrient acquisition, particularly in the case of metals that are mobilized via reaction with superoxide (Kustka et al. 2005). Iron is one such metal; superoxide reduces Fe^{3+} to Fe^{2+} under surface ocean conditions (Kustka et al. 2005; Rose et al. 2008*a*). In addition to Fe, organic carbon, copper (Cu), and manganese (Mn) have been shown to be primary sinks of superoxide in the surface ocean (Wuttig et al. 2013*a*,*b*). Superoxide production in the ocean and its reactivity with these biologically significant nutrients, electron donors, and electron acceptors suggest that superoxide, and ROS in general, provides an abiotic shunt in the cycling of redox active element cycles, the scope of which has yet to be realized.

With this study, we continue to expand the measurements of cell-specific dark extracellular superoxide production rates. Here, we focused on organisms belonging to globally widespread groups to demonstrate the significant potential of superoxide flux in the global ocean. Extracellular superoxide production appears to be widespread throughout the surface and deep ocean. Whether such significant production is largely for physiological benefit or a byproduct of cellular metabolisms remains unclear, but regardless, extracellular superoxide production certainly has consequences for elemental cycling and marine microbial ecology throughout the global ocean.

References

Alvain, S., C. Moulin, Y. Dandonneau, and H. Loisel. 2008. Seasonal distribution and succession of dominant phytoplankton groups in the global ocean: A satellite view. **22**: 1–15. doi:10.1029/2007GB003154.

Andeer, P. F., D. R. Learman, M. McIlvin, J. A. Dunn, and C. M. Hansel. 2015. Extracellular haem peroxidases mediate Mn(II) oxidation in a marine R oseobacter bacterium via superoxide production. Environ. Microbiol. **17**: 3925–3936. doi:10.1111/1462-2920.12893.

Asada, K. 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol. **141**: 391–396. doi:10.1104/pp.106.082040.

Berube, P. M., and others. 2015. Physiology and evolution of nitrate acquisition in *Prochlorococcus*. ISME J. **9**: 1195–1207. doi:10.1038/ismej.2014.211,

Bielski, B. H. J., D. E. Cabelli, R. L. Arudi, and A. B. Ross. 1985. Reactivity of HO2/O−2Radicals in aqueous solution. J. Phys. Chem. Ref. Data Monogr. **14**: 1041–1100. doi:10.1063/1.555739.

Biller, S. J., P. M. Berube, D. Lindell, and S. W. Chisholm. 2015. *Prochlorococcus*: The structure and function of collective diversity. Nat. Rev. Microbiol. **13**: 13–27. doi:10.1038/nrmicro3378.

Brinkhoff, T., H. A. Giebel, and M. Simon. 2008. Diversity, ecology, and genomics of the Roseobacter clade: A short overview. Arch. Microbiol. **189**: 531–539. doi:10.1007/s00203-008-0353-y.

Buetler, T. M., A. Krauskopf, and U. T. Ruegg. 2004. Role of superoxide as a signaling molecule. Phys. Ther. **19**: 120–123. doi:10.1152/nips.01514.2003.

Carini, P., L. Steindler, S. Beszteri, and S. J. Giovannoni. 2013. Nutrient requirements for growth of the extreme oligotroph "Candidatus Pelagibacter ubique" HTCC1062 on a defined medium. ISME J. **7**: 592–602. doi:10.1038/ismej.2012.122.

Carlioz, A., and D. Touati. 1986. Isolation of superoxide dismutase mutants in *Escherichia coli*: Is superoxide dismutase necessary for aerobic life? EMBO J. **5**: 623–630. doi:10.1002/j.1460-2075.1986.tb04256.x.

Carpenter, E. J., A. Subramaniam, and D. G. Capone. 2004. Biomass and primary productivity of the cyanobacterium *Trichodesmium* spp. in the tropical N Atlantic Ocean. Deep-Sea Res. Part I Oceanogr. Res. Pap. **51**: 173–203. doi:10.1016/j.dsr.2003.10.006.

Diaz, J. M., C. M. Hansel, A. Apprill, C. Brighi, T. Zhang, L. Weber, S. McNally, and L. P. Xun. 2016. Species-specific control of external superoxide levels by the coral holobiont during a natural bleaching event. Nat. Commun. **7**. doi:10.1038/ncomms13801.

Diaz, J. M., C. M. Hansel, B. M. Voelker, C. M. Mendes, P. F. Andeer, and T. Zhang. 2013. Widespread production of extracellular superoxide by heterotrophic bacteria. Science **340**: 1223–1226. doi:10.1126/science.1237331.

Diaz, J. M., and S. Plummer. 2018. Production of extracellular reactive oxygen species by phytoplankton: Past and future directions. J. Plankton Res **40**: 655–666. doi:10.1093/plankt/fby039.

Diaz, J. M., S. Plummer, C. Tomas, and C. Alves-de-souza. 2018. Production of extracellular superoxide and hydrogen peroxide by five marine species of harmful bloom-forming algae. J. Plankton Res. **40**: 1–11. doi:10.11821/dlxb201802008.

Dickinson, B. C., and C. J. Chang. 2011. Chemistry and biology of reactive oxygen species in signaling or stress responses. Nat. Chem. Biol. **7**: 504–511. doi:10.1038/nchembio.607.

Flombaum, P., and others. 2013. Present and future global distributions of the marine cyanobacteria *Prochlorococcus* and *Synechococcus*. Proc. Natl. Acad. Sci. USA **110**: 9824–9829. doi:10.1073/pnas.1307701110,

Fridovich, I. 1983. Superoxide radical—an endogenous toxicant. Annu. Rev. Pharmacol. Toxicol. **23**: 239–257. doi:10.1146/annurev.pa.23.040183.001323.

Fridovich, I. 1998. Oxygen toxicity: A radical explanation. J. Exp. Biol. **201**: 1203–1209.

Gast, R. J., Z. M. Mckie-krisberg, S. A. Fay, J. M. Rose, and R. W. Sanders. 2014. Antarctic mixotrophic protist abundances by microscopy and molecular methods. FEMS Microbiol. Ecol. **89**: 388–401. doi:10.1111/1574-6941.12334.

Giovannoni, S. J. 2017. SAR11 bacteria: The most abundant plankton in the oceans. Ann. Rev. Mar. Sci. **9**: 231–255. doi:10.1146/annurev-marine-010814-015934.

Giovannoni, S. J., and others. 2005. Genome streamlining in a cosmopolitan oceanic bacterium. Science **309**: 1242–1245, DOI: 10.1126/science.1114057,

Grote, J., J. C. Thrash, M. J. Huggett, Z. C. Landry, P. Carini, S. J. Giovannoni, and M. S. Rappé. 2012. Streamlining and core genome conservation among highly divergent members of the SAR11 clade. MBio **3**. doi:10.1128/mBio.00252-12.

Guillard, R. R. L. 1975. Culture of phytoplankton for feeding marine invertebrates, p. 29–60. *In* Culture of marine invertebrate animals. Springer. doi:10.1007/978-1-4615-8714-9_3

Gus' kova, R. A., I. I. Ivanov, V. K. Kol'tover, V. V. Akhobadze, and A. B. Rubin. 1984. Permeability of bilayer lipid membranes for superoxide (O2−) radicals. Biochim. Biophys. Acta Biomemb **778**: 579–585. doi:10.1016/0005-2736(84)90409-7.

Hansard, S. P., A. W. Vermilyea, and B. M. Voelker. 2010. Deep-sea research I measurements of superoxide radical concentration and decay kinetics in the Gulf of Alaska. Deep-Sea Res. Part I Oceanogr. Res. Pap. **57**: 1111–1119. doi:10.1016/j.dsr.2010.05.007.

Hansel, C. M., C. Buchwald, J. M. Diaz, J. E. Ossolinski, S. T. Dyhrman, B. A. S. Van Mooy, and D. Polyviou. 2016. Dynamics of extracellular superoxide production by *Trichodesmium* colonies from the Sargasso Sea. Limnol. Oceanogr. **61**: 1188–1200. doi:10.1002/lno.10266.

Hansel, C. M., J. M. Diaz, and S. Plummer. 2019. Tight regulation of extracellular superoxide points to its vital role in the physiology of the globally relevant Roseobacter clade. **10**: 1–13.

Heller, M. I., and P. L. Croot. 2010. Kinetics of superoxide reactions with dissolved organic matter in tropical Atlantic surface waters near Cape Verde (TENATSO). J. Geophys. Res. **115**: 1–13. doi:10.1029/2009jc006021.

Heller, M. I., and P. L. Croot. 2011. Superoxide decay as a probe for speciation changes during dust dissolution in tropical Atlantic surface waters near Cape Verde. Mar. Chem. **126**: 37–55. doi:10.1016/j.marchem.2011.03.006.

Heller, M. I., K. Wuttig, and P. L. Croot. 2016. Identifying the sources and sinks of CDOM/FDOM across the Mauritanian shelf and their potential role in the decomposition of superoxide (O2-). Front. Mar. Sci. **3**: 1–19. doi:10.3389/fmars.2016.00132.

Hess, W. R., G. Rocap, C. S. Ting, F. Larimer, S. Stilwagen, J. Lamerdin, and S. W. Chisholm. 2001. The photosynthetic

apparatus of *Prochlorococcus*: Insights through comparative genomics. Photosynth. Res. **70**: 53–71. doi:10.1023/A:1013835924610.

Johnson, M. D., J. Volker, H. V. Moeller, E. Laws, K. J. Breslauer, and P. G. Falkowski. 2009. Universal constant for heat production in protists. Proc. Natl. Acad. Sci. USA **106**: 6696–6699. doi:10.1073/pnas.0902005106.

Johnson, Z. I., E. R. Zinser, A. Coe, N. P. McNulty, E. M. S. Woodward, and S. W. Chisholm. 2006. Niche partitioning among *Prochlorococcus* ecotypes along ocean-scale environmental gradients. Science **311**: 1737–1740. doi:10.1126/science.1118052.

Kettler, G. C., and others. 2007. Patterns and implications of gene gain and loss in the evolution of *Prochlorococcus*. PLoS Genet. **3**: 2515–2528. doi:10.1371/journal.pgen.0030231,

Kim, D., A. Nakamura, T. Okamoto, N. Komatsu, T. Oda, T. Iida, A. Ishimatsu, and T. Muramatsu. 2000. Mechanism of superoxide anion generation in the toxic red tide phytoplankton Chattonella marina: Possible involvement of NAD(P)H oxidase. Biochim. Biophys. Acta Gen. Subj. **1524**: 220–227. doi:10.1016/S0304-4165(00)00161-6.

Korshunov, S. S., and J. A. Imlay. 2002. A potential role for periplasmic superoxide dismutase in blocking the penetration of external superoxide into the cytosol of Gram-negative bacteria. Mol. Microbiol. **43**: 95–106. doi:10.1046/j.1365-2958.2002.02719.x.

Kustka, A. B., Y. Shaked, A. J. Milligan, D. W. King, and F. M. M. Morel. 2005. Extracellular production of superoxide by marine diatoms: Contrasting effects on iron redox chemistry and bioavailability. Limnol. Oceanogr. **50**: 1172–1180. doi:10.4319/lo.2005.50.4.1172.

Lamb, C., and R. A. Dixon. 1997. The oxidative burst in plant disease resistance. Annu. Rev. Plant Biol. **48**: 251–275. doi:10.1146/annurev.arplant.48.1.251.

Lara-Ortíz, T., H. Riveros-Rosas, and J. Aguirre. 2003. Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate sexual development in Aspergillus nidulans. Mol. Microbiol. **50**: 1241–1255. doi:10.1046/j.1365-2958.2003.03800.x.

Learman, D. R., B. M. Voelker, A. I. Vazquez-Rodriguez, and C. M. Hansel. 2011. Formation of manganese oxides by bacterially generated superoxide. Nat. Geosci. **4**: 95–98. doi:10.1038/ngeo1055.

Marshall, J.-A., M. Hovenden, T. Oda, and G. M. Hallegraeff. 2002. Photosynthesis does influence superoxide production in the ichthyotoxic alga Chattonella marina (Raphidophyceae). J. Plankton Res. **24**: 1231–1236. doi:10.1093/plankt/24.11.1231.

Marshall, J.-A., T. Ross, S. Pyecroft, and G. Hallegraeff. 2005. Superoxide production by marine microalgae: II. Towards understanding ecological consequences and possible functions. Mar. Biol. **147**: 541–549. doi:10.1007/s00227-005-1597-6.

McKie-Krisberg, Z. M., R. J. Gast, and R. W. Sanders. 2015. Physiological responses of three species of Antarctic mixotrophic phytoflagellates to changes in light and dissolved nutrients. Microb. Ecol. **70**: 21–29. doi:10.1007/s00248-014-0543-x.

Mella-Flores, D., and others. 2012. *Prochlorococcus* and *Synechococcus* have evolved different adaptive mechanisms to cope with light and UV stress. Front. Microbiol. 3. doi:10.3389/fmicb.2012.00285.

Moisan, T. A., and B. G. Mitchell. 1999. Photophysiological acclimation of *Phaeocystis antarctica* Karsten under light limitation. Limnol. Oceanogr. **44**: 247–258. doi:10.4319/lo.1999.44.2.0247.

Moore, L. R., and others. 2007. Culturing the marine cyanobacterium *Prochlorococcus*. Limnol. Oceanogr.: Methods **5**: 353–362. doi:10.4319/lom.2007.5.353,

Morris, J. J., R. Kirkegaard, M. J. Szul, Z. I. Johnson, and E. R. Zinser. 2008. Facilitation of robust growth of *Prochlorococcus* colonies and dilute liquid cultures by "helper" heterotrophic bacteria. Appl. Environ. Microbiol. **74**: 4530–4534. doi:10.1128/AEM.02479-07.

Morris, J. J., Z. I. Johnson, M. J. Szul, M. Keller, and E. R. Zinser. 2011. Dependence of the cyanobacterium *Prochlorococcus* on hydrogen peroxide scavenging microbes for growth at the ocean's surface. PLoS One **6**: e16805. doi:10.1371/journal.pone.0016805.

Morris, R. M., M. S. Rappé, S. A. Connon, K. L. Vergin, W. A. Siebold, C. A. Carlson, and S. J. Giovannoni. 2002. SAR11 clade dominates ocean surface bacterioplankton communities. Nature **420**: 806–810. doi:10.1038/nature01240.

Olson, R. J., S. W. Chisholm, E. R. Zettler, and E. V. Armbrust. 1990. Pigments, size, and distribution of synechococcus in the North-Atlantic and Pacific oceans. Limnol. Oceanogr. **35**: 45–58. doi:10.4319/lo.1990.35.1.0045.

Partensky, F., W. R. Hess, and D. Vaulot. 1999. *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. Microbiol. Mol. Biol. Rev. **63**: 106–+.

Powers, L. C., and W. L. Miller. 2014. Blending remote sensing data products to estimate photochemical production of hydrogen peroxide and superoxide in the surface ocean. Environ. Sci. Impacts **16**: 792–806. doi:10.1039/c3em00617d.

Roe, K. L., R. J. Schneider, C. M. Hansel, and B. M. Voelker. 2016. Measurement of dark, particle-generated superoxide and hydrogen peroxide production and decay in the subtropical and temperate North Pacific Ocean. Deep-Sea Res. Part I Oceanogr. Res. Pap. **107**: 59–69. doi:10.1016/j.dsr.2015.10.012.

Rose, A. L. 2012. The influence of extracellular superoxide on iron redox chemistry and bioavailability to aquatic microorganisms. Front. Microbiol. **3**: 1–21. doi:10.3389/fmicb.2012.00124.

Rose, A. L., T. P. Salmon, T. Lukondeh, B. A. Neilan, and T. D. Waite. 2005. Use of superoxide as an electron shuttle for iron acquasition by the marine cyanobacterium *Lyngbya majuscula*. Environ. Sci. Technol. **39**: 3708–3715. doi:10.1021/es048766c.

Rose, A. L., J. W. Moffett, and T. D. Waite. 2008*a*. Determination of superoxide in seawater using 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3(7H)-one chemiluminescence. Anal. Chem. **80**: 1215–1227. doi:10.1021/ac7018975.

Rose, A. L., E. A. Webb, T. D. Waite, and J. W. Moffett. 2008*b*. Measurement and implications of nonphotochemically generated superoxide in the equatorial Pacific Ocean. Environ. Sci. Technol. **42**: 2387–2393. doi:10.1021/es7024609.

Rusak, S. A., B. M. Peake, L. E. Richard, S. D. Nodder, and W. J. Cooper. 2011. Distributions of hydrogen peroxide and superoxide in seawater east of New Zealand. Mar. Chem. **127**: 155–169. doi:10.1016/j.marchem.2011.08.005.

Saito, M. A., J. W. Moffett, S. W. Chisholm, and J. B. Waterbury. 2002. Cobalt limitation and uptake in *Prochlorococcus*. Limnol. Oceanogr. **47**: 1629–1636. doi:10.4319/lo.2002.47.6.1629.

Saragosti, E., D. Tchernov, A. Katsir, and Y. Shaked. 2010. Extracellular production and degradation of superoxide in the coral stylophora pistillata and cultured symbiodinium. PLoS One **5**: 1–10. doi:10.1371/journal.pone.0012508.

Saran, M. 2003. To what end does nature produce superoxide? NADPH oxidase as an autocrine modifier of membrane phospholipids generating paracrine lipid messengers. Free Radic. Res. **37**: 1045–1059. doi:10.1080/10715760310001594631.

Scanlan, D. J., and others. 2009. Ecological genomics of marine picocyanobacteria. Microbiol. Mol. Biol. Rev. **73**: 249–299. doi:10.1128/MMBR.00035-08,

Schneider, R. J., K. L. Roe, C. M. Hansel, and B. M. Voelker. 2016. Species-level variability in extracellular production rates of reactive oxygen species by diatoms. Front. Chem. **4**: 1–13. doi:10.3389/fchem.2016.00005.

Schoemann, V., S. Becquevort, J. Stefels, W. Rousseau, C. Lancelot, V. Rousseau, and C. Lancelot. 2005. Phaeocystis blooms in the global ocean and their controlling mechanisms: A review. J. Sea Res. **53**: 43–66. doi:10.1016/j.seares.2004.01.008.

Storz, G., M. F. Christman, H. Siest, and B. N. Ames. 1987. Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*. Biochemistry **84**: 8917–8921. doi:10.1073/pnas.84.24.8917.

Vogt, M., and others. 2012. Global marine plankton functional type biomass distributions: *Phaeocystis* spp. Earth Syst. Sci. Data **4**: 107–120. doi:10.5194/essd-4-107-2012,

Wang, S. L., and J. K. Moore. 2011. Incorporating Phaeocystis into a Southern Ocean ecosystem model. J. Geophys. Res. **116**: 18. doi:10.1029/2009jc005817.

Waterbury, J. B., S. W. Watson, F. W. Valois, and D. G. Franks. 1986. Biological and ecological characterization of the marine unicellular cyanobacterium Synechococcus, p. 71–120. *In* T. Platt and W. W. K. Li [eds.], Photosynthetic picoplankton. Canadian Bulletin of Fisheries and Aquatic Sciences 214. Friesen Printers.

Wuttig, K., M. I. Heller, and P. L. Croot. 2013*a*. Reactivity of inorganic Mn and Mn Desferrioxamine B with O-2, O-2[–], and H2O2 in seawater. Environ. Sci. Technol. **47**: 10257–10265. doi:10.1021/es4016603.

Wuttig, K., M. I. Heller, and P. L. Croot. 2013*b*. Pathways of superoxide (O-2(–)) decay in the eastern tropical North Atlantic. Environ. Sci. Technol. **47**: 10249–10256. doi:10.1021/es401658t.

Zhang, T., J. M. Diaz, C. Brighi, R. J. Parsons, S. McNally, A. Apprill, and C. M. Hansel. 2016*a*. Dark production of extracellular superoxide by the coral Porites astreoides and representative Symbionts. Front. Mar. Sci. **3**: 1–16. doi:10.3389/fmars.2016.00232.

Zhang, T., C. M. Hansel, B. M. Voelker, and C. H. Lamborg. 2016*b*. Extensive dark biological production of reactive oxygen species in brackish and freshwater ponds. Environ. Sci. Technol. **50**: 2983–2993. doi:10.1021/acs.est.5b03906.

Zhao, X. W., C. L. Schwartz, J. Pierson, S. J. Giovannoni, J. R. McIntosh, and D. Nicastroa. 2017. Three-dimensional structure of the ultraoligotrophic marine bacterium "Candidatus Pelagibacter ubique". Appl. Environ. Microbiol. **83**: 1–14. doi:10.1128/aem.02807-16.

Zinser, E. R., and others. 2009. Choreography of the transcriptome, photophysiology, and cell cycle of a minimal photoautotroph, *Prochlorococcus*. PLoS One 4. doi:10.1371/journal.pone.0005135.

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Conflict of Interest

None declared.

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