APPENDIX S1: DETAILED METHODS

*Reagents and media*

All buffers were from Sigma (St. Louis, MO), except tricine, which was from Fisher (Waltham, MA). All buffers were of ≥ 99.5% purity. Buffers were prepared as 0.5 M stocks in milli-Q water, titrated to pH 8.0, sterile filtered using 0.22 μm PVDF Steriflip units (Millipore, Billerica, MA), and stored at 4ºC in the dark.

Rates of HOOH formation were assessed in Chelex-treated, trace-metal free media without nutrient amendments to avoid unwanted side-reactions between HOOH and media components such as Fe. Experiments were conducted either in 0.22 μm filtered seawater harvested from the continental shelf near the mouth of Chesapeake Bay (37° 6’ N, 75° 42’ W, 32 salinity) or in artificial seawater (Turk’s Island Sea Salts, ([Moore et al. 2007](#_ENREF_4)), 35 salinity). In both cases, water was passed through a Chelex-100 column (Bio-Rad, Hercules, CA) into an acid-washed polycarbonate bottle in a trace metal clean laminar flow hood ([Moore et al. 2007](#_ENREF_4)). Water was subsequently sterilized by boiling in a microwave oven three times on three consecutive days. Each time, the water was allowed to cool in the trace metal clean hood. Dissolved organic carbon (DOC) concentrations of seawater were assayed on triplicate samples using a TOC-VCP analyzer (Shimadzu, Kyoto, Japan) after the above treatments.

*Measurement of HOOH*

HOOH concentration was measured using an acridinium ester chemiluminescence method ([King et al. 2007](#_ENREF_2)) modified for use in an Orion-L microplate luminometer (Berthold Detection Systems, Pforzheim, Germany; [Morris et al. 2011](#_ENREF_5)). 10-methyl-9-(*p*-formylphenyl) acridinium carboxylate trifluoromethanesulfonate (acridinium ester, AE) was a generous gift of M. Gonsior (University of Otago, NZ). Briefly, replicate 200 μL aliquots of sample were loaded onto white, flat-bottomed 96-well plates (Costar #3912, Corning Life Sciences, Lowell, MA) along with HOOH standards (2-fold dilutions ranging from 15.625 nM to 1 μM) prepared using a seawater blank collected from 900 m depth in the oligotrophic South Pacific (32° 25’ S, 159° 5’ E). The blank water was passed through a 0.2 μm polycarbonate filter (GE Osmonics) at the collection site, then treated with 1 unit . mL-1 of catalase (see below) and stored in an opaque bottle at room temperature. The HOOH stock solution was prepared by diluting a 30% solution to approximately 25 mM, and then standardized using its absorbance at 240 nm on a DU-800 spectrophotometer (Beckman Coulter) and the molar absorbance coefficient 38.1 L . mol-1 . cm-1 ([Miller & Kester 1988](#_ENREF_3)). Where necessary, samples were diluted in blank water to bring them into the useful range for the AE response (0-1 μM) and samples of low salinity were diluted into blank water to compensate for the pronounced difference in clarity of seawater and deionized water after alkalinization. After loading, plates were incubated for 10 min in the same room as the luminometer to allow temperatures to equilibrate. The assay consisted of sequential injections of 50 μL 2 M Na2CO3 (pH 11.3) followed by 50 μL of 2.2 mg . L-1 AE (prepared in 1 mM phosphate buffer, pH 3). Immediately after addition of AE, light output was measured for 1 s. HOOH concentrations were determined by comparison of experimental aliquots to a regression line prepared from the HOOH standards run on the same plate. Two mechanical replicates were assayed for each sample.

*Kinetics of HOOH production in sterile media*

We followed the generation of HOOH in test tubes containing seawater amended with a variety of buffers (Table S1). All experiments were conducted on 8 mL of sterile sample in acid-washed borosilicate test tubes incubated at 22ºC under cool fluorescent illumination. Aliquots were removed aseptically periodically over the course of several weeks for HOOH determination. The long-term zero-order rate constant of HOOH formation, kHOOH, was calculated as the slope of HOOH vs. time based on the first 3-8 d of the experiment.

In one experiment, we reduced the dissolved oxygen content of the seawater by incubating the reaction vessels in an airtight anaerobe chamber containing a GasPak Plus O2 removal system (BD). Samples were equilibrated with the anoxic atmosphere in the dark for 1 week and then removed for buffer addition (either 10 mM TAPS or HEPES, or unbuffered control). At this time, HOOH was measured as described and the samples were re-sealed in the anaerobe chamber with a fresh GasPak, then incubated at 22ºC, 65 μmol photons . m-2 . s-1 for 17 d. At the end of this incubation period, HOOH levels were measured again.

*Effects of buffers on growth of* Prochlorococcus

Non-axenic cultures of *Prochlorococcus* were able to grow in media treated with 10 mM of most of the Good’s buffers we tested, but not in bicine or glycylglycine, and growth was highly variable in Tris (Fig. S4). Since heterotrophic contaminants present in these wild-type cultures can confer protection against oxidative stress on *Prochlorococcus* ([Morris et al. 2011](#_ENREF_5" \o "Morris, 2011 #2127)), we used an axenic strain of *Prochlorococcus*, VOL1 ([Morris et al. 2011](#_ENREF_5" \o "Morris, 2011 #2127)) to test the toxicity of buffer-generated HOOH. VOL1 was grown in Pro99 natural seawater medium ([Moore et al. 2007](#_ENREF_4" \o "Moore, 2007 #1111)) supplemented either with HEPES or TAPS under a variety of conditions. Importantly, Pro99 contains 1 μM Fe(III):EDTA as a nutritional supplement, which may enhance the toxicity of HOOH by accelerating the Fenton reaction ([Imlay 2003](#_ENREF_1" \o "Imlay, 2003 #340)). VOL1 was acclimated to growth conditions (other than presence/absence of buffer) over at least two passages into fresh media prior to the start of experiments. Mid-exponential phase cultures (~ 107-108 cells . mL-1) were diluted 200-fold into Pro99 medium either with or without HEPES or TAPS as a buffer and growth was followed by chlorophyll fluorescence using a TD700 fluorometer (Turner Designs, Sunnyvale, CA, USA) equipped with an *in vivo* chlorophyll-a filter set (excitation 340–500 nm; emission ≥ 665 nm). Buffers were added either at 1 mM, the level used in several artificial seawater media recipes for *Prochlorococcus* ([Moore et al. 2007](#_ENREF_4), [Rippka et al. 2000](#_ENREF_7)), or 10 mM, a higher level used for large-volume batch cultures of *Prochlorococcus* in natural seawater ([Moore et al. 2007](#_ENREF_4), [Zinser et al. 2009](#_ENREF_8)). If no growth was detectable after 60 d, the culture was assumed to be dead (growth rate ≤ 0). At the end of each experiment, samples were tested for purity by the addition of 1/10 volume of ProAC medium ([Morris et al. 2008](#_ENREF_6)) to the culture tubes; if no turbidity developed within 60 d, the culture was assumed to be axenic. None of the experiments reported here showed evidence of heterotrophic contamination.

Crystallized bovine liver catalase (1,340 units .  mg protein-1) was resuspended in 1 mM sodium phosphate buffer (pH 7.0) pre-heated to 37ºC at a concentration of 1000+ units . mL-1. The resulting solution was sterilized using a 0.22 μm Millex-GV syringe filter (Millipore) prior to dilution into culture media.

*HEPES as a steady-state HOOH generator*

Using the data presented in Figure 2, we performed a multiple linear regression to attempt to predict kHOOH based on buffer concentration, seawater proportion, and light level. The regression was highly significant (df=45, r2 = 0.958), and all three parameters yielded significant coefficients (Table S2). Our diel experiments used 100% natural seawater, 65 μmol photons . m-2 . s-1, and 10 mM HEPES, which we estimated based on our regression would produce approximately 71 nM h-1 HOOH. We supplemented this medium with a range of catalase concentrations to vary the competing HOOH removal rate and sampled the system for HOOH concentration periodically over two photoperiods of incubation (16:16 h of light:darkness during the first photoperiod, and 12:12 during the second).

We note that HOOH production in our actual diel experiment exceeded our predictions. The values used for our regression model were obtained from long-term incubations under constant illumination, whereas we observed that HOOH was produced much more rapidly during the first few hours of illumination (Fig. 1B). Because the regression model does not accommodate the initial burst of HOOH production, it should underestimate HOOH production in a diel experiment.

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Figure S4. Growth of non-axenic *Prochlorococcus* MIT9215 cultures in Pro99 medium supplemented with 10 mM of various buffers. Dotted lines shows the mean of 3 replicate unbuffered control cultures grown under the same conditions. Error bars are the standard errors of the three control replicates.



Figure S4. Growth of non-axenic *Prochlorococcus* MIT9215 cultures in Pro99 medium supplemented with 10 mM of various buffers. Dotted lines shows the mean of 3 replicate unbuffered control cultures grown under the same conditions. Error bars are the standard errors of the three control replicates.

Table S2. Multiple linear regression of kHOOH vs. medium characteristics.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Parameter** | **Coefficient** | **Std. Error** | **t** | **P** |
| Constant | -18.049 | 1.7415 | -10.364 | <0.001 |
| Buffer (mM) | 6.689 | 0.2455 | 27.247 | <0.001 |
| Seawater (%) | 0.099 | 0.0185 | 5.37 | <0.001 |
| Light level (μmol photons m-2 s-1) | 0.19 | 0.0108 | 17.564 | <0.001 |